CHROMATOGRAPHY '85

Symposia Biologica Hungarica 34

CHROMATOGRAPHY '85

34

Akadémiai Kiadó, Budapest

The Budapest Chromatography Conference was a remarkable event of the year 1985. Chromatographers from Austria, Czechoslovakia, Egypt, Finnland, France, FRG, GDR, Great Britain, Hungary, Italy, Japan, Mexico, The Netherlands, Poland, Rumania, Switzerland, Sweden, USA, USSR, West Berlin and Yugoslavia came to Budapest where they took part in the work of the Conference. Although the scope of the scientific contributions ranged from the theory to practice and from gas-liquid chromatography, through GC–MS, to high-performance liquid chromatography, a considerable portion of the posters and lectures dealt with the biological and medical application of liquid chromatography. The most interesting and up-to-date results and the advances in liquid chromatography have always been on the program of this symposia series (also called American–Eastern European Symposia on Liquid Chromatography), thereby this Budapest Conference yielded such interesting presentations as HPLC of cloned insuline, analyses of free amino acids in a quarter of an hour, determination of various substances from serum or urine, HPLC of ecdysteroids, isotachophoresis of oligopeptides, etc.

The volume presents the majority of these valuable contributions.

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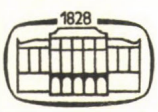
H. KALÁSZ et L. S. ETTRE



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PREFACE

The Budapest Chromatography Symposium was the fifth of the American - Eastern European Symposia on Liquid Chromatography originally initiated by Tibor Dévényi in 1981.

Due to the friendly atmosphere and high scientific level of our early Symposia, the number of papers presented at the recent meeting has grown to over one hundred of which more than half has been published in the Proceedings of the Symposium.

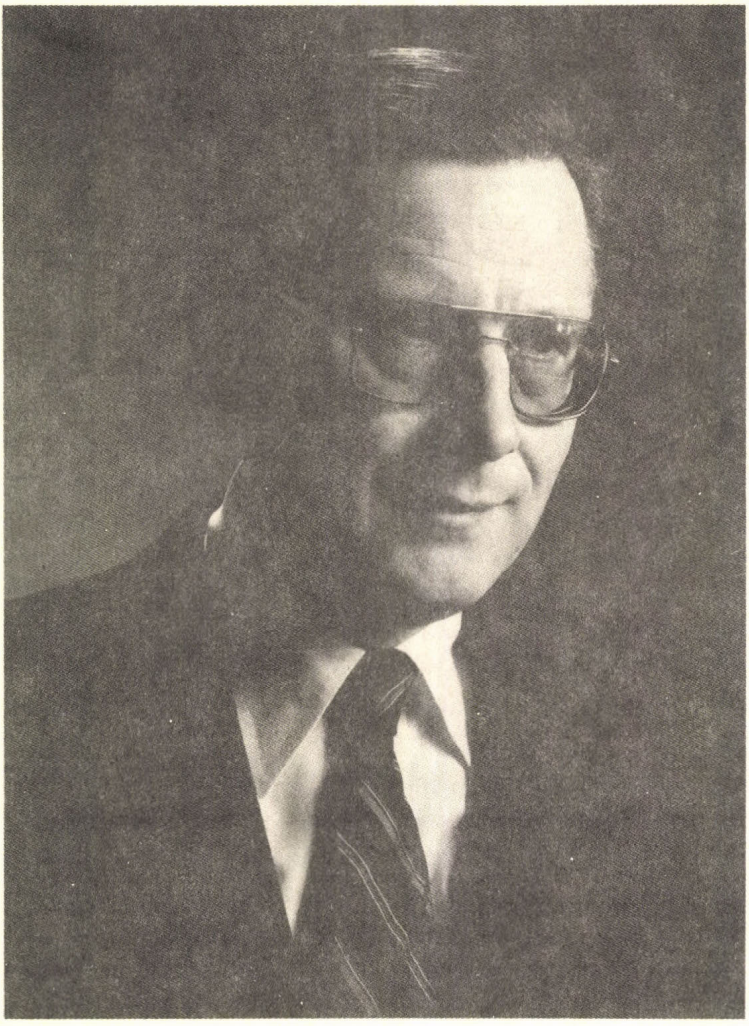
The lectures and poster presentations have dealt with a wide range of topics from theoretical considerations to the chemical, biochemical, biological and medical applications of the various separation methods. The people who are involved in these investigations are employed by various institutions and companies but they have one common characteristic: they apply, develop, utilize or simply use a well-known method called chromatography.

One may raise a theoretical question: are these people "chromatographers"? In 1950, L. Zechmeister, the famous Hungarian-born scientist, objected to the creation of such a category of scientific workers. On the other hand, in 1980, my coeditor, Dr Ettre in a paper about the evolution of chromatography expressed his opinion that today, there is indeed such a discipline. Neither was far from the truth. The rapid progress of chromatography has basically changed the situation and has given a full meaning to this word.

There are many scientists, responsible for this progress, who are well known all over the world and who have contributed to the evolution of various branches of chromatography. Among

them, some are involved in gas chromatography, while others in high-performance liquid chromatography, or thin-layer chromatography. Some of the chromatographers have made contribution to the theory, while others to the day-to-day application of the technique. There is however one person who has contributed to practically almost every field of separation techniques, from gas chromatography to the development of high-performance liquid chromatography, from the separation of amines, amino acids and peptides and proteins to the modern theory of reversed-phase HPLC: Csaba Horváth, Professor of Chemical Engineering at Yale University. He has been the inventor of new methods, mentor of Hungarian scientists. He also contributed to the success of our American - Eastern European Symposia series. He has received numerous awards for his scientific achievements from the Soviet Union to the United States. I would like to dedicate this volume to him with my deepest regards, thanking him for his help, and hoping that his achievements are only the start of a long and continuous professional career, the most important part of which is still to come.

Huba Kalász



Csaba Horváth, Professor of chemical engineering at Yale University, has pioneered the development of HPLC into a widely used analytical tool and made significant contributions to other areas of chromatography.

In the field of gas chromatography he is best known for the introduction of novel column types. As early as 1964 he built a high-pressure liquid chromatograph and was the first to demonstrate the feasibility of high-performance liquid chromatography having a speed and efficiency compared to that of gas

chromatography. His work resulted in wide-ranging technological advances as well as in significant contributions to chromatographic science in almost all areas of modern chromatography. For the separation of complex biological molecules he developed pellicular stationary phases which are comprised of fluid impervious microspheres having an appropriate retentive surface. Dr Horváth has been among the first chromatographers to use ion pair chromatography for the analysis of organic substances. He played a key role in the development of modern reversed-phase chromatography and made major contributions to elucidating the physicochemical basis of the technique which has become the most widely used branch of HPLC.

Professor Horváth's research during the past seven years has led to the development of high-performance displacement chromatography as a highly promising technique for preparative scale separations. He has recently explored novel approaches to the HPLC of proteins and nucleic acids and conducted studies on the fundamentals of biopolymer chromatography, and his work resulted in the introduction of various high-performance columns. Presently he is engaged in research on the theory of non-linear chromatography.

Professor Horváth is author and coauthor of over 170 scientific papers and numerous patents and is the editor of the serial publication "HPLC - Advances and Perspectives". Besides chromatography, his research interests have been in the application of chemical engineering science and principles to life sciences and medicine; presently, his research activities are equally divided between chromatography and various aspects of biotechnology with particular regard to biochemical separations.

Professor Horváth is a native of Hungary who graduated in 1952 with a diploma in chemical engineering from the University of Technical Sciences in Budapest and thereafter served on the faculty of the Department of Organic Chemical Technology. In 1956 he moved to the FRG and gained industrial experience at Farbwerke Hoechst AG in Frankfurt over the next four years. Subsequently he completed his studies at the Johann Wolfgang Goethe University in Frankfurt and received his doctorate in physical chemistry in 1963. The same year he emi-

grated to the USA and became a research fellow at the Harvard Medical School. In 1964 he moved to Yale University where he has held various faculty appointments both in the School of Medicine and in the Department of Engineering and Applied Science. In 1979 he was appointed Professor of Engineering and Applied Science and since 1981 he has been on the faculty of the newly reinstated Department of Chemical Engineering.

Professor Horváth is a widely sought speaker at scientific meetings all over the world and was the organizer of the highly successful 8th International Symposium on Column Liquid Chromatography in New York in 1984. He is the Chairman of the New England Chromatography Council and serves on numerous committees dealing with matters of science policy. He is consultant to several leading industrial organizations in the field of biotechnology, analytical instrumentation and separation technology. He has been a member of the following scientific journals: Journal of Chromatography, LC Magazine, Separation Science and Technology, Enzyme and Microbial Technology, Analytical Chemistry, CRC Handbook of HPLC, Journal of Molecular Catalysis, Reactive Polymers, Ion Exchangers and Sorbents as well as Fuels and Energy.

For his accomplishments in chromatography, Dr Horváth received the Steven Dal Nogare Award in 1978, the M. Tswett Award in 1979, and the Tswett Medal in Chromatography in 1980. He is the recipient of the 1981 U.S. Senior Scientist Humboldt Award, the American Chemical Society Award for Chromatography in 1983, and the Eastern Analytical Symposium Award in 1986.

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Budapest, 1986

Chromatography '85
H. Kalász and L.S. Ettre (Eds)

INTERFERENCE BY ON-COLUMN REACTIONS IN HPLC

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INTRODUCTION

Chemical transformations of the sample in the column are more frequent phenomena in liquid chromatography than is generally believed, and may occur due to a variety of reasons. Untoward reactions caused by active adsorbents have been known for a long time /1/ and the theoretical elucidation of multiple peaks and reaction zones on the chromatogram due to interconverting species commenced with the work of Keller and Giddings /2/ as early as in 1960. In method development the elimination or at least attenuation of such adverse transformations is often necessary in order to obtain chromatograms with well defined and separated peaks and is usually accomplished by manipulating chromatographic systems, mainly the mobile phase composition and operating conditions. This is frequently done by trial and error because the exact nature of the reaction often remains unidentified.

On the other hand it should be noted that on-column reactions are not necessarily bad. Secondary chemical equilibria having fast kinetics /3/, e.g., by pH control, ion-pair or other kind of complex formation, are commonly used to enhance the selectivity of the chromatographic system. In such cases the injected eluite interacts with a particular component of the mobile phase and traverses the column in a partially or fully complexed form as a single peak. This is also an example of interference by the complexation reaction because the conditions are chosen so that the individual interconverting species

are not separated. In many analytical applications the highest degree of such interference is sought in order to obtain sharp peaks. The name hetaeron, from the Greek name for companion, has been suggested /4/ for the complexing agent added for this purpose to the eluent. Such on-column reaction, therefore, can serve as a powerful tool to expand the scope of a given chromatographic system.

In our present discussion emphasis is placed on unbidden chemical transformations which interfere with the chromatographic separation process. As we shall see they occur when the characteristic time of the reaction is commensurable with that of the chromatographic separation process and result in unchromatographic elution profiles with concomitant reduction in the efficacy of the separation. Whereas certain general rules can be followed to diminish the unpropitious consequences of such interference, it is desirable that the nature of the reaction is recognized in order to suppress it in a specific way.

EXAMPLES FOR INTERFERING ON-COLUMN REACTIONS

In reversed phase chromatography protonic equilibria involving ionogenic eluites are ubiquitous /5/. Since the kinetics of the dissociation and protonation reaction is fast the eluite appears on the chromatogram as a single peak with a retention factor which is the weighted sum of the retention factors of the two forms, i.e., the eluite acid and base. The retention of other rapidly interconverting species can also exhibit similar behavior, but if the rate of transformation is relatively slow it interferes with the separation process /6/, and the interpretation of the chromatogram. Such reactions include conformational changes, isomerization, complex formation and reversible agglomeration of the eluite, which can take place either in the mobile phase or on the stationary phase or both. Irreversible reactions such as oxidation, hydrolysis and solvolysis of the sample may also occur in the column. Besides its annoying consequences in analytical work, the combined reaction and separation may be of interest for other reasons, however. For instance it has been exploited in gas chromato-

graphy to study reaction kinetics or formation of a given product in "chromatographic reactors" which have extensive literature /7, 8/.

The adverse effect of such on-column reactions on the separation is illustrated in Fig. 1. The chromatograms obtained under conditions typical in HPLC show that a relatively slow on-column reaction can result in the appearance of unexpected peaks and bizarre elution profiles which are dependent on the flow rate and reduce the efficiency of separation.

INTERPLAY OF REACTION KINETICS AND RETENTION

The combined effect of the reaction and retention in the column is governed by the dimensionless Damkőhler number, Da, which can be considered as the ratio of the residence time of the eluite in the mobile phase to the relaxation time of the reaction /9/. In the case of an irreversible first order reaction A → B, the Damkőhler number is given by

$$Da = Lk_r / u_o \qquad (1)$$

where L is the column length, k_r is the rate constant of the reaction and u_o is the mobile phase velocity.

When the eluite is subject to a reversible interconversion $A \rightleftarrows B$ which takes place in both phases and has an equilibrium constant K_M in the mobile phase, the Damkőhler number is expressed /10/ as

$$Da = (k_{M,f}\, k'_A + k_{S,f})\ (1 + 1/K_M)/u_o \qquad (2)$$

where k'_A is the retention factor of eluite form A, $k_{M,f}$ and $k_{S,f}$ are the appropriate forward rate constants in the mobile and stationary phases, respectively.

It has been shown /10/ that the degree of interference caused by the chemical transformation is a function of Da. The interference manifests itself in additional band spreading, in the conversion of the sample to a product or in the elution of

the interconverting forms of the sample as a single peak. The degree of interference is the greatest at very large values of Da, i.e., when the reaction rate is high, a long column and low flow velocity are used. In this case the reaction does not give rise to unusual peak shapes on the chromatogram. When the reaction is practically irreversible the eluite is completely converted into the reaction product whereas in the case of reversible reaction the two forms elute together without additional band spreading. In the chromatography of interconverting species the maximum interference is sought except when the separation of the two forms is desired that requires minimum interference. This occurs at very small Da numbers, i.e., with slow reaction, short column length and low flow velocity. Under such conditions the eluite passes through the column essentially unreacted or when it is present in two forms in the sample they elute individually according to their prechromatographic equilibrium distribution. The chromatographic results with on-column reaction at the limiting very high or low Da values are summarized in Table I.

At intermediate Damkőhler numbers in the range of $0.1 < Da < 50$, the system is very sensitive to changes in the operating conditions which affect the magnitude of Da because both the chromatographic retention process and the chemical reaction proceed on the same time scale. Interference in this domain of Da usually results in bizarre and unchromatographic elution profiles such as those shown in Fig. 1. In analytical HPLC such situations occur when the characteristic time for the reaction ranges from 10^{-2} to 10^{3} sec and the higher the column efficiency the more appreciable is the effect of the reaction, i.e. the band spreading engendered by the interference on the peak shape and width. Figure 2 illustrates the dependence of the degree of interference on the Damkőhler number in a typical case /10/. Evidently the magnitude of interference also depends on the retention values of the species involved and with reversible reactions on the equilibrium constant as well. The chromatograms in Fig. 1 were obtained with columns having dimensions commonly used in analytical HPLC and the unchromato-

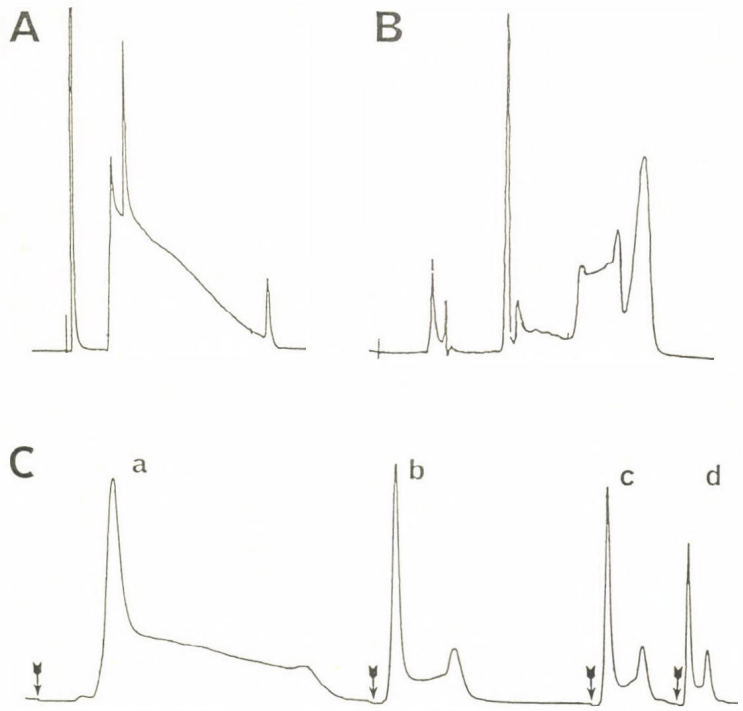

Fig. 1. Chromatograms illustrating the adverse effect of on-column reaction on the separation by reversed phase chromatography standard octadecyl-silica columns under typical conditions used in analytical work. (A) Oxidation of hydroquinone derivatives. (B) Agglomeration of Astrablue dye. (C) Mutarotation of methylfuran-1,2,3,4-tetrahydroxy oxane; chromatograms a to d were obtained at increasing flow rates.

Table I. Conditions for the limiting Damkőhler numbers representing minimal and maximal interference caused by on-column reaction and the effect on the chromatogram.

Damkőhler Number	Reaction Rate	Column	Flow rate	Peaks on the chromatogram	
				A → B	A ⇌ B
Da << 1	slow	short	high	A	A and B separated
Da >> 1	fast	long	low	B	A and B together

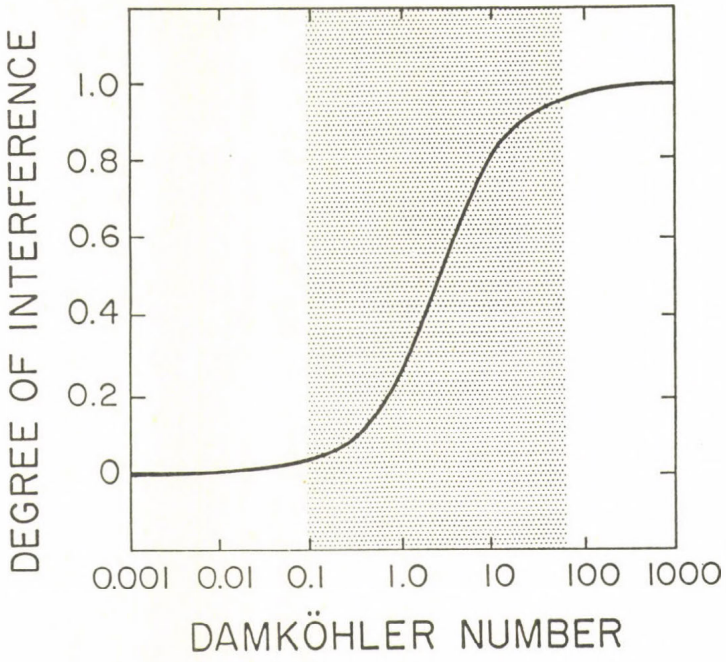

Fig. 2. Graph illustrating the degree of interference by on-column reaction on chromatographic results as a function of the Damköhler number. In the shaded critical domain of Da the interference manifests itself by additional band spreading and/or by unchromatographic elution profiles. At high Da values the maximum interference leads to complete chemical transformation of the sample or elution of its various forms as a single peak. Adapted from data in Ref. 10.

graphic elution profiles indicate the pertinent Da numbers are in the critical range.

Acid dissociation equilibria involving the eluite are perhaps the most common reactions in liquid chromatography as mentioned above. The appropriate rate constants are of the order of $10M^{-1}s^{-1}$ and for carboxylic acids the dissociation constant Ka is typically $2 \times 10^{-5}M$. When the eluent pH and pK_a are about the same the Damköhler number is of the order of 10^6, that is, much too high for the protonic equilibria to have an effect on peak broadening and peak shape. Thus due to the maximum interference the chromatographic system cannot distinguish between

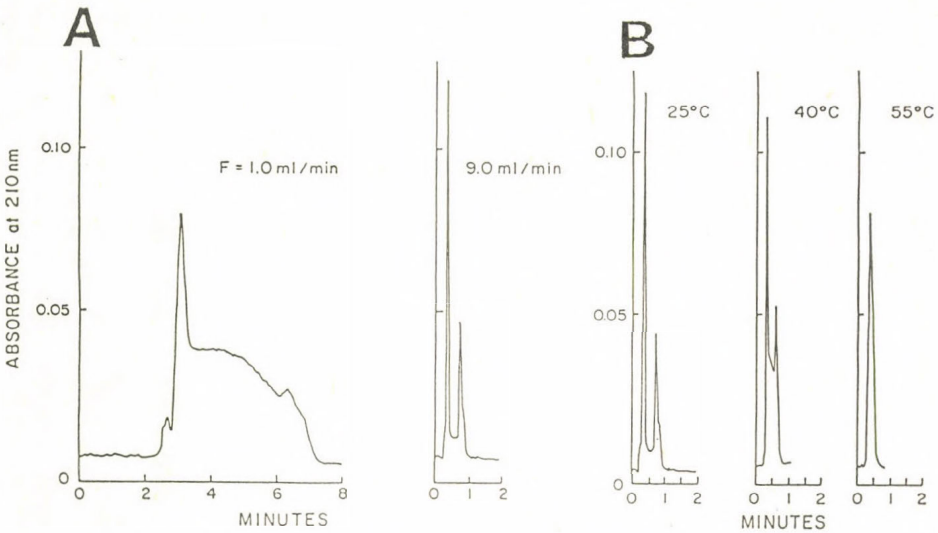

Fig. 3. Effect of flow rate, A, and temperature, B, on the chromatograms of L-alanine-L-proline dipeptide under conditions of reversed phase chromatography. From Ref. 11.

the acid and base forms of the sample. This level of interference is often desirable in chromatographic separations.

The Damkőhler number is proportional to the rate constant of the reaction which usually increases with the temperature much more strongly than the retention factor, and inversely proportional to the flow rate. Therefore, changes in these operational parameters, when Da is around unity, may dramatically affect the elution profiles as suggested by Fig. 2. Under such conditions interference by chemical reaction can conveniently be diagnosed by varying the flow rate, or the temperature. Figure 3 illustrates the effect of such changes on the chromatogram of the dipeptide Ala - Pro, which undergoes cis-trans isomerization in the column /11/.

The degree of interference can often be changed by certain additives to the mobile phase which may act as catalysts in accelerating the reaction. It is frequently found that the nature of the buffer can have a significant effect on separation efficiency and this dynamic role of the buffer /12/, which complements its static role in maintaining the pH of the eluent

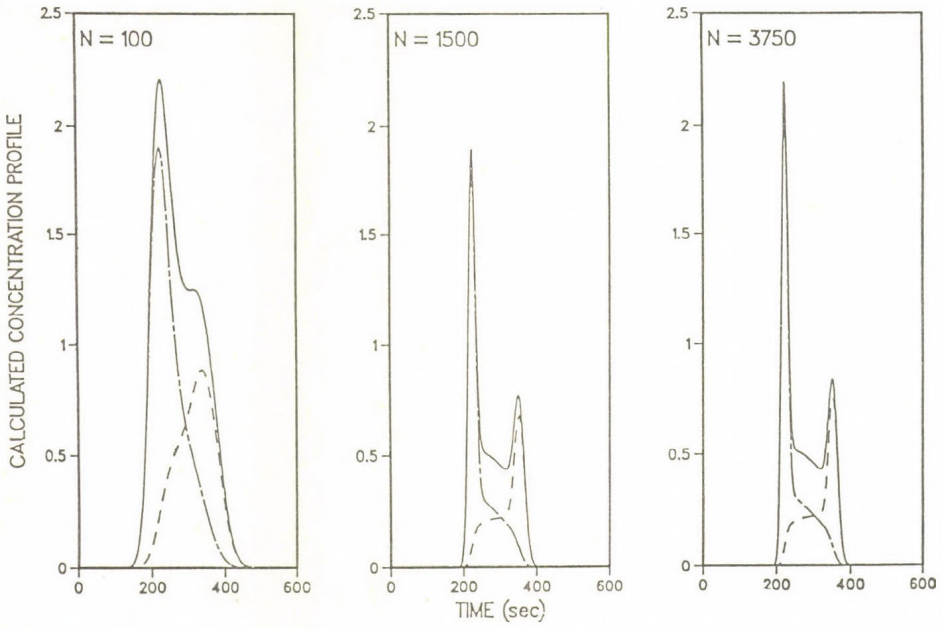

Fig. 4. Simulated chromatograms illustrating the effect of slow kinetics of interconversion at Da = 1.5 in column having different efficiencies as indicated by the plate number, N. From Ref. 15.

constant, is likely to be associated with such "catalytic" effects. For instance, the often observed peak sharpening effect of phosphate at acidic pH which was first found by serendipity at the beginning of modern liquid chromatography /13/ is believed to arise from its involvement in rapid proton transfer reactions. The beneficial effect of trifluoroacetic acid in the mobile phase used for peptide separations by reversed phase chromatography /14/ may also be explained in a similar fashion.

The additional band spreading which arises from secondary equilibria or some other kind of interconversion contributes a certain plate height increment which can be calculated from the appropriate kinetic and equilibrium parameters /10, 15/.

In HPLC, unlike in classical column chromatography, short columns of high efficiency are used at high flow velocities. Consequently in the critical Damkőhler number domain the effect of kinetic interferences on the elution profiles are usually much more pronounced in HPLC than in low efficiency chromato-

graphic systems where the relatively large band spreading from other sources may mask the interferences which arise from slow kinetics /16/. This is illustrated on the simulated chromatograms in Fig. 4 where the elution profiles of two interconverting species having a retention factor ratio of about 1.6, and equilibrium constant of 1.5 are shown at Da = 1.5 in three columns having plate numbers of 100, 150 and 3750, respectively.

The interplay of reaction and retention has been treated extensively in the literature. Most studies were conducted, however, to develop a theoretical basis for elucidation of the nature of the reaction and for extracting physicochemical information from chromatographic data. On the other hand in analytical method development the goal is to find means to minimize or maximize the interference under conditions when no detailed information is available on the nature and kinetics of the reaction. In practice, therefore, each particular on-column reaction is best analyzed on a "case history" basis to find the most appropriate conditions for the separation.

CIS-TRANS ISOMERIZATION OF PROLINE CONTAINING PEPTIDES

Cis-trans isomerization of the peptide bond involving proline residues is implicated in protein denaturation and has therefore received considerable attention in the biochemical literature /17, 18/. Since the relaxation times for the cis-trans isomerization of such proline containing dipeptides are in the order of minutes and the retention factors of the two isomers in reversed phase chromatography are significantly different, they have been used as models for a detailed analysis of the interplay of isomerization and retention in such chromatographic systems.

The chromatograms in Fig. 3 illustrate the effect of flow rate and temperature on the separation. By using an elaborate theoretical analysis /10/ it was possible to calculate the rate and equilibrium constants for the isomerization on the surface of the octadecyl-silica stationary phase from chromatographic data and from the known rate and equilibrium constants of the inverconversion in free solution. The results /15/ are quite

revealing in that the equilibrium constants for the cis-trans isomerization on the surface were found to be 3 to 16 times smaller for small proline peptides than the corresponding equilibrium constants in the mobile phase. This suggests that the stationary phase surface stabilizes the cis isomers which are retained stronger because of their greater hydrophobic surface area than the trans isomers. Such a phenomenon has been observed with larger peptides and proteins as well and found that upon contact with the hydrophobic stationary phase the sample converts to a more hydrophobic conformer which may be the denatured form of the protein /19-21/. Thus, from the kinetic point of view the nonpolar surface of the stationary phase appears to catalyze such isomerization. Indeed, the study with the proline peptides has shown that the rate constants for the isomerization at the octadecyl-silica surface were significantly greater than the corresponding rate constants in free solution. The results of such studies are of biological interest because the stationary phase used in reversed phase chromatography may serve as a model for the low-dielectric environment at the surface of biological membranes.

As far as the HPLC analysis of interconverting substances is concerned the above discussion of system behavior with changing Damkőhler number applies. At sufficiently high temperature and high flow rate the two interconverting species yield a single peak and their total amount can be readily quantified. At sufficiently low temperatures the reaction rate can be attenuated so that the two isomers elute without reaction on the column and can be collected individually in the effluent. Low temperature HPLC has been a powerful tool to minimize the effect of on-column reactions. When the chromatographic separation is carried out at low temperatures where the reaction is so slow that /22/ the value of the Damkőhler number is sufficiently reduced the interference by the reaction can practically be eliminated. Since the relative amounts of the two forms of the eluite are determined by their equilibrium distribution in the sample, the sample history in terms of prechromatographic incubation time, pH and other conditions determines the re-

lative size of the peaks of the two forms. Thus the equilibrium constant can readily be measured by chromatographic analysis.

As mentioned above, when under the chromatographic conditions employed the reaction rate is very slow the two otherwise interconverting species traverse the column independently. However, when they are collected in the effluent each of them may undergo isomerization in postchromatographic treatment, i.e. upon prolonged storage. Then rechromatography of each of the two fractions under the same conditions can yield two peaks again and the pattern repeats itself upon continuing the process. This may be the source of frequently confusing observations and thereby hamper peak identification. In order to diagnose the situation it is necessary to vary the analytical and/or extrachromatographic conditions on the basis of the above physico-chemical considerations.

ON-COLUMN OXIDATION

The stationary phase proper may also catalyze the chemical transformation of the eluite into another chemical substance. Traces of heavy metals, mainly iron, may be extracted from the mobile phase by the siliceous stationary phase /23/ in HPLC and the surface bound metal may catalyze the oxidation of certain eluites.

Such reactions with methoxy substituted hydroquinones were studied in reversed phase chromatography /24/ and salient observations are listed in Table II. Fig. 5 illustrates the effect of the flow rate on the chromatograms of 2,6-dimethoxy hydroquinone which is partially oxidized in the column to the corresponding benzoquinone. From the results we infer that the Damkőhler number is in the critical domain under the conditions of the experiments. Between the peaks of the least retained hydroquinone and most retained benzoquinone there is a "reaction zone" typical for such on-column reactions and in this case believed to contain both species and possibly semiquinone intermediates. At sufficiently low column temperatures and high flow rates the hydroquinone elutes as a single peak since the effect of on-column reaction is negligible, whereas at high

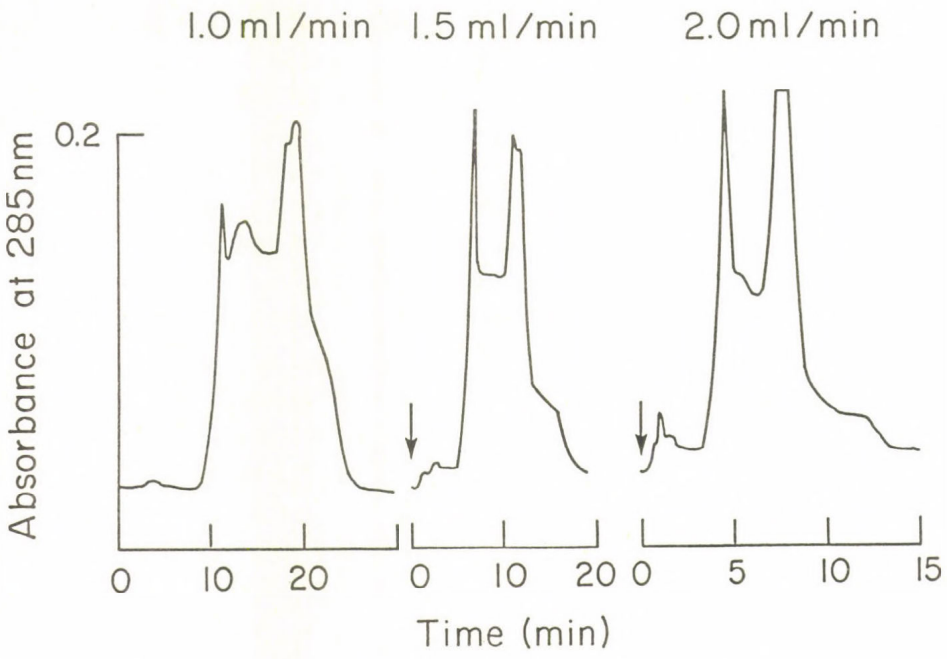

Fig. 5. Chromatograms illustrating the effect of the flow-rate on the elution profiles of 2,6-dimethoxyhydroquinone in reversed phase chromatography. From Ref. 24.

Table II. Features of on-column oxidation of methoxy substituted hydroquinones in reversed phase chromatography /23/

• The siliceous stationary phase with adventitious heavy metals bound to the surface acts as a heterogeneous catalyst in the presence of oxygen in the mobile phase
• Rate of reaction is on the time scale of chromatographic separation
• Reactants and products are continuously separated in the column
• Pretreatment of column with EDTA and reduction of oxygen concentration in the mobile phase attenuate or arrest the reaction
• Pretreatment of column with heavy metals augments the reaction

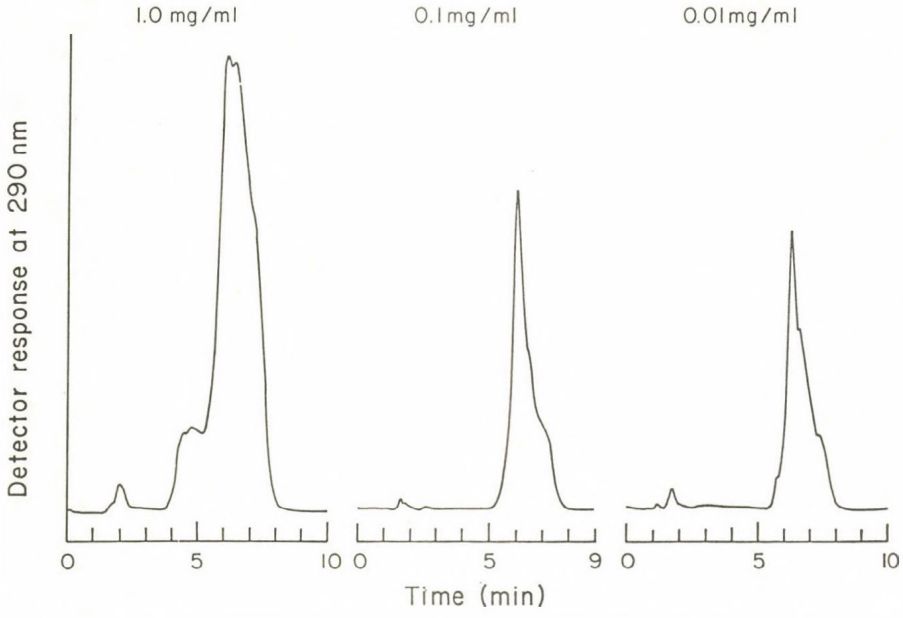

Fig. 6. Effect of sample size on the chromatogram of 2,6-dimethoxy benzoquinone obtained by reversed phase chromatography. From Ref. 24.

temperatures and low flow rates the hydroquinone is completely oxidized and a single peak of benzoquinone appears on the chromatogram. The latter case exemplifies that a peak on the chromatogram may represent a substance which is the product of a chemical reaction and therefore chemically different from the sample injected. Rechromatography by using the same system, however, does not reveal that the original sample has been converted and in the absence of some extrachromatographic identification misleading conclusions may be drawn from chromatographic results. As with other heterogenous catalytic reactions the kinetics of the surface catalyzed oxidation discussed here is expected to follow Langmuir-Hinshelwood kinetics. As a result of the non-linear reaction behavior the elution profile depends on the sample concentration. Fig. 6 illustrates the effect of the sample size on the chromatogram of 2,6-dimethoxy hydroquinone when retention and reaction occur simultaneously on the stationary phase surface. As seen from the chromatograms peak shape changes with the sample load in a

qualitatively different way from that observed when the column is overloaded and due to the high eluite concentration the chromatographic process is governed by the non-linear domain of the adsorption isotherm.

Removal of the metal from the stationary phase surface, e.g., by washing the column with the solution of a chelating agent such as EDTA may eliminate the catalytic sites and facilitate elution of the hydroquinone without reaction. In chromatographic practice the addition of a chelator to the eluent has been frequently found to improve peak shape and eliminate unchromatographic elution profiles. In most cases the chelating agent is believed to preclude catalytic reactions or complexation by the heavy metal ions present in the column.

HPLC OF DEFEROXAMINE

Metals in the column can also give rise to other types of chemical transformation of the sample. An example for the interference by on-column metal complexation has been investigated in reversed phase chromatography of deferoxamine, a chelating drug which binds Fe^{3+} very strongly so that the stability constant of its iron complex, called ferrioxamine, is 10^{-30}. It was found that traces of iron present on the stationary and in the mobile phase can complex with deferoxamine and thus form ferrioxamine in the column /25/. The interference caused by the reaction gave rise to anomalies in the chromatography of deferoxamine. When a small amount of deferoxamine was injected it was converted into ferrioxamine in the column so that only a single peak of the fast eluting ferrioxamine appeared on the chromatogram due to maximum interference as seen in Fig. 7. Upon injecting a large quantity of deferoxamine it mostly eluted without reaction and at the low detector sensitivity again a single peak was only obtained in the absence of interference. Its retention was, however, much greater because deferoxamine is much less polar than ferrioxamine. As depicted also in Fig. 7 at intermediate sample size and detector sensitivity a marked reaction zone was obtained which clearly revealed the interplay of retention and reaction. The appearance

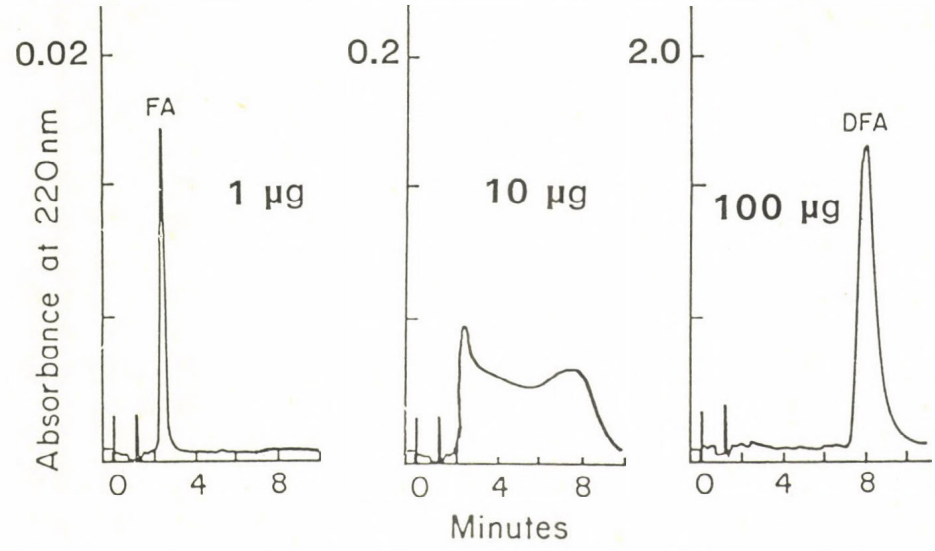

Fig. 7. Chromatogram illustrating the on-column complexation of deferoxamine with iron under conditions of reversed phase chromatography at different sample loading. The amount of deferoxamine injected was 1 µg (a), 10 µg (b) and 100 µg (c).

of the reaction zone suggests that the system is operated within the critical range of the Damkőhler number. Indeed, variations of column temperature and/or flow rate brought about significant changes in the shape of the elution profile at intermediate sample load.

The results illustrate again that fallacious conclusions could readily be drawn from the chromatogram if the on-column reaction were not recognized. In fact the study of this system /25/ was initiated by some confusing observations on the retention behaviour of deferoxamine. Only upon finding that about 150 micrograms of iron was deposited on the stationary phase in a regular size analytical HPLC column could the apparent anomaly be explained. Also in this case washing of the column with EDTA or bicine and maintaining a background chelator in the eluent produced reproducible results as no iron was available for complexation.

CONCLUSIONS

Interfering on-column reactions can give rise to multiple peaks from a single substance, unchromatographic elution profiles and the emergence from the column of a peak which represents the transformed substance rather than the sample compound proper.

In a critical range of the Damkőhler number, whose magnitude governs the chromatographic effect of the interplay between reaction and retention, the efficiency of the separation is greatly impaired. With high efficiency columns used for rapid separations in HPLC the adverse effect of relatively slow kinetic phenomena is generally much more serious than with traditional chromatographic systems of relatively low efficiency. Therefore the use of various precautionary measures such as the adjustment of the mobile phase composition and column temperature is often required to obtain appropriate chromatographic separations. Identification of peaks can also be encumbered by on-column reactions and it has to be realized that the substance which emerges from the column as a single peak may not be same as that injected. It is hoped that with time many of such reactions will be diagnosed and methods developed to reduce their untoward effect on the chromatographic results. Although great advances have been made in the fundamental theoretical understanding of the general phenomena, in practice each requires specific analysis of the factors involved and therefore the compilation of a library of "case histories" may be the most promising aid in coping with interference in chromatographic method development.

As a final note it should be added that preparative liquid chromatography at high concentrations of the feed components is frequently plagued by chemical reactions which may take place either between the components or between different forms of a given component, or both. Due to the high concentrations the reactions are usually non-linear and the analysis of such doubly non-linear systems is rather complicated to say the least. The interference caused by the reaction can be particularly detrimental in displacement chromatography /26/.

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HPLC STUDIES ON THE ORNITHINE-IMBALANCE INDUCED BY DL-α-DIFLUOROMETHYLORNITHINE IN P388 LEUKAEMIA CELLS

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INTRODUCTION

Recently, Weber et al. /1/ demonstrated that depending on the growth rate of tumours ornithine could metabolize either in the urea-cycle to synthesize citrulline with carbamoyl phosphate (by ornithine carbamoyl transferase, OCT EC 2.1.3.3), or to form putrescine by the rate-limiting key enzyme ornithine decarboxylase (ODC, EC 4.1.1.17). Particular significance of the imbalance arising in ornithine metabolism to neoplasia is that carbamoyl phosphate and aspartate could alternatively be utilized as precursors for nucleic acid synthesis /2/. Concerning the polyamines it is considered that the concentration of putrescine (Pu), spermidine (Spd) and spermine (Spn) and their biosynthetic enzymes are high in actively proliferating cells both in vivo and in vitro, and increase rapidly when growth or differentiation is induced /3, 4/. It is generally thought that depletion of cellular polyamines by inhibition of their biosynthetic enzymes is one of the most promising fields in cancer chemotherapy /3, 5/. When P388 leukaemia-bearing mice were treated with DL-α-difluoromethylornithine (DFM), an irreversible inhibitor of ODC, we have observed besides the depletion of polyamines the characteristic increase of some distinct nucleotide phosphates in the tumour cells /6/.

In the present paper the applications of some HPLC methods for monitoring the changes induced by DFMO treatment in the

polyamine and nucleotide concentrations of P388 leukaemia cells are demonstrated.

MATERIALS AND METHODS

All chemicals purchased from commercial sources were analytical grade and purified according to HPLC standards. Reagents were made with water distilled twice in glass and dansylated polyamines were separated with LiChrosolv (Merck No. 6007) methanol. For ion-exchange chromatography of the nucleotides 1 M ammonium phosphate buffer (pH 7) was prepared from 75 % (w/v) orthophosphoric acid and 25 % (w/v) ammonium hydroxyde solutions of analytical purity instead of Na-, or K-phosphates. Reference polyamines (Pu, Spd, Spn) from Calbiochem and nucleotide phosphates from Reanal (Budapest) were used. Dl-α-difluoromethylornithine hydrochloride monohydrate (DFMO, RMI 71,782) was kindly donated by the Centre de Recherche Merrell International (Strasbourg, France).

Apparatus

A Hewlett-Packard Model 1084B liquid chromatograph equipped with a Model 79785A variable wavelength UV detector and a 7985OLC terminal was applied throughout. Calibrations for quantitative measurements were performed with reference compounds used as external standards. Chromatographic parameters were calculated in the conventional way /7, 8/.

P388 leukaemia cells and in vivo DFMO treatment

P388 leukaemia cells were maintained in BDF_1 inbred male mice by serial transplantation of 5.10^6 cells per animal in our Institute as described earlier /9/. DFMO was given to BDF_1 mice (60-70 per group) in 2 % water solution as sole drinking fluid two weeks before the i.p. inoculation of P388 leukaemia cells and continued through tumour growth. Another group of mice served as control and received tap water. At the beginning of the experiments 5-6-day-old tumour cells were transplanted into

the animals of both groups. P388 leukaemia cells were harvested from 5-7 animals at every day of tumour growth by rinsing the intraperitoneal cavity of mice with 2 ml ice-cold 0.33 % citrate-0.15 M NaCl solution. Tumour cells were pooled, counted and samples containing appropriate number of cells ($50\text{-}100.10^6$) for polyamine and nucleotide determinations were centrifuged.

HPLC determination of polyamines

P388 leukaemia cells ($50\text{-}100.10^6$) were homogenized in 2 ml 0.1 M phosphate buffer (pH 7) by sonication (MSE P6100, at max. output for 1 min) and precipitated with 2 ml 0.7 N perchloric acid. After centrifugation the supernatant was collected and the precipitate was extracted again with 4 ml perchloric acid. Supernatants were pooled and the polyamines were pre-separated on a Dowex 50 x 8 column (40 x 5 mm I.D.) eliminating the contaminants and other amines by step-wise elution according to Seiler and Knödgen /10/. Dansylation of polyamines was carried out as previously described /7/. Dansylated polyamines were separated on a LiChrosorb RP-8 reversed phase column (200 x 4.6 mm I.D., 7 µm) and measured at 254 nm using a programmed linear gradient of methanol in water ranging from 70 % to 100 % (w/v) at a rate of 3 % methanol per minute. A mixture of Pu, Spd and Spn containing 6.66, 3.33 and 3.33 nmol per 10 µl, respectively, was treated identically with the biological samples and used for reference and quantitation.

High-performance ion-exchange liquid chromatography of nucleotide phosphates

Sedimented tumour cells were precipitated without previous homogenization with 0.7 N ice-cold perchloric acid and agitated vigorously for 3 x 1 min with a Vortex mixer. After standing for 10 min at $0^{\circ}C$ the samples were centrifuged (5000 rpm, $0^{\circ}C$, 10 min), the supernatants were measured and poured into conical centrifuge tubes. Ten mg of $KHCO_3$ was added to each 100 µl of supernatant and mixed. After centri-

fugation the clear supernatants were collected and stored for HPLC analysis at -20°C. High-performance ion-exchange chromatography of nucleotide phosphates was performed on a Polyanion SI HR 5/5 column (50 x 5 mm I.D., 8 µm, Pharmacia Biotechnology International AB, Uppsala, Sweden) using special adapters for fitting. The chromatograms were developed by a programmed linear gradient of ammonium phosphate buffer (pH 7) ranging from 0.01 to 1 M. Adenosine-, cytidine-, uridine- and guanosine-5'-mono-, di- and triphosphates (AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP and GTP) were identified and measured at 254 nm. Results were expressed in nmol nucleotides per 10^6 tumour cells. Mean values and standard errors were statistically evaluated.

RESULTS AND DISCUSSION

A continuously increasing number of publications devoted to the separation and measuring of metabolites from various biological sources indicates the importance and superior applicability of high-performance liquid chromatographic techniques in biomedical research /11, 12/. Investigation of polyamines and nucleotides in relation to the metabolic events proceeded during proliferation and/or drug treatment of different tumour cell lines has been effectively promoted by HPLC methods /7-12/. Figs 1 and 2 show that HPLC of dansylated polyamines on LiChrosorb RP-8 reversed phase packing and of nucleotides on Polyanion SI HR 5/5 ion-exchanger provided excellent resolution for both groups of compounds and allowed their quantitation in P388 leukaemia cells in a range of 0.1-10 nmol. Accuracy and advantages of liquid chromatographic determination of polyamines have been reported earlier /7, 8/. Regarding the separation of nucleotides phosphates it has to be noted that commercial analytical grade sodium or potassium phosphates were not suitable for gradient formation because of their relatively high content of UV-absorbing impurities and because, unfortunately, these contaminants could deteriorate the ion-exchanger in a short time. The best results were obtained when ammonium phosphate buffer prepared from its

constituents, e.g. from analytical grade phosphoric acid and ammonium hydroxide was used. The separation of nucleotide phosphates on the Polyanion SI HR 5/5 column was definitely specific of the quality of counter-anion. Only phosphate buffers provided adequate resolution, while increase of the ionic strength with acetate, chloride, sulphate or carbonate ions did not result in appropriate separations. The linear program of ammonium phosphate concentration gradient that proved to be the most suitable for the fractionation of some critical pairs of monophosphates (CMP-UMP, AMP-IMP), as well as for the elution of GTP (at 0.8 M approx.) is listed in Fig. 2. However, the impurities of the buffer sometimes resulted in an extra peak at the end of the chromatogram that might cause an inaccuracy in measuring GTP.

In the present work we applied successively two HPLC methods, reversed-phase separation of dansylated polyamines (Fig. 1) and ion-exchange chromatography of nucleotide phosphates (Fig. 2) for monitoring the in vivo metabolic changes in P388 leukaemia cells. These changes were induced either by the tumour growth itself, or by the peroral DFMO treatment of leukaemia-bearing animals. We have shown earlier that P388 leukaemia cells grown intraperitoneally in BDF_1 mice have an active polyamine metabolism mostly at the early phase of cell proliferation (1-2 days), while a continuous decrease of all polyamine fractions could be observed through tumour growth /9, 13/. It has also been reported that competitive or irreversible inhibitors of biosynthetic enzymes of polyamines deplete the cellular polyamine content, slow the growth and division of cells and produce marked antitumour effect in different in vivo and in vitro models /3, 4, 5/. In an attempt to demonstrate the antitumour effect of DFMO in P388 leukaemia cells we have shown the inhibition of ODC activity as well as the depletion of some polyamines due to the DFMO treatment of tumour-bearing animals /6/. However, the changes in cellular polyamines went along with tumour progression and scheduled for the late exponential period of tumour growth (3-6 days). Fig. 3 shows that DFMO caused either a 30-45 per cent lowering of cell count per animal, or 30-60

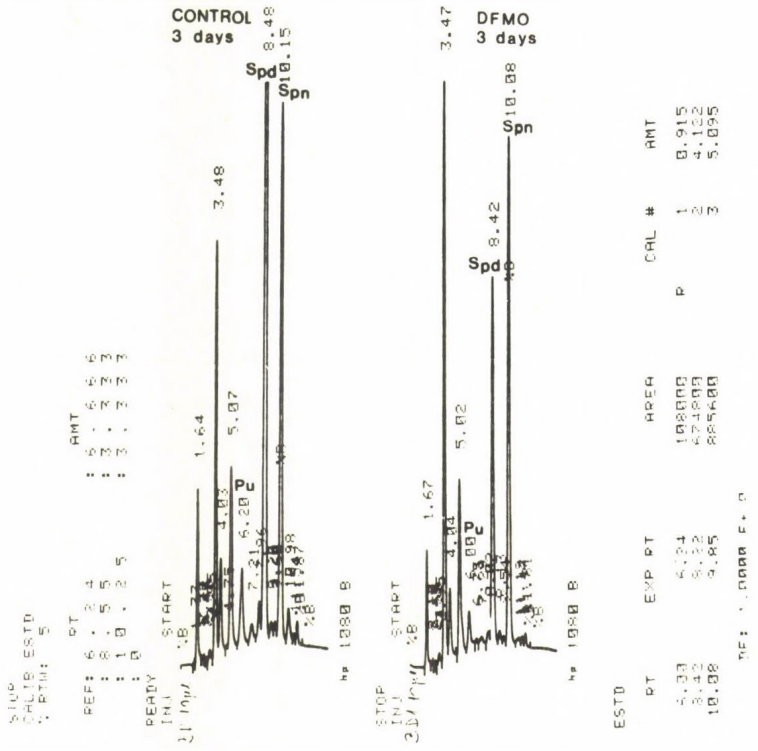

Figure 1. Reversed-phase (LiChrosorb RP-8, 20 x 0.46 cm, 7 µm) separation of dansylated polyamines (Pu, Spd, Spn) originated from 3-day old control (CONTROL) and DFMO-treated (DFMO) P388 leukaemia cells.

per cent depletion of Pu and Spd, respectively. On the contrary, Spn increased continuously with 20-50 per cent in comparison to the controls of the same age in accordance with some other tumour models investigated /3, 4/.

Determination of nucleotide phosphates in P388 leukaemia cells originated either from tumourous control or DFMO-treated animals led to two further conclusions:

(a) The predominant nucleotides (AMP, ADP, ATP, UTP, CTP and GTP), like polyamines, displayed characteristic growth-dependent changes related, in principle, to the shift from aerobic to anaerobic glycolysis in leukaemia cells proceeded during the progression of tumour /6/;

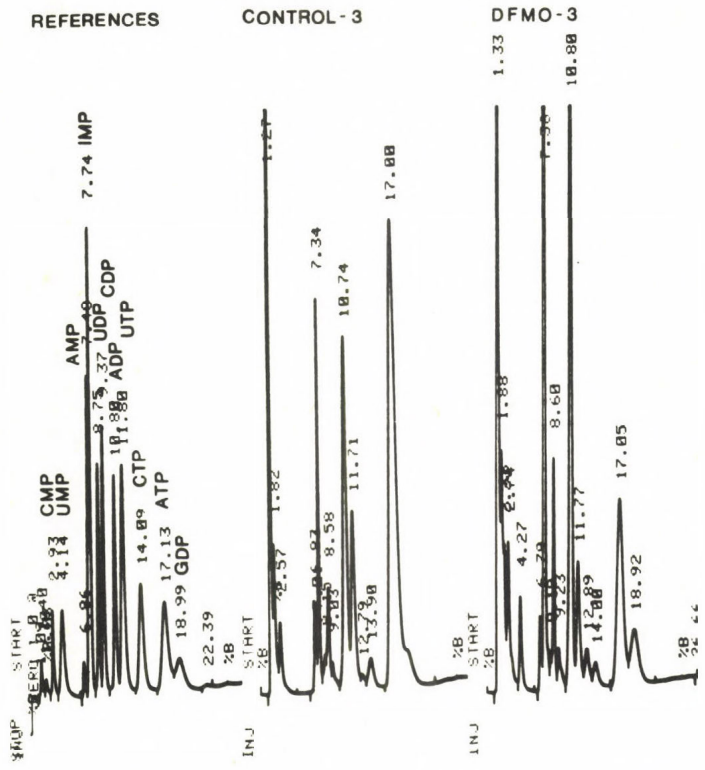

Figure 2. Ion-exchange HPLC of nucleotide phosphates on Polyanion SI HR 5/5 column (50 x 5 mm, 5 µm). Separation of REFERENCES is compared with the nucleotide patterns of 3-day old control (CONTROL-3) and DFMO-treated (DFMO-3) P388 leukaemia cells. Total nucleotides correspond to 5.10^6 tumour cells. Abbrevations see in Methods. Gradient program:

t (min)	0	2	25	28	30	31	35
Buffer (Mol)	0.01	0.15	0.30	1.00	1.00	0.01	STOP

(b) DFMO treatment resulted in significant elevation of uridine-, cytidine- and adenosine-phosphates in leukaemia cells on days 3-4 following transplantation of the tumour (see Figs 2 and 4) that reflected on the effect of DFMO on the ornithine metabolism.

In the studies of Weber et al. /1, 2/ it has been demonstrated that beyond the biosynthesis of polyamines (Pu) via ODC the alternative pathway for ornithine utilization is

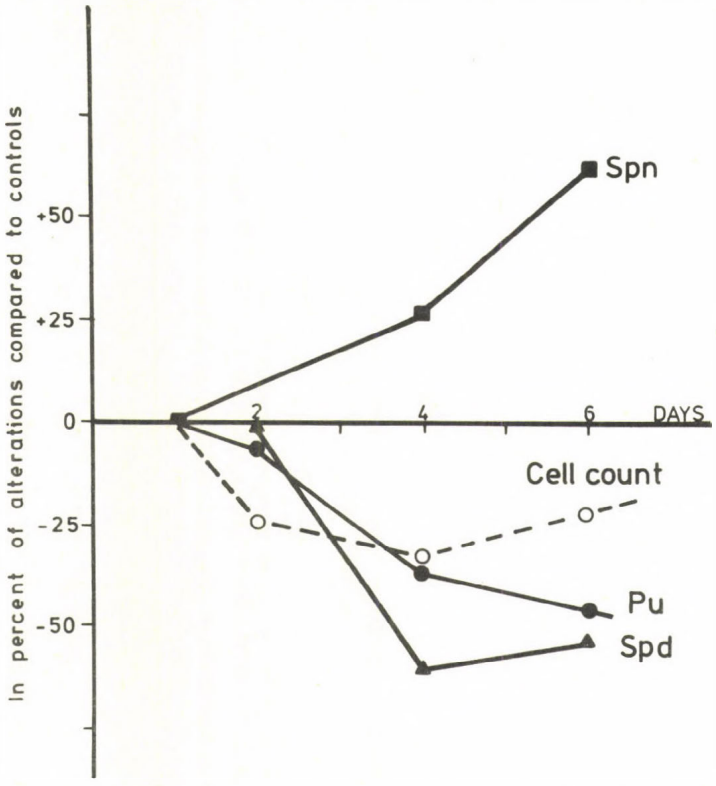

Figure 3. Changes induced by DFMO treatment in the putrescine (Pu), spermidine (Spd) and spermine (Spn) content of P388 leukaemia cells and in the cell count per animal (dotted line) depending on the growth of tumour.

the urea-cycle, in which citrulline is formed from ornithine and carbamoyl phosphate by ornithine carbamoyl transferase (OCT, EC 2.1.3.3). Considering the results presented here it is suggested, therefore, that a simultaneous inhibition of ornithine-requiring enzymes (ODC and OCT) by DFMO may prevent the access of ornithine for the biosynthesis of polyamines and citrulline as well, and may result in enhanced utilization of the metabolites carbamoyl phosphate and aspartate for the synthesis of uridine and cytidine nucleotides.

Our in vivo observations on P388 leukaemia cells arein accord with the in vitro results of Heby et al. /4/, who also

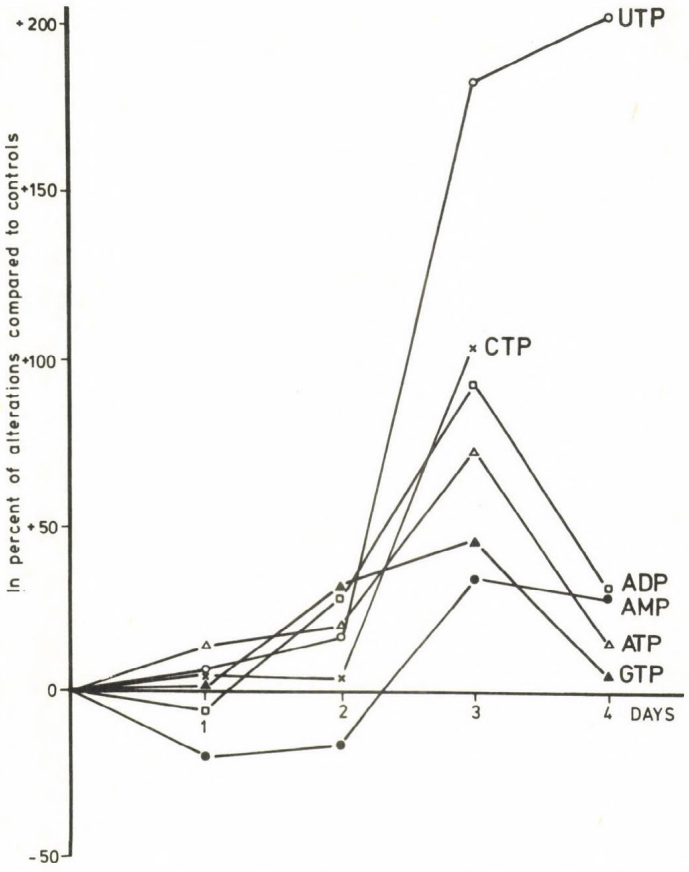

Figure 4. Changes induced by DFMO treatment in the nucleotide phosphates of P388 leukaemia cells depending on the growth of tumour. Abbreviations see in Methods.

found a significant elevation of UTP, CTP, ADP and ATP in Ehrlich ascites carcinoma cells treated in tissue culture with 5 mM DFMO. The practically identical results obtained with different tumour lines and experimental conditions may suggest that there is a common feature of rapidly growing tumours related to the metabolic imbalance of ornithine. Little is yet known about the activity of urea-cycle and related enzymes in the fast-growing tumour cells, but our results look as to give an indirect evidence for the mechanism mentioned above and for the varied effects of DFMO on the ornithine imbalance in tumour cells. Further investigations are

required to support this interpretation, and to clarify the significance of DFMO treatment in the ornithine metabolism of neoplastic cells.

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HPLC-ANALYSIS OF FREE AMINO ACIDS IN BIOLOGICAL FLUIDS. METHODOLOGICAL ADVANTAGES, SHORTCOMINGS AND TECHNICAL PITFALLS

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SUMMARY

In repeated reports the potential use of HPLC in analyzing free amino acids (AA) in biological fluids is claimed. However, it appears that the validity of this approach has not yet been adequately controlled. Improper sample handling results in unreliable results and inappropriate identification which invalidate quantitation and recovery of major free AA constituents in plasma, tissue or urine.

By carefully controlling factors and limitations inherent in methodology, we developed an ultra rapid and sensitive automated system enabling precise, specific, and routinely manageable measurement of the 23 major free AA in tissues and plasma in the lower picomole range precolumn derivatization with OPA/3-mercaptopropionic acid (OPA/3-MPA) or 9-fluorenylmethyloxycarbonylchloride (FMOC) in less than 13 and 25 min, respectively. The combined error of the method ranged between 2-6% (C.V.) with either methods. The HPLC results were in excellent agreement with those earlier acquired by using alternative conventional methods in plasma, muscle and liver.

It is to conclude that, although HPLC provides a powerful tool in AA research, adequate use of the method requires the exercise of great caution in controlling factors which may jeopardize the reliability of analyses.

Simultaneously with all technical developments, which made HPLC-amino acid analyses easily available, a new danger occurred. As more use is made of extensive analytical programs, there

must be an equally wide awareness of limitations inherent in these new methods and in the interpretation of their findings. It is regrettable that the attention that must be paid to details in handling the samples are sadly often neglected resulting in certain artifacts and thus, in unreliable results. Consequently, in this report certain shortcomings and technical pitfalls will be commented upon which easily can lead to errors invalidating proper identification and adequate quantitation of amino acids.

THE SAMPLE

Indeed, it is important to decide, when to take the sample, how to perform sampling and what kind of sample will be used for the analyses (1). A common misconception manifests itself that the amino acid concentrations in serum are equal with those of plasma (2, 3). Unfortunately the majority of free amino acid determinations with HPLC is performed in serum (4-8). However, as repeatedly reported, most of the free amino acid concentrations in serum are not only considerably higher than those in plasma, but in real extent they exhibit substantial variations compared with those measured in plasma (2, 3).

SAMPLE HANDLING

Adequate deproteinization is one of the most important factors, unfortunately often neglected. The best recommendation is to use 5-sulfosalicylic acid (9, 10). Another popular deproteinization is the so-called picric acid method (11). This method suffers, however, from the disadvantage that the recovery of tryptophane, aspartic acid and citrulline and to some extent glutamic acid, hydroxy-proline and proline is not complete (12, 14).

It is known that precipitation with PCA, TCA and organic solvents such as acetonitrile and methanol may result in proteolysis, and/or incomplete extraction (15, 16). Thus, it is not surprising that the use of such precipitation methods has

resulted in concentrations either highly exceeding (6, 8, 17) or far below (5, 7) the true physiological values.

There are several reports available showing that glutamic acid and aspartic acid rise slowly and glutamine and asparagine fall equivalently in samples stored at -20° C. Additionally, losses of tryptophane and cystine are reported. These changes are minimized by storage at -70° C at which temperature protein free filtrate samples can be stored over a year period, without appreciable alterations of amino acid concentrations (9, 12, 18, 19). It might be remembered that the same considerations must be made when analyzing amino acids with an automated device. Thus to avoid loss of glutamine and asparagine one must keep sample-injectors at a temperature range of +4 - +6°C.

IDENTIFICATION AND QUANTITATION

A considerable problem seems to regard proper identification of free amino acids in biological fluids based on a corresponding standard run. It is remarkable that in a recent work the most important free amino acid in serum, glutamine could not be detected and other amino acids known to be present in measurable amounts (1-MeHis, 3-MeHis, ADA) could not be recovered. Rather more striking is the fact that the authors were not able to identify and recover the major urinary amino acid constituents, carnosine and 3-MeHis, whereas certain other urinary amino acids are obviously wrongly identified (7).

Thus it might be concluded that despite the advanced basic technology currently achieved, which may enable proper application of HPLC in routine biological practice, the validity and value of this approach has not yet been adequately controlled and verified. Thus, the majority of HPLC applications dealing with biological samples obviously reports erroneous concentrations.

Rapid, sensitive and specific HPLC method for routine determination of tissue free amino acids

Our main intentions were to develop a routinely manageable HPLC method by considering first: various derivatization methhods, the mode of detection and the use of different columns; second: to optimize the analytical conditions, preferentially tailored for automated analyses of free amino acids in human plasma and tissue specimens, and third: to carefully control the validity of the results obtained.

As earlier reported excellent separation of 26 major physiological amino acids was obtained in biological samples (plasma, muscle and liver) in the lower pmol range. As derivatization agent OPA/3-mercaptopropionic acid was used and the detection was made with fluorescence. The separation was performed on a Superspher CH-8 column (4 µm) with a length of 250 x 4 mm eluting with a sodium-phosphate-buffer and acetonitrile gradient at a flow rate of 1.2 ml/min. The linearity of individual free amino acids was calculated at various concentrations (125 - 1000 pmol/ml) by considering the relative fluorescence of each amino acid. As shown in Fig. 1, the obtained linearity was good for all amino acids analyzed, irrespective of fluorescence yield. By using these conditions, the method facilitates precise measurement of free amino acids in human biological tissues in less than 40 min. A comparison between HPLC amino acid results and those acquired from the conventional amino acid analyzer for the same samples is illustrated in Fig. 2. As shown the HPLC results compared favourably with those obtained with the conventional amino acid analyzer. The actual correlation between the two methods was highly significant ($r = 0.97$, $p < 0.001$) in 120 comparative analyses (20).

In a current work this method (20) has been developed to an ultra rapid and sensitive HPLC-system for measuring individual free amino acids in biological fluids by using similar derivatization conditions by employing shorter columns (125 x x 4.6 mm) with 3 µm particle size at an enhanced flow-rate and steeper gradient slope. These new conditions facilitate separation of the 23 major tissue free physiological amino acids in

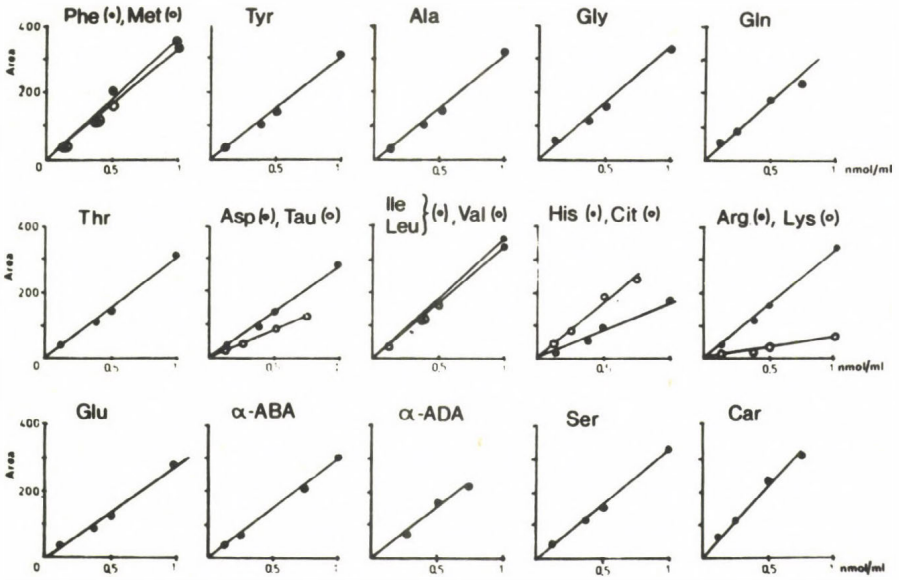


Fig. 1. The linearity obtained for 21 amino acids in the concentration range of 125-1000 pmol/ml (injection volume 20 µl). (Reprinted from Godel et al (20) with permission)

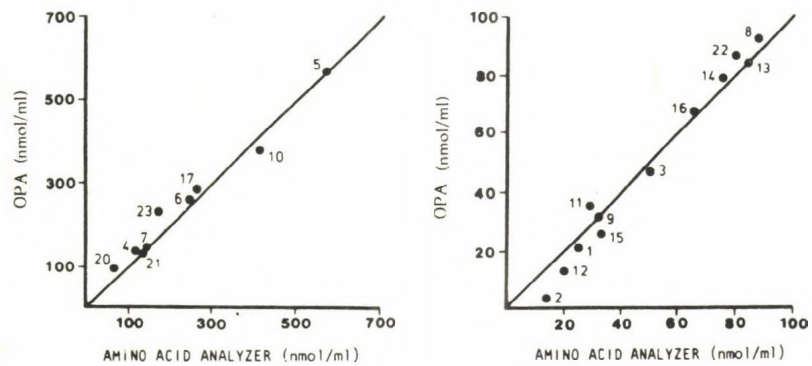
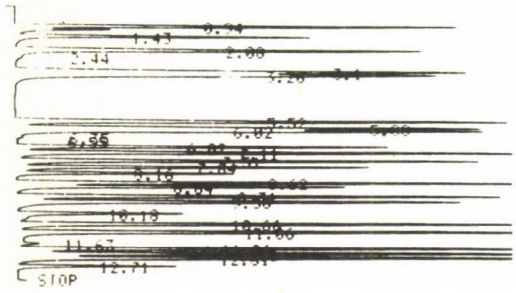


Fig. 2. Comparison between results derived from OPA-method by HPLC and a conventional amino acid analyzer (ninhydrin method).
Key: 1 Glu, 2 aAda, 3 Arg, 3 Ser, 5 Gln, 6 Gly, 7 Thr, 8 His, 9 Cit, 10 Ala, 11 2-Me-His, 12 3-Me-His, 13 Arg, 14 Tau, 15 aAba, 16 Tyr, 17 Val, 18 Met, 19 Ile, 20 Phe, 21 Leu, 22 Orn, 23 Lys
(Reprinted from Godel et al (20) with permission)



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METHOD 41

#	NAME	TIME	CONC	MK	AREA
0	Asp	0.94	4.4368		198057
0	Glu	1.43	2.231	V	99591
0	α-AAA	2.08	4.3883		195890
0	Asn	2.1	4.7753		213166
0	Ser	2.26	5.327	V	237793
0	Gln	5.52	5.1985		232058
0	Gly	5.88	4.4819	V	200067
0	Thr	6.02	5.0063	V	223475
0	His	6.82	3.4899	V	155789
0	Cit	7.11	5.149	V	229848
0	Ala	7.51	4.4696	V	199519
0	Car	7.84	3.4122	V	152317
0	Ala-Gln	8.16	1.9203	V	85722
0	Arg	8.62	4.6811	V	208961
0	3-Me His	8.84	3.3369	V	148956
0	α-ABA	9.31	4.5774	V	204330
0	Tyr	9.58	4.6922	V	209457
0	NH_3	10.18	1.6727	V	74668
0	Val	10.66	4.99	V	222751
0	Met	11.06	5.1659	V	230603
0	-	11.63	0.3108	V	13874
0	Ile	11.86	4.8605	V	216970
0	Phe	12.1	4.9223	V	219728
0	Leu	12.31	5.1502	V	229901
0	Lys	12.71	1.3525	V	60376
	TOTAL		100.		4463875

Fig. 3. Standard elution profile of 23 major free physiological amino acids (Spherisorb ODS II, 3 µm, 125 x 4.6 mm flow-rate 1.5 ml/min). Each peak represents 30 pmoles (21).
(Reprinted from Graser et al (21) with permission)

the lower pmol range in less than 13 min, as shown in Fig. 3 (21). Muscle, plasma and liver free amino acid concentrations (Fig. 4), as determined by HPLC, are in the expected physiological range (21) and compare favourably with those obtained by conventional amino acid analyzer (22, 23).

The reproducibility of the method, calculated as the deviation in a single determination from the mean fluorescence yield, ranged between 4.2 and 6.8%, whereas the reliability of the method ranged between 1.6 and 6%. The error of the method

in a single determination based on duplicates ranged between 4.5 and 8.2%, whereas in duplicate determination it was estimated to yield between 3.2 and 6.1%. In recent ongoing studies the error of the method could be considerably improved by using automated on-line derivatization and subsequent injection (to be published).

One may thus conclude that the use of OPA/3-MPA method has discernible advantages such as high sensitivity, feasibility of automated on-line derivatization and short analysis time. However, one has to keep in mind that the instability of the OPA adducts requests great care in standardizing the entire analytical procedure. Moreover, as known, derivatization with OPA cannot be used to detect proline, hydroxy-proline and other secondary amines.

We are in progress to use an alternative derivatization agent - 9-fluorenylmethylchloroformate (FMOC) - enabling the detection of secondary amines, especially those of proline and OH-proline (24). The derivatization with FMOC requires manual extraction with pentane, whereas the injection of the sample in the present work was automated. FMOC reacts with amino acids beneficially in sodium-phosphate-buffer at pH 7.7 within 45 sec. Immediately after derivatization, the reaction mixture must be repeatedly (6-fold) extracted with 1 ml of pentane in order to minimize the formation of a FMOC-hydrolysis product. Excellent separation of 23 physiological amino acids including proline was obtained in standard runs and biological material (human plasma) as exemplified in Figs 5 and 6, respectively. The separation was performed on a LiChroCART Superspher CH-8 column (4 µm) with a length of 125 x 4 mm, capped with a guard column 10 x 4 mm (7 µm particle size). The elution was carried out with a sodium-acetate-buffer/ acetonitrile gradient at a flow-rate of 1.5 ml/min. By using these conditions, the method facilitates precise measurement of free amino acids in less than 25 min.

One may conclude that the superior sensitivity, stability and the feasibility to detect secondary amines (Pro and HO-Pro) favour the FMOC method against the OPA-method, whereas the labourous extraction procedure is to be rendered as a clear

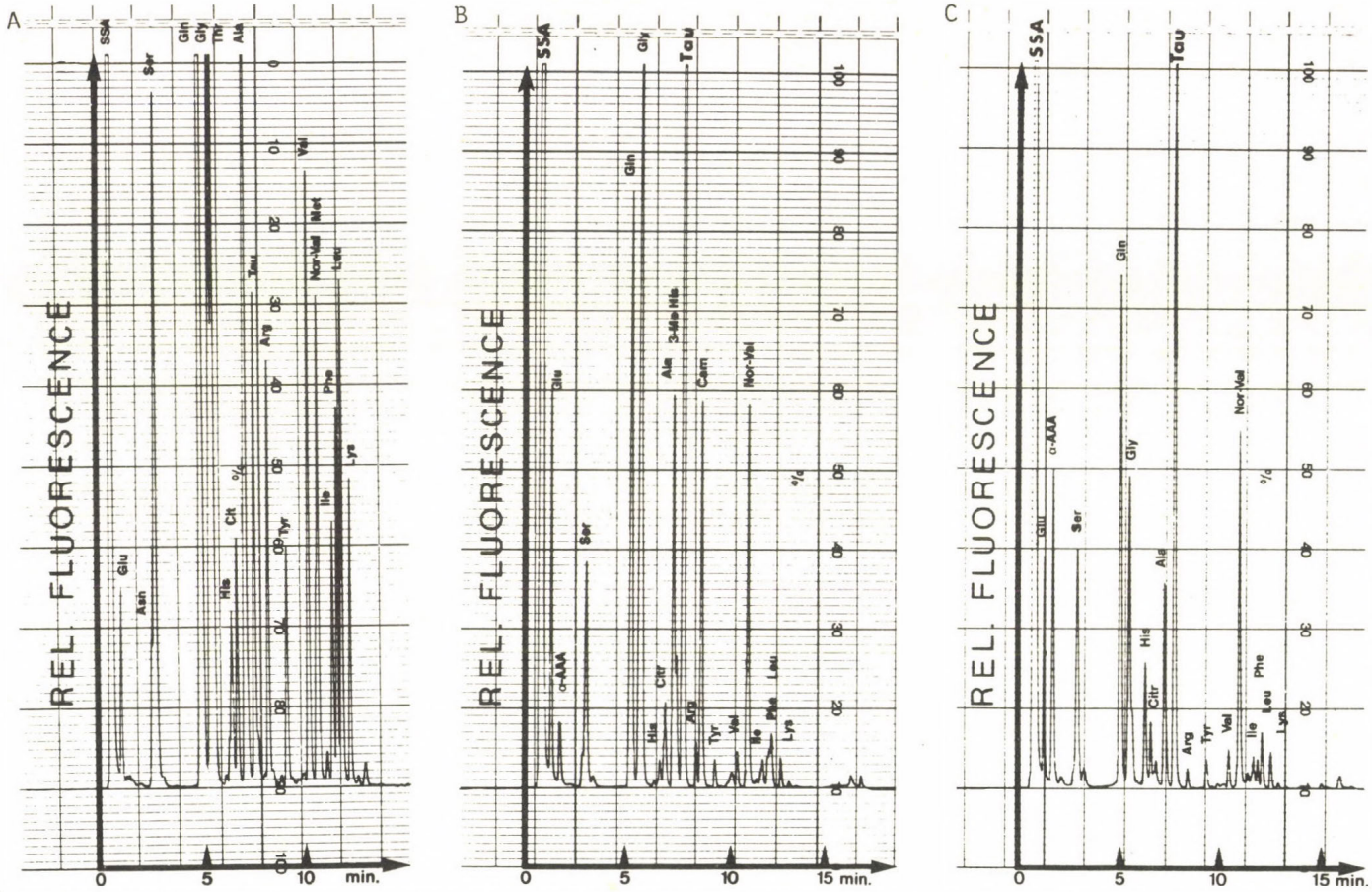

Fig. 4. Elution profiles of OPA-derivatized amino acids of biological samples from rat plasma (A), rat muscle (B) and rat liver (C). Analytical conditions see Fig.3. (Reprinted from Graser et al. (21) with permission)

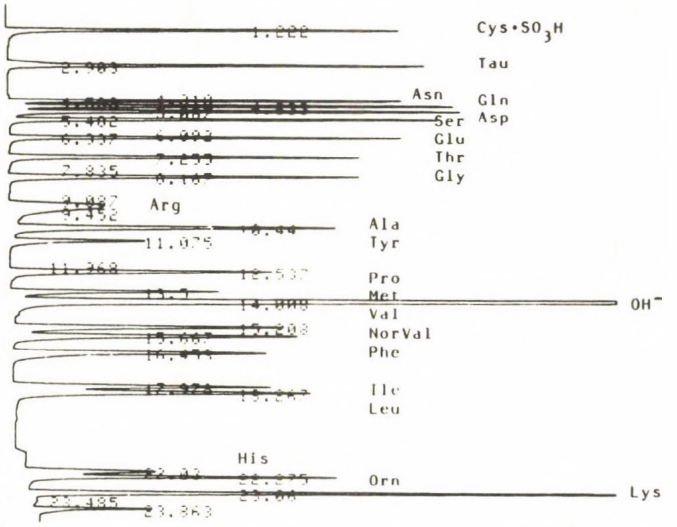

Fig. 5. Standard elution profile of 23 major free physiological FMOC-derivatized amino acids including proline (LiChro-CART Superspher CH-8 column 125 x 4 mm; flow-rate 1.5 ml/min). Each peak represents 10 pmols

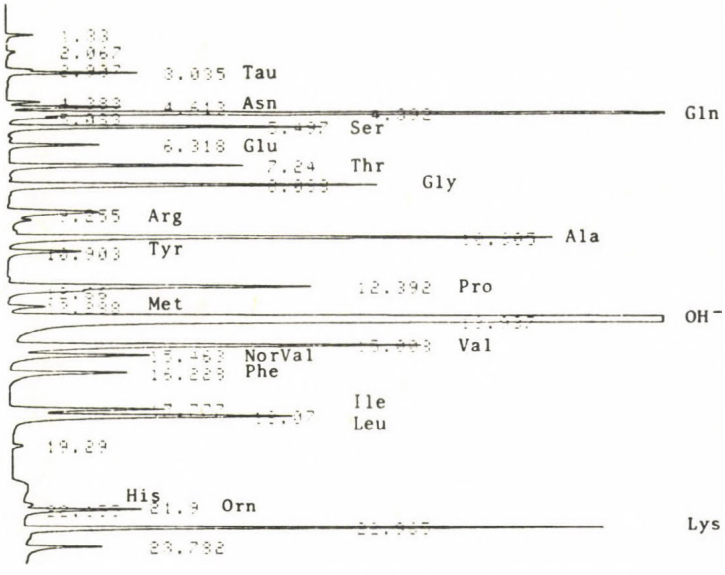

Fig. 6. Elution profile of FMOC-derivatized amino acids in human plasma. Analytical conditions see Fig. 5

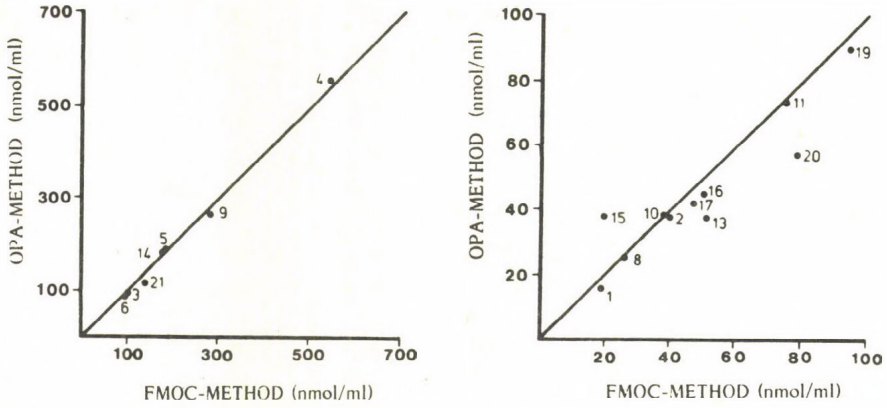

Fig. 7. Comparison between HPLC results derived from OPA-method and FMOC-method.
Key: 1 Glu, 2 Asp, 3 Ser, 4 Gln, 5 Gly, 6 Thr, 8 Cit, 9 Ala, 10 Tau, 11 Arg, 13 Tyr, 14 Val, 15 Met, 16 Ile, 17 Phe, 19 Leu, 20 Orn, 21 Lys.
Pro could not be elected by OPA method, whereas His, Cys and Trp could not be detected by FMOC-method

disadvantage. The combined error of the method based on duplicates ranged between 2-6%. A comparison of the results acquired from the OPA- and the FMOC methods shown in Fig. 7 illustrats the excellent agreement between the two analytical systems.

As an overall conclusion our results indicate that HPLC provides a powerful tool in the study of amino acid metabolism. There are factors, however, which make the blind approval of uncontrolled results dangerous thus the adequate use of the method requires that the results obtained should be accepted with caution.

ACKNOWLEDGEMENTS

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SEPARATION OF ACIDIC AMINO COMPOUNDS USING GUARD COLUMNS IN ION-EXCHANGE CHROMATOGRAPHY

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INTRODUCTION

Samples from biological material, animal brain tissue extracts in particular, contain a number of acidic amino compounds: amides, amines, peptides and their derivatives, which are only poorly separable in ion-exchange chromatography. Since highly ionized moieties (carboxyl-, sulfonyl- or phosphoryl-groups) are their main chromatographic determinants, different compounds elute in the same fractions (1 - 3). For example, Mangan and Whittaker (4) found at the beginning of the chromatograms, in addition to glycerophosphocholine, glycerophosphoethanolamine and phosphocholine, 18 other unidentified non-phosphorus-containing ninhydrin-positive substances in guinea-pig brain subcellular fractions. Some of these were presumed to be acidic peptides. Recently, we have identified from the brain with mass spectrometry and amino acid analysis after thin-layer chromatography and various hydrolytic and derivatization methods several small acidic taurine-containing peptides (5 - 9). In this connection we also tried to refine amino

acid analyzer methods to separate these peptides from each others and from other interfering acidic compounds.

Traditional amino acid analysis, based on ion-exchange chromatography, has very slowly adopted recent advances made in liquid chromatography. A guard column, inserted between the sampling valve and the analytical column, has only with the latest technology become applicable, though it effectively can protect the analytical column against damage by trapping highly retentive substances and particles which reduce column performance. A guard column with the same stationary phase as the analytical column has generally been recommended. However, any dissimilar separation principle might prove advantageous in enhancing the selectivity of substances. This is particularly desirable with sensitive detection of important compounds in trace amounts.

MATERIAL AND METHODS

In order to obtain an enhanced selective separation in the area of acidic amino compounds, Brownlee Labs, Inc., standard guard cartridges (Alumina and Silica adsorption columns, RP-18 and RP-300 reverse phase columns, 4.6 mm id x 3 cm with 5 and 10 µm sorbants) were used in front of the 20 cm long Kontron AS70 ion-exchange column kept at 313 K in a Kontron Chromakon 500 amino acid analyzer. The elution buffer was the first lithium citrate buffer, pH 2.6 (5.05 g LiOH x H_2O, Merck, 17.4 g citric acid x H_2O, Merck, 70 ml methanol, Merck 9.9 ml HCl Suprapur, Merck, in 1 liter of distilled

water with 2 ml Brij 35, Sigma, added after filtration through a Millipore 0.22 µm filter). The detection was the fluorescence method after the reaction with o-phthalaldehyde according to the instrument manual.

The following peptides used in the present study were synthesized by us: α-glutamyl-taurine, γ-glutamyl-taurine, β-alanyl-taurine, γ-aminobutyryl-taurine, β-aspartyl-N-methyl-taurine and α,γ-glutamyl-ditaurine. In addition, only commercial reference substances were used as 50 µmol/l solutions in various combinations and they were obtained from Fluka, Merck, Sigma and Vega-Fox Biochemicals.

RESULTS AND DISCUSSION

Cysteate derivatives

The first compounds eluting from biological samples in an amino acid analyzer are carbamylphosphate, an intermediate of the urea cycle, and cysteate, homocysteate and cysteinesulfinate. Cysteate and cysteinesulfinate are the major intermediates in taurine biosynthesis from cysteine. Pasantes-Morales et al. (10) have shown their presence also in brain extracts with enzymatic determination. The simultaneous determination of cysteate and cysteinesulfinate, separately, can be done with high-performance liquid chromatography (11) and with adsorption chromatography (3). None of the four guard columns used in the present study improved significantly their separation from each others.

Phosphohydroxy amino compounds

O-Phosphates are the major class of phosphorylated amino compounds in proteins (12). They include phosphoserine, phosphothreonine and phosphotyrosine. In addition to two-dimensional thin-layer electrochromatography (13) and paper electrophoresis (14), which fail to give quantitative results, nigh-performance liquid chromatography (15) has been applied to determination of these phosphohydroxy amino acids. Also ion-exchange chromatography has been used (16-18). Phosphoserine is the compound eluting first in most commercial standard reference mixtures. Phosphothreonine has the same retention time, but phosphotyrosine is eluted somewhat later. Glucosaminephosphate also belongs to this group and it normally comes out shortly after phosphoserine.

According to Martensen and Levine (19) lowering of the pH of the elution buffer to 2.0 resulted in somewhat improved separation. The guard columns tested here did not affect the separation of phosphoserine from phosphothreonine. However, most impressive results were achieved with the lithium citrate buffer, pH 2.6, used throughout this study, and the Alumina type guard column, which retained all of these phosphohydroxy amino compounds. In the case of overlapping substances, such as phosphoserine, phosphothreonine and γ-glutamyl-taurine, it is advantageous to bind interfering phosphohydroxy compounds to the Alumina guard column and quantitate thereafter γ-glutamyl-taurine.

Substances interfering with taurine determination

The complexity of the taurine fraction in automated amino acid analysis was encountered in our systematic evaluation of free amino acids in bovine brain synaptosomes and synaptic vesicles (20). The peak with the retention time of authentic taurine has also been found by other investigators to be contaminated with urea (21), glycerophosphoethanolamine (4,22,23), and taurine-containing peptides (3).

James (21) added ethanol to the samples after precipitation of proteins with sulfosalicylic acid, in order to improve the separation of taurine from urea. Although the fluorescence of urea is very low, it is a source of error in taurine determination. Erbersdobler et al. (24) have suggested a decreased elution temperature and optimization of the buffers to improve the separation of taurine from phosphoethanolamine, glycerophosphoethanolamine and urea. Only a lowering of the elution temperature to the ambient one did not improve the separation in our system, but the combination to another conventional practice, viz. to the usage of a 5 cm longer column, a good separation was achieved (Figure 1). Gurusiddaiah and Bromser (25) found that in three from four amino acid analysis systems checked by them taurine and glycerophosphoethanolamine did not coelute. They also suggested a Na^+ system for analysis of taurine in brain and an accelerated Li^+ system for the analysis of the other amino acids. With this praxis the resulting taurine value is still erroneously high - whether the sample is hydrolyzed or

not - because of the presence of small acidic taurine-containing peptides (6-9, 26, 27).

The synthetic taurine-containing peptides tested here could be separated by two-dimensional thin-layer chromatography (Figure 2) using 0.25 mm thick silica gel (Kieselgel G) plates and 70 % (v/v) ethanol (I): 75 % (w/v) phenol (II) in water. In the amino acid analysis some peptides were always eluted together and only three peaks were obtained (Figure 3). This remained the general picture also with

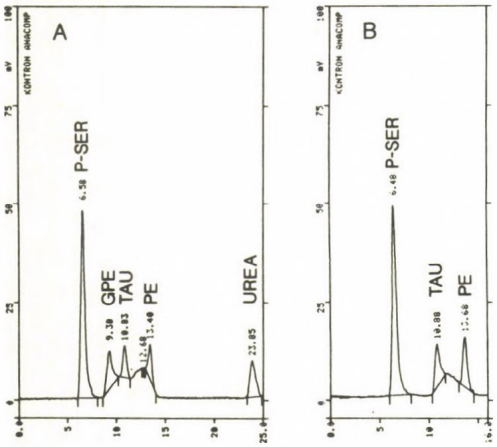


Figure 1. Analysis of phosphoserine (P-SER), glycerophosphoethanolamine (GPE), taurine (TAU), phosphoethanolamine (PE) and urea in Kontron Chromakon 500 automatic amino acid analyzer using a longer column (25 cm instead of the 20 cm column) at ambient temperature.

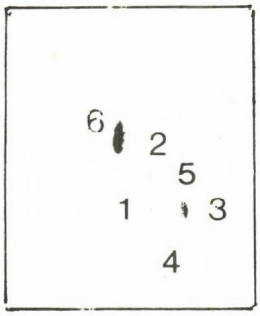


Figure 2. Analysis of taurine and synthetic taurine-containing peptides with two-dimensional thin-layer chromatography using 0.25 mm thick silica gel plates and 70 % (v/v) ethanol (I): 75 % (w/v) phenol (II) in water. 1 = taurine (I:II = 0.54:0.19), 2 = γ-glutamyl-taurine (0.72:0.17), 3 = γ-aminobutyryl-taurine (0.54:0.26), 4 = β-alanyl-taurine (0.49:0.25), 5 = β-aspartyl-N-methyltaurine (0.58:0.23), 6 = α,γ-glutamyl-ditaurine (0.69:0.13). α-Glutamyl-taurine reacted very poorly with ninhydrin, hence it is not visible.

Figure 3. Separation of a mixture of taurine and synthetic taurine-containing peptides in an automatic amino acid analyzer with Kontron AS70 resin as three peaks. 1 = γ-glutamyl-taurine and β-aspartyl-N-methyltaurine, 2 = taurine, γ-aminobutyryltaurine, β-alanyl-taurine and α,γ-glutamyl-ditaurine, 3 = α-glutamyl-taurine.

different guard columns. The acidic groups in these peptides determined their elution order. The pK-values of the carboxyl groups in glutamate are 2.19 and 4.25 (28). The two possible configurations of glutamyl-taurine possess entirely different retention times, viz. 4.6 and 107.1 min.

Hypotaurine and aspartylglucosamines

Hypotaurine (30 µmol/kg wet weight in mouse brain; 29), the immediate precursor of taurine, is completely overlapped in many amino acid analyzers by the large peak of urea (7 mmol/kg wet weight in mouse brain; 3). On the other hand, urea co-elutes with aspartylglucosamines with some other resins (30). Aspartylglucosamines are abundant in aspartylglucosaminuria, an autosomal, recessively inherited inborn error of metabolism predominantly occuring in Finnish population (31,32). They can be hydrolyzed away and then the small peak of hypotaurine can be analyzed alone (3). For qualitative purposes the separation can also be done with thin-layer chromatography with subsequent scraping off and extraction of the zone containing glycoasparagines (30). The guard columns tested in this study affected particularly the retention time of hypotaurine improving its separation from the reference substance 2-acetamido-1-N-(4'-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine. Thus, with the resin Kontron AS70 combined with any of the guard columns tested here, it should be possible to quantitate without prior extra purification the amount of aspartylglucosamines eluting in this peak.

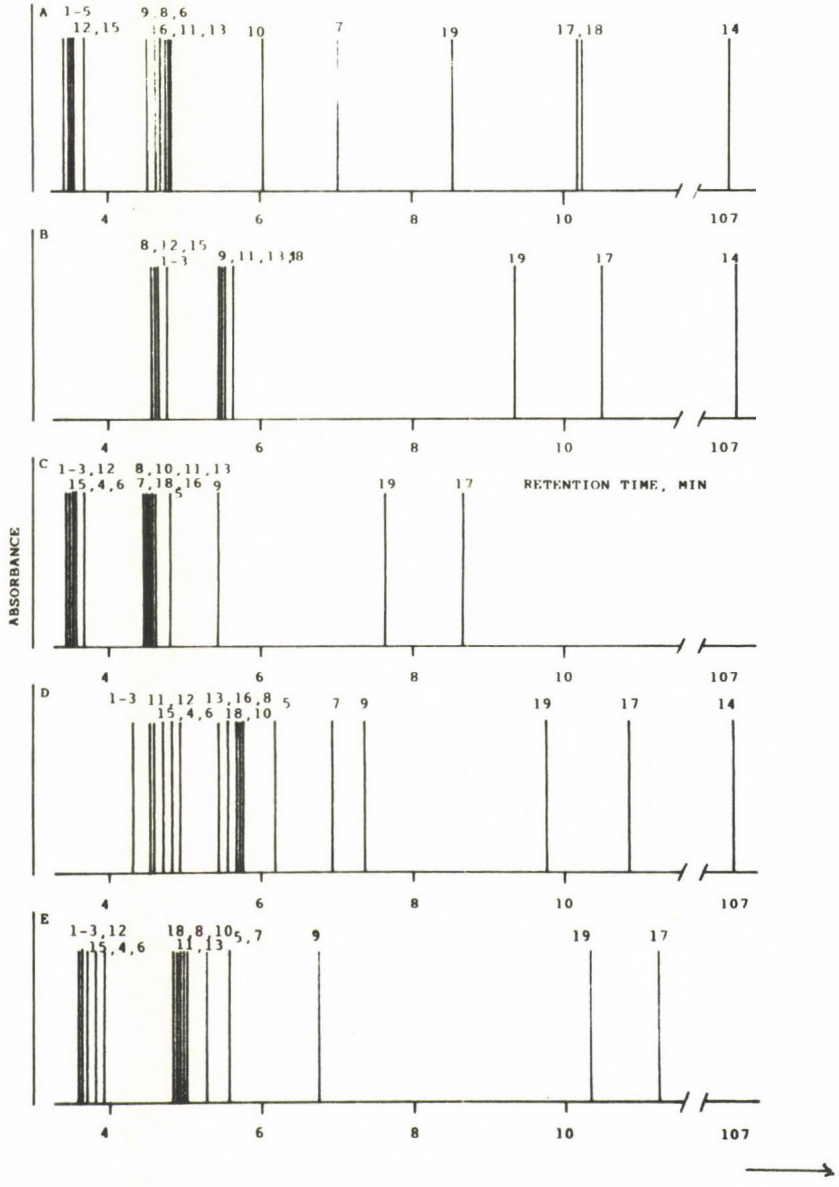
CONCLUDING REMARKS

The present results are compiled in Table 1 and Figure 4. The most important finding is probably the retention of all the phosphohydroxy amino compounds by Alumina-type guard column. Two of these, phosphoserine and phosphothreonine, are co-eluted with γ-glutamyl-taurine. After their removal the amount of γ-glutamyl-taurine can be determined. β-Aspartyl-N-methyltaurine has not been detected in brain samples so far. Fluorescence detection with increased sensitivity is

Table 1. Effects of various guard columns on ion-exchange chromatographic separation of acidic amino compounds

Compounds	Guard column type			
	Alumina	Silica	RP-18	RP-300
Cysteate derivatives	0	0	(+)	0
O-Phosphate group	+	-	+	(+)
Taurine group	-	-	+	0
Hypotaurine & glycoasparagines	+	+	+	+

0 no change
(+) slight improvement
\+ improvement
\- decrease


A
1-5
12,15
9,8,6
16,11,13
10
7
19
17,18
14
4
6
8
10
107
B
8,12,15
1-3
9,11,13,18
19
17
14
4
6
8
10
107
C
1-3,12
15,4,6
8,10,11,13
7,18,16
5
9
19
17
RETENTION TIME, MIN
ABSORBANCE
4
6
8
10
107
D
1-3
11,12
13,16,8
15,4,6
18,10
5
7
9
19
17
14
4
6
8
10
107
E
1-3,12
15,4,6
18,8,10
11,13
5,7
9
19
17
4
6
8
10
107

particularly applicable to these determinations. The results can also be used with benefit with amino acid analyzers equipped with a stream divider to collect the separate fractions in the elution order.

The silica-based quard column did not generally affect the separation very much. Slight positive effects could be obtained using the reverse phase guard column RP-18 which is silica with a covalently bond short alkyl chain. Its separation is based on differences in the hydrophobicity of samples, and in lieu of amino acid analyzers using water-based buffers does not show its full power. The hydrophilic components pass through the reverse phase column with little if any retention. However, the peaks ranging from cysteate to phosphoethanolamine could be dispersed as function of retention time using RP-18 (Figure 4). A longer column would probably still enhance this effect.

←———

Figure 4. Relative retention times of various acidic amino compounds in an automatic amino acid analyzed with Kontron AS70 resin (A), and with different guard columns: Alumina (B), Silica (C), RP-18 (D) and RP-300 (E). 1 = cysteate, 2 = cysteinesulfinate, 3 = homocysteate, 4 = phosphoserine, 5 = phosphothreonine, 6 = phosphotyrosine, 7 = glucosamine-phosphate, 8 = taurine, 9 = glycerophosphoethanolamine, 10 = phosphoethanolamine, 11 = β-alanyl-taurine, 12 = β-aspartyl-N-methyltaurine, 13 = γ-aminobutyryl-taurine, 14 = α-glutamyltaurine, 15 = γ-glutamyl-taurine, 16 = α,γ-glutamyl-ditaurine, 17 = hypotaurine, 18 = urea, 19 = aspartylglucosamines

When using a guard column in front of the analytical ion-exchange column the choice of the resin should be carefully considered. An identical resin can be used if no changes in separation are desired. If increased selectivity in separation is endeavoured, Alumina, Silica, RP-18 or RP-300 may be considered, in particular, when accurate and precise quantitative estimation assumes paramount importance.

Acknowledgements

We thank the Hungarian Academy of Sciences and the Academy of Finland for exchange scholarships for K.-M.M. and P.L. Furthermore, we thank Mrs. Raija Repo and Miss Leena Vuolas for skilful technical assistance, Mr. Ari-Pekka Viirtelä for preparation of the illustrations and Miss Eija Kyrölä for secretarial help.

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Chromatography '85
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N^G-HYDROXYMETHYL-L-ARGININES: NEW DISCOVERED SERUM AND URINE COMPONENTS AND THEIR CHARACTERISATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In biological systems endogenous formaldehyde can react with L-arginine and, due to this interaction, different N^G-hydroxmethyl derivatives can be produced. The serum and urine samples free from proteins were dissolved in distilled water and were applied to a Sephadex G-15 column equilibrated with distilled water. The combined and dried fractions were dissolved in distilled water and an aliquot of this solution was injected into a modified normal-phase HPLC column. Ethyl acetate was used as the eluent. The separation parameters of hydroxymethyl-L-arginines were determined.

INTRODUCTION

In biological systems a "pool" of C_1 methyl groups has a basic significance. Formation of endogenous formaldehyde, which can have a purely chemical, enzymatically not supported, methylating effect, can also take place from nitrosamines. This reaction is catalysed by N-demethylase /1, 2/. Environmental factors like viral infections also have an influence on the in vivo formation of formaldehyde /3/. Endogenous releasable formaldehyde is known to occur at low concentrations in biological systems. Thus, in normal human blood 0.4 - 0.6 µg/ml and in urine 2.8 - 4.0 µg/ml have been detected with ^{14}C-dimedone reagent. However, significant deviations have been observed in different tumorous conditions, with values ranging from 8-10-fold higher to 10-fold lower than the reported normal formaldehyde concentration /4/. Recent results on the carcinogenicity

of formaldehyde /5/ suggest that formaldehyde might contribute to the carcinogenic action of dimethylnitrosamine. A thiazolidine derivative is formed when formaldehyde reacts in vivo with cysteine /6/. A thiazolidine derivative has also been implicated in the biological reactions of aldehyde, as cysteine is reported to alleviate the toxicity of aldehyde in experimental animals /7/. A significant increase in urinary alkali-hydrolysable thiols has been observed after administration of aldehyde to experimental animals /8/. There seems to be a mechanism that prevents the accumulation of endogenous formaldehyde. This mechanism works so that the undesirable surplus of formaldehyde should react with some formaldehyde acceptor and is eliminated after having been structurally changed due to this interaction. Our experiment has proven that in this respect first arginine and then certain mobile compounds, e.g. cysteine or glutathione containing thiol groups, and the formaldehyde-reducing ascorbic acid should be taken into consideration /9/. The guanidine groups of arginine react rapidly with formaldehyde. The product of this interaction is the hydroxymethyl derivative of arginine. The rate of this reaction is higher than that of any other reaction of formaldehyde taking place in biological systems. The rate constant was 0.035 min^{-1}. It was proven that hydroxymethylation takes place first on the imino part and then on the primary amino group of the guanidino group. The hydroxymethyl structure was proven by NMR-spectroscopy /10/.

We have isolated the hydroxymethyl-L-arginines from human serum and urine.

EXPERIMENTAL

Materials and Methods

The standard amino acids, L-arginine (Arg), $N^{G}N^{G}$-dihydroxymethyl-L-arginine (DHMA), N^{G}-monohydroxymethyl-L-arginine (MHMA) and $N^{G}N^{G},N'^{G}$-trihydroxymethyl-L-arginine (THMA) were purchased from Reanal, Budapest, Hungary, and were prepared according to our method described earlier /10/. Sephadex G-15 and

Visking dialysis tubing 20/32 were purchased from Pharmacia, Uppsala, Sweden, and from Serva, Heidelberg, F.R.G., respectively. A Liquochrom 2010 (Labor MIM, Hungary) liquid chromatograph was used in our work; OE 320 Labor MIM injector fitted with a 20 µl sample loop was used for injection. The separation was carried out on a 25 cm x 4 mm I.D. column packed with modified Chromspher-Sil (Labor MIM) normal silica (10 µm). The modification of the column consisted of the production of a covalent bond between the silicate and stearic acid with the help of carbodiimidazole. The new semi-nonpolar system was used for the separation of the antibiotics /11/. All other materials were analytical grade, and were obtained from Reanal, Budapest, Hungary.

Analysis of serum and urine

Human serum and urine samples from five normal adults were collected individually. 20.0 ml of serum, or urine was equilibrium dialysed against 200 ml of distilled water. The protein-free solution was evaporated to dryness at room temperature. The rest was dissolved in 3 ml of distilled water, applied to a Sephadex G-15 column equilibrated with distilled water and eluted with distilled water.

The column was calibrated with the preparation of hydroxymethyl-L-arginines. On the basis of calibration the corresponding fractions were combined and dried in vacuum at room temperature.

The rest was dissolved in 1.0 ml of distilled water and an 20 µl aliquot of this solution was injected into the HPLC column. The sample contains the hydroxymethyl-L-arginines from 0.4 ml biological fluid.

The Chromatographic parameters were:

Eluent: ethyl acetate (Lichrosolv^{R} - Merck)

Absorption wavelength: 265 nm

Absorbance unit: 0.02 a.u.f.s.

Flow rate: 0.8 ml/min

Temperature: 25^{o}C.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical chromatogram of the standard hydroxymethyl-L-arginine mixture.

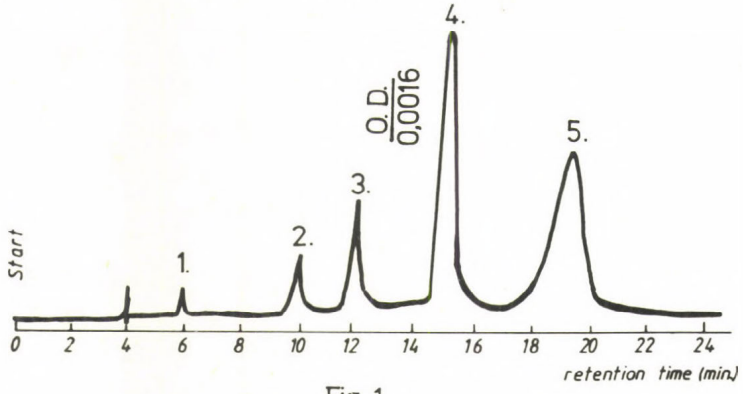

Fig. 1.

Fig. 1. Chromatogram of a N^G-hydroxymethyl-L-arginine standard solution (concentration: 10 µg/ml). Column, 25 cm x 4 mm ID Chromspher-Sil (Labor MIM), covered by stearic acid; eluent, ethyl acetate (Lichrosolv^R-Merck); flow rate, 0.8 ml/min; detection at 265 nm; a.u.f.s. = 0.02. Peaks: 1 unidentified L-arginine derivative; 2 trihydroxymethyl-L-arginine; 3 dihydroxymethyl-L-arginine; 4 L-arginine; 5 monohydroxymethyl-L-arginine.

Table I shows the separation parameters of a standard hydroxymethyl-L-arginine mixture measured in the specified HPLC system.

Five urine samples contained 14.0 $\pm$ 5.0 µg/ml (≈14 mg/day) L -arginine, 4.5 $\pm$ 0.9 µg/ml (≈4.5 mg/day) MHMA, 2.6 $\pm$ 1.1 µg/ml (≈2.6 mg/day) DHMA and zero THMA.

Five serum samples contained 15.5 $\pm$ 4.5 µg/ml L-arginine, 2.6 $\pm$ 0.7 µg/ml MHMA, 3.7 $\pm$ 0.5 µg/ml DHMA and 1.0 $\pm$ 0.5 µg/ml THMA.

Formaldehyde, mobilized in the form of hydroxymethylated arginine which is present in both blood and urine, can have access to cell components it could not attain spontaneously. In this respect methylol-arginine can be regarded as a formaldehyde donor. The hydroxymethyl derivative of arginine can thus bring formaldehyde into a biologically effective state (biophase) which it could not otherwise reach, being hindered by

Table I. Capacity factor (k'), separation factor (α) and relative separation factor (resolution, R_s) values for hydroxymethyl-L-arginines on modified semi-nonpolar silica packings
Eluent: ethyl acetate (Lichrosolv^R-Merck); flow rate: 0.8 ml/min.

Substance	k'	α	R_s
Unidentified L-arginine derivative	0.50		
Trihydroxymethyl-L-arginine (THMA)	1.50	3.0	10.7
Dihydroxymethyl-L-arginine (DHMA)	2.00	4.0	12.0
L-arginine	2.75	5.5	13.3
Monohydroxymethyl-L-arginine (MHMA)	3.75	7.5	10.6

various barriers. Under a given ionic strength and pH conditions, the methylol derivative is likely to be in equilibrium with the concentration of the intact arginine and free formaldehyde in the system. In our model, the endogenous or exogenous accumulation of arginine in a biological system would result in a decrease of the free endogenous formaldehyde concentration or in an increase of the methylol-derivative of arginine. A decrease of arginine would cause the amount of free endogenous formaldehyde to increase or that of the methylol derivative to decrease.

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Chromatography '85
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HPLC STUDY OF BINDING OF L-TRYPTOPHAN DERIVATIVES TO BOVINE SERUM ALBUMIN

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Abstract

Size-exclusion chromatography was carried out at room temperature for the identification of binding from non-binding; and, if there was binding, for the determination of binding constants. Two techniques were developed: internal calibration and external calibration. The systems chosen for study were the binding of L-tryptophan methyl ester and of 5-hydroxy-L-tryptophan to bovine serum albumin. However, only L-tryptophan methyl ester was investigated in detail and the data plotted in the form of scatchard graph. Unlike L-tryptophan, its derivative, L-tryptophan methyl ester, does not give a straight line, indicating that bovine serum albumin has more than one binding site. Curve fitting analysis yields the binding constants as follows: $k_1 = 6.0 \times 10^4$, $n_1 = 1$; $n_2k_2 = 4.0 \times 10^3$, $n_2 = 25$. **Due to the uncertainty in the measurement of the peak area (negative), the error is expected to be relatively large.**

INTRODUCTION

Based upon the classical method of gel filtration by Hummel and Dreyer[1] for the determination of the binding parameter $\bar{r}$ (the number of moles of ligand (small molecules) bound per total number of moles of protein (macromolecule)), we have developed two techniques in HPLC (high performance liquid

chromatography): internal calibration and external calibration. The two techniques may be characterized by two simple mathematical equations, respectively:

$$\bar{r} = \frac{L_b}{P} \qquad \text{(internal calibration)}$$

$$\bar{r} = \frac{[L]_b}{[P]} \qquad \text{(external calibration)}$$

Where L_b is the absolute amount of ligand in /umoles, P the absolute amount of protein, also in /umoles; [ ] refers to molar concentrations, and b in both equations refers to "bound". Our goal, as with other analytical methods, is to make the measurements accurate, simple and fast. Both techniques have already been tested on two different systems, the binding of warfarin (an anticoagulant drug) to serum albumins and that of L-tryptophan (an essential amino acid) to serum albumins[3]. The results were found to be highly satisfactory. This paper reports our efforts to extend the two techniques to the binding of L-tryptophan methyl ester to bovine serum albumin, and to describe qualitatively binding and non-binding elution profiles.

EXPERIMENTAL

Materials

L-tryptophan methyl ester (lot no. 32F-0327) was obtained from Sigma (St. Louis, Missouri, U.S.A.), 5-hydroxy-L-tryptophan (lot no. 15414) from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.), and bovine serum albumin (lot no. J-72212) from Metrix, Armour Pharmaceutical Co. (Chicago, Illinois, U.S.A.). All other chemical were reagent grade and were used as received. Glass distilled water was used in all experiments.

Preparation of Solutions

Solutions for the mobile phase and for injected samples were prepared according to the methods described in detail in the previous publications[2,3]

Chromatography

For size-exclusion chromatography the apparatus is usually very simple. We only used a Waters Assoc. pump A-6000, and injector UK-2, a UV variable wavelength detector and a recorder. The column used was Waters I-125 (30 cm x 7.8 mm I.D., particle size 37-53 /um).

RESULTS

Table I describes the preparation of protein samples to compare the two techniques. Samples A, B, C, D and E were prepared for internal calibration and F and G for external calibration.

Table I. Preparation of Protein Samples

Sample	Protein stock solution (160 /uM) /ul	Ligand solution (47 /uM) /ul	Phosphate buffer (0.05 M, pH 7.4) /ul
A	500	125	625
B	500	250	500
C	500	375	375
D	500	500	250
E	500	750	0
F	500	0	750
G	0	0	1250

It is now a well-established conclusion that, if a ligand is contained in the mobile phase, the appearance of two peaks (usually a positive peak followed by a negative peak) is an indication of the binding of ligand (small

molecules) to macromolecules. The first peak (e.g., the positive one) represents the complex, whereas the second peak (e.g., the negative one) represents the loss of free ligand. The area of the second peak is a measure of the amount of the ligand bound to the macromolecule. Figure 1 shows the elution profiles to compare binding with non-binding. Three experiments were run under identical conditions, i.e., same column, same flow rate, same wavelength and same a.u.f.s. The mobile phase was also the same: 9.4 μM 5-hydroxy-L-tryptophan in 0.05 M phosphate buffer, pH 7.4. In each experiment, 100 μl of sample was injected. The samples were: a. 0.03 M KCl, b. 66 μM BSA in 0.03 M KCl and c. 66 μM methylated BSA in 0.03 M KCl. As shown in Fig. 1, we found only one peak (negative) in methylated BSA (Fig. 1 c) similar to the profile in 0.03 M KCl (Fig. 1 a), whereas we found two peaks,

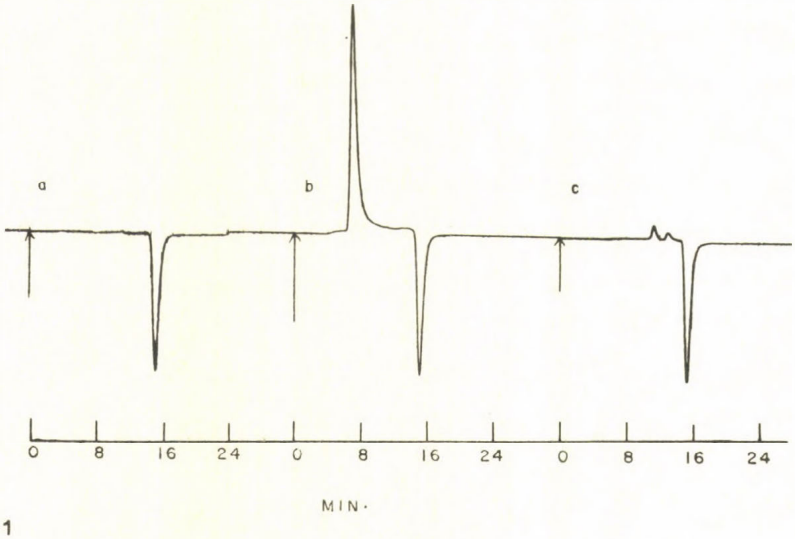

Fig. 1
Elution profiles of the binding or non-binding of the ligand to protein. Detector: UV at 280 nm, 0.4 a.u.f.s. Flow rate: 1.0 ml/min. Mobile phase: 9.4 μM 5-hydroxy-L-tryptophan in 0.03 M KCl aqueous solution. Samples: 100 μl of: a. 0.03 M KCl, b. BSA in 0.05 M sodium phosphate buffer, pH 7.4, and c. methylated BSA in 0.03 M KCl aqueous solution.

a positive peak followed by a negative peak in BSA. This unequivocally indicates that 5-hydroxy-L-tryptophan does not bind to methylated BSA. The removal of approximately 77 free carboxyl groups out of 100 from the protein results in the change in conformation[4], thereby preventing the binding of 5-hydroxy-L-tryptophan. Similar results were obtained when we tested with L-tryptophan, i.e. the ligand binds to BSA, but not to methylated BSA. (Chromatograms are not shown.)

In Figures 2 and 3 we give an example of the binding of L-tryptophan methyl ester to bovine serum albumin. Fig. 2 illustrates the method of internal calibration, whereas Fig. 3 illustrates the external calibration. Such an example was also shown in previous publications[2,3]. In comparison we found a small peak appearing in front of the second peak (negative) in the present case. This makes the second peak less symmetrical near the base line. Such kind of asymmetry was not found in warfarin binding and L-tryptophan binding. Both bovine serum albumin and L-tryptophan methyl ester (L-tryptophan too) are absorbed in the region around 280 nm. It obviously shows some interference in absorption of uncomplexed serum albumin on the L-tryptophan methyl ester. For the first approximation we treated the data in the present work in the same way as L-tryptophan or warfarin peak. The uncertainty in the measurement of the area is, of course, greater.

In Fig. 4 we present the binding data in a Scatchard plot. In contrast to the binding of L-tryptophan to serum albumins, the curve is not linear, indicating that there are more than one binding site in BSA for L-tryptophan methyl ester. The shape of the plot is in good agreement with that reported in literature[5] where the classical equilibrium dialysis method was employed. Our values of the ordinate, $\frac{\bar{r}}{[L]_f}$, are slightly higher than those in the literature, possibly due to the greater uncertainty in the measurement of the area of the second peak (negative).

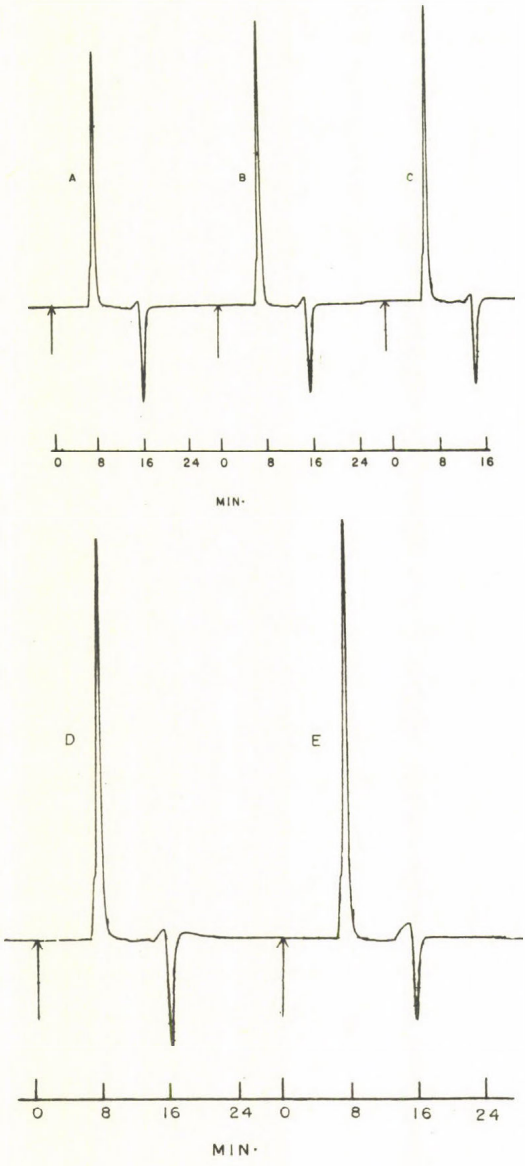

Fig. 2

Elution profiles of the binding of L-tryptophan methyl ester to BSA. Experimental condition same as in Fig. 1. Mobile phase: 28.3 μM L-tryptophan methyl ester in 0.05 M sodium phosphate buffer, pH 7.4. Samples: 100 μl of A, B, C, D, and E (see Table I).

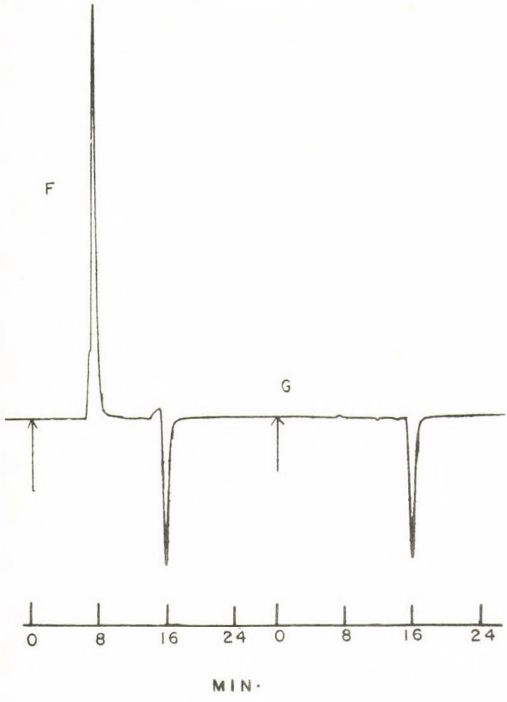

Fig. 3

Elution profiles of the binding of L-tryptophan methyl ester to BSA.

Experimental condition including the mobile phase same as in Fig. 2.

Samples: 100 µl of F and G.

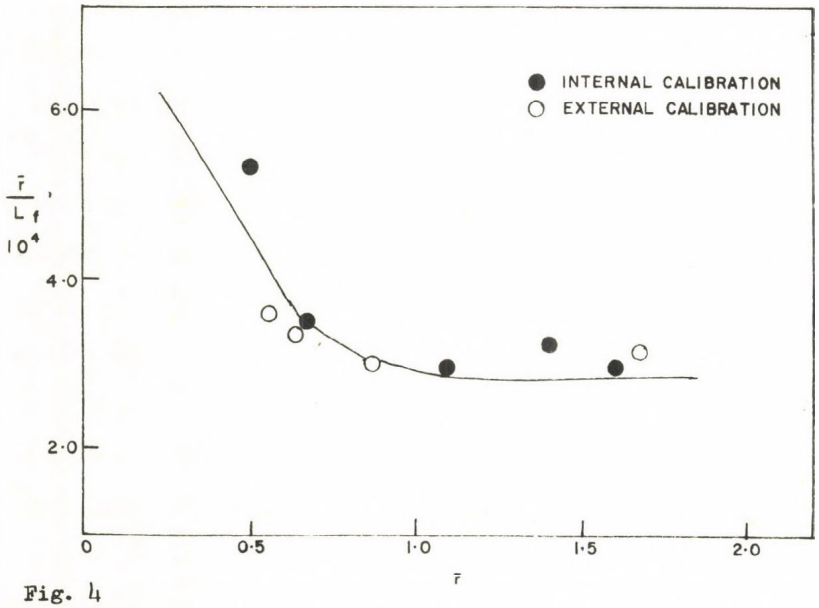

Fig. 4

Scatchard plot for the binding of L-tryptophan methyl ester by BSA.

DISCUSSION

Assuming that BSA has two binding sites $n_1 = 1$ and $n_2 = 25$ for L-tryptophan methyl ester[5], we used the same curve fitting method for analysis as we did for warfarin.[2] The constants estimated are: $k_1 = 6.0 \times 10^4$, $n_2k_2 = 4.2 \times 10^3$, in comparison with the literature values[5]: $k_1 = 3.1 \times 10^4$, $n_2k_2 = 1.6 \times 10^3$. The agreement is not as satisfactory as in the case of binding of L-tryptophan or warfarin to serum albumin.

The blockage of the negative charge carried by the carboxylate of L-tryptophan by methyl ester makes the molecule more ionic in character (i.e., a cation). The application of an electrostatic correction to the data $\bar{r}/[L]_f$ is perhaps more important in L-tryptophan methyl ester than in L-tryptophan. The correction factor that McMenamy and Oncley[5] used is exp $(2 Z_p Z_A W)$, where Z_p and Z_A are the net charges on the protein molecule and L-tryptophan methyl ester, respectively, and W is the electrostatic parameter of the Delye-Hückel theory. We did not make such a correction because neither we nor McMenamy- Oncley have enough experimental data for accurate analysis. McMenamy and Oncley had only four experimental points in their plot; we had nine. We believe that the application of the electrostatic correction on the limited amount of data, $\bar{r}/[L]_f$, does not necessarily lead to the estimation of accurate binding sites and binding constants. Recently, Klotz sreiously questioned the validity of the Scatchard plot[6]. He stated, ".... it is apparent that extrapolations in the Scatchard graph to yield a total number of receptor sites are generally not correct." It is our general impression that unless additional studies at higher and lower ligand concentrations are carried out, that is, unless more experimental points are obtained in the two extreme regions (very low and very high, particularly very high to reach a plateau), the Scatchard plot can only provide a rough estimate; it cannot lead to accurate calculations. It is our effort and goal to run HPLC experiments faster and simpler in order to get more experimental points

distributed in wider regions. At present, we are satisfied by demonstrating that not only can the HPLC method obtain values of $\bar{r}$ as well as the classical method equilibrium analysis, but that the HPLC method is obviously faster and simpler.

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LIPOPHILICITY DETERMINATION OF AMINO ACIDS BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Chromatographic data have been successfully applied in Quantitative Structure - Activity Relationship (QSAR) studies /1/. Lipophylicity as an important physico-chemical property of bioactive molecules has been frequently used in QSAR studies /2, 3, 4/. It can be determined by the classical partition method between water and n-octanol /5/, by reversed-pnase thin-layer chromatography (RPTLC) /6, 7, 8/ and by high-performance liquid chromatography /9, 10, 11/. Recent research indicates that the chromatographic methods, although they are rapid and simple, do not need pure compounds and use only a few micrograms of material, have some drawbacks: the support may partially retain its original adsorptive character even after coating /12/ and the R_M values depend on the adsorption behaviour and on the surface pH value of the support /13, 14/. The R_M values generally linearly decrease with increasing concentration of the organic phase of the eluent; however, in some cases, the R_M values increased at higher organic phase concentration /15/. This phenomenon has been explained by dual retention mechanism /16/: the free silanol groups of the support influence the retention mainly at higher organic phase concentrations.

In spite of their biological importance the number of chromatographic methods developed to determine the lipophilicity of amino acids is surprisingly small. This is probably related to the fact that on an impregnated silica support most frequently used in reversed-phase TLC, the retention of amino

acids is low /17, 18/. Therefore, mainly, various partition methods have been used to determine the lipophilicity of amino acids /19, 20/. Until now only some a limited number of reversed-phase TLC methods have been described, all, using water-insoluble alcohols saturated with water, with acetic acid or with ammonia solutions /21, 22/.

The objectives of our work were to find a simple reversed-phase TLC system for the determine action of the lipophilicity of amino acids and to compare the R_M values with the partition coefficients.

MATERIALS AND METHODS

Layers of 0.25 mm thickness were prepared on glass plates (20x20 cm) from the following supports and from their mixtures: Kieselgel G after Stahl (Merck) Darmstadt, FRG), MN-Aluminium-oxide G (Macherey-Nagel, Düren, FRG), Cellulosepulver MN 300 (Macherey-Nagel) and Kieselgur G (Merck). The composition of the mixed supports is given in Table I.

After drying the plates were impregnated by immersion overnight in the solution of 10% paraffin oil in n-hexane. Amino acids were dissolved in distilled water at a concentration of 2 mg/cm^3 and 3 mm^3 of each solution was spotted on the plates. Various water: methanol mixtures (up to 60% methanol concentration) served as the eluent for mixed supports, while water and 1:1 water:methanol were used as the eluent for pure supports. After development the plates were dried and the spots were detected by the standard ninhydrin technique.

The actual R_M values were extrapolated to zero methanol concentration separately for each mixed support according to the following equation:

$$R_M = R_{M(O)} + b \cdot C \qquad (1)$$

where R_M = the actual lipophilicity value of an amino acid determined at C% methanol concentration, $R_{M(O)}$ = the R_M value for O% methanol concentration, and C = methanol concentration in

Table I. Composition of the mixed supports

No of support	Kieselgel* %	Aluminium oxide %	Cellulose %	Kieselgur** %
I	70	30	-	-
II	50	50	-	-
III	30	70	-	-
IV	-	30	70	-
V	-	50	50	-
VI	-	70	30	-
VII	-	30	-	70
VIII	-	50	-	50
IX	-	70	-	30

*Silica gel.

**Diatomaceaus-earth support.

the eluent (vol %). In order to facilitate the calculations $100 \cdot R_M$ values were used instead of the R_M values.

As both reserved-phase TLC parameters (intercept = $R_{M(O)}$ and slope = b) may correlate with the log P values /23, 24/, linear correlations were calculated between the reversed-phase TLC parameters and the log P values taken from ref. /20/:

$$\log P = a + b_1 \cdot R_{M(O)} + b_2 \cdot b \qquad (2)$$

RESULTS AND DISCUSION

In most cases a higher methanol concentration increased the retention (higher R_M values) of amino acids (Fig. 1). The effect was the same for basic (Fig. 2) and acidic amino acids (Fig. 3.). The behaviour of amino acids under these reversed-phase TLC conditions differed from the expected general behaviour. According to our knowledge the R_M value of all compounds investigated until now decreased at higher organic phase concentration; however, the R_M values of amino acids increased

linearly with increasing methanol concentration in the eluent. This somewhat surprising finding can be explained by the assumption that the decreasing dielectric constant (increasing methanol concentration)of the eluent suppresses the dissociation of the polar groups of amino acids, resulting in higher lipophilicity.

The parameters of Eq. 1. are compiled in Table II. The regression coefficients of all equations in Table II indicate a significance level higher than 95%, except His on support I (90% significance level). As Asp and Cys were too strongly retained on supports II and III, we did not have sufficient data for calculation according to Eq. 1. In some cases the R_M values of amino acids did not significantly depend on the methanol concentration in the eluent (Trp on supports I, II, III, IV, VI, IX; Leu on support I; Ile on supports I, VIII; Phe on supports II, III, IV, V, VI, VII, IX; Pro on supports III, VIII; and Tyr on supports III, V, VI, VIII, IX); therefore, these data were excluded from the further calculations.

Both $R_{M(O)}$ and the slope (b) values depended heavily on the composition of the support. This finding again proves that the character of the suppor considerably influences the retention. We assume that only mixed supports of these types are suitable for the determination of the lipophilicity of the amino acids, because the retention is negligible on silica cellulose and diatomaceous earth supports while it is too strong on alumina, independently of the organic phase concentration in the eluent. The correlation between the R_M values and the composition of the mixed supports is generally non linear (Fig.4.), Gly, His and Lys remained on the start on alumina support in an eluent consisting of 1:1 water:methanol. The dependence of the R_M values on the composition of the support is easily understandable; the more alkaline is the overall surface pH value of the support, the more considerable the retention. Glu and Lys exhibited similar retention behaviour; thus, this finding suggests that the carboxyl groups have a preponderant role in the retention, while the impact of amino groups is of secondary importance.

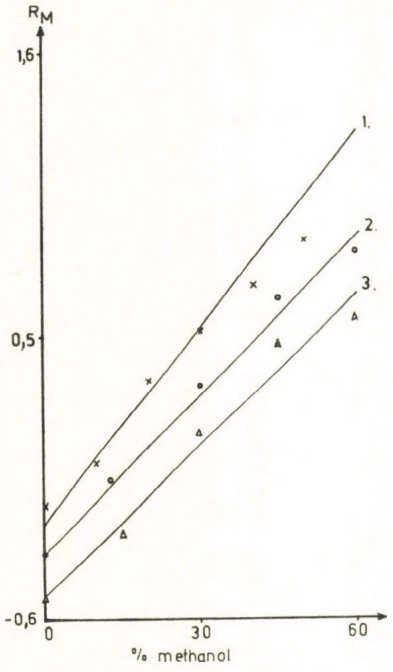

Fig. 1. Dependence of the R_M values of glycine on the methanol concentration in the eluent
1. support III.
2. support II.
3. support I

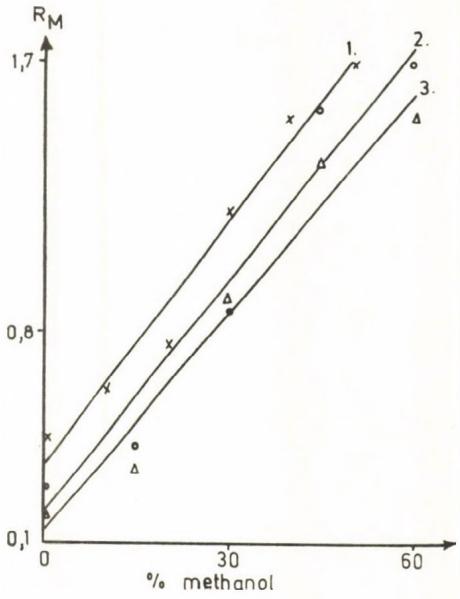

Fig. 2. Dependence of the R_M values of lysine on the methanol concentration in the eluent
1. support III.
2. support II.
3. support I.

The slope values (b) also increased at increasing alkalinity of the support. We could not find any plausible explanation for this phenomenon; perhaps the alkaline environment promotes the suppression of dissociation of the hydrophilic polar groups.

The parameters of Eq. 2. are summarized in Table III. Since for uncharged Arg we did not find partition data, it was omitted from the calculations.

The reversed-phase TLC parameters correlated well with the partition data on all mixed supports; the significance level was over 99.9% in most cases, proving that correct lipophilicity values can be obtained in these reversed-phase TLC systems. However, we could not find a reversed-phase TLC system which was suitable to simultaneously determine the lipohilicity of

Table II. Parameters of the linear correlation (Eq.1) between the eluent

Amino acids	No. of support								
	I		II		III		IV		V
	$R_{M(O)}$	b	$R_{M(O)}$	b	$R_{M(O)}$	b	$R_{M(O)}$	b	$R_{M(O)}$
Ala	-70.60	1.547	-54.20	1.687	-48.14	2.071	-85.80	1.313	-65.39
Arg	20.80	1.887	34.00	1.880	42.29	2.349	-40.50	1.848	-7.20
Asn	-32.60	2.227	-7.40	2.267	13.43	2.063	-35.99	1.562	63.73
Asp	14.60	2.407	-	-	-	-	13.95	1.581	43.17
Cys	35.17	3.167	-	-	-	-	-2.16	0.952	69.20
Gln	51.00	1.947	-29.80	1.933	-23.36	2.264	-69.50	1.582	-19.97
Glu	-36.60	2.693	-3.80	2.607	32.81	1.594	-38.04	2.038	0.37
Gly	-50.40	1.953	-33.80	2.047	22.46	2.539	-66.04	1.514	-32.03
His	2.80	1.067	19.80	1.367	31.93	1.621	-32.24	1.368	1.21
Ile	-	-	8.20	0.487	21.36	0.536	-54.79	0.660	-11.03
Leu	-	-	14.00	0.493	26.00	0.729	-44.02	0.753	-4.57
Lys	13.40	2.440	18.20	2.613	36.24	2.677	-57.44	1.942	-26.55
Met	-16.80	0.713	4.60	0.847	21.50	0.993	-26.79	1.077	-12.83
Phe	24.00	0.240	-	-	-	-	-	-	-
Pro	-36.50	0.653	-45.50	0.920	-	-	-96.40	1.025	-64.07
Pro(OH)	-63.20	1.393	-59.40	1.593	-55.75	2.096	-87.78	1.362	-78.23
Ser	-49.60	2.147	-32.40	2.427	-23.43	2.771	-53.32	1.661	-16.04
Thr	-40.60	1.940	-15.60	1.980	1.89	2.132	-45.58	1.440	- 4.88
Trp	-	-	-	-	-	-	-	-	81.89
Tyr	-23.50	0.860	0.50	0.827	-	-	-26.00	0.533	-
Val	-40.00	0.927	-34.00	1.213	-21.46	1.467	-75.50	0.979	-54.87

the R_M values of amino acids and the methanol concentation of

No. of support									
V		VI		VII		VIII		IX	
b	$R_{M(O)}$	b	$R_{M(O)}$	b	$R_{M(O)}$	b	$R_{M(O)}$	b
1.442	-47.20	1.586	-105.84	2.049	-72.72	1.391	-33.43	1.554
1.504	-5.66	1.745	-46.37	1.772	-10.32	1.613	-1.37	1.922
1.144	35.51	1.056	-17.44	1.868	60.01	1.211	4.25	1.450
1.470	68.72	1.882	39.24	1.803	43.16	1.488	82.23	2.039
2.093	38.95	0.629	-4.82	1.151	93.71	1.322	44.64	0.711
0.861	-16.04	1.597	-71.11	2.024	-26.22	1.095	-0.55	1.426
1.295	20.57	2.055	-20.97	2.207	12.22	0.917	37.16	1.483
1.332	-18.83	1.617	-71.80	2.020	-33.59	1.254	-9.28	1.718
1.047	19.31	1.040	-15.96	1.292	-4.85	1.039	32.35	1.290
0.379	7.42	0.683	-42.25	0.901	-	-	22.83	0.677
0.402	23.98	0.512	-35.26	1.178	-2.14	0.344	37.80	0.581
1.827	-23.64	2.311	-76.97	2.540	-35.89	2.011	-11.27	2.171
0.749	40.04	0.724	-6.26	1.022	-9.17	0.547	52.62	0.874
-	-	-	-	-	56.83	-0.570	-	-
0.583	-69.01	1.016	-127.68	1.343	-	-	-72.60	1.272
1.455	-66.25	1.790	-107.99	1.616	-93.82	1.452	-51.86	1.633
1.208	6.47	1.479	34.08	1.571	-24.71	1.373	10.66	1.839
0.901	11.12	1.574	-20.34	1.275	-11.09	1.181	30.23	1.430
-0.734	-	-	33.90	-0.360	79.36	-0.921	-	-
-	-	-	-18.61	0.734	-	-	-	-
0.960	-18.98	0.941	-81.26	1.318	-45.21	0.648	-2.35	0.805

Table III. Correlation between the partition coefficients and RPTLC parameters of amino acids (Eq.2.)

Parameters	No. of support								
	I	II	III	IV	V	VI	VII	VIII	IX
n	18	17	15	18	18	17	19	17	17
$b_1 \cdot 10^3$	0.30	-3.30	-7.25	-0.21	-0.37	-1.52	-2.82	-1.25	0.15
b_2	-1.02	-1.14	-1.20	-1.13	-0.67	-0.90	-0.80	-0.78	-1.13
a	-0.25	-0.16	-0.13	-1.28	-2.01	-1.56	-1.64	-1.97	-1.22
r^2	0.8402	0.9034	0.9188	0.6479	0.4455	0.6198	0.5966	0.7078	0.8075
r	0.9166	0.9505	0.9586	0.8049	0.6674	0.7873	0.7724	0.8413	0.8986
b'_1 %	1	6	14	2	4	11	20	10	2
b'_2 %	99	94	86	98	96	89	80	90	98
s	0.59	0.42	0.41	0.59	0.63	0.61	0.62	0.66	0.61
$s_1 \cdot 10^3$	6.06	4.80	3.82	3.12	2.70	2.69	2.39	2.05	1.85
s_2	0.12	0.10	0.11	0.21	0.19	0.19	0.17	0.14	0.15
F	36.80	60.80	62.25	13.82	6.07	11.41	11.80	17.06	29.40
t_1	0.05	0.69	1.90	0.07	0.14	0.57	1.18	0.61	0.08
t_2	8.56	11.02	10.74	5.25	3.44	4.74	4.75	5.42	7.50
$F_{99.9\%}$	11.34	11.78	12.31	11.34	3.74*	6.51**	10.97	11.78	11.78
$t_{99.9\%}$	4.07	4.14	4.32	4.07	2.95**	4.14	4.02	4.14	4.14

*$F_{95\%}$ **$F_{99\%}$, $t_{99\%}$

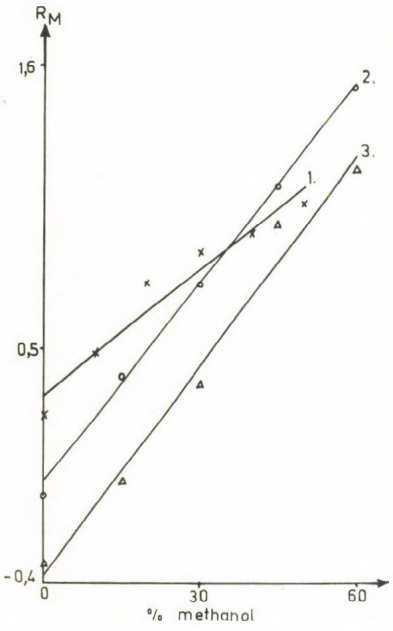

Fig. 3. Dependence of the R_M values of glutamic acid on the methanol concentration in the eluent
1. support III.
2. support II.
3. support I

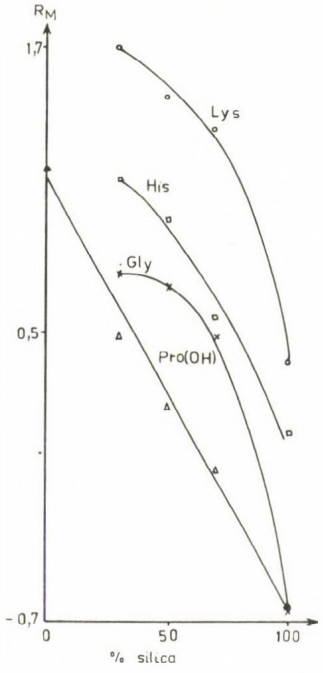

Fig. 4. Dependence of the retention of some amino acids on the composition of the support.
Eluent: 1:1 water-methanol

all amino acids. The normalized slope (b_1') and the t_1 values clearly indicate that the contribution of $R_{M(O)}$ to the correlation between the partition coefficients and the reversed-phase TLC parameters of amino acids is negligible and insignificant. This means that the $R_{M(O)}$ value of amino acids changes according to the surface pH of the supports depending on the pK values of the carboxyl and amino groups and it changes independently of the partition coefficients. This fact also confirms the validity of the approaches described by Cserháti /23/ and Valkó /24/, i.e., that the slope (b) values contain valuable information concerning the lipophilic character of the compounds investigated.

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HPLC WITH FLUORESCENCE DETECTION FOR THE ANALYSIS OF DECARBOXYLATED S-ADENOSYLMETHIONINE AND ITS ANALOGUES

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INTRODUCTION

Decarboxylated S-adenosylmethionine (dc-SAM) plays a key role in the biosynthesis of the polyamines spermidine and spermine as the donor of the propylamine portion (1). In previous publications (2, 3), we described an HPLC method based on UV detection at 254 nm for the determination of dc-SAM and its precursor S-adenosylmethionine (SAM) in various tissues of rats. This HPLC method allowed us to show that the treatment with α-difluoromethylornithine (DFMO), a potent enzyme-activated inhibitor of ornithine decarboxylase (ODC), lead to a pronounced increase of dc-SAM in the prostate of rats (2). The sensitivity of the method, however, proved unsatisfactory for the determination of dc-SAM in urine, plasma or such tissues as thymus or lung with low dc-SAM content. Therefore, we described recently how the well-known reaction of chloroacetaldehyde with the adenine moiety (4, 5, 6) has been used to advantage for the analysis of dc-SAM in urine (7). The precolumn derivatization with chloroacetaldehyde lead to the formation of the $1,N^6$-etheno derivatives of dc-SAM and its analogues which were then analysed by HPLC with fluorometric detection. A similar derivatization procedure had previously been described for the

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analysis of methylthioadenosine (MTA) in urine samples, but no use had been made of the separation efficiency of actual HPLC procedures (8).

In this paper, we shall describe the application of this HPLC procedure with precolumn derivatization and fluorometric detection to the analysis of dc-SAM in urine and in an other biological fluid i.e. plasma and in certain tissues with low dc-SAM content. The gain in sensitivity of the new method will be illustrated by the study of the effect of a chronic treatment with the ODC inhibitor, (2R,5R)-6-heptyne-2,5-diamine, MDL 72175 (9,10) on the dc-SAM levels in urine of mice and in urine, plasma and several tissues of rats.

MATERIALS AND METHODS

Chemicals

With the exception of S-(5'-deoxy-5'-adenosyl)-3-methylthiopropyl -amine bisulfate (dc-SAM), S-(5'-deoxy-5'-adenosyl)-3-thiopropylamine bisulfate (dc-SAH), S-(5'-deoxy-5'-adenosyl)-3-ethylthiopropylamine bisulfate (dc-SAE) prepared according to published procedures (11,12), all other SAM analogues were products of Sigma (St.Louis, MO, USA). D,L-α-difluoromethylornithine (DFMO, MDL 71782) and (2R,5R)-6-heptyne-2,5-diamine (MDL 72175) were synthetised in our Centre (9,13). Octanesulfonic acid sodium salt (OSA) was from Eastman Kodak (Rochester, NY, USA) and ortho-phthalaldehyde (OPA) was obtained from Carl Roth (Karlsruhe, FRG). All other chemicals, including the 45% aqueous solution of 2-chloroacetaldehyde, were from E. Merck (Darmstadt, FRG).

Animals

Female albino mice (30-40 g) and male Sprague-Dawley rats (350-400 g) were from Charles River (Saint-Aubin-les-Elbeuf, France).

Chromatographic system

. Instruments : The HPLC system used was similar to the one previously described (3,7) and was comprised of two M6000A pumps, a M440 UV detector, a M680 solvent programmer and a WISP injector, all from Waters Associates (Milford, Mass. USA). The fluorescence of the etheno derivatives was monitored with a Kontron instrument SFM 23 LC (Zürich, Switzerland). The excitation wavelength was set at 270 nm with the emission wavelength held at 410 nm. The column was an Ultrasphere-IP (25 cm x 4.6 mm) packed with 5 µm spherical particles from Beckman (Berkeley, CA, USA). The main column was protected with a guard column (7 cm x 2 mm) filled with pellicular ODS media (particle size 37 - 53 µm) from Whatman (Clifton, N.J., USA). The two columns, main and guard, were thermostated at 40°C in a jacket with a circulating water-bath.

Mobile phases

Two different mobile phases and gradient profiles were used. System I was identical to the one previously described (3). System II differed mainly by the pH's of the eluents and by the gradient profile used. Mobile phase A consisted of a 98/2 (v/v) solution of 0.1M NaH_2PO_4 and acetonitrile and contained 8 x 10^{-3}M OSA. The pH of eluent A was adjusted to 3.65 with 1 ml of 3M H_3PO_4 per liter. Mobile phase B was obtained by mixing 740 ml of 0.2M NaH_2PO_4 with 260 ml of acetonitrile and contained 8 x 10^{-3}M OSA. Its pH was adjusted to 4.3 with 1 ml of 3M H_3PO_4 per liter. The two mobile phases were filtered with Millipore filters, a HA filter for eluent A and a FHUP for eluent B as previously described (3, 7). A linear gradient was used, leading in 30 min from 75% of eluent A and 25% B to 24% of eluent A and 76% of B. The gradient controller then triggered a linear return, in 0.5 min, to the initial conditions, followed by a stabilization period of 10 min before the next injection proceeded. The flow rate was 1.5 ml/min.

Sample preparation and derivatization procedures

Standard Samples

The derivatization conditions were similar to those recently described (7). Five ml of a 5-10 µM standard solution of the different SAM analogues were reacted at 40° with 0.5 ml of the 45% aqeous solution of chloroacetaldehyde and at a pH of 3.5-3.8 after addition of 100 µl 3M sodium acetate. After 14 hours, the reaction was stopped by cooling in ice and the solution kept at 0 to 4°C in the refrigerator before injection.

Preparation of the biological samples

* Mouse urine : The urine of mice was collected from individual mice over a three day period. At the end of each day, the urine collected was diluted with an equal volume of 0.4M $HClO_4$. In view of the low volumes obtained, the urine of the three consecutive days was pooled for each mouse and filtered over a 0.22 µm Millipore Millex filter. The sample preparation then followed the procedure described for rat urine (7). One and a half ml of this filtered urine, to which was added 1 ml of a 0.66 µM solution of dc-SAE, the internal standard, were purified over a cation-exchange column, Dowex AG 50W-X 8 (5 cm x 1.2 cm). After washing with 10 ml of H_2O and 25 ml of 2M HCL, dc-SAM and dc-SAE along with the polyamines were eluted with 25 ml of 6M HCl. After evaporation on a rotavapor, the residue was dissolved in 1.5 ml of 0.2M $HClO_4$. This solution was then used in part for the analysis of dc-SAM and the polyamines by direct injection onto the HPLC using the procedure described (3) and in part for the derivatization with chloroacetaldehyde. Typically 500 µl of this 0.2M $HClO_4$ extract were reacted with 50 µl of chloroacetaldehyde solution at a pH of 3.5 - 3.8 after addition of 50µl of 3M sodium acetate. The samples were heated for 12 - 16 hours at

40°C. The reaction was stopped by cooling and stored at 0 to 4°C. Usually 10 to 20 µl of the reaction mixture were injected onto the column.

* Rat urine : The urine collection, prepurification and derivatization were similar to that previously described (7). Urine was collected individually over an equal volume of 0.4M $HClO_4$ for a 24 h period and filtered over a 0.22 µm Millipore Millex filter. One and a half ml of this acidified urine, to which were added 3 ml of a 0.69 µM solution of the internal standard dc-SAE, were purified over the Dowex column in the same way as for the mouse urine samples. After evaporation of the 6M HCl eluate, the residue was dissolved in 1.5 ml of 0.2M $HClO_4$. A part of this solution was then directly injected onto the HPLC for the determination of dc-SAM by UV detection and for the analysis of the polyamines after post-column derivatization with OPA, by using chromatographic system I as previously described (3). Another fraction, 500 µl, was derivatized with chloroacetaldehyde by using the same conditions as for the mouse urine samples and analysed by using chromatographic conditions II.

* Plasma samples : The rats were killed by decapitation and 8 to 10 ml of whole blood were withdrawn into heparinazed tubes. After centrifugation at 700 g for 15 min, the plasma was collected and an equal volume of 0.4M $HClO_4$ was added. After centrifugation at 18000 g for 20 min, the supernatant was removed and filtered over a 0.22 µm Millipore Millex filter. To a 500 µl aliquot were added 100 µl of a 3.3 µM solution of dc-SAE, the internal standard, and this solution was derivatized with 50 µl of chloroacetaldehyde and 50 µl of 3M sodium acetate at pH 3.5 - 3.8 and at 40°C for 12 - 16 h. The samples were stored at 0 - 4°C before injection onto HPLC proceeded.

Tissue analysis

The different tissues of rats were homogenized in 0.2M $HClO_4$, usually 5 ml, for the following tissue weights, testis (1.0 - 1.5 g), thymus (0.3 - 0.6 g), duodenum (0.3 - 0.5 g), prostate (0.5 - 0.6 g), pancreas (0.8 - 1.2 g), and parts of the lung (0.3 - 0.5 g). After centrifugation at 18000 g during 20 min, the supernatant was filtered over Millipore Millex filter. To a 500 µl aliquot were added 100 µl of a 3.3 µM solution of dc-SAE and 50 µl of chloroacetaldehyde. The pH was then adjusted to 3.4 - 3.8 by addition of 25 to 50 µl of 3M sodium acetate (depending on the tissue) and samples heated at 40°C for 12 to 16 h, as for the urine samples. The samples were then stored at 0 - 4°C and 10 to 50 µl injected.

Calculations

The calculations for the dc-SAM values were performed by the internal standard method. The amount of dc-SAE added was precisely determined by separate HPLC analysis using HPLC systems I or II with UV detection. Previous calibration and recovery studies had shown that indeed dc-SAM and dc-SAE display the same recovery and derivatization kinetics (7).

RESULTS

1) Chromatographic separation

Eluent system I was identical to the one previously described (3) for the simultaneous analysis of dc-SAM and the polyamines. It was mainly used for the analysis of the polyamines after post-column derivatization with ortho-phthalaldehyde. It was also applied for the determination of dc-SAM by UV detection at 254 nm for the tissue samples with sufficiently high dc-SAM content. This system also allows a separation of the different etheno derivatives, as shown by the chromatogram of a standard sample in Fig. 1. This system I, however, was not found suitable for the analysis of

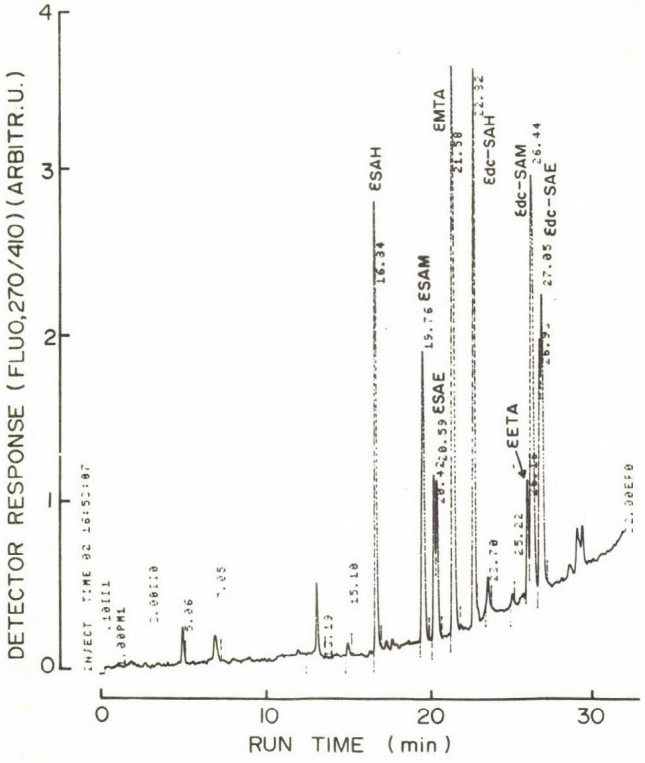

Figure 1 :

Chromatogram with fluorescence detection (λ_{exc} = 270 nm, λ_{em} =410 nm) of a standard solution of the etheno derivatives of dc-SAM and its analogues. Chromatographic system I was used with pH = 2.65 of eluent A and pH = 3.25 of eluent B, and a gradient leading in 30 min from 85% of eluent A and 15% of B to the final conditions of 100% of eluent B.

Amounts in pmoles : εSAH (5.0), εSAM (5.4), εSAE (3.5), εMTA (6.1), εdc-SAH (5.3), εETA (1.1), εdc-SAM (5.4), εdc-SAE (4.3).

The sensitivity of the SFM 23 fluorescence detector was set at high and the attenuation of the SP4100 integrator at 8 with the input selector at 1V.

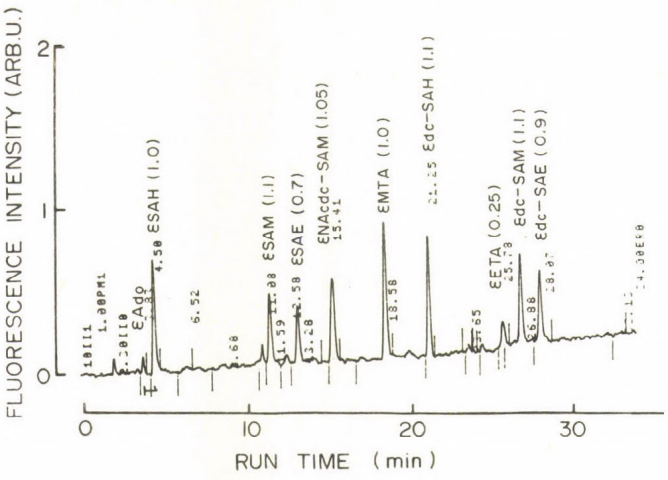

Figure 2 :

Chromatogram of a standard solution of the etheno derivatives of dc-SAM and analogues with fluorescence detection. Chromatographic system II was used with pH = 3.65 of eluent A and pH = 4.3 of eluent B as described in Materials and Methods. Amounts in pmol for the different compounds are given between brackets. The conditions used for the detection and recording were the same as in Fig. 1.

εdc-SAM in biological samples because of interferences by unknown compounds. Therefore, system II was developed which differes mainly by its higher pH values and its gradient composition. Fig. 2 shows a typical chromatogram obtained under these conditions, by fluorescence detection, with a standard mixture of about 1 pmol of the different etheno derivatives. This chromatogram clearly illustrates the sensitivity of this new procedure with a gain in sensitivity comprised between 10 and 20 times as compared to previous methods (3, 14). The gain in sensitivity is further illustrated in Fig. 3 which shows the chromatograms of rat urine samples obtained by UV detection without derivatization (A) and by fluorescence detection after derivatization to the etheno analogues (B).

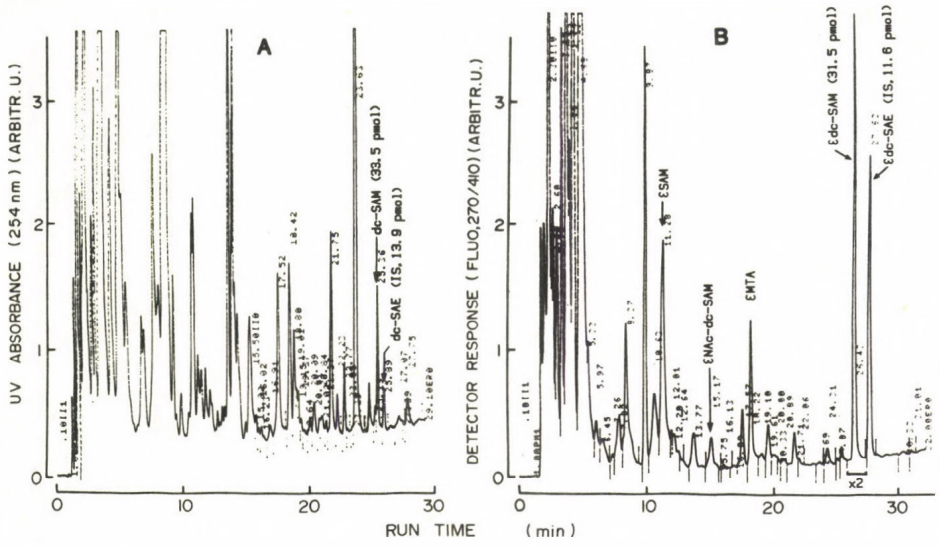

Figure 3 :

Chromatograms of urine samples of a control rat after Dowex prepurification. Chromatogram A was obtained by direct injection of 10 µl of the 0.2M $HClO_4$ solution obtained after Dowex prepurification and evaporation, with chromatographic conditions I and UV detection at 254 nm. Chromatogram B was obtained with the urine sample of the same rat, by fluorescence detection after derivatization with chloroacetaldehyde and injection of 10 µl of the reaction mixture. The chromatographic conditions used for B were the same as in Fig. 2 with the attenuation set at 16 or 32 (x2). The amounts in pmol of εdc-SAM and εdc-SAE for the 10 µl injected are given between brackets.

For the underivatized samples, dc-SAM is detectable by UV absorbance but several other peaks are observed in the vicinity. However, the precolumn derivatization of the same rat urine sample, leading to the etheno derivative, gives a peak corresponding to εdc-SAM which is well detected and free of observable interferences. The peak of εdc-SAM in this sample

of urine of control rats corresponds to 31.5 pmol of dc-SAM for the 10 µl injected. The gain in sensitivity and in selectivity obtained by the precolumn derivatization into the etheno derivatives with subsequent fluorescence detection, has, therefore, been extensively used for the analysis of dc-SAM and its analogues in all the urine, plasma and tissue samples having a low dc-SAM content.

Effect of a chronic treatment with (2R,5R)-6-heptyne-2,5-diamine (MDL 72175) on the dc-SAM level in urine of mice

In order to study the cumulative effect of a chronic treatment with a potent inhibitor of ornithine decarboxylase, mice were treated for 4 months with 0.2% (w/v) MDL 72175 in the drinking water. Individual 72 h urine samples were collected on the three last days of the treatment and were purified as described in the methods. Fig. 4 shows typical chromato-

Table I Effect of a chronic treatment a) with MDL 72175 ont the dc-SAM levels in urine of mice b).

	nmol/ml	nmol/day
Control	4.5 + 0.4	5.1 + 0.6 (n = 10)
MDL 72175 treated	13.0 + 2.3 (**)	19.9 + 4.5 (n = 9) (**)

a)
0.2% (w/v) in drinking water for 4 months.

b)
Values are expressed in nmol/ml or nmol/day + SEM. Statistically significant differences compared to controls as determined by Student's t-test are shown by (**), $p < 0.005$.

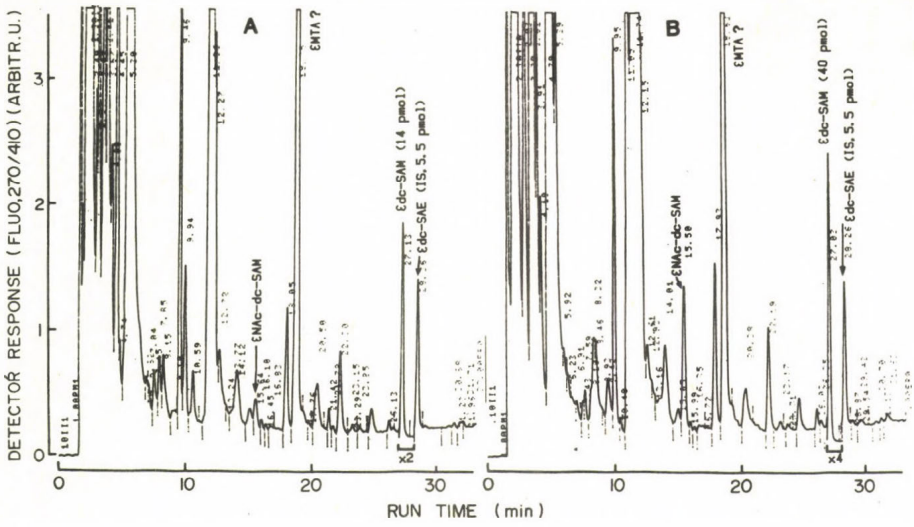

Figure 4 :

Chromatograms of the εdc-SAM analogues with fluorescence detection of mouse urine : A, control and B, mouse treated for 4 months with 0.2% (w/v) MDL 72175 in the drinking water. After prepurification over Dowex and evaporation, a 0.5 ml aliquot of the 0.2M $HClO_4$ solution was reacted with 50 µl of chloroacetaldehyde and 50 µl of 3M sodium acetate at pH 3.5 - 3.8 and 40°C for 14 h. A 10 µl aliquot was injected onto the HPLC with chromatographic conditions identical to those of Fig. 2 and the attenuation set at 16. The attenuation was increased by a factor of 2 (x2) or 4(x4) and the amounts in pmol of εdc-SAM and εdc-SAE for the 10 µl injected are indicated between brackets.

grams obtained with fluorescence detection of mouse urine samples after derivatization with chloroacetaldehyde. In the control sample (A), εdc-SAM is clearly detected, 15.5 pmol for the 10 µl injected, and corresponds to 4.5 nmol of dc-SAM per ml of crude urine. This value is comparable to the values found for dc-SAM in urine of rats (7). After treatment (B), the level of dc-SAM is markedly increased. As described in the experimental procedure, dc-SAE was used as internal standard. Table I summarizes the

values obtained. The data show that the treatment with the ODC inhibitor leads to a four-fold increase of the dc-SAM values in urine as expressed in nmol excreted per day. It is noteworthy that the increase in level of dc-SAM, after treatment with MDL 72175, is accompanied by the concomittant increase of the peak with a retention time of 15.5 min. This peak has been fully characterized and shown to correspond to the N-acetylated metabolite of dc-SAM (NAc-dc-SAM) (15). Other peaks, corresponding probably to εSAM, εMTA are also observed but no full characterization of the identity of these peaks has been made.

2) Effect of four days treatment with MDL 72175 on the dc-SAM levels in plasma, urine and different tissues of rats.

Previous studies have shown that the treatment with ODC inhibitors leads to a marked increase of dc-SAM levels in various organs (2) and urine (7) of rats and mice (see above). Therefore, it was of interest to study if similar increases would be observed in other body fluids such as plasma. Rats were treated for 4 days with 0.2% (w/v) of MDL 72175 in the drinking water. On day 4 blood was withdrawn after decapitation, as described above. Whereas the urine samples were purified and derivatized as described, the plasma and other tissue extracts in 0.4N $HClO_4$ were simply reacted with chloroacetaldehyde at a pH of 3.3 to 3.8, without any prepurification. The crude reaction mixtures were injected onto the HPLC column. The chromatograms obtained by the injection of 100 µl of the derivatized plasma samples are shown in Fig. 5. In the control rat, (A), a small peak of εdc-SAM is identified and corresponds to 0.7 pmol of dc-SAM for the 100 µl injected and to a dc-SAM level of 19 pmol for 1 ml plasma. After treatment with MDL 72175, (B), its height clearly increases and amounts to 3.5 pmol for the 100 µl injected. This result shows that

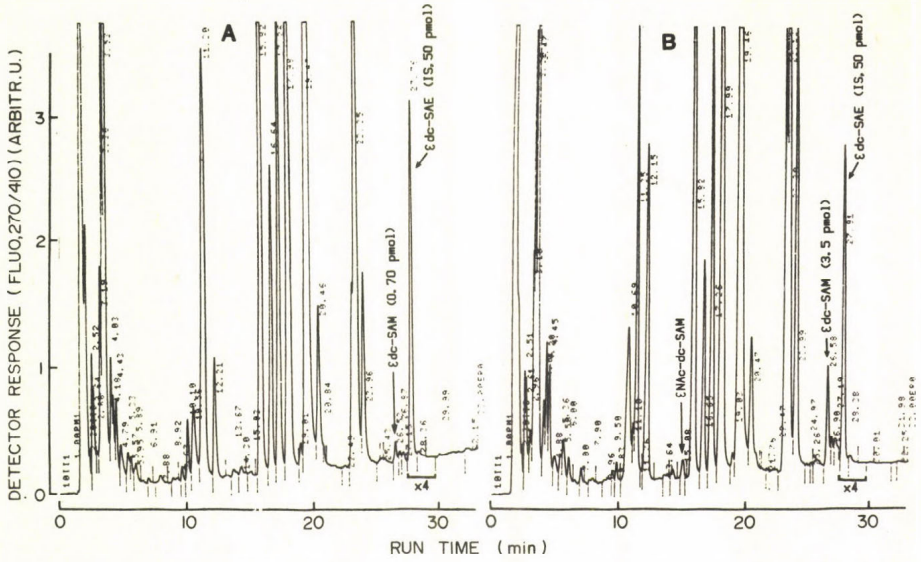

Figure 5 :

Chromatograms of the εdc-SAM analogues with fluorescence detection of rat plasma : A, control and B, rat treated for 4 days with 0.2% (w/v) MDL 72175 in the drinking water. The derivatization was performed as described in Materials and Methods, by reacting a 500 µl aliquot, to which were added 100 µl of the 3.3 µM solution of dc-SAE, with 50 µl of chloroacetaldehyde and 50 µl of 3M sodium acetate at pH 3.5 - 3.8 and 40°C for 14 h. A 100 µl aliquot of this reaction mixture was injected into the column with chromatographic conditions identical to those of Fig. 4 and attenuation increased by a factor of 4 (x4). The amounts in pmol of εdc-SAM and εdc-SAE for the 100 µl injected are listed between brackets.

although the level of dc-SAM in plasma is rather low, it is significantly increased after treatment with an ODC inhibitor. Similar increases, 3 to 5 times, are also observed in urine samples of the same rats.

Fig. 6 presents the chromatograms of the derivatized extracts of duodenum samples of the same rats. Although εdc-SAM is quite low, 0.75 pmol

Table II Effect of repeated treatment with MDL 72175, 0.2% (w/v) in drinking water for 4 days, on the dc-SAM level in plasma, urine and various tissues of rats.

Body Fluid or Tissue	Control	Treated MDL 72175
Plasma	17.2 ± 0.6^{a}	86.5 ± 5.7^{a} (***)
Urine	$8.6 \pm 0.5 \times 10^{3a}$ $91 \pm 10 \times 10^{3b}$	$34.3 \pm 7.5 \times 10^{3a}$ (*) $319 \pm 29 \times 10^{3b}$ (***)
Prostate[c]	$3.4 \pm 0.5 \times 10^{3}$	$1290 \pm 98 \times 10^{3}$ (***)
Duodenum[c]	550 ± 56	$63 \pm 5.5 \times 10^{3}$ (***)
Thymus[c]	234 ± 16	658 ± 78 (***)
Testis[c]	$1.45 \pm 0.15 \times 10^{3}$	$1.92 \pm 0.16 \times 10^{3}$ (NS)

Means $\pm$ SEM for five rats in each series.

a) Values are expressed in pmol/ml.

b) Values in pmol/total urine excreted in 24 h.

c) Values in pmol/g net weight.

Statistically significant differences from controls determined by Student's t-test are shown by (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$ (NS) not significant.

for the 25 µl injected, in the control rat (A), its level is markedly increased in the treated rat with a parallel increase of the peak corresponding to εNAc-dc-SAM. Two other peaks have been identified as corresponding to εSAM and εMTA. Other representative tissues presenting different ODC activities (16) have also been analysed, i.e. prostate, thymus and testis. The results are summarized in Table II along with those obtained for urine and plasma. The value found for dc-SAM in the prostate thymus and testis of control rats are in overall good agreement with those published (2). No reference values could be found for the dc-SAM levels in plasma and in the duodenum. These results listed clearly show that in the tissues with high ODC activity, i.e. duodenum and prostate (16), the treatment with an ODC inhibitor leads to a an enormous increase of the dc-SAM levels, 110 and 385-fold respectively. Marked increases in the dc-SAM levels had also been previously observed in various cell cultures with high ODC activity (2, 17) after treatment with α-difluoromethyl-ornithine, another ODC inhibitor. For tissues with lower ODC activity (16), i.e. thymus and testis, the dc-SAM level is much less affected. More interestingly, in urine and in plasma a similar 3 to 5-fold increase of the dc-SAM level is observed after treatment with MDL 72175. These results suggest that the analysis of dc-SAM in urine and in plasma may be proposed as a non-invasive method for the estimation of the ODC inhibition after treatment with various inhibitors. The analysis of dc-SAM in the plasma and urine of patients undergoing therapy with ODC inhibitors may, therefore, be an index of the biochemical efficiency of the treatment.

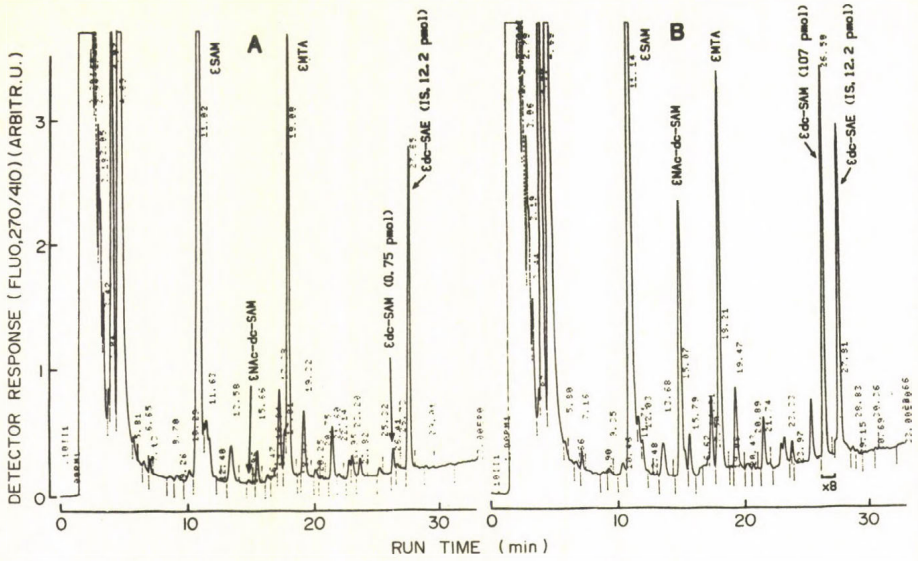

Figure 6 :

Chromatograms of the εdc-SAM analogues with fluorescence detection from rat duodenum : A, control and B, rat treated for 4 days with 0.2% (w/v) MDL 72175 in the drinking water. To a 500 µl aliquot of the tissue extract were added 100 µl of a 3.3 µM solution of dc-SAE and 50 µl of chloroacetaldehyde. The pH was adjusted to 4 with 40 µl of 3M sodium acetate and the mixture heated at 40°C for 15 h. A 25 µl aliquot was injected with the chromatographic conditions identical to those of Fig. 4 and the attenuation increased by a factor of 8 (x8) for the peak of εdc-SAM in (B). The amounts in pmol of εdc-SAM and εdc-SAE for the 25 µl injected are given between brackets.

CONCLUSION

Our results clearly demonstrate that this new HPLC procedure for the analysis of dc-SAM through its fluorescent $1,N^6$-etheno derivative is a highly sensitive and selective method. The sensitivity obtained, in the subpicomole range, allows an unambiguous determination of the dc-SAM

levels in plasma and urine for which the previously used HPLC procedure with UV detection was not sensitive enough. The derivatization procedure with the use of dc-SAE, a close structural analogue of dc-SAM, as internal standard, is shown to be easily applicable for the analysis of dc-SAM in various tissues. The application of this method to the analysis of dc-SAM levels in human urine (7) and plasma samples should allow us to monitor the treatments with various ODC inhibitors in clinical studies.

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DETERMINATION OF HISTIDINE, 1-METHYLHISTIDINE AND 3-METHYLHISTIDINE IN BIOLOGICAL SAMPLES BY HPLC. CLINICAL APPLICATION OF URINARY 3-METHYLHISTIDINE IN EVALUATING THE MUSCLE PROTEIN BREAKDOWN IN UREMIC PATIENTS

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SUMMARY

A high-performance liquid chromatographic method based on precolumn derivatization of amino acids with o-phthalaldehyde/mercaptopropionic acid which permits determination of histidine, 3-methylhistidine and 1-methylhistidine and 18 other amino acids in small volume of plasma and urine. The separation is conducted on a reversed-phase C_{18}-column by using a two-solvent system by a multi-step gradient under 50 min.

The method was applied to study 4 uremic patients kept on meat-free diet for 16 days to evaluate the time required to obtain a steady-state condition with regard to plasma concentration and urinary excretion of 3-MH.

INTRODUCTION

3-methylhistidine (3-MH) is produced in actin of all muscle fibers and in myosin of white muscle fibers by post-translational methylation of peptide-bound histidyl residues /1-3/. When these proteins are catabolized 3-MH is liberated and excreted quantitatively and unchanged in the urine /4-6/.

Therefore, the urinary excretion of 3-MH is considered to be an index of the in vivo rate of endogenous myofibrillar protein breakdown /7-9/, provided that the individual is maintained on a meat-free diet (i.e. a diet not containing 3-MH) under steady state conditions.

Young and Munro /7/ showed that 80% of the excreted 3-MH comes from actin and the remaining 20% from myosin in adults. Actin and myosin comprises 65% of the total muscle protein and 30-35% of the total body protein pool, thus being the two most abundant proteins in the body.

Unlike 3-MH, little is known about the metabolism of 1-methylhistidine (1-MH) apart from that it exists in dipeptide, anserine.

Numerous methods have been put forward for the quantitation of 3-MH based on ion-exchange chromatography /10-14), high performance liquid chromatography /15-18/ and gas chromatography /19-21/. These methods mostly suffer from the lengthy preparation of the samples and subsequent separation and detection. Besides, the methods allow only the determination of 3-MH and only in one study /17/ a limited number of other amino acids could be quantitated.

In this communication, we present a rapid procedure for the separation of 21 amino acids of protein-free biological specimens by HPLC method. We have optimized our experimental conditions for the resolution of histidine (His), 3-MH and 1-MH from other amino acids. The method is based on the pre-column derivatization of amino acid with orthophthalaldehyde (OPA) in presence of 3-mercaptopropionic acid (3-MPA) and the detection has been made at E_{ex} = 340 nm and E_{em} = 450 nm. The separation is performed on a reversed-phase column with two-solvent system. The protocol also allows for the quantitation of tyrosine (Tyr), phenylalanine (Phe) and 16 other amino acids.

This method of analysis was applied for the quantitation of 3-MH in plasma and urine of uremic patients kept on meat-free diet for 16 days to measure the muscle protein break-down.

EXPERIMENT

Chemicals

Individual crystalline samples of L-amino acids were obtained from Pierce (Rockford, IL, USA) (AMAC Standard Kit No. 20065). 1-MH, 3-MH, citrulline, phosphoserine, carnosine and

taurine were obtained from Sigma (St. Louis, MO, USA). Individual standard stock solutions (1 µM) were prepared in distilled water by addition of a few drops of 0.1 M HCl. A standard mixture containing 23 amino acids was prepared to a concentration of 0.1 µM and stored at -70°C until analyzed. This standard mixture was diluted as required with distilled water. Methanol "HPLC grade" was obtained from Rathburn Chemicals (Walkerburn, Scotland). Sodium dihydrogenphosphate and disodium hydrogenphosphate and boric acid all "Anala R" grade were obtained from Merck (Darmstadt, FRG). Sodium hydroxide, hydrochloric acid and perchloric acid (70%) in pure form were obtained from B.T. Baker. 3-Mercaptopropionic acid (3-MPA) was obtained from Fluka (Bucks, Switzerland) whereas orthophthalaldehyde (OPA) was obtained from Sigma. Brij (30%) was obtained from Pierce. Water used for preparation of buffers and standards was ion-exchange and sterile (Milli-Q Water Purification System, Millipore).

OPA-reagent

50 mg of OPA were dissolved in 2 ml of methanol, to this 8 ml of 0.4 M borate buffer (pH 10.4 containing 0.6% of 30% Brij) was added. To this mixture, 100 µl of 3-MPA was added. The reagent mixture was kept at 4°C for 24 hrs before use. The reagent is stable for a week.

Preparation of buffer and the gradient used

7.1 g anhydrous Na_2HPO_4 and 6.9 g $NaH_2PO_4 \cdot H_2O$ were dissolved separately in 1 l deionized H_2O to make solutions of 0.05 M concentration. A buffer of pH 7.2 was made by mixing NaH_2PO_4 to Na_2HPO_4 gradually. This buffer is diluted to the concentration of 0.02 M with deionized H_2O. For pump A, mobile phase consisted of 20 mM phosphate buffer: Tetrahydrofuran (99:1) (solvent A) and for pump B 20 mM phosphate buffer: Methanol (30:70)(solvent B) was used. Solvent A and B were filtered through 0.45 µm type HA and HV (Millipore, Bedford, MA, USA), respectively. The gradient used is shown in Fig. 1. The flow

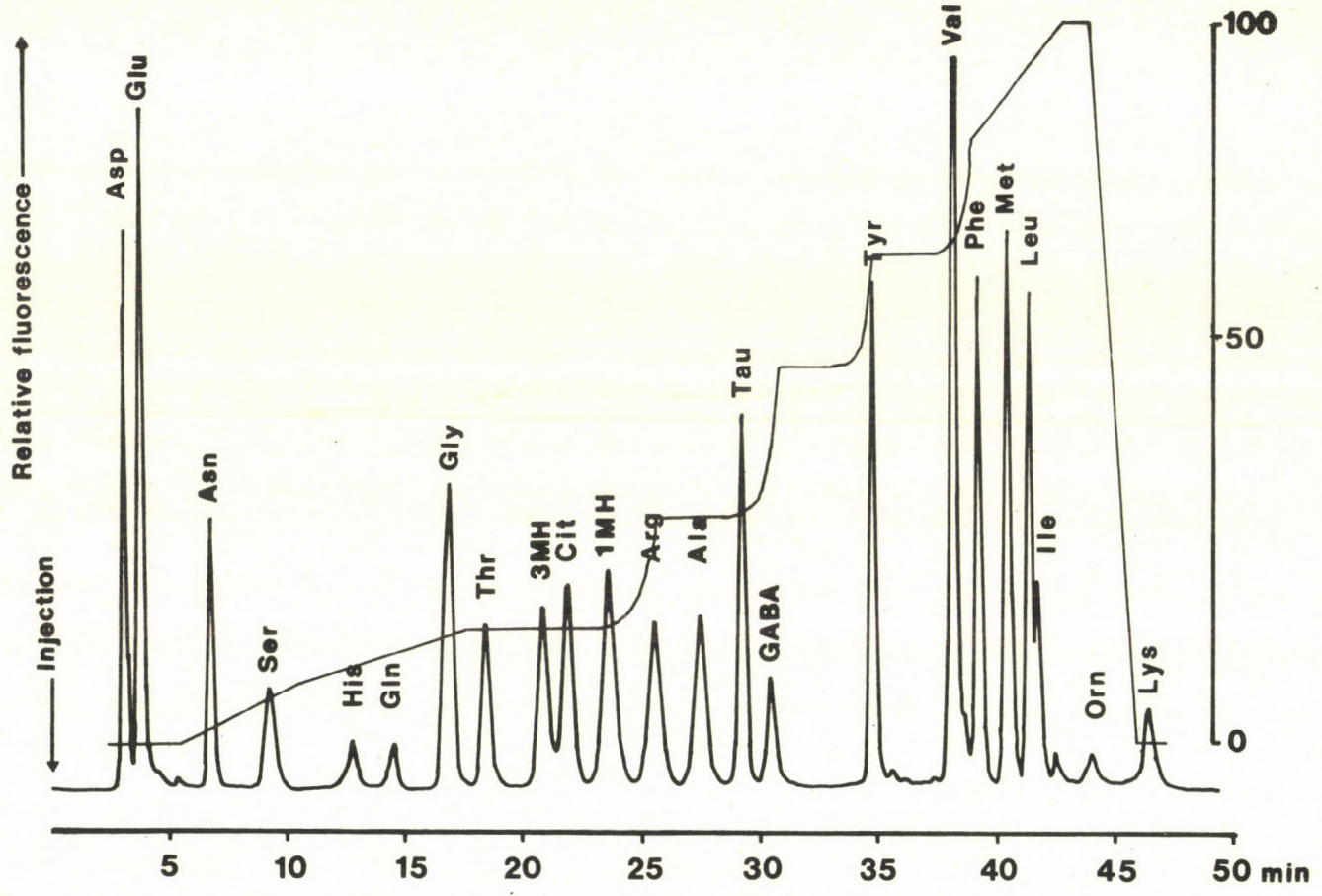

Fig. 1. Chromatogram of a standard mixture of 23 amino acids (20 nm/ml) as OPA-MPA derivatives. Chromatographic conditions are given under experimental.

rate was maintained at 1 ml/min during the run apart from the first 3 min when the flow rate was increased linearly from 0.2 ml to 1.0 ml/min. The column is equilibrated with 100% solvent A for 5 min before and after every run.

Handling of biological samples

200 µl standard mixture of amino acid (100 nm/ml), urine or plasma was treated with the similar volume of either 30% SSC or 1 M $HClO_4$ and centrifugated at 5000 rpm for 15 min. 200 µl of supernatant was added to 300 µl of double distilled H_2O. If not immediately analyzed, this solution was stored at -70°C.

The derivatization of OPA with sample was done according to ref. 18.

Apparatus

The chromatograph consisted of two solvent delivery pumps 6000 A and M 45, multiple sampler 710 B, data module, system controller 730 B, a 420 fluorescence detector equipped with an excitation monochromator (340 nm) and an emission cut-off filter (450 nm), all manufactured by Waters Associates (Millford, MA, USA). Separation of amino acids was carried out on a 5 µm Hypersil-ODS column with 150 x 4.6 mm dimension obtained from Shandon Product Ltd. (Cheshire, England). A pre-column (50 x x 4.6 mm) packed with C_{18} material obtained from Waters Associates was connected to the analytical column. The new analytical column was conditioned first with 50:50 H_2O: Methanol and then with 100% solvent A for 1 hr each before use.

Patients

Plasma and urine were collected from 4 patients with chronic renal failure over 19 days (creatinine clearance < 10 ml/min). The patients were given a diet containing 80 g of meat and providing 40 g of protein per day for the first two days. From the third day, meat was excluded from the diet but the

protein intake remained the same i.e. 40 g per day for the following 17 days. Plasma samples were collected on 3rd, 8th, 10th, 12th, 15th, 17th and 19th day. Urine samples were collected during 24 hrs on 3rd, 7th, 9th, 11th, 16th and 18th day.

All samples collected were kept at -70°C if not analyzed immediately.

RESULTS AND DISCUSSION

3-mercaptopropionic acid (3-MPA) /22, 23/ is recently introduced as an alternative to 2-mercaptoethanol (3-ME) and Ethadiol (E) where it has been shown that OPA in presence of 3-MPA reacting with amino acids forms more stable derivatives with their fluorescence response comparable to 2-ME and E. The reaction of OPA with amino acid in presence of 3-MPA is rapid and takes 2-3 min to complete at room temperature. However, the time-dependence factor is proved to be a hinder in the reproducibility of the results. Thus, using an automatic injector avoids the unstability of derivatives with time factor which otherwise is observed with a manual injector /24/. Besides, the time of the reaction between OPA and amino acids can exactly be controlled. This procedure shows consistent results with SD of 1-2% over triple analyses.

A typical chromatogram of standard amino acid and plasma sample of an uremic patient is shown in Fig. 1 and Fig. 2, respectively. The whole separation takes less than one hour. Apart from the quantitation of 3-MH, His and 1-MH, this method also allows quantitation of 18 other amino acids. As the changes in urinary and plasma levels of several of these amino acids have been correlated with various disease states /25/, this method of analysis could be used for diagnostic purposes.

The correlation coefficient (r) between area response and the molar concentration in range of 5-100 nm/ml per 25 µl injection of His, 3-MH, 1-MH, Tyr and Phe is between 0.983 to 0.998. Fig. 3 shows a linear relationship between the area and concentration of five amino acids.

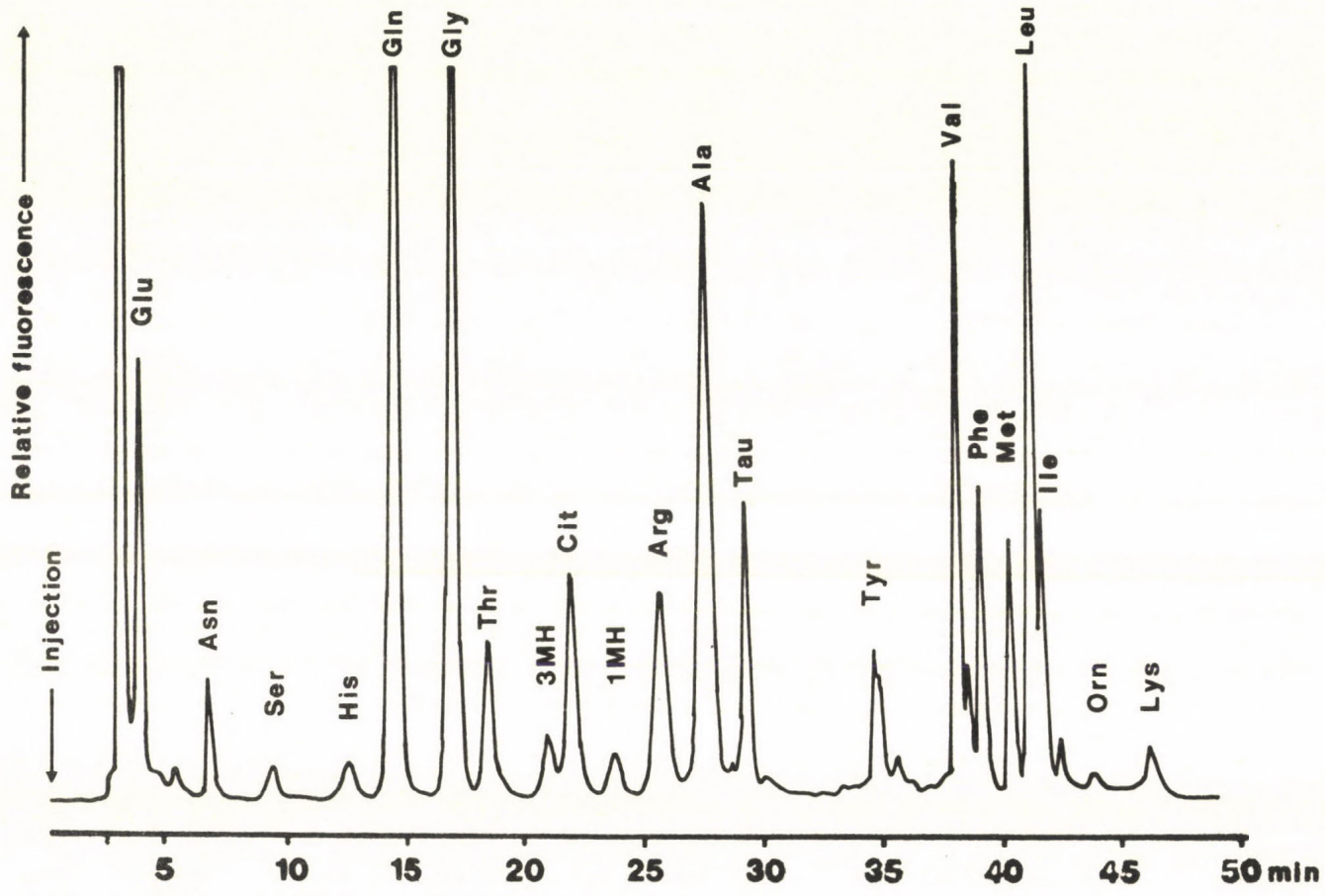

Fig. 2. Chromatogram of plasma sample of a uremic patient under the similar experimental conditions as Fig. 1.

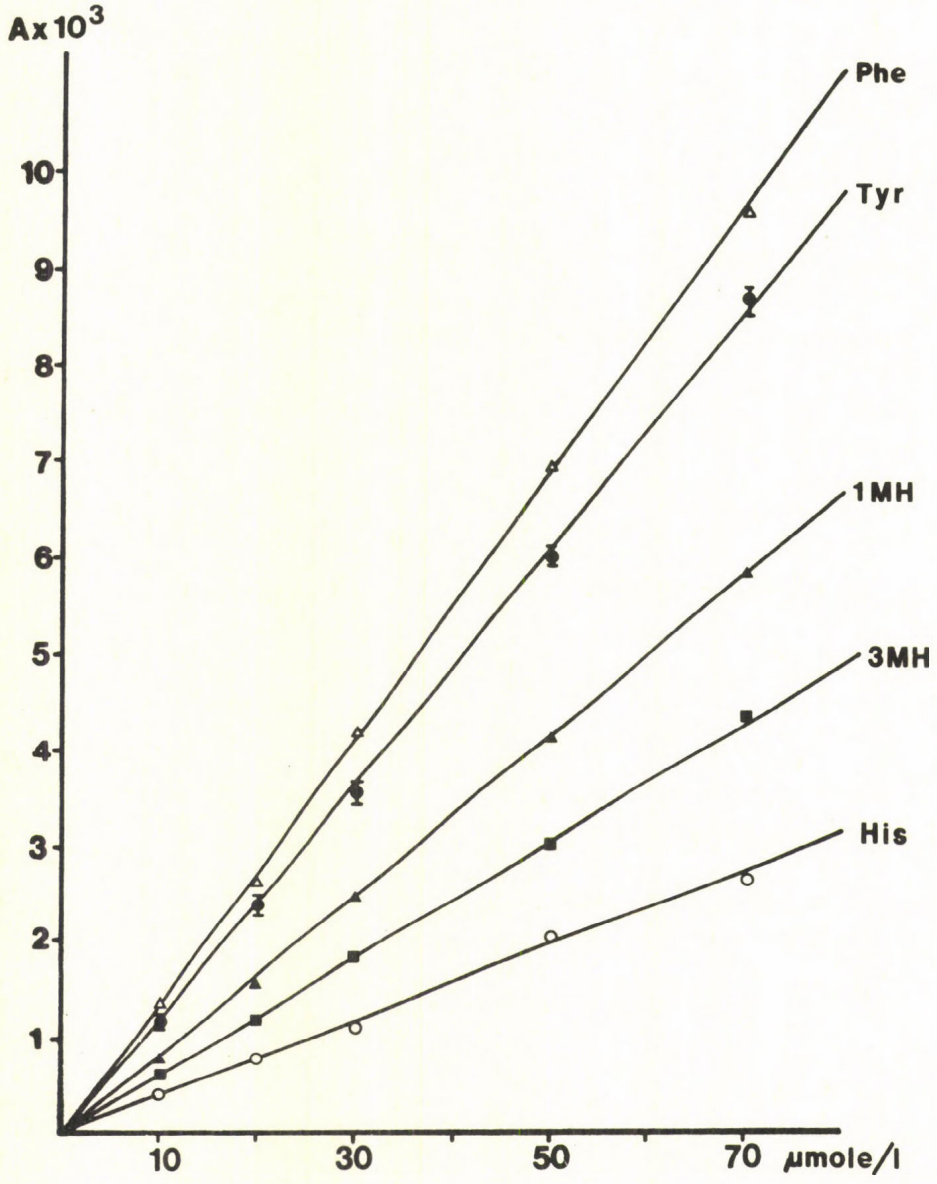

Fig. 3. Representative standard curves for OPA-MPA derivatives of His, 3-MH, 1-MH, Tyr and Phe.

However, by variety of criteria this method of analysis appears to adequately resolve 3-MH, 1-MH and His from other amino acids. No preliminary sample purification other than deproteinization is necessary, thus avoiding the possibilities for technical errors.

Besides, the use of an automatic injector permits reproducible results and eliminates the need for tedious manual injector which may create variable time factor which otherwise gives unreliable results in recording the fluorescence intensity of OPA amino acid adduct.

This method of analysis was applied to study 4 patients with chronic renal failure kept on meat-free diet for 16 days. In all these 4 patients the 3-MH urinary excretion reached a plateau value between 12-14th day of meat-free diet as is shown in Fig. 4. The plasma concentration of 3-MH also showed the same tendency, whereas the concentration of 1-MH and His remained unchanged (Fig. 5). In a previous study /18/ it was shown that patients with chronic renal failure kept on meat-free diet for 8 days showed a continuous decrease in plasma concentration and urinary excretion of 3-MH without reaching a plateau. In healthy individuals the urinary 3-MH excretion equilibrates within 3 days under the similar conditions /27, 28/.

The reason why a prolonged period of adaptation (12-14 days) on meat-free diet is required in patients with severe renal failure is that the renal clearance of 3-MH is reduced in proportion to the reduction in glomerular filtration rate /26/. This long adaptation period may restrain the use of 3-MH excretion for the evaluation of muscle protein breakdown in such patients.

ACKNOWLEDGEMENTS

We are thankful to Ms Monica Larsson for the technical assistance. Thanks are also due to Ms Ann Hellström for typing the manuscript.

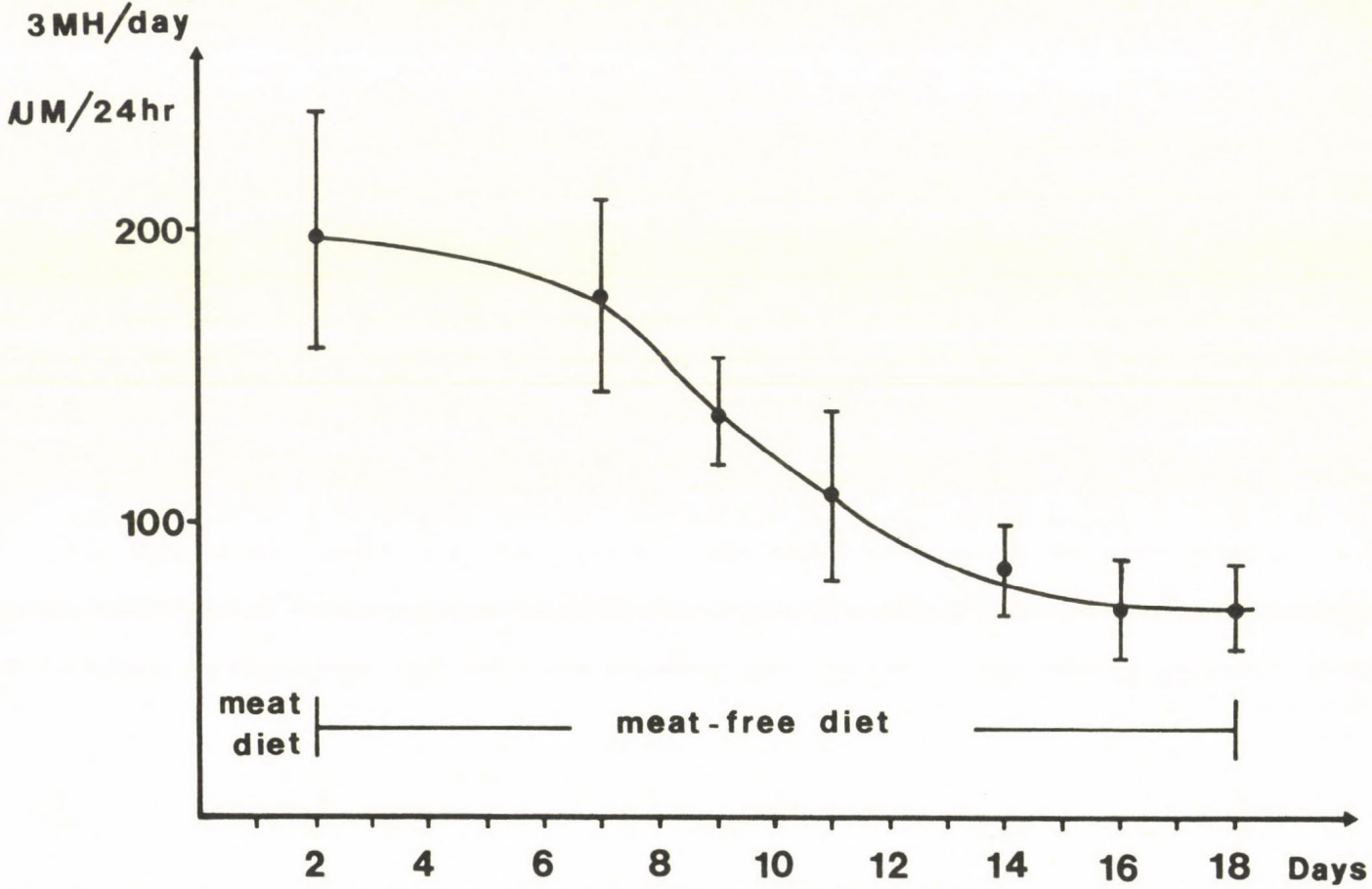

Fig. 4. Urinary excretion of 3-MH in four patients on meat-free diet.

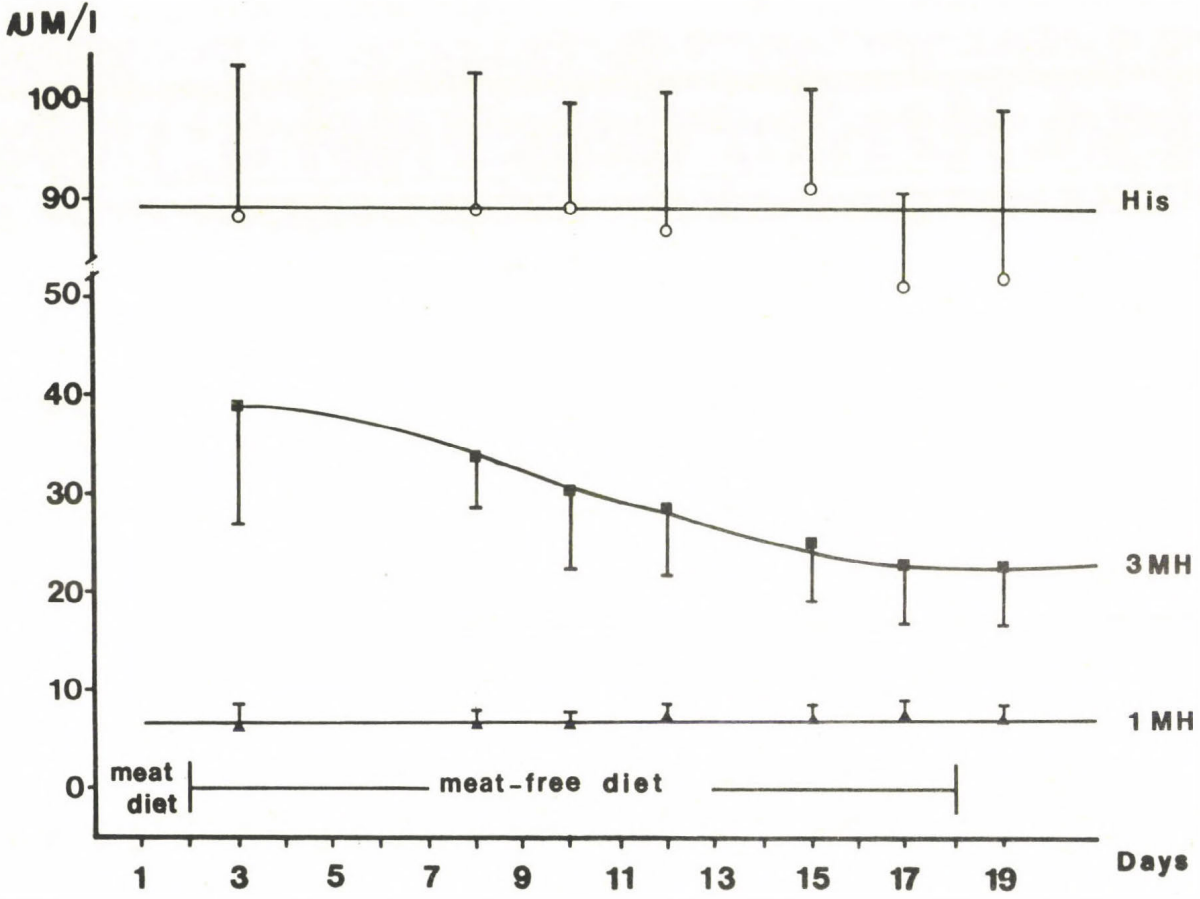

Fig. 5. Dependence of the plasma concentration of His, 3-MH and 1-MH in four uremic patients on meat-free diet.

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H. Kalász and L.S. Ettre (Eds)

HPLC OF PEPTIDES CONTAINING BASIC NON-PROTEINOGENIC AMINO ACIDS

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INTRODUCTION

Non-proteinogenic amino acids incorporated into the sequence of a peptide hormone can be responsible for changes in the biological effects. If the side-chain of an amino acid contains an amino acid-amide bond, it seems to be less flexible and the biological effect can be stronger or more selective. If the peptide binding in the sequence can be substituted for a unit of $-CH_2-NH-$, the peptide becomes also less rigid, is enzyme-resistant and causes different biological effects /1, 2/.

Arginine and lysine often play a key role at the active centre of some peptides. Amino acid analogues containing ethylenediamine (EDA) have been applied to replace these basic amino acids. For this reason they can be used as non proteinogenic amino acids with a basic character. Aminoethyl-glutamine and -isoglutamine derivatives (α- and γ-GluEDA) have been prepared from glutamic acid, and α-GluEDA has been built into position 8 in the sequence of vasopressin.

An amino acid-amide derivative (GlyEDA) has been made from glycine, as the equivalent of a des-carboxyl-lysine analogue. This basic aminoethyl-N^{CO}-glycine amide gives the C terminal part of the analogue: a 9-des $GlyNH_2$, 8-GlyEDA vasopressin, (9-des glycineamide, 8-aminoethyl N^{CO}-glycine amide) vasopressin.

In the same manner the C terminal end of a tuftsin analogue has been formed: Thr-Arg-Pro-GlyEDA, Threonyl-arginyl-prolyl-(aminoethyl)-N^{CO}-glycine amide.

Moreover, we have used a substituted glycine analogue, aminoethyl-glycine (AEG), which is a special amino acid having two basic amino groups and is similar to glycylglycine. This dipeptide was replaced in the sequence of Leu-enkephalin.

These peptides have been prepared by solid-phase synthesis and by known coupling methods.

Their purities were checked by RP-HPLC, on RP C-18 column cartridges /3/. Methanol-water or acetonitrile-water mixtures containing phosphate, acetate or carbonate buffers (pH 2-9) and trifluoroacetic acid were used as the mobile phases.

The purity control of peptides prepared for biological investigations is very important (the HPLC purity grade).

For this, the most suitable procedure is reversed-phase HPLC. The reaction time during the whole synthesis can be followed with this analysis, or the HPLC can be directed to the purification or further separation of given end-products.

EXPERIMENTAL PROCEDURES*

One chromatograph used was a laboratory assembled instrument. Its principal components were an Orlita DMP 1515 (Giessen F.R.G.) pump and variable wavelength photometer fitted to an 8 µl flow cell (CECIL Model 212, Cambridge, Great Britain). The column was 125 mm long, with an i.d. of 4 mm. The column packing material was ODS Hypersil, with a particle size of 5 µm /4/. Injection was performed with a 10 µl 7011 loop injector (Rheodyne, Inc. Berkeley, California, USA).

An LKB instrument with an Uvicord S 2138 photometer, a 2150 HPLC pump and a 10 µl loop injector (Rheodyne) was also used. A 250 mm long Ultropack column, with an i.d. of 10 µm was utilized for separation.

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*ABBREVIATIONS

EDA=ethylenediamine, BOC=tert.-butyloxycarbonyl, Z=benzyloxycarbonyl, M.A=mixed anhydride, Bzl=benzyl, MeCN=acetonitrile, MeOH=methanol, TFA=trifluoroacetic acid, Aeg=aminoethyl-glycine

1. 8-aminoethyl-N^{CO}-isoglutamine vasopressin, (8-GluEDA) vasopressin.

This analogue was prepared by solid-phase synthesis. First it was necessary to synthesize the adequately protected derivative. We used tert.-butyloxycarbonyl α- and γ-benzyl-L-glutamate as the starting materials /5/. From these the amide analogues were synthesized by mixed anhydride methods with monobenzyloxycarbonylethylenediamine /6/.

The BOC-protected derivatives of the two amide analogues were obtained by catalytic hydrogenolysis. In the last step the protected α analogue was produced in a larger quantity by acylation with isobutylchloroformate, while in the case of the γ analogue two derivatives were obtained because of transpeptidation (See SCHEME 1).

The two derivatives IV and VIII were investigated by HPLC (See Fig. 1).

BOC-glycine resin was subjected to eight cycles of deprotection, neutralization and coupling to yield the protected nonapeptidyl resin Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)--Pro-GluEDAZ
|
Gly-R

The resin R was ammonolysed in methanol. The peptide derivative was reduced with sodium in liquid ammonia and reoxidized with potassium ferricyanide /7/. (See SCHEME 2.)

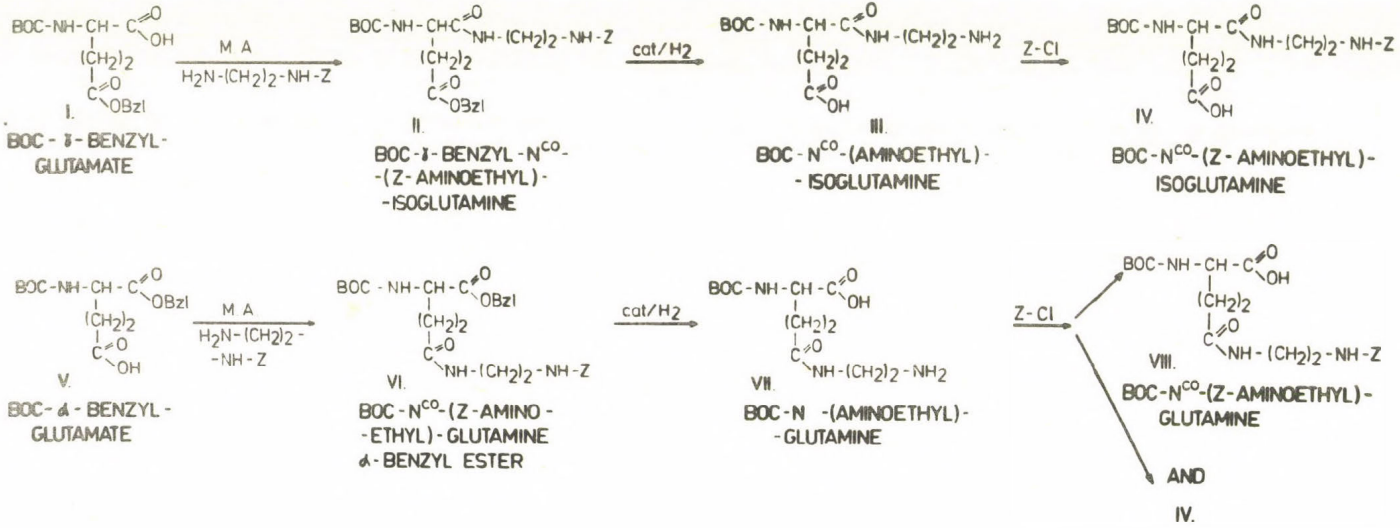
This lyophilized peptide analogue was purified by moderate-pressure liquid chromatography /8/.

LiChroprep RP C-18 (Merck) with a particle size of 25-40 µm was used as the column packing. The column was 610 mm long with an i.d. of 15 mm.

The following gradient was used: buffer A: 0.1 M ammonium acetate (pH=6.5); buffer B: 0.1 M ammonium acetate containing 60% MeCN. A gradient was applied from 0% to 100% B in 192 min. Flow rate: 3.2 mL/min; pressure 1.2 MPa; absorbance:x1 at 277 nm. Fractions were collected manually. The fractions of the main peak (t_R=117 min) were collected and lyophilized.

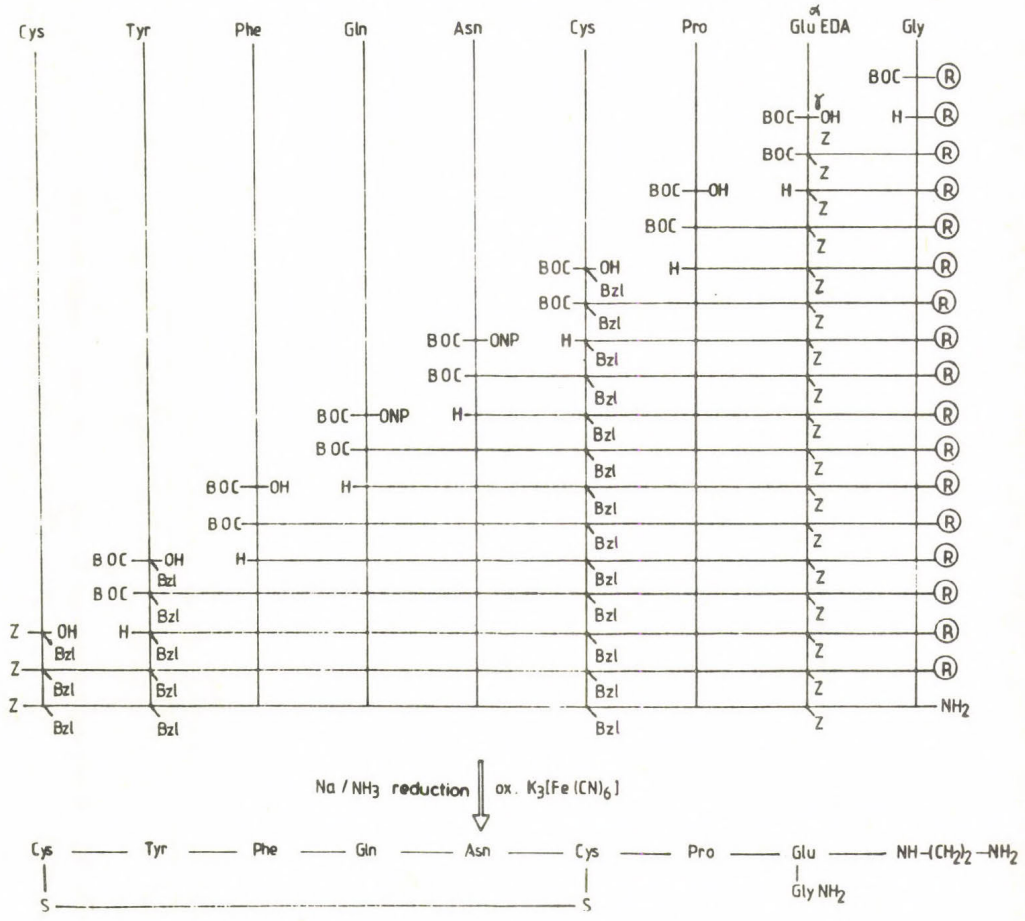
SCHEME 1.

REACTION ROUTES OF α AND γ-GLUEDA DERIVATIVES



SCHEME 2.

THE SYNTHESIS OF [8-GluEDA] VASOPRESSIN



The purity of this analogue was controlled by HPLC using the LKB instrument. (See Fig. 2).

2. 9-des glycineamide, 8-N^{CO}-aminoethyl-glycineamide vasopressin, (9-des $GlyNH_2$, 8-GlyEDA) vasopressin.

This analogue was also prepared by solid-phase synthesis. BOC-glycine-resin was used and the reaction route was the same as for the previous analogue resulting in
Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Gly-R'

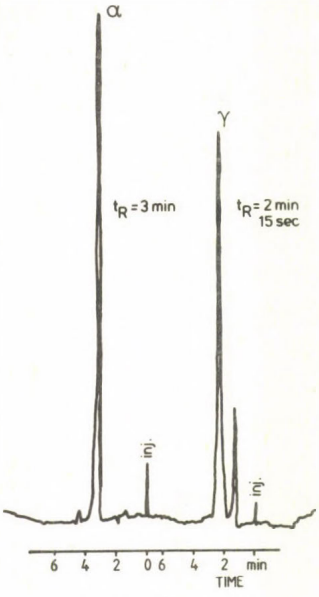


Fig. 1. Separation of derivatives (α) IV and (γ) VIII by HPLC on an ODS Hypersil (125x4 mm) column.
Eluent: 70% MeOH - 30% 0.01 M/L sodium acetate buffer (pH=4); flow rate 0.8 mL/min; 2500 p.s.i; absorbance 0.02 at 254 nm; chart speed 30 cm/hour

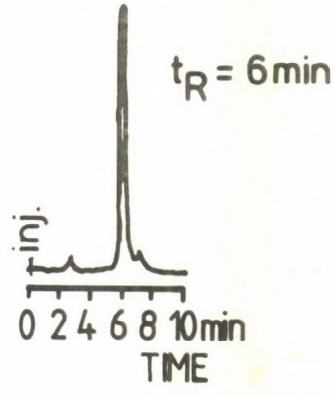


Fig. 2. HPLC control of (8-GluEDA) vasopressin.
LiChrosorb (250x4 mm) column.
Eluent: 20% MeCN - 80% water (0.1% TFA); flow rate 1 mL/min; absorbance: 0.2 at 226 nm; chart speed 12 cm/hour

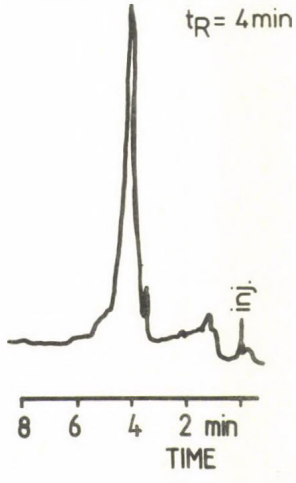


Fig. 3. HPLC control of (9-des $GlyNH_2$, 8-GlyEDA) vasopressin; ODS Hypersil (125x4 mm) column.
Eluent: 50% MeOH - 50% 0.02 M ammonium carbonate buffer (pH=8.5); flow rate 1.3 mL/min; 1900 p.s.i.; absorbance: 0.02 at 254 nm; chart speed: 30 cm/hour

From this the protected octapeptide was split off with ethylenediamine in methanol. The reduction and reoxidation were performed under the same conditions as earlier. (See SCHEME 3.)

Desalting of the product was achieved on Sephadex G 15, elution being carried out with aqueous acetic acid (20%), and purification was completed by gel filtration on Sephadex G 15, elution being achieved with 0.2M aqueous acetic acid.

The purity of this compound was controlled on an ODS column (See Fig. 3) and on a Lichrosorb column (Fig. 4).

Fig. 4. HPLC control of (9-des $GlyNH_2$, 8-GlyEDA) vasopressin; LiChrosorb (250x4 mm) column.
Eluent: 20% MeCN - 80% water (01% TFA); flow rate 1 mL/min; absorbance 0.2 at 226 nm; chart speed 12 cm/hour

3. Threonyl-arginyl-prolyl-N^{CO}-aminoethyl-glycineamide, Thr-Arg-Pro-GlyEDA

Z-Thr-Arg-Pro-GlyEDAZ, the protected tuftsin analogue
NO_2 was synthesized by mixed anhydride and dicyclohexylcarbodiimide (DCC) coupling methods.

SCHEME 3

THE SYNTHESIS OF [9 des Gly NH_2,8-Gly EDA] VASOPRESSIN

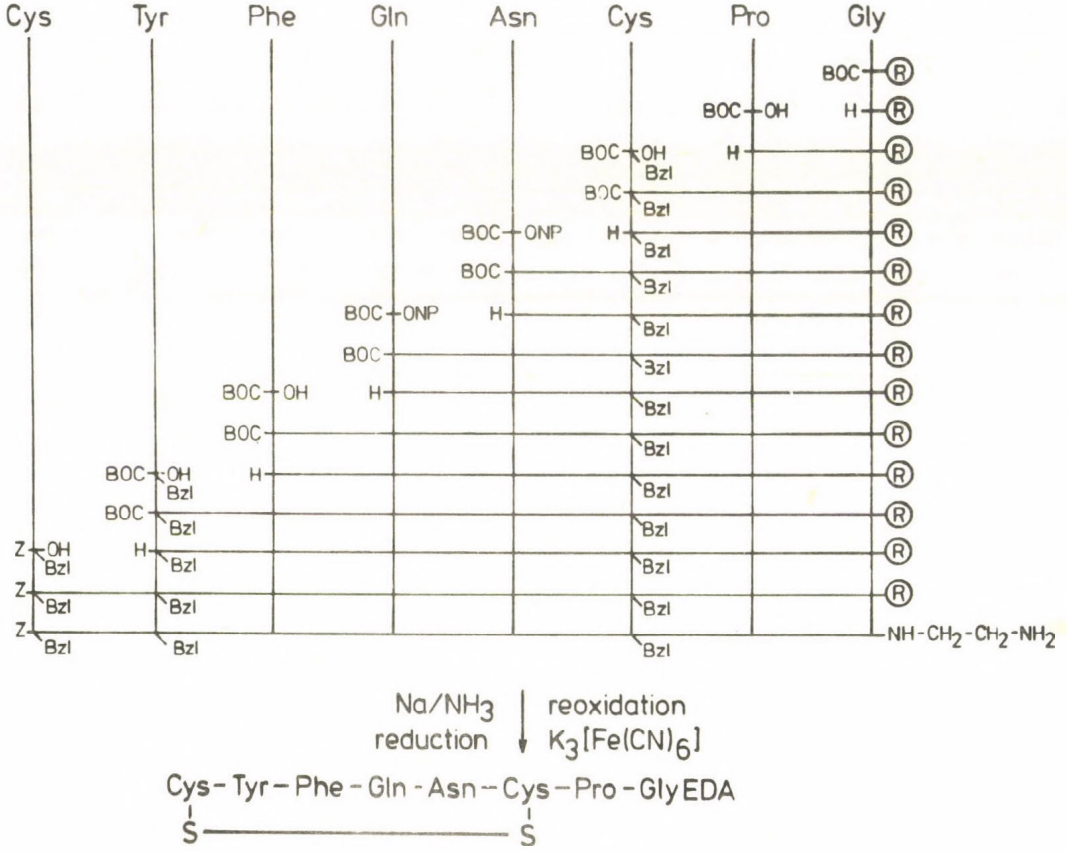


Fig. 5. HPLC control of Thr-Arg-ProglyEDA, a tuftsin analogue. LiChrosorb (250x4 mm) column.
Eluent: 20% MeCN - 80% water (0.1% TFA); flow rate 1 mL/min; absorbance 0.2 at 226 nm; chart speed 12 cm/hour

BOC-protected amino acids were used and BOC was removed with TFA or HCl. H-Thr-Arg-Pro-GlyEDA was obtained by catalytic hydrogenolysis with Pd black.

The purity of this analogue was controlled on a LiChrosorb column /9/ (See Fig. 5).

4. Tyrosyl-aminoethylglycyl-phenylalanyl-leucine-trifluoro-acetate, TFA x H-Tyr-Aeg-Phe-LeuOH

This basic non-proteinogenic amino acid AEG can be substituted for the glycylglycine in Leu-enkephalin. First of all Aeg was first prepared from EDA and monochloracetic acid, and then protected with BOC /10/.

The protected tetrapeptide BOC-Tyr-AEG-Phe-Leu-OMe was prepared by mixed anhydride and DCC coupling methods. BOC protected amino acids were used and, after coupling, BOC was removed with TFA. Hydrolysis was carried out with NaOH.

The purity of this product was controlled on a Hypersil column (see Figs 6 and 7) /11/.

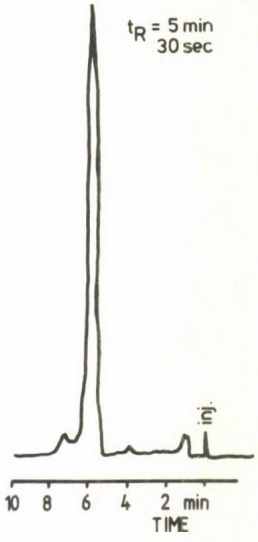


Fig. 6. HPLC control of TFA x Tyr -AEG-Phe-Leu, a Leu-enkephalin analogue; ODS Hypersil (125x4 mm) column. Eluent: 50% MeOH - 50% 0.02 M ammonium carbonate buffer (pH=8.5); flow rate 1.3 mL/min; absorbance 0.05 at 254 nm; 1900 p.s.i.; chart speed 30 cm/hour

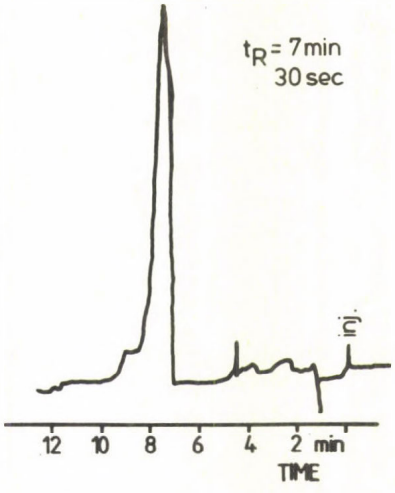


Fig. 7. HPLC control of TFA x Tyr-AEG-Phe-Leu a Leu-enkephalin analogue; ODS Hypersil (125x4 mm) column. Eluent: 60% mEOH - 40% 0.02 M phosphate buffer (pH=2.5); flow rate 1.26 mL/min; absorbance 0.05 at 254 nm; 1450 p.s.i.; chart speed 30 cm/hour

SUMMARY

N^{CO} aminoethyl glutamine and isoglutamine (α- and γ-GluEDA), N^{CO} aminoethyl glycine amide (GlyEDA) and aminoethyl-glycine (AEG), as amino acid derivatives, can replace the basic arginine and lysine in the sequences of the following peptides: vasopressin, tuftsin, Leu-enkephalin.

Liquid chromatographic systems with ODS Hypersil and LiChrosorb reversed-phase materials were used for the separation of these peptide derivatives.

Methanol-water and acetonitrile-water mixtures containing acetate, carbonate and phosphate buffers or trifluoroacetic acid were used as the mobile phase. These methods are applicable for the purity control of these derivatives.

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DECOMPOSITION PRODUCTS OF (-)DEPRENYL REACTED BY HYDROGEN PEROXIDE

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SUMMARY

Basic decomposition products of (-)deprenyl can be detected from the reaction between hydrogen peroxide and (-)deprenyl carried out in a test tube. This reaction may serve as a model for the metabolism of (-)deprenyl and gives the possibility of screening the tentative metabolic pathways prior to the "in vivo" experiments; the results also support some recent data on the oxidative dealkylation of tertiary amines during their "in vitro" transformation.

INTRODUCTION

(-) Deprenyl (Jumex^{R}, Eldepryl^{R}) the firstly described selective inhibitor of B-type monoamino oxidase (MAD) and still the only one in clinical practice (for review see /1/), is now widely used as adjuvant to levodopa, thereby in the treatment of Parkinson's disease (for review see /2/).

The basic metabolites of (-)deprenyl, published earlier, are amphetamine, methamphetamine /3/, as well as propargylanara /4-6/. These metabolites were identified using gas chromatography /3/, thin-layer chromatography (TLC) /4/, spacer-displacement thin-layer chromatography (SD-TLC) /5, 6/. A recent report in the literature describes the metabolism of (-) - deprenyl by non-mammalian organisms, namely the metabolic profile of (-)deprenyl in Cunninghamella echinulata, fungi which possess a cytochrome P-450 system /7/. At the same time, novel

data strongly suggest an alternative way of demethylation of tertiary amines that is the oxidative demethylation utilizing the peroxidase/H_2O_2 system /8/.

MATERIALS AND METHODS

Solvents and chemicals as chloroform, diethylether, sodium hydroxide, sodium chloride, triethanolamine were purchased from Reanal (Budapest), (-)deprenyl [N-methyl-N-propargyl-(2-phenyl-1-methyl)-ethyl ammonium chloride]was obtained from Chinoin (Budapest, Hungary).

TLC silica plates F_{254} were purchased from E. Merck (Darmstadt, FRG). Test Dye Mixture II was obtained from CAMAG Inc. (Muttenz, Switzerland).

Spacer displacement thin-layer chromatography was performed using silica gel stationary phase, the running solvent was chloroform - triethanolamine (95:5 v/v), the samples were spotted at a distance of 30 mm from the bottom edge of the plate and the Dye-Mixture II was lined parallel to the bottom edge of the TLC plate at a distance of 25 mm.

Gas chromatography - mass spectrometry was performed using a Hewlett-Packard HP-5985B instrument. The stationary phase was methylsilicone, the diameter and length of the capillary tube were 0.5 mm and 25 meter, respectively, the carrier gas was helium.

Reaction of (-)deprenyl with hydrogen peroxide: 1 mg of (-)deprenyl was dissolved in 0.4 ml phosphate buffer, and 0.2 mg of hydrogen peroxyde was added. In the 5th hour of the reaction 0.1 g of sodium chloride, 1 ml of 0.5 mol/l sodium hydroxide solution and 1 ml of diethyl ether was added to the reaction mixture. The mixture was shaken for 5 minutes and the aqueous layer was frozen at -35°C. The ether layer was removed from the top of the aqueous layer and was taken into dryness.

RESULTS

The pH optimum of the reaction was checked using spacer displacement thin-layer chromatography. The results are given

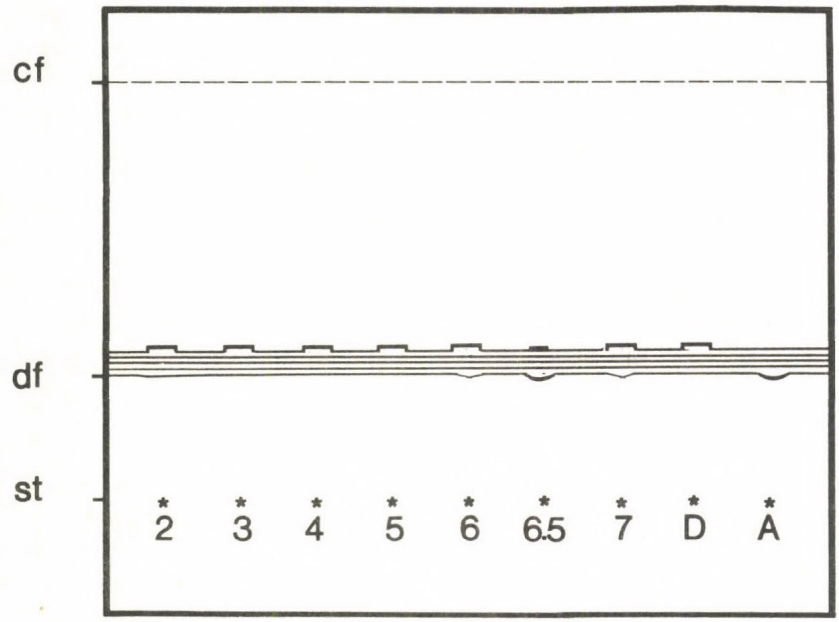


Fig. 1. Optimization of pH for reaction between deprenyl and hydrogen peroxide. Abbrevations: st = start; df = displacer front; cf = carrier front; 2, 3, 4, 5, 6, 6.5, 7 = spots of products of reactions performed at pH 3, 4, 5, 6, 6.5 and 7, respectively; The stationary phase, carrier and displacer were silica plate, chloroform and 5% triethanolamine in chloroform. Camag Test Substance No. II was used as spacer.

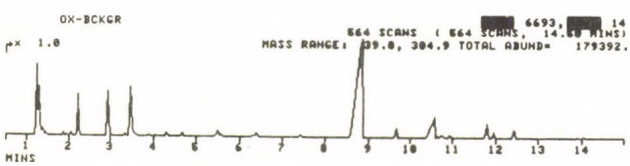
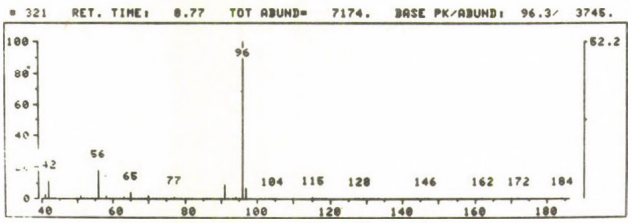


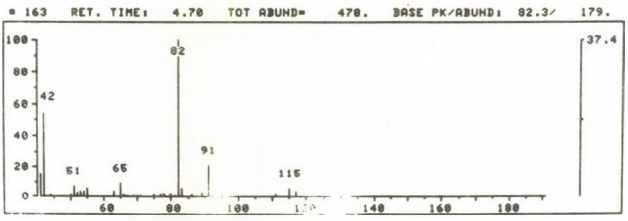
Fig. 2. Gas chromatogram of the reaction mixture.

in Fig. 1. The fragmentation of (-)deprenyl may be best observed when a pH = 6.5 buffer was used.

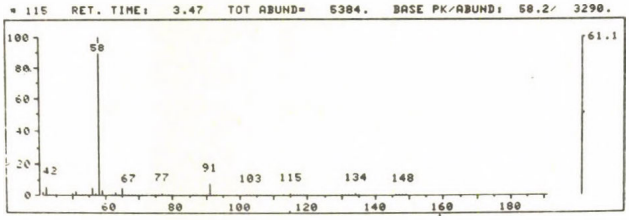
Reaction products were investigated by gas chromatography-mass spectrometry. Fig. 2 depicts the gas chromatogram recorded by total ion monitor, while Fig. 3 gives the mass spectra of the substances present in the reaction mixture, i.e. that of



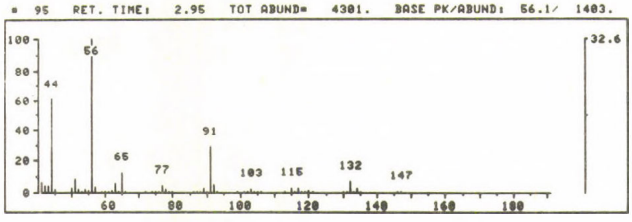
3/a



3/b



3/c



3/d

Fig. 3. Mass spectra of the individual peaks of the gas chromatogram. a: (-)deprenyl, b: propargylanara, c: methamphetamine, d: amphetamine.

unaltered (-)deprenyl, propargylanara, methamphetamine and amphetamine can be seen in Figs 3a, 3b, 3c and 3d, respectively.

DISCUSSION

The N-dealkylation of tertiary amines is generally regarded as the consequence of action of oxidizing enzymes /9/ when the cytochrome P-450/NAD(P)H is also involved in the procedure.

The presence of hydrogen peroxide in the reactions with participation of some oxidative enzymes, e.g. MAO has also been known /9/. Data in the literature support this view proving that the endproducts of biotransformation of (-)deprenyl by Cunninghamella echinulata ATCC 9244 are amphetamine and methamphetamine /7/. These results were explained /7/ by the similarity of cyctochrome P-450 system of C. echinulata to that of the mammalian organisms. A recent publication /8/ suggested that in addition to oxidative reactions catalyzed by the cytochrome P-450 system. An alternative way can be the transformation facilitated by the peroxidase/H_2O_2 systems, both types of reactions yield the corresponding secondary amine and formaldehyde /8/. Similar reaction route was shown in the cases of O-demethylation /8/.

The presence of hydrogen peroxide in the reactions with participation of some oxidative enzymes, e.g. MAO has also been observed /9/.

In our recent work, the identification of the metabolites of (-)deprenyl was done using spacer-displacement thin-layer chromatography (SD-TLC) /5-6/. These methods were also used in this experimental series, where the pH optimum was checked by SD-TLC, and the metabolites were identified by GC-MS. In the course of the identification work, not only the mass-fragments of the generated compounds were taken into consideration, but also the mass spectra of the standard compounds were compared to that of the reaction products.

The results indicate that the pH optimum of the oxidative dealkylation reaction generated by hydrogen peroxide shows a definite optimum at pH = 6.5. Furthermore, each of the known

metabolites of (-)deprenyl can be identified from the reaction products, that is propargylanara, methamphetamine and amphetamine.

CONCLUSIONS

(-)Deprenyl can be transformed into its main metabolites by the reaction with hydrogen peroxide. In the case of similar types of tertiary amines, this reaction gives the possibility of scouting the potential metabolites prior to the "in vivo" experiments. At the same time, these experiments render indirect proof that the formation of amphetamine, methamphetamine and propargylanara are the results of the oxidative dealkylation of (-)deprenyl during its metabolism.

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SEPARATION OF ARYLPROPARGYLAMINES

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The present paper concerns the development of methods for the separation and identification of aromatic propargylamines.

The synthesis of amines was carried out by interaction of primary or secondary aromatic amines with propargylbromide. The reaction proceeds according to the following scheme:

$$R_1\text{-}\underset{\displaystyle R_2}{\underset{|}{N}}\text{-}H + BrCH_2\text{-}C{\equiv}CH \longrightarrow R_1\text{-}\underset{\displaystyle R_2}{\underset{|}{N}}\text{-}H^{+} \ldots CH{\equiv}C\text{-}CH_2Br^{-}$$

$$\xrightarrow{NaOH + H_2O} R_1\text{-}\underset{\displaystyle R_2}{\underset{|}{N}}\text{-}CH_2\text{-}C{\equiv}CH + NaBr + H_2O$$

R_1 = H, or C_6H_5; R_2 = C_6H_5, or $C_6H_4COCH_3$

In such a way phenyl-, diphenyl- and acetylphenylpropargylamines were prepared (for abbreviations see Table I). As a result a mixture of aromatic amines is obtained.

Table I. Abbrevations used to identify the amines

NN	Abbrevation	Chemical name
1	PPA	phenylpropargylamine
2	DPPA	diphenylpropargylamine
3	APPA	acetylphenylpropargylamine
4	DPA	diphenylamine
5	APA	acetylphenylamine

The amines were separated by adsorption chromatography. The applied glass columns had a length of 80 cm, an I.D. of 1.5 cm, and a flow rate ~1 ml/min. Brockman II neutral Al_2O_3 was used for the separation. Accordingly a weakly polar eluent (mixture of 10 % acetone and 90 % heptane) was selected /1/. This eluent was used for the separation of aniline - PPA and diphenylamine (DPA) - DPPA. A mixture of 30 % acetone and 70 % heptane as the eluent was used for the separation of acetylphenylamine (APA) and APPA. The isolated products were identified by thin-layer chromatography using glass plates with an Al_2O_3 layer or Silufol UV plates. Aniline, DPA and APA were used as the test mixture.

The development of the plates consists of treatment of the plates by CBr_4 with subsequent UV-irradiation. CBr_4 forms donor-acceptor complexes with the aromatic amines.

The photochemical reactions of the donor-acceptor complexes occurring under UV-irradiation lead to the formation of coloured products.

We have investigated the formation of donor-acceptor complexes based on aromatic propargylamines and CBr_4 /1/. The ratio of the components is identified by means of the isomolar series technique /2/. It is equal to 1:1. The coefficient of molar extinction of the complexes and the complex formation constant K is estimated using the Benesi-Hildebrand relation /3/.

Parameters of the donor-acceptor complexes of PPA, DPPA and APPA with CBr_4 are shown in Table II.

Table II. Parameters of donor-acceptor complexes of PPA, DPPA, and APPA with CBr_4

Amine	Ratio of the components	hν, ev	ε, l $mol^{-1}cm^{-1}$	K, l mol^{-1}
PPA	1:1	3.90	$5.0 \cdot 10^2$	0.61
DPPA	1:1	3.40	$3.3 \cdot 10^2$	1.10
APPA	1:1	3.65	$1.9 \cdot 10^2$	0.11

hν - energy transfer, ε- coefficient of molar extinction, K - equilibrium constant.

The values of K suggest that the bond between the amine and CBr_4 in these complexes is weak. In the case of weak donor-acceptor complexes electron transfer from the donor to the acceptor can only be realized under photoexcitation.

The photoinduced electron transfer from amine to CBr_4 leads to the formation of coloured products. The colour of the resulting products depends on the nature of the amines under study. The structure of the coloured products was investigated in the reaction between DPPA and CBr_4 /4/.

The coloured product was isolated and its structure and properties investigated. It is a green compound with a melting point of 30°C. The absorption spectra of the initial DPPA (curve 1), the donor-acceptor complex of DPPA with CBr_4 (curve 2) and the coloured product formed (curve 3) are shown in Fig. 1. The coefficient of molar extinction at 670 nm is equal to 4.0×10^3 $l \cdot mol^{-1} cm^{-1}$. With the help of this value, the quantum efficiency of the dye formation was determined. It is found to be equal to 0.18.

The products absorbs at a greater wavelength than the initial compound, which means that the length of effective conjugation increases.

Proton magnetic resonance investigation indicates two phenyl and one acetylene groups, the disappearance of the methylene group and the appearance of a wide signal of one proton. The carbon-carbon triple bond is also evidenced by the presence of bands at 650 cm^{-1}, 2120 cm^{-1} and 3300 cm^{-1} in IR-spectroscopy data.

These data suggest that the photochemical reaction involves the loss of a hydrogen atom by the methylene-group and the formation of the carbon-nitrogen double bond.

Accordingly, the propargyl group becomes conjugated with the phenyl ring and the length of effective conjugation increases. These experimental data allowed to conclude that the coloured product has the structure of $(C_6H_5)_2\overset{+}{N}(\bar{Br})=CH-C\equiv CH$. The presence of bromine is estimated by mass-spectral investigation: the molar mass is equal to 286.

The mechanism according to which this compound is formed depends on whether the incident light wavelength is situated

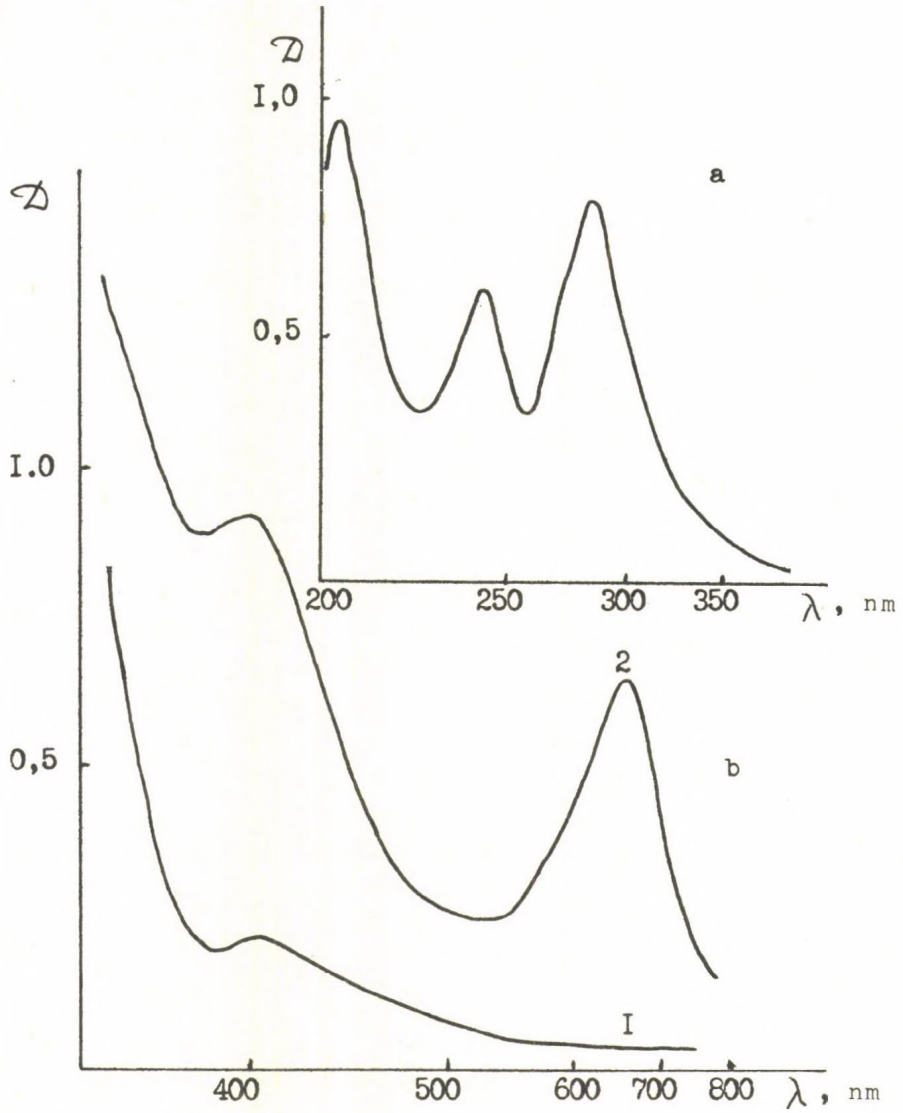

Fig. 1. The absorption spectra of DPPA (a), the donor-acceptor complex of DPPA with CBr_4 (b-1) and the resulting coloured product (b-2).

within the DPPA own absorption band (λ = 280 $\pm$ 20 nm) or the charge transfer band (λ = 365 $\pm$ 15 nm).

The photoexcitation in the charge transfer band appears to lead to the occurrence of energy transfer from the excited donor-acceptor complex to free DPPA molecules with the formation of a "collision" complex /5/.

We have shown /4/ that the formation of the coloured product from DPPA by photoexcitation in the charge transfer band occurs as follows:

(a) $DPPA \cdot CBr_4 \xrightarrow{h\nu} {}^1(DPPA \cdot CBr_4)^*$ - photoexcitation of the complex.

(b) ${}^1(DPPA \cdot CBr_4)^* \xrightarrow{k_1} DPPA \cdot CBr_4$ - deactivation of the singlet state of the complex.

(c) ${}^1(DPPA \cdot CBr_4)^* \xrightarrow{k_{s-t}} {}^3(DPPA \cdot CBr_4)^*$ - singlet-triplet transition.

(d) ${}^3(DPPA \cdot CBr_4)^* \xrightarrow{k_2} DPPA \cdot CBr_4$ - deactivation of the triplet state.

(e) ${}^3(DPPA \cdot CBr_4)^* + DPPA \xrightarrow{k_3} DPPA + {}^3DPPA^*$ - triplet energy transfer from the donor-acceptor to DPPA.

DPPA in the triplet state forms a "collision" complex with a nonexcited CBr_4 molecule:

(f) ${}^3DPPA^* + CBr_4 \xrightarrow{k_c} {}^3(DPPA)^* \cdot CBr_4$

The photodissociation of this complex results in a coloured product whose structure was discussed above:

(g) ${}^3(DPPA)^* \cdot CB_r4 \xrightarrow{k_d} (DPPA^+ \cdot + Br^- \ldots CBr_3 \cdot) \xrightarrow{k_p}$

$(C_6H_5)_2\overset{+}{N}(Br^-)=CH-C\equiv CH + CHBr_3$

(k_1, k_{s-t}, k_2, k_3, k_c, k_d, k_p are the rate constants of the corresponding reactions).

As shown above, the colour of the resulting product depends on the chemical constitution of the initial time. Thus, the colour of the products is as follows:

PPA - brown	aniline - orange
DPPA - green	diphenylamine - blue
APPA - lemon	APA - yellow

Additionally we have shown that the adsorption power of aromatic amines is defined by the structure of the amine. It is decreased on passing from primary to tertiary amines. The presence of conjugated double bonds or polar groups in the amine molecules leads to an increasing of absorption power. The following sequence is valid:

$(C_6H_5)_2NCH_2-C{=}CH$ (DPPA) < $(C_6H_5)NH-CH_2-C{\equiv}CH$ (PPA) <

$(C_6H_4COCH_3)NH-CH_2-C{\equiv}CH$ (APPA) < $(C_6H_5)_2NH$ (DPA) <

$(C_6H_5)NH_2$ (aniline) < $(C_6H_4COCH_3)NH_2$ (APA)

Thus the formation of donor-acceptor complexes of aromatic amines with CBr_4 and the subsequent photochemical transformation permits the clear identification of the aromatic propargylamines.

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RETENTION REGULARITIES OF AMINO COMPOUNDS ON STATIONARY PHASES CONTAINING METAL STEARATES

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Retention of substances in stationary liquid phase containing a component being able to form complexes with sorbate molecules depends on the concentration of the complexing agent /1, 2/. If polar substances are analyzed, the influence of interfacial sorption on retention must also be considered. A method for the evaluation of complex formation and interfacial sorption effects on retention of substances has been offered in the literature /3-5/. This method requires the measurements of retention volumes on two sets of columns (with and without the complexing agent); moreover, this model also accounts for adsorption on the surface of the uncovered solid support directly from the gas phase /6/.

In the present study we consider both the influence of complexation and interfacial sorption in typical conditions of gas-liquid chromatography - when the support surface is completely coated with the stationary phase - using the general approach developed by Berezkin et al. /7, 8/. Assumptions made are that in the complexing process only the molecules distributed in the liquid phase volume take part and that only the solute molecules not fixed in the complex are adsorbed on the support surface.

In the full differential equation of equilibrium elution chromatography of a single substance under the action of longitudinal elution factors the term $\beta_k \frac{\partial c_k}{\partial t}$ was introduced by Andersons and Mekšs /9/ to account for complex formation:

$$u\frac{\partial c_G}{\partial x} + \beta_G\frac{\partial c_G}{\partial t} + \beta_{GL}\frac{\partial c_{GL}}{\partial t} + \beta_L\frac{\partial c_L}{\partial t} + \beta_k\frac{\partial c_k}{\partial t} + \beta_S\frac{\partial c_S}{\partial t} = D^*\frac{\partial^2 c_G}{\partial x^2} \quad (1)$$

where u - carrier gas linear velocity, x - the coordinate, t - the time, c_G, c_L, c_{GL}, c_S, c_k - the concentrations of the chromatographed substance in the gas and liquid phase, at the gas-liquid and liquid-solid interfaces and fixed in the complex, respectively; β_G, β_L, β_k - the fractions of the gas, liquid phase and complexing agent in the cross-section of the column, respectively; β_{GL} and β_S - the ratios of the gas-liquid and liquid-solid interfaces to the sorbent volume, D* - the effective longitudinal diffusion coefficient. Combining eq.(1) with the corresponding equations of the sorption isotherms:

$$c_L = f_L(c_G) \quad (2)$$

$$c_{GL} = f_{GL}(c_G) \quad (3)$$

$$c_k = f_k f_L[A](c_G) \quad (4)$$

$$c_S = f_S f_L(1-f_k[A])(c_G) \quad (5)$$

and solving the obtained equation by the methods described by Rachinskii /10/ and assuming that the isotherms are linear we obtain:

$$V_N = K_L V_L + K_{GL} S_L + K^* K_L m_k \frac{V_k}{V_L} + K_S K_L (1-K^*\frac{m_k}{V_L}) S_S \quad (6)$$

(V_N - the net retention volume, V_L and S_L - the volume and the surface area of the liquid phase in column, V_k and m_k - the volume and the mass of the complexing agent in column, K_L, K_{GL}, K_S - partition and interfacial adsorption coefficients, K* - complex formation constant).

Equation (6) offers the possibility of simultaneous evaluation of the contributions of the separate sorption process to V_N and the determination of the sorption coefficients and complexation constants assuming a linear dependence of V_N on the concentration of the complexing agent /A/. This equation is valid if the sorbate forms 1:1 complexes with the complexing agent.

We have applied equation (6) to the investigation of the sorption of C_4-C_{15} n-di- and trialkylamines, arylamines, heterocycles of pyrrole, pyridine, piperidine, and pyrazine series

at 125, 140 and 150°. The stationary phases used were Apiezon M (15 % on Celite 545, 40-60 mesh) modified with copper, nickel and cobalt stearates (0.1; 0.18 and 0.24 mole/l). Experiments were carried out using gas chromatographs Varian Aerograph 2068 and Yanagimoto GCG-5DH; helium was used as the carrier gas at 40-50 ml/min. Data compiled in the tables are based on experiments carried out at 140°; retention of amines at 125 and 150° was analogous to the data established at 140°.

Sorption of amino compounds in binary liquid phases consisting of Apiezon M and transient metal stearates is mainly determined by the processes occurring in the volume of the liquid phase - by complexation and physical intermolecular interactions - and to some extent by adsorption on the solid support surface. Adsorption of amines on the gas-liquid interface is negligible (its contribution to V_N is about 0.1 %).

Complex stability constants K* (Table I) show that the ability of transient metals to form complexes with amines increases in the series Ni<Cu<Co - along with the increase of the ionic radii, ionization energies and potentials of the metals (excepting primary arylamines that form the most stable complexes with nickel). The more stable complexes with metal stearates are formed by dialkylamines and methylpyridines, the less stable complexes are characteristic for trialkylamines and N-alkylanilines, as ligands. The stability of the complexes is lower when the nitrogen atom in the amine molecule is sterically hindered or when the electron density at the nitrogen atom is decreased.

The influence of physico-chemical and structural parameters of amines on their complexing ability was evaluated by means of regression analysis of the K* values. As follows from the data given in Table II, the basicity of the amines is of great importance at complexing with transient metal stearates. For the alkylamines studied the value of the ionization potential, IP, is the dominating factor in the complexing process (correlation coefficient r for single-factor regression equation amounts to 0.95-0.96). In the case of nitrogen heterocycles the K* values are better correlated with the energy values of the highest occupied molecular orbital (HOMO) of the

Table I. Complex formation constants and relative contributions of the complex formation process to the total net retention volumes of amines at 140°

Amines	K*, l/mole			$K^*K_L \frac{V_k^s}{V_L^s} m_k^s \frac{100}{V_N^s}$		
	Apiezon M +			Apiezon M +		
	$Ni(C_{17}H_{35}COO)_2$	$Cu(C_{17}H_{35}COO)_2$	$Co(C_{17}H_{35}COO)_2$	$Ni(C_{17}H_{35}COO)_2$	$Cu(C_{17}H_{35}COO)_2$	$Co(C_{17}H_{35}COO)_2$
n-Dialkylamines	53 - 55	62 - 63.5	70.8 - 70.9	62 - 63	65 - 66	64
n-Trialkylamines	1.2 - 2.0	1.5 - 2.2	4.5 - 4.8	3 - 5	4 - 6	10 - 11
Aniline	14.0	8.2	10.8	30.2	20.2	21.5
o-Toluidine	10.8	7.3	7.4	25.2	18.4	15.2
m-Toluidine	17.1	8.9	11.2	34.5	21.9	22.4
p-Toluidine	26.2	12.5	14.9	44.5	28.0	28.1
N-Alkylanilines	1.8 - 2.3	2.6 - 2.8	4.2 - 4.3	5.0 - 6.5	7.5 - 7.9	9 - 10
N,N-Dialkylanilines	1.5 - 1.6	1.8 - 1.9	3.1 - 3.5	4.3 - 4.5	5.0 - 5.5	6.1 - 7.5
Pyrroles	1.7 - 2.2	2.4 - 3.0	4 - 5	5 - 6	7 - 8	10 - 11
2-Methylpyridine	21.4	29.4	30.2	39.8	47.6	43.4
2,4- and 2,5-Dimethyl-pyridines	38 - 55	42 - 52	45 - 55	54 - 64	56 - 62	52 - 58
2,6-Substituted methylpyridines	3 - 4	8 - 9	4 - 6	10	21 - 22	9 - 10

donor E_{HOMO}. The importance of amine basicity in the complexing conforms with the data of Mulliken /11/, Rose /12/, and Laub and Pecsok /13/ establishing the existence of a correlation between the values of the stability constants of charge-transfer complexes and the electron-donor activity of the donor molecules. The sterical hindrance to the nitrogen atom seems to be more important in the case of arylamines.

Bier /14/ established that in general, no correlation was observed between the complexing ability and the dipole moments, μ, of the molecules for charge-transfer complexes. This factor (Table II) is significant only for complexes formed by linear alkylamine molecules. The coefficients of the ln K* vs. μ^2 correlation increase in the series Co<Ni<Cu which corresponds to the increase of the polarizing ability of the ions.

Gas chromatography data also permit to make some assumptions concerning the type of the bonding in the formed complexes. The existence of correlation between K* and the E_{HOMO} values for heterocycles (Table II) indicates that the formation of the donor-acceptor bond is realized by a transfer of the electron density from the highest occupied molecular orbital of the nitrogen base molecules to the vacant d-orbitals of the metals. On the other hand, correlation of K* with the energy of the lowest vacant molecular orbital E_{LVMO} indicates the possibility of the formation of the dative bond by transfer of electrons from the metal to the lowest vacant molecular orbital (LVMO) of azaheterocycles. In the cases of complexing with cobalt stearate the formation of the donor-acceptor bond seems to be prevailing, the dative bond with participation of LVMO of amine being far less significant.

Model equations representing ln K* as a linear combination of the values of ionization potentials (excess charge on the N atom), dipole moments and the sum of Palm's steric constants ΣE_S^o (Table II) are found to adequately describe the behaviour of the studied set of amines.

Contribution of the complexing process to V_N of amines (Table I) constitute 50-70 % only in the case of dialkylamines, 2,4- and 2,5-dimethylpyridines; only in these cases (at K*>40 l/mole) the complexation process is the dominating type of the

Table II. Characteristics of the correlation between the sorption coefficients, and the physico-chemical and structural parameters of amino compounds[a)]

	Apiezon M +					
	$Cu(C_{17}H_{35}COO)_2$		$Ni(C_{17}H_{35}COO)_2$		$Co(C_{17}H_{35}COO)_2$	
	r	F	r	F	r	F
1	2	3	4	5	6	7
Alkylamines						
$\ln K^* = b_o + b_2\mu^2$	0.930	39	0.910	34	0.890	27
$\ln K^* = b_o + b_3$ IP	0.720	6	0.643	5	0.743	9
$\ln K^* = b_o + b_2\mu + b_3$ IP $+ b_4\Sigma E_S^o$					0.996	196 0.155
($b_o = -9.21$; $b_2 = 3.40$; $b_3 = 1.21$; $b_4 = 0.27$)						
Arylamines						
$\ln K^* = b_o + b_4\Sigma E_S^o$	0.911	29	0.875	20	0.865	18
$\ln K^* = b_o + b_3$ IP	0.962	74	0.955	62	0.949	55
$\ln K^* = b_o + b_2\mu + b_3$ IP					0.981	63 0.140
($b_o = -25.09$; $b_2 = 1.87$; $b_3 = 3.25$)						
Azaheterocycles						
$\ln K^* = b_o + b_6 E_{HOMO}$	0.875	25	0.820	14		
$\ln K^* = b_o + b_7 E_{LVMO}$	0.895	31	0.810	13		
$\ln K^* = b_o + b_2\mu + b_4\Sigma E_S^o + b_8 q_N$					0.994	153 0.148
($b_o = -2.49$; $b_2 = 1.75$; $b_4 = 5.24$; $b_8 = -6.54$)						
Alkylamines						
$\ln K_L = b_o + b_1 M$	0.75	9	0.79	12	0.91	34 0.70
$\ln K_L = b_o + b_5 pK_{BH^+}$	0.66	6	0.57	3	0.63	4 1.35

Table II. continued

1	2	3	4	5	6	7
Arylamines						
$\ln K_L = b_o + b_1 M$	0.60	3	0.51	2	0.91	30 0.15
$\ln K_L = b_o + b_5 pK_{BH^+}$	0.61	4	0.55	3	0.79	10 0.28
Azaheterocycles						
$\ln K_L = b_o + b_1 M$	0.89	18	0.88	24	0.87	16 0.43
$\ln K_L = b_o + b_2 \mu$	0.81	10	0.81	10	0.83	11 0.50
$\ln K_L = b_o + b_7 E_{LVMO}$	0.81	10	0.83	11	0.81	10 0.53
Alkylamines						
$\ln K_S = b_o + b_5 n_{H(N)}$	0.94	44	0.88	22	0.94	44 0.20
$\ln K_S = b_o + b_2 \mu$	0.92	35	0.94	54	0.94	44 0.20
Arylamines						
$\ln K_S = b_o + b_5 n_{H(N)}$	0.89	23	0.92	32	0.94	44 0.90
$\ln K_S = b_o + b_3$ IP	0.94	43	0.95	56	0.96	80 0.68
Azaheterocycles						
$\ln K_S = b_o + b_6 E_{HOMO}$	0.82	10	0.75	6		
$\ln K_S = b_o + b_7 E_{LVMO}$	0.98	97	0.89	19		

a) Specification: M - molecular mass, μ - dipole moment, IP - ionization potential, ΣE_S^o - sum of Palm's sterical constants, E_{HOMO} - energy of the highest occupied molecular orbital, E_{LVMO} - energy of the lowest vacant molecular orbital, $n_{H(N)}$ - number of hydrogen atoms in amino group, q_N - excess partial charge on the nitrogen atom, b_o, b_1, b_2, b_3, b_4, b_5, b_6, b_7, b_8 - coefficients at corresponding terms in regression equations, r - coefficient of multiple regression, F - value of F-test for r[$F = \frac{r^2(n-2)}{1 - r^2}$, where n is the number of measurements].

intermolecular interaction at sorption in metal stearate solutions.

The existence of a linear dependence between the complexing enthalpies and entropies (see Fig. 1) indicates the similarity of the structures of the complexes formed by 1) dialkylamines, 2) trialkylamines, pyrroles, indoles, 2,6-dimethylpyridine, and 3) methyl- and dimethylpyridines, pyrazines.

Processes caused by physical intermolecular interactions in the bulk of the stationary phase (Apiezon M + stearates) constitute 33-38 % of the value of V_N for dialkylamines and up to 90 % of the value of V_N in the case of trialkylamines, alkylanilines and pyrroles exerting a slight trend to complex formation (Table III). The partition coefficient K_L mainly depends on the molecular masses of the amines; at this type of sorption the dispersion intermolecular interactions are dominating (r up to 0.91; see Tables II, III). The K_L values (Table III) are much higher than the value of K_L determined on the columns with pure Apiezon M /15/ indicating that here some specific interactions also take place. Correlation of $\ln K_L$ on E_{LVMO} (heterocycles) or on pK_{BH^+} (alkyl- and aryl-amines) indicates the possibility of hydrogen bond formation N-H ... O (or involving LVMO of the heterocycle). In physical interactions of the nitrogen bases with the stearoyl radical the influence of the dipole moments of molecules seems to be also significant.

Adsorption of amino compounds on the stationary liquid-solid support interface (Table IV) was found to be suppressed in comparison with the system without the complexing agent/15/. Contributions of adsorption to V_N do not exceed 5-6 % (instead of 14-15 % on pure Apiezon M); in systems containing cobalt stearate they constitute only 3 % of V_N. The decrease of adsorption can be partially assigned to the modifying effect of the polar component of the liquid phase covering the active sites on the support surface. However, increased contributions of adsorption to the V_N values in the case of trialkylamines, alkylanilines and pyrroles (cf. Table IV and /15/) allows to assume that concurrent interactions are taking place in the volume of the stationary phase. If the nitrogen base has a

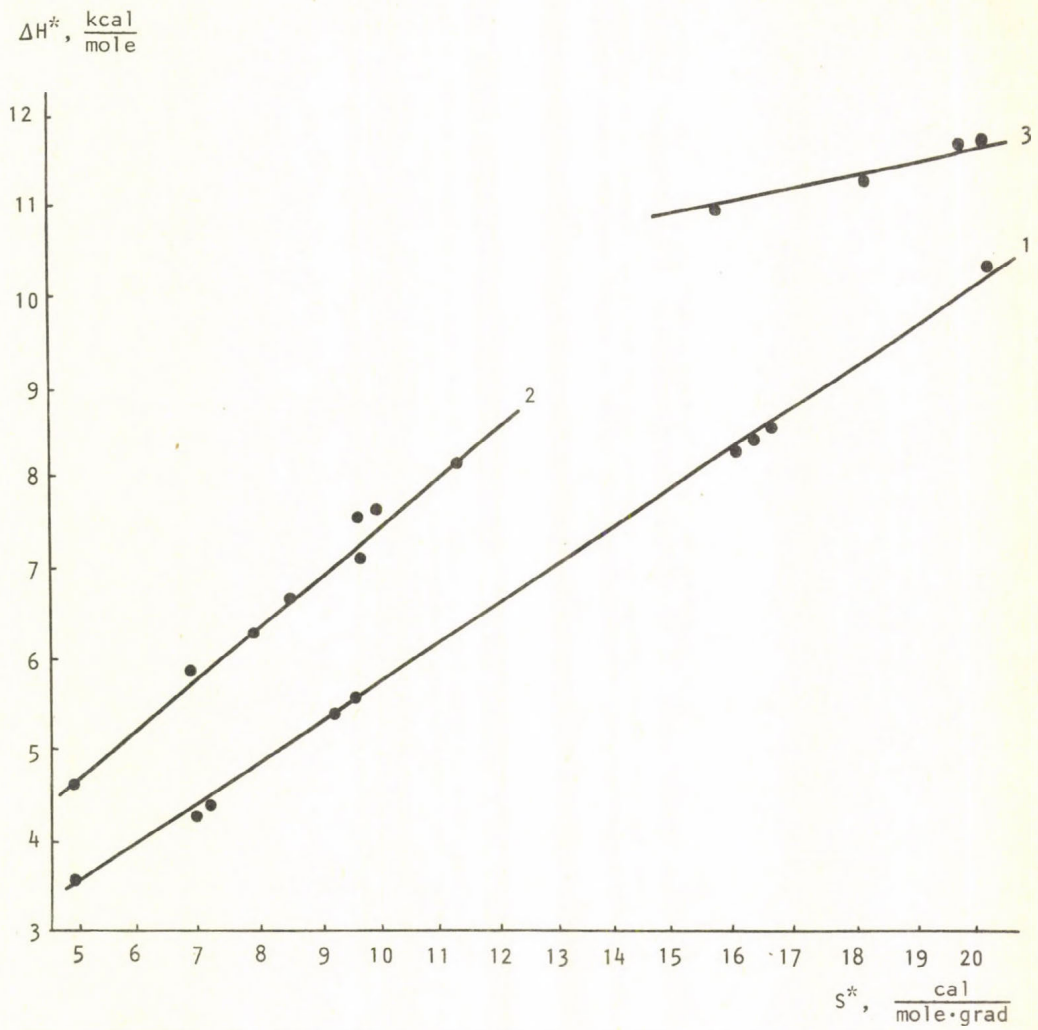

Fig. 1. Relation between the complex formation enthalpy and entropy
1 - Trialkylamines, 2,6-dimethylpyridine, pyrroles, indole;
2 - Methylpyridines, pyrazines; 3 - Dialkylamines.

Table III. Partition coefficients and relative contributions of the process to the total specific retention volumes of amines

Amine	K_L			$K_L V_L^s \cdot 100/V_N^s$		
	Apiezon M + stearate of			Apiezon M + stearate of		
	Ni	Cu	Co	Ni	Cu	Co
Diethylamine	57.9	69.3	38.3	36.0	33.1	34.3
Dipropylamine	121.4	124.7	82.2	35.8	32.7	35.3
Dibutylamine	304.0	435.0	301.3	36.5	33.1	34.3
Diamylamine	847.0		1069.2	37.6		34.5
Triethylamine	16.1	23.5	29.4	90.8	91.7	75.0
Tripropylamine	70.0	82.7	113.2	90.1	91.3	88.7
Tributylamine	342.4	373.4	540.2	88.2	89.1	88.7
Triamylamine	1566.5	1786.7	2610.1	88.1	88.8	88.7
Aniline	191.9	177.6	180.5	68.3	78.4	78.3
o-Toluidine	297.5	293.0	329.6	73.7	80.2	84.7
m-Toluidine	384.7	367.6	351.0	63.7	77.4	77.6
p-Toluidine	372.0	390.9	345.7	53.6	70.8	71.7
2,4-Xylidine			601.4			76.6
2,5-Xylidine			605.2			80.2
2,6-Xylidine			587.2			86.8
N-Methylaniline	185.1	222.3	283.2	90.3	89.9	88.8
N-Ethylaniline	258.4	269.1	401.3	89.7	88.5	89.6
N,N-Dimethylaniline	220.0	236.4	331.4	89.4	90.9	90.2
N,N-Diethylaniline	440.2	452.6	642.8	90.1	90.6	90.2
Pyrrole	18.0	19.0	31.2	87.3	87.3	87.2
1-Methylpyrrole	20.8	22.3	31.8	91.0	90.9	88.1
2,5-Dimethylpyrrole	59.7	64.9	93.5	87.3	88.1	88.3
2-Methylpyridine	83.6	80.7	110.8	58.7	51.1	54.8
2,3-Dimethylpyridine	188.8	185.5	258.9	56.4	48.6	53.5
2,4-Dimethylpyridine	206.6	194.7	292.4	35.6	37.2	38.4
2,5-Dimethylpyridine	191.8	183.0	260.3	45.1	42.5	45.3
2,6-Dimethylpyridine	62.7	64.2	92.5	89.4	77.5	88.5
2,4,6-Trimethylpyridine			201.5			87.7
Pyrazine			58.9			53.1
Methylpyrazine			86.5			56.6
2,5-Dimethylpyrazine			123.8			65.5
1-Methylpiperidine	57.2	80.1	76.4	88.9	57.1	60.3

Table IV. Adsorption coefficients of amines on the stationary phase - solid support interface and relative contributions of the process to the total net retention volumes

Class of amines	K_S			$\frac{K_S K_L (1-K^* \frac{m_k^s}{V_L^s}) S_S}{V_N^s} \cdot 100$		
	Apiezon M +			Apiezon M +		
	$Ni(C_{17}H_{35}COO)_2$	$Cu(C_{17}H_{35}COO)_2$	$Co(C_{17}H_{35}COO)_2$	$Ni(C_{17}H_{35}COO)_2$	$Cu(C_{17}H_{35}COO)_2$	$Co(C_{17}H_{35}COO)_2$
C_4-C_6 n-Dialkyl-amines	$(7.7-11)10^{-8}$	$(5.7-7)10^{-8}$	$(7.1-7.6)10^{-8}$	1.8 - 2.5	1.4 - 1.7	1.7 - 1.9
C_8-C_{10} n-Dialkyl-amines	$(5.5-14)10^{-9}$	$2.8 \cdot 10^{-8}$	$(6-7)10^{-8}$	0.1 - 0.3	0.4 - 0.5	1.4 - 1.7
C_6-C_{15} n-Trialkyl-amines	$(1.8-2.8)10^{-6}$	$(1.3-2.4)10^{-6}$	$(1.0-1.1)10^{-7}$	6-7	4-5	0.2 - 0.7
Arylamines	$(1.3-1.6)10^{-7}$	$(1.2-1.6)10^{-7}$	$(2.4-2.7)10^{-8}$	1.1 - 1.9	0.8 - 1.4	0.1 - 0.2
N-alkylanilines	$(1-7-2.2)10^{-6}$	$(1.5-1.9)10^{-6}$	$(1.6-3.3)10^{-6}$	3.3 - 6.1	2.2 - 4.4	1.1 - 3.3
Pyrroles	$(1.5-3.3)10^{-6}$	$(1.5-1.2)10^{-7}$	$(1.2-1.7)10^{-6}$	4.6 - 6.8	2.1 - 4.4	1.2 - 1.9
Alkylpyridines	$(1.2-5.0)10^{-8}$	$(1.7-8)10^{-8}$	$(1.2-12)10^{-7}$	0.3 - 1.6	0.8 - 1.3	1.8 - 3.3
Pyrazine bases			$(1.1-1.7)10^{-7}$			

strong affinity to the complexing agent, interactions between the latter and the sorbate in the bulk liquid are predominant. On the other hand, if the affinity of the amine to the complexing agent is only minor, the amine molecules are able to interact with the support surface.

Correlation analysis of the K_S values (Table II) gives evidence for the existence of interaction of the amines with two types of active sites on the support surface: with the silanol hydrogen atoms (correlation of ln K_S on IP or on E_{HOMO}) and with sites possessing increased electron density (correlation of ln K_S on the number of hydrogen atoms bonded to the nitrogen atom or on E_{LVMO}).

The decrease in the interfacial sorption in systems with binary stationary phases including the polar component seems to be a rather general phenomenon. The polarity of the liquid phase causes a modification of the active sites on the support surface but the superficial activity of the polar component leads to a decrease in the adsorption on the gas-liquid interface.

As a conclusion, the use of an extended equation (6) along with regression analysis provides the evaluation of the separate sorption processes that take place in the gas-binary stationary phase system containing a complexing agent and a solid support; it allows to establish that the intermolecular interactions are responsible for each definite process.

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GAS CHROMATOGRAPHIC INVESTIGATION OF CHANGES IN THE VOLATILE AMINE COMPOSITION DURING STORAGE OF BAKERY YEAST AUTOLYSATE

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SUMMARY

The composition of volatile amines from dry bakery yeast autolysate and changes occurring during five-year storage have been studied. Volatile amine concentrates isolated by two methods (extraction and vacuum distillation) were compared. A total of 54 compounds were identified by gas chromatography utilizing the retention indices, while the structures of 11 heterocyclic compounds were confirmed by gas chromatography-mass spectrometry. The gas chromatographic data have shown that the amount of dimethyl-, trimethyl-, butyl-, propyl-tert-butyl-, dimethylisopropyl-, dimethylisobutyl-, and dimethyl-n-heptylamines in the autolysate increased after storage and this correlated with the deterioration of the organoleptic properties of the autolysate and with a decrease in its amino acid content.

INTRODUCTION

Amino acids obtained from natural raw materials by hydrolysis are used in the medical and food industries as a major ingredient in nutritive mixtures and as additives in the production of dry soups, briquetted meat and fish, canned meat and sauces. They are also utilized as a reactant in the production of artificial flavouring materials by the Maillard reaction. At present hydrolysates of plant and yeast proteins are extensively used. Belikov and coworkers /1/ have developed a procedure for the preparation of a mixture of amino acids and lower peptides by the autolysis of the bakery yeast Saccharomyces cerevisiae. Autolysates to be used as an additive in foodstuff have to meet strict requirements concerning their

organoleptic properties. It is generally known that amino acids are precursors of volatile amines that can influence the organoleptic properties of foods. Investigation of the volatile amine composition of used autolysate is also important in view of the problem of determination of amines as possible precursors of carcinogenic N-nitroso compounds in foods /2/.

Volatile components of the basic fraction of bakery yeast autolysate have been studied by gas chromatography-mass spectrometry /3/. However, only heterocyclic bases, viz., eight alkyl pyrazines and a thiazole derivative were detected. The absence of primary aliphatic amines such as methyl-, ehtyl-, isobutylamines, etc. resulting from the decarboxylation of amino acids may have been due to the procedures used for the isolation and analysis of the organic bases. Changes in the properties of bakery yeast autolysate during its storage have not been studied.

In order to prove the presence of aliphatic amines in the volatiles of the basic fraction of bakery yeast autolysate we applied two methods for the isolation of the volatile amines: extraction of the dry autolysate powder and vacuum distillation of the 10 % aqueous hydrolysate solution. Changes in the composition of the volatile amines and amino acids and in the organoleptic properties of the autolysate upon prolonged storage were also studied.

EXPERIMENTAL

Reagents and Materials

The autolysate of bakery yeast Saccharomyces cerevisiae treated to remove peptides, nucleic components and fat impurities was studied as powder with 8 % moisture content /1/. The same sample was investigated after five-year storage at room temperature in glass vessel with a ground stopper.

Freshly distilled solvents and recrystallized chemically pure grade reagents ("Reakhim") were utilized. Volatile amines were extracted from the bakery yeast autolysate with freshly distilled absolute ether. In preparing the aqueous solutions, double-distilled water purified from organic bases was used.

Isolation of the Volatile Amines

We have utilized two methods for the isolation of the volatile amines:

I: extraction with absolute diethyl ether from the dry autolysate powder followed by distillation of the volatile amines and their separation from nonvolatile substances;

II: distillation from a 10 % aqueous autolysate solution in vacuo.

The procedures are detailed in Fig. 1. The volatile amines were concentrated in the form of their HCl salts.

Sample Preparation for GC and GC-MS Analysis

The hydrochloric salts of the volatile amines were washed with 3x3 ml absolute hexane to remove the organic compounds belonging to other classes which could be absorbed by the hydrochlorides and thus distort the results of the GC analysis. Just prior to the analysis, the volatile amines were regenerated from 5-10 mg of the salts with an alkali under a layer of chromatographically pure n-pentane or n-hexadecane according to the procedure described earlier /4/. The sample size for GC analysis on packed columns was 4-7 µl.

The salts isolated by method II contained a large amount of ammonium chloride. In order to increase the relative concentration of the salts of organic volatile amines in the sample, the hydrochlorides were extracted with glacial acetic acid (3x2 ml) /5/. From 0.3 g salts 0.06 g was obtained after the extraction. The sample size for GC-MS analysis on a capillary column was 0.5-1.5 µl.

Instrumentation

The volatile amines were analysed with a Model 104 Pye Unicam (U.K.) gas chromatograph with a flame ionisation detector. Separation of the amine mixtures was carried out at 100° on three glass columns (270 cm x 4 mm i.d.) packed with Chromosorb G AW (80-100 mesh) coated with 4 % Apiezon L + 1 %

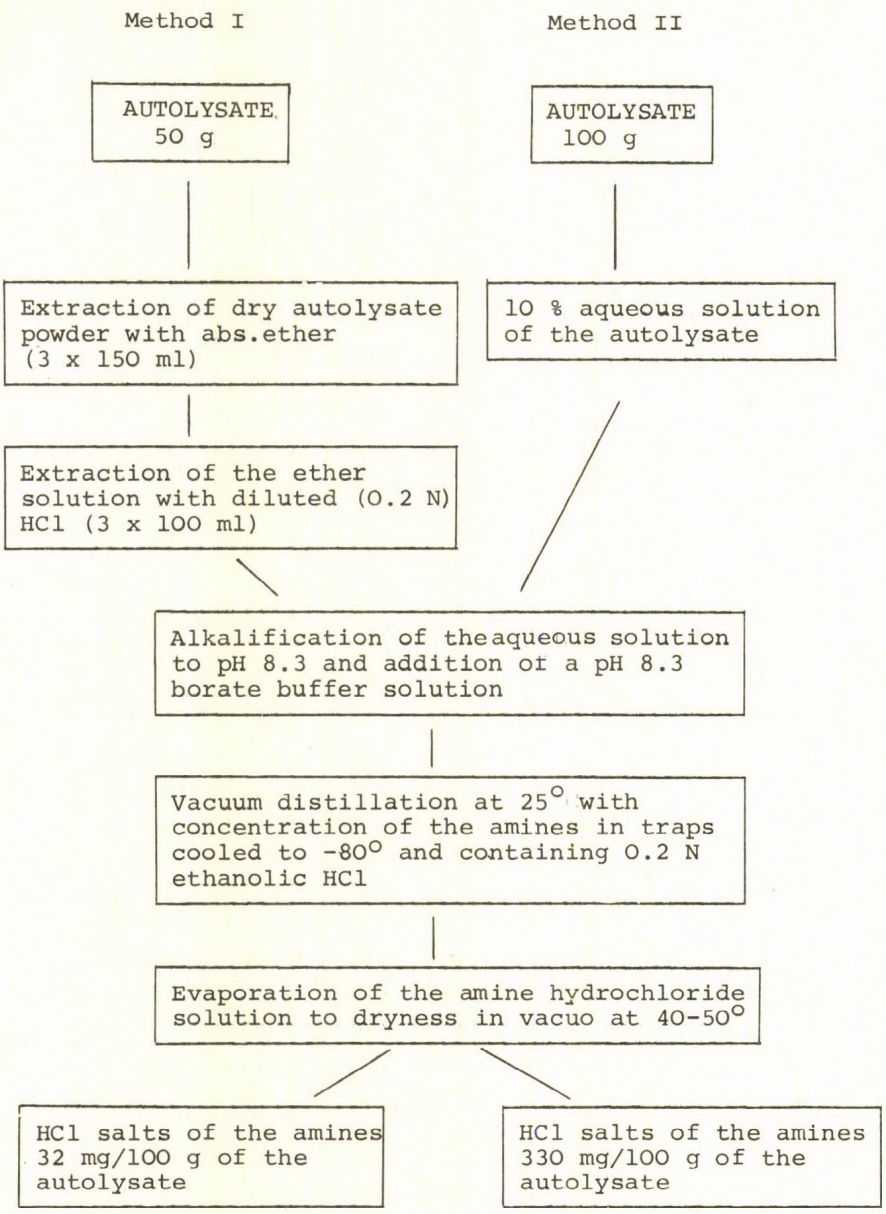

Fig. 1. The scheme of the isolation and concentration of volatile amines from bakery yeast autolysate.

KOH, 5 % Triton X-305 + 0.5 % Na_3PO_4, or 5 % PEG-1000 + 0.5 % Na_3PO_4. High-purity nitrogen was used as the carrier gas, with flow rates in the range of 15-50 ml/min. The mass spectra were obtained in the electron impact mode (70 eV) with a Model R-10-10B Nermag (France) gas chromatograph-mass spectrometer equipped with a computer. The mixture was separated at 100°C using a PEG-40M + KF coated capillary column (40 m x 0.27 mm i.d.) prepared by the high pressure static method /6/. The injected sample was split 1:20.

Identification

The volatile amines were identified by gas chromatography, on the basis of retention data. We have utilized a BESM computer with a program described earlier /7/. Retention indices of more than 250 organic bases were stored and the actual data compared with the stored data. The program also takes into account the possible deviations between the stored and measured retention indices /8/.

Identification of the compounds by the mass spectra was performed via comparison with spectra known from the literature /9-11/.

RESULTS AND DISCUSSION

The composition of the identified substances can depend on the procedure of their isolation and their original concentration. When studying the flavour components of vegetable hydrolysates Withcombe, Mookherjee and Hruza /12/ utilized the technique of gas extraction (75° for 24 hrs) with concentration of the volatiles on porous polymer sorbents and GC-MS for their identification. They discovered more than 70 compounds which included none of the alkyl amines. A possible reason for the absence of aliphatic amines was the severe conditions used in their isolation which mainly resulted in the formation of heterocyclic compounds. It is known that the reaction of pyrazine formation occurs easily in the 70-120° temperature range /13/. Withcombe, Mookherjee and Hruza /12/ showed that

the composition of the identified substances from the same isolation method will be different if different sorbents (Tenax, Porapaks Q or R) are used in their concentration.

As mentioned earlier we have studied the relative advantages of two methods used for the extraction of volatile amines. Method I was time consuming but it reduced the possibility of emergence of additional volatile amines during distillation from the alkaline medium. Method II was simpler; however, here the whole autolysate was reacted with alkali. Comparing the recoveries of volatile amines we have found that in the distillation method (Method II) 10 times more products were obtained than with the extraction method (Method I). Comparison of the qualitative composition after GC analysis on the Triton X-305 + Na_3PO_4 column has shown that the composition of the volatile amines was close: 24 substances out of the 28 volatile amine peaks isolated by extraction were also found among the 31 peaks obtained by distillation (see Table 1). As shown in Table 1 the amount of the amines isolated by distillation and detected in peaks No. 3, 7, 10, 14 and 18 exceeds by an order of magnitude the quantity of substances isolated by extraction. The relative volatile amine content present in one of the two investigated samples was not large; therefore, the distillation method was selected for further investigation.

After the analysis of the volatile amines on three columns with stationary phases of different polarity (Apiezon L + KOH, Triton X-305 + Na_3PO_4 and PEG-1000 + Na_3PO_4), we have identified 54 compounds by computer-processing of the GC data. These are listed in Table 2. A total of 34 compounds out of these 54 compounds are aliphatic amines while the other 20 are heterocyclic bases. Among the primary amines found methyl-, ethyl-, isobutyl-, n-butyl-, and isopentyl-amines were probably the products of Strecker cleavage of the α-amino acids, while n-heptyl- and n-octylamines can result from the cleavage of the higher fatty acids present as traces in the autolysate. Among secondary aliphatic amines we have found methylated, ethylated and propylated amines although n-propylamine itself could not be identified in the mixture. Among the tertiary amines six methylated amines, four propylated amines, and triethylamine

Table I. Comparison of the composition of volatile amines isolated by extraction (extr.) and distillation (dist.) methods

Peak No.	Retention indices*		Ratio of peak area (A) $A_{dist.}/A_{extr.}$
	extraction (Method I)	distillation (Method II)	
1	514	521	7.5
2	602	596	2.8
3	654	644	12.0
4	693	708	2.3
5	724	-	-
6	750	750	0.1
7	804	799	29.3
8	847	842	0.8
9	-	884	-
10	922	918	10.8
11	-	949	-
12	964	963	1.6
13	1000	999	1.4
14	1031	1027	17.8
15	1058	1052	0.9
16	1079	1083	6.9
17	-	1095	-
18	1119	1115	12.1
19	1129	1130	3.9
20	1150	1146	8.3
21	-	1157	-
22	1178	1174	Trace
23	1187	-	-
24	1203	1198	9.7
25	-	1214	-
26	1224	1221	9.4
27	1245	1242	2.4
28	-	1254	-
29	1264	-	-
30	1280	1275	6.0
31	-	1296	-
32	1307	1307	2.8
33	1324	1319	6.0
34	1346	-	-
35	1366	1365	3.2

*Measured on a 270 cm x 4 mm i.d. packed column containing 5 % Triton X-305 and 0.5 % Na_3PO_4 on Chromosorb G AW 80-100 mesh, at 100°C.

Table 2. Volatile nitrogen-containing bases present in the bakery yeast autolysate

Group	Compound
Primary amines	Methylamine
	Ethylamine
	Isobutylamine
	n-Butylamine
	Isopentylamine
	n-Heptylamine
	n-Octylamine
Secondary amines	Dimethylamine
	Methyl-sec-heptylamine
	Ethyl-n-pentylamine
	Ethylcyclohexylamine
	Ethyl-n-octylamine
	Di-n-propylamine
	n-Propylallylamine
	n-Propyl-tert-butylamine
	n-Propyl-n-heptylamine
	n-Butyl-sec-butylamine
	Isobutylisopentylamine
	sec-Butyl-n-pentylamine
	tert-Butyl-n-hexylamine
	Diisopentylamine
Tertiary amines	Trimethylamine
	Dimethylisopropyl amine
	Dimethylisobutyl amine
	Dimethyl-n-heptylamine
	Dimethyl-n-octylamine
	Methyldi-n-pentylamine
	Triethylamine
	Ethyldi-n-propylamine
	Di-n-propylisopropylamine
	Di-n-propyl-sec-butylamine
Heterocyclic compounds	Ethylene imine
	Pyridine*
	2-Picoline*
	3-Picoline
	4-Ethylpyridine*
	2,4-Lutidine*
	2,6-Lutidine*
	Piperidine
	N-Methylpiperidine*
	2,6- and/or 2,5-Dimethyl-pyrazine*
	2,3-Dimethylpyrazine
	Trimethylpyrazine
	Tetramethylpyrazine
	2-Methoxypyrazine
	Piperazine*
	2-Methylpiperazine*
	N,N-Dimethylpiperazine*
	N-Propylpyrrole*
	Pyrrolidine
Amino alcohols	2-Amino-propanol-1
	Aminoethanol

*Identification through the retention indices confirmed by GC-MS data.

were also detected. The heterocyclic bases were represented by ethylene imine, pyrrolidine, N-propyl pyrrole, alkyl substitued pyridine, piperidine, pyrazine and piperazine derivatives. Unfortunately, only 11 substances were at the level of 10^{-7} g/ml providing interpretable mass spectra which confirmed the identification on the basis of GC data. The mass spectra of aliphatic amines were absent. The GC-MS analysis of a model mixture containing di-n-propyl- and dimethylisopropylamines, pyridine and 2-picoline at the 10^{-8} g/ml level showed that the mass spectrometer records only the spectra of heterocyclic basis. Aliphatic amines, perhaps, are completely adsorbed on the surface of the metal transfer lines, ionisation cell walls, etc.This is possibly also the reason why aliphatic amines have not been found in the study of odour components of vegetable protein hydrolysate /12/ and bakery yeast autolysate /3/. The aliphatic amines could only be identified on the basis of the GC data.

Change in the composition of the volatile amines during protein food storage is often responsible for the appearance of off-flavour /14/. It was observed that the autolysate colour is changing from white to yellow-brown after five year storage while undesirable properties notes are enhanced and unpleasant components are emerging in changing the odour of the material. We have compared the volatile amine composition of freshly prepared and stored samples both qualitatively and quantitatively; the Triton X-305 column was used in these measurements. Table 3 lists the information obtained. As seen there is no appreciable change in the qualitative composition of the volatile amines although some new compounds could be identified (see e.g., peaks Nos 22, 33, and 34). However, the total amount of these compounds is insignificant and does not exceed 0.1 %. It should be noted that the trimethyl amine (peak No. 1), dimethyl amine (peak No. 2), dimethylisopropyl-, and dimethylisobutyl amine (peak No. 4), n-butyl- and n-propyl-tert-butyl amine (peak No. 7) and dimethyl-n-heptylamine (peak No. 15) contents increased 11.8; 21.3; 57.5; 17.1 and 74.8 times respectively. The presence of aromatic heterocyclic bases is not typical of the stored autolysate. For example,

Table 3. Changes in the volatile amine content during the storage of the bakery yeast analysate

Peak No.	Identified substance	Retention index* Fresh (fr.)	Stored (st.)	Ratio of peak area (A) $A_{st.}/A_{fr.}$
1	Trimethylamine	521	556	11.8
2	Dimethylamine	596	610	21.3
3	Methyl- and ethylamine	664	653	5.3
4	Dimethyl-isopropyl- and dimethylisobutylamine	708	719	57.5
5	Triethyl- and diisopropyl amine	750	758	9.0
6	Isobutyl-, ethylpentyl- and methyl-sec-heptylamine	799	806	2.6
7	n-Butyl- and n-propyl-tert-butylamine	851	856	17.1
8	Diisopropyl- and di-n-propyl-ethylamine	884	-	-
9	Isopentyl-, n-propylallyl- and N-methylpiperidine	918	923	3.9
10	Di-n-propylisopropylamine	949	955	10.2
11	Pyrrolidine,ethylene imine	963	-	-
12	n-Butyl-sec-butylamine, piperidine	999	1003	4.1
13	Di-n-propyl-sec-butylamine	1027	1035	0.8
14	N,N-Dimethylpiperazine, isobutylisopentylamine	1052	1053	3.6
15	Dimethyl-n-heptylamine	1083	1083	74.8
16	n-Pentyl-sec-butylamine	1095	-	-
17	Pyridine	1115	-	-
18	tert-Butyl-n-hexylamine	1130	-	-
19	2-Picoline	1146	1143	1.4
20	Ethylcyclohexyl- and heptylamine	1157	1152	11.4
21	Dimethyl-n-octylamine, N-propylpyrrole	1174	-	-
22	Not identified	-	1185	-
23	Methyldi-n-pentylamine	1198	1195	1.3
24	3-Picoline and 2,6-lutidine	1214	-	-
25	Not identified	1220	1217	0.3
26	Not identified	1236	-	-
27	2,5- and/or 2,6-Dimethyl-pyrazine and 2-methoxypyrazine	1242	1240	2.0
28	2,3-Dimethylpyrazine, 2,4-lutidine and n-octylamine	1257	1260	1.7
29	Piperazine, 2-methylpiperazine and n-propyl-n-heptylamine	1275	1274	6.0
30	Ethyl-n-octylamine	1296	1292	Trace
31	4-Ethylpyridine, trimethylpyrazine	1307	1305	Trace
32	2-Aminopropane-1-ol, aminoethanol	1319	-	-
33	Not identified	-	1327	-
34	Not identified	-	1332	-
35	Not identified	1365	1360	0.07

*Measured on a 270 cm x 4 mm i.d. packed column containing 5 % Triton X-305 and 0.5 % Na_3PO_4 on Chromosorb G AW 80-100 mesh, at 100°C.

peaks Nos 17 and 24 corresponding to pyridine and to the mixture of 3-picoline and 2,6-lutidine disappeared during storage. The pyrazine base content increased only insignificantly (see Table 3).

It is interesting to note that the opposite took place upon the storage of dried milk protein concentrates (casein and co-precipitate): the concentration of pyridine and alkyl pyrazines increased /14/. The amount of heterocyclic bases increased from 42 to 79 % during casein storage, while in the case of the autolysate the percentage of these substances decreased from 15 to 5 %. The accumulation of aliphatic amines from 69 to 95 % may be considered as a criterion of the quality of the autolysate during its storage in contrast to the protein material.

In order to establish the relationship between the instrumental and organoleptic data and to elucidate the role of the volatile amines in the autolysate odour we evaluated the odour of the components eluted from the Apiezon L + KOH, after separation at 100°. The "aromagram" of the volatile amines is shown in Fig. 2. We established the fractions responsible for the following odours; bread, chocolate, and musty. In the fraction with bread odour we identified piperazine, 2,6-lutidine and N-propyl pyrrole. In dry autolysate the fraction with the odour of dried mushrooms has a major role; however, we could not find it here among the volatile amine fractions. Probably components belonging to other classes of organic compounds are responsible for the mushroom odour. For example, it is known that the components of the neutral fraction, octene-1-01-3, have a mushroom-like smell /15/. The significance of the neutral and acidic fractions in the odour of the yeast autolysate Gistex X-II has been emphasized by Hajslova and coworkers /3, 16/.

Since amino acids can be the precursors of volatile amines we have assumed that the accumulation of volatile amines during storage will correlate with the change in the amino acid composition of the autolysate. The data we have obtained for 16 amino acids indicated a 10.6% decrease in the total amount of amino acids and a 6.1% decrease in the amount of the essential acids after three-year storage.

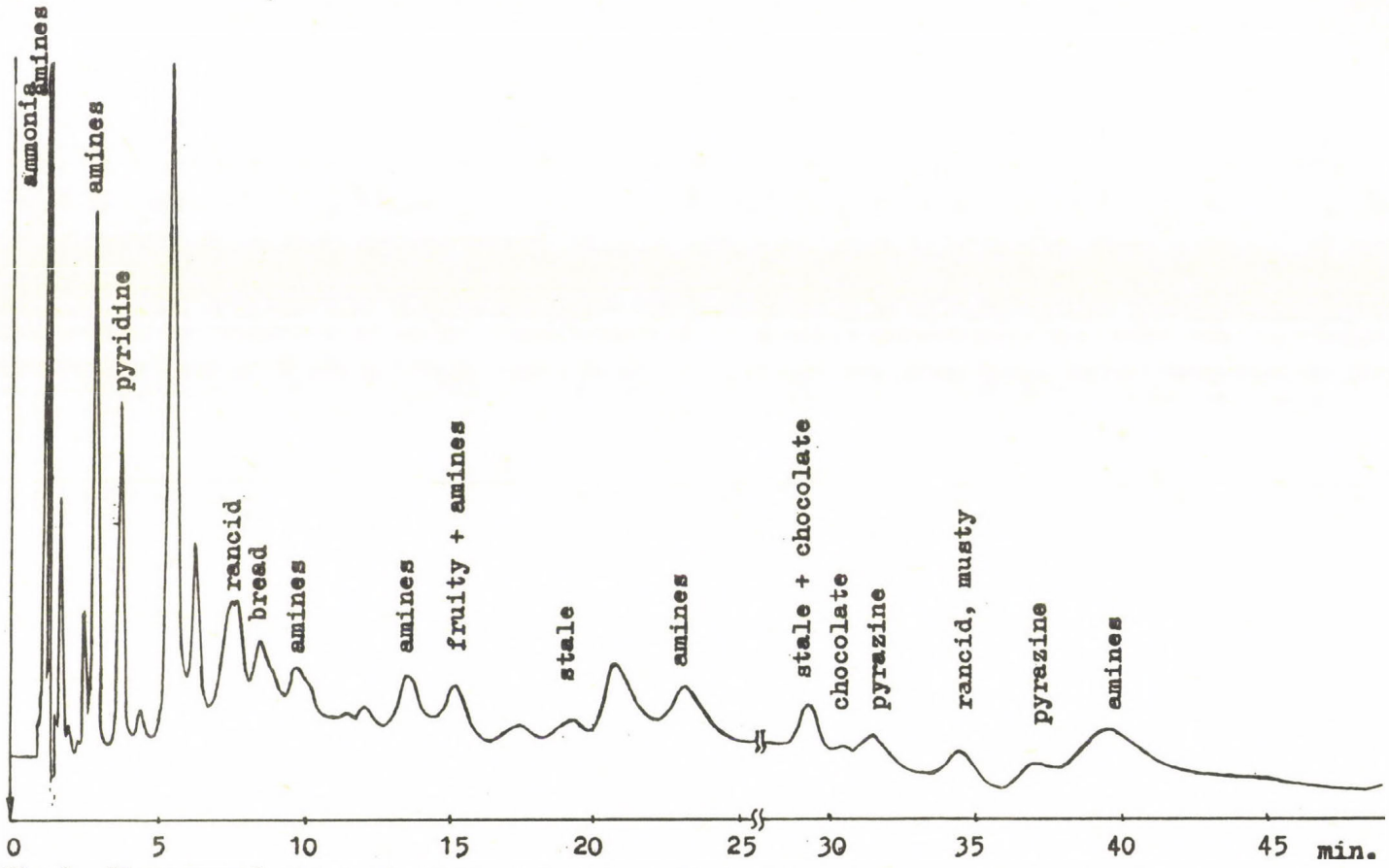

Fig.2. "Aromagram" obtained of the volatile amines of the bakery yeast autolysate, after a five-year storage period. Column: 270 cm x 4 mm. i.d., containing 4% Apiezon L + 1 % KOH. Temperature: 100°C.

The most profound transformations were observed for lysine, arginine, leucine and isoleucine. The decrease in the leucine content may be explained by its decarboxylation: isobutylamine and dimethylisobutylamine, its methylated derivative, are the degradation products.

CONCLUSIONS

As a conclusion we can state that aliphatic amines and heterocyclic bases of the pyridine and pyrrole series have been identified for the first time in the odour concentrate of the bakery yeast autolysate. It has been shown that an accumulation of certain aliphatic amines takes place upon prolonged storage which correlates with the deterioration of the organoleptic properties of the material and with a decrease in the amino acid content. The accumulation of the aliphatic amines increases the carcinogenic risk due to the emergence of N-nitroso compounds in foods containing the autolysate as an additive.

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AN ANALYTICAL HPLC METHOD FOR THE ISOLATION AND CHARACTERIZATION OF INSULIN

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SUMMARY

A method for the isolation and quantitative determination of insulin is described. Genes for the production of A and B human insulin chains were cloned and introduced separately into *E. coli* for the production of two hybrid polipeptides. A and B insulin chains were isolated, purified, and thereafter joined by disulfide bridge formation *in vitro*. Two different chromatographic systems were used to verify the reproducibility of the method. The peaks corresponding to insulin were analyzed by polyacrylamide gel electrophoresis and the amino acid composition was determined. The results were in good agreement with literature values.

INTRODUCTION

To study the chemical constitution of insulin /1/ from its constituent peptides A and B, a method of reversed-phase HPLC was developed, which permitted the isolation and quantification of the correctly reassociated insulin molecules from the incorrectly reassociated products and unreacted peptides. From the hydrophilic reagents which form ion-pairs used in the separation of peptides /2/, formic acid was selected for the following reasons:

1) The products of reassociation of the A and B chains are quite insoluble in phosphoric acid, for the ion-pairing reagent /2, 3/ which is used in the analytical method for the identification of insulin. Use of formic acid eliminates this problem.

2) by using formic acid in eluent A (the resolution having been optimized) with a linear gradient of acetonitrile (effluent B), a capacity factor was obtained which was sufficient /4/ to isolate the insulin during its recovery. The solid residues obtained after lyophilization dissolved easily in a variety of solvents, thereby yielding products with characteristics that not only made their characterization by amino acid analysis and by polyacrylamide gel electrophoresis easier but also permitted an easier handling in later processing. This method made possible the isolation and characterization of the human insulin molecules which resulted from the correct association in vitro of A and B chains which had been produced in bacteria.

MATERIAL AND METHODS

Apparatuses

Two different chromatographic systems, both from Spectra-Physics, were used. The first was a modular analytical system: SP8750 combined with the Rheodyne Injector Loop (10 µl), the SP8700 solvent delivery system, the SP8400 variable wavelength detector, and the SP4100 computing integrator. The second was the Performance SP8000B semi-preparative system with a 100-µl injector loop. In both systems, the same Water Radial Pak µBondapak C_{18} cartridge was used. Sample and solvents were clarified by filtration through porous membranes (0.22 µm) from Millipore.

Reagents

HPLC grade water was obtained from pre-treated water by using an organic filter apparatus (cat. No. XX1504-710) from Millipore. Acetonitrile (lichrosolv grade art. 30) was from Merck. Formic acid (Baker 0128) was redistilled under vacuum (20 mm Hg). Insulin from bovine pancreas (Sigma I-550) was used as the standard.

Procedure

Both eluent A [0.5 % (v/v) distilled formic acid pH 2.7 in ultra-pure water] and eluent B (acetonitrile) were filtered through a porous membrane (0.22 µm) and degassed with helium. The insulin standard was dissolved in eluent A, filtered, and chromatographed to determine its retention time (Figs 1 and 2). Two to 22 µg of this standard were used to determine the linearity of response (Fig. 3). The external standard procedure was used to calculate the yield. To calculate the confidence limits of the method /5/ the standard was run three times at each concentration (Table I).

Chromatographic conditions

The Spectra-Physics system was programmed to provide a linear gradient elution in order to separate the correctly associated peptides from other products (Figs 4 and 5). The mobile phase consisted of eluent A and eluent B. The linear gradient was from 20 % to 40 % eluent B. The mobile phase was pumped at a flow rate of 2.5 ml/min and the column effluent was monitored at 280 nm. Chromatography was performed at room temperature in a µBondapak C_{18} cartridge. The injection volume of the sample was 10 µl for the quantitative determinations and 100 µl for the isolation and characterization of the desired product.

RESULTS AND DISCUSSION

The newly developed method permitted the isolation of insulin correctly reassociated from the cleaved A and B chains of the commercial product, with a high degree of purity as determined by amino acid analysis (Table II). The purity was determined by polyacrylamide gel electrophoresis in which only one band, co-migrating with that of the standard, was observed (Fig. 6).

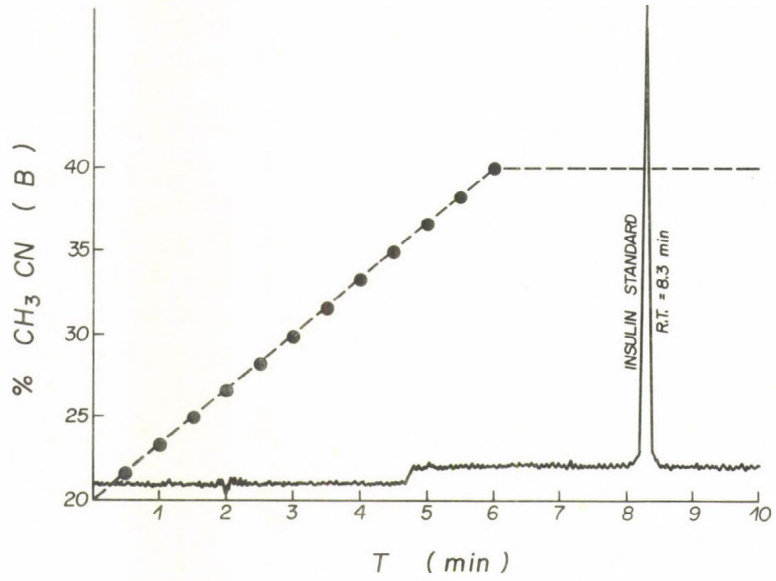

Fig. 1 Schematic chromatogram showing the retention time of standard insulin; Spectra-Physics System; column: Radial Pak μBondapak C_{18} (8 mm x 10 μm x 10 μm) mobile phase: linear gradient from 20 % to 40 % CH_3CN in 0.5 % formic acid pH 2.7, flow rate = 2.5 ml/min. UV = 280 nm.

Table I. Statistical results from the HPLC analysis of insulin standard

Insulin sample (μg)	S.D.[a]	C.L.[b]
2 - 5	0.03504	± 0.0398
6 - 10	0.06565	± 0.074511
11 - 14	0.2129887	± 0.2417
15 - 23	0.121417	± 0.1378

S.D.[a] = Standard deviation of five runs at each concentration

C.L.[b] = Confidence limit

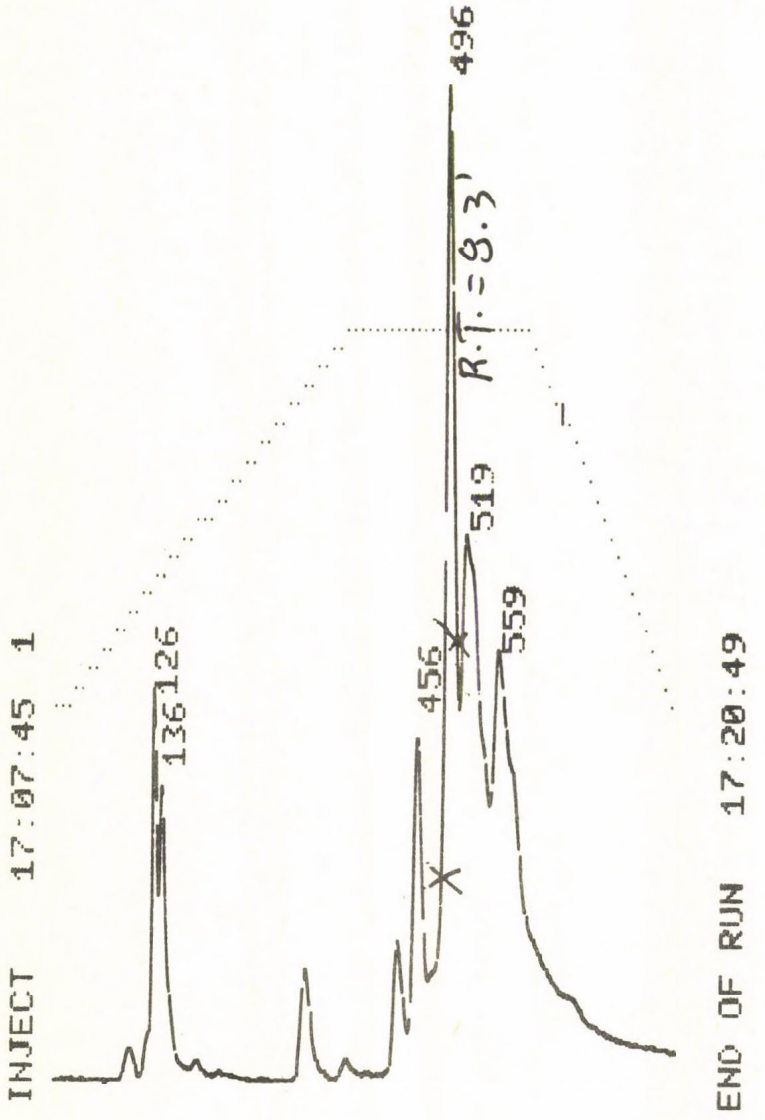

Fig. 2. Full chromatogram of bovine insuline standard. Retention time of the insulin peak: 8.3 min. Conditions as given in Fig. 1. Sample amount: 25.9 µg bovine insulin.

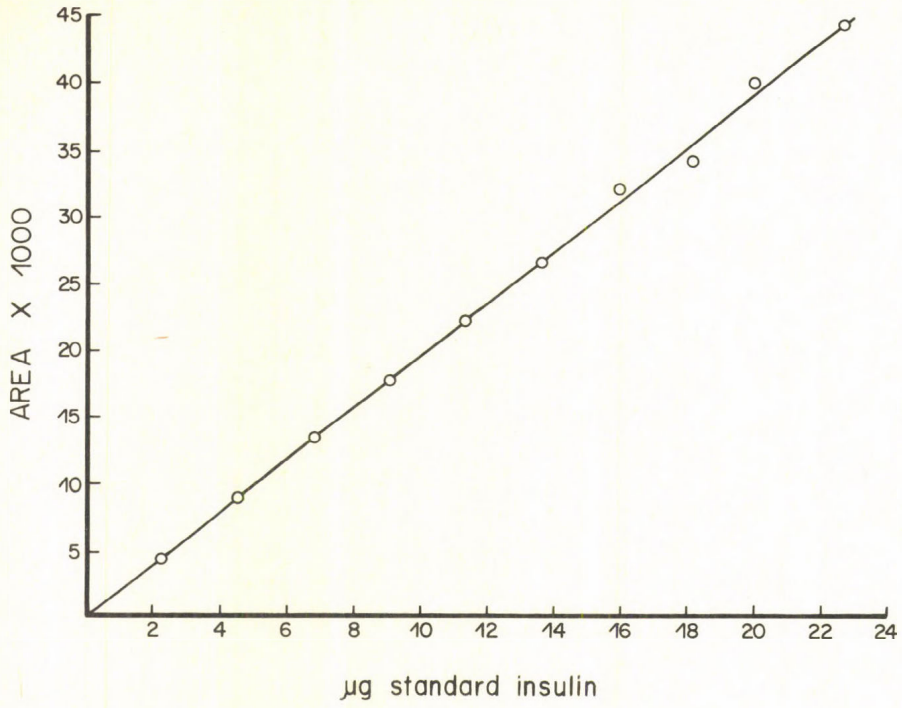

Fig. 3. Linearity of response of standard insulin (2-22 µg).

Table II. Amino acid analysis of insulin

Amino acid	Literature values	Commercial insulin	Reassociated insulin
aspartic acid	3	3.05	2.98
threonine	1	1.03	0.98
serine	3	3.02	2.78
glutamic acid	7	6.9	7.1
proline	1	0.3	0.68
glycine	4	3.96	3.94
alanine	3	3.0	3.23
cysteine	6	N.D.[a]	N.D.[a]
valine	4	4.3	4.39
isoleucine	2	0.2	0.07
leucine	6	5.1	5.86
tyrosine	4	3.37	4.52
phenylalanine	3	2.32	3.0
histidine	2	2.07	1.9
lysine	1	0.83	0.79
arginine	1	0.81	0.88

[a] N.D. = Not determined

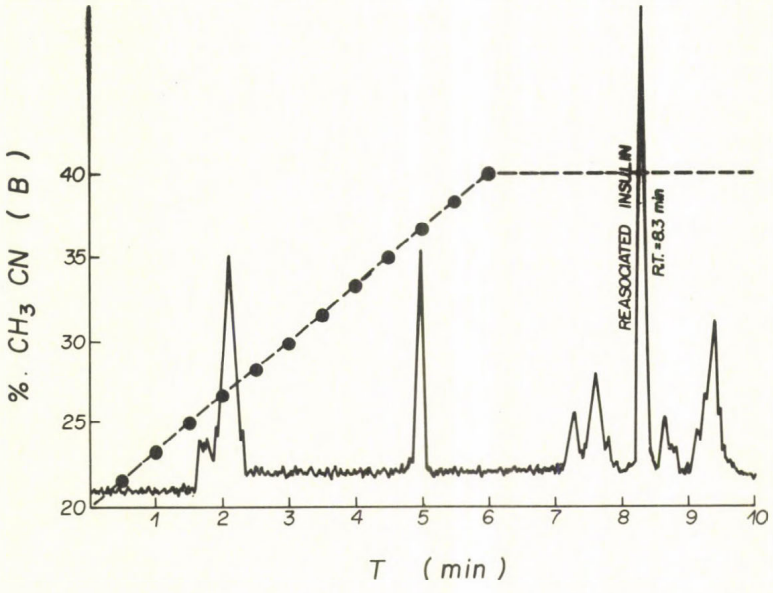

Fig. 4. Chromatogram of reassociated insulin isolated in the Spectra-Physics Systems. Chromatographic conditions: same as in Fig. 1.

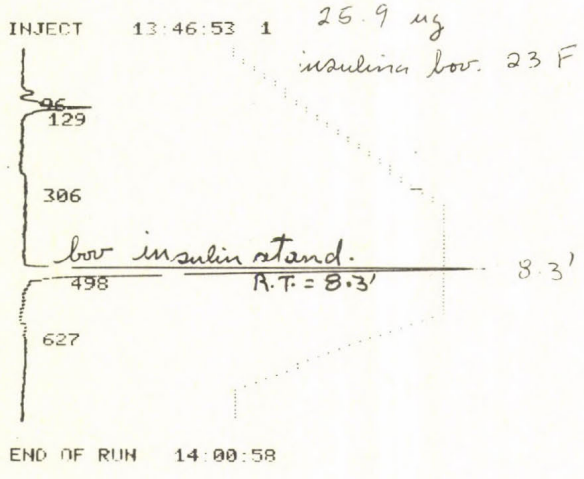

Fig. 5. Full chromatogram of reassociated insulin. Retention time of the insulin peak: 8.3 min. Conditions: as given in Fig. 1.

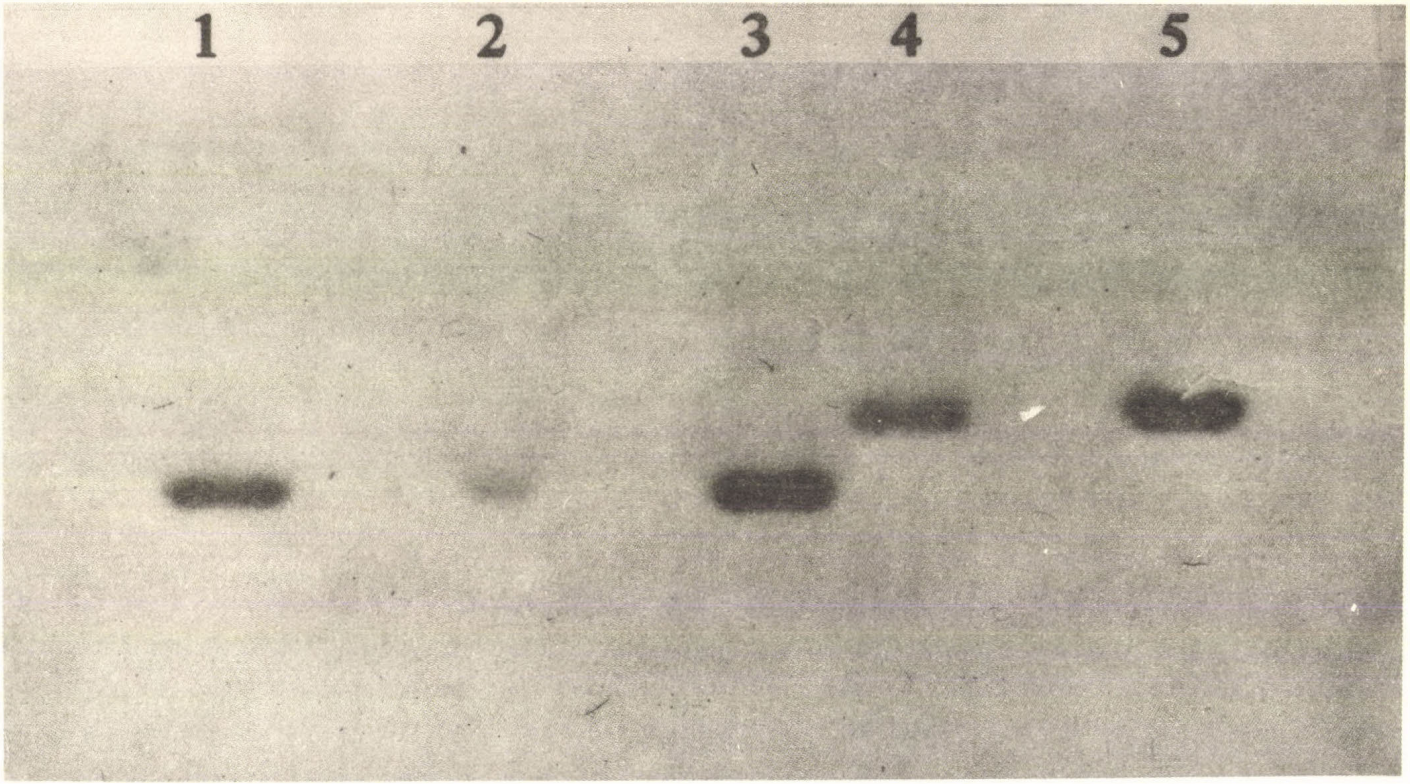

Fig. 6. Electrophoretic analysis of HPLC-purified and reassociated insulin in polyacrylamide gel (5 % stacking; 12 % separating). Run at pH 9.5 in a discontinuous electrolyte borate-sulfate-chloride; Jovin J4179 system.
Tracks: 1) associated human insulin purified by HPLC after concentration, 2) associated human insulin purified by HPLC, 3) porcine insulin standard, 4) porcine insulin peptide B standard, 5) porcine insulin peptide B standard.

From the agreement between the data obtained for the test and standard samples, in both chromatographic systems, and from the acceptable confidence limit values, we conclude that this new method is reproducible.

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ISOTACHOPHORETIC CONTROL OF PEPTIDE SYNTHESIS AND PURIFICATION

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SUMMARY

The intravenous application of glutamine-, tyrosine- and cystine-containing short chain peptides is specially indicated in postinjury conditions, in childhood and in uremia. We have synthesized di- and tripeptides including the above amino acids by employing the N-carboxy anhydride method.

In the present study, synthesis and purification progress were controlled by analytical capillary isotachophoresis (ITP) The separations were made in PTFE capillaries attached to a newly developed detector block allowing detection of the linear stepwise conductivity signal and UV detection at 254 and/or 206 nm. The peptide fractions were analyzed by using anionic electrolyte systems at pH 6.0 and 9.1. Isotachophoretic analyses after synthesis and during purification progress served as a rapid, sensitive, specific and quantitative determination of the single peptides. Additionally, simultaneous detection of free amino acids and its derivatives as well as contaminating organic and inorganic ions was feasible, enabling optimization of synthesis and purification procedures. The new detector block used allowed UV detection at 206 nm, facilitating specific determination of certain peptides not absorbing at the commonly used higher wavelengths of 254 and 280 nm.

Advantages of analytical isotachophoresis over other techniques (e.g. HPLC):

- simultaneous determination of various anionic and cationic compounds (salts) in a single run
- no column packing material and no derivatization step are necessary
- short time of analysis

INTRODUCTION

As recently emphasized, intravenous application of a so-called "complete-well balanced" amino acid solution including all physiological amino acids in physiological proportions might be a mandatory component of an optimum clinical nutrition in various diseased states (1-4). However, poor solubility and/or instability of certain amino acids as glutamine (Gln), tyrosine (Tyr) and cystine ($[Cys]_2$) hindered the formulation and preparation of such solutions (4-8).

In order to facilitate inclusion of the above amino acids to parenteral nutrition, we have synthesized highly soluble and stable Gln-, Tyr- and $(Cys)_2$-containing di- and tripeptides, L-alanyl-L-glutamine (Ala-Gln), L-aspartyl-L-glutamine (Asp-Gln), N^2-L-tyrosinyl-N^6-L-tyrosinyl-L-lysine (Tyr-Lys[Tyr]) and L-cystinyl-bis-L-alanine ($[Cys\text{-}Ala]_2$) as previously described (9, 10). In subsequent reports, the potential use of analytical capillary isotachophoresis (ITP) in characterizing these synthetic peptides after purification was demonstrated (11-13).

In the present study, ITP was successfully used to monitor synthesis and purification progress of the above short chain peptides. Applying conductivity and UV detection (254 nm and/or 206 nm), satisfactory analysis of peptide material in amounts of less than 200 ng was feasible. Apart from the qualitative approach, ITP provided proper quantitative information about the composition of the samples analyzed without the requirement of analyzing suitable reference substances, thereby promoting optimization of peptide synthesis and purification process.

MATERIALS AND METHODS

Peptide synthesis and purification

Ala-Gln, Asp-Gln, Tyr-Lys(Tyr) and $(Cys\text{-}Ala)_2$ were synthesized by applying the N-carboxy anhydride (NCA)-method and subsequently purified as previously described (9, 10). Crude materials, intermediate and end products obtained during synthesis and purification were isotachophoretically analyzed in concentrations and amounts given in the figures.

Table 1. Operational systems for anionic separations

	Leading electrolyte	Terminating electrolyte
System 1	0.01 M Cl^- Ammediol 0.4% HPMC pH 9.1	0.01 M β-alanine Ammediol $Ba(OH)_2$ pH 10.2
System 2	0.005 M MES Ammediol 0.4% HPMC pH 9.1	0.01 M β-alanine Ammediol $Ba(OH)_2$ pH 10.2
System 3	0.01 M Cl^- Bis-Tris 0.4% HPMC pH 6.0	0.01 M MES Tris pH 6.0

Analytical isotachophoresis

ITP analyses were performed by using a LKB 2127 Tachophor equipped with a PTFE capillary (230 mm x 0.55 mm ID) as previously described (13, 14). Conductivity and UV signal was monitored by a two channel recorder (Kipp & Zonen, Delft, The Netherlands) with a chart speed of 6 cm/min. The separations required about 10 min, and current at detection was generally 60 µA.

The operational systems used are outlined in Table 1. For preparing these solutions analytical grade chemicals and ultrapure water were used as previously described (13, 14).

The peptide $(Cys\text{-}Ala)_2$ (reference substance) was purchased from Bachem (Bubendorf, Switzerland). Free amino acids Ala, Gln, Tyr and $(Cys)_2$ as well as L-alanyl-L-alanine (Ala-Ala) were obtained from Sigma (St. Louis, MO, USA).

RESULTS AND DISCUSSION

It is well known that the course of peptide synthesis depends on the chosen reaction conditions as pH, temperature, blocking groups, solvents and especially the ratio of the starting amino acid materials. Thus, for optimization of synthesis procedures various trials under modified reaction variables must be carried out. Furthermore, suitable analytical techniques are necessary to elucidate the amounts of peptide(s) formed and resting starting material in the respective crude products.

Applying electrolyte system 1, three crude materials of $(Cys\text{-}Ala)_2$ obtained under different reaction conditions were analyzed. The respective isotachopherograms are depicted in Fig. 1 showing the presence of at least seven discrete zones in various amounts in both conductivity and UV signals. In these analyses UV detection was made at 254 nm. It is clearly visible that several compounds present in the crude preparations exhibited characteristic UV levels at this wavelength. This enabled a direct classification of the sample components since only the free amino acid $(Cys)_2$ as well as $(Cys)_2$-containing peptides might show specific UV absorption according to the presence of disulfide bonds.

These assumptions could be verified by analyzing commercially available reference substances. Compared to the isotachophoretic pattern of electrolyte system 1 (A), the respective isotachopherogram (B) is illustrated in Fig. 2. Free $(Cys)_2$ as well as $(Cys\text{-}Ala)_2$ revealed characteristic UV levels, whereas for free Ala no UV absorption could be measured. Indeed, it is evident that the characteristic conductivity step heights and UV levels derived on one hand from the crude preparations and on the other hand from the standard substances are identical. Further minor UV absorbing zones detected in the crude materials must correspond to $(Cys)_2$-containing side products as, by example, di-or tetrapeptides.

Apart from the qualitative approach, ITP provides quantitative information about the composition of the samples analyzed (11-13, 15). According to the basic theory firstly de-

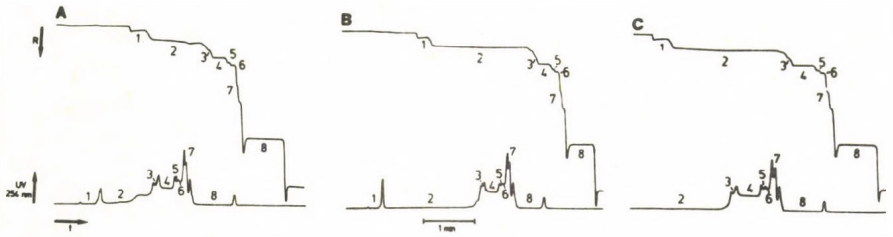

Fig. 1. Isotachophoretic analyses (electrolyte system 1) of three crude samples of $(Cys\text{-}Ala)_2$ (A-C). For each separation, 10 µl were injected, corresponding to 7.64 µg of material.
Key: 1 = SO_4^{2-}; 2 = HCO_3^-; 3,5,7 = unknown side products; 4 = $(Cys\text{-}Ala)_2$; 6 = $(Cys)_2$; 8 = Ala

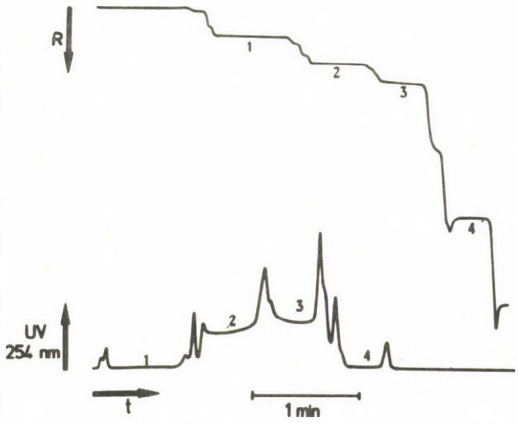

Fig. 2. Isotachophoretic analysis (electrolyte system 1) of a reference mixture of $(Cys\text{-}Ala)_2$ (2), $(Cys)_2$ (3) and Ala (4). (A) electrolyte system; (B) reference mixture (2.5 µl injected, corresponding to 1.91 µg $(Cys\text{-}Ala)_2$, 1.20 µg $(Cys)_2$ and 0.45 µg Ala). 1 = HCO_3^-

scribed by Kohlrausch (16) in the so-called regulating function, the concentration in a separated sample zone is directly proportional to the leading ion concentration. Since the ion concentration in each zone is constant, quantitative evaluation of isotachopherograms is possible by measuring single zone lengths and total zone lengths, respectively.

Table 2. Zone lengths of $(Cys\text{-}Ala)_2$, $(Cys)_2$, Ala and unknown side products in the different crude preparations in percent of total zone length

	$(Cys\text{-}Ala)_2$	$(Cys)_2$	Ala	Sum of side products
Crude material I	21.0	7.0	62.2	9.8
Crude material II	26.5	6.9	58.8	7.9
Crude material III	29.1	6.1	54.6	10.2

Consequently, the respective zone lengths of $(Cys\text{-}Ala)_2$, $(Cys)_2$, Ala and side products in the different crude preparations were calculated in percent of the total zone length (Table 2). Obviously, a considerable increase in the formation of $(Cys\text{-}Ala)_2$ accompanied by a proportional decrease in unreacted free amino acids could be achieved by varying the reaction conditions.

As mentioned above, the measurement of characteristic UV levels provides information about the chemical structure of the separated compounds thereby allowing a direct differentiation of the detected ions. In this context it is to be noted that only peptides containing $(Cys)_2$ and aromatic amino acids revealed characteristic UV levels at the commonly used wavelengths of 254 nm and/or 280 nm. As shown in Fig. 3, analysis of the crude material of Tyr-Lys(Tyr) applying electrolyte system 1 (Table 1) revealed 8 fully separated zones and one mixed zone. In addition to the main zone corresponding to Tyr-Lys(Tyr) six of the minor zones exhibited characteristic high UV levels thus indicating the presence of Tyr-residues in these compounds.

In contrast to the Tyr-containing peptide, analysis of the crude material of Ala-Gln applying electrolyte system 2 resulted in one main zone and four minor zones, none of them showing characteristic UV levels at 254 nm (Fig. 4A). Only the occurrence of so-called "marker or separation peaks" (presumably due to different refractive indices of the separated zones) (10) between non-UV absorbing zones facilitated the evaluation of the UV signal. Nevertheless it is to mention that in certain

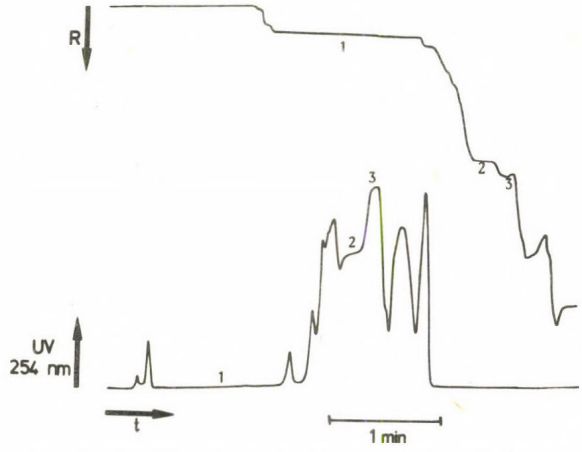

Fig. 3. Isotachophoretic analysis (electrolyte system 1) of a crude sample of Tyr-Lys(Tyr) (5 µl injected, corresponding to 4.73 µg of material)
Key: 1 = HCO_3^-; 2 = Tyr-Lys(Tyr); 3 = Tyr

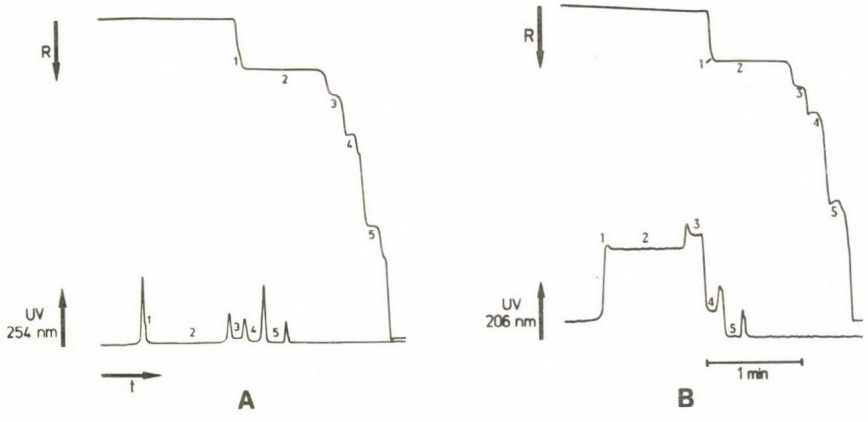

Fig. 4. Isotachophoretic analyses (electrolyte system 2) of a crude sample of Ala-Gln: UV detection at 254 nm (A) and 206 nm (B). For each separation, 20 µl were injected, corresponding to 8.68 µg of material.
Key: 1 = Ala-Ala; 2 = Ala-Gln; 3 = unknown peptide; 4 = Gln; 5 = Ala

operational systems no such marker peaks are detectable thereby invalidating the evaluation of the UV signal (10, 15). Further-

more, the lack of characteristic UV levels complicates the identification and classification of unknown sample compounds.

As currently reported, adequate solution of this problem could be achieved by using a new available detector block which allows UV measurement at 206 nm (14). In all previous types of capillary plates, UV signal was measured directly through the capillary wall. Thus, the high absorption and dispersion of the capillary material (generally PTFE) hindered the use of wavelengths below 230 nm. The great improvement of the new UV detector lies in the fact that the fibre optics leads the light directly into the interior of the liquid system resulting in a significantly smaller loss of UV light intensity. Consequently, this detector system facilitates UV measurement at wavelengths below 230 nm.

The great advantage of measuring UV absorption at 206 nm in the analysis of crude Ala-Gln material is illustrated in Fig. 4B. In contrast to 254 nm (Fig. 4A), four of the separated zones exhibited characteristic UV levels at 206 nm. According to the high absorption coefficient of peptide bonds at 206 nm (17), the main peptide Ala-Gln as well as two minor peptides show high UV absorption, whereas low (owing to the presence of the amide group) or no UV-absorption could be measured for free Gln and Ala, respectively.

As demonstrated for Ala-Gln, UV detection at 206 nm allows a direct classification of unknown sample ions enabling a direct differentiation between peptides formed during synthesis and unreacted free amino acids. Thus, ITP analysis of crude peptide materials provide valuable information about the progress of peptide synthesis thereby highly facilitating the optimization of the synthesis procedure.

The suggestion that ITP could be useful as a monitor for controlling peptide purification progress was first made by Kopwillem and colleagues (18-20). They analyzed the crude material as well as peptide containing fractions obtained during the purification procedure of synthetic tetradeca-, deca- and undecapeptides. Recently, Friedel and Holloway employed ITP in the analysis of intermediate and end products during synthesis of a pentapeptide (21). In these studies detection was made by

monitoring thermometric signal and UV detection at 254 and/or 280 nm. Owing to the relatively low sensitivity of thermometric detection, high amounts (about 30 µg) of peptide material were necessary to obtain satisfactory results. Additionally, the characteristic wave form of the thermometric signal rendered a quantitative and qualitative evaluation difficult. Thus, only the enrichment of the main products during the purification process could be followed.

In the present study, crude materials, intermediate and end products obtained during the development of purification of Ala-Gln and Asp-Gln were analyzed applying conductivity detection and UV measurement at 206 nm. Compared to the analysis of the crude Ala-Gln material (A) both free amino acids Ala and Gln could be removed during the first gel-chromatographic purification step (B) as illustrated in Fig. 5. Furthermore, the zone length for the main product was markedly increased and moderate increases were also noted for Ala-Ala and the unknown side product. This might be due to the simultaneous removal of salts during the first purification step. The majority of the two contaminating products could be removed applying further gel filtration purification steps (C, D). In the final product an enrichment of Ala-Gln approaching 100% could be achieved. Only traces of Ala-Ala were detectable.

The crude material (A) and the different purified peptide fractions (B, C) of Asp-Gln were analyzed similarly but applying electrolyte system 3 (Table 1) as depicted in Fig. 6. In addition to Asp-Gln only traces of Asp and small amounts of overreaction products could be detected after the first purification step (B). After the second gel chromatographic separation (C) an enrichment approaching 100% was observed for Asp-Gln.

A further valuable advantage of ITP compared with chromatographic techniques is well emphasized in Fig. 6. In addition to the respective starting material and the synthetic peptides formed, ITP allowed a simultaneous detection of contaminating inorganic ions in the same run. Obviously in the crude material as well as in the intermediate product of Asp-Gln a further zone, only detectable in the conductivity signal was observed,

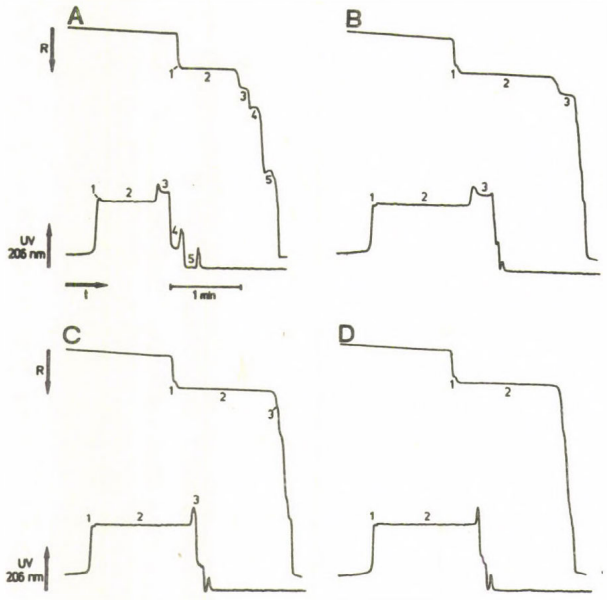

Fig. 5. Isotachophoretic analyses (electrolyte system 2) of Ala-Gln before (A) and after (B-D) the different purification steps. For each separation, 20 µl were injected, corresponding to 8.68 µg of material.
Key as in Fig. 4.
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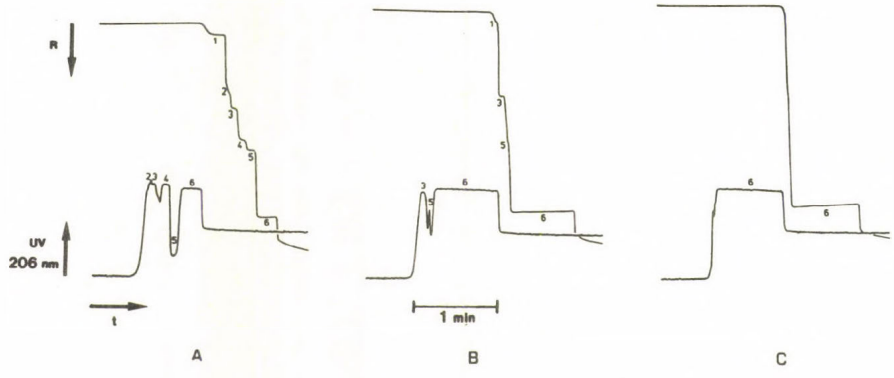

Fig. 6. Isotachophoretic analyses (electrolyte system 3) of Asp-Gln before (A) and after (B, C) the different purification steps. For each separation, 10 µl were injected, corresponding to 5.49 µg of material.
Key: 1 = SO_4^{2-}; 2,3,4 = unknown peptides; 5 = Asp; 6 = Asp-Gln.
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suggesting the presence of contaminating sulphate derived from the peptide synthesis procedure.

In the present study, it may be concluded that ITP was found to be a rapid and reliable method in the analysis of synthetic short chain peptides. Compared with previously used detection systems, application of unspecific conductivity detection and UV monitoring at 206 nm considerably increased the resolution and sensitivity, enabling qualitative and quantitative measurement of peptide material in amounts of less that 200 ng.

Compared to HPLC, certain advantages of ITP are obvious: (1) simultaneous detection of starting material, peptides formed during synthesis and contaminating inorganic ions is feasible; (2) quantitative information is provided about the composition of the sample analyzed without the mandatory use of suitable reference substances; (3) no column packing material and no derivatization step are necessary. Consequently, the application of analytical capillary isotachophoresis might improve the control of synthesis and purification progress in peptide chemistry.

ACKNOWLEDGEMENTS

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STUDY OF PROTEIN ASSOCIATION AND MICELLE FORMATION IN SURFACTANTS BY MICROCOLUMN EXCLUSION CHROMATOGRAPHY

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ABSTRACT

The application of microcolumn exclusion chromatography to the determination of equilibrium and kinetic association constants of proteins is described. Taking phospholipase A_2 as the example the relationship between association and enzymatic activity was investigated. A method was developed for the determination of the critical concentration of micelle formation (CCM) in surfactants.

INTRODUCTION

Problems related to the determination of physico-chemical constants of samples available only in very small amounts often occur in molecular biology. Microcolumn chromatography which makes it possible to reduce the amount of the substances investigated by about two orders of magnitude is an invaluable method for the investigation of such biological samples available in small amounts. Liquid exclusion chromatography is traditionally used for the separation of substances according to the size of their molecules but can also be applied to the study of physico-chemical properties of reacting systems, e.g. of protein associations or micelle formation in surfactants.

The mechanism of the catalytic action of proteins is often based on their association. Of particular interest are systems consisting of two kinetically different components which can reversibly pass from one form to the other. The process depends on the content of various ligands and the concentration or the

aggregate state of the substrate. This mechanism of the catalytic action of enzymes is widely observed in nature /1/.

Natural diphilic substances capable of forming micelles are not biologically inert because they participate in the process of suction and metabolism. Evidence exists that the property of forming ordered aggregates is a fundamental factor in the biological activity of surfactants. Liquid size exclusion chromatography (SEC) permits the evaluation of the main parameter characterizing the micellar system: the critical concentration of micelle formation (CCM)/2/.

MATERIALS AND METHODS

Experiments were carried out using a model KhZh-1305 microcolumn chromatograph (Special Design Bureau of Analytical Instruments, USSR Academy of Sciences) equipped with a double-beam spectrophotometer with a scanning range of 220-600 nm. The optical path length of the cell was 1.5 mm with 1-µl volume. The cell volume of the precision syringe micropumps was 2 ml with the flow rate of the eluent ranging from 8 to 4000 µl/h.

Glass microcolumns (250 mm x 1 mm I.D.) used for the study of the proteins were packed with Sephadex G-75, superfine (Pharmacia, Sweden) or Bio-gel P-60, >400 mesh (Bio-Rad Lab., USA). The same columns packed with Sephadex G-25 or G-75 were used for the determination of CCM of the surfactant.

A phospholipase sample and a model substrate, dihexanoyl lecithin, were obtained from the Institute of Bioorganic Chemistry, USSR Academy of Sciences (Moscow). The following standard proteins were used for calibration: cytochrome C, ribonuclease, myoglobin, chymotrypsinogene A, ovalbumin and bovine serum albumin (Serva, FRG). Sodium dodecyl sulphate (Fluca, Denmark) was used without additional purification. Other reagents were of "pure for analysis" grade.

RESULTS AND DISCUSSION

Determination of equilibrium and kinetic association constants for phospholipase A_2

Four levels of sample study exist in the investigation of association: 1) association diagnostics, 2) determination of the number of components, determination of 3) equilibrium and 4) kinetic constants, and 5) study of the relationship between association and the functional activity of the enzyme.

Transport methods (sedimentation, chromatography and electrophoresis) sensitive to the size of molecules are widely used in the study of association. Size-exclusion chromatography has an advantage over other methods because it may be used in all the stages of association investigations. In many cases a comprehensive analysis of protein association is possible without the application of other methods. The study of association in stages 1-3 has been very soundly based theoretically by Gilbert /3/ and Ackers /4/. The methodologic approach in stages 4 and 5 has been developed by us /5, 6/. The concentration dependence of the elution volume of the associating protein has been used for the determination of equilibrium constants. The concentration dependence of dispersion of the chromatographic boundary permits the determination of the kinetic constants for association. For this purpose stepwise chromatography ensuring the constant composition of components in the plateau region has been used /7/. Moreover, the centre of gravity of the leading and tailing fronts of the stepwise chromatogram corresponds to the weight-average retention volume of reaction components /7/. When a series of chromatographic experiments is carried out stepwise over a wide concentration range, large quantities of protein are consumed. In order to form a wide protein zone it is necessary to apply a sample with a volume of about the column volume. Hence, when the experiments are carried out, for example on a 60-cm long column with 7 cm diameter, approximately 500 mg protein is needed, and when a 25-cm long column with 4 cm diameter is used, about 100 mg protein is employed. Moreover, one series of experiments is sufficient for the study of association on

levels 1-4, whereas the establishment of the mechanism for enzymatic activity usually requires several series of experiments in a chromatographic system saturated with some ligands. In this case the major factor is the consumption of the expensive eluent.

When SEC is carried out on columns with 0.5-1 mm I.D., protein consumption is greatly reduced (by approximately two orders of magnitude). Hence, microcolumn chromatography provides a real possiblity to study the association reaction and its relationship to the enzymatic activity of proteins only available in small amounts.

During the SEC of the reacting system, separation into components occurs only for slow reactions. If the reaction proceeds rapidly, the chromatogram shows only a single peak with a sharp leading boundary and a spread trailing boundary. When the rates of the reaction and of the separation process are comparable, polymodal distribution can be observed. However, this distribution does not correspond to individual components. The most complex problem in the study of association is the determination of the number of components. However, there are criteria making it possible to distinguish between a two-component and a multicomponent system. If only two components are present, the problem is considerably simplified. Let us consider the application of SEC to the determination of the equlibrium and the rate constants taking as an example the simplest monomer - n-mer reaction:

$$nA \overset{K_{1,n}}{\longleftrightarrow} A_n \qquad (1)$$

Since the elution volume of the centroid of the front is the weight-average elution volume of the reaction components, the concentration dependence may be used to find the equilibrium constant:

$$\bar{V}_W(C_O) = \frac{V_1 + K_{1,n}C_1V_n}{1 + K_{1,n}C_1} \qquad (2)$$

where C_1 is the concentration of the monomer and C_O is the overall protein concentration. By varying $K_{1,n}$ it is possible to achieve the best coincidence between the curve satisfying eq. (2) and the experimental dependence $\bar{V}_W(C_O)$. The elution

volume of the monomer (V_1) may be found by the extrapolation of the dependence $\bar{V}_W(C_O)$ to infinite dilution and the elution volume of the n-mer (V_n) can be calculated from V_1 and the calibration dependence $V(M)$ or $V(R_{St})$ where M is the molecular weight and R_{St} is the Stokes radius of the protein.

Hence, the equilibrium constant $K_{1,n}$ and the degree of association n for a two-component system is uniquely determined from the concentration dependence $\bar{V}_W(C_O)$.

For equilibrium the following relationship are valid:

$$k_1 C_1^2 = k_2 C_2, \quad K = k_1/k_2 = C_2/C_1^2 \qquad (3)$$

where k_1 and k_2 are the kinetic constants for the direct and the reverse reactions, respectively. It is desirable to use the rear fronts of the chromatographic zones for the determination of k_1 and k_2 because they provide more information about the reaction kinetic than the leading fronts do /8/. Zone spreading may be described with the aid of a dimensionless dispersion coefficient

$$L_V = \sigma_V^2/V^2 = 1/N \qquad (4)$$

where σ_V^2 is the dispersion of the elution volume and N is the plate number. If the reaction takes place, zone spreading depends not only on purely chromatographic factors but also on the recombination of the monomer and the associate. Hence, the measured dispersion coefficient is summed up from dispersion and a certain added kinetic value depending on the rate constants and the reaction equilibrium /8/.

This method was used by us for studying the association of phospholipase A_2.

Phospholipase A_2 hydrolyzes the ester bond of fatty acids. Moreover, the presence of Ca^{2+} ions is absolutely indispensable for the activity of this enzyme and the hydrolysis rate depends on the aggregate state and increases markedly when the substrate exists in the micellar phase /9/. There are reasons to assume that the active form of the enzyme is an associate, probably a dimer /10/. Hence, it is of interest to characterize the equilibrium and the kinetics of phospholipase A_2 association and their dependence on the presence of Ca^{2+} ions

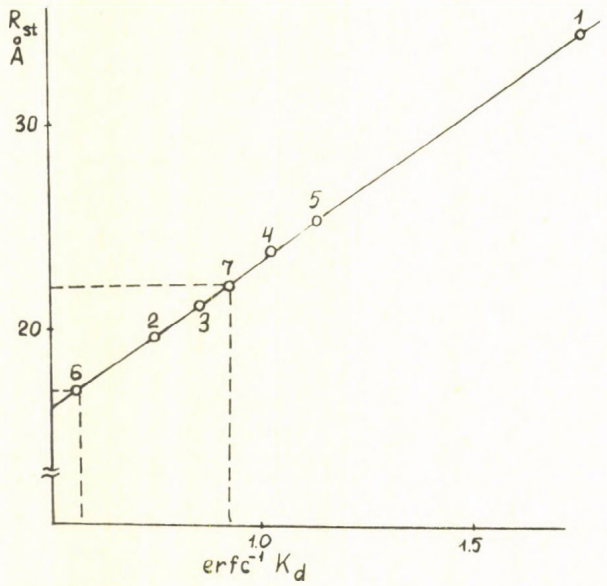

Fig.1. Calibration dependence of the distribution coefficient K_d on Stokes radii of proteins. 1 - cytochrome C, 2 - ribonuclease, 3 - myoglobin, 4 - chymotrypsinogen A, 5 - ovalbumin, 6 - phospholipase A_2 (monomer), 7 - phospholipase A_2 (dimer).

and of the substrate in the monomeric and the micellar forms.

In order to determine the degree of association or the size of the associate Ackers'calibration dependence /11/ may be used:

$$R = a + b\ erfc^{-1}\ K_d \qquad (5)$$

where a and b are constants, K_d is the distribution coefficient and $erfc^{-1}K_d$ is the inverse function (Fig. 1). The degree of association may be determined according to a model for the merging of two globular proteins into a single sphere from the values of $R_1 = (16.8 \pm 0.3)10^8$ cm and $R_2 = (21.0 \pm 0.3)10^8$ cm. Then we have $n = (R_n/R_1)^3 = 1.95 \pm 0.08$. Hence, the reaction proceeds according to the monomer-dimer scheme.

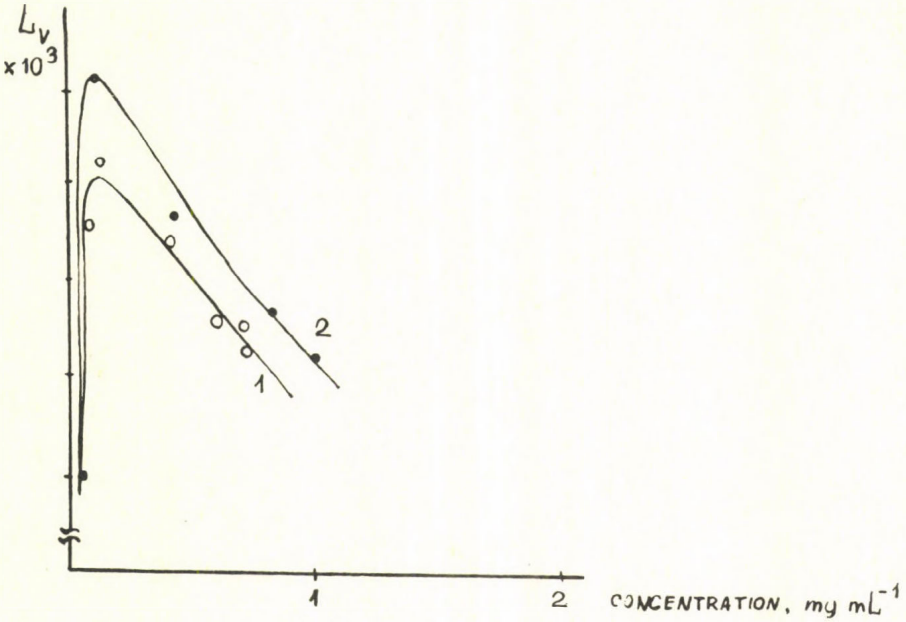

Fig. 2. Concentration dependence of the dispersion coefficient $L_{V(M)} = {}^2V/V_w{}^2$ for phospholipase A_2 leading boundary of chromatogram. (1) $C_{Ca}2+ = 0$; (2) $C_{Ca}2+ = 0.02$ M.

Fig. 2 shows the experimental concentration dependences of dispersion coefficients for the rear fronts of the chromatograms for spontaneous association and for that induced by Ca^{2+}. These dependences are of the extreme character. This fact is due to the mixed kinetics of the chromatographic process at intermediate concentrations when association and mass transfer provide comparable contributions to front spreading. The rate constants determined on the basis of experimental data and eq. 8 are given in Table 1.

The evidence obtained shows that Ca^{2+} ions do not change the position of the monomer-dimer equilibrium and only somewhat retard the rates of dissociation and association. Hence, Ca^{2+} ions alone cannot serve as agents stabilizing the active dimeric form of phospholipase A_2. Since data are available showing that dimerization rate increases markedly in the presence of a micellar substrate, the effect of the substrate in the monomeric and the micellar forms on the dimerization

Table 1. The equilibrium and the rate constants for phospholipase A_2

Ca^{2+} concentration	$K_{1,2} \times 10^{-4}$, M^{-1}	$K_1 \times 10^{-4}$, $M^{-1}s^{-1}$	k_2, s^{-1}
0	6.0	4.2	0.7
0.02	6.3	1.27	0.2

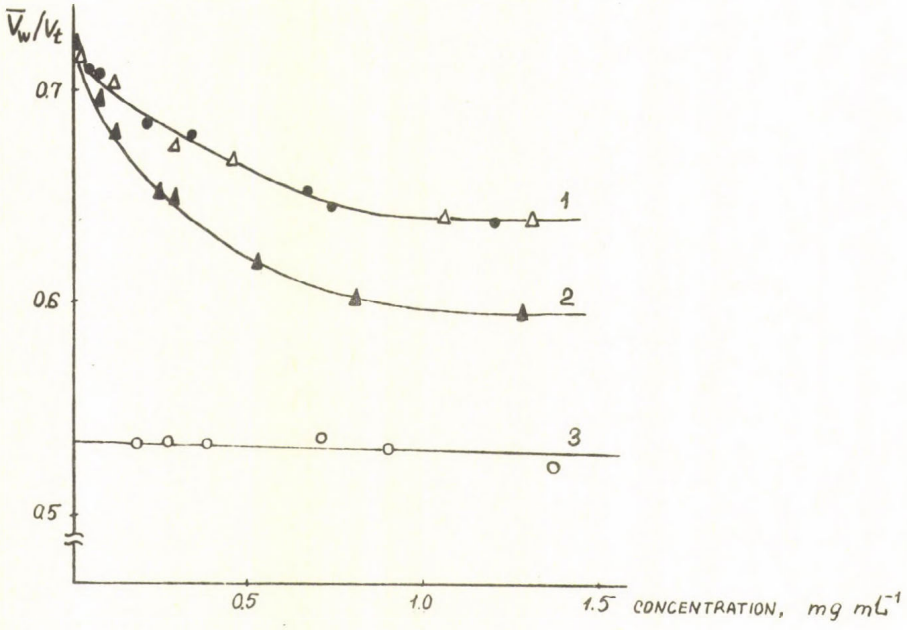

Fig. 3. Concentration dependence of the reduced weight-average elution volume V_w/V_t for phospholipase A_2 (Sephadex G-75 column) obtained in the systems: Tris-HCl-KCl (2, x), Tris-HCl-KCl-$BaCl_2$ (1, O); Tris-HCl-KCl-$BaCl_2$-S ([S] = 1 mg/ml < CCM)(1,Δ); Tris-HCl-KCl-$BaCl_2$-S ([S] = 20 mg/ml > CCM)(3, O); S - substrate (dihexanoyl-lecithin), CCM - critical concentration of micelle formation.

equilibrium was investigated. For this purpose the chromatographic system was saturated with the substrate at appropriate concentration in the presence of an inhibitor and the dependences of reduced weight-average retention volumes were obtained. These data are given in Fig. 3. Dimerization constants K = (4.5 $\pm$ 0.2) ml/g were found for spontaneous association and for association in the presence of an inhibitor, and K = (1.6 $\pm$ 0.1) ml/g was determined for a system containing the inhibitor and the monomeric substrate. Hence, it can be seen that the inhibitor slightly shifts the equilibrium towards the monomer, and the substrate in the monomeric form does not affect the association equilibrium.

Fig. 3 shows the concentration dependence of reduced weight-average elution volume of phospholipase A_2 in the presence of the micellar substrate ([S] = 20 mg/ml) and the inhibitor Ba^{2+}. This dependence is linear. Extrapolation to zero concentration gives the value of 0.54 which corresponds to the reduced elution volume of the dimer.

Hence, the chromatographic data shown in Fig. 4 lead to the unequivocal conclusion that the micellar substrate completely shifts the association equilibrium of phospholipase A_2 towards the dimer. However, this dimer forms a complex with the micelle only in the presence of Ca^{2+}, whereas Ba^{2+} precludes the formation of the complex. After the completion of the enzymatic reaction the enzyme is regenerated in the form of the monomer.

Chromatographic method also permits a direct observation of the reaction between the enzyme and the substrate in a system saturated with the substrate and various ligands. In this case differential detection may be used in which the detector is insensitive to one of the components.

Thus, the product of the reaction between phospholipase A_2 and dihexanoyl lecithin is not detected at the wavelength of 290 nm. Fig. 4 shows the stepwise zone of the micellar substrate which is eluted in the form of a complex polymodal chromatogram. Furthermore, the peaks of the leading front are due to the formation of thermodynamically stable micelles with a large size and the plateau region at the rear front corres-

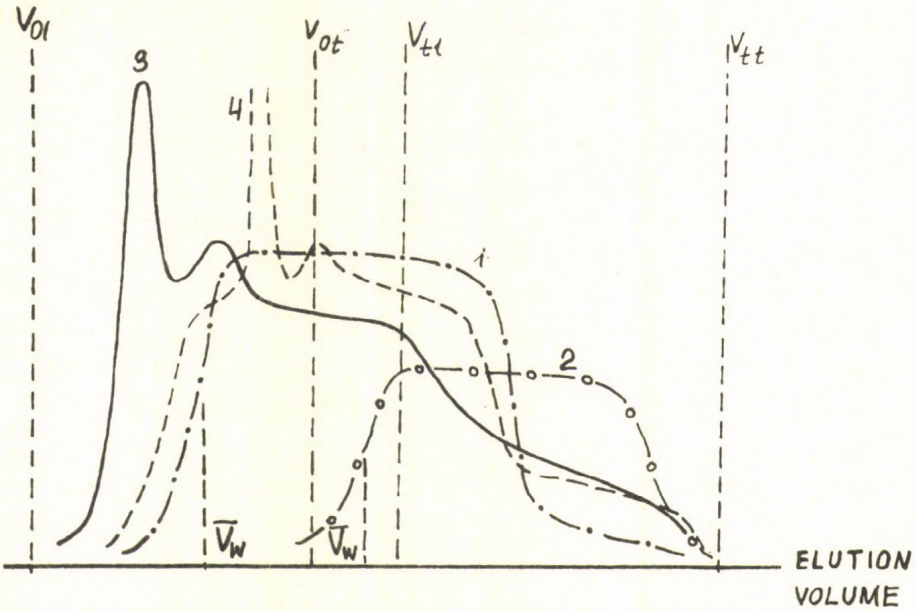

Fig. 4. Frontal chromatograms of phospholipase A_2 (C = 0.2 mg/ml) (1,4) and the substrate in the range of true solutions (2) and micellar concentrations (3). Buffer solutions containing a micellar substrate and 20 mM of Ba^{2+} or Ca^{2+} were used for the elution of 1 and 4 respectively; detection was carried out at 290 nm. A buffer solution containing Ba^{2+} was used for the elution of 2 and 3; detection was carried out at 230 nm. V_{ol} and V_{ot} are elution volumes for the substance excluded from the gel pores for the leading and the tailing fronts of chromatograms (blue dextran). V_{tl} and V_{tt} are complete elution volumes for the leading and the tailing fronts of chromatograms with tryptophan. V_w - the centre of gravity of the leading front.

ponds to the equilibrium concentration of the monomeric substrate.

The reduced elution volume for micelles determined from the centre of gravity of the leading front $\bar{V}_w/V_t$ is 0.52, i.e. the micelles are of an average size close to that of the dimeric phospholipase A_2 (R = 21 Å) because the presence of the complex would lead to a considerable decrease in retention

volume as compared to that for the dimer.

If phospholipase A_2 is eluted in a system with a promoter (Ca^{2+}) and a micellar substrate, in the course of chromatography the substrate undergoes hydrolysis and the monomeric enzyme is regenerated. Therefore the centroid of the leading front is displaced toward the reduced weight-average value for the monomer.

It is important that in this case in the presence of Ca^{2+} (Fig. 4/4) the chromatogram of phospholipase A_2 reproduces the shape of the elution curve for the micellar substrate (Fig. 4/3) and in the presence of Ba^{2+} is of the usual shape (Fig. 4/1).

Hence, by using microcolumn SEC alone it was possible to calculate the equilibrium and kinetic association constants for phospholipase A_2 and to establish the mechanism for the catalytic action of this protein.

Determination of critical concentration of micelle formation in surfactants by liquid size-exclusion chromatography

Experiments with a diphilic substrate of phosphoplipase A_2 showed that in elution exclusion chromatography of surfactants the zone of the monomer is separated from that of micelles and the area of the former is not related to the general concentration of the surfactant but depends only on the thermodynamic properties of the solution: the critical concentration of micelle formation (CCM). When the proposed procedure is used, a single chromatogram of the surfactant at a concentration exceeding CCM is sufficient for the evaluation of CCM from the following ratio:

$$\frac{\text{surface under the monomer peak}}{\text{total surface under the chromatogram}} \times \text{overall concentration of the surfactant} = \text{CCM}$$

A throroughly investigated substance, sodium dodecyl sulphate (SDS), was used to check this method. Fig. 5 shows SDS chromatograms at various concentrations. The calculated

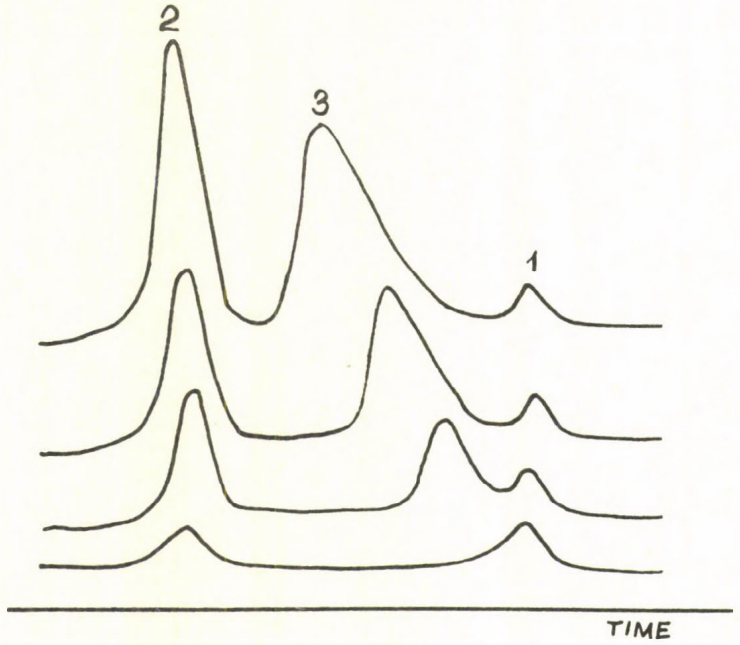

Fig. 5. Chromatograms of sodium dodecyl sulphate on a 25 cm x 0.1 cm I.D. column packed with Sephadex G-75 at various surfactant concentrations. 1 - monomeric surfactant, 2 - thermodynamically stable micelles, 3 - micelles undergoing fast recombination.

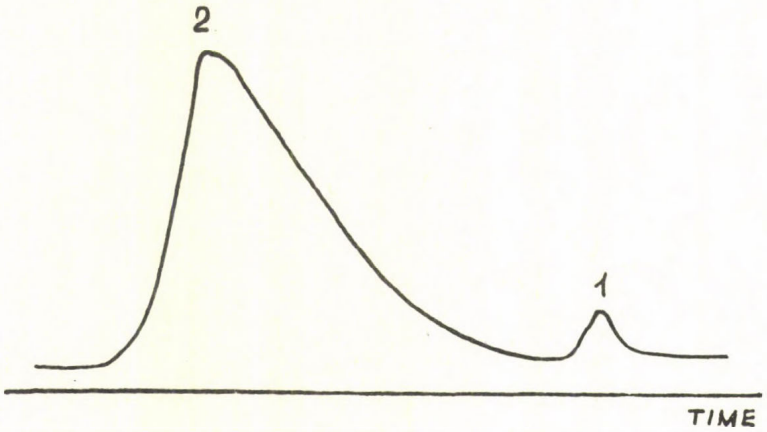

Fig. 6. Hypothetical chromatogram of fast recombinating surfactant. 1 - zone of monomer.

values of CCM are in good agreement with those obtained by other methods /2/.

The chromatograms of the surfactant are characterized by some specific features reflecting the dependence of the rate of micelle dissociation on their size. A range of thermodynamically stable micelles exists (Fig. 5/2), the monomer peak retains constant surface regardless of the overall concentration and the peak of thermodynamically unstable micelles is characterized by a train (the rear front is spread as a result of micelle dissociation in the column). For the latter peak retention volume depends on the overall concentration of the surfactant (Fig. 5/3).

The shape of the chromatogram in Fig. 5 suggests that the micelles of the surfactant form relatively stable structures of large size. They are separated according to size during chromatography and exhibit a long half-life period. If the recombination of micelles had proceeded rapidly, the chromatogram should have had the form shown in Fig. 6, i.e., would have had a broad train of dissociating micelles passing into the monomer zone. The size of micelles was evaluated on a column packed with Sephadex G-75 calibrated with proteins.

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PLASMA PROTEIN SEPARATION BY ION EXCHANGE AND AFFINITY CARTRIDGES

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I. INTRODUCTION

The broad applicability of ion-exchange chromatography, which ranges from separation of inorganic and organic ions to that of protein molecules and other biomolecules, has made it a powerful and versatile tool for chemical and biochemical separations. The technique was originally limited to the use of natural products such as cellulose, clay and other minerals containing mobile ions that would exchange with ionic materials in the surrounding solute phase. Because of the low exchange capacity of these natural products, however, practical utilization thereof was limited, and synthetic organic polymers capable of exchanging ions were developed.

Among the first generation of synthetic ion exchange materials were the ion-exchange resins /1/. The fundamental framework of these ion-exchange resins is an elastic three-dimensional hydrocarbon network comprising ionizable groups, either cationic or anionic, chemically bonded to the backbone of a hydrocarbon framework. The network is normally fixed, insoluble in common solvents and is chemically inert. The ionizable functional groups attached to the matrix carry active ions which can react with or can be replaced by ions in the solute phase. Therefore, the ions in the solute phase can be easily exchanged for the ions initially bound to the polymeric resins. Typical examples of commercially available ion-exchange resins are the polystyrenes cross-linked with divinylbenzene (DVB), and the methacrylates copolymerized with DVB. The ion-exchange resins

made from polymeric resins have been successfully applied for the removal of both organic and inorganic ions from aqueous media but they are normally unsuitable for the separation of biopolymers such as proteins. This is due, among other things, to their narrow pores which are unable to accommodate the diffusion of large protein molecules and their hydrophobic nature leading to high non-specific adsorption and the eventual denaturing of protein molecules.

The next generation of chromatographic materials useful for separation of proteins and other labile biological substances was based on cellulose ion exchangers. /2/ These lacked nonspecific adsorption and had practicable pore structure. Such prior art ion-exchange celluloses are made by attaching substituent groups with either basic or acidic properties to the cellulose molecule by esterification, etherification, or oxidation reactions. However, only limited capacity can be achieved by such derivatization method without destruction of the cellulose structure.

A third generation of ion-exchange materials, which were developed to solve some of these problems, were the ion-exchange gels. Those gels are cross-linked polysaccharides /3, 4/. They exhibit very low nonspecific adsorption which makes them ideal as a matrix for biological separations. However, the porosity of ion-exchange gels is critically dependent on their swelling properties, which in turn are affected by the environmental ionic strength, pH and the nature of the counterions. Swelling of gels in a buffer is caused primarily by the tendency of the functional groups to become hydrated. The amount of swelling is directly proportional to the number of hydrophilic functional groups attached to the gel matrix, and is inversely proportional to the degree of crosslinking present in the gel. This characteristic swelling is a reversible process, and at equilibrium there is a balance between two forces: The tendency of the gel to undergo further hydration, and hence to increase the osmotic pressure within the gel beads, and the elastic forces of the gel matrix. Gels without crosslinking provide large pores and high capacity due to maximum swelling. They suffer, however, from the weakness of structure integrity

and can easily be crushed with a minimum amount of pressure. Removal of the solvent from the gels often results in collapse of the matrix. Highly crosslinked gels have mechanical strength, but lose capacity and pore size due to restrictions in swelling.

The failure of single components to have both capacity and dimensional stability led to yet another generation of ion-exchange materials comprising composite structures, e.g., hybrid gels. Hybrid gels are made by combining a semi-rigid component for the purpose of conferring mechanical stability with a second component, a softer network, which is responsible for carrying functional groups. Cellulose, which would otherwise be very soft and compressible, as well as low in capacity, can be made stronger through hybridizing with cross-linked polyacrylates. The cellulose-acrylate composite matrices developed by us and applied in the present study are based on this principle. The media can be made to carry either anionic functional groups such as DEAE and QAE or cationic groups such as CM and SP, as well as epoxy groups for affinity. The fabrication of the media to a thin sheet paper form was performed by conventional paper making technology.

The composite sheet of active chromatography media is spirally wound around a perforated cylindrical core having a longitudinal axis to form a plurality of layers around the axis. The cylindrical core is provided with apertures near the top for the flow of samples from the circumferential channels into the open interior of the core and then exiting through the top aperture. The stationary phase and caps of this subassembly are sealed by thermoplastic fusion to the core and also to the ends of the composite media.The whole unit is then slipped into a cylinder and thermoplastically fused to the cylinder top edge to form a cartridge. Fluid can thus flow radially from the outside through the multiple layers of media to the center core and exit through the top open channel.

Human plasma was chosen as the bio-model to study the performance and efficiency of protein separation by this cartridge due to its importance in the bio-medical field.

II. METHODS AND MATERIALS

The method involves three basic manipulations. The first step consists of plasma dilution to reduce the electrolyte concentration to the ion-exchange operating range. The second step consists of filtration to remove lipidic substance and fibrin clots. Finally the third step consists of ion-exchange adsorption and elution.

Pre-Treatment of Plasma

Outdated human plasma was received from the local Red Cross. Plasma was first diluted with low-molarity buffer at 1 :10 ratio in order to bring the electrolyte concentration down to the level where ion-exchange process can be conducted effectively. Otherwise, the counter ion of the electrolytes in plasma will mask the charge sites causing interference on protein adsorption. Diluted plasma was applied to a prefiltration column packed with depth type of filters in stacked form. The pre-filter material was pure cellulose (ZetaPlus 01A).

Ion Exchange Procedure

The preparation of albumin /5/, IgG /6, 7/, and prothrombin complex /8, 9/ by using combination of anionic and cationic exchangers of Sephadex® and Sepharose® was reported. The ion exchange property of the present matrix applied in this study differ considerably from Sepharose, as well as the pure cellulosic type as shown from the pH effect on protein adsorption capacity in Figure 1. We discovered that the chemical behavior of the ion-exchange groups can be governed by the following factors: the chemical nature of the solid support where ionic groups are linked, the length of the spacer where ionic groups are located, as well as the neighboring chemical groups around the charge sites. Since the composite matrix we synthesized has functional groups introduced to the solid structure by polymerization instead of derivatization, the optimal condition

for ion-exchange chromatography on binding and elution of proteins are slightly different than in other matrices. The best results can be obtained only by observing the pH effect on matrix adsorption capacity with BSA as an example shown in Figure 1. In the present study, a 5-in. diameter QAE cartridge containing approximately 300 grams of matrix material is preequilibrated by three cartridge volumes of 10 mM phosphate buffer at pH 7.2. IgG shows minimum interaction toward the matrix under such condition and thus filtered through the cartridge leaving the other proteins adsorbed on the cartridge. A stepwise elution was performed to elute the adsorbed proteins separately according to their relative strength of interaction toward QAE groups.

Affinity Chromatography

The affinity matrix was made by grafting the epoxy containing acrylate to the cellulose. The composite structure containing high number of epoxy groups is oxidized by 2% sodium periodate at room temperature for converting the epoxy to aldehyde groups. Ligands such as lysine /10/, arginine /11, 12/, and benzamidine /13, 14/ can be coupled to the matrix through Schiff base formation by reacting their respective amino groups with the aldehyde groups generated in the matrix. The unreacted aldehyde groups are reduced by 1% sodium cyanoborohydride. 20-30 mg of ligands were generally bound per gram of matrix.

Assay Methods for Plasma Proteins

Protein separation was continuously monitored by measuring optical density at 280 nm by Gilson flow cell model. Protein concentration was determined by UV absorption at 280 nm and checked by both Biuret Standard Assay from Bio-Rad and also by nitrogen analysis with Brinkmann Kjeldahl nitrogen analyzer. Albumin was measured by cellulose acetate membrane electrophoresis. IgG, IgA and IgM were determined by radial immune diffusion kits from Miles Laboratory. Transferrin and α_1 antitrypsin were determined by radial immuno diffusion assay from

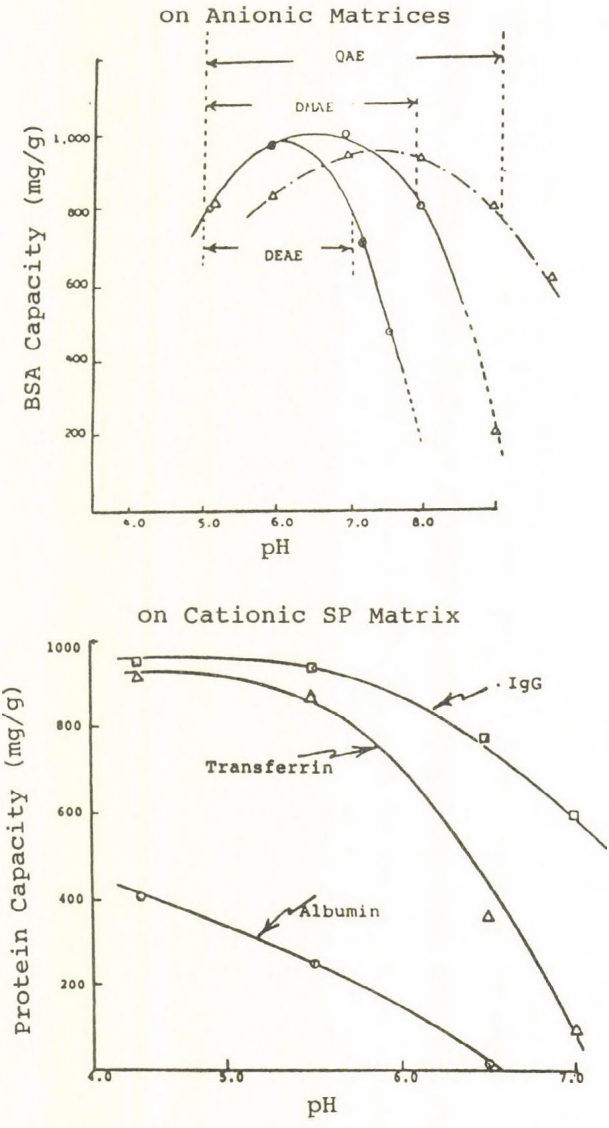


Figure 1. Effect of pH on protein adsorption by cellulose-acrylate matrices

Kallestad Laboratories of Austin, Texas. The sensitivity of these kits is approximately 2.73 mg/dl for α, anti-trypsin and 1.5 mg/dl for transferrin. The purity of IgG and albumin were checked by HPLC using a Beckman Model 100 A liquid chromatograph.

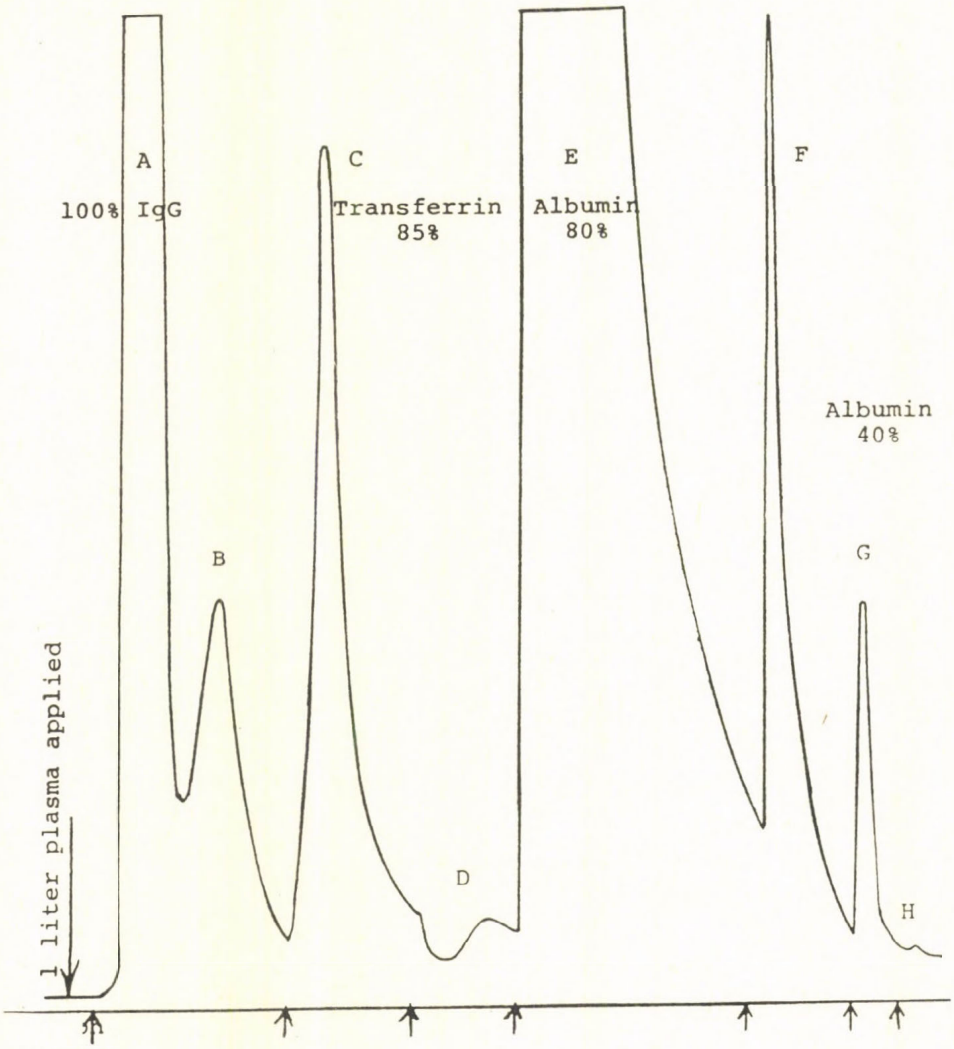
The protein adsorption capacity study was conducted by mixing 100 ml of bovine serum albumin, or other proteins of interest, at 10 mg/ml concentration dissolved in 10 mM phosphate buffer of specific pH with 1 gram of media for 1 hour. After centrifugation the protein loss in the supernatant was measured by O.D. 280. 10 mM acetate buffer was used at pH below 6.0.

SDA-PAGE was performed on 5-15% gradient gels (0.075 cmx60 cm^2) and run on a Bio-Rad Mini Vertical Slab Cell for 40 minutes at 200 V (constant voltage). The gel was stained with Coomassie Blue. Percent purity of various fractions was determined by densitometry (Helena Quick Scan).

The plasminogen concentration was determined by the chromogenic substrate S-2251 and the Kallikrein by S-2302 from Kabi Stockholm, Sweden, and by following the procedure prescribed in the work of Alving et al. /17/.

III. RESULTS

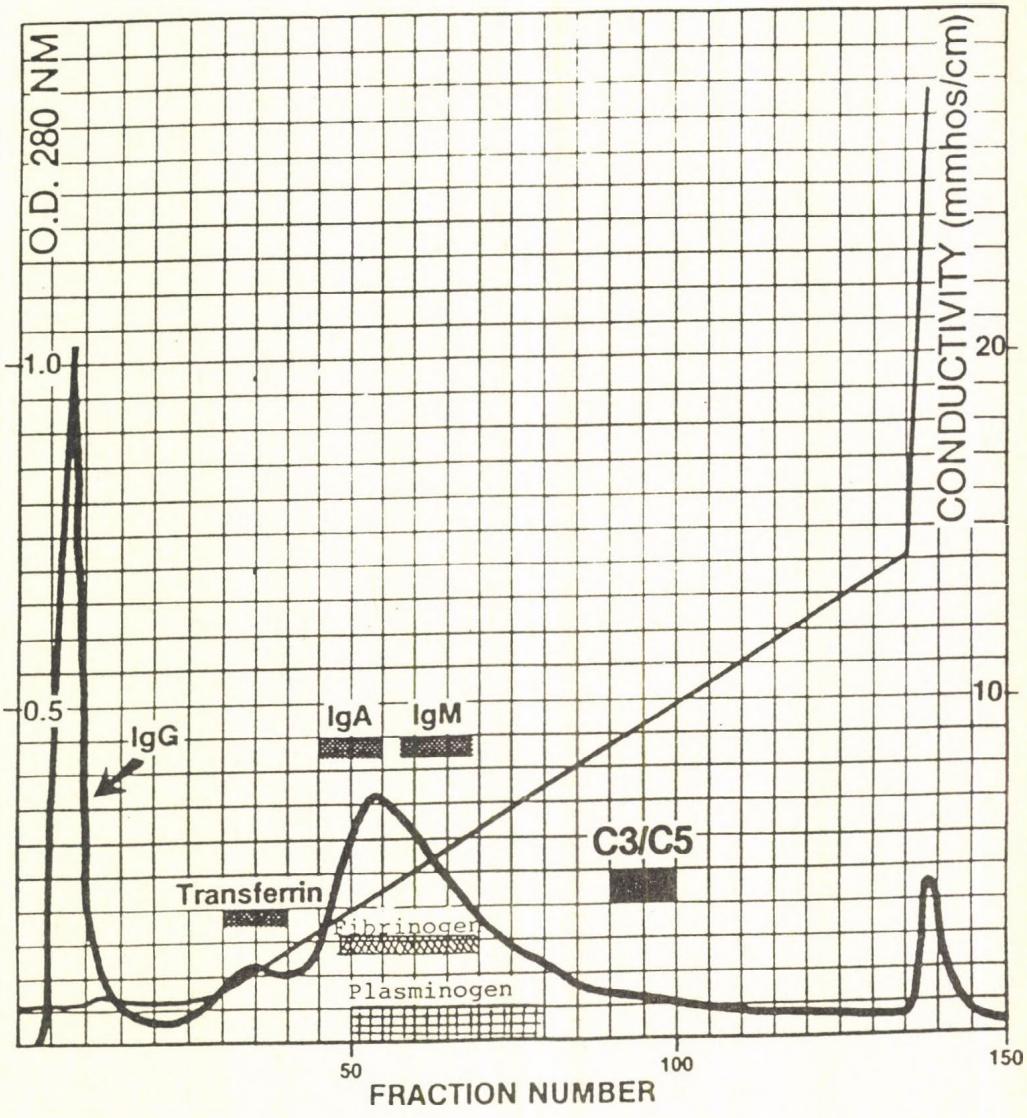
One liter of diluted and filtered plasma containing 62 grams of total protein was applied directly to the preequilibrated cartridge (ZetaPrep HP 3200 QAE) at 400 ml/min. The pressure drop across the cartridge was 5 psi. The filtrant was found to be highly pure IgG as measured by electrophoresis on cellulose acetate and further confirmed by SDS-PAGE. More than 80% of IgG was recovered. The cartridge was then eluted stepwise according to the conditions specified in Figure 2 and also in Table 1. Peak B in Figure 2 immediately following the filtrant was also identified as IgG. It is tentatively identified as IgG subclass 4. Peak C was found to be transferrin of 85-90% purity. The majority of plasma proteins were eluted in Peak E which was a mixture of α_1 antitrypsin, and albumin with IgA appearing at the beginning of the peak and IgM at the tail part of the peak. Figure 3 illustrates the relative locations where



Elution Condition								
pH	7.16	7.14	6.53	6.05	4.45	4.4	4.15	4.0
Conductivity (MS)	3.05	3.15	4.30	4.60	5.65	12.9	39.0	80.0

Cartridge size: 5 in. dia. x 9.5 in. length
Flow Rate: 400 ml/min

Figure 2. Plasma fractionation pattern on QAE cartridge (HP3200)



Cartridge type: DEAE 100
Plasma applied: 5 ml (1:10 dilution) by 0.015 M phosphate buffer
Volume collected: 15 ml per fraction
Gradient: 0.015 M NaH_2PO_4, pH 6.4 to 0.2 M, pH 6.8.

Figure 3. Identification of minor components eluted from DEAE cartridge

Table 1. Plasma Protein Recovery from the QAE Cartridge

Sample Identification	Elution Condition pH	Conductivity	Total Protein Recovery	IgG	Plasma Protein Components Transferrin	AT	Albumin
Peak A	7.18	3.05	4.92 g	4.92 g	0	0	0
B	7.14	3.15	0.35	0.35	0	0	0
C	6.53	4.30	2.64	traces	2.45	0	0
D	6.05	4.60	0.24	traces	0.23	0	0
E	4.45	5.65	21.80	traces	0.17	1.76 g	20.0
F	4.40	12.9	8.14 g	0	0	0	8.0
G	4.15	39.0	3.86 g	0	0	0	1.7
H	4.0	80.0	0.1 g	0	0	0	0

the minor protein components were found. Both fibrinogen and plasminogen were detected in Peak E. Haptoglobin and complement C_3 and C_5 were found to be present in Peak G. The material balance shown in Table 1 was estimated from the combination of electrophoresis pattern, RID and Bio-Rad protein assay. Transferrin recovered in Peak C can be further purified on a DEAE cartridge at pH 6.4. When the albumin fraction (Peak E) is applied to an SP cartridge at pH 6.4, the albumin should pass through unadsorbed, while transferrin and other proteins are retained on the cartridge.

Proteolytic enzymes such as Kallikrein have been identified as a major cause of IgG instability due to cleavage of IgG molecules (15). The removal of Kallikrein by benzamidine and soybean trypsin inhibitor (STI) was performed by coupling the ligands on the epoxy containing matrix shown in Figure 4. Better results were obtained with STI ligand than with benzamidine under similar conditions. However, it was found that the STI ligands are more sensitive to environmental conditions probably due to their relatively complex structure as compared to benzamidine. The removal of plasminogen from albumin eluted in Peak E by a lysine affinity column was performed under the conditions also prescribed in Figure 4. The sequence of the entire plasma process is shown in Figure 5. The yield of each protein component was proven to be over 80% from a number of tests conducted in the laboratory.

IV. DISCUSSION

The major advantage of applying ion-exchange chromatography in protein separation is that high purity products can be obtained by avoiding protein denaturation caused by excessive steps of solvent treatments involved in the Cohn process. The formation of aggregates and degradates in proteins received from Cohn process are eliminated in the AMF chromatography process.

The major drawback in applying chromatography to large volume plasma fractionation has been the limitation in flow rate and the pressure build-up due to the swelling of the con-

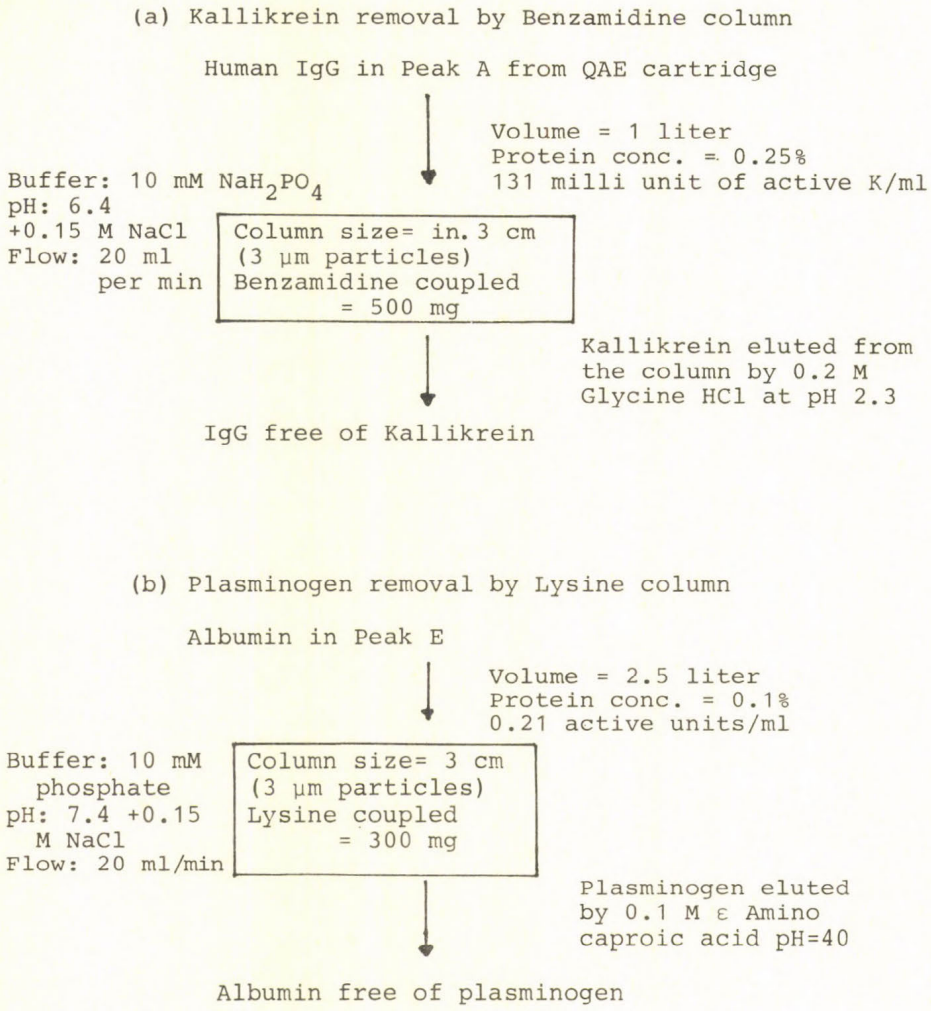


Figure 4. Proteolytic enzyme removal by affinity chromatography

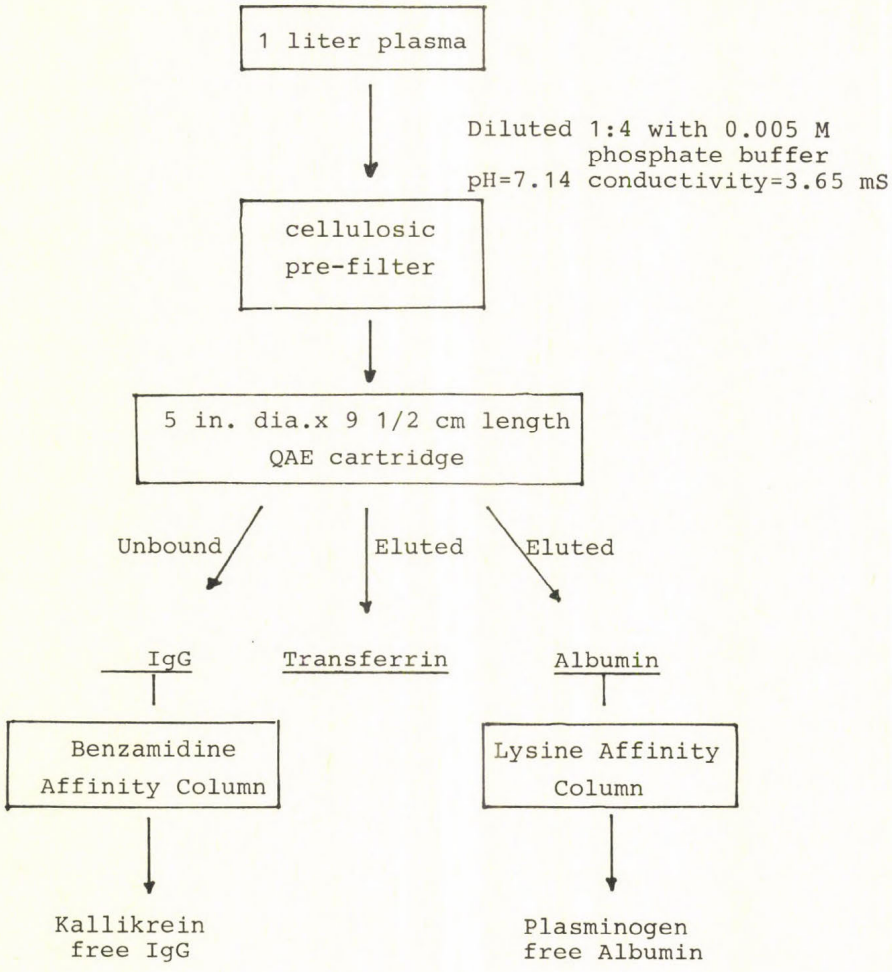


Figure 5. Sequence for separating plasma proteins by ion exchange and affinity cartridges

ventional matrices. The general approach on resolving this problem is to strengthen the matrix through cross-linking. The composite matrix reported here demonstrated the applicability of this semi-rigid thin sheet media for large scale operations. The cartridges can be connected either in parallel or in series or in combination for handling large volumes of bio-fluids. Contact time required for adequate adsorption of proteins on ZetaPrep cartridges was found to be in the order of 1 to 3 minutes. Adequate resolution can be achieved by using a 5-inch diameter cartridge as demonstrated from the present study. High protein yield are usually obtained when the protein of interest passes through the cartridge as a filtrant with the impurities adsorbed to the cartridge.

The simplicity of the cartridge system eliminates the cumbersome procedures of column packing and pre-conditioning ordinarily involved in chromatography. Since very little is known about the kinetics and radial diffusion of the cartridge structure, the best flow condition for maximum utilization of capacity can only be learned from field testing.

Acknowledgements

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THE OPTIMIZATION OF THE SEPARATION OF FUNGAL PROTEINS BY AFFINITY CHROMATOGRAPHY ON CONTROLLED-POROSITY GLASS MODIFIED BY BORON AND WITH VANILLIN AS A LIGAND

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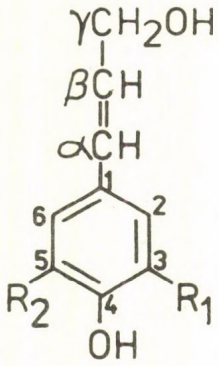
SUMMARY

Controlled pore glass activated with vanillin was chosen for the separation of anionic proteins from Trametes versicolor fungus by affinity chromatography. The chromatographic separation was optimized by the regulation of the boron concentration on the surface of the controlled-porosity glass by heat treatment. Better resolution of anionic proteins was obtained by the modification of the $MgCl_2$ concentration in the linear gradient. The separated anionic protein fractions contained peroxidase, laccase and glucose oxidase. It is assumed that the vanillin affinity of these enzymes is significant for their physiological role in lignin biodegradation.

INTRODUCTION

The woody plant tissues contain about 20-30% lignin. Lignin is a phenylpropane biopolymer (Fig. 1). The phenylpropane subunits form lignin. The phenylpropane subunits of lignin are coupled by a number of linkage types. They are bound to each other via aliphatic chains or aromatic rings. Due to that lignin is a very complex and variable molecule. Therefore, the enzymatic pathway of lignin biodegradation is not yet well understood. Many authors agree that fungi possess enzymatic abilities for lignin biodegradation /1, 2, 3/. The so-called "white rot" fungi which are especially good lignin degraders have been described. Amoung them there are two types of fungi, Trametes versicolor and Phanerochaete chrysosporium /1, 2, 3/.



(1) $R_1 = R_2 = H$
(2) $R_1 = OCH_3$, $R_2 = H$
(3) $R_1 = R_2 = OCH_3$

Fig. 1. Cinnamyl unit-precursor of lignin.

The products of lignin biodegradation by "white rot" fungi were discovered and described by Chen et al. /4/ and Leatham and Kirk /5/ (Fig. 2). As shown in Figure 2 one of the enzymatic products of lignin biodegradation is vanillic acid. Usually the products of the enzymatic reaction form complexes with corresponding enzymes.

The present paper describes the possibility of a specific separation of fungal enzymes from the mycelium of Trametes versicolor fungus through affinity chromatography using vanillin as the ligand. The fungal enzymes separated by the optimized affinity procedure are probably useful in lignin biodegradation.

MATERIAL AND METHODS

Cultures

Pure culture of Trametes versicolor (L.ex Fr/Quel) was supplied by the Museum of Fungi in Paris. The mycelium of Trametes

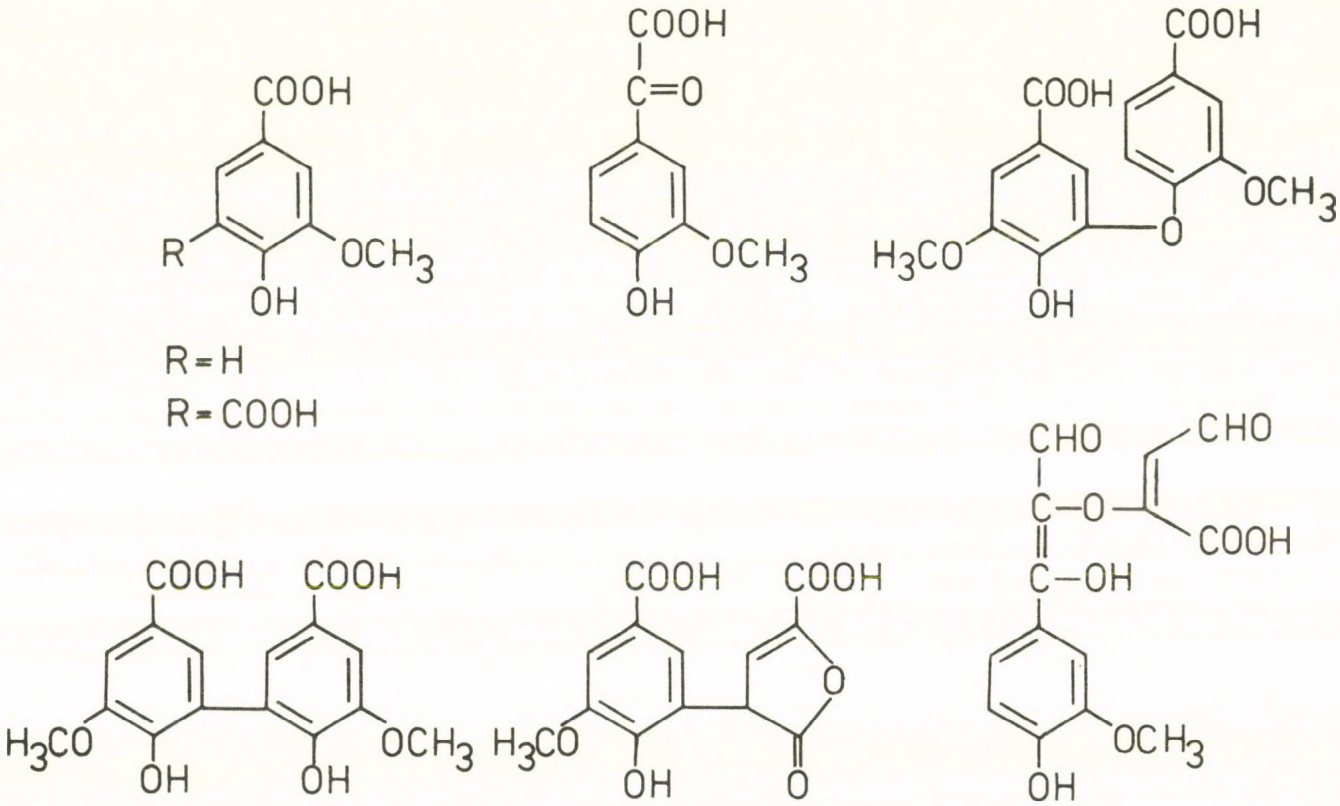


Fig. 2. Carboxylic acids identified in extracts of white-roted spruce wood /4, 5/.

versicolor was grown in submerged cultures on the mineral medium as described earlier /6/. Five-day-old mycelia were homogenized and proteins from the homogenate were precipitated using 80% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 0.005 M acetate buffer, pH 5.4, dialyzed and lyophilized before further use.

Methods

Protein was determined according to Lowry et al. /7/. Peroxidase (EC 1.11.1.7) and laccase (EC 1.10.3.2) activities were determined as described earlier /6/. Glucose oxidase (EC 1.1.3.4) activity was estimated after Svoboda and Massey /8/. The electrophoretic separation of proteins was carried out in the system of Laemmli /9/ with Na_2SO_4. The gels were loaded with 100 µg protein samples. After electrophoresis the gels were stained with Coomasie Blue G 250 and destained as described earlier /10/.

Controlled porous glass activation

Controlled-porosity glass (CPG) was prepared from sodium borosilicate Vycor glass and kindly supplied for our experiments by Dr. A.L. Dawidowicz (Department of Physical Chemistry, M. Curie-Sklodowska University, Lublin, Poland). In affinity experiments two types of CPG were used:

1. Sodium borosilicate Vycor glass (100-200 µm fraction) was prepared as previously described /11/. The physico-chemical properties of CPG were: specific surface area 171.6 m^2/g and average pore diameter 79 Å (CPG - No 1).

2. Sodium borosilicate Vycor glass (100-120 µm fraction) had the same physico-chemical properties as the glass described above but it was modified by further thermal treatment (20 hrs at 700°C) which led to the enrichment of their surface in boron atoms /12/. This material was also additionally hydroxylated by etching in 3N HCl at 100°C for 1 hr (CPG No 2).

The CPG was further activated with V-aminopropyltriethoxysilane and vanillin before use for chromatography column pre-

paration as described earlier /11/. Each of the CPG activated with vanillin was packed in a 6.0 cm x 0.5 cm I.D. column. The columns were equilibrated with 0.02 M acetate buffer, pH 5.4. A sample containing 15 mg of fungal proteins dissolved in this buffer was added to the top of the column. Proteins were eluted and collected in three fractions as shown in Figures 3 - 6. The eluate was always collected in 5 ml portions.

RESULTS AND DISCUSSION

In earlier papers it has been shown that the heat treatment and hydroxylation of CPG can modify the elution profile of fungal proteins in the chromatographic procedure using vanillin

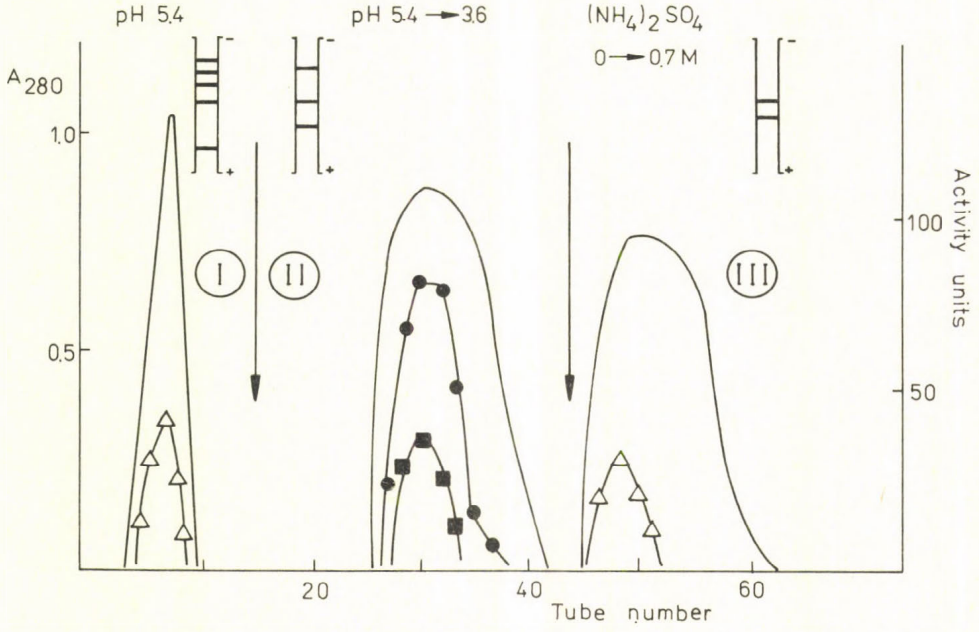


Fig. 3. Affinity chromatography of fungal proteins activated with vanillin. The matrix was sodium borosilicate Vycor glass (100 µm) heated at 500°C for 4 hrs. Specific surface area, 171.6 m^2/g. Average pore diameter, 79 Å. The column (6.0 x 0.5 cm I.D.) was equlibrated using 0.05 M acetate buffer, pH 5.4, and then two linear gradients of pH and $(NH_4)_2SO_4$ were used in 2 x 50 ml volumes.

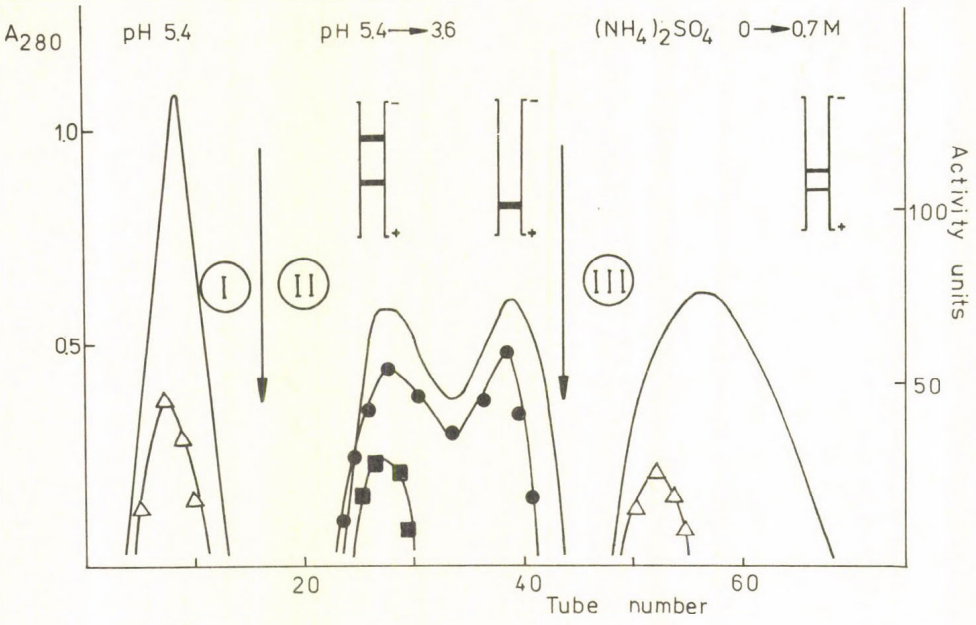


Fig. 4. Affinity chromatography of fungal proteins activated with vanillin. The matrix was sodium borosilicate Vycor glass, 100 µm, additionally heated at 700°C for 20 hrs and hydroxylated. The column was equilibrated using 0.05 M acetate buffer, pH 5.4, and then two linear gradients of pH and $(NH_4)_2SO_4$ in 2 x 50 ml volumes.

as the ligand /10, 11/. This paper presents results or further experiments with two types of CPG matrices and vanillin as the ligand for the optimization of the affinity chromatography process of fungal enzymes from Trametes versicolor.

As shown in Figure 3 the proteins from the mycelium homogenate of Trametes versicolor are separated into three main fractions of the chromatography column using CPG No 1. The first fraction contains proteins which have no affinity to vanillin. The second and third protein fractions are separated on CPG No 1 but they are not homogenous in polyacrylamide gel electrophoresis (Fig. 3). It should be mentioned that the latter fractions are interesting in regard to the main topic of this paper (Fig. 3).

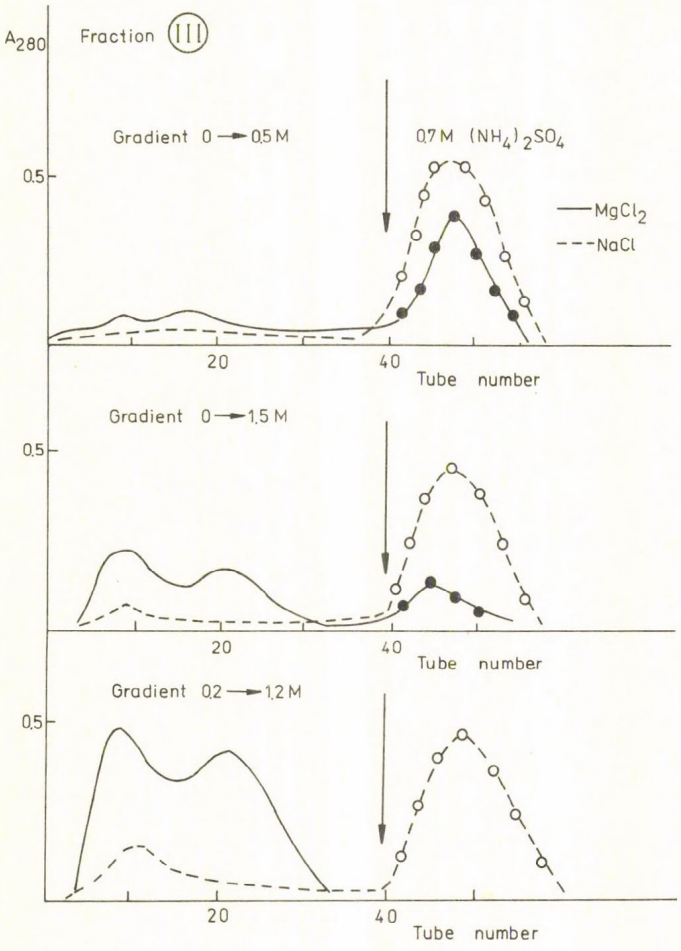


Fig. 5. Affinity chromatography profile of the third fraction of fungal proteins on the CPG column activated with vanillin. The CPG matrix is as given in Fig. 2. The first two steps of chromatography are identical to in Fig. 2 and are omitted. The Figure shows the optimization process of the elution using NaCl or $MgCl_2$ gradients.

By changing the CPG matrix (CPG No 2) it has been possible to separate the proteins of fraction II into 2 peaks (Fig. 4). It was then checked out by means of $MgCl_2$ and NaCl concentration gradients replacing $(NH_4)_2SO_4$ gradient elution for a better

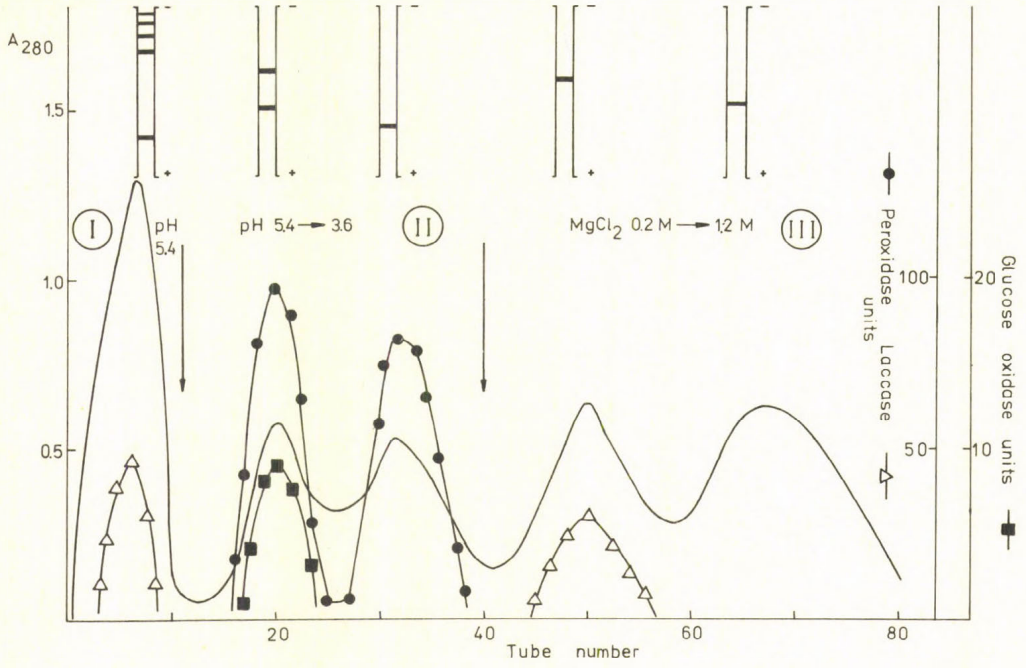


Fig. 6. Affinity chromatography of fungal proteins activated with vanillin. The CPG matrix is as given in Fig. 2. The sequence of the elution was as follows: I) 0.05 M acetate buffer, pH 5.4; II) linear pH gradient 0.05 M acetate buffer, pH 5.4 - 3.6 (2 x 50 ml); III) $MgCl_2$ gradient 0.2 - 1.2 M. Protein heterogeneity is shown in the form of polyacrylamide gel electrophorograms. The dimensions of the column size are the same as in the other figures.

separation of fraction III of the fungal proteins. The results are shown in Figure 5. The elution profile of fraction III proteins from the column was optimalized using $MgCl_2$ gradient (Fig. 5). The same concentrations of NaCl as $MgCl_2$ used for fungal fraction III elution give no positive results. Only 0.7 M $(NH_4)_2SO_4$ enables elution of fraction III from the vanillin-activated CPG column (Fig. 5). Figure 6 shows the fully optimized elution profile of fungal proteins from the chromatography column. This figure also presents the discovered enzyme activities and electrophorograms. In the flow-through fraction I

a laccase-type enzyme was discovered. It is interesting that another form of a laccase type enzyme was present in fraction III which possesses a high affinity to vanillin.

The most interesting finding is the separation profile of fraction II (Fig. 6). Two forms of peroxidase have been observed. One form of peroxidase was also homogenous in the electrophoresis. The other isoform of peroxidase was eluted together with the glucose oxidase activity. In this elution peak two protein forms have also been observed in disc electrophoresis (Fig. 6). It is significant that glucose oxidase activity accompanies the peroxidase. Glucose oxidase catalyzes glucose oxidation and in this reaction H_2O_2 is the first substrate of peroxidase while the second products are phenolic compounds. It is possible that glucose oxidase forms an enzymatic complex with peroxidase and thus these enzymes could have better steric cooperation in the oxidation of phenolic compounds.

As conclusion, it should be stated that in the mycelium of Trametes versicolor fungus some anionic proteins have differentiated affinity to vanillin. These proteins also have enzymatic activities connected to the oxidation of phenolic compounds which implies their physiological role in the biotransformation process of lignin.

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Chromatography '85
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INVESTIGATION OF CEREAL PROTEINS BY HPLC

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Wheat proteins have been extensively studied since a long time ago and several very important porblems were cleared concerning the baking quality of proteins, nutritional value, effect of fertilization, variety identification on the basis of electrophoretic protein spectrum, etc. Nevertheless, our understanding of its properties is at least incomplete. The cereal chemist must attempt to better understand the basis of functionality and quality, just as the baker must know proteins so that he may control, select or modify processing conditions to produce optimum products.

"Gluten" generally refers to the major protein of wheat. It is, however, an extremely complex material, consisting of numerous proteins of two major classes gliadin and glutelin as well as their complexes with other, non-protein compounds of flour. Some gliadin and glutelin proteins are directly associated with good gluten quality. The way in which proteins interact with each other and other flour constituents and the resulting molecular size distribution are especially important.

Most of these information have been obtained through two major techniques, electrophoresis and chromatography.

The investigation of cereal proteins by HPLC dates back to but a fex years. More recently the new wide-pore or large-pore columns have become available to separate proteins on the basis of ionic characteristics or size. Proteins can penetrate these packings and no irreversible binding occurs.

Our aim is to use the size exclusion and reverse phase HPLC columns for the analysis of cereal proteins, to identify varieties and predict wheat and flour quality.

Baking properties and structure of the protein-lipid complexes of wheat flour are highly related. Polar lipids (mainly glycolipids) interact with both gliadin and glutenin during doughmaking, forming a membrane-like structure which is responsible for the gas retaining capacity of dough. Thus loaf volume - as a critical characteristic of wheat products - depends on the ability of protein and lipid components to form complexes.

On the other hand lipids bound to the surface of proteins modify their surface charge distribution and hydrophobicity, therefore mediate the protein-protein interactions. This effect explains the role of lipids in the regulation of mixing time, mixing tolerance and rheological properties of dough.

The aim of this study was to investigate the nature of protein-lipid complex of gluten formed during doughmaking. We intended to search for those gliadin and glutelin subunits which are built into these complexes.

The complexity of problem is shown in Fig. 1. in which the separation of the alcoholic soluble fraction (gliadin) from 4 wheat varieties is presented on reverse phase column.

MV-9 and MV-12 cultivars were crossed from the same cultivar but MV-12 is selected later. They show only little differences, the other two reveal cultivars different picture.

The chromatographic conditions are presented in Table 1. In the solvent - acetonitrile modified by trifluoracetic acid - the gliadin complex disintegrates to polypeptides.

The easiest way to isolate protein-lipid complexes is the fractionation of 70% ethanol soluble wheat proteins (gliadin) according to their molecular weight. Elution profiles of gliadin (Fig. 2.) isolated from wheats with different baking properties show characteristical differences. In the gliadin extracted from the wheat with better baking properties peak I, which is the protein-lipid complex has a higher proportion.

The gliadin presented in Fig. 2. was separated on TSK3000SW gel by size-exclusion chromatography. The chromatographic conditions are presented in Table 2.

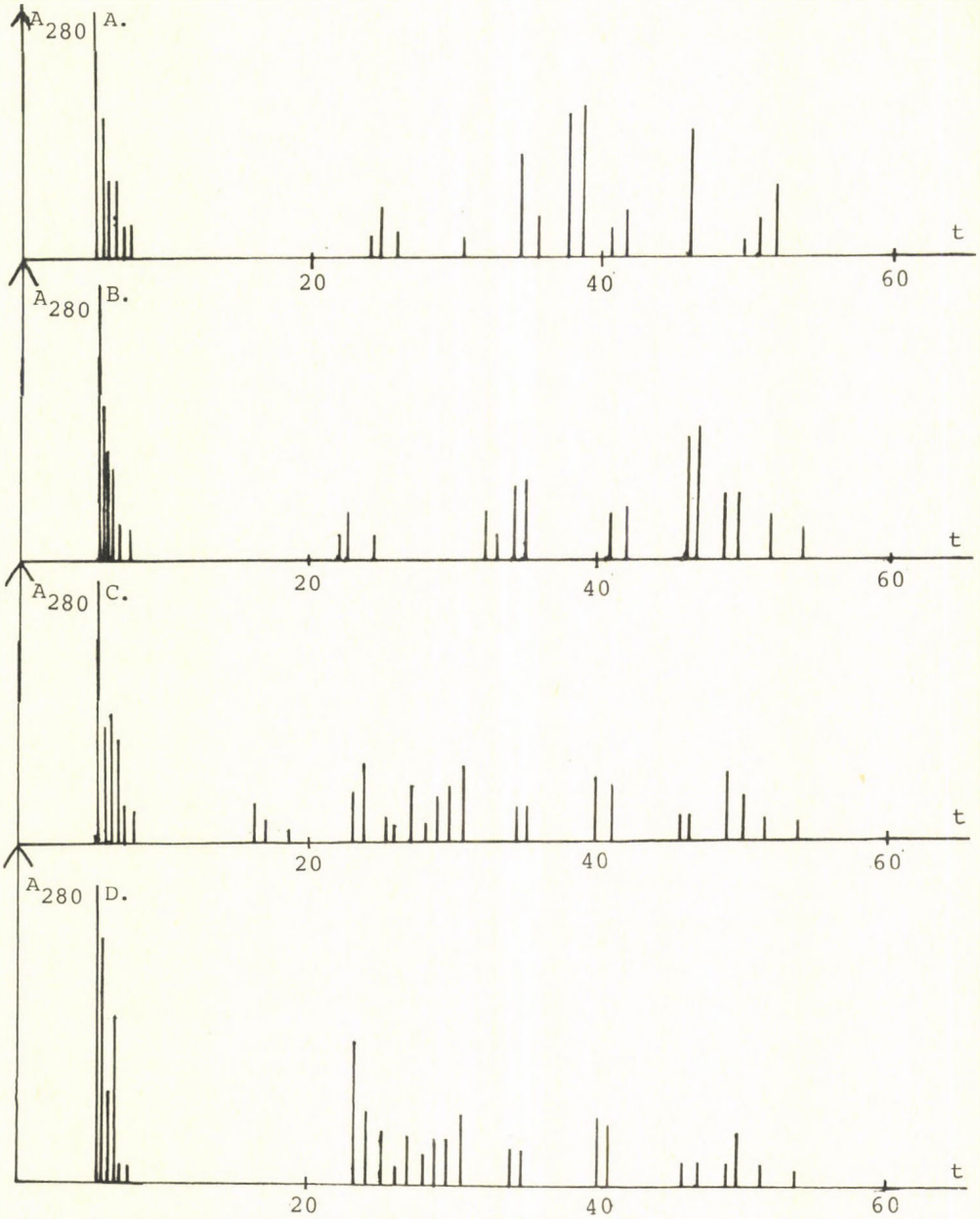

Fig. 1. Comparison of A, B, C and D ethanol soluble gliadin proteins of wheats separated by RP-HPLC. 0.1 mg protein was solved in 100 mm^3, 70% ethanol.
Name and pedigree of wheats:

A. MV-08/-82:/ At166xKr1F1/xKr1F2/x/Bz1-Kr1/
B. MV-10: Moi-Art
C. MV-12: Mrn-Bz1xBz1$^2_2$ - Prod
D. MV- 9: Mrn-Bz1xBz1^2 - Prod

Table 1. Reverse phase HPLC

INSTRUMENTATION

Pump: Waters 6000A HPLC Pump
Waters 4500 HPLC pump
Injector: Waters U6K injector
Solvent programer: Waters M660
Detector: Waters adsorbance detector M440 at 280 nm
Recorder: 4 channel recorder of KUTESZ type 175
Integrator: Digint 35 CHINOIN product

SEPARATION CONDITION

Column: Sinchropack 10-C18 250 mm long and 4.6 mm ID
Eluent: A: 15% acetonitrile + 0.1% TFA
B: 90% acetonitrile + 0.1% TFA
Program: 15% B in 60 min to 100% B with curve 6
Flow rate: 1 cm^3/min
Pressure: 70 bar

Table 2. Size-exclusion HPLC

INSTRUMENTATION

Pump: Waters 6000A HPLC pump
Injector: Waters U6K injector
Detector: Waters adsorbance detector M440 at 280 nm
Recorder: 4 channel recorder of KUTESZ type 175
Integrator: Digint 35 CHINOIN product

SEPARATION CONDITIONS

Column: Micropack TSK 03000SW 300 mm long 7.5 mm ID
Eluent: 29/dm^3 SDS + 0.2 M Na_2HPO_4 pH = 4.5
Flow rate: 1 cm^3/min
Pressure: 19 bar
Temperature: 22 C
Injection vol.: 20-50 mm^3
Sensitivity of detector: 0.1-0.02 AUFS

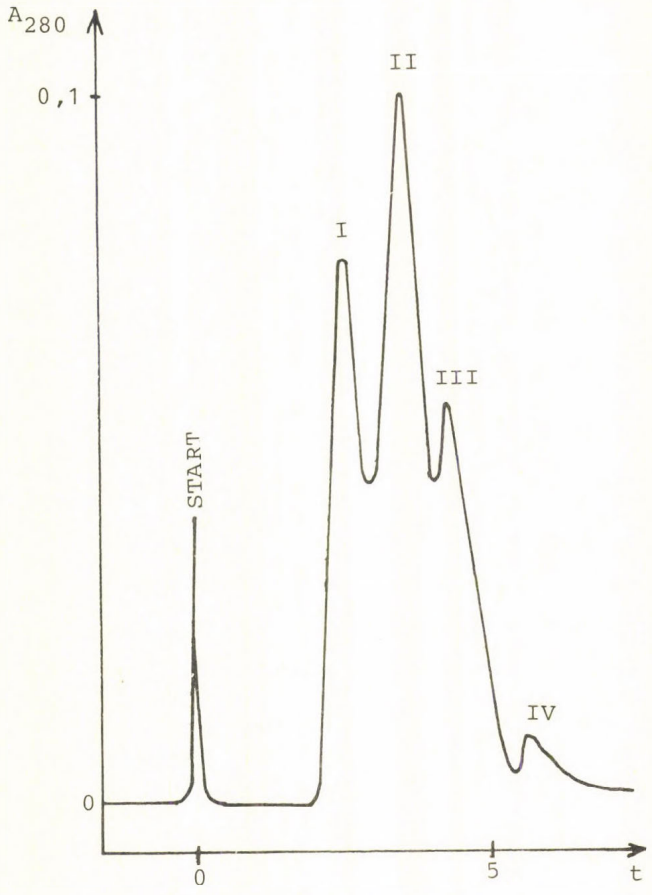

Fig. 2. SE-HPLC of gliadins on TSK300SW
Molecular weight of fractions:
I:126kD; II:86kD; III:23kD; IV:10kD

Comparing elution profiles of gliadin isolated from flour and gluten of the same wheat, a drastical increase of the amount of this fraction can be observed in the gluten. If gliadins are prepared from control flour and from defatted flour, elution profiles show that the presence of lipids is necessary for the appearance of the increase of this fraction.

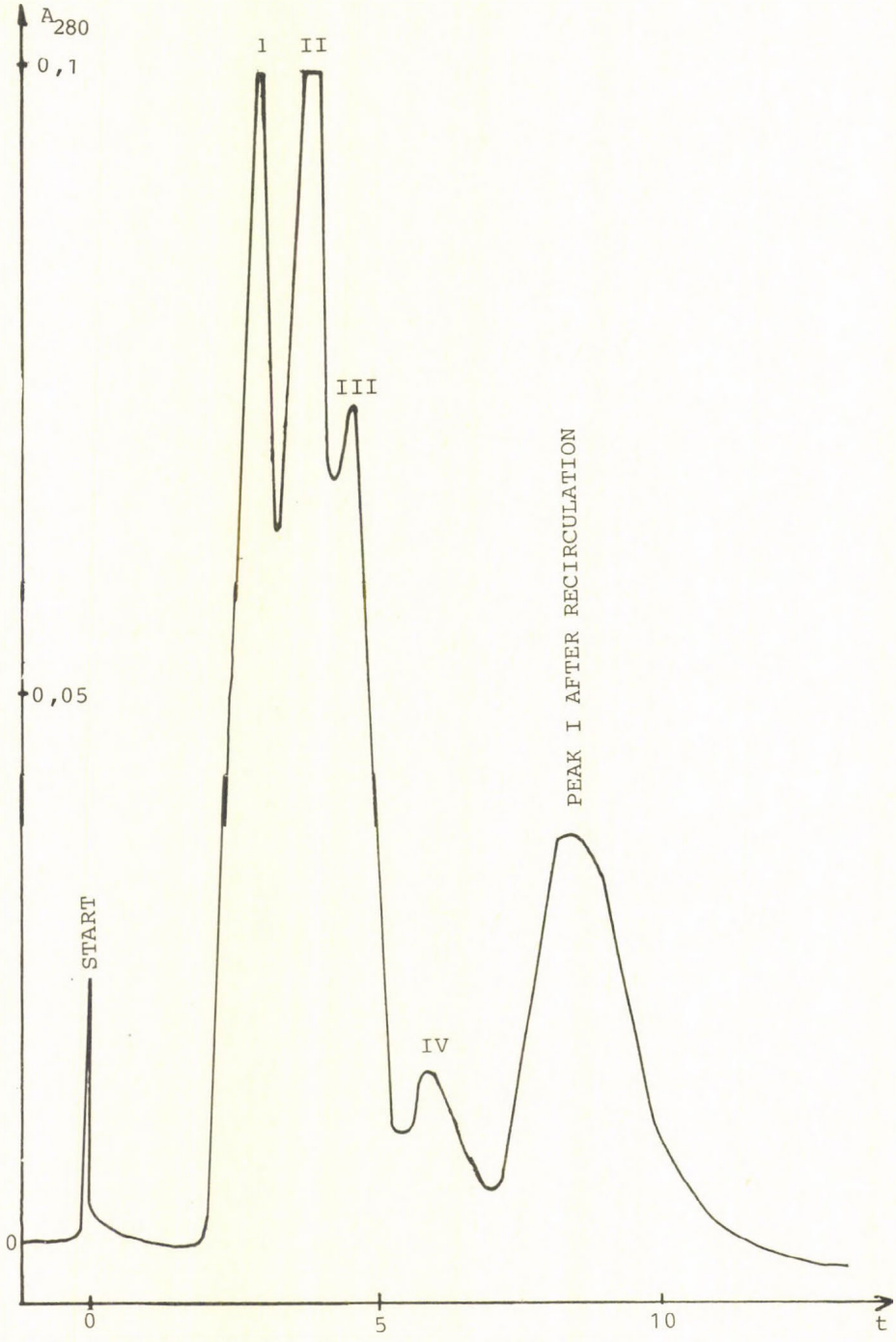

Fig. 3. SE-HPLC of gliadins on TSK3000SW and peak I after recirculation.

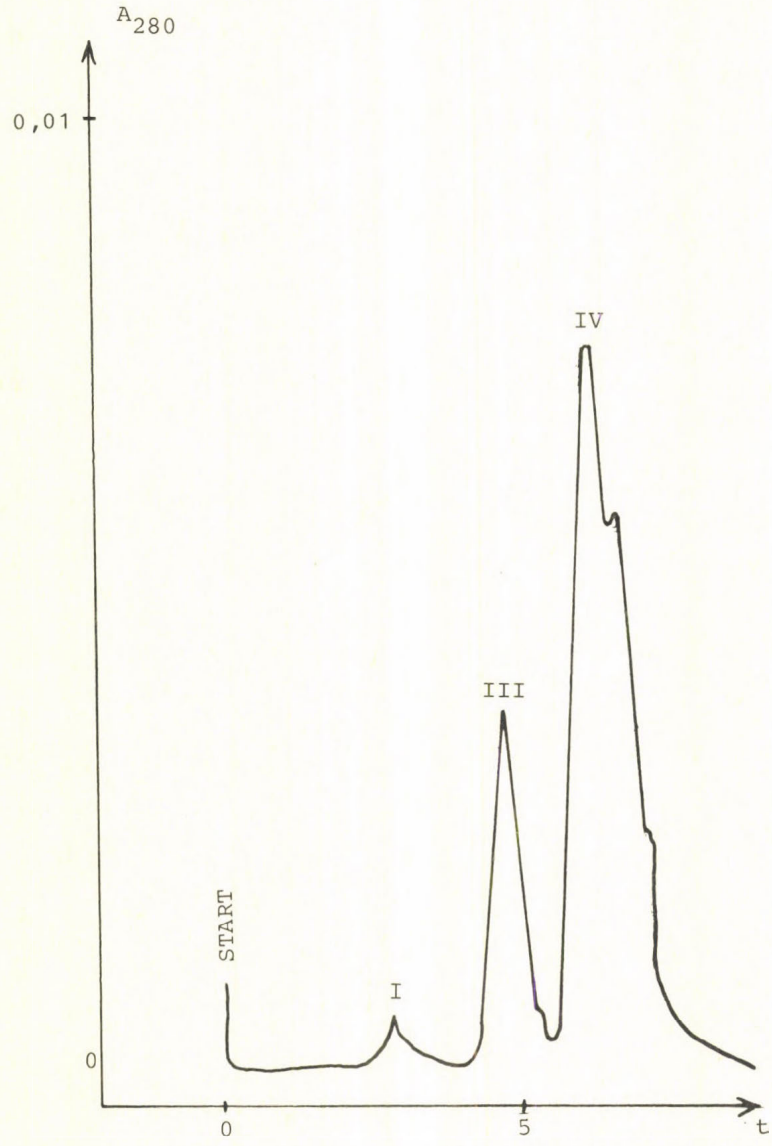

Fig. 4. SE-HPLC of protein collected, defatted from peak I.

SDS gel electrophoresis showed that almost all the types of subunits of the original gliadins can be built in the complex (not shown).

The protein-lipid complex of gliadin was isolated from gluten, prepared from original (not defatted) flour. Fig. 3. shows the separation on size-exclusion chromatographic column. The first peak was purified by recyclic procedure to eliminate all of the residual fractions with lower molecular weight.

Collected, rechromatographed peak I was dialyzed, freeze-dried and defatted with water-saturated butanol, then chromatographed using the same separation conditions as before the defatting procedure. The chromatogram is presented in Fig. 4. The figure shows that as a result of defatting the complex which previously gave a uniform single peak fell into its parts resulting an elution profile with five peaks different in molecular weight.

The protein-lipid complex can be reconstituted if a given amount of wheat lipids with an optimal composition is given to the separated protein fractions. It proves the fact that lipids can mediate the aggregation of gluten proteins reversibly.

Protein fractions of protein-lipid complex of gluten will be further investigated, comparing the subunit composition of lower molecular weight fractions (peaks II and III) of whole gliadin and those of isolated from the complex.

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ECDYSTEROIDS FROM SILENE NUTANS L.

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INTRODUCTION

The significant ecophysiological role that ecdysteroids play in plant-plant and plant-animal interactions is strongly underlined by their multiple biochemical and physiological functions both in arthropods and plants. According to a recent review on the distribution and role of ecdysteroids in plants /1/ over 60 ecdysteroid-type compounds have been described from various species of Pteridophyta, Gymnospermae and Angiospermae. The ecdysteroids form a fairly homogenous group within natural steroids with distinct structural features such as cis A/B-ring fusion, the presence of 6-one-7-ene moiety and of a full sterol side as well as several hydroxy groups at various sites of the molecule.

Besides various genera of ferns and the genus Podocarpus in Gymnospermae, the greatest number and the highest structural diversity of phytoecdysteroids was found in Angiospermae, and this is also reflected in the wide distribution among the taxonomic entities; although less than 1% of all higher plant species have been as yet screened for the presence of ecdysteroids, several hundred species belonging to more than 70 families of Angiospermae were found to contain such substances. Their distribution does not seem to follow any phylogenetic lines. From the practical point of view it is of interest, however, that in certain taxa of Caryophyllales and Lamiales, high frequency of occurrence and large structural diversity have been seen. In the continuation of our earlier work /2-6/ on Hungarian native

species a frequency of about 22.8% was found for ecdysteroid containing species within the Caryophyllaceae family /7/. This, together with the fairly high concentration of 20-hydroxyecdysone in several species makes Caryophyllaceae an attractive target for detailed chemical study. In this paper the analysis, isolation and identification of the major ecdysteroid components of Silene nutans L., a Europe-wide native species on rocky and sandy soils is presented.

EXPERIMENTAL PART

Materials

Solvents and chemicals were purchased from Reanal (Budapest, Hungary) with the exception of 1-butanesulfonic acid sodium salt (Aldrich, Milwaukee, USA); silica gel 60 F_{254} TLC plates, aluminium oxide, Brockmann II (Merck, FRG); polyamide (Woelm, FRG), Kieselgel (Merck, FRG). The ecdysteroid standard compounds (1,20-dihydroxyecdysone 1, 5,20-dihydroxyecdysone 2, 20-hydroxyecdysone 3), were earlier isolated at the Department of Pharmacognosy, University Medical School, Szeged, Hungary /2, 3, 6/.

Thin-layer chromatography (TLC)

Desaga TLC chambers were used. The developing systems were:

1. dichloromethane-ethanol (96%), (85:15),
2. ethylacetate-methanol-ammonia (85:10:5).

The spots were detected by vanilline-sulphuric acid reagent and in UV light.

High-performance liquid chromatography (HPLC)

Liquochrom OE 312 consisting of a solvent delivery system, an injector with 20 µl sample loop, a variable wavelength ultraviolet detector and a recorder were used. The stationary phase was Chromsil C-18, 6 µm particle size (both the liquid

chromatograph and the stationary phase from Labor MIM, Budapest, Hungary).

The mobile phase was a mixture of triethylamine-phosphate buffer pH = 1.8 and methanol in ratios 60:40, or 55:45 where the buffer contained 50 ml phosphoric acid, 10 ml triethylamine and 50 mg 1-butanesulfonic acid sodium salt per liter. The flow rate of the mobile phase was 1.2 ml/min or 0.7 ml/min, as it will be given.

Plant materials

Silene nutans L. was collected in the vicinity of Budapest, Hungary, in the month of May 1982 and 1983. (Voucher species are deposited in the Herbarium of Szeged University.)

Table 1. TLC characteristics of ecdysteroids from Silene nutans (L.)

Name of compounds	R_f x 100 Eluent: 1.	2.	Colour on TLC with vanilline-sulfuric acid reagent in UV_{254} nm	day-light
1. Sn_7	24.7	18.5	violet	green
2. Sn_3 (5.20-dihydroxy-ecydsone)	52.6	29.8	violet	green
3. Sn_5 (20-hydroxy-ecydsone)	35.7	27.8	violet	green
4. Sn_4	44.8	33.8	orange	orange

Isolation

The substances were isolated from fresh plant material in the following procedure (Fig. 1):

4.85 kg of fresh plant material (whole plant) was extracted with methanol in a blender, then percolated with 32 liter of methanol at room temperature. The extract was concentrated

under reduced pressure. The solid residue (377 g) was taken up in 3500 ml of methanol when a precipitate was formed. The precipitate was filtered and washed with methanol. The methanol soluble portion was evaporated in vacuo, and the residue (325 g) was taken up in 1200 ml of methanol and 600 ml of acetone was added to the solution. The precipitate was filtered; the filtrate was concentrated in vacuo resulting 276 g of substance. This procedure was repeated twice with different ratios of methanol and acetone (1:1 and 1:2 in the 2nd and 3rd precipitations; the residues were weighting 236 and 201 g, respectively). The last residue (201 g) was chromatographed on a column of Brockmann grade II, neutral alumina (1000 g). (The main steps of the isolation are shown in Fig. 1.) Column chromatography was monitored by thin-layer chromatography (TLC). The elution was done with dichloromethane-methanol (9:1), and the fractions 5-8 yielded a crude ecdysteroid mixture. They were concentrated in vacuo and the residue (56 g) was rechromatographed on 115 g polyamide. Water and 50% aqueous methanol eluates yielded fractions containing ecdysteroids. The combined fractions were concentrated in vacuo and the residue (31 g) was rechromatographed on 684 g of silica. Fractions eluted with chloroform-ethanol (9:1) contained the substances Sn_3 and Sn_4. Fractions eluted with chloroform-ethanol (87:13) and (85:15) contained substance Sn_5 as main compound. From these fractions, white needles of Sn_3 (417 mg), that of Sn_5 (4.9 g) and that of Sn_4 (61 mg) were separated by repeated crystallization.

Fractions eluted with chloroform-ethanol (8:2) were containing the substance Sn_7. These fractions were taken into dryness and purified further on a Sephadex LH-20 column (elution with ethylacetate-ethanol-water(8:1:1)) giving the pure compound of Sn_7 (900 mg).

According to their melting points, TLC and spectral data, the compounds called Sn_3 and Sn_5 were identical with standard samples of 5,20-dihydroxyecdysone (2) and that of 20-hydroxyecdysone (3), respectively (Figs 2, 4, 5 and Table 1).

20-hydroxyecdysone (Sn_5) (3): white needles from ethylacetate-methanol (2:1); its melting point is 237-239 C^{o}, the same as given in the literature /8/, the melting point of its

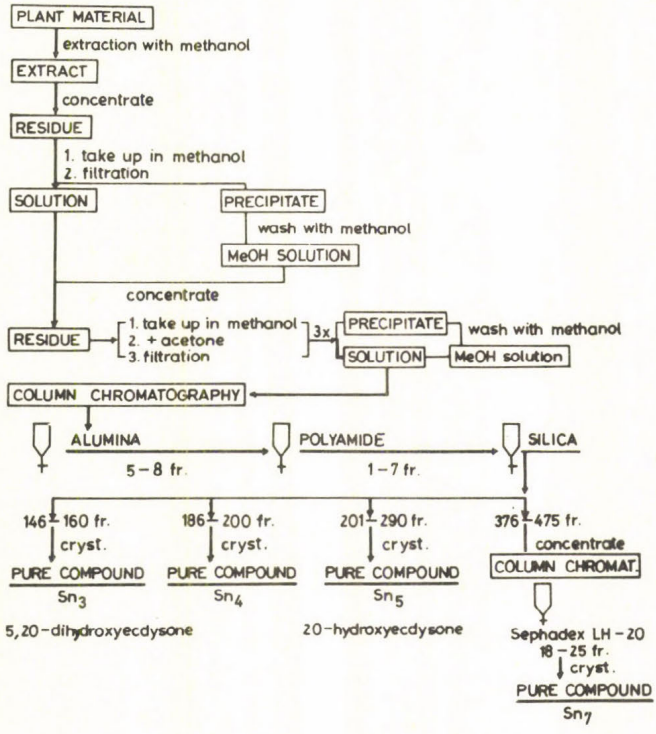


Fig. 1. Steps of the isolation.

triacetate-monohydrate is 151-154°C (crystallized from methanol); it gives an olive-green colour and identical R_f values with authentic 3 on TLC plates after spraying with vanilline-sulfuric acid reagent (Fig. 2 and Table 1). Its spectra (UV, IR, ^{1}H-NMR, ^{13}C-NMR and MS /9/ were identical with those of the standard 3 sample.

5,20-dihydroxyecdysone (Sn_3) (2): white needles from aqueous methanol, melting point is 253-257 C° (literature /8/ gives 254-257°C). Olive-green colour is given on TLC after spraying the spots with vanilline-sulfuric acid reagent (Fig. 2). Its R_f-values (Table 1) and UV, IR, ^{1}H-NMR, ^{13}C-NMR /9/ and mass spectra are identical with those of the standard sample.

The infrared, nuclear magnetic resonance and mass spectra as well as the chromatographic parameters (TLC and HPLC) of Sn_7 and Sn_4 show similarities to the corresponding characteristics

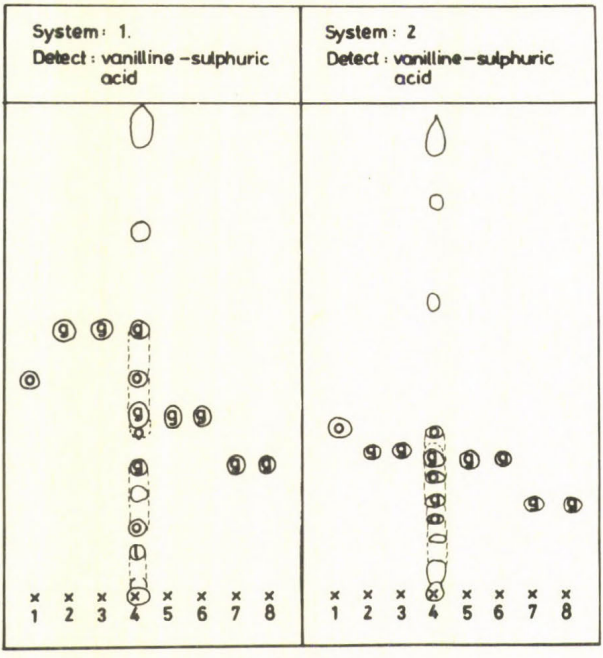


Fig. 2. TLC of the extract, isolated substances and standard samples in solvent systems Nos 1. and 2.

Abbreviation: o: orange
g: green

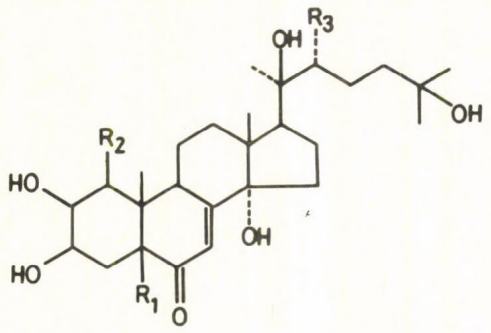
Samples:

1. Sn_4
2. Sn_3
3. 5,20-dihydroxyecdysone 2
4. Plant extract
5. Sn_5
6. 20-hydroxyecdysone 3
7. Sn_7
8. 1,20-dihydroxyecdysone 1

of 1,20-dihydroxyecdysone (1) and 22-deoxy-20-hydroxyecdysone (4), respectively (Figs 2, 4, 5 and Table 1).

DISCUSSION

All samples of Silene nutans from various geographic locations showed the presence of a series of ecdysteroids when tested by the usual TLC screening method /3, 10/. As in most plant species, 20-hydroxyecdysone was always present as the most abundant component. In addition, two distinct series of compounds consisting of at least 7 components could be distin-



1. Sn_7	R_1=H	R_2=OH	R_3=OH
2. Sn_3 /5,20-dihydroxyecdysone/	R_1=OH	R_2=H	R_3=OH
3. Sn_5 / 20-hydroxyecdysone /	R_1=H	R_2=H	R_3=OH
4. Sn_4	R_1=H	R_2=H	R_3=H

Fig. 3. Structures of the substances isolated from Silene nutans

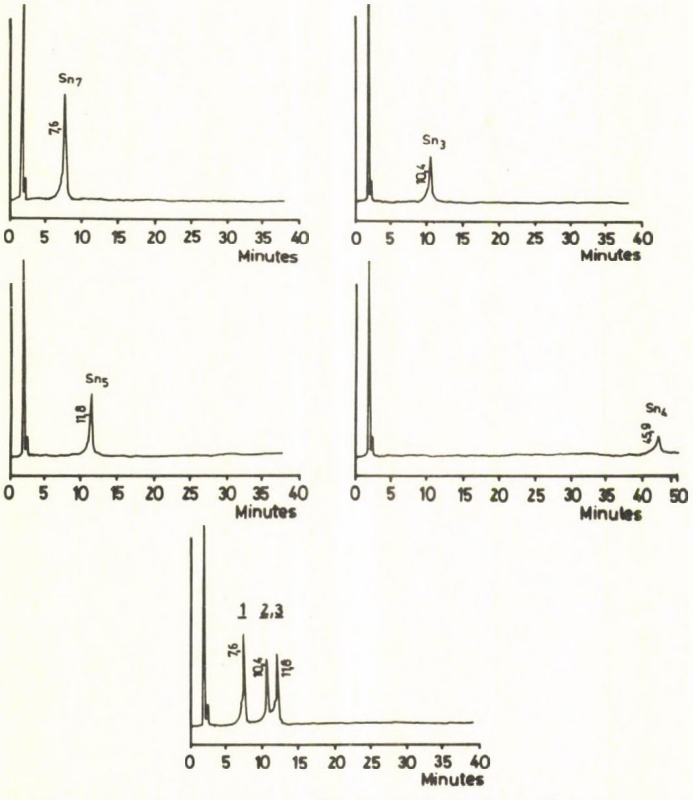


Fig. 4. HPLC of the substances isolated and reference samples in system No. 1.

guished using vanilline-sulphuric acid reagent, one with a colour and fluorescence similar to 20-hydroxyecdysone, the other having a characteristic orange fluorescence and orange colour (Fig. 2). Since the latter type has not been seen previously in any other species, a more detailed analysis of the extracts using HPLC and isolation work was initiated.

For the isolation the fresh plant material was extracted with methanol. Four pure compounds designated as Sn_3, Sn_4, Sn_5 and Sn_7 were isolated in sufficient quantities for identification or structure determination.

The pure ecdysteroids were analyzed by TLC (Fig. 2) and by HPLC (Figs 4-5) and identified on the basis of their physical constants and spectral characteristics as well as by direct comparison with standard reference samples (see Experimental part).

Of the four compounds isolated Sn_3 was identified as 5,20-dihydroxyecdysone (2), Sn_5 as 20-hydroxyecdysone (3) by direct comparison with authentic reference samples. On the basis of their chromatographic characteristics and spectral data Sn_7 seems to be identical with 1,20-dihydroxyecdysone (1), while the very characteristic ecdysteroid of Silene nutans, Sn_4, showing an orange colour and orange fluorescence after using vanilline-sulfuric acid reagent for the detection (Fig. 2) seems to be 22-deoxy-20-hydroxyecdysone (4) (see Fig. 3). A detailed study on their structure is still in progress and will be the subject of a subsequent publication.

The purity of the isolated compounds and their analytical scale separation from crude methanolic extracts was controlled on a reverse-phase column in isocratic mode of elution using two different ratios of a buffer and methanol (see Experimental and Figs 4, 5). While the 40% methanol-60% buffer system gives better separation of the more polar components but is inconvenient for the separation of less polar ones, with increased methanol ratio the separation of the more polar group becomes less complete. With the successive use of the two systems a fairly good separation of all major components of the crude extract can be achieved (Fig. 6).

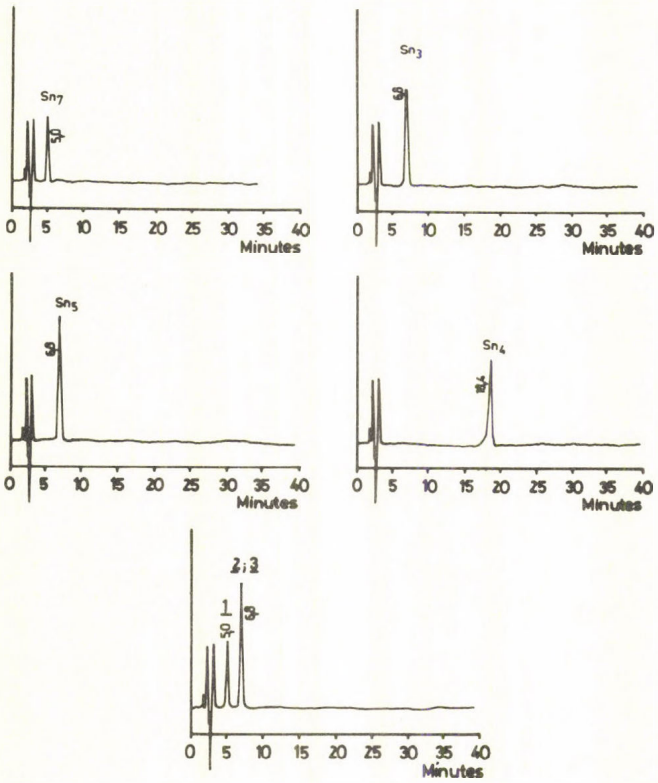


Fig. 5. HPLC of the substances isolated and reference samples in system No. 2.

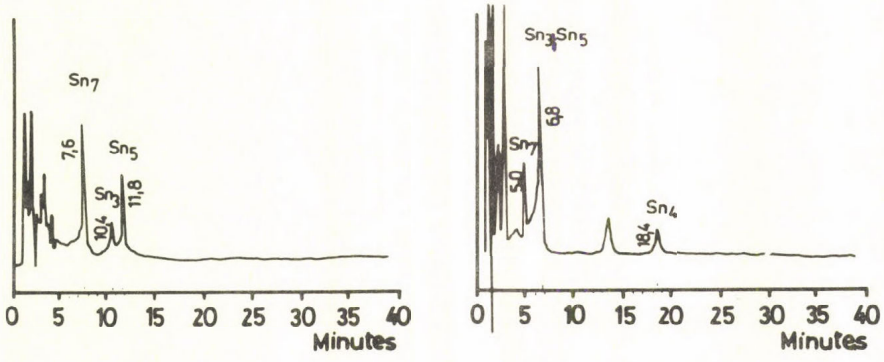


Fig. 6. HPLC of the extract in systems Nos 1 and 2.

While our isolation work was in progress, two publications appeared in the literature from Baltaev et al. /11-12/. In the first paper /11/ an HPLC method was published for the analysis of ecdysteroids in Melandrium nutans L. (= Silene nutans L.). In addition, two components were isolated using preparative HPLC and identified as 20-hydroxyecdysone (3) and 5,20-dihydroxyecdysone (2). A third compound showed chromatographic parameters similar to 1,20-dihydroxyecdysone (integristerone A) (1). The second paper /12/ described the isolation and characterisation of a further rare phytoecdysone, 22-deoxy-20-hydroxyecdysone (4) as a characteristic component of the species.

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ISOLATION OF COUMARINS FROM THE FRUITS OF PASTINACA SATIVA L. BY COLUMN CHROMATOGRAPHY AND MODIFIED THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The isolation of coumarins from the fruits of Pastinaca sativa L. was carried out in three stages:

1. Selective extraction of Pastinaca sativa fruits using extractants of increasing polarity.
2. Separation of extracts by column chromatography.
3. Analysis of the eluate fractions and their separation by preparative TLC.

The use of a horizontal sandwich chamber and some methodological variants such as band application of large sample volumes, and two-dimensional TLC with continuous elution, permitted the isolation of a number of pure coumarins, including some compounds occurring in trace amounts.

INTRODUCTION

The plants of Umbelliferae family contain great amounts of substances of therapeutic effect. Among these substances the derivatives of coumarin and furocoumarin dominate with a wide spectrum of biological activity, e.g. anticoagulant, spasmolytic, sedative, bacteriostatic, fungistatic, anti-cholinergic and photodynamic action /1-5/. There are also some reports about the cytostatic and the cancerogenic activities of these plants /6, 7/. The earlier examination of compounds of this group in the fruit of the Umbelliferae plants led to the investigation of Pastinaca fruits which are rich in these compounds /8/. Pastinaca fruits contain the derivatives of psoralene such as imperatorin, bergaptene, xanthotoxin, xanthotoxol, isopimpinellin and sphondin /9-11/. This plant material is

utilized in the production of drugs: Pastinacin with spasmolytic activity and Beroxan with photodynamic activity. At the same time Beroxan contains the furocoumarin fractions obtained by the chromatographic separation of Pastinaca fruits extract.

The present paper summarizes the results of systematic studies on the choice of fruit extraction conditions and on the optimization of liquid column and thin-layer chromatographic systems for the separation of coumarin derivatives.

EXPERIMENTAL

The extraction of powdered parsnip fruit grown in the pharmacognostic garden of the Medical Academy in Lublin in 1984, was carried out in a Soxhlet-Type extractor with a closed solvent (eluent) cycle and, on a larger scale, in a Quickfit Ex-11 extraction apparatus. Several pure solvents of increasing polarity were used as extractants (see Table 1). The technique of gravity column chromatography was applied: the glass columns (600 x 15 mm I.D.) have been packed with silica gel 100-200 mesh (Koch-Light Laboratory Ltd.) and aluminium oxide (Woelm, acidic, for column chromatography Activity Grade I). The columns were packed by tapping. The following solvents were employed as eluents for the aluminium oxide column: benzene (fractions 1-3); benzene + 5 % v/v ethyl acetate (fractions 4-10); benzene + 10 % v/v ethyl acetate (fractions 11-20); benzene + 15 % ethyl acetate (final fractions). A mixture of benzene and 15 % ethyl acetate was applied to the development of the column packed with silica gel.

Analytical and preparative thin-layer chromatography was carried out on 100 x 200 mm or 200 x 200 mm glass plates, covered by 0.25 mm or 0.5 mm layers of silica gel Si 60 H (Merck, Darmstadt, FRG) or alumina 60, type E, neutral (Merck, Darmstadt, FRG). The plates were covered by using the Jobling-Quickfit apparatus; they were dried and activated in the usual way. Thin-layer chromatography was performed in sandwich type flat chambers (POCh, Poland). Diisopropyl ether and ethyl acetate mixed with n-heptane, dichloromethane and toluene were used as binary and ternary eluents.

RESULTS AND DISCUSSION

Extraction of parsnip fruits

Larger amounts (100 g) of the powdered fruit samples were extracted using pure solvents in order to establish which would allow mainly the separation of the furocoumarin fraction and some of the less polar coumarin derivatives with a good yield. The results are presented in Table I and in the schematic diagram of the thin-layer chromatogram (Fig. 1).

Table I. Conditions for the extraction of the coumarin fraction from parsnip fruits by using organic solvents of different polarity

Solvents	Extraction yield of coumarins g/100 g of Pastinaca fruits		Total yield
	After 3 h of extraction	After exhaustive extraction	
n-hexane	0.37	0.25	0.62
petroleum ether	1.07	0.63	1.70
chloroform	0.78	0.37	1.15
methylene chloride	1.12	0.64	1.76
methanol	0.62	0.50	1.12

The analysis of the above results leads to the conclusion that extraction of the fruits with n-heptane mainly yielded great amounts of ballast substances (lipids, chlorophyll).

The greatest yield was obtained by using dichloromethane (1.76 %) or petroleum ether (1.70 %); at the same time the coumarin compounds dominate in the etheric extract. The methanolic extract contained the most components and compounds of higher polarity (coumarins, flavonoids).

Analyzing the obtained data it may be stated that the fraction of coumarin compounds containing mainly furocoumarins may be separated from parsnip fruit by exhaustive extraction with petroleum ether (alternatively with gasoline) after a previous, short-duration degreasing of the fruits with n-hexane.

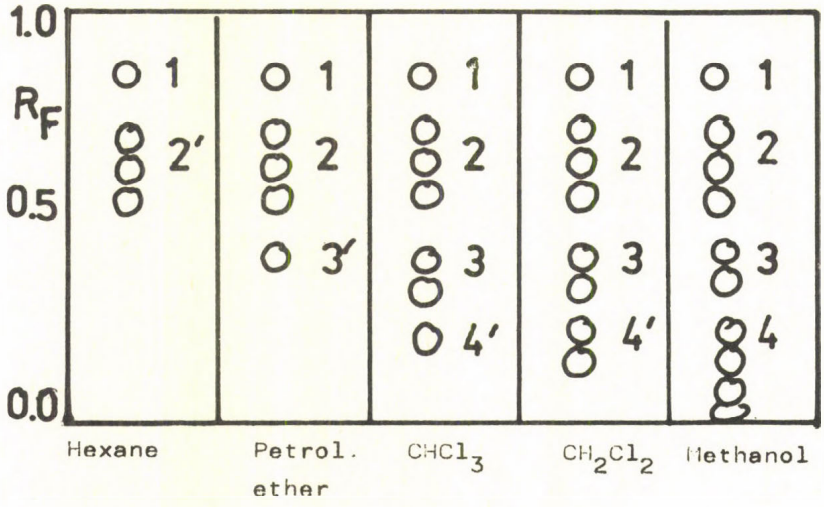


Fig.1. Schematic representation of the chromatograms of various extracts of Pastinaca fruits (see Table I). System: 15 % v/v ethyl acetate in benzene/silica gel. 1. lipids; 2. furocoumarins; 3. less polar coumarins; 4. polar coumarins; 5. 2',3',4' trace amounts of above fractions.

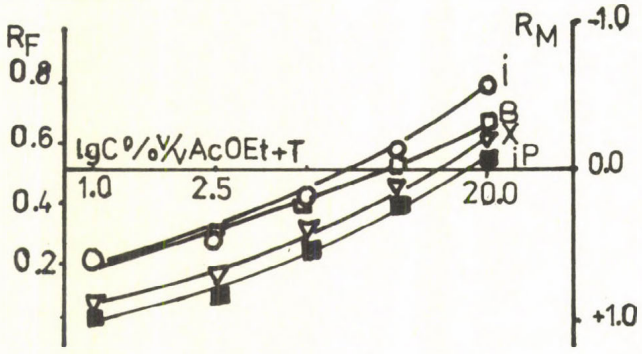


Fig.2. R_M vs. log $C_{modifier}$ plots of furocoumarin standards on silica gel using ethyl acetate + toluene as the solvents. I - imperatorin; B - bergaptene; X - xanthotoxin; iP - isopimpinellin.

Optimization of the chromatographic systems

In order to be able to select the optimum system, systematic chromatographic analysis of basic standards of the furocoumarins occurring in parsnip fruits (imperatorin, bergaptene, xanthotoxin, isopimpinellin) was carried out in binary solvent systems consisting of a polar solvent ethyl acetate, diisopropyl ether and a nonpolar or a weakly polar diluent n-heptane, or n-hexane, benzene or toluene and dichloromethane. Typical $R_M(R_F)$ vs. log C vol-% relationship are presented for silica gel in Figs 2 and 3.

The behaviour of standards in the other solvent systems is illustrated in Fig. 4 by showing R_F vs. eluent composition relationship. The positions of the spots after the continuous elution are also indicated (6-8).

As can be seen from Fig. 4 none of the solvent systems is satisfactory, due to the too small separation coefficients. Better results were obtained using benzene, toluene or dichloromethane as the solvents rather than n-heptane. In analytical thin-layer or in milligram-scale chromatography it is profitable to carry out continuous eluation with several interstitial volumes of the solvent. Considering the analogous systems for alumina, considerably worse separation has been found. Better effects may be obtained by employing continuous elution with solvents of low elution strength, for example 5:40:55 diisopropyl ether - dichloromethane - heptane or 5:95 diisopropyl ether or ethyl acetate - benzene /12/.

Column chromatography

Based on the thin-layer chromatography data, two types of adsorbents have been applied to separate the coumarin fraction on a preparative scale. First a column packed with alumina was used, with a concentration gradient of ethyl acetate in the eluent. The first fraction contained imperatorin with trace impurities. Further fractions containing mixtures of components were introduced onto a second column packed with silica gel and eluted with a solution of 15 % ethyl acetate in

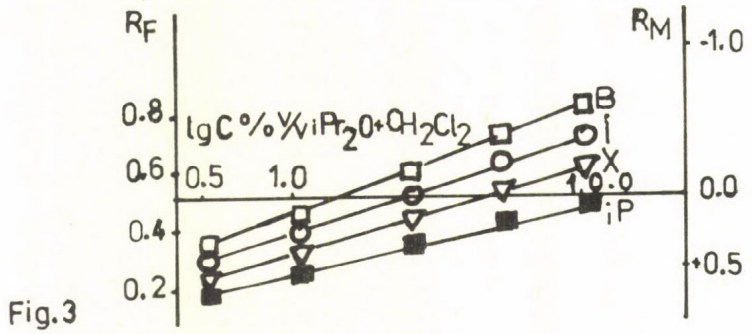


Fig.3

Fig.3. R_M vs. log $C_{modifier}$ plots of furocoumarin standards on silica gel using diisopropyl ether + dichloromethane as the solvents. For compound identification see Fig. 2.

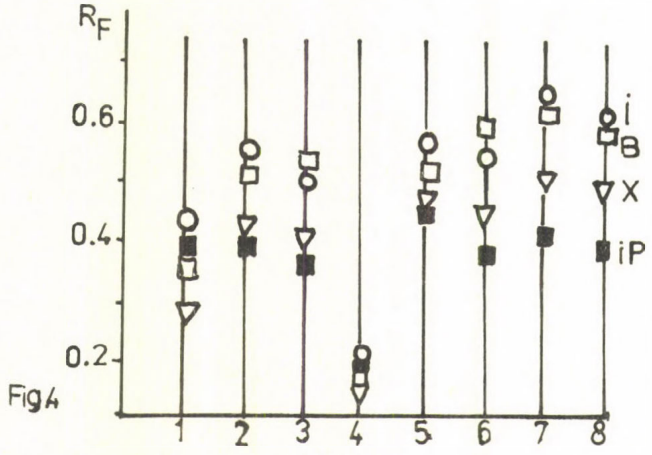


Fig.4

Fig.4. R_F values of furocoumarin standards for various eluents on silica gel with comparison to continuous development using the sandwich chamber (6, 7, 8). Solvents: 1) 30 % v/v ethyl acetate + hexane; 2) 10 % v/v ethyl acetate + toluene; 3) 2 % v/v ethyl acetate + CH_2Cl_2; 4) 70 % v/v diisopropyl ether + hexane; 5) 40 % v/v diisopropyl ether + toluene; 6) 2.5 % v/v diisopropyl ether + CH_2Cl_2; 7) 2.5 % v/v ethyl acetate + benzene: 8) 5 % v/v diisopropyl ether + benzene.

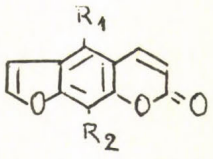
benzene (Table II). From the fractions the compounds I-VIII were obtained by multiple crystallisation. Their identity has been confirmed by comparison with thin-layer chromatography of standards. In addition the melting points of compounds I-V and VII have also been measured and their IR spectra and elementary analyses obtained.

Table II. Column chromatography of the coumarin extract from Pastinaca fruits

Column A (Al_2O_3) and eluents:

Benzene	5 % v/v AcOEt + B		10 % v/v AcOEt + B		15 % v/v AcOEt + B
fractions 1 - 3	fractions 4 - 7	fractions 8 - 10	fractions 11 - 12	fractions 13 - 20	final fractions comp. VIII
comp. I (imperatorin) m.p. 99-100°			comp. V (xanthotoxol) m.p. 248-9°		(8-hydroxybergaptene) m.p. 221-3°
	Column B (SiO_2)	Column B (SiO_2)		Column B (SiO_2)	
	eluent 15 % AcOEt + B	eluent 15 % AcOEt + B		eluent 15 % AcOEt + B	
fractions 11 - 15 comp. II (bergaptene) m.p. 188-90°	fractions 20 - 22 comp. III (xanthotoxin) m.p. 145-6°	fractions 24 - 31 comp. IV (isopimpinellin) m.p. 148-9°		fractions 20 - 27 comp. VI m.p. 225-8°	fractions 33 - 39 comp. VII m.p. 184-6° (sphondin)

In this way the following pure compounds were isolated from the coumarin fraction: imperatorin, bergaptene, xanthotoxin, xanthotoxol, sphondin and probably 8-hydroxybergaptene (Table II).



Psoralene

$R_1 = -H \quad R_2 = -O-CH_2-CH=C(CH_3)(CH_3)$ imperatorin

$R_1 = -H \quad R_2 = -O-CH_3$ xanthotoxin

$R_1 = -OCH_3 \quad R_2 = -H$ bergaptene

$R_1 = R_2 = -OCH_3$ isopimpinellin

Preparative thin-layer chromatography

The construction of the flat sandwich chamber with a distributor which can play the role of a flat pipette, makes it possible to dosage a large volume of the sample as a band on the edge of a layer /13/, and next the elution with a chosen solvent. While introducing successive solvent volumes, the mutual displacement of substances may be expected /14/. Using large chromatographic plates (0.5 x 200 x 200 mm) and ternary solvents, the chromatographic separation of coumarin fraction of parsnip fruit was carried out and illustrated by the diagram of two chromatograms for silica gel and alumina (Fig. 5a, b). On the silica gel layer partial separation of imperatorin from bergaptene was obtained, however, the extreme zones (of different fluorescence) contained pure substances. From other bands pure xanthotoxin, isopimpinellin and sphondin were eluted. The separation on alumina was worse but using the presented system (Fig. 5b) pure isopimpinellin may be isolated.

In two-dimensional preparative chromatography, a two-piece distributer has been applied (Ref. /15/, Fig. 2). Using two different eluents, the incomplete separation of the coumarin fraction was obtained (Fig. 6). The design of sandwich chamber permits the dosage of large volume of sample. The following continuous chromatography under equilibrated conditions with several interstitial volumes of eluent makes it

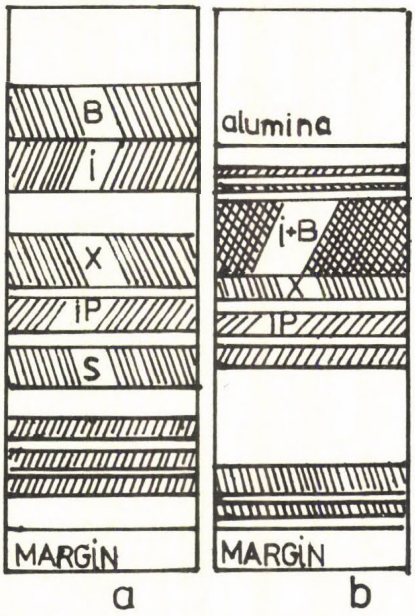


Fig.5. Comparison of preparative TLC chromatograms of an extract of Pastinaca fruits. System: a) 3:67.9:29.1 diisopropyl ether - dichloromethane - heptane/silica gel (continuous development); b) 10:27:63 diisopropyl ether- - dichloromethane - heptane/alumina (continuous development). For compound identification see Fig. 2 (S-sphondin).

possible to acquire milligram amounts of pure furocoumarins. At the same time the overloaded system allows to separate and isolate the trace components which are the impurities in the column effluent. The analysis of column effluent containing bergaptene and xanthotoxin by using the overloaded system and zonal TLC allowed to separate bergaptene and xanthotoxin and to detect the trace impurities which is difficult in the case of chromatography on an analytical scale (Fig. 7).

The presented results have demonstrated that the use of different chromatographic techniques together with selective extraction of a plant material allows to isolate the pure derivatives of psoralene. It ought to be emphasized that the thin-layer chromatography carried out in sandwich chamber makes it possible to isolate the compounds under discussion by using the minimum amounts of solvents.

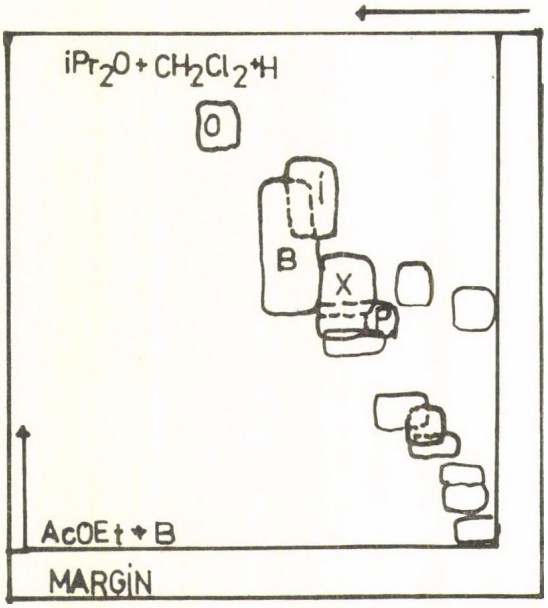


Fig.6. Two-dimensional preparative chromatogram of an extract of Pastinacea L. (100 µl) on silica gel 200 x 200 x 0.5 mm layer: a) elution in the first direction with 5 % v/v ethyl acetate in benzene; b) elution in the perpendicular direction: 2.5:70:27.5 diisopropyl ether - dichloromethane - heptane (o-osthol; for other notation see Fig. 2).

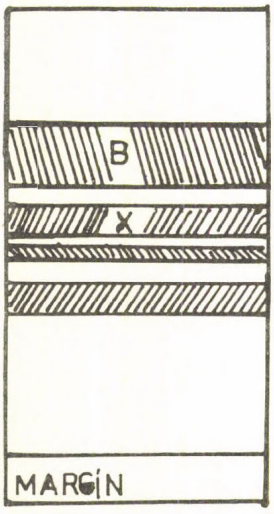


Fig.7. Zonal TLC chromatogram of column fraction: bergaptene (B) - xanthotoxin (X) on the preparative silica gel layer. Eluents: benzene (1 interstitial volume) and 2 % v/v AcOEt + benzene (2 interstitial volumes).

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THIN-LAYER CHROMATOGRAPHY AND HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF DIFFERENT ACRIDINE-GLYCOSIDES

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INTRODUCTION

It is known from the literature that the most important and interesting derivatives of acridine are the amino-acridines. They are particularly interesting because of the wide variety of their physical and chemical properties, biological activity as well as because most of acridine medicines and dyes also belong to this group /1/.

The best-known representative of amino-acridines is 3,6-diamino-acridine, i.e., proflavine. Because of its wide antimicrobic spectrum, proflavine, as an antisepticum, has been used in therapy for a long time but genetical and biochemical research also utilizes its biological activity. It can be intercalated into DNA, inhibits DNA-depending RNA-polymerase, affects cellular respiration, and its mutagenic and antimutagenic effects are also known /2/.

Our experiments deal with proflavine glycosides, a new group of amino-acridine derivatives, a representative of amino-acridine-N-glycosides.

It is known that under certain conditions the reaction between carbohydrates and primary aromatic amines results in the formation of so-called secondary glycopyranosil-amine derivatives. Till the early 60s this reaction was examined by a number of teams, mainly because of an analogy with nucleosides /3/.

In the literature only the reactions of homoaromatic monoamines were discussed. At first Antal Kovács, a researcher at

BIOGAL Pharmaceutical Company, noticed that the reaction is carried out with heteroaromatic diamines: N-glycoside and N-ramnoside of proflavine were produced by him in 1978. Later, by a procedure developed together with researchers (Nánási-Lipták-Szurmai) at the Biochemical Institute, Kossuth Lajos University, Debrecen, a number of anomer-pure derivatives were produced and the compounds were patented in 35 countries /4/.

Amino-acridine-N-glycosides have biological activity of a fairly wide spectrum: they are valuable compounds and compared to the basic compound, they have very different or new effects. Among them there are substances of antitumour, immunmodulatory and stimulative effects. N-glycosides of proflavine, the mono- and di-derivatives can affect the growth and development of plants and the yield of agricultural plants can be increased by them. On the basis of the measurements in an American research institute, in wheat 120%, in soybean 130% and in rice higher than 130% increase in yield could be reached as compared to control groups (100%) which were not treated with proflavine N-glycoside.

This paper discusses the chromatographic behaviour of representatives of these compounds, i.e. proflavine, proflavine monoglycoside (PMG) and proflavine diglycoside.

INSTRUMENTS, COLUMNS, ELUENTS AND SOLUTIONS

The experiments were carried out with a Hewlett-Packard Liquid Chromatograph, Model 1084 B, using a variable wavelength detector. Components were detected at 430 nm (Attenuation: 2^8 x AU x 10^{-4} cm, slope sensitivity: 0.1, oven temperature: $35^{\circ}C$.)

Separations were attempted on Lichrosorb-Si-60, Lichrosorb-NH_2, Lichrosorb-RP-2, Lichrosorb-RP-8, Lichrosorb-RP-18 columns.

The columns, produced by Merck (Darmstadt, FRG), had 20 cm length, 4.6 mm inner diameter and a particle size of 10 µm.

Organic solvents used in the analyses were of HPLC purity, produced by Merck. Water was distilled twice.

$(NH_4)_2CO_3$ used in buffer solutions was of analytical purity. From the ammonium carbonate stock solution a solution with 0.1% concentration was made and the pH was adjusted to 7.5, 6.5, 5.0 and 3.0 by concentrated phosphoric acid (eluent A). At separations on reversed-phases the eluent contained 20-40-60-80% acetonitrile besides the aqueous buffer (eluent B).

During the analyses on reversed-phases the flow rate was 1.5 ml/min. The concentrations of the substances to be determined were as follows: PDG: 0.04006 mg/ml, PMG: 0.04068 mg/ml and proflavine: 0.03996 mg/ml. The components can be formed as bases as well as salts, but in our investigations the chlorides of all three components were used.

A 10 µl aliquot of the solutions was injected into the chromatograph.

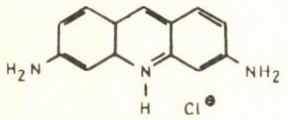
EXPERIMENTAL

Figure 1 shows the structure of the starting substances, i.e. proflavine, PMG and PDG.

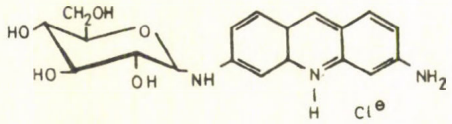
During the production the final product contains all three components. The quantities of PDG and PMG, or their ratio is important concerning the effect, while the amount of proflavine, present as impurity, shows the purity of the product.

We have investigated these compounds for two and half years and developed a number of chromatographic methods for their determination. The first experiments were carried out on a normal phase, a Lichrosorb-Si-60 column with eluents of different strength. When using eluents of variable strength, several suitable solvent pairs were found satisfactory for the separation. In the direction of the strength of the elution series, the capacity factor (k') values are strongly decreased. Taking into consideration the time of separation, i.e. the k' values, and the resolution, suitable separation was obtained on a normal phase in two systems. Figure 2 shows chromatograms obtained in the two systems.

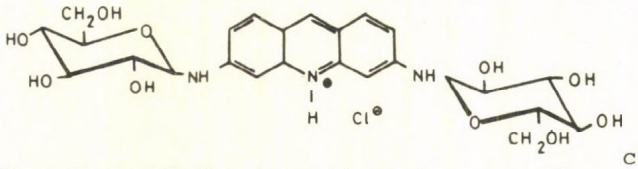
Experiments were carried out on a Hewlett-Packard-NH_2 column. Eluents of variable compositions were used also here. The



a)



b)



c)

Fig. 1. Structure of the investigated compounds.
a) proflavine HCl [3,6-diaminoacridine HCl]. MW = 245.6; mp = 288°C; pK_a = 9.7.
b) proflavine monoglycoside (PMG) [3-amino-6-(α, β-D-glycopyranosyl-amine)-acridine HCl]. MW = 407.7; mp = 190°C; $[\alpha]_D$ = -95.3°.
c) proflavine diglycoside (PDG) [3,6-di(α, β-D-glycopyranosylamine)-acridine HCl]. MW = 569.8; mp = >210°C (dec.); $[\alpha]_D$ = -225.8°.

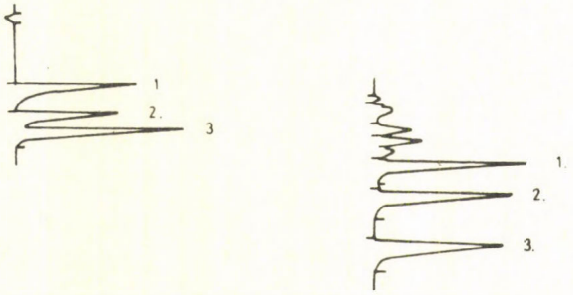


Fig. 2. Chromatograms of proflavine glycosides in two chromatographic systems. Column: 20 cm x 4.6 mm I.D., containing Lichrosorb Si-60. Mobile phase: top chromatogram, 20:80 water-methanol, 2 ml/min; bottom chromatogram, n-heptane, 2 ml/min. The elution sequence is PDG, PMG, proflavine. The numbers above the peaks represent: top chromatogram, 1. PDG (9.77 min), 2. PMG (13.34 min), 3. proflavine (15.63 min): bottom chromatogram, 1. PDG (4.15 min), 2. PMG (4.92 min), 3. proflavine (6.09 min). The relative standard deviations of the quantity of the compounds determined from 9 measurements in the first system are: PDG, 1.9%, PMG, 2.8%, proflavine, 3.5%. Recovery data: PDG, 98.1-100.9%; PMG, 97.9-104.5%; proflavine, 96.1-103.4%.

The order of elution is reversed on this column; it is: proflavine-PMG-PDG. When changing the strength of the eluent, the k' values did not change in such a high degree as on a normal-phase column. With respect of the capacity factor and resolution the best separation was obtained in the following system: A: 0.5% ethanolamine in methanol, B: 60% acetonitrile. Figure 3 shows the chromatogram obtained under these conditions.

Separation on thin layers was also attempted. More than 50 eluent systems of different polarity were tried on different normal and reversed-phases. The best separation was obtained on a Merck 5721 silica layer (ready layer), using 55:20:20:5 isobutanol-ethanol-water-cc. NH_3 as the eluent. In this system the R_f values of the components are as follows:

proflavine: 0.67;
PMG: 0.53;
PDG: 0.39.

The quantitative evaluation of the thin-layer chromatographic plates was carried out by a Shimadzu CS 920 spectrodensitometer and the components were detected at 430 nm. This method gives quick information about the composition of the product, but the standard deviation values and the accuracy of the measurements do not comply with the analytical requirements.

Our experience showed that the lifetime of the amino- as well as the normal-phase columns was short, especially when the samples contained components which could not be identified and some of these remained on the column. In this case the selectivity and resolution of the columns rapidly deteriorate; on the normal-phase column the peaks of all three components, while on the amino column the PDG peak became broad.

The behaviour of proflavine glycosides was examined in detail on different reversed-phases. Experiments were conducted on Lichrosorb-RP-2, RP-8 and RP-18 columns; ammonium carbonate buffers of different pH and acetonitrile concentration were applied as the eluents.

The effect of the alkyl chain on the capacity factor (k'), selectivity factor (α), and resolution (R_s) was investigated. Furthermore the effects of the pH of the buffers on the peak shape, retention time and resolution were also studied.

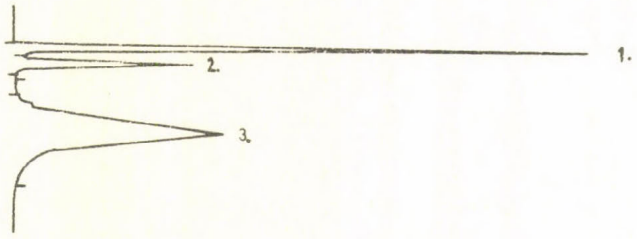


Fig. 3. Chromatogram of proflavine glycosides on a Lichrosorb-NH_2 column (20 cm x 4.6 mm I.D.; particle size: 10 µm). Mobile phase: 40:60 methanol: acetonitrile; the methanol contained 0.5% ethanolamine. Flow rate: 1 ml/min. Detection at 430 nm. Elution sequence: 1. proflavine (1.83 min), 2. PMG (2.56 min), 3. PDG (5.79 min).

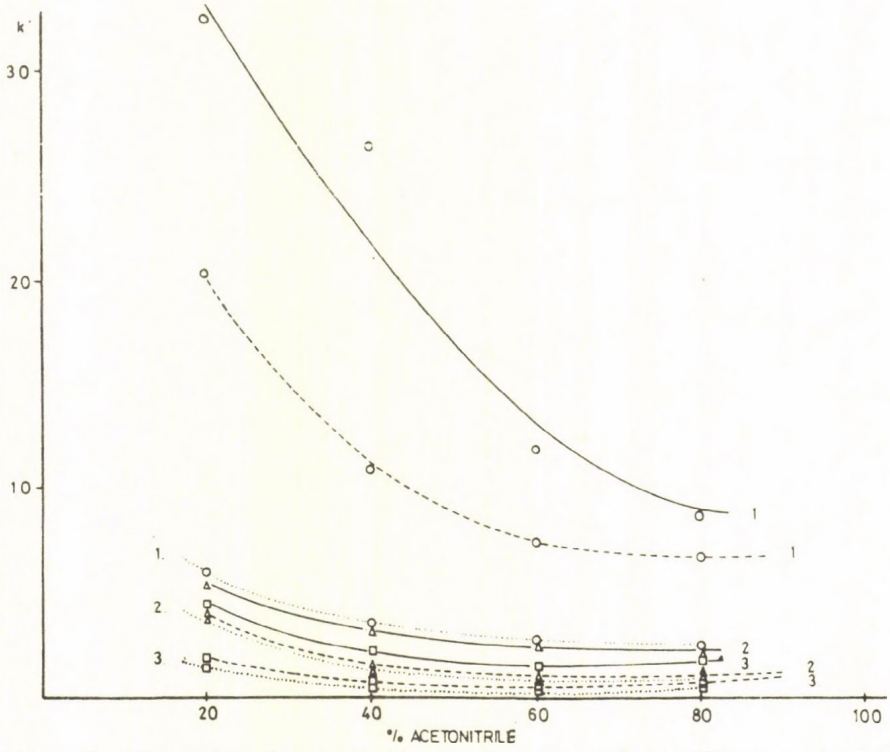


Fig. 4. The k' values of the PDG, PMG and proflavine on columns RP-2, RP-8, RP-18 at pH = 3. 1. proflavine, 2. PMG, 3. PDG. ——— = RP-18, ------- = RP-8, ... = RP-2.

DISCUSSION

Change of the capacity factor as a function of the pH and acetonitrile concentration of the eluent

These investigations were carried out on columns containing Lichrosorb-RP-2, RP-8 and RP-18.

Figure 4 plots the k' values obtained on these columns against the acetonitrile concentration in a pH = 3 buffer. As seen, the shape of the curves for proflavine, PMG and PDG is similar on all three columns. At this pH, when increasing the acetonitrile concentration of the eluent, the k' values decrease.

In the case of a non-polar phase, the separation mechanism can be explained partly by the so-called solvophobic theory /5/.

According to Horváth et al. /6/, as a result of hydrophobic interactions, repulsive forces are formed among the polar solvent, the non-polar dissolved substance and the stationary phase. The elution order in our case is: PDG-PMG-proflavine.

When the composition of the eluent changes from the aqueous buffer to acetonitrile, by increasing the quantity of the organic solvent, the k' values decrease. On the basis of this theory the size of the substance to be analyzed affects the slope of the plot: the slope increases with an increase of the specific surface.

In the case of the three investigated substances the greatest slope is shown in the case of proflavine: its retention decreases most on all three columns. The slope of the plot is the smallest for PDG. From this point of view PMG is between proflavine and PDG. A difference in the slope of the plots is also observed on different reversed-phases: the slope decreases in the direction of RP-18 → RP-8 → RP-2.

This observation can probably beexplained by the fact that the polarity of the three compounds investigated increases in the above-mentioned order. When introducing a sugar substituent having greater polarity, the surface size of the basic

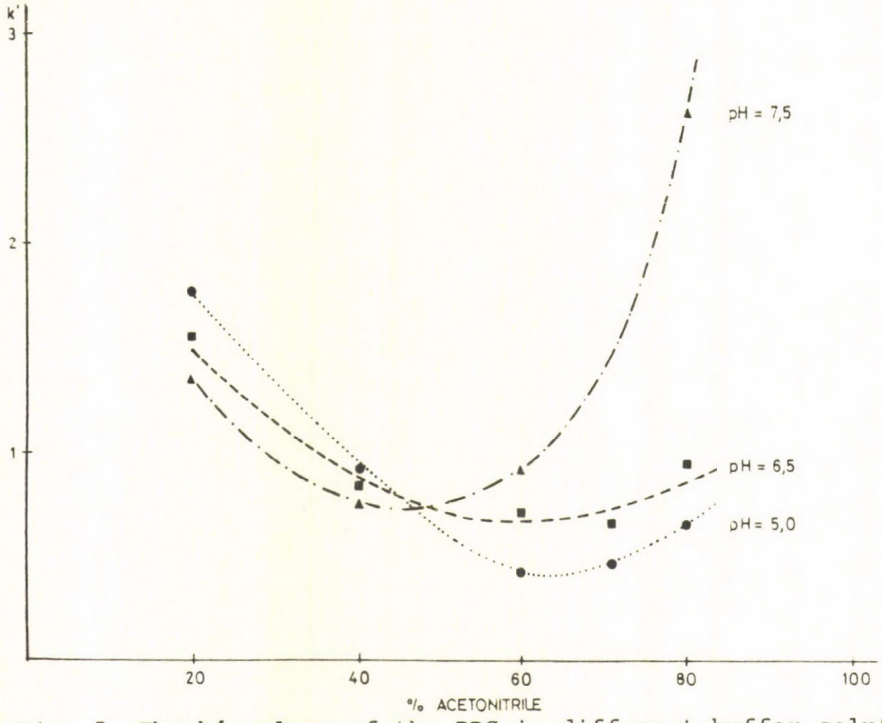


Fig. 5. The k' values of the PDG in different buffer solutions on column RP-8.

molecule bonding to the stationary phase as well as the release of free energy after complex formation decrease. This is reflected in the reduction of the slope of the plots in the direction of proflavine-PMG-PDG.

On RP-8 and RP-18 columns, at pH = 5, 6.5 and 7.5, and when increasing the acetonitrile concentration of the eluent, the shapes of the plots are different from those obtained in a pH = 3 buffer and are similar on both columns. For this reason only the results obtained on a RP-8 column are shown (Figs 5, 6 and 7).

It can be seen in these figures that for all substances, the plots decrease to a critical acetonitrile content, then increase. The critical values are different at different pH values but are the same for all the substances. The position of the minimum is: at pH = 7.5 about 40-45%, at pH = 6.5 about 65% and at pH = 5.0 about 65-70% acetonitrile content.

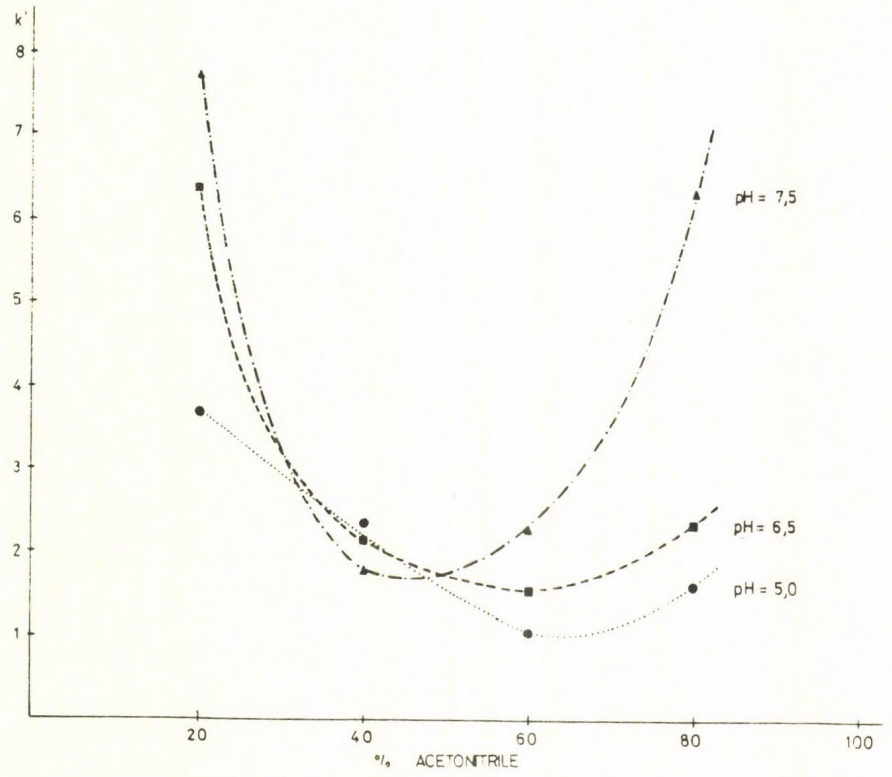


Fig. 6. The k' values of the PMG in different buffer solutions on column RP-8.

In the case of a pH = 3.0 buffer there is also a critical minimum in the plot, but it is placed at a higher acetonitrile concentration (higher than 80%). In this case the k' values, i.e. retentions already become very high; therefore we have not studied this range in detail. Under the effect of different buffers and acetonitrile concentrations the change in the k' values is the greatest in the case of proflavine, about 20 units; it is about 6 units for PMG, and it is the smallest, about 1.5 units, for PDG.

This means that the retention of proflavine can be influenced in many ways while the retention time of PDG is in a narrow range even in the case of eluents of different pH and composition.

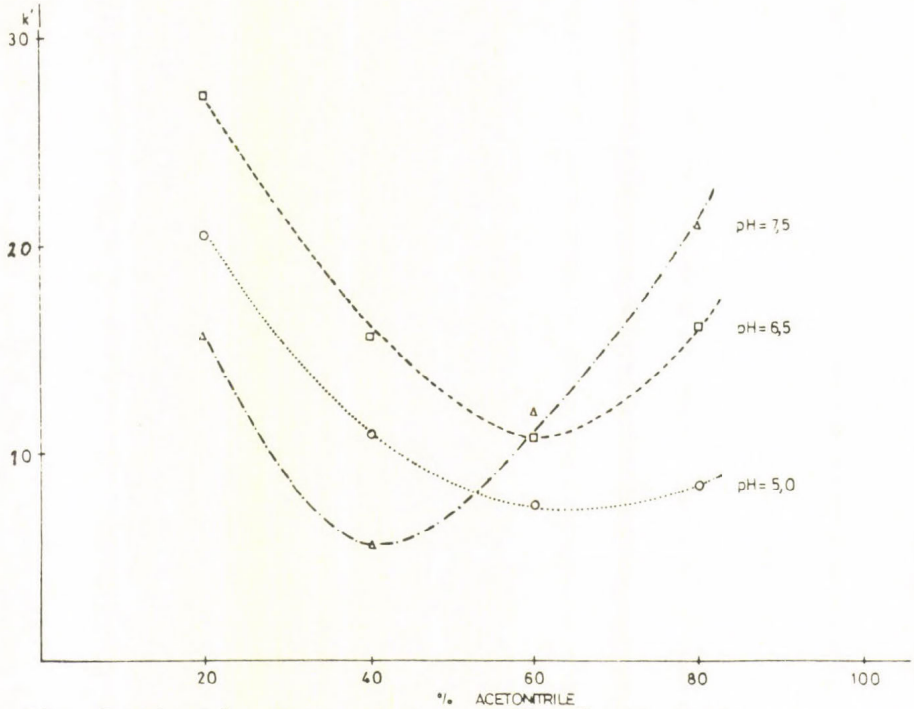
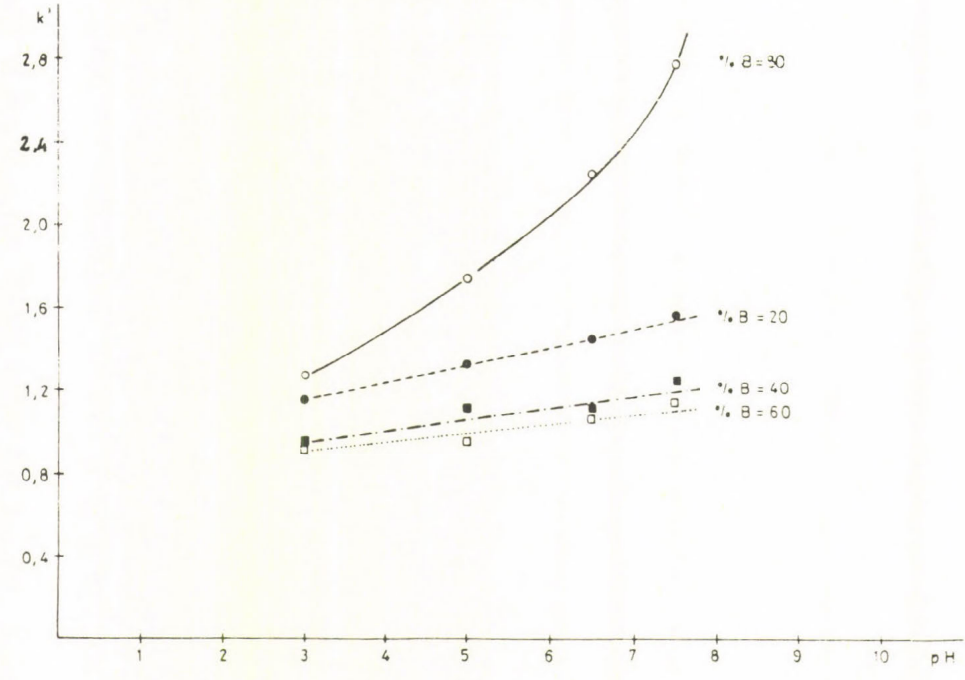


Fig. 7. The k′ values of the proflavine in different buffer solutions on column RP-8.



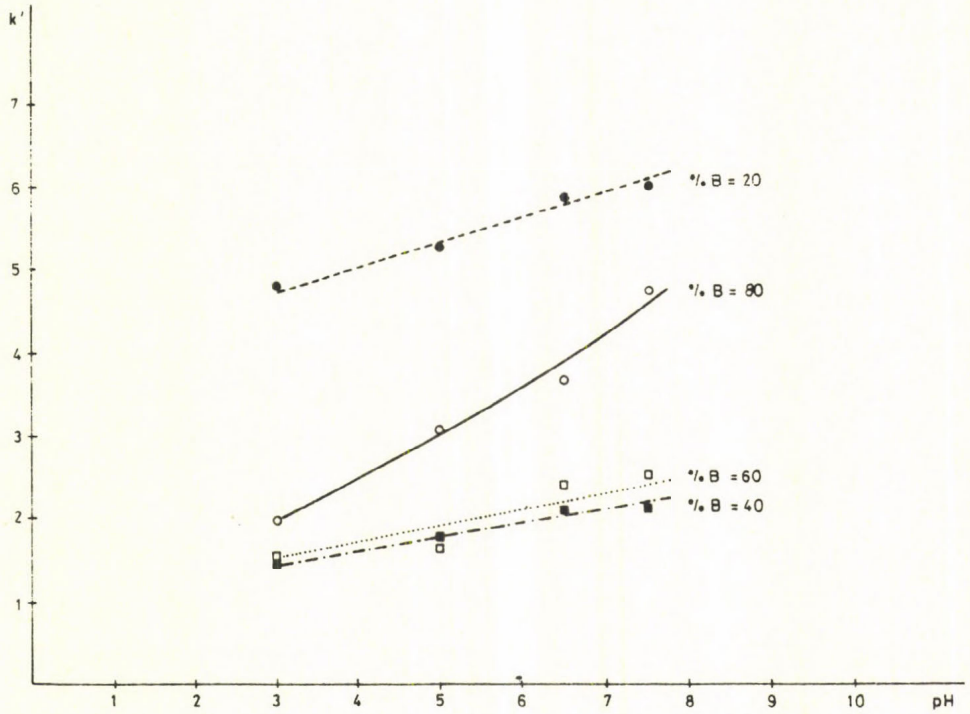


Fig. 9. The k' values of the PMG in different buffer solutions on column RP-18.

Figures 8, 9 and 10 plot the capacity factor as a function of the pH.

The shapes of plots on RP-2 and RP-8 are similar to those obtained on RP-18. The absolute values of k' for PDG and PMG on the RP-2 column are nearly the same as the values obtained on RP-18 (except for the eluent containing 20% acetonitrile), however, on the RP-8 column, the k' values vary in a wider range.

The trend of the plots for proflavine is more uniform: the k' values increase quasi-linearly in the direction of RP-2-RP-8 - RP-18. The slope of the plots is affected by the acetonitrile content of the eluent and by the pH of the buffer.

←

Fig. 8. The k' values in different buffer solutions on column RP-18 refer to PDG.

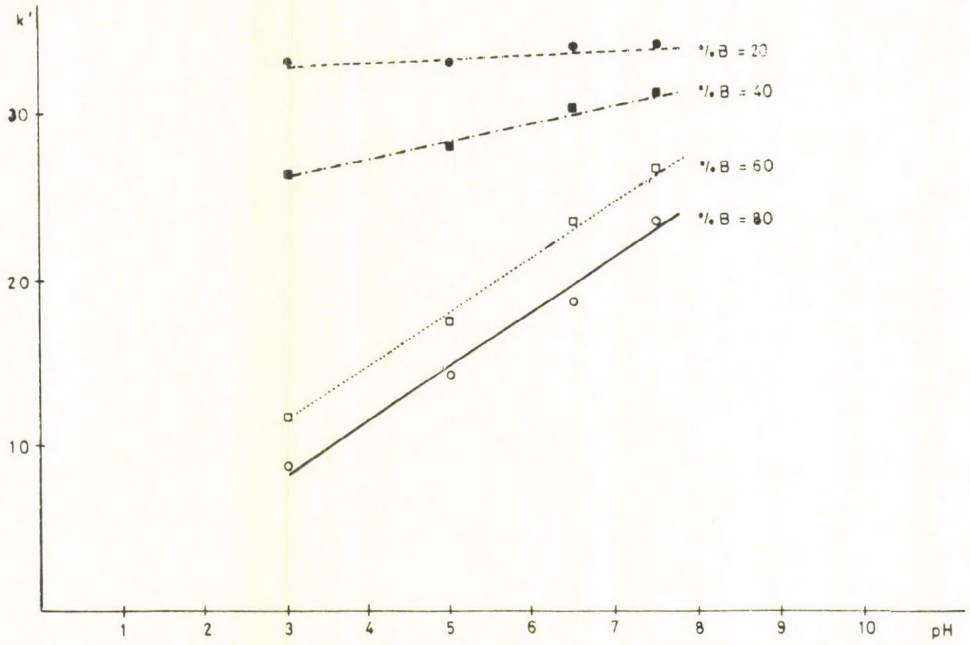


Fig. 10. The k′ values of the proflavine in different buffer solutions on column RP-18.

It can be concluded from the figures that the pH affects the k′ values of the PDG only in a very small degree (Fig. 8): they are within a 1.5 units intervallum and while the slope of the plots depends on the acetonitrile content of the eluent, a significant difference can be found only in the case of 80% acetonitrile content.

PMG shows a more variable picture (Fig. 9); the k′ values are placed in a wider (4.5 units) range. The values of slopes are here similar to those obtained for PDG.

In the case of proflavine, increasing the acetonitrile content of the eluent, the slope of the k′ plots increases more definitely; at 60% and 80% acetonitrile concentration the k′ values can be affected in a high degree by the pH of the buffers (Fig. 10).

Change of the capacity factor as a function of the pH-pK_a

Knowing the pK_a value of proflavine (9.7) /1/, we can prepare the pH-pK_a plot, or its portion corresponding to the experimental range. At the pH values of the applied buffers, which naturally (because of the organic solvent content) do not represent the true pH values, the degree of ionization can be calculated in the following way (see Fig. 11):

$$\% \text{ of } [HB^+] \text{ form} = \frac{100}{1 + \text{antilog } (pH - pK_a)}$$

We have worked below the pK_a value; at pH = 7.5. 99% of the material was in the protonated form, while in other cases it was present as 100% (calculated according to the formula above) /7/. As shown by the plots, where the effect of pH is the greatest, i.e. at 20% acetonitrile concentration, the k' values change only by a very small degree as the function of pH, and in this system the affinity of the protonated proflavine base to the stationary phase is very high. The k' values are more desirable at higher acetonitrile concentrations and lower pH values.

Unfortunately, the columns cannot be applied near the pK_a value or with an eluent having a pH higher than the pK_a. For the time being the pK_a constants of PDG and PMG are not available; we hope to be able to determine them, because this can make clearer the effect of pH. The knowledge of the pK_a of PDG and PMG is desirable not only from the chromatographic point of view, since the biological effect of these compounds is connected to the pK_a values: the ionization degree of the cation, i.e. of the protonated base at physiological pH is important. Albert and Serjeant /7/ conducted experiments with compounds similar to proflavine and they showed that the nearer are the pK_a and the pH to each other the smaller is the minimum bacteriostatic concentration and the more effective is the compound.

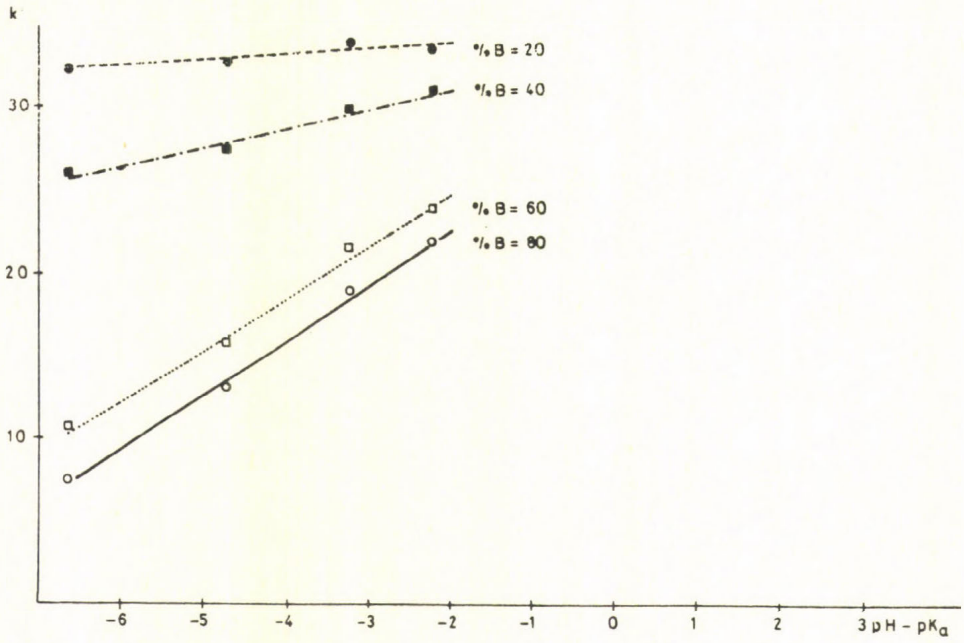


Fig. 11. The k' values of the proflavine versus pH-pK_a on column RP-18. The pK_a of the proflavine = 9.7.

$$\% \text{ of } [HB^+] \text{ form} = \frac{100}{1 + \text{antilog } (pH - pK_a)}$$

Effect of the alkyl chain length on the capacity factor

The structure of the alkyl chains usually plays an important role in retention and selectivity: increasing the length of the chain or the number of carbon atoms at a given mobile phase, retention increases /8/. However, Berendsen and de Galan obtained different results /9/: retention was found to increase to a "critical length of the carbon chain" (C_6-C_{10}) and then a constant value is reached. Different substances can have different critical values. After reaching the critical value, i.e. using a stationary phase with a longer carbon chain, the capacity factor remains approximately constant.

Since for a given substance the critical chain length does not depend on the composition of the mobile phase, this means that the length of the alkyl chain affects separation only up to a certain limit.

Experiments conducted with proflavine glycosides prove only partly this finding. In Figs 12, 13 and 14 the k' values measured in a pH = 3 buffer are plotted as a function of the length of the alkyl chain using eluents with different acetonitrile concentration.

In the case of PDG (Fig. 12) the broadest k' range was found on a RP-8 column, while the absolute values of the capacity factors are nearly equal on the RP-2 and RP-18 columns.

It seems that for PDG with eluents containing 20% and 40% acetonitrile, the "critical chain length" is 8. Above this value the k' values do not increase, but do not reach a constant value: they even decrease.

In the case of PMG (Fig. 13) the picture is more variable. Under the influence of an eluent containing 20% acetonitrile the k' values increase with increasing alkyl-chain length. In the case of 40% acetonitrile the k' values correspond to a plot similar to that obtained for PDG; 60% and 80% acetonitrile causes a slight decrease in the plot for RP-8 and then a little more abrupt increase for RP-18. In the case of proflavine a uniform picture is obtained (Fig. 14) and the findings of Hemetsberger et al. /10/ are partly confirmed: in the case of eluents containing 20% and 40% acetonitrile the k' values are quasi-linear, while at 60% and 80% acetonitrile content they follow the findings of Berendsen and de Galan /9/, i.e. the plot linearly increases to RP-8 and then becomes almost parallel to the abscissa, having the k' values for RP-18 almost the same as for RP-8. These data also show that the slope of the plots depends on the concentration of the organic solvent in the eluent.

When applying a pH = 7.5 buffer, the shapes of the k' vs. chain length plots for PDG and PMG somewhat change, however, for proflavine the slope and the trend of the k' values is similar to the plots obtained in a pH = 7.5 buffer (Figs 15, 16 and 17).

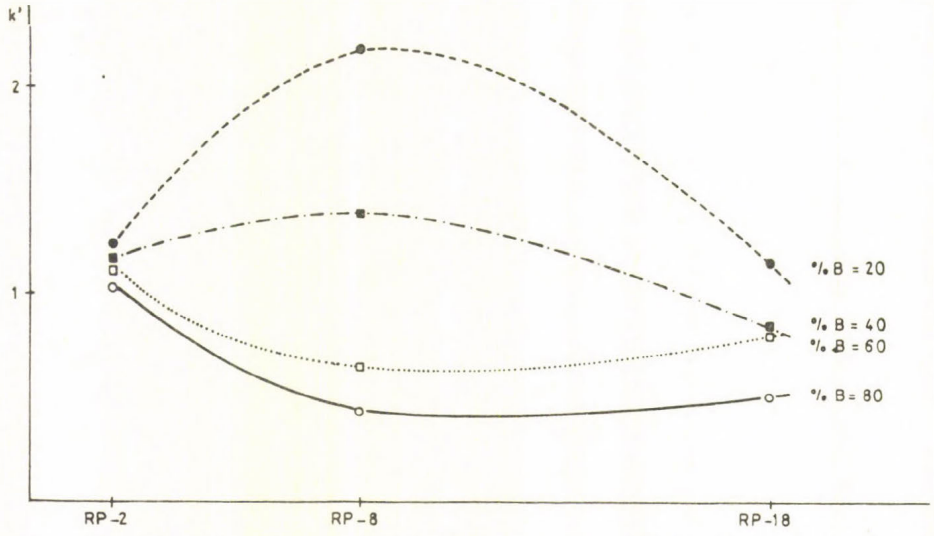


Fig. 12. The k' values of the PDG versus alkyl chain length at pH = 3.

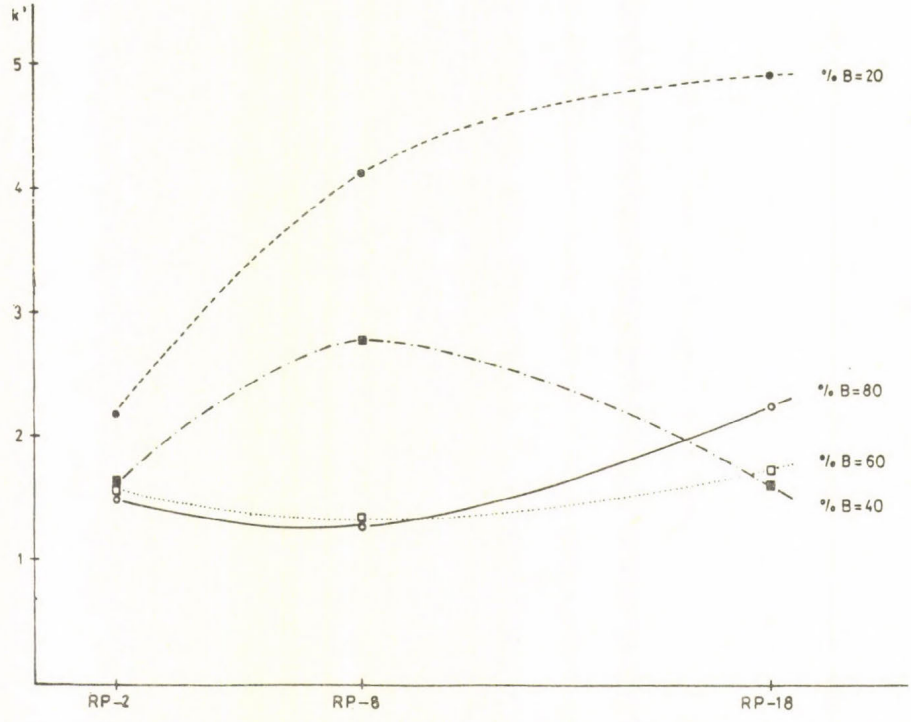


Fig. 13. The k' values of the PMG versus alkyl-chain length at pH = 3.

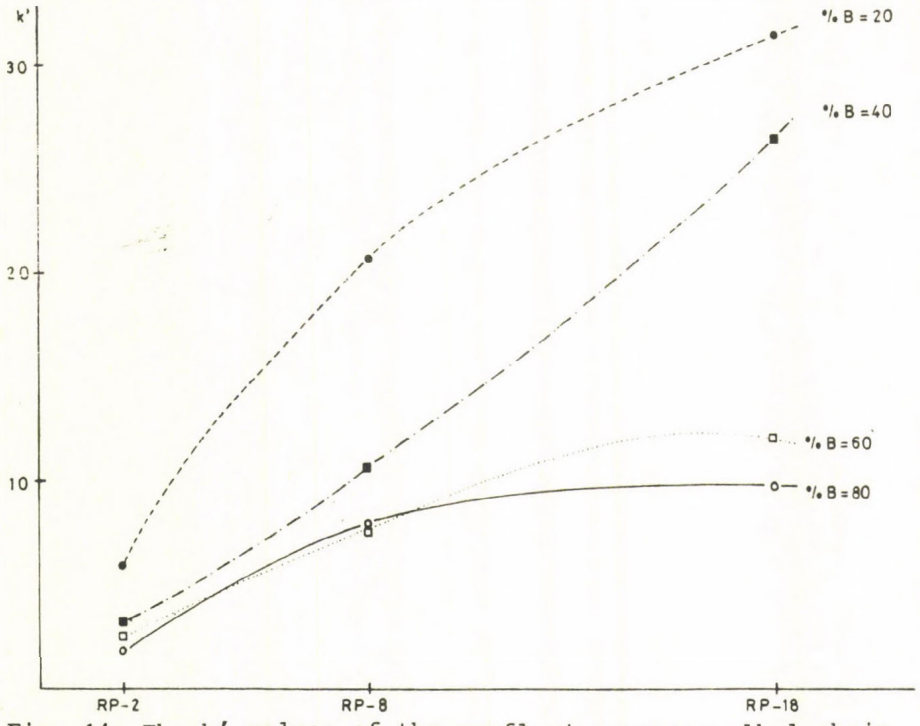


Fig. 14. The k' values of the proflavine versus alkyl-chain length at pH = 3.

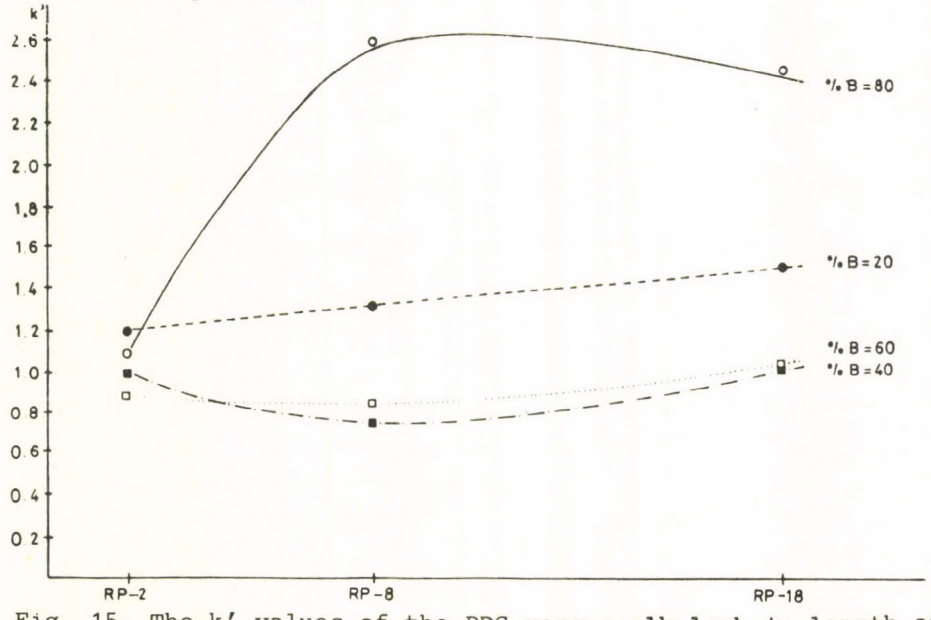


Fig. 15. The k' values of the PDG versus alkyl-chain length at pH = 7.5.

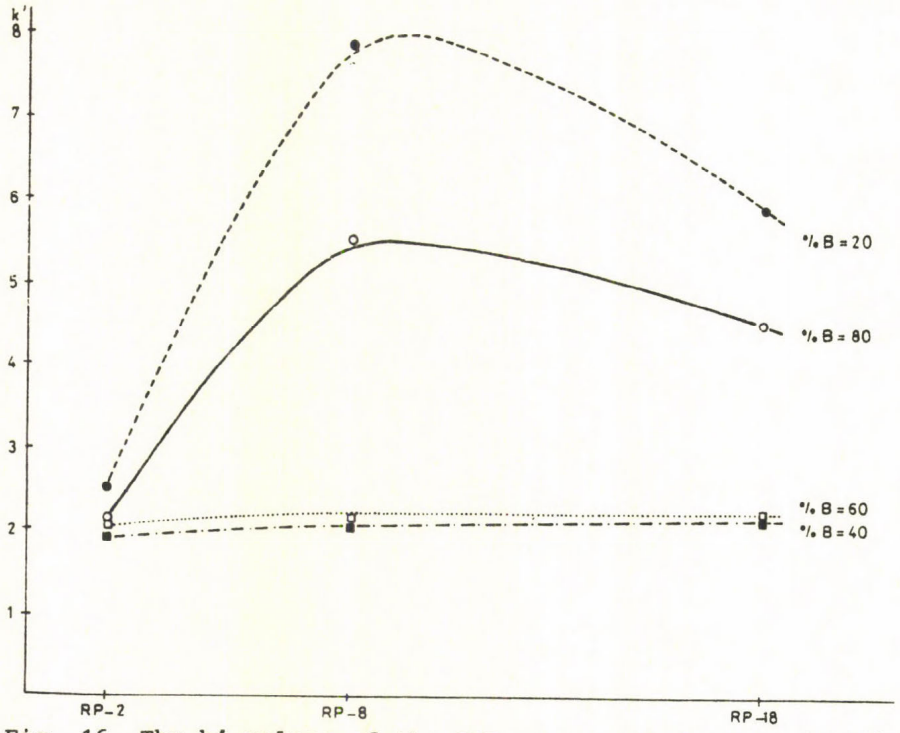


Fig. 16. The k' values of the PMG versus alkyl-chain length at pH = 7.5.

Effect of the alkyl chain length on selectivity

Some authors have found that selectivity depends on the structure of the molecules and the composition of the mobile phases, but it is relatively independent on the length of the alkyl chain /10, 11/.

According to other outhors the selectivity, after an initial increase(C_6-C_{14}), remains constant when the length of the carbon chain increases /9, 12/. Figures 18 and 19 plot selectivity at pH = 3 and at different acetonitrile concentration of the eluent as a function of the carbon chain; selectivity is given for the proflavine-PMG and PMG-PDG peak pairs.

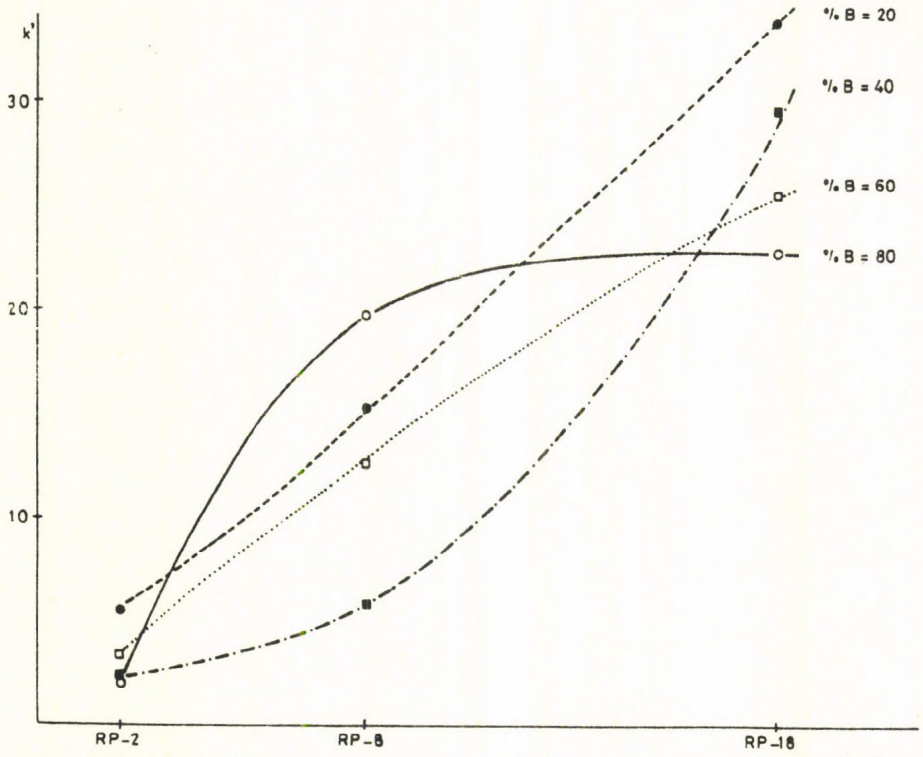


Fig. 17. The k' values of the proflavine versus alkyl-chain length at pH = 7.5.

In the case of 20% acetonitrile the selectivity for PDG and PMG (Fig. 18) increases slightly for RP-8, and significantly for RP-18. At 40% and 60% acetonitrile concentrations it reaches a plateau at RP-8 and then practically remains constant for RP-18. In the case of 80% acetonitrile the plot has a maximum at RP-8.

For proflavine-PMG, the selectivity reaches a plateau at about 20%, 40% and 60% acetonitrile concentrations. In the case of an eluent containing 80% acetonitrile the plot has a maximum at RP-8 (Fig. 19).

These figures show that the physical-chemical properties of the molecules, the length of the alkyl chain as well as the composition and pH of the mobile phase can significantly affect selectivity.

Even in the case of such similar compounds it is impossible to predict with absolute certainty from previous results

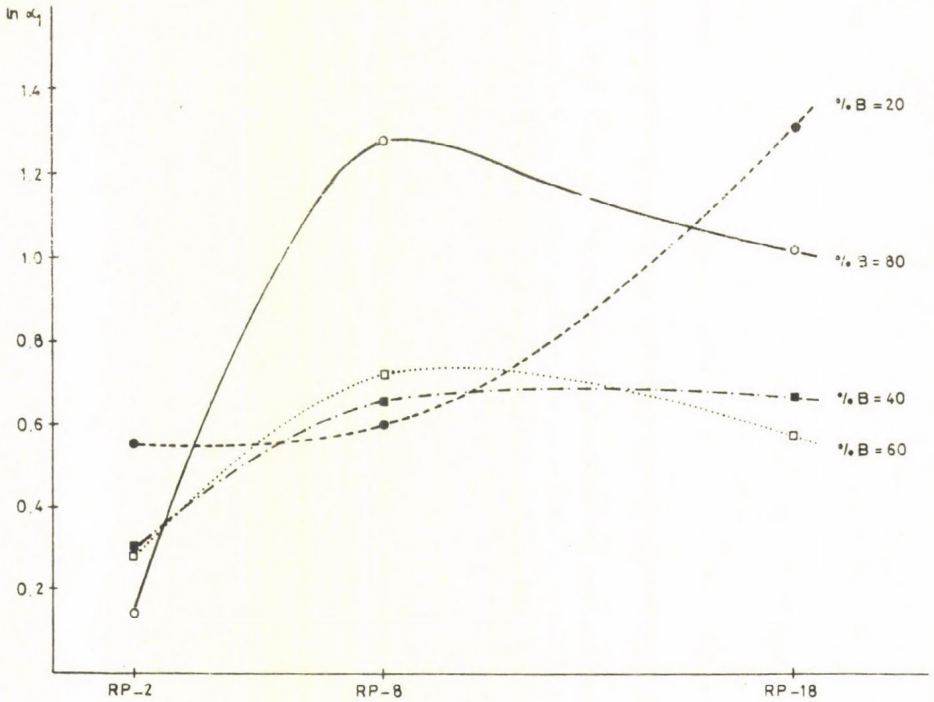


Fig. 18. Relationship between the selectivity (α) of the chromatographic systems and the length of the alkyl chain of the stationary phase for binary mobile phases having a pH = 3 and the given acetonitrile concentration. Peak pair: PMG-PDG.

what kind of retention can be expected at a given pH and eluent compositon, and for a stationary phase with a certain alkyl chain length.

Change of resolution when using different pH and acetonitrile concentration

Since, in addition to the chromatographic study of the compounds our task was also their quantitative determination, we evaluated the most suitable reversed-phase for quantitative measurements. At pH = 5, 6.5 and 7.5 the peaks of proflavine glycosides showed a rather strong asymmetry (tailing). At pH = 3 the peaks became symmetrical at all acetonitrile concentrations; therefore, we settled at pH = 3 and studied the

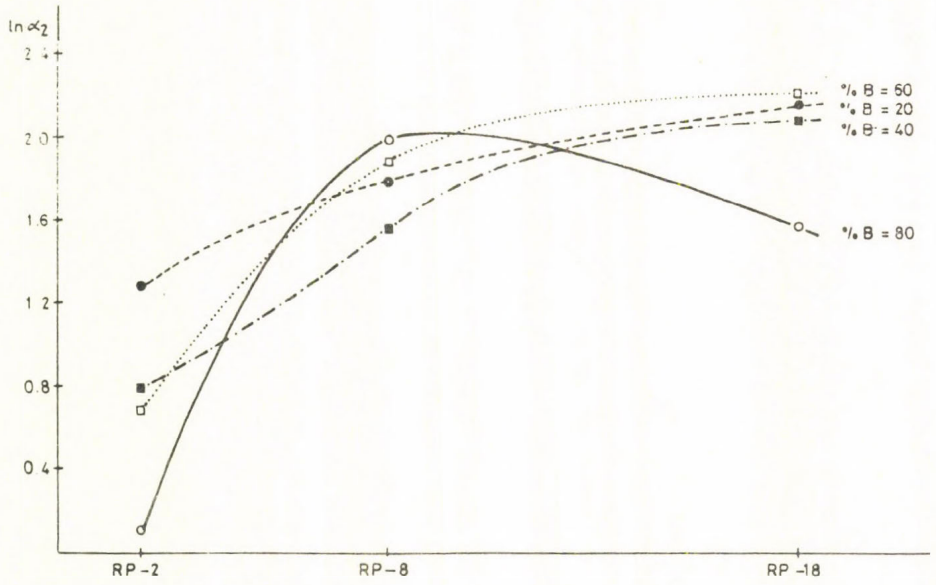


Fig. 19. Relationship between the selectivity (α) of the chromatographic systems and the length of the alkyl chain of the stationary phase for binary mobile phases having a pH = 3 and the given acetonitrile concentration. Peak pair: proflavine-PMG.

change of resolution as a function of the k' values on columns containing different lengths of carbon chains, with eluents containing different acetonitrile concentrations. Figures 20, 21 and 22 show our results obtained under these conditions, plotting resolution (R_s) against the k' values.

Capacity factor values up to 15 can still be accepted in practice. Since proflavine glycosides have to be determined simultaneously and in very different quantities, the R_s value has to be 1.5 or higher.

RP-2 column (Fig. 20): for the PDG-PMG pair only 20% acetonitrile concentration, while in the case of the proflavine-PMG pair, both 20% and 40% acetonitrile give appropriate resolution. On this column all three components can be determined simultaneously using an eluent containing 20% acetonitrile.

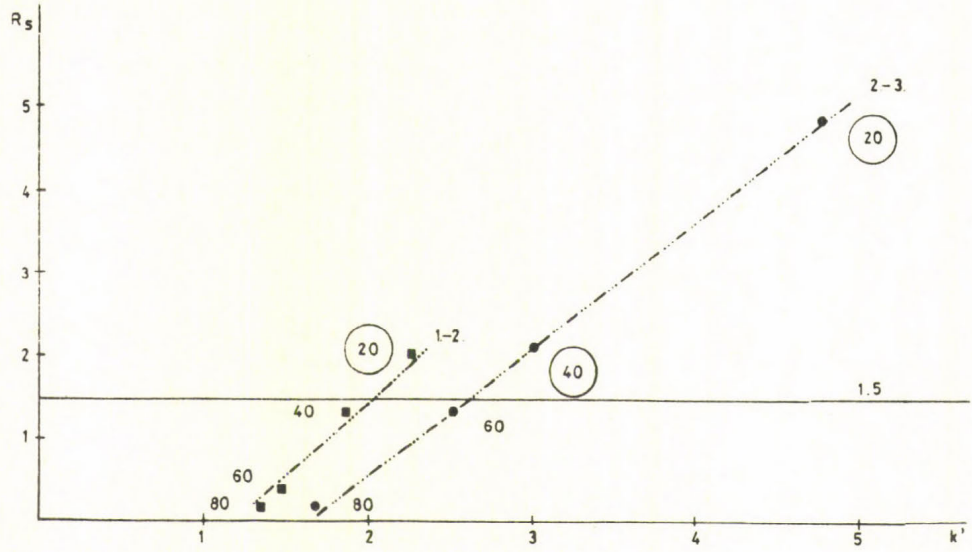


Fig. 20. Relationship between resolution (R_s) of the chromatographic system and the capacity factor values. Peak pairs: 1-2. PMG-PDG, 2-3. proflavine-PMG; stationary phase: RP-2. The numbers in circle indicate the suitable acetonitrile concentration in the mobile phase for the resolution of the components.

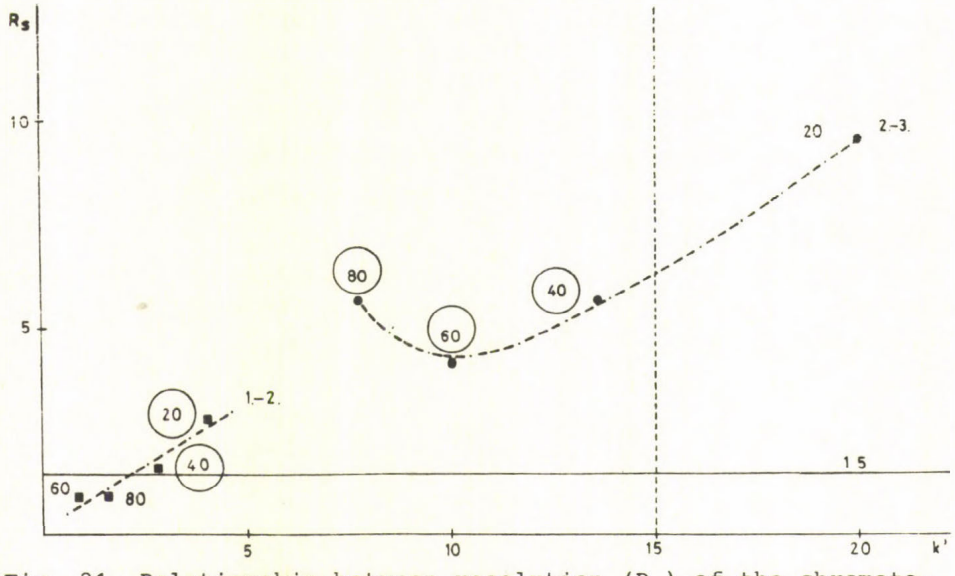


Fig. 21. Relationship between resolution (R_s) of the chromatographic system and the capacity factor values. Peak pairs: 1-2. PMG-PDG, 2-3. proflavine-PMG; stationary phase: RP-8. The numbers in circle indicate the suitable acetonitrile concentration in the mobile phase for the resolution of the components.

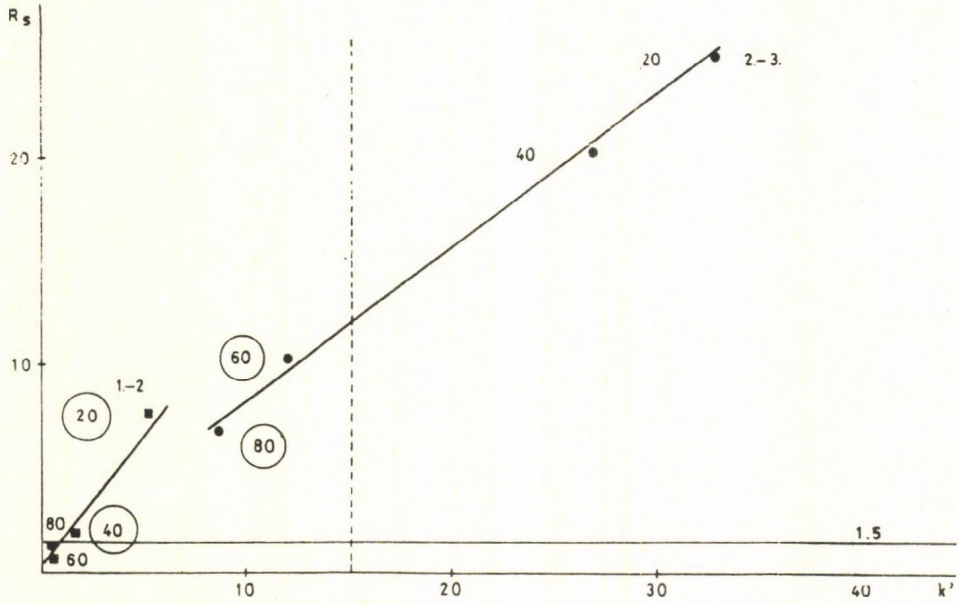


Fig. 22. Relationship between resolution (R_s) of the chromatographic system and the capacity factor values. Peak pairs: 1-2. PMG-PDG, 2-3. proflavine-PMG; stationary phase: RP-18. The numbers in circle indicate the suitable acetonitrile concentration in the mobile phase for the resolution of the components.

RP-8 column (Fig. 21): For the PDG-PMG pair both 20% and 40% acetonitrile concentration is satisfactory while in the case of the proflavine-PMG pair all four acetonitrile concentrations give appropriate resolution. However, at 20% concentration, the retention of proflavine becomes too long. On this column all three components can be measured simultaneously by an eluent containing 40% acetonitrile.

RP-18 column (Fig. 22): For the PDG-PMG pair both 20% and 40% acetonitrile concentration is satisfactory, while in the case of proflavine-PMG pair all four acetonitrile concentrations give appropriate resolution. In the latter case, however, the k' values became too large for 20% and 40% acetonitrile concentration. Therefore on this column the three components cannot be determined simultaneously in a practical analysis.

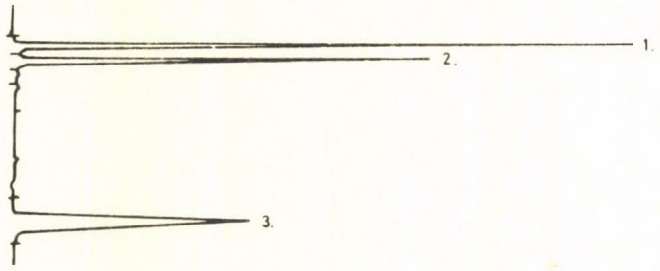


Fig. 23. Chromatogram of proflavine glycosides on an RP-8 column (20 cm x 4.6 mm I.D.). Mobile phase: 60:40 A:B; A = 0.1% $(NH_4)_2CO_3$ solution, pH = 3 (corrected with H_3PO_4); B = acetonitrile. Flow rate: 1.5 ml/min. Detection at 430 nm. Elution sequence: 1. PDG (1.84 min), 2. PMG (2.66 min), 3. proflavine (13.71 min).

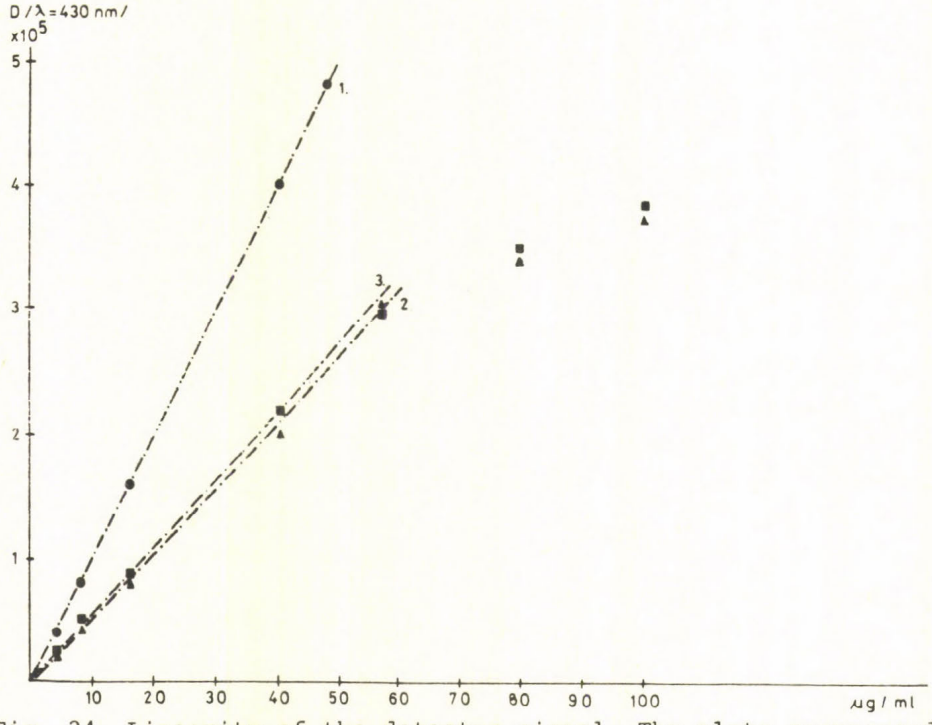


Fig. 24. Linearity of the detector signal. The plots correspond to the following equations:
1. proflavine: y = 9825 x + 1548
2. PMG: y = 5211 x - 2058
3. PDG: y = 5328 x + 4989
The correlation coefficients are: proflavine, r = 0.9997; PMG, r = 0.9960; PDG, r = 0.9975. Recovery data (n = 7): proflavine, 96.9-102.1%; PDG, 98.9-101.1%, PMG, 98.3-102.2%.

On the basis of these results we can state that the RP-8 column is the most suitable for the practical analysis of these compounds. A RP-2 column would also be suitable for the simultaneous determination of the three components, however, its efficiency is much smaller. This is probably due to the fact that free silanol groups are present and they take part in the separation mechanism /13/. We also have to pay attention to the fact that besides the three main components, the experimental samples may also contain other similar compounds as impurities; they require a "place" on the chromatogram and they have to be separated from proflavine, PMG and PDG. Figure 23 shows a chromatogram which was obtained on the RP-8 column, using an eluent containing a pH = 3 buffer and 40% acetonitrile. The experimental conditions are given in the figure caption.

Figure 24 demonstrates the detector signals as a function of sample concentration.

SUMMARY

On the basis of the results the RP-8 column was found to be the most effective from both analytical and economical point of view. An RP-2 column results in a relatively poor separation but gives quick information RP-18 is also suitable for the measurement of proflavine glycosides but because of the long retention of proflavine in eluents containing low acetonitrile concentrations, simultaneous analysis is not practical.

Our study showed that the effect of the pH also is very interesting. However, final conclusion can only be drawn if the pK_a values of PDG and PMG are known, and if further experiments are conducted in a wider pH range, even above the pK_a.

There are still a number of open questions concerning chromatography of proflavine glycosides. The results will contribute to the theoretical interpretation of the separation mechanism on different stationary phases.

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COMPARATIVE INVESTIGATION OF BRAIN LIPIDS OF FISH WITH DIFFERENT TYPES OF FEEDING BEHAVIOUR

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SUMMARY

The brain lipids were compared in fish with two different types of feeding behaviour, to show how the feeding conditions influence the main lipid classes of the brain tissue, namely the neutral lipids and the polar lipids, and the amounts of fatty acids. The two groups consisted of fish eating mixed food (omnivorous), and fish eating mainly food of plant origin, that is phytoplankton (herbivorous). The lipids were separated by thin-layer chromatography and were quantified by densitometry. After hydrolysis of the lipids in the samples, methyl esters were formed and measured quantitatively by means of gas liquid chromatography. The results supported the assumption that the constitutions of the brain lipids of fish with different feeding behaviours differ basically from each other. The structural lipids were present in larger amounts in the brains of the omnivorous group, while the depot lipids of the herbivorous fish are increased relative to the other group. There was also a difference in the ratio of saturated and unsaturated lipids in the different groups on the basis of the feeding behaviour of the fish.

INTRODUCTION

For mammalian organisms, it is known that the fatty acid synthesis and the stearic acid delta-9 desaturase and acetyl coenzyme A carboxylase activities depend on the feeding conditions /1, 2/. In fish, there is a definite dependence of the lipid metabolism on the consumed food /3/, and it has been shown that the type of food consumed influences the biosynthesis of lipids /4/. The metabolic pathways of the lipids of fish are generally similar to those of other mammalian organisms, that is the lipids in the food go first of all into the

depots and the tissues, and are later mobilized and pass to the general metabolic routes for further consumption. The lipids, in addition to the supply of energy, can play the main role in the building-up of the tissues /5/.

Omnivorous fish consumes large amounts of unsaturated lipids in the food; this increases the amount of structural lipids and the incorporation of polar lipids into the tissues of this type of fish. The other group of fish dealt with in this study eats phytoplankton (algae, other plankton or plant parts). The carbohydrate content of their food is larger than its lipid content and therefore the incorporation of neutral lipids becomes conspicuous /5/. Our target was the quantitative investigation of the brain lipids of omnivorous and herbivorous fish.

MATERIALS AND METHODS

Fish

Omnivorous fish

Common carp (Cyprinus carpio L. morpha), crusian carp (Carassius carassius L.).

Herbivorous fish

grass carp (Chenopharyngodon idella), carp fish (Hypophthalmichthys molitrix).

The fish, which weighed 450-500 g, originated from the Fishing Research Institute at Szarvas. For two days before the investigations, 5 fish were kept in each 100 L aquarium, thermostated at 20°C. Our experiments were generally performed in March or April. The brains of the fish were taken after decapitation.

Extraction and separation of lipids

Total lipid content

The brain tissue was homogenized and extracted according to the method of Folch /6/, that is with chloroform-methanol (2:2). The extract was filtered and reduced to 1 mL with a

stream of nitrogen. A few dibutylhydroxytoluene crystals were put into the solution and it was kept at -30 °C in frozen state.

Neutral lipid content

The neutral lipids were analysed according to Malins and Mangold /7/. They separated on silica gel plates; the running solvent was petroleum ether - diethyl ether - acetic acid (85:15:1). The separated spots were visualized with iodine vapour.

Analysis of phospholipids

Phospholipids were separated by two-dimensional thin-layer chromatography according to Lepage /8/. The 1st-dimensional development was done in chloroform - methanol - water (65:25:4), and the 2nd-dimensional run in diisobutyl ketone - acetic acid - water (8:5:1). Spots were detected either with iodine vapour or with ammonium molybdate dissolved in perchloric acid /9/.

Analysis of fatty acids

The spots detected with iodine vapour were scraped from the plates and esterified in dry methanol containing 5% hydrogen chloride, under a nitrogen atmosphere. The fatty acids were analysed with a JGC 110 apparatus (JEOL, Tokyo, Japan). Glass columns packed with 11% or 15% DEGS on Gas Chrom P, 100-200 mesh, were used.

A temperature-programmed separation from 120°C to 175°C at a rate of 2.5°C/min was used. The spots and peaks were identified by using standard substances, as well as by taking into consideration the logarithm of the relative retention time and the number of carbon atoms. The quantitative ratios of the fatty acids were calculated according to the triangle technique of Farkas et al. /10/.

The measurements were evaluated semiquantitatively with a Telechrom OE 976 densitometer (Chinoin, Budapest, Hungary). The measurements were also subjected to statistical evaluation. The data in the Tables and Figures are given as means and SEM.

Table I. Comparative study on the neutral lipid content from freshwater fish brain tissue

Fractions	Common carp Cyprinus carpio L.	Crucian carp Carassius Carassius L.	Silver carp Hypophthal-michthys molitrix	Grass carp Chenopharyn-godon idella
Cholesterol esters (ChE)	5.6±1.8	6.9±0.2	2.4±0.4	3.3±0.3
Triglyceride (TG)	37.4±3.4	39.2±1.2	55.1±1.4	58.4±4.9
Fatty acids (FA)	10.5±1.2	8.8±1.2	4.3±0.4	5.8±0.4
1,3-diglyceride	0.9±0.3	1.7±0.2	0.6±0.01	0.7±0.1
1,2-diglyceride	0.6±0.01	0.4±0.01	0.8±0.2	N.D.
Cholesterol (Ch)	14.8±2.1	16.1±0.5	17.2±0.9	12.2±0.2
Monoglyceride	0.3±0.03	0.4±0.01	0.3±0.1	0.2±0.01
Phospholipid (PL)	31.2±2.5	24.2±2.8	18.3±1.3	19.9±0.4

Table II. Comparative data on the polar lipid content from freshwater fish brain tissue

Fractions	Common carp	Crusian carp	Silver carp	Grass carp
NL	2.2±0.32	6.5±1.8	15.3±1.5	11.6±1.2
ChE	18.2±1.7	17.0±1.9	24.8±2.5	21.5±2.1
PA	1.9±0.1	2.6±0.2	3.5±0.66	2.8±0.1
Ch	1.6±0.06	2.2±0.5	0.7±0.07	1.8±0.12
PE	25.1±0.4	26.9±3.1	20.6±3.1	24.1±2.4
LPE	2.4±0.2	2.8±0.4	3.0±0.22	2.4±0.1
PS+PI	6.8±1.3	9.5±0.6	9.2±1.3	8.9±0.2
PC	31.8±0.3	22.8±3.7	14.3±1.9	15.4±0.7
SM	9.5±1.4	10.1±1.0	8.5±0.7	9.9±0.9
LPC	0.7±0.02	N.D.	0.7±0.1	1.3±0.01

RESULTS

Tables I and II show the results of quantitative analyses of neutral and polar lipid fractions originating from the brains of the four species of fish. The first two and the last two columns of both Tables give the results of quantitative analyses of the neutral and polar lipids of the omnivorous and the herbivorous fish, respectively. The fatty acids of the neutral lipid fractions from the fish with different feeding behaviours show a significant difference. Such a difference can also be found for the triglyceride or ChE contents. Similar distinctions are observed in the case of the polar lipids in Table II.

Generally, the ChE and total polar lipid contents of the neutral lipid fractions extracted from the brain of the herbivorous fish are significantly lower than those of the omnivorous fish. At the same time, the triglyceride content of the brain of the herbivourous fish is definitely higher than that of the omnivorous fish. In contrast, the ChE and NL contents of the brain of the herbivorous fish are significantly higher than those of the omnivorous ones.

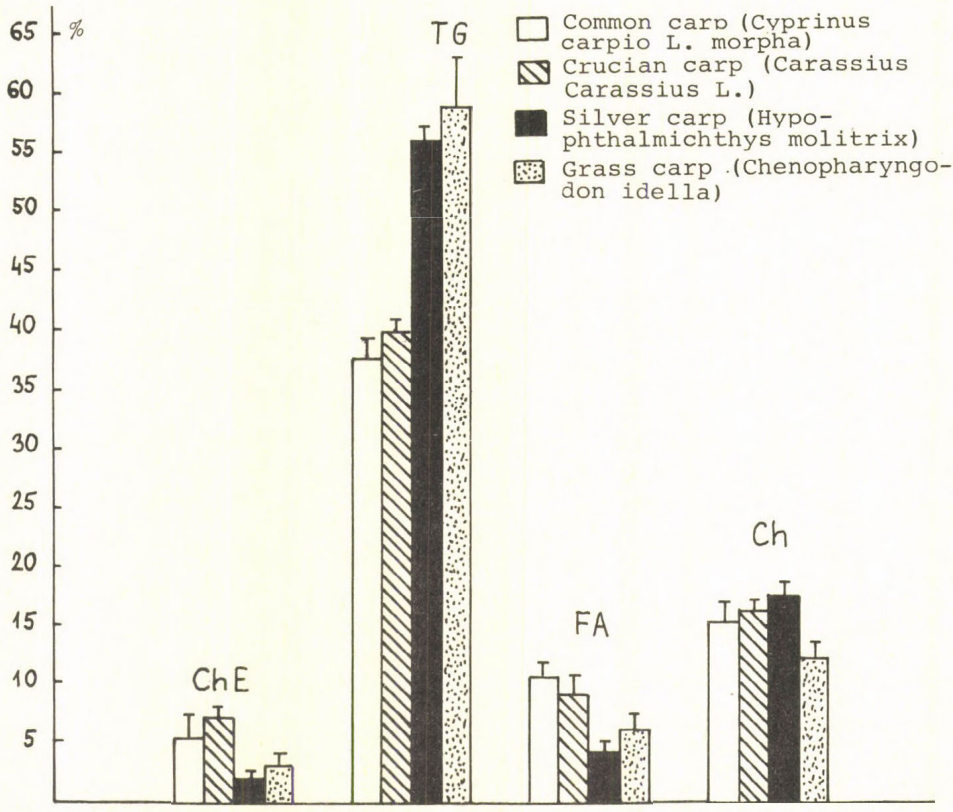

Figure 1. Comparison of the main neutral lipid fractions (weight %) of the brain extracts of different freshwater fishes.

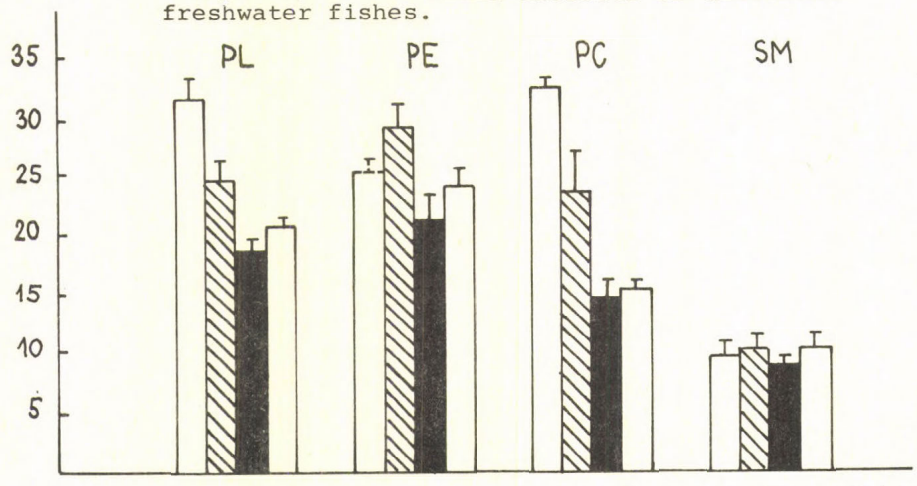

Figure 2. Comparison of the main polar lipid fraction of brain tissues (weight %) of different freshwater fishes. (For explanation see Fig. 1.)

The most characteristic data from Tables I and II, the amounts of neutral and polar lipids, are depicted in Figs 1 and 2, respectively.

The amounts of fatty acids after hydrolysis and esterification of the PE, PC, TPL (total PL), TG and TL contents are also indicated. Tables III, IV, V and VI show these values for the common carp, crusian carp, silver carp and grass carp, respectively.

With the help of column-graphics, the numerical data on the four fish are compared in Figs 3-7. The fatty acid contents of the total lipid fractions are depicted for PE, PC, TPL, TG and TL in Figs 3, 4, 5, 6 and 7, respectively. The essential characteristics of the individual Figures can be explained as follows:

1. Docosahexaenic acid (22:6ω3) is the only fatty acid of the PE of the fish brain which shows a significant difference between the two different groups. Its content is higher in the omnivorous group.

2. The amount of arachidonic acid is also higher in the PE and PC fractions than in the other fractions. In the PC, the amount of palmitoleic acid (16:1ω9) is significantly higher in the brain of the herbivorous fish than in the omnivorous ones.

3. As concerns the TPL fraction, palmitic acid (16:0) and 16:1ω9 are found in larger amounts in the omnivorous fish than in the other type.

4. The triglyceride fatty acids of fish brain also show a difference depending on the feeding type. The amount of palmitic acid is higher in the herbivorous species than in the omnivorous fish. There is a totally contrary situation for oleic acid. The differences between the various species are also high with respect to the fatty acids of the TG fraction.

5. As concerns the contents of individual fatty acids, the amount of docosahexaenoic acid seems to be very high. Furthermore, its content is higher in the herbivorous species than in the omnivorous fish. Otherwise, the 18:2ω6 fatty acids show an essentially higher occurrence in common carp and crusian carp than in the other two fish species.

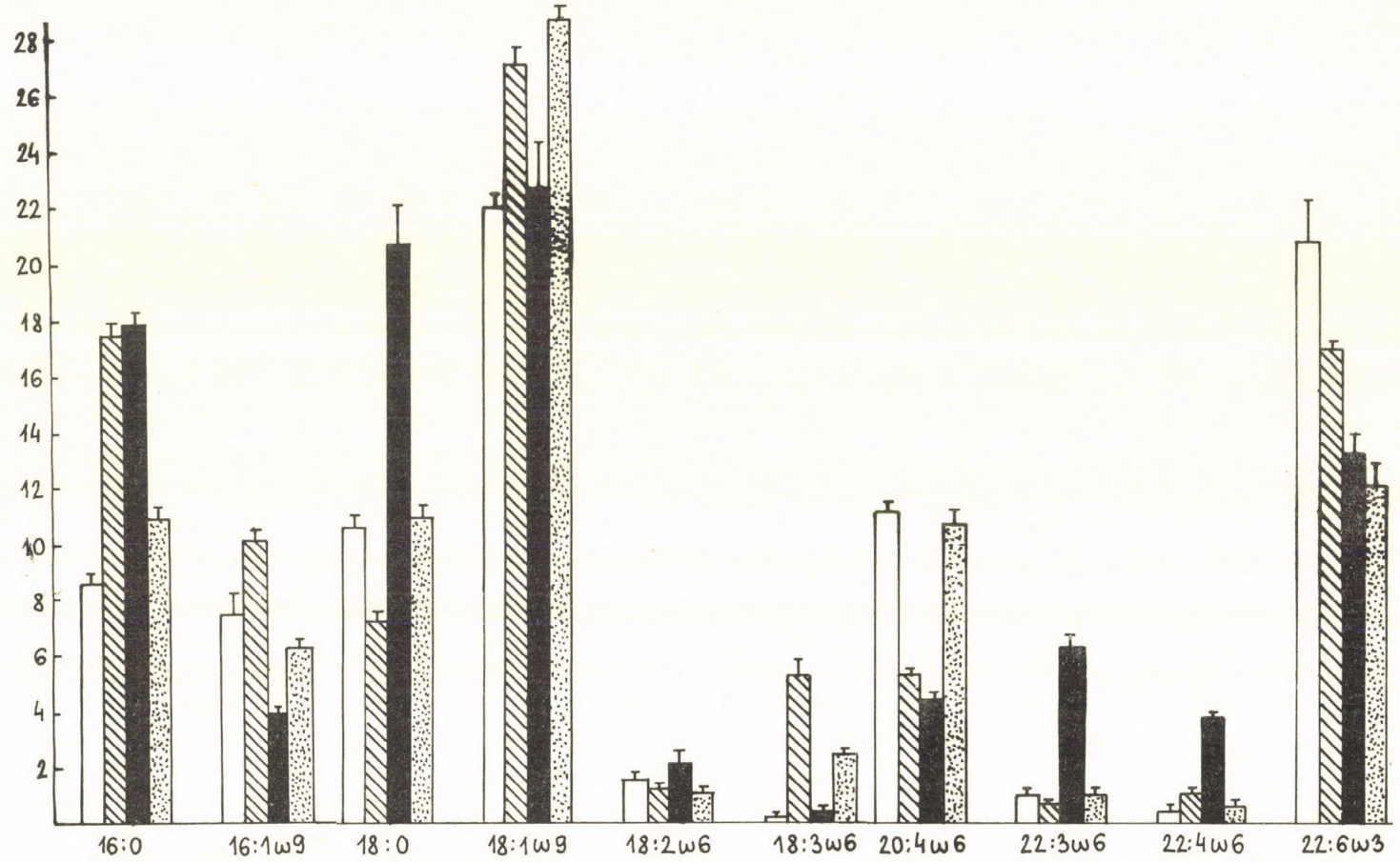

Figure 3. Comparison of the main PE fatty acids composition (weight %) of the brain tissues from different freshwater fishes. (For explanation see Fig. 1.)

Table III. Levels of fatty acid components in common carp lipid fractions

Fatty acids	PE	PC	T PL	TG	TL
16 : 0	8.34 ±0.30	23.38 ±0.33	18.52 ±1.90	18.72 ±1.20	19.04 ±1.20
16 : 1ω7	7.27 ±1.10	9.42 ±0.01	11.20 ±0.40	7.30 ±0.20	9.31 ±0.30
16 : 2ω7	5.24 ±0.30	0.50 ±0.03	0.11 ±0.02	0.76 ±0.14	0.93 ±0.03
16 : 3ω3	2.90 ±0.14	0.28 ±0.06	-	0.13 ±0.10	-
18 : 0	10.41 ±0.90	9.93 ±2.70	7.63 ±0.30	4.03 ±0.10	7.49 ±0.45
18 : 1ω9	21.69 ±0.70	25.10 ±0.01	23.87 ±0.10	35.50 ±1.90	26.10 ±1.40
18 : 2ω6	1.20 ±0.40	0.81 ±0.04	0.81 ±0.06	6.71 ±0.14	4.20 ±0.30
18 : 3ω6	0.10 ±0.05	0.12 ±0.20	-	0.64 ±0.02	0.10 ±0.04
18 : 3ω3	2.80 ±0.10	2.80 ±0.05	2.48 ±0.10	2.56 ±0.10	2.42 ±0.20
20 : 2ω6	0.15 ±0.10	0.19 ±0.04	0.60 ±0.05	0.20 ±0.02	1.05 ±0.04
20 : 3ω6	-	0.30 ±0.03	0.52 ±0.03	0.30 ±0.04	0.68 ±0.06
20 : 4ω6	11.10 ±0.50	5.72 ±0.33	5.25 ±0.24	3.30 ±0.32	6.37 ±1.10
20 : 5ω3	0.24 ±0.10	0.45 ±0.20	0.55 ±0.06	0.72 ±0.10	0.55 ±0.11
22 : 1ω9	0.36 ±0.20	2.81 ±0.14	1.32 ±0.20	1.46 ±0.31	0.49 ±0.12
22 : 3ω6	1.10 ±0.03	0.46 ±0.05	0.42 ±0.07	4.94 ±0.31	1.01 ±0.04
22 : 4ω6	0.49 ±0.10	0.54 ±0.05	5.10 ±1.02	0.52 ±0.10	4.50 ±0.30
22 : 5ω6	0.64 ±0.09	0.45 ±0.02	5.59 ±0.30	0.52 ±0.08	2.75 ±0.09
22 : 5ω3	1.29 ±0.21	-	0.41 ±0.05	-	0.67 ±0.03
22 : 6ω3	20.71 ±1.90	13.74 ±0.84	12.31 ±1.40	8.19 ±1.20	10.46 ±1.30

Table IV. Fatty acid composition of crusian carp lipid fractions

Fatty acids	PE	PC	TPL	TG	TL
16 : 0	17.26 ±1.20	21.43 ±0.50	15.13 ±0.30	15.53 ±2.40	18.71 ±1.20
16 : 1ω7	9.77 ±0.52	11.57 ±0.55	12.61 ±0.64	8.27 ±0.20	10.39 ±0.91
16 : 2ω7	-	-	-	0.73 ±0.05	0.74 ±0.14
16 : 3ω3	0.71 ±0.01	0.30 ±0.03	1.34 ±0.03	2.34 ±0.30	2.24 ±0.20
18 : 0	7.13 ±0.90	8.48 ±1.60	10.22 ±0.61	20.63 ±3.20	11.34 ±1.30
18 : 1ω9	24.70 ±1.50	20.42 ±0.52	25.66 ±2.70	27.73 ±1.30	22.53 ±2.60
18 : 2ω6	1.10 ±0.03	0.94 ±0.10	2.80 ±0.30	2.75 ±0.02	4.10 ±0.24
18 : 3ω6	5.20 ±0.71	0.73 ±0.04	0.62 ±0.25	4.94 ±0.54	0.12 ±0.10
18 : 3ω3	0.77 ±0.10	2.11 ±0.02	2.34 ±0.25	1.09 ±0.20	2.61 ±0.30
20 : 2ω6	2.66 ±0.10	0.61 ±0.10	0.43 ±0.09	0.42 ±0.15	0.37 ±0.05
20 : 3ω6	2.21 ±0.20	-	0.76 ±0.06	1.42 ±0.20	0.71 ±0.10
20 : 4ω6	5.31 ±0.52	3.40 ±0.14	5.11 ±0.75	2.75 ±0.25	5.65 ±0.40
20 : 5ω3	0.30 ±0.03	-	0.43 ±0.09	0.32 ±0.05	0.25 ±0.02
22 : 1ω9	1.12 ±0.03	1.76 ±0.10	1.36 ±0.30	0.96 ±0.05	-
22 : 3ω6	0.74 ±0.12	1.40 ±0.05	0.67 ±0.08	2.78 ±0.40	3.29 ±1.30
22 : 4ω6	1.10 ±0.10	0.81 ±0.01	2.61 ±0.06	-	0.51 ±0.11
22 : 5ω6	1.08 ±0.11	0.81 ±0.01	2.61 ±0.06	-	0.51 ±0.07
22 : 5ω3	0.43 ±0.02	-	0.62 ±0.04	-	0.51 ±0.07
22 : 6ω3	16.58 ±0.69	22.32 ±0.60	12.42 ±1.03	2.70 ±0.33	11.29 ±0.94

Table V. Fatty acid components of silver carp lipid fractions

Fatty acids	PE	PC	TPL	TG	TL
16 : 0	17.61 ±1.10	19.31 ±0.30	22.10 ±2.80	28.18 ±0.62	15.03 ±1.50
16 : 1ω7	3.51 ±0.13	8.29 ±1.50	7.98 ±0.30	7.20 ±0.04	9.54 ±0.84
16 : 2ω7	0.05 ±0.01	0.59 ±0.11	0.87 ±0.02	0.10 ±0.01	1.26 ±0.12
16 : 3ω3	-	1.34 ±0.28	-	1.86 ±0.54	0.76 ±0.05
18 : 0	20.40 ±2.80	9.38 ±1.30	14.76 ±0.31	13.85 ±0.80	8.78 ±0.40
18 : 1ω9	22.50 ±2.49	26.83 ±1.50	18.22 ±0.21	16.38 ±1.15	22.82 ±0.90
18 : 2ω6	2.03 ±0.01	0.33 ±0.15	2.43 ±0.20	2.05 ±0.01	0.59 ±0.10
18 : 3ω6	0.10 ±0.01	1.30 ±0.07	4.20 ±0.40	4.10 ±0.10	0.26 ±0.10
18 : 3ω3	0.10 ±0.01	2.18 ±0.11	0.99 ±0.14	-	1.39 ±0.20
20 : 2ω6	0.07 ±0.01	1.13 ±0.04	2.40 ±0.30	0.78 ±0.01	1.10 ±0.10
20 : 3ω6	0.20 ±0.01	1.05 ±0.01	1.84 ±0.20	0.75 ±0.49	3.23 ±0.50
20 : 4ω6	4.22 ±0.12	2.77 ±0.02	4.20 ±0.30	7.53 ±0.50	5.49 ±0.40
20 : 5ω3	0.72 ±0.10	0.04 ±0.01	0.92 ±0.10	0.20 ±0.01	1.48 ±0.27
22 : 1ω9	1.04 ±0.10	1.90 ±0.01	2.50 ±0.04	3.56 ±0.33	2.49 ±0.20
22 : 3ω6	6.30 ±0.25	1.60 ±0.01	-	-	0.86 ±0.12
22 : 4ω6	3.80 ±0.30	-	3.48 ±0.40	-	3.40 ±0.26
22 : 5ω6	3.80 ±0.30	0.24 ±0.15	0.75 ±0.01	-	1.06 ±0.05
22 : 5ω3	0.17 ±0.01	0.44 ±0.03	0.33 ±0.03	-	0.76 ±0.09
22 : 6ω3	13.11 ±1.80	16.48 ±2.60	11.79 ±0.30	6.30 ±0.64	17.93 ±0.23

Table VI. Fatty acid of grass carp lipid fractions

Fatty acids	PE	PC	TPL	TG	TL
16 : 0	10.67 ±0.72	23.85 ±0.65	19.47 ±0.50	22.88 ±1.80	17.37 ±2.20
16 : 1ω7	6.00 ±0.21	6.80 ±1.10	8.27 ±0.80	6.36 ±0.02	7.79 ±1.10
16 : 2ω7	3.62 ±1.20	0.61 ±0.01	0.86 ±0.04	3.10 ±0.77	0.15 ±0.01
16 : 3ω3	-	-	-	1.43 ±0.13	1.89 ±0.21
18 : 0	10.61 ±1.02	9.66 ±0.23	9.50 ±0.40	10.88 ±0.11	10.02 ±0.43
18 : 1ω9	28.53 ±0.13	27.76 ±1.03	27.91 ±2.70	12.22 ±3.90	23.48 ±2.40
18 : 2ω6	0.85 ±0.04	0.72 ±0.03	0.87 ±0.04	2.30 ±0.10	1.01 ±0.10
18 : 3ω6	2.37 ±0.10	1.59 ±0.11	0.10 ±0.01	8.73 ±1.50	0.10 ±0.01
18 : 3ω3	0.10 ±0.04	0.44 ±0.01	1.74 ±0.04	0.38 ±0.04	1.28 ±0.12
20 : 2ω6	0.63 ±0.04	0.54 ±0.02	0.20 ±0.03	0.10 ±0.01	0.60 ±0.10
20 : 3ω6	0.64 ±0.04	0.87 ±0.30	0.72 ±0.03	1.61 ±0.03	0.73 ±0.10
20 : 4ω6	10.67 ±0.72	5.45 ±0.71	4.94 ±0.40	5.75 ±1.50	5.69 ±1.60
20 : 5ω3	0.35 ±0.03	0.54 ±0.30	0.87 ±0.34	0.37 ±0.02	0.33 ±0.01
22 : 1ω9	2.88 ±0.33	1.18 ±0.21	1.50 ±0.10	2.35 ±0.68	2.60 ±0.35
22 : 3ω6	1.30 ±0.94	0.54 ±0.03	0.79 ±0.01	0.61 ±0.03	1.46 ±0.20
22 : 4ω6	0.62 ±0.10	-	5.52 0.60	2.31 ±0.03	9.13 ±0.24
22 : 5ω6	-	2.33 ±0.20	1.33 ±0.13	0.56 ±0.01	1.88 ±0.14
22 : 5ω3	-	0.45 ±0.08	0.17 ±0.52	-	0.71 ±0.10
22 : 6ω3	12.03 ±0.49	14.85 ±0.44	12.98 ±2.80	14.40 ±1.10	16.62 ±1.50

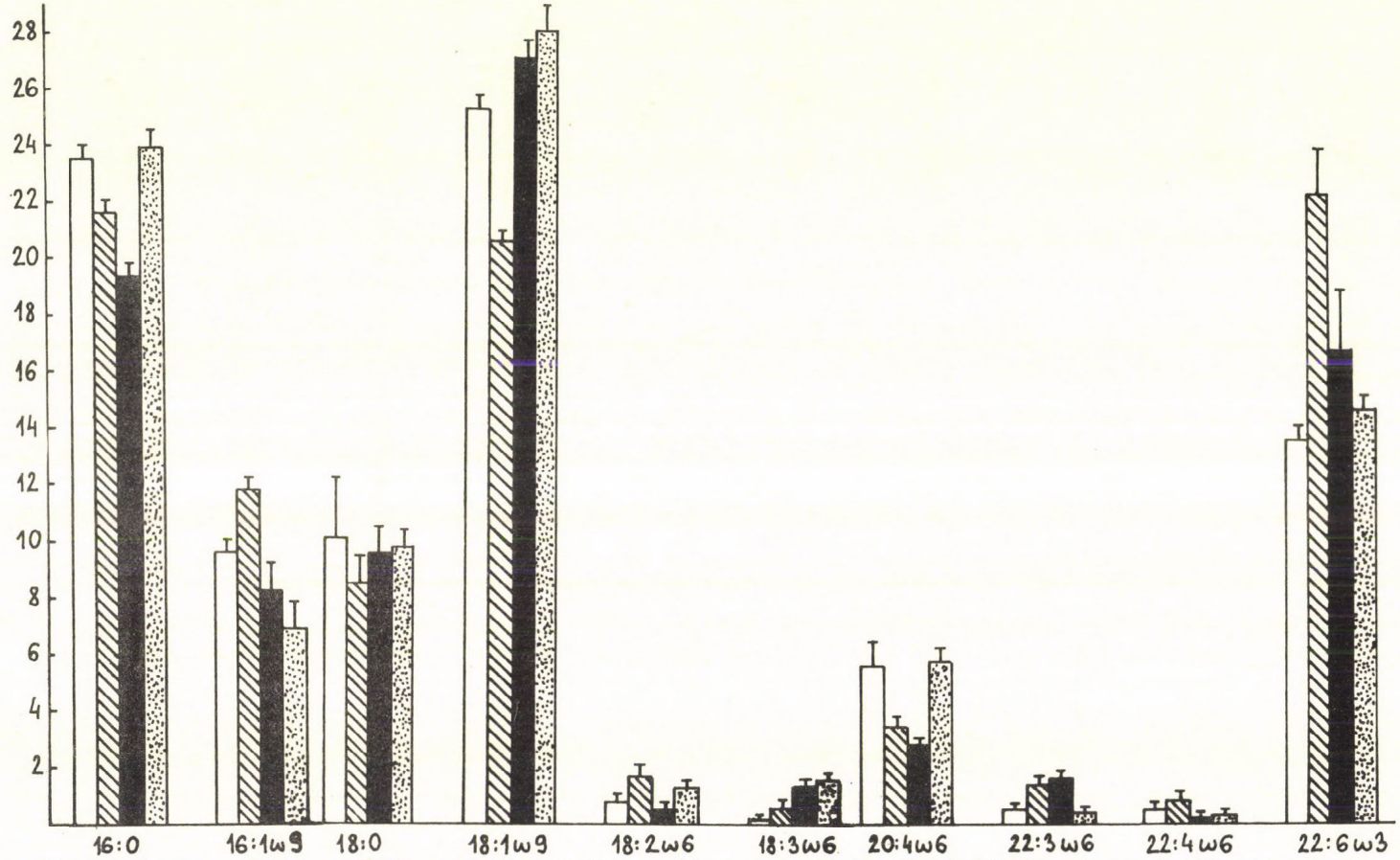

Figure 4. Comparison of the main PC fatty acids composition (weight %) of the brain tissues from different freshwater fishes. (For explanation see Fig. 1.)

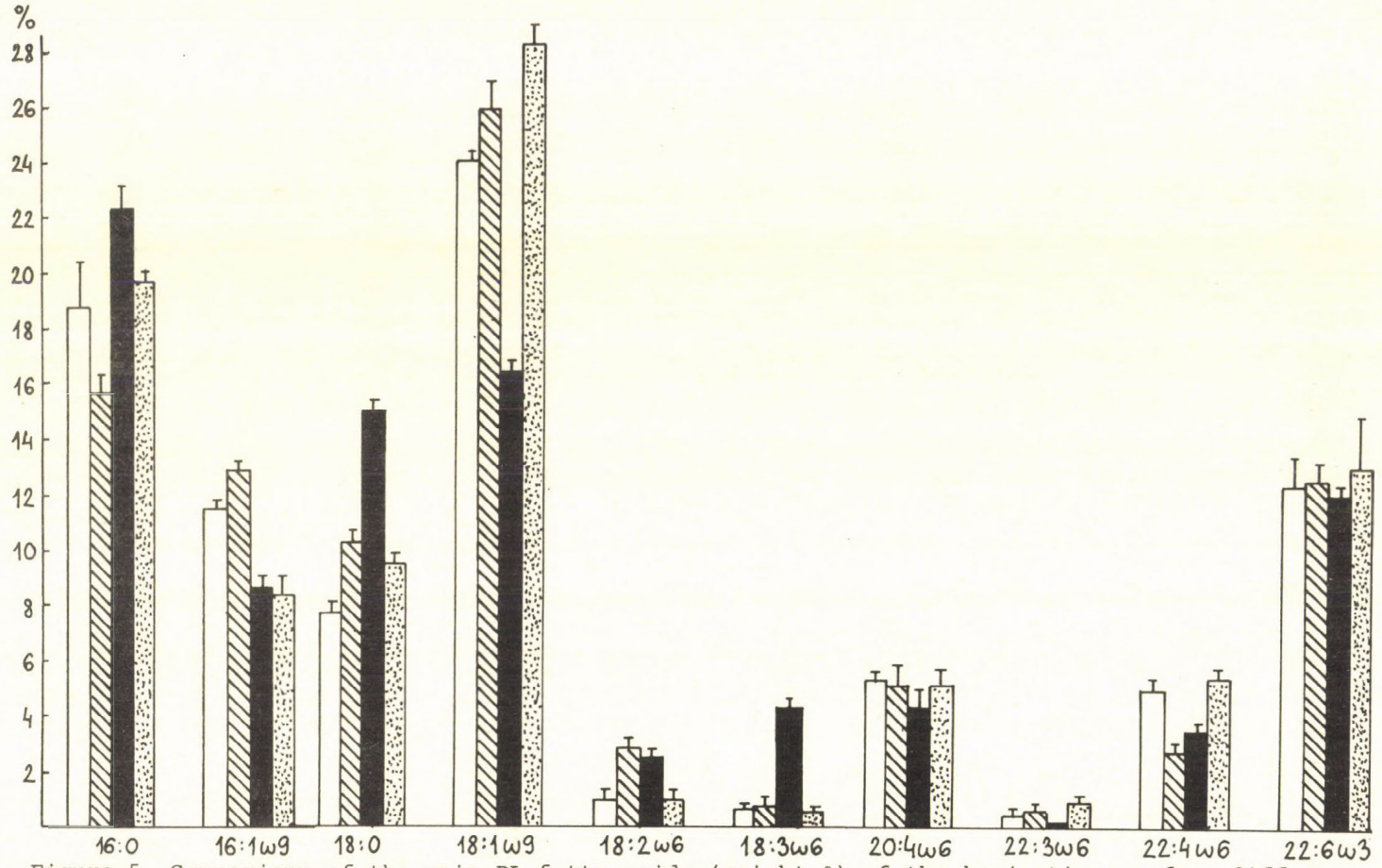

Figure 5. Comparison of the main PL fatty acids (weight %) of the brain tissues from different freshwater fishes. (For explanation see Fig. 1.)

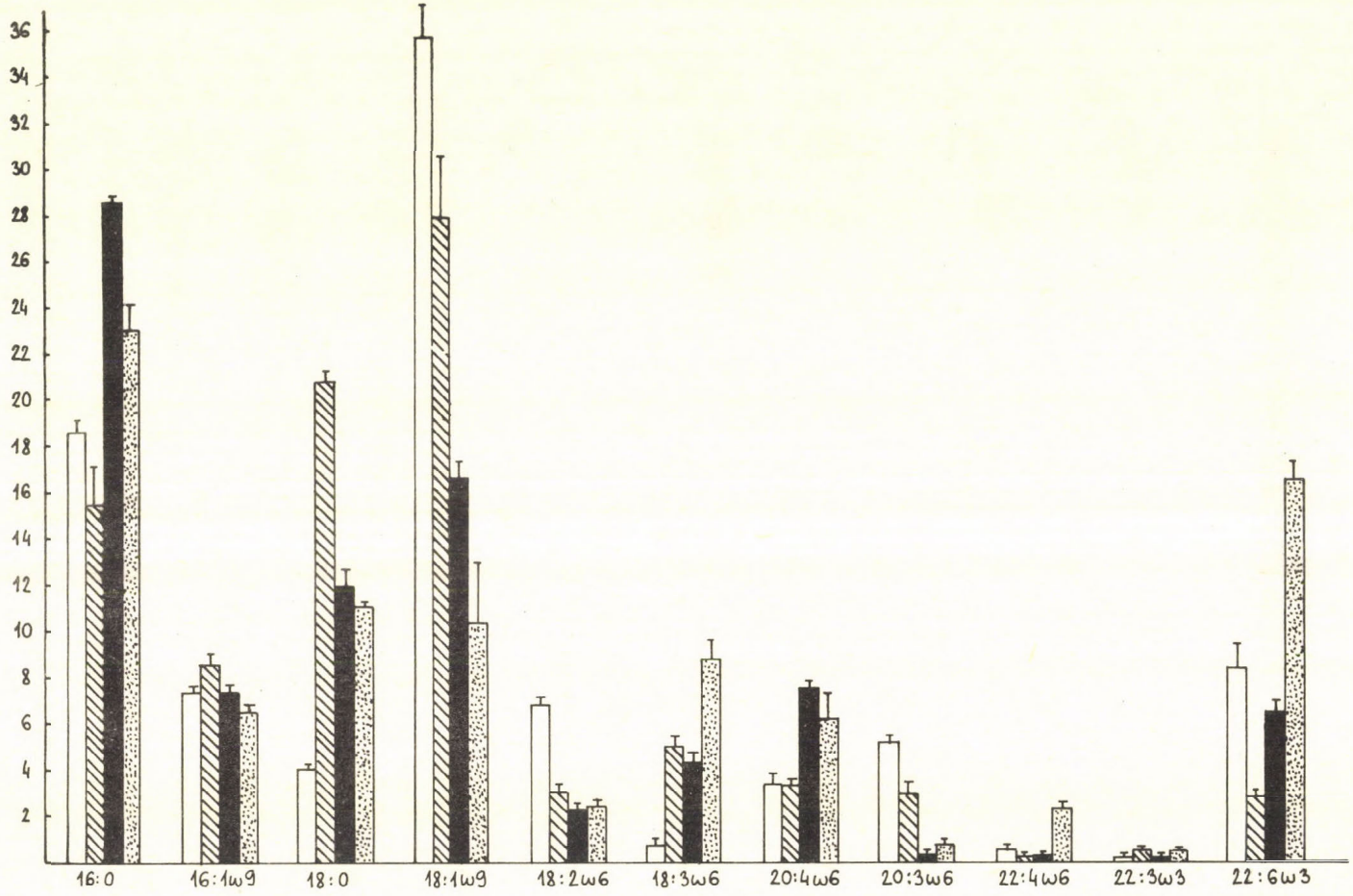

Figure 6. Comparison of the main TG fatty acids (weight %) of the brain tissues from different freshwater fishes. (For explanation see Fig. 1.)

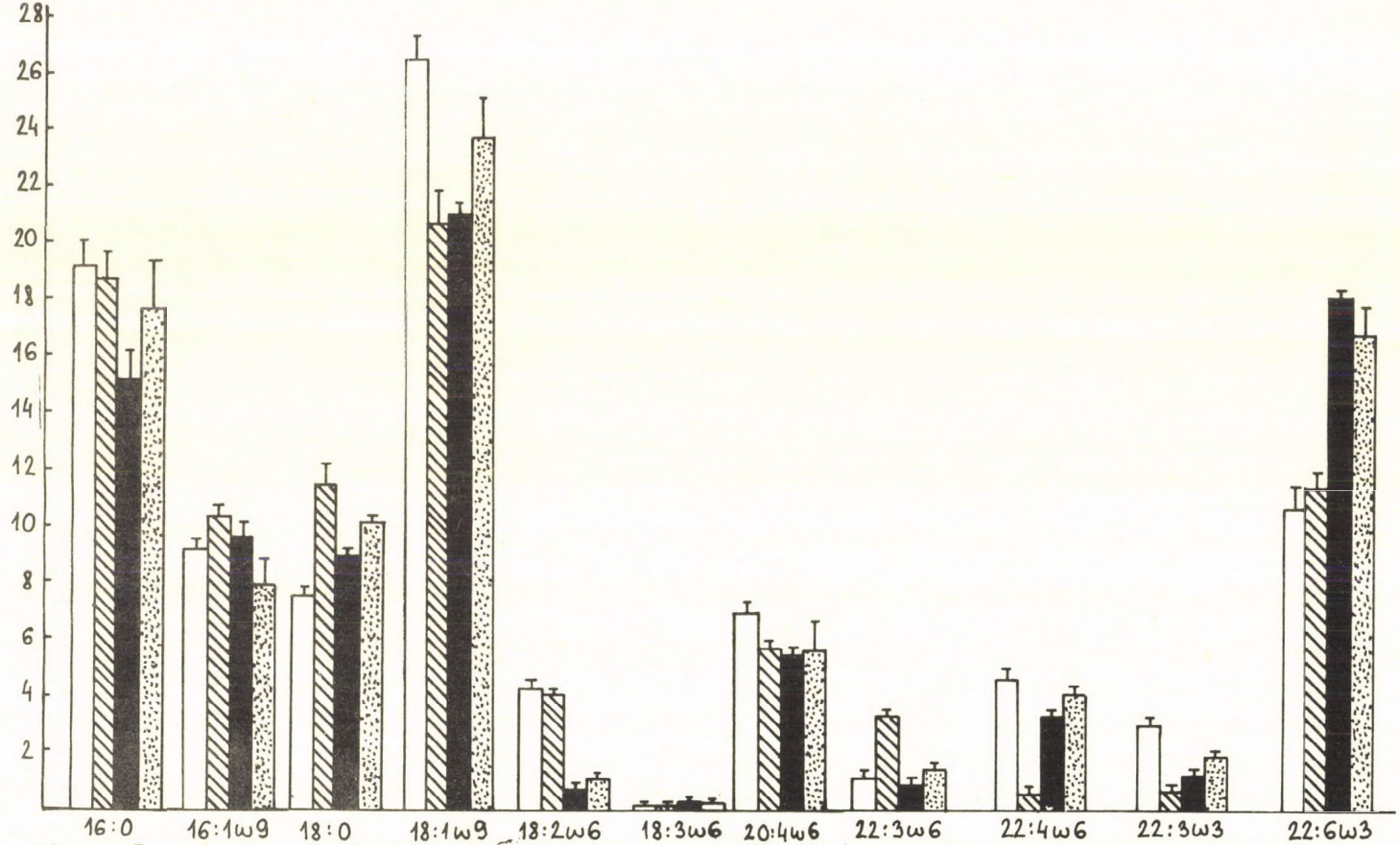

Figure 7. Comparison of the main TL fatty acids (weight %) of the brain tissues from different freshwater fishes. (For explanation see Fig. 1.)

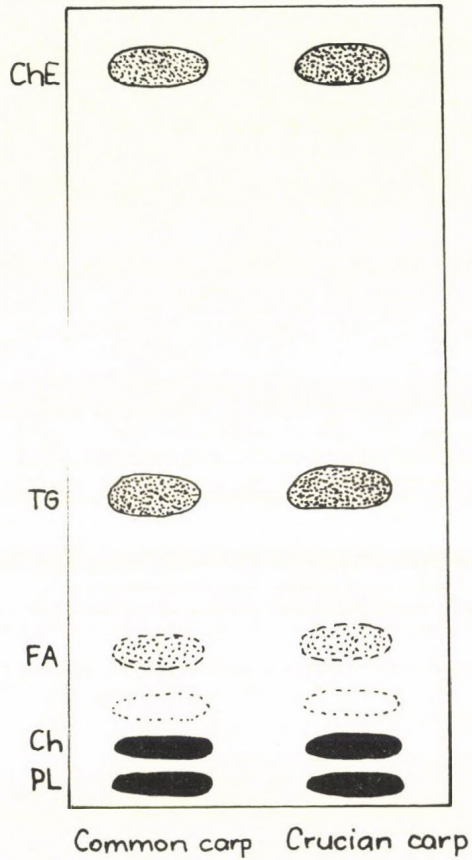

Figure 8. Characteristic one dimensional TLC of the neutral lipids of omnivorous fish brain.

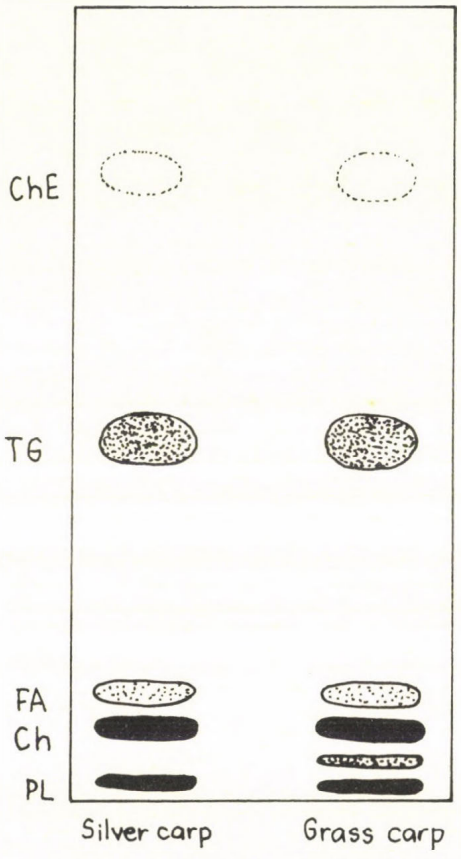

Figure 9. The same lipids as Fig. 8, but for herbivorous fish brain

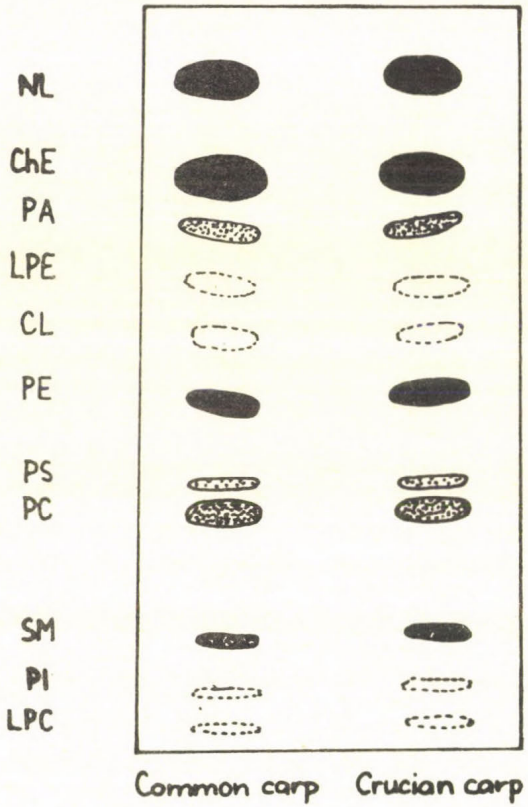

Figure 10. Characteristic one dimensional TLC of the polar lipids of omnivorous fish brain.

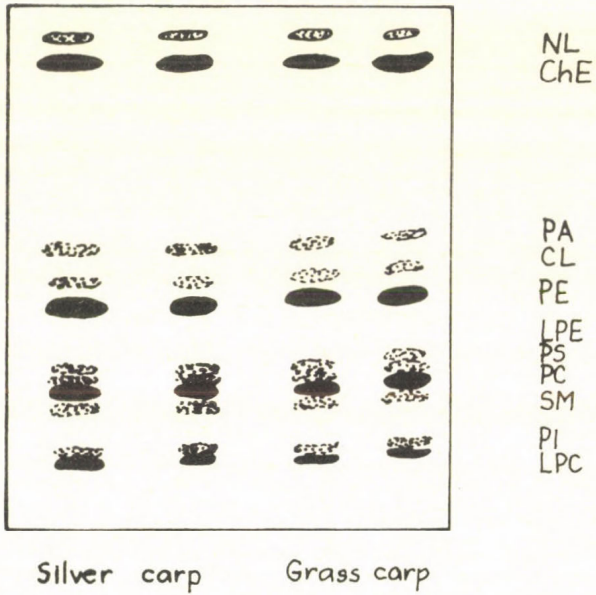

Figure 11. The same lipids as Fig. 10, but for herbivorous fish brain.

Characteristic thin-layer chromatograms are depicted in Figs 8-11.

Figures 8 and 9 show chromatograms of the neutral lipid extracts of the two omnivorous fish and of the two herbivorous fish, respectively. The thin-layer chromatogram of the polar lipid fractions of the omnivorous and herbivorous fish can be seen in Figs 10 and 11, respectively.

DISCUSSION

Differences have been found with respect to the amounts of neutral lipids, polar lipids and the main fatty acid ester fractions in the brain tissues of various fish species and fish with different feeding behaviours. The differences found here are partially connected with the mode of feeding.

The basic results of this investigation are summarized in Tables VII and VIII.

Table VII. Characteristic lipid fractions of fishes

Lipid fractions		Omnivorous fish	Herbivorous fish
Neutral lipids			
	ChE	+	-
	TG	-	+
	FA	+	-
	PL	+	-
Polar lipids			
	NL	-	+*
	ChE	-	+
	PE	+	-
	PC	+	-

The symbol + in Tables VII and VIII means that the component in question is in larger, and in most cases in significantly larger amount.

From the data presented here and from references (11-14), it is clear that the feeding conditions have a basic influence on the lipid constitution of the brain of fish. Generally:
(a) In the extracts of the brains of the omnivorous fish, the amounts of polar lipids, structural lipids and mono- or poly-

Table VIII. Lipid fractions occurring in significant amount

Lipid fractions	Characteristic fatty acids	Omnivorous fish	Herbivorous fish
PE	22:6 ω 3	+	-
PC	22:6 ω 3	+	-
	18:1 ω 9	-	-
TPL	16:0	-	+
	16:1 ω 9	+	-
TG	16:0	-	+
	18:1 ω 9	+	-
TL	22:6 ω 3	-	+
	18:2 ω 6	+	-

unsaturated lipids are higher than in those of the herbivorous fish. The amounts of singly and multiply unsaturated fatty acids exceed the total amount of the saturated fatty acids. In the TPL fraction, through the increase of the 16:1 ω 9 fatty acid, this ratio is further shifted in the direction of unsaturated fatty acids in the case of the omnivorous fish.
(b) In the evaluation of the TL fraction, the amounts of oleic acid are alike; However, in the cases of the herbivorous fish there is a definite correlation between the plant origin of the food and the significantly high content of the 22:6 ω 3 fatty acid. The observations of Farkas et al. /3/ have made clear the correlations between the lipogenesis in the liver of the fish and the carbohydrates taken up in the food. A similar consideration is probably valid for the lipids of the brain tissue of fish.

ABBREVIATIONS

Ch: cholesterol
ChE: cholesterol esters
CL: cardiolipin
LPA: lysophosphatidic acid
LPC: lysophosphatidyl choline
LPE: lysophosphatidyl ethanolamine
PA: phosphatidic acid
PC: phosphatidyl choline
PE: phosphatidyl ethanolamine
PI: phosphatidyl inositol
PL: phospholipid
PS: phosphatidyl serine
TPL: total phospholipid
TG: triglycerate
TL: total lipid

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SEPARATION OF PROSTAGLANDIN INTERMEDIATES BY COLUMN LIQUID CHROMATOGRAPHY

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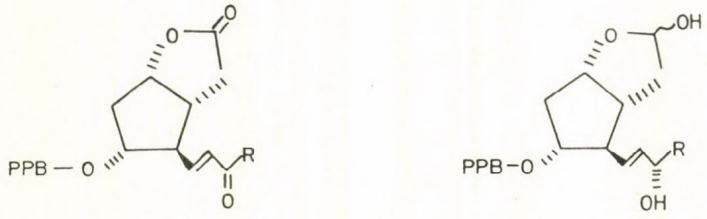
INTRODUCTION

Until now, relatively little work had been carried out on the theoretical aspects of selecting the best sorbent for a particular separation /1, 2/. In the present paper various aluminas are tested for the isolation of two different $PGF_{2\alpha}$ intermediates from their industrial reaction mixtures. Formulas of the two intermediates are shown in Fig. 1.



PG-VI PG-VII

Fig. 1. Compounds to be separated by chromatography

Both of the mixtures contained additional 6-8 components. The desired product should be separated even from its stereo-isomer, which can be found among these compounds in the PG-VII mixture.

The tested aluminas were different in pore size, particle size, specific surface area and pH.

Fractions taken from model columns were analyzed by TLC and weighing.

MATERIALS AND METHODS

a) Adsorbents

Special products of Almásfüzitő Alumina Plant were mainly used for the investigations. They were compared with some types of Woelm aluminas and the Merck silica, which is widely used in the industry. The investigated adsorbents were acidic, neutral and basic, all of them with wide and narrow pore size. Their other morphological properties were also different, according to the pore structure. Properties of the investigated adsorbents are summarized in the Table 1.

The following equipment and methods were used for the determination of the tabulated data.

Universal pH-meter, Type: OP-204/1 was used for the determination of the pH after filtration of the suspensions (10 % adsorbent in water). The specific surface areas were measured by the BET method using the Micrometrics 2205 Surface Area Analyser. Determination of the average particle size and the particle size distribution was carried out in a Coulter Counter Model TA II. The amount of particles above 45 μm was determined with an Alpin air pump. The porosity was determined by Micrometrics Acu Sorb 2100 E equipment, measuring the adsorption and desorption isotherms.

b) Mixtures to be separated

The PG-VI and PG-VII compounds are successive intermediates of the multistep synthesis of $PGF_{2\alpha}$. Fig. 2 shows the thin-layer chromatograms of the reaction mixtures. On the TLC chromatogram of the PG-VI mixture there is a spot, closely under the spot of the desired product. This is an unsaturated compound, which is formed during the decomposition of PG-VI, as it can be seen in the Fig. 3.

Table 1. Characterization of the adsorbents

Adsorbent	pH	Specific surface area m^2/g	Particle size μm	Pore size nm	Pore volume cm^3/g	Phases X-ray diffraction
TK-1	8.8	238	76	7.4	0.182	γ boehmit(α)
F-2	8.3	115	61	10.7	0.260	γ χ boehmit (gibbsit)
TK-3	6.4	162	77	9.9	0.272	γ χ κ(boehmit)
TK-4	4.5	139	77	10.2	0.269	γ χ κ(boehmit)
TK-5	6.1	304	83	4.8	0.284	γ (boehmit)
TK-6	4.0	311	80	4.6	0.295	γ (boehmit)
TK-7	9.0	308	61	4.8	0.192	γ χ (boehmit)
WA-18*	5.5	117	24	8.3	0.279	γ χ κ
WN-18*	6.9	130	25	7.7	0.258	γ χ κ
WB-18*	8.6	139	26	8.2	0.264	γ χ κ
Silica	6.9	482	84	8.2	0.795	amorphous silic acid

*For HPLC

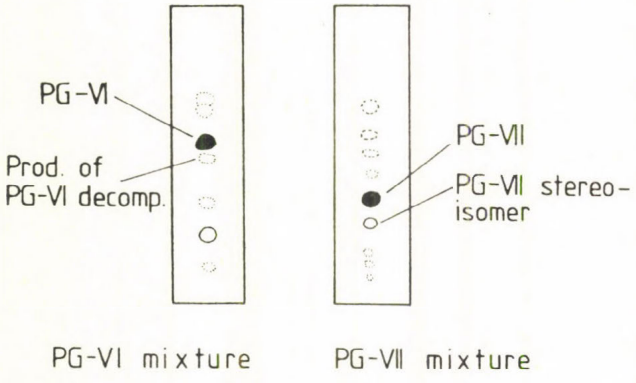


Fig. 2. Thin layer chromatograms of the reaction mixtures, obtained on silica plates

In the reaction mixture of PG-VII two stereoisomers of PG-VII can be found, which should also be separated. The desired product, the S-configuration alcohol (Fig. 1) is the upper spot of the stereoisomers in the TLC chromatogram.

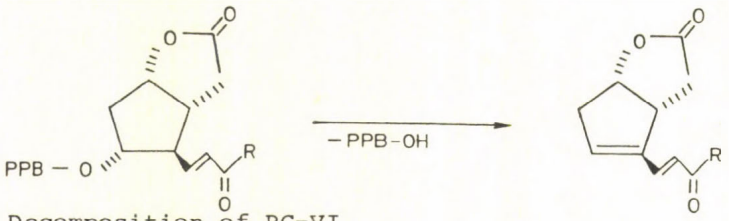


Fig. 3. Decomposition of PG-VI

c) Method

The L/d ratio of the columns prepared from the adsorbents suspended in the selected eluent varied in the range of 15-20. The ratio of the mass of adsorbent/adsorbate was equal to 100, but in the course of the loading tests we applied with success even a ratio of 30. Qualitative analyses of fractions of same volume were made by TLC, quantitative analyses by weighing after evaporation of the solvent.

RESULTS AND DISCUSSION

a) The mobile phase

Experiments were carried out by TLC on alumina plates (DC Fertigplatten Aluminiumoxid 60 F_{254} Typ E, Art. 5731) for the selection of the mobile phase. Several solvent combinations proved to be suitable as mobile phases for the separation. The mixtures applied in the series of tests for the separation of both reaction mixtures consisted of benzene and ethyl acetate. The optimum composition of the mixture highly depends on the nature of the compounds to be separated and the activity and pH of the adsorbents.

b) The role of pH

According to the experiments PG-VI is adsorbed more weakly and PG-VII is absorbed more strongly on alumina than on silica. As can be seen in Fig. 4 the strength of the adsorption forces is increasing with increasing pH of the alumina in the case of PG-VII. Fig. 4 shows the TLC chromatograms of the fractions of

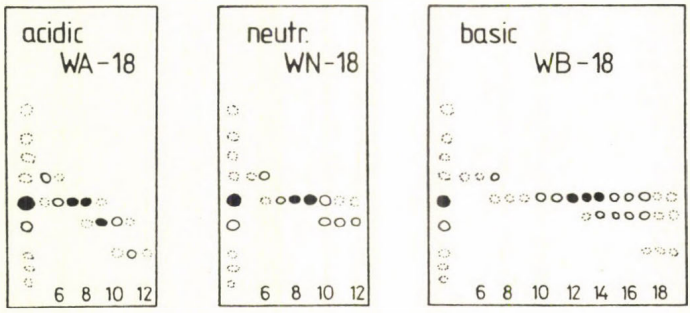


Fig. 4. TLC analysis of the fractions of the PG-VII reaction mixture on acidic, neutral and basic aluminas

the PG-VII mixture taken from columns containing acidic, neutral or basic alumina. One can see in the figure that the chromatogram is getting wider and the spots gradually overlap as the pH is increased. This is the reason why primarily acidic, or by chance neutral aluminas can be proposed for the isolation of PG-VII.

In the case of PG-VI only acidic aluminas were suitable for the separation. Adsorbents with basic or neutral character cannot be applied at all, because the otherwise slight decomposition of PG-VI (Fig. 3) is highly increased. Fig. 5 shows that adsorbents with increasing pH cause an increase in the quantity of the decomposition products at the expense of PG-VI. Majority of PG-VI decomposed in the fractions when using more active and basic columns.

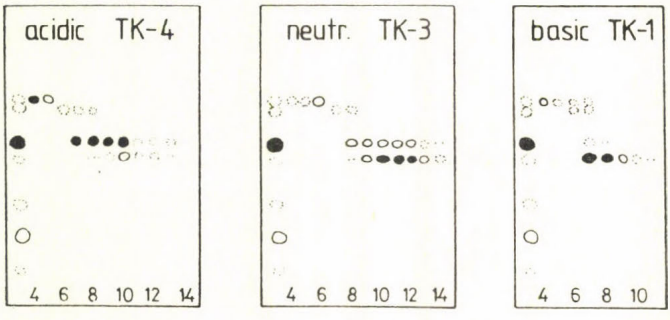


Fig. 5. TLC analysis of the fractions of the PG-VI reaction mixture on acidic, neutral and basic aluminas

c) Activity of the adsorbent

Adsorbents with moderate activity are frequently preferred to high activity adsorbents /2/. In the case of recent investigations the efficiency of the separation has an optimum as a function of the activity. This is shown in Fig. 6. Chromatograms of the separation of PG-VII are shown on acidic alumina having a specific surface area of 139 m^2/g, with the addition of 5 % water.

Reducing the activity during the separation of PG-VI has the additional advantage that in this case the decomposition of PG-VI (Fig. 3) significantly decreases, mainly on the acidic adsorbents.

d) Effect of porosity and specific surface area

It can be seen in Table 1 that the tested aluminas of the Almásfüzitő Alumina Plant can be divided into two groups by their porosity having pore diameters of about 5 nm and 10 nm, with nearly identical particle sizes (average about 75 µm). Due to the different porosity their specific surface area are about 300 and 150 m^2/g, respectively.

The separations were best for both reaction mixtures on the adsorbents with large average pore diameter, in spite of their smaller specific surface area. At the same column loading the separation was significantly less efficient on the aluminas with larger specific surface area: the peaks were widening and overlapping.This fact is due to the narrow pores, because the large adsorbate molecules are diffusing deeper into these pores and spend more time there, and therefore, they travel slower along the column.

However, the numerical data referring to the porosity do not give any information about the geometry of the pores. There are pores with V form, and the so-called "ink-pot"-form /3/. The latter pores are not accessible for the larger molecules.

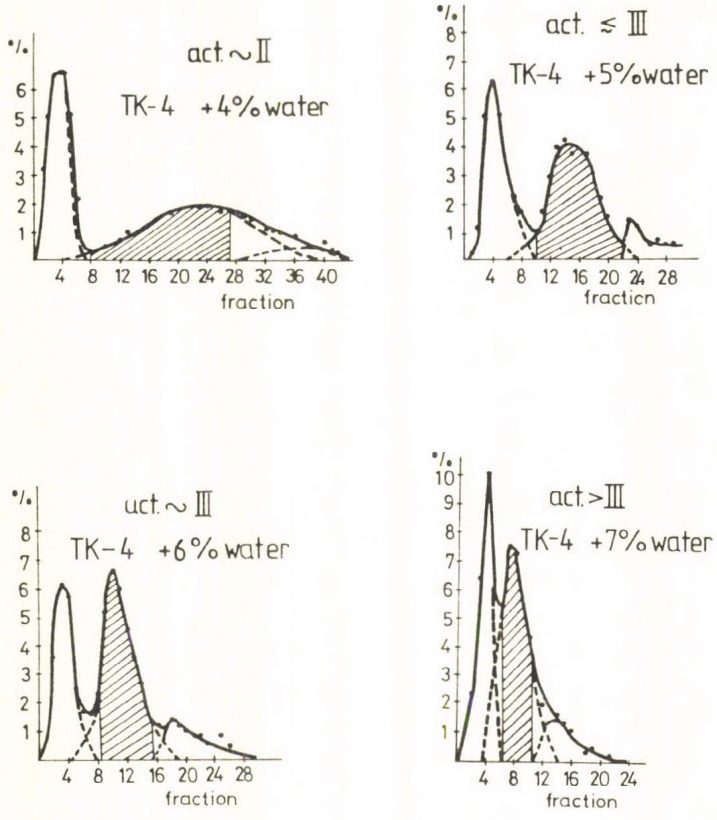


Fig. 6. Chromatograms of the PG-VII reaction mixture on acidic alumina columns with different activities.

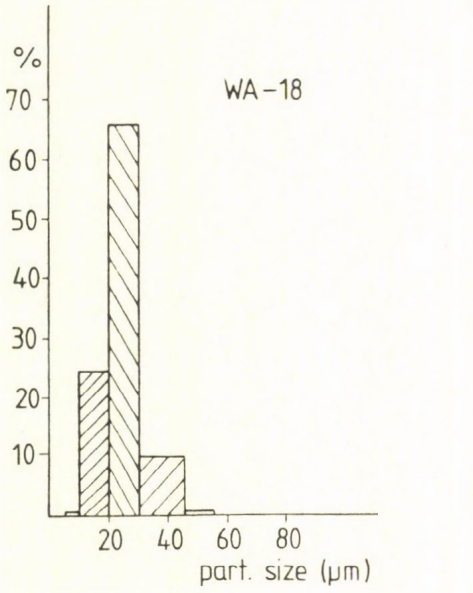
e) Other adsorbents

In the previous part various aluminas having different pH, activity and porosity obtained from the Almásfüzitő Alumina Plant were compared. The WA-18, WN-18 and WB-18 aluminas are special products of Woelm Company, offered for HPLC. Their particular properties are mainly due to the homogenic distribution of their particle size and pore size as it is shown in Figs 7 and 8. The corresponding data of TK-4 -the adsorbent among the Almásfüzitő aluminas most suitable for gravitation columns - are also shown in the same figures. It can be seen that the pore size distribution of TK-4 is as homogenic as that of WA-18, but its particle size distribution is less good. One can see from the TLC analysis (Fig. 9) that the separation of the desired PG-VII isomer is nearly complete on WA-18 alumina.

It can be noted that there is a close relationship between the homogenity and the price of the adsorbent.

In this paper we shall not deal with the morphological properties of Merck silica widely used in the industry. The nature of the adsorption forces on the silica surface covered with hydroxyl groups differs from that of the aluminas. The interaction between the silica and adsorbate takes place mainly by hydrogen bridges. One can find much less hydroxyl groups on the surface of aluminas. These sites behave as Brönsted-acid sites; in addition, the exposed Al-atoms act as Lewis acid sites due to their electron-deficient character. The role of the lattice vacancies of alumina (type of transitional Al_2O_3 phases) in the adsorption is not yet exactly clarified.

These differences in the properties of the surfaces of the adsorbents can give an answer to our earlier results that the carbonyl-group containing PG-VI adsorbs stronger on silica, while the hydroxyl-group containing PG-VII adsorbs stronger on alumina. This difference can be seen in Fig. 10, in the case of the separation of the PG-VI reaction mixture. Benzene-ethyl acetate mixtures were used as the mobile phase. The composition of the eluents was changing as follows increasing their strength:



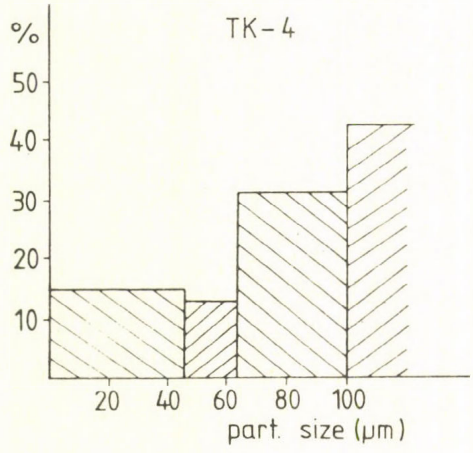
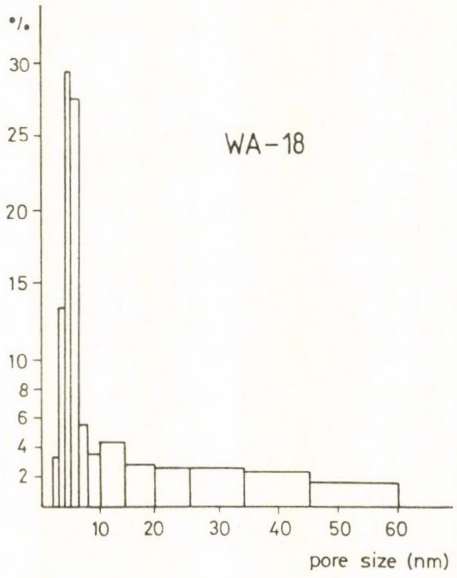


Fig. 7. Particle size distribution of WA-18 and TK-4 aluminas



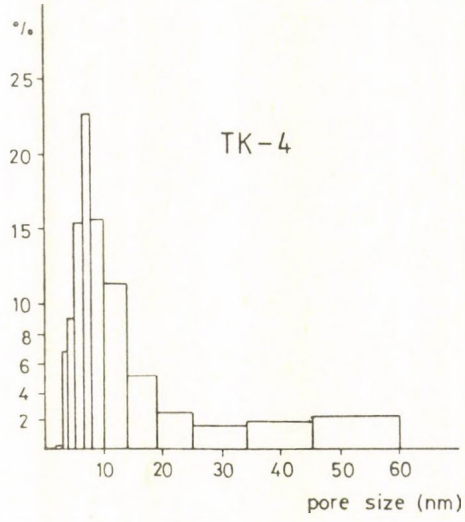


Fig. 8. Pore-size distribution of WA-18 and TK-4 aluminas

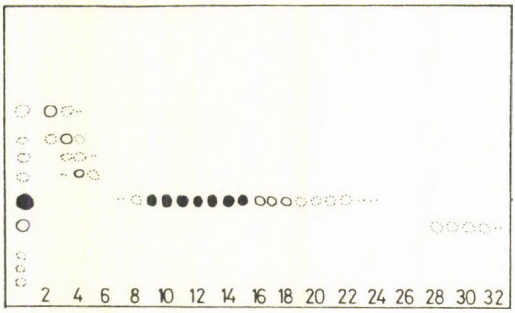
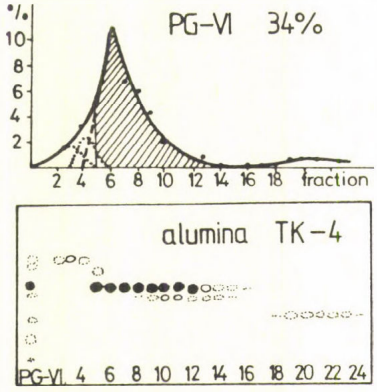


Fig. 9. TLC analysis of the fractions of a PG-VII mixture on the WA-18 column



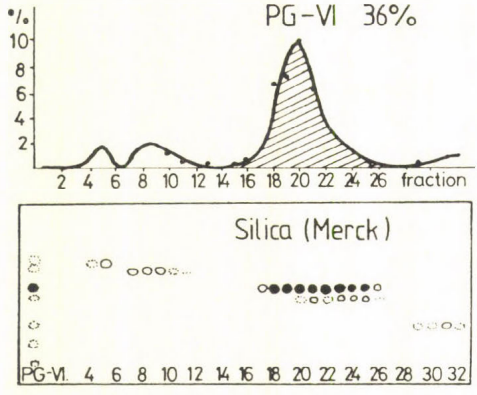


Fig. 10. Comparison of the chromatograms obtained by analyzing the reaction mixture of PG-VI on silica and alumina columns

fractions	benzene	-	ethyl acetate
1-12	14		1
13-22	5		1
after 22	1		1

Columns packed with the same adsorbent volume were loaded with the same amount of adsorbate. It is necessary to state that the mass of a given volume of alumina is twice of that of an identical volume of silica.

The following adsorbent/adsorbate ratios were obtained as minimum values when the separation efficiency was acceptable:

	adsorbent/adsorbate (m/m)	
	PG-VI	PG-VII
Silica	~30	~20
Alumina	~60	~30

CONCLUSIONS

On the basis of the detailed investigations it can be stated that in addition to the widely used silica some types of alumina with special properties can be competitively applied for the separation of some $PGF_{2\alpha}$ intermediates. For PG-VI the most favourable adsorbent proved to be the acidic alumina with wider pores and medium activite (act.$\leq$ III according to Brockmann). More active and more basic aluminas result in the decomposition of PG-VI.

For PG-VII the aluminas with wider pores, either acidic or neutral, can be applied. With increasing pH and activity the adsorption forces are increasing. These factors determine the amount of water to be added for deactivation and the optimum strength of the mobile phase which may consist of mixtures of benzene and ethyl acetate for both intermediates.

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STABILIZATION OF PROSTACYCLIN AND FUROSEMIDE BY CYCLODEXTRIN

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SUMMARY

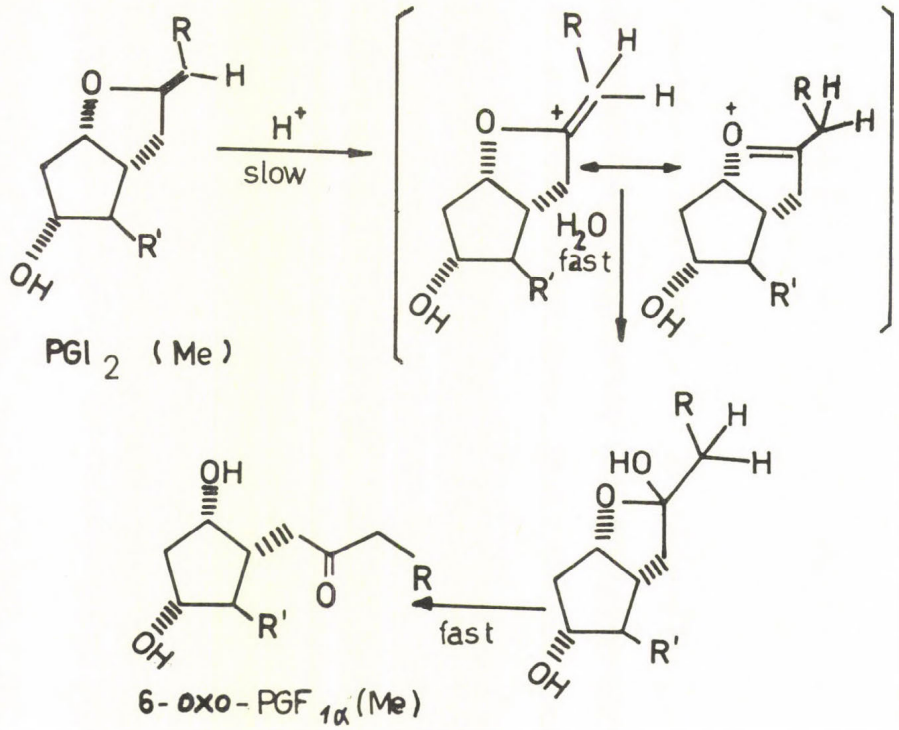
Stability of prostacyclin methyl ester and furosemide has been studied both in solid and dissolved, as well as in free and in cyclodextrin complexed form. The degradation (hydrolysis and light-induced decomposition) has been followed with HPLC. Prostacyclin methyl ester and its decomposition products were well resolved with simple reversed-phase HPLC, while for the separation and determination of furosemide and its photolytic and hydrolytic products an ion-pair reversed-phase HPLC method has been developed. The stabilizing effect of β-cyclodextrin on two drugs has been established.

INTRODUCTION

Cyclodextrins (CD-s) as host molecules may entrap a great variety of molecules forming inclusion compounds. This type of molecular encapsulation generally protect the sensitive substances against decomposition /1/. Prostacyclin methyl ester PGI_2(Me), an effective antiplatelet and cytoprotective agent, is an extremely instable compound: it decomposes rapidly to 6-oxo-$PGF_{1\alpha}$-(Me)/2/ (Scheme 1).

Furosemide (FS, a frequently used diuretic, is sensitive to light-induced decomposition. Its possible degradation products are shown in Scheme 2 /3, 4/.

The stabilizing effect of CD-s on these two drugs was investigated both in an aqueous system and in solid state. High-performance liquid chromatography can be successfully used to follow the degradation of these drugs.



Scheme 1. Hydrolysis of prostacyclin methyl ester /2/

MATERIALS

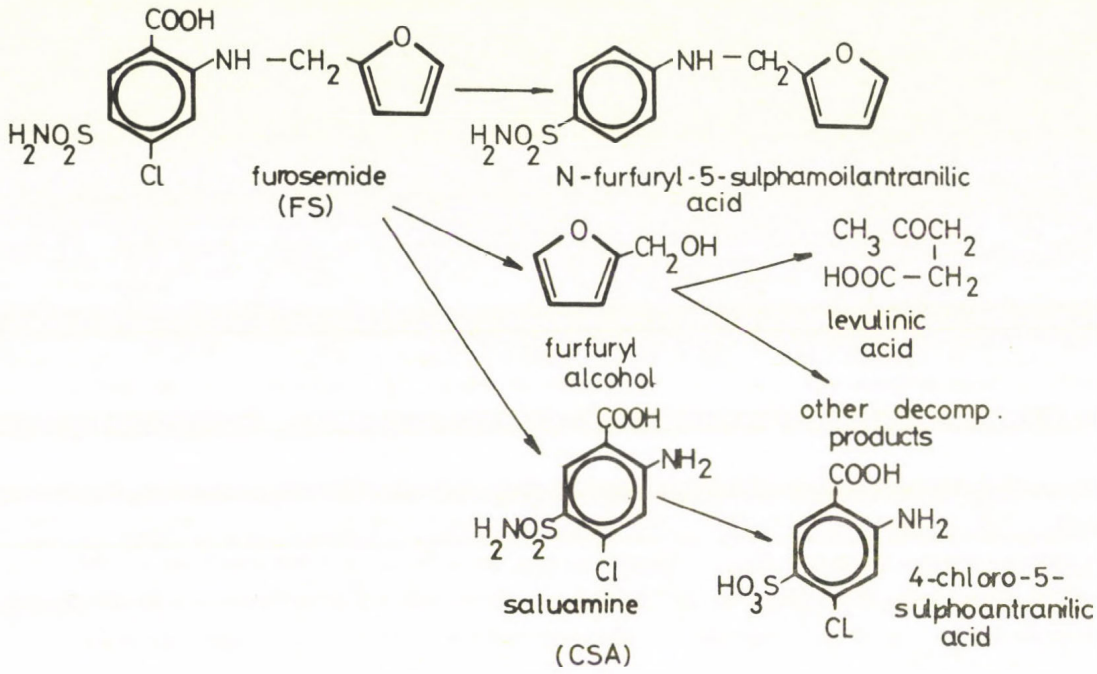
PGI_2Me, furosemide (pharmaceutical grade) and CD-s were products of Chinoin Pharmaceutical Works Ltd., Budapest.

Acetonitrile and methanol were of HPLC grade (Merck, Darmstadt, FRG, LiChrosolv). All other materials were of analytical reagent grade.

METHODS

HPLC apparatus:

Beckman Model 114 Solvent Delivery Module and Model 165 Variable Wavelength Detector, Digint L 80 Integrator (Chinoin Instrument), Radelkis Recorder (type OH-814/1). Ultrasphere ODS columns (Beckman).



Scheme 2. Structures of furosemide and its known photolytic products /3, 4/.

STUDIES OF THE STABILITY OF PGI_2Me

HPLC method:

The chromatograph was operated isocratically at ambient temperature. The instrument was fitted with a 7.5 cm x 4.6 mm I.D. precolumn (particle size: 10 μm), and a 25 cm x 4.6 mm I.D. analytical column (particle size: 5 μm).

The mobile phase consisted of 70:30 (v/v) acetonitrile 0.01 M phosphate buffer pH 7.6; the flow rate was 1.0 ml/min. Detection was carried out at 205 nm.

Preparation of the solid complex:

The PGI_2Me-βCD complex was prepared from a homogeneous solution by freeze-drying /5/. PGI_2Me content: 2.78 %, molar ratio of PGI_2Me:CD = 1:10.

Stability in solid state:

The PGI_2Me-βCD complex samples were stored in sealed vials at room temperature and at $+4^{\circ}C$ for months. Samples were dissolved in 1:1 (v/v) borate buffer (pH 9.2) - ethanol solution, and the PGI_2Me content was determined by HPLC.

Stability in solution:

The PGI_2Me and the PGI_2Me-βCD complex were dissolved in 0.01 M buffers (pH 1.2; 2.6; 5.0; 6.2; 7.6; μ = 0.2) at $37^{\circ}C$; at appropriate time intervals samples were taken and their PGI_2Me content was determined. The initial PGI_2Me concentration was 0.1 mg/ml in each run.

STUDY OF THE STABILITY OF FUROSEMIDE

HPLC method:

Columns: 7.5 cm x 4.6 mm I.D. precolumn (particle size: 5 μm) and 15 cm x 4.6 mm I.D. analytical column (particle size:

5 m). Mobile phase: 38:62 (v/v) methanol - 20 mM KH_2PO_4 (Ph 6.4) containing 0.1% (v/v) tetramethyl ammonium hydroxide as a pairing agent (in isocratic mode). Flow rate 1 ml/min. Detection was carried out at 275 nm.

Preparation of the solid complex:

The solid complex of furosemide with βCD was crystallized from a homogeneous solution. The furosemide content of the complex is 11.5 %. The molar ratio of FS to βCD is 1:2.

Stability in solid state:

Furosemide-βCD mechanical mixture and their complex were subjected to day-light at room temperature (in Petri dishes, covered by glass-plate, in 0.5 mm layers). Samples were dissolved in 1:1 (v/v) 0.01 M NaOH/methanol mixture, and the furosemide content was determined by HPLC.

Stability in solution:

The photochemical degradation of furosemide was studied in 0.01 M NaOH solution. Initial concentration of furosemide was 0.02 mg/ml. Molar ratios of FS and CD-s were 1:10. The samples were irradiated at 254 nm (under an analytical UV-lamp) for 3 hours at 25°C. The concentration of FS was determined by HPLC.

RESULTS AND DISCUSSION

Prostacyclin methyl ester

The influence of CD-s on the rate of hydrolysis of PGI_2 and PGI_2Me was studied by Uekama /6/ monitoring the decomposition by UV-spectrophotometry. The reversed-phase HPLC method applied in our investigations more selectively resolves the PGI_2Me from its hydrolytic product and impurities (Fig. 1).

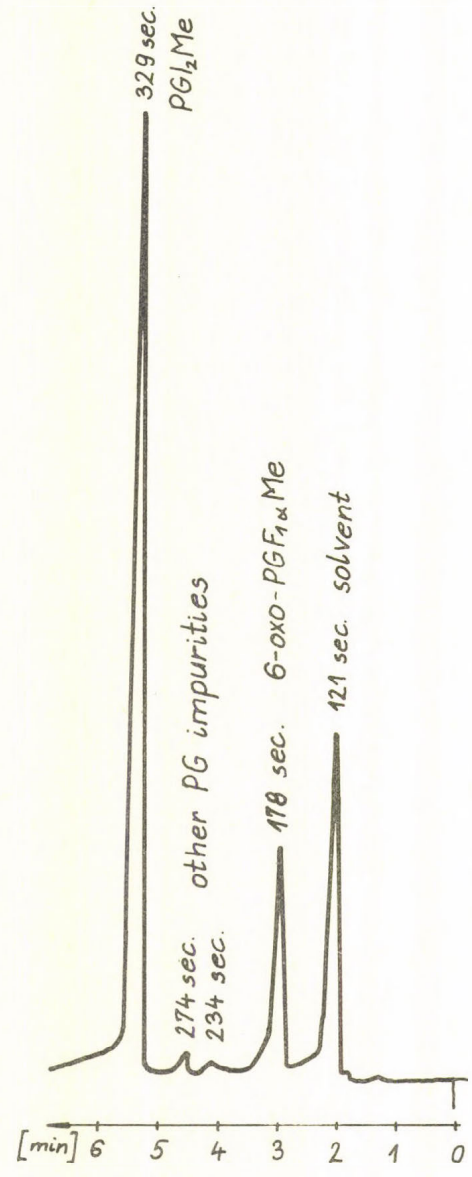


Fig. 1. Typical chromatogram of PGI_2Me and its decomposition product (6-oxo-$PGF_{1\alpha}Me$) and impurities. Chromatographic conditions as in the text.

Table I. Rate constants and half life times of PGI_2Me and PGI_2Me-βCD systems at 37°C in 0.01 M buffers (μ= 0.2)

pH	k'_{obs} x10^4 sec^{-1}		$t_{½}$ min	
	PGI_2Me	PGI_2Me-βCD	PGI_2Me	PGI_2Me-βCD
1.2	7000	860	0.016	0.134
2.6	1915	513	0.056	0.205
5.0	344	117	0.334	0.983
6.2	39.7	18.9	2.90	6.08
7.6	4.08	2.49	28.18	46.16

Table II. Degree of decomposition of PGI_2Me and PGI_2Me-βCD after storage for 20 months

Condition of storage	Degree of decomposition %	
	PGI_2Me	PGI_2Me-βCD
+4°C	5	0
at room temperature in sealed vials	100	40-45
at room temperature rel. moisture 75 %	100	85

The decomposition of PGI_2Me in the absence and in the presence of βCD in aqueous solution at different pH values is shown in Fig. 2. The βCD decreases the rate of hydrolysis of PGI_2Me.

The observed hydrolytic rate constants (k'_{obs}) and the half life times ($t_{½}$) are summarized in Table I. The extremely fast decomposition of the free PGI_2Me made possible to calculate only approximative values for k'_{obs} and $t_{½}$ at pH 1.2.

The stabilizing effect of β-cyclodextrin is more expressed at low pH values (Fig. 3); the ratio of the $t_{½}$ values for the complexed and the free PGI_2Me is 1.64 at pH 7.6, while it is 3.66 at pH 2.6.

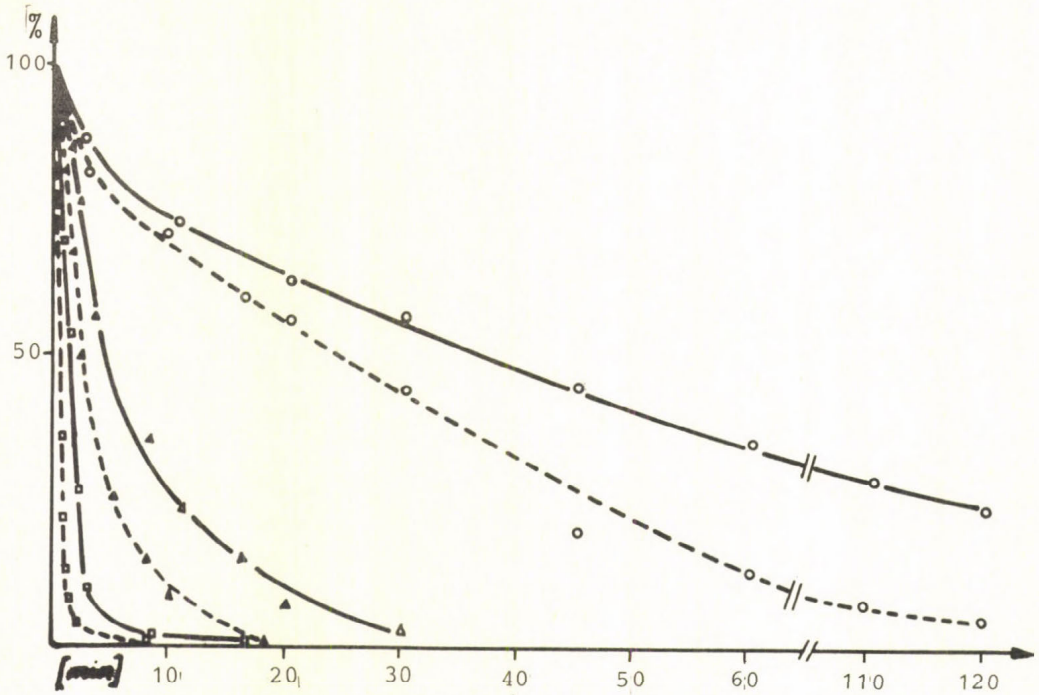


Fig. 2. Decomposition of PGI_2Me in the absence (- - -) and in the presence (——) of β-cyclodextrin in 0.01 M buffers (μ = 0.2) at 37°C.

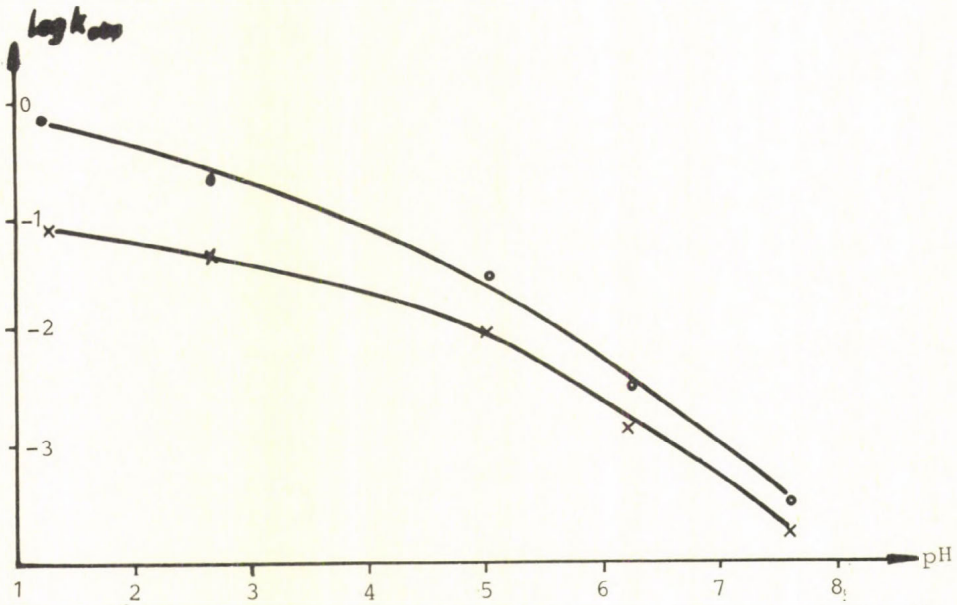


Fig. 3. Dependence of log k'_{obs} of PGI_2Me and PGI_2Me-βCD on the pH at 37°C.

The solid PGI_2Me-βCD complex can be stored without significant extent of decomposition for 20 months at +4°C. The humidity accelerates the decomposition of PGI_2Me in complexed form (Table II).

Furosemide

An ion-pair reversed-phase HPLC method was developed for the separation of furosemide from 4-chloro-5-sulphamoylantranilic acid (CSA), its main hydrolytic product, and from the other products (Fig. 4).

Table III. Influence of α-, β-, γ-cyclodextrin and β-cyclodextrin derivatives on the rate constants of decomposition of furosemide on 0.01 M NaOH solution at 25°C.

	FS	FS-αCD	FS-βCD	FS-γCD	FS-diMe βCD*	FS-triMe βCD**
			S y s t e m s			
k'_{obs} x 10^4 sec^{-1}	2.09	1.90	1.79	1.71	1.88	1.82

*diMe βCD: heptakis-(2,6-di-O-methyl)-β-cyclodextrin
**triMe βCD: heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin

Table IV. Degree of light-induced decomposition in FS-CD mechanical mixture and in their complex

Duration of storage	Degree of decomposition (CSA content %)	
	FS-βCD mechanical mixture	FS-βCD complex
3 months	4.62	0.72
4 months	6.52	0.98

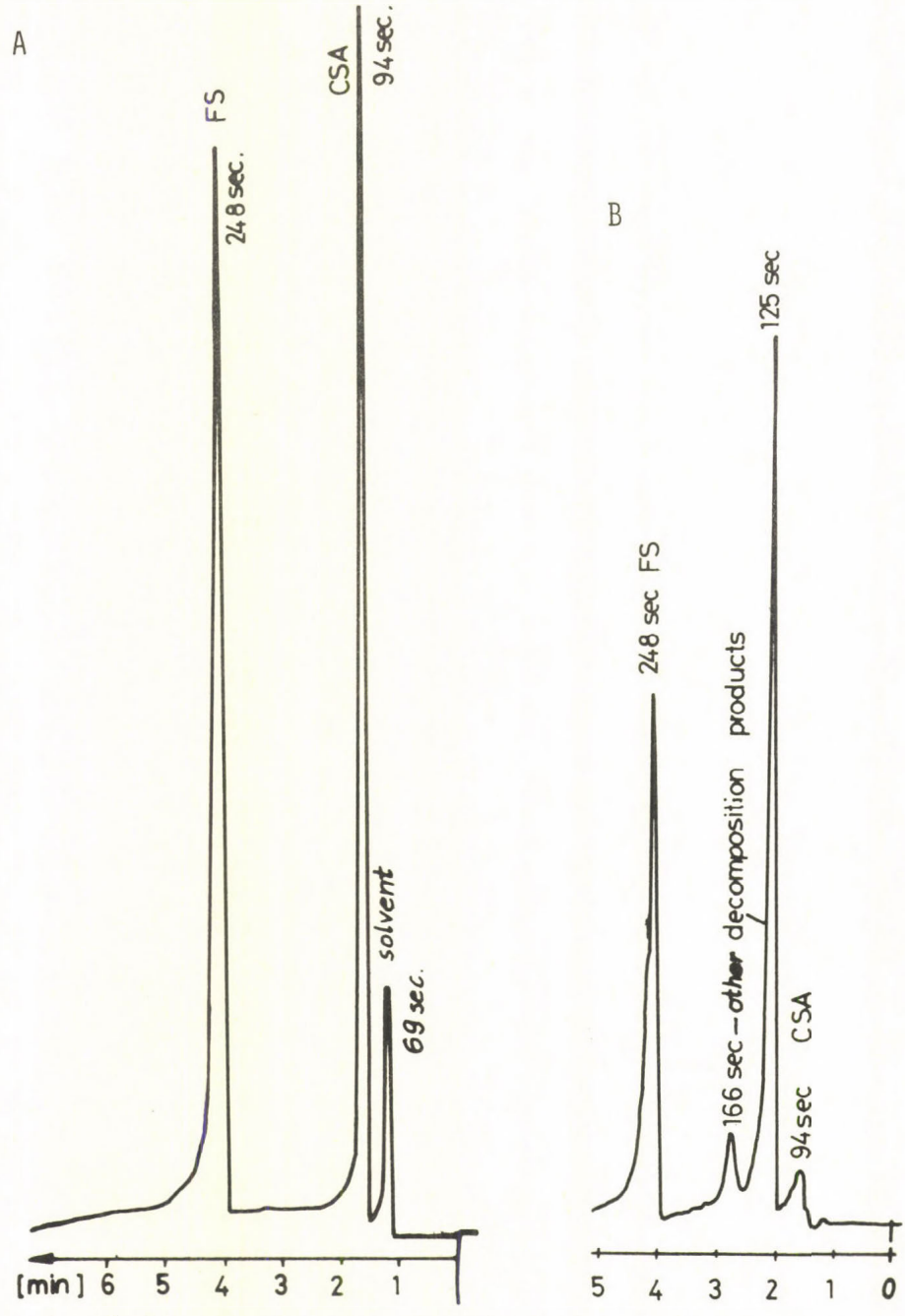
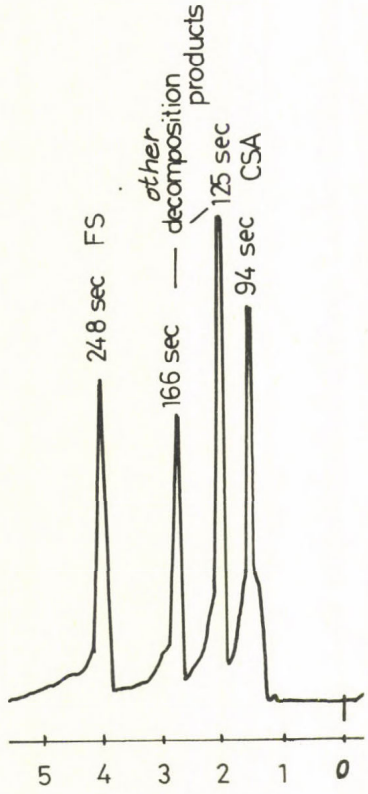


Fig. 4. A/ Chromatogram of a synthetic mixture of furosemide
C/ Chromatogram of a FS sample exposed to UV light
Chromatographic conditions as

Cyclodextrins showed some light-protective effect on furosemide in aqueous solution (Fig. 5). The observed rate constants of decomposition (k'_{obs}) are shown in Table III.

In solid state only the CSA was detected as a decomposition product. The quantity of CSA was more than six-fold in the mechanical mixture than it is in the FS-βCD complex after storing for 4 months (Table IV).

C



(FS) and its main decomposition products (CSA) B/ and
(254 nm) for an hour (B) and for 3 hours (C).
in the text.

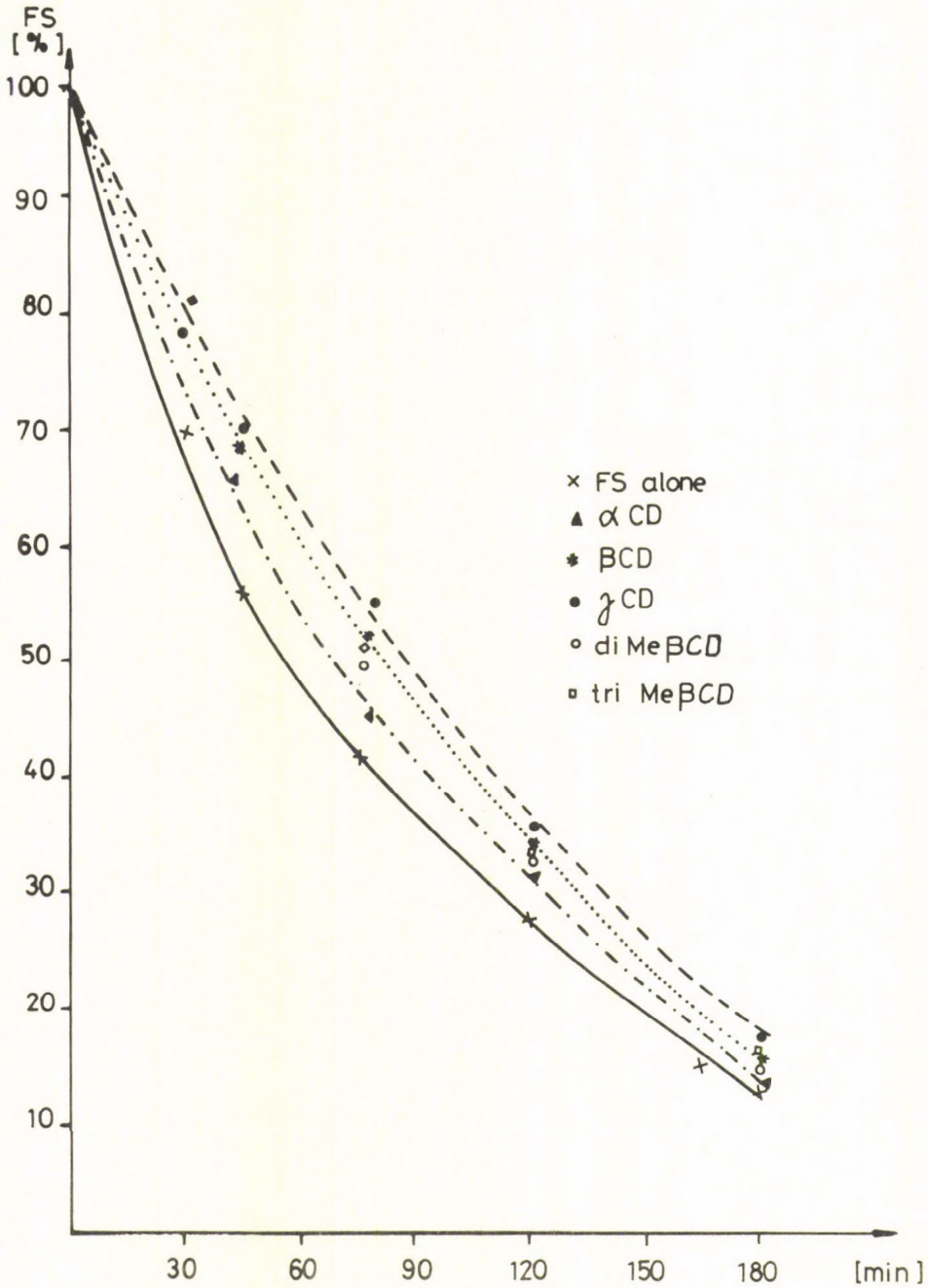


Fig. 5. Photolytic decomposition of furosemide samples exposed to UV light in 0.01 M NaOH solution at 25°C in the absence and in the presence of cyclodextrins and cyclodextrin derivatives.

ACKNOWLEDGEMENT

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ANALYSIS OF URINARY NEOPTERINE IN AUTOIMMUNE DISEASES WITH HPLC

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INTRODUCTION

Neopterine (2-amino-4-hydroxy-6-trihydroxypropyl-pteridine) originates biosynthetically from guanosine triphosphate as an intermediate in the biosynthesis of biopterine.

It had been demonstrated that the compound is a specific indicator of the activation of T-lymphocytes /1, 2/.

An increased elimination of neopterine was found in several diseases coupled with an activation of the T-lymphocyte-macrophage axis.

Elevated urinary neopterine levels were observed in patients suffering from allograft rejection /3/, infections with viruses /4/, autoimmune diseases /5/ and a large number of neoplastic diseases /6/. Due to the increase of neopterine excretion which usually precedes the clinical manifestation and its correlation with the activity of the underlying disease, a rapid determination of this compound is of great clinical interest. In this paper we present a modification of the method originally introduced by Hansen et al. /7/ for the determination of neopterine by reversed-phase HPLC.

The modified method was used to quantify neopterine in healthy subjects and in patients with autoimmune diseases.

HPLC-separation

HPLC separations were carried out on a Hewlett-Packard Model 1084 B high-performance liquid chromatograph equipped with a reversed phase column (200 x 4.6 mm) RP 18 (10 µm). The analytical column was fitted with a 30 mm guard column. An aqueous 15 mmol/l potassium phosphate buffer pH 7.4 was used as the eluent at a flow rate of 0.8 ml/min. The effluent was monitored by fluorescence detection using the Fluorichrom (Varian, Los Altos, CA, U.S.A.) fluorescence detector equipped with a glass-band filter (350 nm) for excitation and an interference filter (430 nm) for emission. The column was cleaned after the application of about 20 samples by flushing with 20 ml of methanol.

RESULTS AND DISCUSSION

The experimental conditions were optimized by altering the concentration and pH of the aqueous potassium phosphate buffer. The best separation was obtained at a concentration of 15 mmol/l and a pH of 7.4. Under these conditions a good separation of neopterine from other fluorescent compounds and a sufficient stability of the retention times was observed.

Run-to-run precision was evaluated by repeated injections of a urine sample containing 10 pmol neopterine resulting in a relative standard deviation of 3.8 % (n = 10). Day-to-day precision was observed for 10 days by analysing aliquots of a urine sample containing 15 pmol neopterine per injection (rel. st. dev.: 8.6 %).

Samples were either analysed immediately or stored at $-20^{\circ}C$, protected from light. Usually 10 µl of the urine sample diluted with water (1:1) were injected (Fig. 1).

Final results were expressed in µmol neopterine per mole creatinine. Using the ratio between neopterine and creatinine physiologically variable concentrations of urine were taken into consideration.

We investigated the neopterine levels of 81 healthy persons (61 women and 20 men).

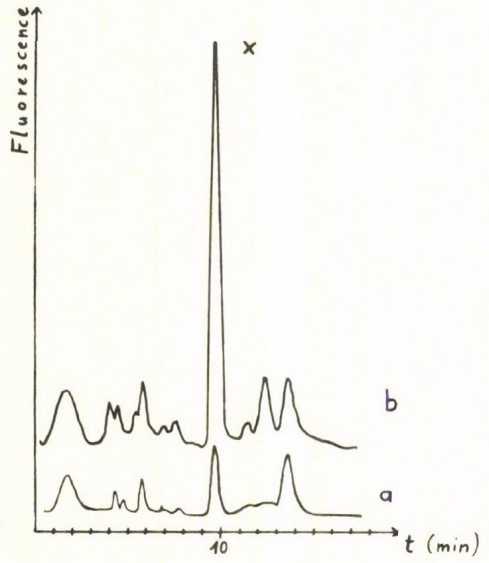

Figure 1. Determination of neopterine (x) in human urine (5 µl)
a) Healthy person with neopterine level in the normal range
b) Patient with an active Lupus erythematodes visceralis (LEV)

The mean values found in women were 159 µmoles neopterine/mole creatine (rel. st. dev.: 42.8 %) and 123.5 µmoles neopterine/mole creatine (rel. st. dev.: 39.2 %) in men. These mean values differed significantly as demonstrated by the Student t-test ($p<0.05$).

The determination of urinary neopterine was applied to 20 patients with Lupus erythematodes visceralis, two patients with dermatomyositis and one person with sclerodermia. As demonstrated in Fig. 2, 17 of the 23 patients had elevated levels of neopterine up to 200 µmoles/molecreatinine. The remaining six patients with normal nopterine values showed no activity of their disease.

Thus, we can find a good relation between the neopterine level and other activity parameters as well as clinical signs. Fig. 3 shows the time course of neopterine values over a two-month period in relationship to the therapy with immuno-suppressants.

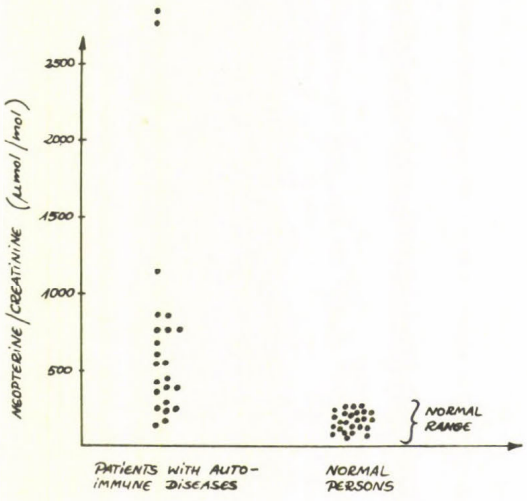

Figure 2. Urinary neopterine levels in normal persons (n = 81) and in patients with autoimmune diseases (LEV n = 20, dermatomyositis n = 2, sclerodermia n = 1)

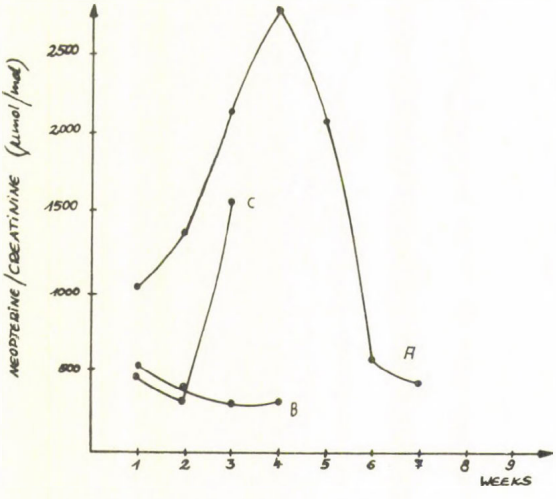

Figure 3. Course of neopterine values over a longer period a) Dermatomyositis, b and c) LEV. Patients were treated with high doses of "Imurek" and "Prednisolon".

It becomes obvious that during therapy the maximum values are significantly reduced indicating the suitability of the neopterine value for monitoring the activity of these diseases. Summarizing our data, we hope that neopterine can be used as potent indicator for optimizing the therapy of autoimmune diseases.

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CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS AND ITS PHARMACEUTICAL APPLICATIONS

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There are two modes of chromatographic resolution of enantiomers: the indirect and the direct mode. The indirect mode is represented by the following scheme:

Unresolved enantiomers (racemate) + optically active reagent $\xrightarrow{\text{formation of}}$ diastereoisomers

$\rightarrow$ unresolved diastereoisomers $\xrightarrow{\text{chromatographic separation}}$ resolved diastereoisomers $\rightarrow$

$\xrightarrow{\text{recovery of}}$ resolved enantiomers

Diastereoisomers formed in the first step differ in their physico-chemical properties, contrary to the enantiomers, and their chromatographic separation is relatively easy. For this purpose both gas and liquid cromatography may be used.

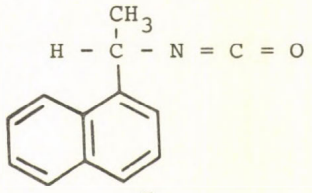
In the indirect mode each sequence has its own source of pitfalls and errors. The racemate must be able to react with the derivatizing reagent, this reaction should be quantitative, and the reagent should be of high optical purity. The reaction rate should be equal for both enantiomers and no racemization should occur. Chromatographic separation of diastereoisomers may give satisfactory results when the proper chromatographic system is used but one must be sure that the detector response is identical for both diastereomers if quantitative analytical conclusions are to be drawn. The last step, necessary for preparative purposes, calls for efficient chemical reaction usually under mild conditions, in order to retrieve the separated enantiomers and sometimes additional purification is also required.

Some of these disadvantages are avoided when the direct mode of chromatographic resolution of enantiomers is employed. In this approach, given by the scheme:

Unresolved enantiomers (racemate) $\xrightarrow[\text{separation}]{\text{chromatographic}}$ resolved enantiomers

the derivatization step is omitted and the enantiomers form unstable complexes with a chiral stationary phase or with a chiral resolving reagent added to the mobile phase. In the first case, the stability of these complexes may be different and the less stable complex results in faster elution of this enantiomer which is engaged in its formation. Diastereomeric complexes formed between the enantiomeric solute and the chiral resolving reagent may result in the separation of enantiomers due to different stability of these complexes or to their different affinity to and adsorption on the achiral stationary phase.

In the scheme above the derivatization and dederivatization steps are omitted; however, sometimes derivatives are formed for the sake of better resolution or better detectability of the chromatographed compounds. However, we do not use chiral derivatizing agents.



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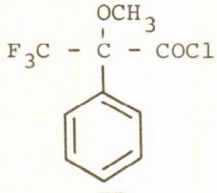
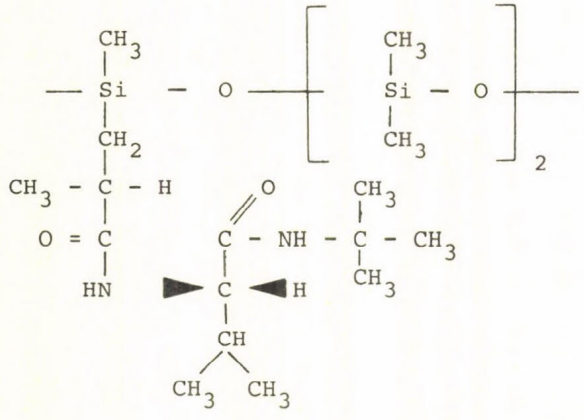


II

Compounds I and II represent widely used chiral derivatizing reagents. R-(-)-1-(1-naphthyl)ethyl isocyanate, I, or its phenyl analogue, react with chiral alcohols to form diastereoisomeric carbamates, which are well separated by liquid chromatography /1/. (-)-α-Methoxy-α-trifluoromethylacetyl chloride, II (MTPA chloride) reacts with alcohols, amines or acids to form appropriate derivatives which can be successfully resolved by different chromatographic techniques /2/. Some of the other derivatizing agents will be mentioned later, when discussing the separation of enantiomers of different drugs.

There are many types of chiral stationary phases /3-5/ and here only a few examples will be presented. Fig. 1 shows the structure of a thermally stable chiral stationary phase often used in gas chromatography for the separation of enantiomers /6-11/. It is a derivative of L-valine t-butyl amide covalently bonded to the polysiloxane polymer.

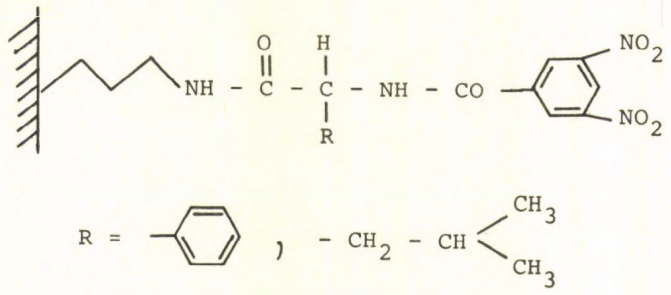


III

Fig. 1. The chiral stationary phase Chirasil-Val.

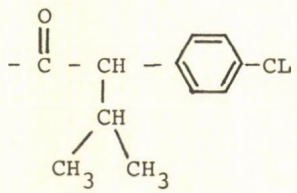
Pirkle and coworkers developed chiral stationary phases comprised of N-3,5-dinitrobenzoyl derivatives of phenylglycine or leucine covalently (Fig. 2) or ionically bonded to aminopropyl silica /12-16/. These phases provide resolutions through a combination of π-π, hydrogen bond and steric interactions according to the "three point attachment" principle of Dalgliesh /17/. Columns with these phases are claimed to separate different types of compounds and are commercially available for both analytical and preparative purpose (J.T. Baker, USA).

Examples of chiral stationary phases prepared by Oi et al. /18-20/ are presented in Fig. 3. Racemates of alkyl esters of N-acetylamino acids were separated successfully on phase VII, while amines, amino acids, carboxylic acids and alcohols were

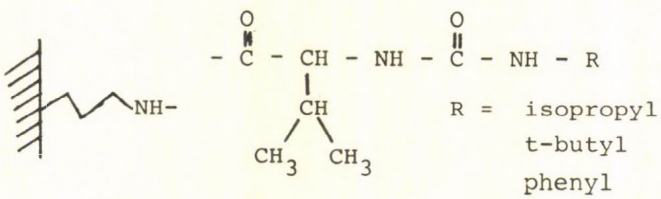


IV V

Fig. 2. Pirkle's covalently bonded chiral stationary phases.



VI



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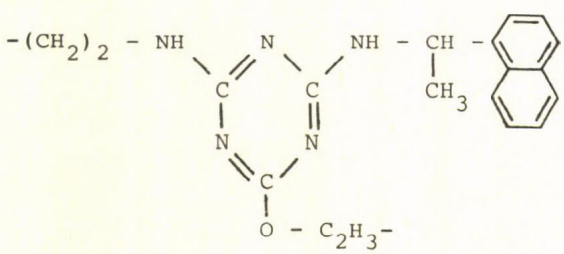
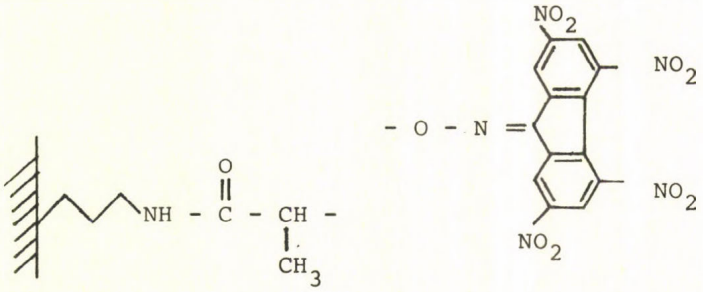


VIII

Fig. 3. Chiral stationary phases described by Ôi et al./18-20/.

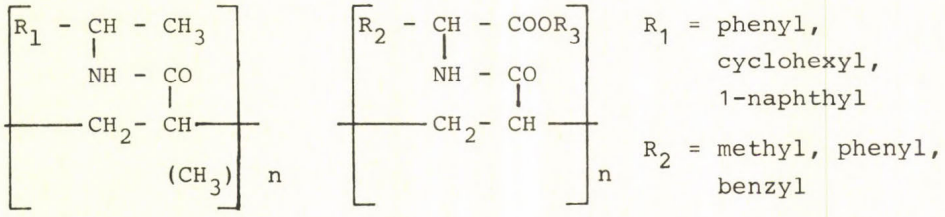
resolved on phase VIII as derivatives with the 3,5-dinitrophenyl moiety.

Chiral stationary phases, IX (Fig. 4) show stereochemical recognition of some chiral helicenes, mainly due to the charge transfer type of interactions /21, 22/.



IX

Fig. 4. Chiral stationary phases for the resolution of helicenes.



X XI

R_1 = phenyl, cyclohexyl, 1-naphthyl

R_2 = methyl, phenyl, benzyl

R_3 = ethyl, t-butyl

Polyacrylamides and polymethylacrylamides of chiral amines, X, or amino acid esters, XI, were used by Blaschke et al. /3,23-25/ for the separation of enantiomers of different drugs, but the exact mechanism of the chiral recognition is still not fully understood.

Microcrystalline swollen triacetyl cellulose was used for the successful resolution of many compounds, mainly by

Mannschreck and coworkers /26-36/. Recently, a review article on the preparation and use of this material was published /37/. Also in this case, the mechanism of chiral recognition is uncertain and a lot of empiricism is involved. The comparison of the resolving power of triacetyl cellulose and polyacrylamides was investigated /23/ and it was found that these materials often complement each other in the separation of enantiomers.

Cyclodextrins are chiral molecules formed from glucose units closed in a ring by α-1,4 bonds. Chiral stationary phase with β-cyclodextrin (cycloheptaamylose) is available commercially and separates enantiomers /38-40/ and other structural isomers /41, 42/ due to the formation of inclusion complexes and their different stability and to the interactions of enantiomers with structural units of chiral cyclodextrin cavity.

Some chiral crown ethers bonded to silica gel or polystyrene were used for the separation of enantiomers of compounds which may form alkyl ammonium ions with the asymmetric center adjacent to the amino group /43-46/.

Bovine serum albumin /47-50/ and α_1-acid glycoprotein /51-53/ were also used as chiral components of the stationary phases used for optical resolutions. Ligand-exchange stationary phases were developed and used for chromatographic resolutions mainly by Davankov and coworkers /54-60/.

There are many other chiral stationary phases of natural (cellulose, starch) and synthetic origin and new developments in this field are continuously reported in the literature /61-73/. They differ in their efficiency, resolving power, the type of the mobile phase and the type of the compounds which can be separated. For some chiral phases the mechanism of chiral recognition is well understood but quite often we cannot predict the successful separation of enantiomers on a given phase. Therefore, further studies on their scope and limitations are in high demand.

Many of the reagents which were immobilized as chiral stationary phases may be used as well as chiral resolving reagents added to the mobile phase. Many successful resolutions were accomplished with reagents such as cyclodextrins /74-78/,

proteins /79, 80/, ligand exchangers /81-85/, ion exchangers /86-90/ and others.

Although both gas and liquid chromatography are extensively used for chiral resolution, HPLC seems to gain in popularity for this purpose. The reason for this is the general development of modern HPLC methodology and instrumentation, the use of low temperature during separation and the possibility of effective preparative operation. Gas chromatography, used rather as an analytical tool, requires the thermal stability and volatility of the samples analyzed and their thermal racemization may be a prohibitive factor for its application. However, some GC chiral stationary phases proved to be quite stereoselective /91-93/. Thin layer chromatography is rather scarcely used for the separation of enantiomers, but some attempts have been published recently /94-100/. Studies such as the determination of enantiomeric purity in spite of incomplete chromatographic separation /101, 102/, selection of the mobile phase for enantiomeric resolution on chiral stationary phases /103/, or enantiomerization during complexation gas chromatography /104/ may be useful in practical applications. These applications comprise monitoring of asymmetric syntheses, analysis of enantiomeric excess, determination of absolute configuration and separation of pure enantiomers, to mention just a few. They may help to investigate the stereochemical course of chemical and biochemical reactions, to check the optical purity of reagents and compounds of natural and synthetic origin, or to solve some problems in geochronology, analysis of extraterrestrial materials, pharmacology, etc.

The determination of absolute configuration may be easily accomplished when we have an appropriate standard of known configuration and compare its chromatographic parameters with those of the unknown enantiomer. We may also anticipate absolute configuration from the elution order predicted theoretically or similar to structurally closely related enantiomers of known configuration and the chromatographic behavior in the same system.

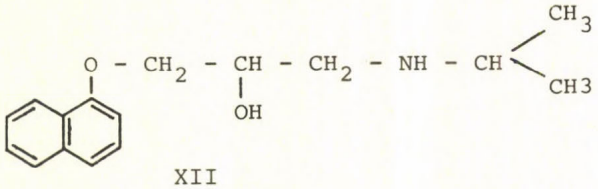
The separation of pure enantiomers is of crucial pharmacological importance, because they may differ in the potency and

effectiveness for chiral drugs, such as, for example, S-(-)-propranolol, which is much more potent than its R-(+) enantiomer /105/. Some enantiomers are entirely lacking the desired or undesired biological effects in comparison with their counterparts. Thus, only levorotatory methadone is analgesic /106/ and only the S-(-) enantiomer of thalidomide is teratogenic in rats and mice /107/. Sometimes enantiomers show different types of activity. For instance, N-methyl-5-phenyl-5-propylbarbituric acid with R-(-) configuration is narcotic, while the S-(+) enantiomer is convulsive /108/. Therefore, the preparation of optically pure chiral drugs, screening of their pharmacological activity and eventual production of the more desirable enantiomer for therapeutic use is very important. Closely associated with these are problems of the stereochemical fate of chiral drugs and other biologically active substances in the body, their bioavailability, pharmacokinetic parameters, metabolic pathways, in vivo racemization, etc., which may be different for optical antipodes.

Quite often the clinical analysis of the enantiomers of drugs in body fluids and tissues uses chromatographic methods as the methods of choice, due to their high sensitivity and other well-known advantages.

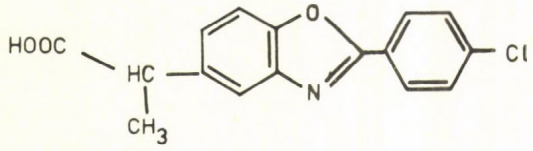
Here we present some examples of recently published chromatographic resolution of different drugs.

Propranolol, XII is the β-adrenergic blocking drug extensively used in therapy. Wainer et al. /109/ described the direct determination of propranolol enantiomers in human serum. The enantiomers were derivatized with phosgene to the 2-oxazolidone derivatives and resolved on a chiral stationary phase with ionically bonded (R)-3,5-dinitrobenzoyl phenylglycine. The simultaneous determination of the enantiomers of propranolol and its 4-hydroxy metabolite by HPLC, after derivatization with 1-phenylethyl isocyanate was also studied /110/. The results indicate stereoselectivity of the metabolism of these compounds in the human body.



XII

Sedman and Gal /111/ resolved the enantiomers of propranolol and other β-adrenergic agents by HPLC after derivatization with 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl isothiocyanate (GITC). Ion-pair chromatography /86/ and gas chromatography /112, 113/ was also used for the analysis of these drugs.



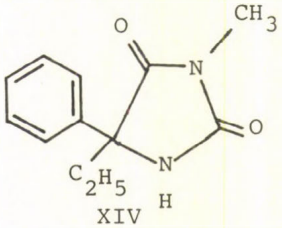
XIII

Benoxaprofen, XIII is a member of the large family of nonsteroidal antiinflammatory agents which are derivatives of α-methylarylacetic acid. It was resolved on the covalently bonded Pirkle's columns with phenylglycine /114, 115/, as the 1-naphthalenyl-methyl amide derivative. An alternative approach was the indirect separation of diastereomeric α-methylbenzyl amide derivatives on an achiral column by preparative HPLC. Then, after conversion to chlorides, the enantiomers of benoxaprofen served as derivatizing reagents for the stereoselective determination by TLC and HPLC of chiral amines, such as amphetamine, methamphetamine and tranylcypromine in biological fluids /116/.

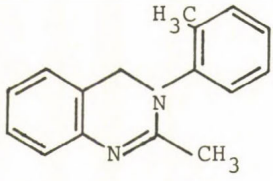
Closely related to benoxaprofen is ibuprofen [2-(4-isobutylphenyl)-propionic acid]. For both of these drugs pharmacological antiinflammatory activity is associated with S-(+) enantiomers, however, in vivo R-(-) enantiomers are stereoselectively converted to their counterparts /117/. Ibuprofen enantiomers were separated on Pirkle's column /114/ and were determined in equine urine by HPLC coupled with mass spectrometry /115/. Indirect HPLC determination of ibuprofen enantio-

mers in human plasma and urine via their diastereomeric esters with (+)-2-octanol was also recently reported /118/.

An elegant method for the derivatization of a similar drug, indoprofen (2-/4-(1,3-dihydro-1-oxo-2H-isoindol-2-yl)phenyl] propionic acid), by coupling it to leucinamide by ethyl chloroformate, was used for the determination of its enantiomers in human blood and permitted some pharmacokinetic conclusions /119/. The derivatization step requires only 3 minutes for completion.



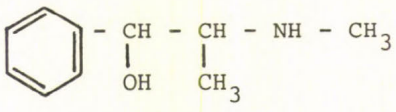
XIV



XV

The anticonvulsant drug mephenytoin, XIV, along with its demethylated metabolite, may be directly assayed in plasma and blood by gas chromatography on Chirasil-Val. The method revealed stereoselective differences in the pharmacokinetic parameters and the metabolism of enantiomers of this drug /120/. The same drug, some chiral barbiturates and succinimides were resolved by HPLC on chiral stationary phases /121/.

Methaqualone, XV, is a hypnotic and anticonvulsive drug. Its chiral properties are associated with the hindered rotation around the nitrogen-aryl bond. This compound was subjected to resolution on triacetyl cellulose /34/. Only partial preparative separation of the enantiomers was achieved, but enriched samples were used for pharmacological screening, which revealed statistically significant higher potency of the (-) enantiomer.

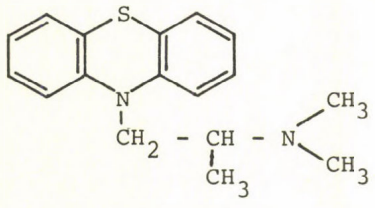


XVI

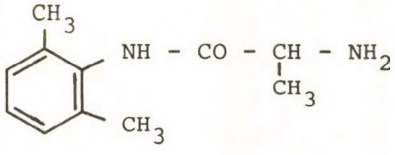
Ephedrine, XVI has the absolute configuration of 1(R), 2(S) for the levorotatory isomer, which is more active than its dextrorotatory antipode. Since ephedrine has two chiral centers it

may occur as a diastereoisomer called pseudoephedrine with 1(R), 2(R) configuration for the (-)isomer. Another closely related compound is norephedrine without the methyl group at the nitrogen atom. All these compounds are adrenergic agents used as vasopressors, bronchodilators and ophthalmic and nasal decongestants. The enantiomers of these compounds were resolved directly by GC /112, 113, 122/ and HPLC /123, 124/ chiral stationary phases. Gal derivatized ephedrine and related compounds with GITC reagent, which forms diastereomeric thiourea derivatives subsequently resolved by reversed-phase HPLC /125/. Pettersson and Stuurman successfully employed the di-n-butyl ester of (+) tartaric acid adsorbed on the solid phase as a liquid stationary phase able to complex different aminoalcohols and among them ephedrine type drugs /90/. Elution was achieved by phosphate buffer solution containing hexafluorophosphonate as a counter ion.

Promethazine, XVII, is a highly potent antihistaminic agent used in allergies. It was resolved on a column containing α_1-acid glycoprotein as the stationary phase /126/; other drugs, including some local anesthetics were also separated on this chiral stationary phase /51, 53, 127/.



XVII



XVIII

Tocainide, XVIII, a new antiarrhythmic drug was resolved by GC after derivatization with MTPA chloride /128/. The use of electron-capture detector gave a high sensitivity, with a minimum detectable limit of 10 ng/ml, permitting an assay in blood plasma for pharmacokinetic purposes.

Determination of the enantiomers of 1,4-benzodiazepine drugs was achieved by reversed-phase HPLC of their glucuronide derivatives assayed in human plasma and urine /129/. Other compounds of this type were resolved on chiral stationary

phases by HPLC /130/. Other examples include the indirect HPLC resolution of racemates of warfarin, an oral anticoagulant, as the diastereomeric carbonates formed with 1-methyl chloroformate, or mexiletine, an antiarrhythmic agent, after derivatization with GITC /131, 132/. Wainer et al. /133/ directly resolved some derivatives of tropic acid, among them the anticholinergic agent tropicamide. Enantiomeric resolution of derivatives of polycyclic aromatic hydrocarbons /134/, abscisic acid /135/ and carotenoids /136/ allows the investigation on the stereoselective metabolism of the parent compounds. Pharmaceutical analysis of enantiomers in different drug formulations is exemplified by the determination of methamphetamine in an illicit preparation /137/, levodopa in tablets and capsules /138/, or adrenaline (epinephrine) in an ophthalmic formulation /193/ or in combinations with lidocaine /140/.

All these examples clearly show that the analysis of enantiomers of chiral drugs is possible and will be further developed in the future. The results will broaden our knowledge and may bring better therapeutic use of pharmaceuticals. Chromatographic methods will certainly play an important role in achieving these goals.

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HPLC ASSAY OF CHINOIN-127 IN BLOOD AND ITS APPLICATION IN THE PHARMACOKINETICS OF RAT

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SUMMARY

A simple, rapid, sensitive and specific high-performance liquid chromatographic method was developed for the determination of CHINOIN-127 and its active metabolites in serum and urine.

The method involved rapid quantitative extraction without precipitation or centrifugation with absolute recoveries better than 85%.

The separation was carried out on Nevisorb Si-60 column using hexane-dioxane-tetrahydrofuran-methanol-acetic acid-water as the mobile phase with UV-detection at 242 nm. The lowest sensitivity of the assay was 20 ng per ml serum extracted for the drug, and 50 ng and 100 ng per ml serum extracted for the metabolites I and II, respectively. Linear concentration ranges used in the assay (referred to 50 mg/kg individual doses) were 1.875 -60.0 µg/ml for CHINOIN-127, 0.125 - 4.0 µg/ml for metabolite I, and 0.25 - 8.0 µg/ml for metabolite II. The low relative standard deviations (<3.0%) for samples spiked with the drug and metabolites I, and II in these concentration ranges demonstrate good reliability and reproducibility.

This method was used for the "single-dose" studies on rats. It is also applicable to human pharmacokinetics and to therapeutic monitoring of CHINOIN-127.

INTRODUCTION

CHINOIN-127 (1,6_{ax}-Dimethyl-4-oxo-1,6,7,8,9,$9a_{ax}$-hexahydro-4H-pyrido [1,2-a] pyrimidine-3-carboxamide) represents a new class of antiinflammatory agents with a non-narcotic analgesic activity, and it has better therapeutic index than acetylsalicylic acid, phenylbutazone or indometacin. /1/.

CHINOIN-127 undergoes an extensive transformation to produce several metabolites. The possible transformation of the drug into metabolite I (1,6-dimethyl-4-oxo-1,6,7,8-tetrahydro-4H-pyrido-[1,2-a]pyrimidine-3-carboxamide) and metabolite II (6_{ax}-Methyl-4-oxo-1,6,7,8,9,$9a_{ax}$-hexahydro-4H-pyrido-1,2-a pyrimidine-3-carboxamide) is shown in Fig. 1.

In order to characterize the extent of exposure and disposition of the drug and metabolites I and II their determination in various biological fluids is required.

HPLC with UV-detection represents a particularly sensitive technique. The best separation of compounds I, II, CHINOIN-127 and internal standard III was achieved on a silica column with isocratic elution. The mixture of hexane-dioxane-tetrahydrofuran-methanol-acetic acid-water was used as the mobile phase. UV-detection at 242 nm provides the necessary sensitivity and specificity.

We have also utilized the HPLC assay for the determination of the drug in rat serum, urine, and also in human blood samples.

The method described here is used for the routine monitoring of CHINOIN-127 during "single-dose" pharmacokinetic studies in rats because of its specificity, practicability, easy sample clean-up without precipitation or centrifugation using solvent extraction on Extrelut columns, good recoveries and reliability. The silica column for HPLC separation gives the opportunity to inject large number of samples without noticeable effect on pressure drop and column performance.

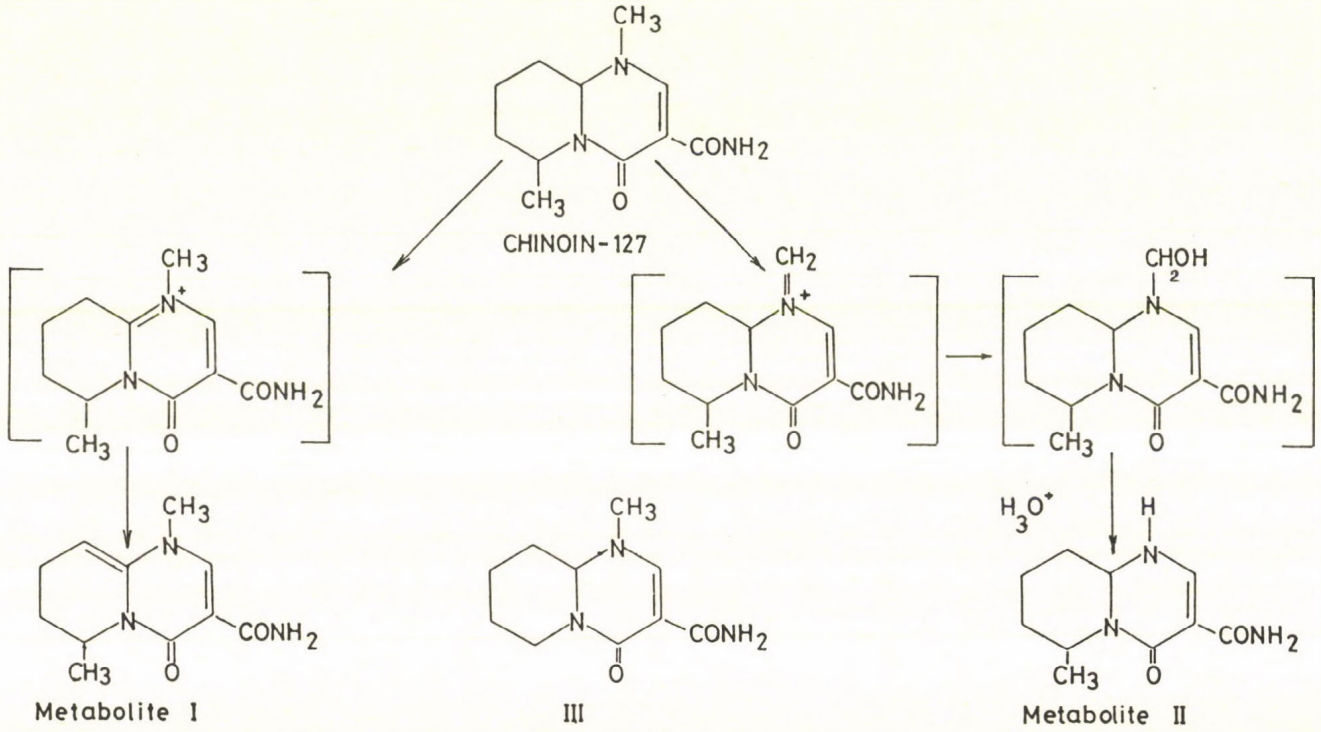

Fig. 1. Molecular structure of CHINOIN-127 and a possible passway of the formation of its metabolites I and II. The internal standard III was used in the HPLC assay

EXPERIMENTAL

Materials

The following compounds:

- CHINOIN-127 (1,6_{ax}-dimethyl-4-oxo-1,6,7,8,9,$9a_{ax}$-hexahydro-4H-pyrido[1,2-a]pyrimidine-3-carboxamide),
- metabolite I (1,6-dimethyl-4-oxo-1,6,7,8-tetrahydro-4H-pyrido-[1,2-a] pyrimidine-3-carboxamide),
- metabolite II (6_{ax}-methyl-4-oxo-1,6,7,8,9,$9a_{ax}$-hexahydro-4H-pyrido [1,2-a] pyrimide-3-carboxamide), and
- internal standard III (1-methyl-4-oxo-1,6,7,8,9,9a-hexahydro-4H-pyrido [1,2-a] pyrimidine-3-carboxamide) were synthesized in our factory according to the methods described in the literature |2|.

All chromatographic solvents were of HPLC grade. The inorganic reagents were prepared in distilled, deionized water. All reagents used were analytical grade. Degassing of the mobile phase was carried out by sonication for 20 min.

Extrelut (E. Merck, Darmstadt FRG) was used for sample extraction.

Apparatus

A Biotronik Model 3020 pump (Biotronik, Wissenschaftliche Geräte GmbH, Maintal, FRG), and a Rheodyne Model 2125 valve-loop injector fitted with a 20 µl loop were employed. A Nevisorb Si-60 7-µm 250 x 4.0 mm I.D. column (BST Budapest, Hungary) was connected to a Biotronik Model 3030 UV Detector used at 242 nm (0.08 AUFS and 0.16 AUFS). Data was recorded with a SP-4100 10 mV computing-integrator, (Spectra-Physics GmbH, Darmstadt, FRG.

A 90:50:16:20:0.2:0.4 (v/v) mixture of hexane:dioxane:tetrahydrofuran:methanol:acetic acid:water was used as the mobile phase, at a flow rate of 2.4 ml/min.

Chromatography was carried out at ambient temperature.

Standard solutions

Stock solutions of the internal standard (III), and CHINOIN-127 at a concentration of 1 mg/ml were prepared in methanol and stored at -30°C. Stock solutions of metabolite I at a concentration of 2 mg/ml and metabolite II at 4 mg/ml in methanol were mixed in a 1:1 (v/v) ratio and stored also at -30°C.

Further serial dilutions were made in methanol while calibrations were prepared each day.

These reference standard solutions contained 3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 µg of metabolite I per ml and 6.25, 12.5, 25.0, 50.0, 100.0, and 200.0 µg of metabolite II per ml. Appropriate serial dilutions of CHINOIN-127 were made to obtain the standard concentrations of 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg/ml.

The stock solution of the internal standard (III) was diluted with methanol to the concentration of 100.0 µg/ml.

Extraction of CHINOIN-127

To the unknowns and the control samples of rat serum or urine (0.25 - 0.25) 15 µl of the internal standard (III) and 0.1 ml of a 0.2 M phosphate buffer (pH 7.4) were added. The mixture was agitated on a vortex mixer for 15 sec. Then the samples were adjusted on the glass column (130 x 7 mm I.D.) packed with 2 ml Extrelut. For 5 min the samples were left to stand. Subsequently, the Extrelut layer was washed with 0.5 ml chloroform. The extraction was repeated three times. The solution was evaporated to dryness under a stream of nitrogen, then dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot of this solution was injected onto the column.

Extraction of metabolites I and II

The standard samples were extracted identically to the unknown samples. The procedure was similar to that described above for the extraction of CHINOIN-127.

To 0.5 ml of the rat serum or urine 40 µl of a diluted internal standard solution (100.0 µg/ml) and 0.2 ml of the 0.2 M phosphate buffer (pH 7.4) were added, mixed by vortex for 15 sec and then adjusted on 2 ml Extrelut. The samples were left to stand for 5 min. Then, the compounds were extracted with chloroform (0.5 ml, 4 times). The combined organic extracts were evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 1.0 ml of the mobile phase and an aliquot of this solution was injected into the chromatography.

Chromatography

CHINOIN-127, metabolites I and II, and the internal standard (III) gave sharp symmetrical peaks under the described experimental conditions, with retention times of 5.45, 2.70, 4.24 and 6.58 minutes, respectively (peaks a, b, c, d in Figs 2-10).

Calibration curves

Calibration curves were prepared each day when samples were analyzed to establish the linearity and reproducibility of the assay.

Serum standard curves for each compound determined were constructed (at each run) over the concentration ranges of 1.875-60.0 µg/ml for CHINOIN-127, 0.125-4.0 µg/ml for metabolite I and 0.25-8.0 µg/ml for metabolite II.

In each case, the peak height ratios of each compound to the internal standard were plotted against the concentration of CHINOIN-127, and metabolites I and II in 1 ml serum or urine.

In vivo studies

Male CFY rats weighing 207.03 $\pm$ 15.5 SD were used for the studies. Six groups were made, each of them containing 10 rats.

50 mg/kg dose of CHINOIN-127 was dissolved in distilled water, in a volume of 1 ml per 100 g body weight and was administered orally. Rats were fasted for 18 h prior to the experi-

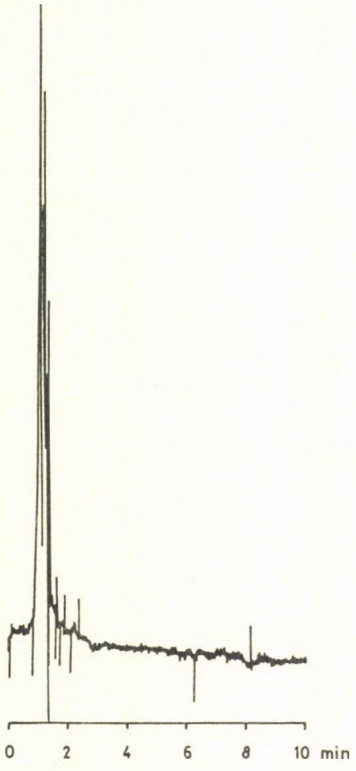

Fig. 2.

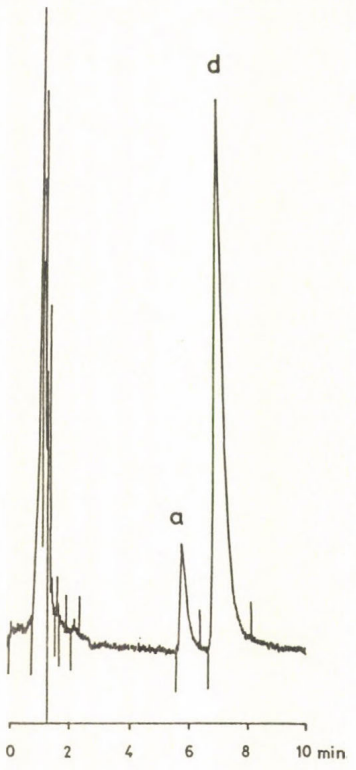

Fig. 3.

Fig. 2. Chromatogram of a 0.25 ml blank rat serum sample. Extracted residue was dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot injected

Fig. 3. Chromatogram of a 0.25 ml blank rat serum sample spiked with CHINOIN-127 (7.5 µg/ml serum) and the internal standard (60 µg/ml serum. Extracted residue was dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot was injected

ment. During the experiment water was provided ad libitum. The blood was obtained from the inner corner of the eye using a heparinised glass capillary tube.

Bleeding was carried out after the drug administration at 12 different points of time as follows: 0, 10, 20 and 30 min., 1, 2, 3, 4, 6, 9, 12, 24 and 36 hours. Each group had two different points of bleeding time.

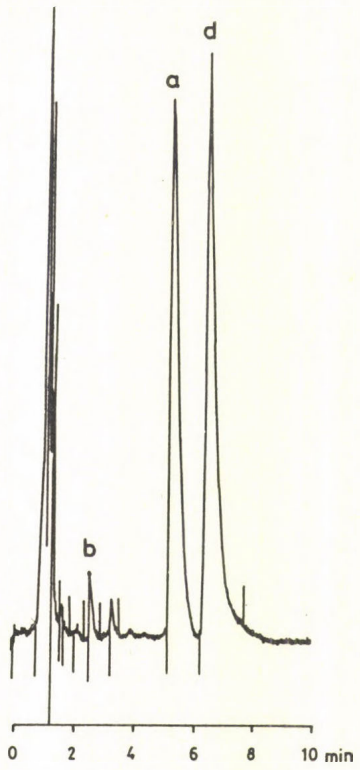

Fig. 4.

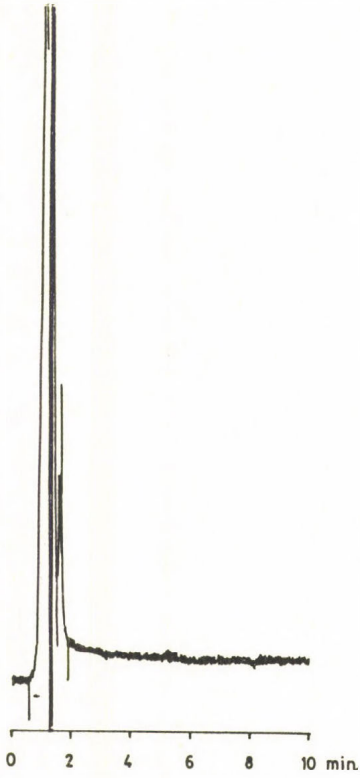

Fig. 5.

Fig. 4. Chromatogram of 0.25 ml of rat serum collected at 10 min after an oral dose of 50 mg/kg of CHINOIN-127. Extracted residue was dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot was injected

Fig. 5. Chromatogram of a 0.5 ml blank rat serum. Extracted residue was dissolved in 1.0 ml of the mobile phase and a 20 µl aliquot was injected

Blood was centrifuged at 3000 rpm and the collected serum samples were stored frozen at -30°C until their analysis. Urine was collected during 36 h at 6-h intervals, and stored at -30°C.

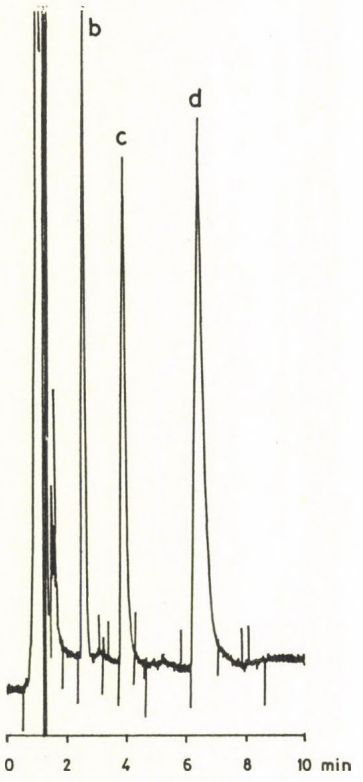

Fig. 6.

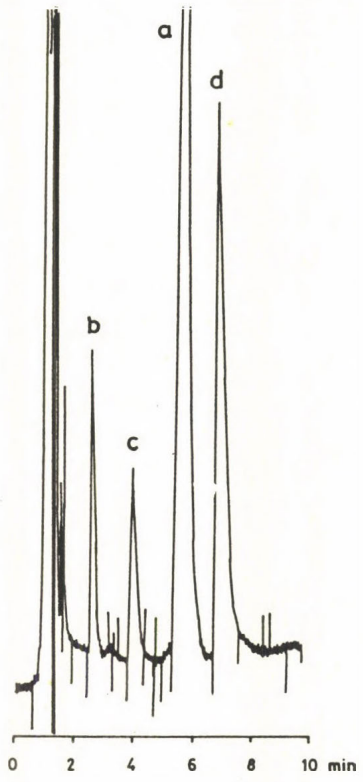

Fig. 7.

Fig. 6. Chromatogram of a 0.5 ml blank rat serum spiked with metabolites I and II (4.0 µg/ml and 8.0 µg/ml). Extracted residue was dissolved in 1.0 ml of the mobile phase and a 20 µl aliquot was injected

Fig. 7. Chromatogram of 0.5 ml of rat serum collected at 4h after an oral dose of 50 mg/kg of CHINOIN-127. Extracted residue was dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot was injected

RESULTS AND DISCUSSION

Figs 2-10 illustrate the chromatograms of CHINOIN-127 and its metabolites obtained after processing rat serum and urine samples. The drug and its metabolites present in biological fluids eluted in a reasonable period of time.

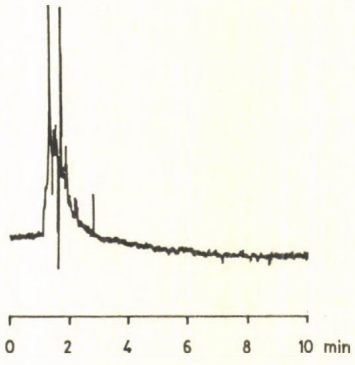

Fig. 8.

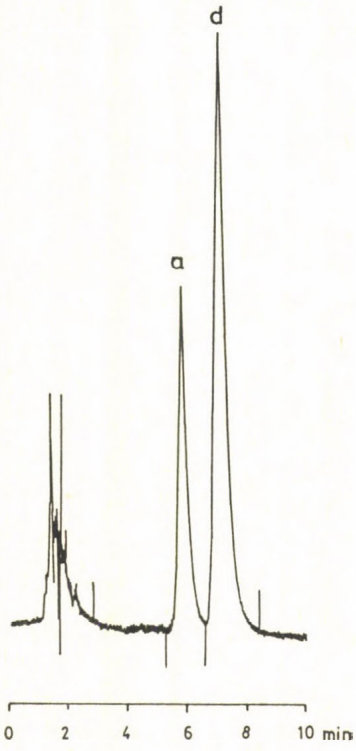

Fig. 9.

Fig. 8. Chromatogram of a 0.5 ml blank rat urine sample. Extracted residue was dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot was injected

Fig. 9. Chromatogram of a 0.5 ml blank urine sample spiked with CHINOIN-127 and the internal standard. Extracted residue was dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot was injected

The extraction procedure described in this paper is rapid and practical, especially for routine monitoring of the drug. The resultant dissolved residues are free of visible impurities and normal-phase HPLC analysis indicates no interference from endogenous materials for the compounds studied.

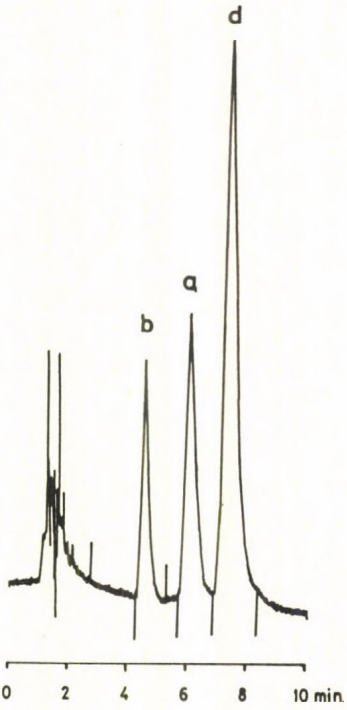

Fig. 10. Chromatogram of a 0.5 ml rat urine sample collected 24h after an oral dose of 50 mg/kg of CHINOIN-127. Extracted residue was dissolved in 1.75 ml of the mobile phase and a 20 µl aliquot was injected

Assay recovery and sensitivity

The absolute recovery of CHINOIN-127, metabolites I and II from serum or urine were determined by comparing the peak heights obtained when the redissolved serum residue and standards were chromatographed.

For all concentrations, no statistically significant differences in the recovery were found ($p<0.05$) with the average recovery of CHINOIN-127 being 85.98% (See Table I).

The detection limit (calculated for a peak height corresponding to twice the noise level) of CHINOIN-127 was 20 ng/ml, of metabolite I 50 ng/ml, and of metabolite II 100 ng/ml; these values refer to the extracts, having the residue dissolved in 50 µl of the mobile phase.

Table 1. Precision of the assay of CHINOIN-127, based on peak height ratios. Recovery of CHINOIN-127 from 1 ml of serum*

No.	µg/ml added	µg/ml detected	N	S.D.	rel.S.D. %	recovery % ± S.D.
1.	1.875	1.7370 ± 0.0597	4	0.0844	6.420	82.35 ± 5.36
2.	3.75	3.5412 ± 0.0507	4	0.1004	2.836	82.61 ± 3.55
3.	7.5	7.6394 ± 0.0222	4	0.0445	0.583	83.57 ± 1.11
4.	15.0	15.7075 ± 0.1438	4	0.2876	1.831	81.47 ± 0.51
5.	30.0	31.0378 ± 0.1238	4	0.2477	0.798	89.89 ± 4.71
6.	60.0	59.3152 ± 0.5001	4	0.8662	1.460	96.03 ± 0.82
average					2.32	

*S.D. = standard deviation;
rel. S.D. = relative standard deviation;
N = number of samples extracted.

Table 2. Interassay precision for CHINOIN-127 for standard curves run oven a 10-day period and having concetrations ranging 0-60 µg/ml

Day	Slope	Intercept	r^2
1	0.018685	0.003618	0.99920
2	0.019249	0.008965	0.99969
3	0.017670	0.010801	0.99957
4	0.020626	0.005368	0.99971
5	0.019668	0.010998	0.99916
6	0.021296	0.015403	0.99855
7	0.021061	0.012941	0.99926
8	0.019968	0.020311	0.99852
9	0.020436	0.012436	0.99961
10	0.020378	0.018025	0.99697

Assay precision

Linear regression analysis of the data included in the calibration curves indicated no significant deviation from

Table 3. Interassay precision for metabolite I for standard curves run oven a 6-day period and having concentrations ranging 0 - 4.0 µg/ml.

Day	Slope	Intercept	r^2
1	0.510165	0.001493	0.99922
2	0.416294	0.028956	0.99846
3	0.369789	0.061087	0.99249
4	0.49460	0.0197	0.98734
5	0.434608	0.017829	0.98910
6	0.538315	0.030568	0.99907

Table 4. Interassay precision for metabolite II for standard curves run oven a 5-day period and having concentrations ranging 0 - 8.0 µg/ml.

Day	Slope	Intercept	r^2
1	0.119462	-0.007611	0.99946
2	0.114361	0.002615	0.99948
3	0.108431	0.013305	0.99406
4	0.149723	0.028547	0.99674
5	0.139880	0.03576	0.99620

linearity for concentrations of CHINOIN-127 up to 60 µg/ml, of metabolite I up to 4.0 µg/ml and of metabolite II up to 8.0 µg/ml serum or urine (See Tables 2-4).

Correlation coefficients for the standard curves run over a 10-day period and having the CHINOIN-127 concentration ranging from 0 up to 60 µg were better than $r^2 = 0.99697$, with the mean correlation coefficient being $r^2 = 0.99900$ (See Table 2).

The standard curve intercepts were not significantly different ($p<0.01$) for all curves.

No statistically significant differences were found in the relative standard deviation values, during a 10-day period ($p<0.025$), with the average relative standard deviation being 2.289%.

Correlation coefficients for the standard curves for metabolites I and II run over a 6-day period and having concentrations ranging from 0 - 4.0 µg/ml and 0 - 8.0 µg/ml, respectively, were better than r^2 = 0.98735 for I, and r^2 = = 0.99407 for II; the mean correlation coefficient being r^2 = = 0.99428 for metabolite I and r^2 = 0.99720 for metabolite II.

An estimate of the interassay reproducibility and precision was obtained by comparing the standard curves prepared on different days (See Tables 2-4). The slopes of these curves ranged from 0.01767 to 0.02129 ml/µg for CHINOIN-127, 0.369789 to 0.538315 ml/µg for metabolite I, and 0.1084 to 0.1398 ml/µg for metabolite II; the mean standard deviation values were 0.019921 ± 0.001142 SD for CHINOIN-127, 0.460628 ± 0.06409 SD for metabolite I and 0.130072 ± 0.018178 SD for metabolite II.

METHODOLOGY APPLICABILITY

The applicability of the assay was demonstrated by a preliminary study of the serum concentration profiles of CHINOIN-127 and metabolites I and II in rats.

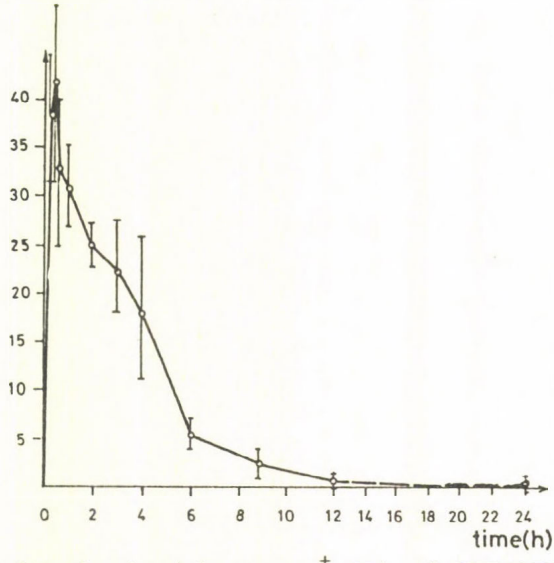

Fig. 11. Serum levels (µg/ml serum ± SD) of CHINOIN-127 following oral administration of 50 mg/kg dose of the drug dissolved in distilled water

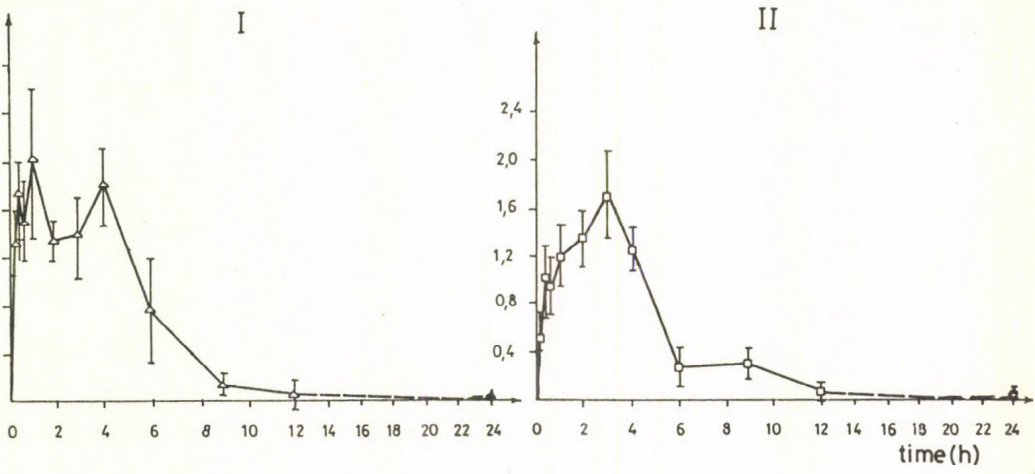

Fig. 12. Serum levels (µg/ml serum ± 50) of metabolites I and II following oral administration of 50 mg/kg dose of the drug dissolved in distilled water

Absorption after oral administration of 50 mg/kg of CHINOIN-127 was rapid. Peak levels of the drug and metabolites I and II were attained within 10 min. (See Figs 11-12). The maximum serum concentration (C_{max}) was 41.01 µg/ml ± 9.28 SD at 20 min. After 24 h no drug could be detected in the serum with HPLC.

Maximum serum concentrations of metabolites I and II were obtained at t_{max} = 1h and t_{max} = 3h, respectively, being C_{max} = = 2.032 µg/ml ± 0.569 SD for I and C_{max} = 1.684 µg/ml ± 0.3926 SD for II.

The complete pharmacokinetic modelling and studies of CHINOIN-127 and its metabolites are under way.

ACKNOWLEDGMENT

Help from Dr. I. Hermecz for generously supplying CHINOIN-127, the metabolites and the compound used as the internal standard, and his continuing advice and support is especially acknowledged.

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DETERMINATION OF COMPOUNDS EGYT 1855 AND GYKI 51189 IN RAT AND MOUSE FOODS BY TLC

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INTRODUCTION

The concentration of the active ingredient should be determined in the food given to laboratory animals in toxicological experiments.
TLC methods were developed for this purpose.

The food with 50-2000 ppm active substance concentration was extracted by ethyl acetate.

Two developments were applied to separate the active substance from the other extracted components of the food.

After visualization with Dragendorff-reagent the quantitation of EGYT 1588 was carried out by densitometric method.

Compound GYKI 51189 was quantified by direct photometry using the densitometer at 300 nm.

MATERIALS AND METHODS

Extraction with ethyl acetate

Sample preparation

Grind about 100 g of the food sample and mix it thorougly. Weigh 5 or 10 g of the food prepared in the described way and add 25 or 50 ml ethyl acetate to it. Stir the mixture for half an hour and filtrate it with a glass filter. Evaporate the ethyl acetate in a ROTA-VAPOR.

Add a known amount of ethyl acetate to the evaporated residue of the extract so that the concentration of the active

ingredient in the solution should be 100 or 200 µg/ml, depending on the active ingredient concentration of the food.

Chromatography

Chromatographic plates

200 x 200 mm, 0.25 mm, Precoated Silica Gel 60 HF_{254}

Mobile phase

for GYKI 51189: (A) benzene
(B) 20:80 benzene:ethyl acetate

for EGYT 1855 : (A) chloroform
(B) 20:20:10 ethyl acetate:10 % n-propanol acetic acid

Sampling

Transfer 10 or 20 µl of the food extract to the plate by Linomat III. automatic spotter.

For the calibration curve: transfer 0.5, 1.0, 2.0, 3.0 µg of the compound GYKI 51189 or EGYT 1855.

Chromatographic procedure

After spotting place the plates in the chromatographic tank equilibrated before use with 100 ml of the prepared solvent system.

Apply two consecutive developments in systems A and B to separate the active ingredient from the other extracted components of the food.

After the first development dry the plates for a quarter of an hour in stream of air.

Visualization

For GYKI 51189: fluorescence quenching

Place the plate under UV-lamp and at 254 nm mark the height of the examined compound at both edges of the plate.

For EGYT 1855: Dragendorff-reagent

Solution No.I: Dissolve 0.85 g bismuth(III) nitrate in the mixture of 10 ml glacial acetic acid and 40 ml water

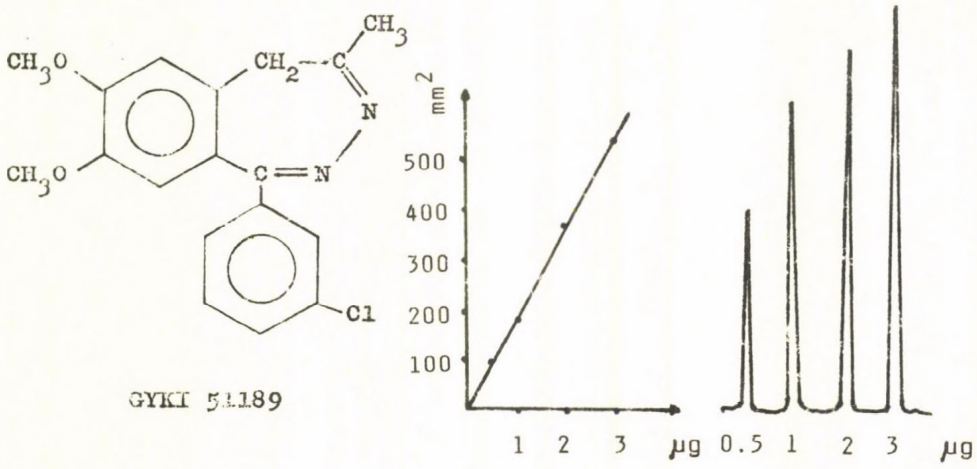
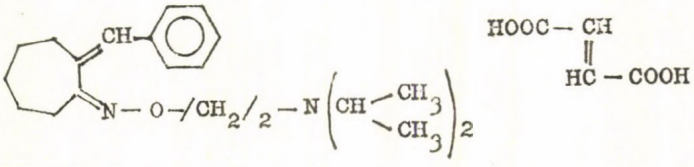


Fig. 1. Calibration curve of compound GYKI 51189



EGYT 1855

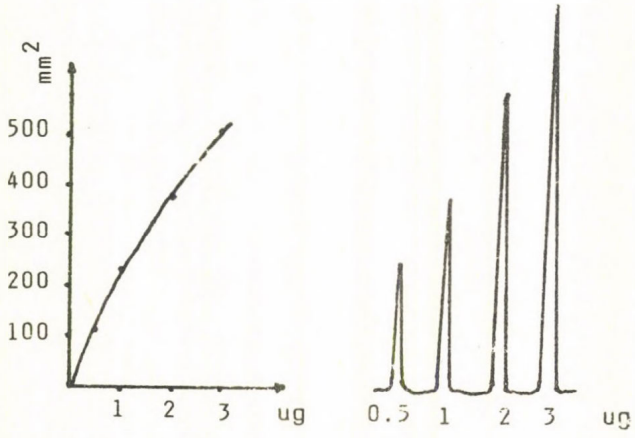


Fig. 2. Calibration curve of compound EGYT 1855

Solution No.II: Dissolve 8 g potassium iodide in 20 ml water

Spray solution: Mix 5 ml solution No.I, 5 ml solution No.II, 20 ml 0.1 N sulphuric acid and 70 ml water before use.
This reagent forms orange spots in white background, with compound EGYT 1855.
In a few minutes after spraying cover the plate by a glass plate, so that the coloured spots will remain unchanged for at least one hour.

QUANTITATION BY DENSITOMETRIC METHOD

Camag-Z-Scanner

Working wavelength: 450 nm for coloured spots of EGYT 1855
300 nm for GYKI 51189
Monochromator slit: 1 mm
Length of slit: 10 mm

Densitometric procedure

Place the plate into the plate-holder of the Scanner. The plate containing EGYT 1855 should be covered by a glass plate during the measurement.
The direction of working is at right angles to the direction of the development.

Evaluation

Determination of the peak area: peak height x peak width at half height.

For the calculation of the results draw a calibration curve using the average of areas of the standard compounds.

Table I. Results of Parallel Measurements

Samples	Active ingredient content %	
	EGYT 1855	GYKI 51189
1.	94	91
2.	90	93
3.	90	93
$\bar{x}$	91.3 ± 2.30	92.3 ± 1.15

RESULTS AND DISCUSSION

The efficiency of the extraction with ethyl acetate was found to be near 95 percent. The results were modified according to this measured yield in order to obtain the real concentration of the active substance.

In order to apply TLC to determine the active substance content of the ethyl acetate extract the majority of interfering extracted components had to be separated. The use of Al_2O_3 column or a preliminary extraction with light petroleum to remove fats and some other substances which have already been described /1/ caused serious losses of the examined compound. Developing the plates twice proved to be the best method to solve this problem. During the first development in solvent A we could separate a large number of interfering compounds while the spots of the active ingredient remained in the start point. After second development the plates were suitable for scanning.

Beside 10 µl or 20 µl of the sample extract we have applied to every plate 0.5 µg, 1.0 µg, 2.0 µg, and 3.0 µg of the standard compound for drawing the calibration curve. In the case of GYKI 51189 scanning was carried out at 300 nm, near to the maximum absorption. In the mentioned interval the obtained calibration curve was linear (Fig. 1.).

The operating wavelength for coloured spots of EGYT 1855 was 450 nm. In spite of the fact that the calibration curve of EGYT 1855 in the given interval is nonlinear(Fig. 2.),reproducibility was very good. The relative standard deviation of five parallel applications of the same sample was not more than $\pm$ 1 %.

Homogenity, which is very important not only in analytical but also in toxicological experiments, was also found to be suitable. Table I gives the results of some parallel measurements, the samples were taken from different parts of the food.

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Budapest, 1986

Chromatography '85
H. Kalász and L.S. Ettre (Eds)

HPLC INVESTIGATION OF CHLORAMPHENICOL STEREOISOMERS

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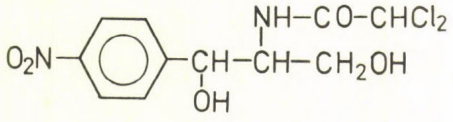
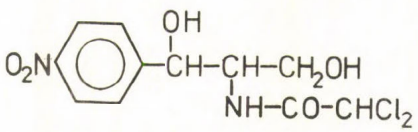
Chloramphenicol is one of the important antibiotics. After the discovery of the natural form the total synthesis was successfully made by Rebstock and Crooks in 1949 /1/. Chloramphenicol is manufactured by EGIS Pharmaceuticals Co. by a diastereospecific synthesis, after resolvation the only pharmacologicaly active D(-) threo-1-p-nitrophenyl-2-dichloroacetamido-propane-1,3-diol (1R. 2R) is formed nearly completely.



DL-threo-chloramphenicol



DL-eritro-chloramphenicol

The molecule contains two asymmetric centrums and thus, four enantiomers exist. Kawamura et al. prepared in 1975 the cyclodextrin inclusion complex of chloramphenicol in order to obtain a better solubility in water /2/.

In our examinations we tried to separate the stereoisomers of chloramphenicol by HPLC using cyclodextrin dissolved in the eluent. Cyclodextrines (CyD) are cyclic oligosaccharides built up from six, seven or eight glucopyranose units, according to the number of the units, they are called α-β-γ-cyclodextrins. They are water-soluble since all free hydroxyl groups are on the outer surface of the ring. Their well-known properties to form inclusion complexes with many molecules and ions and the

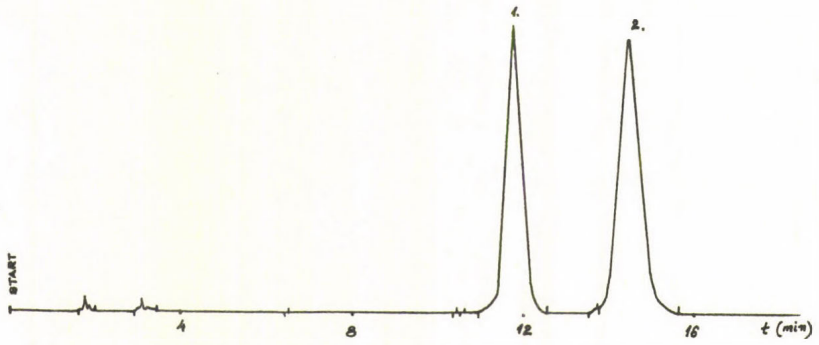


Fig. 1a. Separation of DL-eritro and DL-threo chloramphenicol. Chromatographic conditions: column: 250 x 4.6 mm. ID, packed with LiChrosorb 10 RP-18; mobile phase: 30:25:50 ethanol-water-phosphate-buffer (pH 7.6); flow rate: 1 ml/min; detection at 278 nm.
Peaks: *1* DL-eritro-chloramphenicol (8 µg) (K′ = 5.5)
2 DL-threo-chloramphenicol (8 µg) (K′ = 7.00)

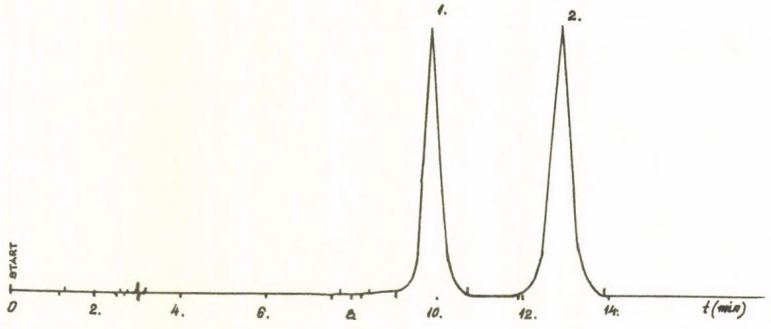


Fig. 1b. Separation of DL-eritro and DL-threo-chloramphenicol. Chromatographic conditions: column: 250 x 4.6 mm. I.D., packed with LiChrosorb 10 RP-18; mobile phase: 30:25:50 ethanol-water-phosphate-buffer (pH 7.6); flow rate: 1 ml/min.; detection at 278 nm.
Peaks: *1* DL-eritro-chloramphenicol (8 µg) (K′ = 4.5)
2 DL-threo-chloramphenicol (8 µg) (K′ = 5.8)

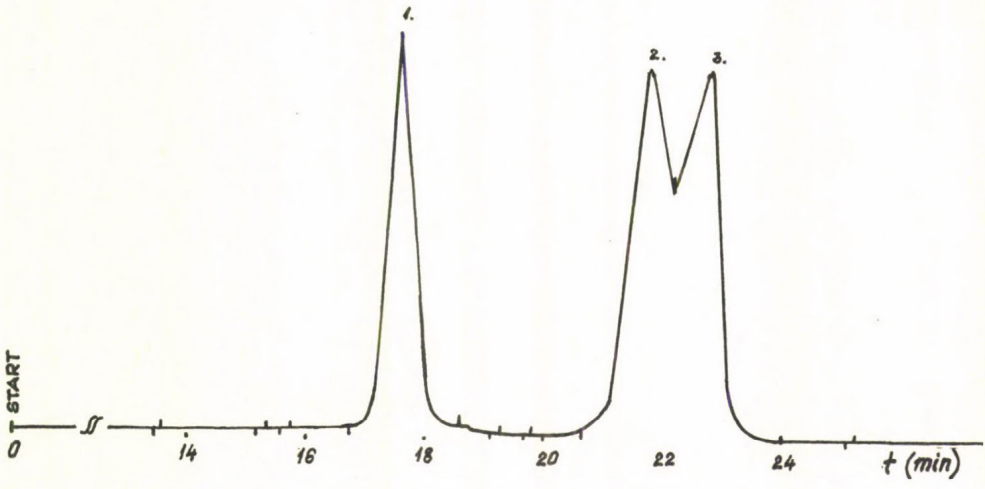


Fig. 2. Separation of DL-eritro and DL-threo-chloramphenicol. Chromatographic conditions: column: 250 x 4.6 mm I.D., packed with LiChrosorb RP-18; mobile phase 2% β CyD in 10:50:40 ethanol-phosphate-buffer (pH 7.6) -water; flow rate: 1 ml/min.; detection at 278 nm.
Peaks: *1*. DL-eritro-chloramphenicol (8 µg) (K′ = 8.94)
2. L (+) threo-chloramphenicol (K′ = 11)
3. D (-) threo-chloramphenicol (K′ = 11.62)
Resolution of 2/3 : 0.71

formation of an inclusion complex with CyD alter some physical and chemical properties of the guest molecule /3/.

The analytical application of cyclodextrins has rapidly developed during the last years. They are supplied as a stationary phase as well as a component of the mobile-phase in chromatography.

The β-CyD has 35 chiral atoms and therefore it is useable as an enantioselective agent. It was easy to separate DL-eritro and DL-threo-chloramphenicol from each other without any CyD.

According to preliminary investigations β-CyD was the best for the separation of the enantiomers in comparison with α and γ-CyD. In selecting the proper composition of the eluent we had to take into consideration the solubility of CyD and its ability to form inclusion complex. The solubility of β-CyD is the best in 70:30 water:ethanol and since the phosphate ions do not interact with it we used a phosphate buffer /4/.

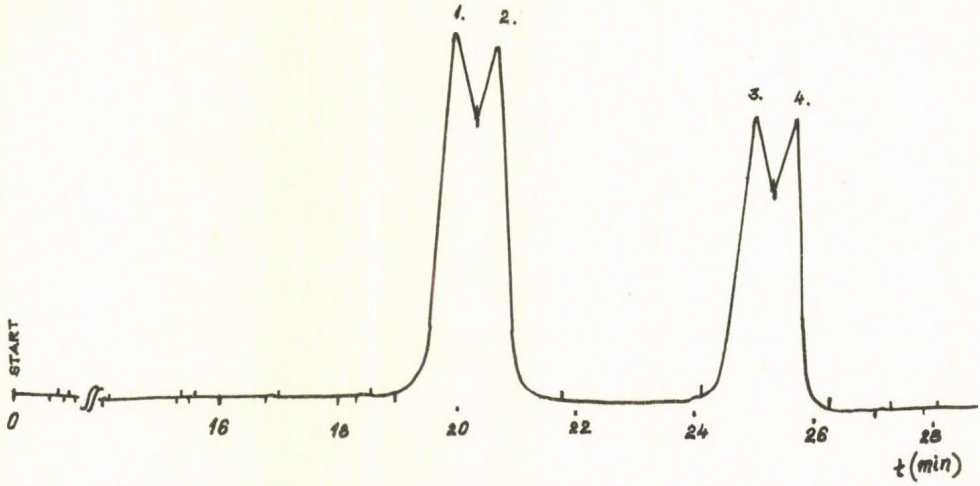


Fig. 3. Separation of DL-eritro and DL-threo chloramphenicol stereoisomers. Chromatographic conditions: Column: 250 x 4.6 mm I.D. packed with LiChrosorb 10 RP-18; mobile phase: 1.6% β CyD in 8:10:80 ethanol-0.1 M sodium acetat-buffer (pH: 5.6) - water; flow rate: 1 ml/min; detection at 278 nm
Peaks. *1*. L(+) eritro-chloramphenicol (K' = 9.76)
2. D(-) eritro-chloramphenicol (K' = 10.2)
3. L(+) threo-chloramphenicol (K' = 12.50)
4. D(-) threo-chloramphenicol (K' = 13.00)
Resolution of 1/2 = 0.77
Resolution of 3/4 = 0.80

2% of β-CyD was added to the same eluent as listed in Fig. 1, however, while we observed a shorter retention time, no resolution of the enantiomers was obtained.

We have changed the composition of the eluent and we have found that while ethanol is necessary for the elution, above 10% the system becomes unfavourable for the chloramphenicols to keep the β-CyD complex forms.

Dissolving 2% of β-CyD in a 10:50:40 mixture of ethanol-phosphate buffer (pH 7.6) - water, we only obtained same resolution of the D and L-threo-chloramphenicols.

Next we change the pH (pH 4-7.6) and the relative volume of the buffer solution but no important improvement was found in the resolution. We also tried to use 0.1 M sodium acetate solution instead of the phosphate buffer and it resulted in a better resolution between the eritro and threo-chloramphenicols.

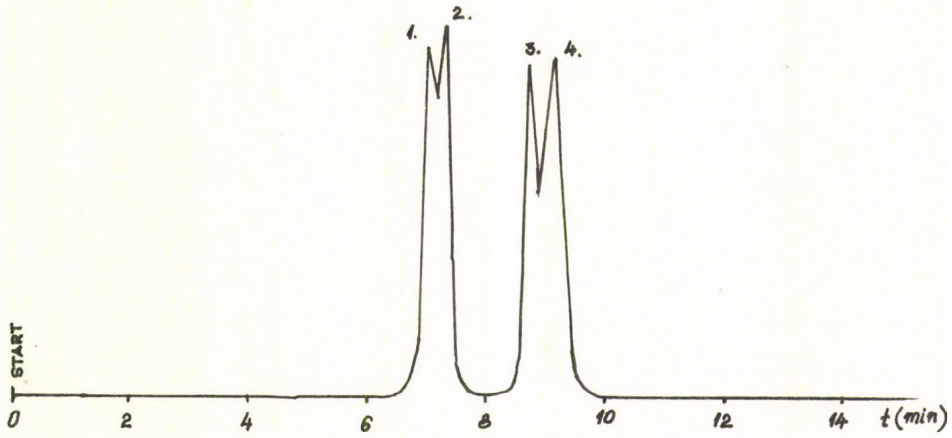


Fig. 4. Separation of DL-eritro and DL-threo-chloramphenicol steroisomers. Chromatographic conditions: Column: 125 x 4 mm I.D. packed with LiChrosorb 5 RP-18 (HIBAR); mobile phase: 2% β CyD in 10:50:40 ethanol-phosphate-buffer (pH 7.6)-water; flow rate: 1 ml/min. detection at 278 nm.
Peaks: *1*. L(+) eritro-chloramphenicol (K′ = 5.8)
2. D(-) eritro-chloramphenicol (K′ = 6.0)
3. L(+) threo-chloramphenicol (K′ = 7.4)
4. D(-) threo-chloramphenicol (K′ = 7.8)
Resolution of 1/2 = 0.54
Resolution of 2/3 = 0.70

Using a 125 x 4 mm I.D. C_{18} column with 5 µm particle, we obtained better results. Working with the same eluent as for Fig. 2, the retention times decreased by 50% and even the eritro enantiomers were slightly separated.

Applying the conditions given in Fig. 3, we also obtained a decreased retention and a certain resolution which is good enough for our primary purpose.

As shown could achieve the separation of the DL-eritro and DL-threo diastereomers from each other and the method is satisfactory for the identification of the four enantiomers. However, the obtained resolution is not enough to determine the optical purity of chloramphenicol. It is very difficult to work with an eluent containing dissolved CyD because this macromolecule can precipitate very easily. The precipitation causes clogging on the top of the column, in the tubings or in the detector. Sometimes it is difficult ot know whether the in-

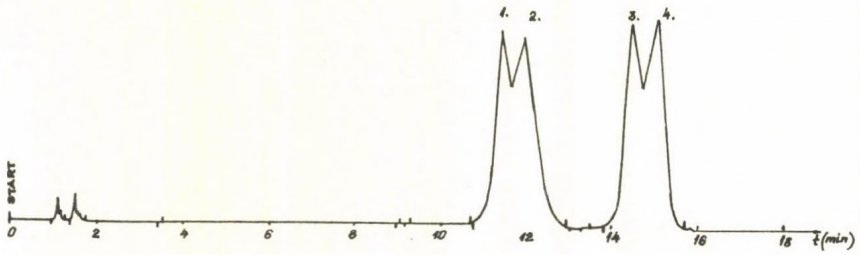


Fig. 5. Separation of DL-eritro and DL-threo-chloramphenicol stereoisomers. Chromatographic conditions: Column: 125 x 4 mm I.D. packed with LiChrosorb 5 RP-18 (HIBAR); mobile phase: 1.6% β CyD in 8:10:80 ethanol- 0.1 M sodium acetat(-buffer (pH 5.6)-water: flow rate: 1 ml/min.; detection at 278 nm.
Peaks: *1.* l(+) eritro-chloramphenicol (K' = 9.50)
2. D(-) eritro-chloramphenicol (K' = 9.90)
3. L(+) threo-chloramphenicol (K' = 12.20)
4. D(-) threo-chloramphenicol (K' = 12.8)
Resolution of 1/2: 0.68
Resolution of 3/4: 0.80

proper composition of the eluent or the clogging in the system results in peak deformation and poor resolution.

ACKNOWLEDGEMENT

We should like to thank Dr. J. Szejtli and Mrs. Wichmon for their kind help in providing the cyclodextrins and for useful advices.

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Budapest, 1986

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INVESTIGATIONS OF THE STABILITY OF CYTOSINE ARABINOSIDE IN INJECTION SOLUTIONS BY HPLC METHOD

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SUMMARY

Dependence of the stability of cytosine arabinoside (4-amino-1- β -D-arabinofuranosyl-2/1H/ pyrimidone = Ara C) in injection solutions and in relation to temperature and pH has been investigated.

The deamination rate of Ara-C to arabinosyluracyl was controlled by HPLC using a 250 x 4 mm stainless steel column packed with LiChrosorb RP-18 (particle size 10 um).

A 0.2 M aqueous KH_2PO_4 solution was used as the mobile phase, the flow rate was 2 ml min^{-1}.

INTRODUCTION

Cytosine arabinoside (cytarabine, Ara-C) is used for the treatment of leukemia. It is especially effective against myeloblastic leukemia but its scope is broader when applied in combination with other antitumor drugs.

Susceptibility of the drug towards deamination, which leads to inactive uracil derivative (Ara-U), /1, 2/ constitutes some inconvenience in its clinical application.

There are two pharmaceutical forms of Ara-C: the injection solution and after freeze drying. Both of them were investigated for stability in relationship to technological requirements.

METHODS AND RESULTS

Analytical methods for the determination of Ara-C are given

in BP/3/ and USP XXI/4/. According to BP-80 Ara-C is assayed by nonaqueous titration with perchloric acid in the presence of 2-naphthylbenzoine. Impurities are determined by TLC in reference to a standard Ara-C solution; the level of impurities should not exceed 0.5%.

Determination of the Ara-C content in the substance as well as in the lyophilisate is, according to USP XXI, based on UV measurement, preceded by TCL purification.

HPLC (on anion exchangers of reversed-phase columns) has also been applied /5, 6/ for the determination of Ara-C derivatives in body fluids.

These methods proved as inadequate for the analytical control of Ara-C synthesized according to the IPF method and of its pharmaceutical forms. The compound being obtained from cytidine via acetylsalicyloyl chloride isomerisation may contain the following contaminations: cytidine, 2,2'-anhydro-1-β-d-arabinofuranosylcytosine and 1-β-D-arabinofuranosyluracyl.

It has been found that such a mixture can be efficiently analysed under the following conditions:

- LKB modular liquid chromatograph, pump model 215 equipped with Spectra Rapid detector and M-20 Olivetti computer,
- stainless steel column, 250 x 4 mm I.D., packed with Lichrosorb RP-18,
- mobile phase, 0.2 M aqueous KH_2PO_4. Solution flow rate 2 ml/min,
- injections: 20 µl of 1 mg/ml solution in the mobile phase.

Retention times:	2,2'-anhydrc Ara-C	- 3.04 min
	cytidine	- 4.40 min
	cytarabine	- 5.45 min
	arabinosyluracyl	- 8.14 min

Quantitative determination of Ara-C concentrations was carried out by external calibration with a reference compound (Fluka). In absence of arabinouracyl reference the degradation product concentration is expressed as % of the total peak area. The results are presented in Table I.

Table I. Determination of cytosine arabinoside (cytarabine)

Sample	time storage in months	temperature °C	pH	Assay Cytarabine in mg/ml*	Assay Ara-U
1	2	3	4	5	6
1+buffer	0	-	6.60	20.5	absence
1 "	3	8	6.80	20.3	absence
1 "	6	8	7.30	20.4	< 0.5%
1 "	12	8	7.35	20.3	< 0.5%
1 "	1	20	6.80	20.4	< 0.5%
1 "	3	20	7.00	20.3	< 0.5%
1 "	6	20	7.10	20.2	> 0.5%
1 "	1	37	8.45	19.5	> 0.5%
1 "	3	37	8.65	18.0	> 0.5%
2+buffer	0	-	8.50	20.2	absence
2 "	3	8	8.55	20.1	absence
2 "	6	8	8.55	20.0	< 0.5%
2 "	12	8	8.65	20.0	< 0.5%
2 "	1	20	8.55	20.1	< 0.5%
2 "	3	20	8.60	20.1	< 0.5%
2 "	6	20	8.75	19.9	> 0.5%
2 "	1	37	8.85	19.1	> 0.5%
3 "	0	-	6.70	20.4	absence
3 "	3	8	6.60	20.2	absence
3 "	6	8	6.80	20.3	< 0.5%
3 "	12	8	6.90	20.2	0.5%
3 "	1	20	6.80	20.3	0.5%
3 "	3	20	6.90	2.02	0.5%
3 "	6	20	7.40	20.0	0.5%
3 "	1	37	8.65	19.8	0.5%
4 lyophil.	0	-	7.35	103 mg/amp	absence
4 "	12	8	7.45	104 mg/amp	absence
4 "	12	20	7.55	103 mg/amp	absence
4 "	12	37	7.45	102 mg/amp	0.5%
4 "	12	50	7.40	99 mg/amp	0.5%

*Precision of the Procedure
Concentration of the cytarabine (mg/ml)
20.25; 20.00; 20.15, 19.90; 20.50; 20.54; 19.60; 19.85; 19.75
Number of Measurements 9
Mean 20.06
Standard Deviation 0.325
Relative Standard deviation 1.33%

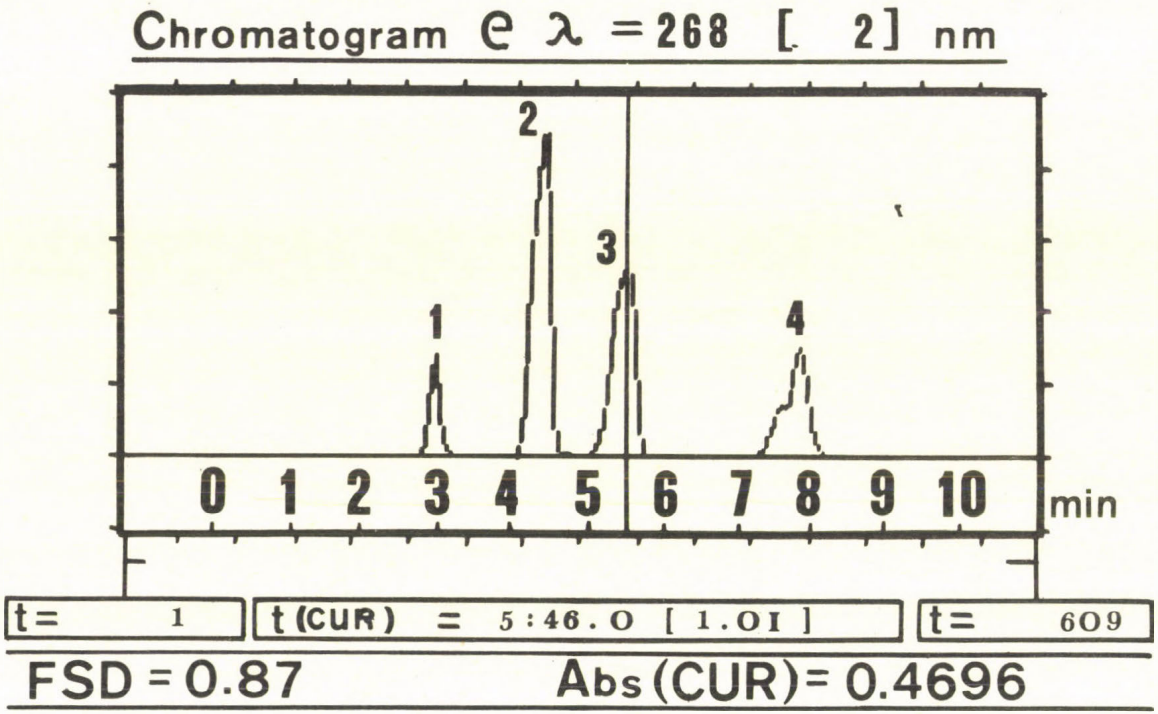

Fig. 1. Chromatogram of a mixture of 2,2'-anhydro Ara-C (1), cytidine (2), cytarabine (3) and arabinosyluracyl (4)
Column: 250 x 4 mm, Lichrosorb RP-18; mobile phase: 0.2M KH_2PO_4 water solvent, flow rate 2 ml min^{-1}

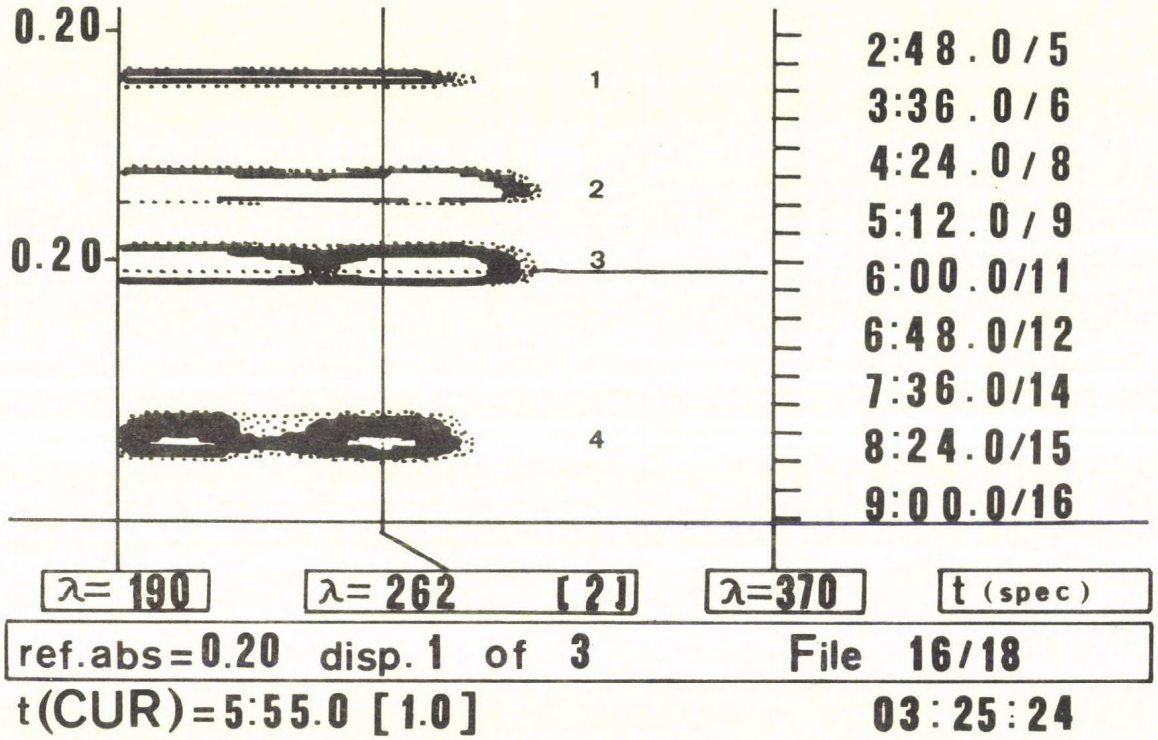

Fig. 2. Isogram of a mixture of 2,2'-anhydro Ara-C (1), cytidine (2), cytarabine (3) and arabinosyluracyl (4)

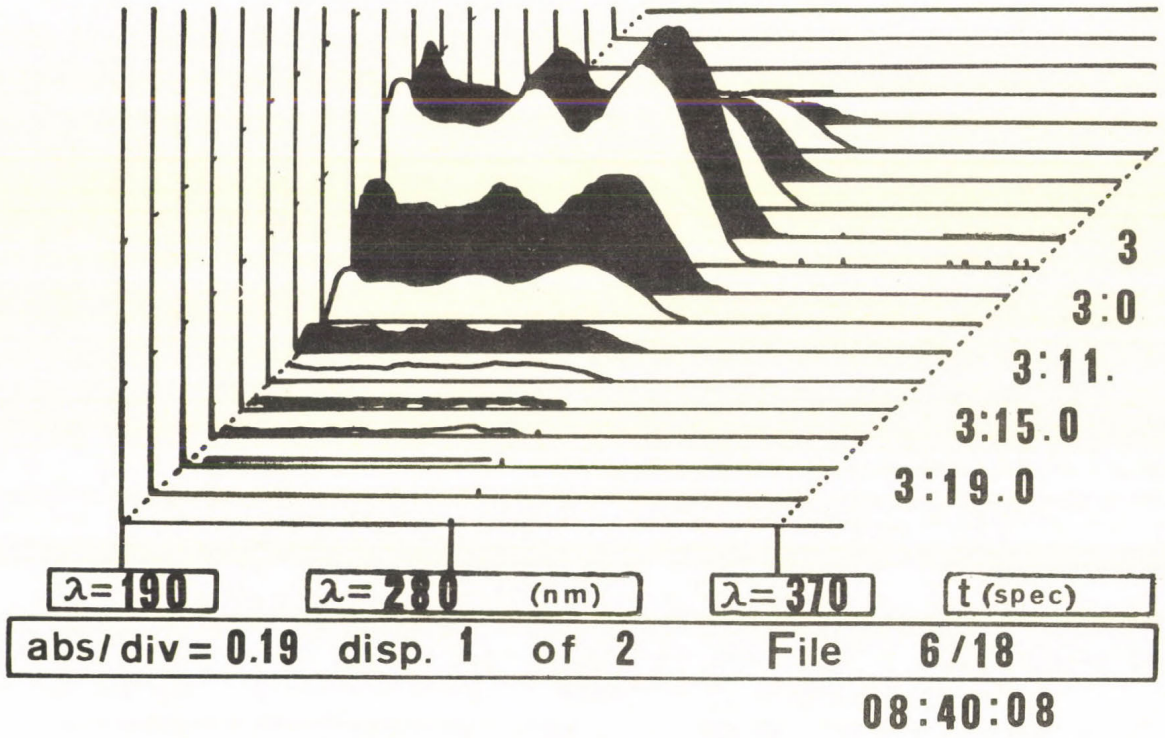

Fig. 3. Topogram. Spectrum of 2,2'-anhydro Ara-C

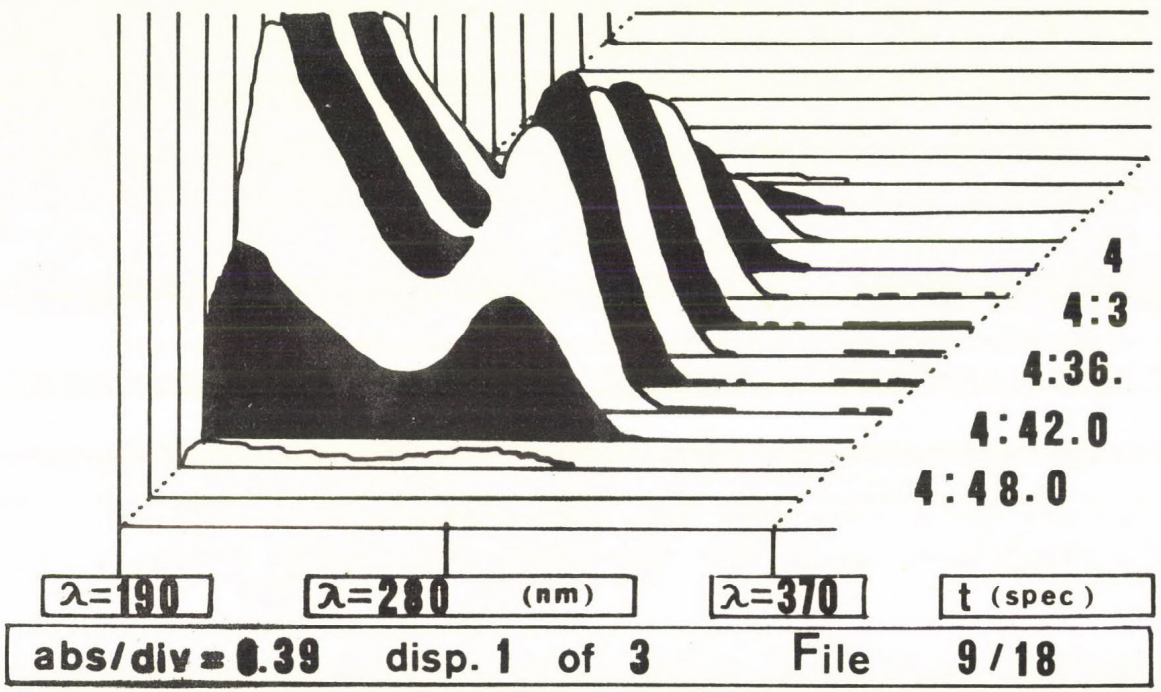

Fig. 4. Topogram. Spectrum of cytidine

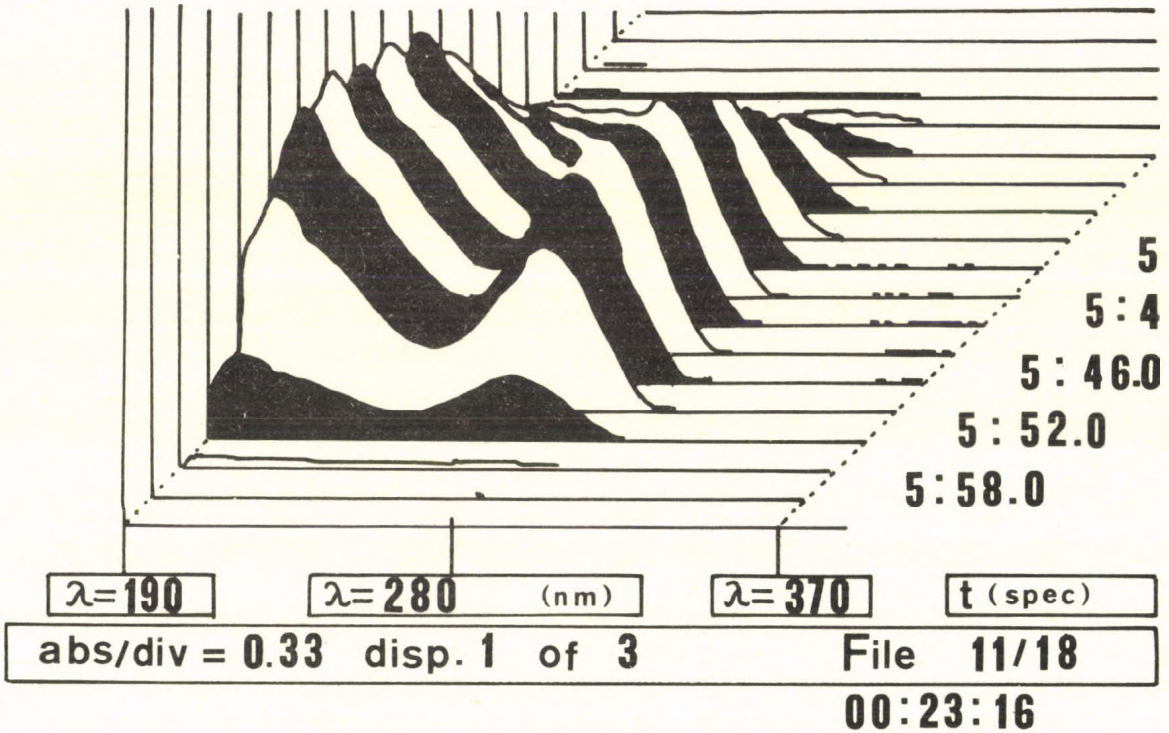

Fig. 5. Topogram. Spectrum of cytarabine

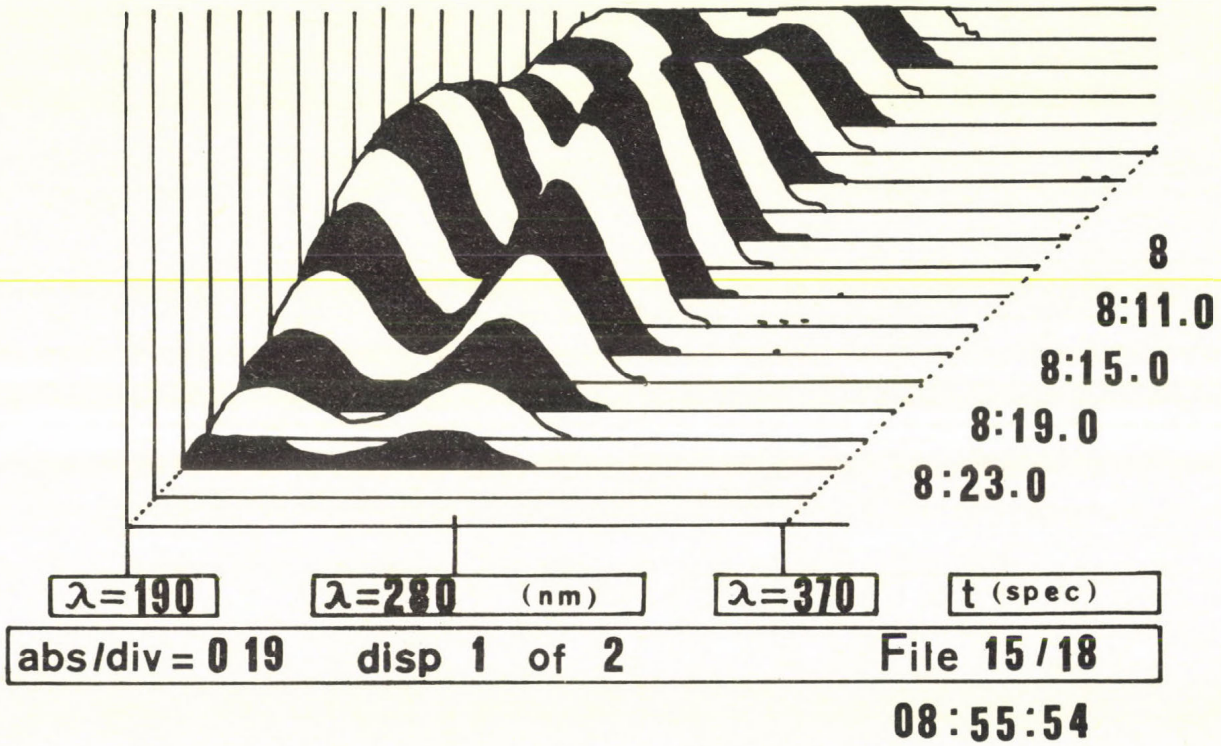

Fig. 6. Topogram. Spectrum of arabinosyluracyl

CONCLUSIONS

Conditions for the HPLC determination of Ara-C in substance and in pharmaceutical forms have been elaborated. It has been determined that the storage temperature is critical for the stability of the injection solutions. When stored at room temperature the samples, buffered or not, contain about 0.5% of arabinosyluracyl after 3 months. Samples stored below 8°C showed good stability through a 12 months period (less than 0.5% contaminant).

The lyophilisate was shown to be stable at room temperature as well as at 37°C during a 1-year storage.

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HPLC DETERMINATION OF FUROSEMIDE IN BLOOD

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INTRODUCTION

Furosemide is a wide-spectrum diuretic agent, its active ingredient is 4-chloro-N-furfuryl-5-sulphamoyl-anthranilic acid, a light sensitive substance, stable in alkaline media/1/.

It inhibits the Na^+ and Cl^- ion transport in the ascending branch of the Henle's loop and its efficacy is independent of the acid-base conditions. Upon oral administration it is absorbed rather rapidly and its diuretic effect appears within 30 minutes. According to Andreasen's /2/ investigations 90-95 % of the substance is bound to proteins,and almost 60 % is excreted in the urine, part of which in the form of glucuronide. Its main metabolite is 4-chloro-5-sulphamoyl-anthranilic acid (CSA) /3/.

DETERMINATION OF FUROSEMIDE BY HPLC

For the determination of furosemide the following methods are described in the literature:

1. Colorimetry /4/
2. UV Spectrophotometry /5/
3. Fluorometry /6/

Furosemide at the detection by UV spectrophotometry in acidic media shows wavelength maxima at 235, 275 and at 340 nm while in alkaline media the maxima are at 229, 272 and 335 nm.

Ghanekar and coworkers /1/ used a 75:25 mixture of 0.01 M $(NH_4)HPO_4$:methanol as the mobile phase for stability investiga-

tion of furosemide tablets and injection. According to Lin and coworkers /7/ better results are obtained with a pH 5 eluent (acetate buffer: methanol), although at this pH value the detectability limit of furosemide is higher (1000 ng/ml), while at the same time, the stability of the solution is greater. They have used this method for the determination of furosemide in urine and in plasma, after suitable extraction.

Most of the investigators have used more acidic conditions, for example, Blair and coworkers /8/ carried out the separation with 2.5 phosphate buffer solution using an ion-exchange column. Lagerström /9/ used acetonitrile:phosphate buffer mixture as the mobile phase and RP-8, or RP-18 as stationary phase, respectively. No appreciable differences were found in the sensitivities between fluorescence and UV detection (0.2 µmol/l).

Nation and coworkers /10/ achieved the same detection limit of plasma concentrations of furosemide as above (0.1 µg/ml) using a Schoeffel fluorometer. Rapaka and coworkers /11/ also used a Schoeffel fluorometer, but their detectability limit of furosemide was significantly lower, at 20 ng/ml, utilizing phosphoric acid:acetonitrile mobile phase on a column packed with RP-18 stationary phase.

HPLC determination of drug contents in biological samples were carried out by applying routine precleaning in order to prevent contamination of the column. A number of papers deal with the removal of proteins by means of solvents. Nation and coworkers /10/ use acetonitrile, while Lin and coworkers /7/ use methanol for the removal of proteins. Rapaka and coworkers /11/, apart from the removal of proteins by acetonitrile, use a precolumn, in order to remove the interfering materials from the sample prior to its application onto the analytical column. In the case of urine samples direct injection has also been applied, e.g., by Lagerström /9/. The minimum detection level of furosemide was 100 ng/ml, with an error of $\pm$ 10 %.

Hajdu and Haussler /12/ used ether after acidification to extract furosemide from serum. Forrey and coworkers /13/ also used ether for the extraction of the acidified sample and considered further extractions with 0.1 M phopshate buffer

(pH:7) necessary. After acidification they directly injected the solution on the column.

Swezey and coworkers /14/ also considered purification by stepwise extraction necessary. After acidifying the biological fluid with HCl following ether extraction it is separated by freezing and the aqueous layer is discarded. This aqueous alkaline solution (containing the furosemide) is acidified and is injected on the column.

Katsuyoshi Uchino and coworkers /15/ utilize a more complex extraction purification method. Dichlormethane is used as the organic phase while a pH 1.5 phosphate buffer is used for acidification. Finally the organic phase is evaporated and furosemide is dissolved in ethanol. In spite of the lengthy procedure 95-100 % recovery is achieved.

According to MacDougall and coworkers /16/ the sample is first treated by zinc sulphate - barium hydroxide to remove protein. Subsequently, the supernatant is diluted with NaCl, acidified, extracted with ether and alkalified. Ether extraction is repeated, the sample is evaporated to dryness, and dissolved in phosphate buffer. Both the furosemide and CSA contents of the sample can be quantitatively measured.

EQUIPMENTS AND MATERIALS

Equipments

Centrifuge, Model K 70 (Janetzky, Leipzig, GDR)
Vortex (KUTESZ, Budapest, Hungary)
pH-meter, Model OP 208 (Radelkis, Budapest, Hungary)
HPLC pump, Model BT 3020 (Biotronik, Maıntal, FRG)
Detector: fluorimeter, Model FP 550 (Jasco, Tokyo, Japan)
Recorder: Model 120 Servogor (Goerz, Wien, Austria)
Injector: Model 7125, 20 µl loop (Rheodyne, California, USA)
Column: 25 cm x 4 mm I.D. (Bio-Separation-Technologies, Budapest, Hungary)

Materials

All reagents used were of analytical grade.
Furosemide (Batch No 840304) was obtained from Chinoin.
4-Chloro-5-sulphamoyl-anthranilic acid was prepared by the research laboratory of Chinoin.

METHOD OF HPLC DETERMINATION

Based on the literature a method was developed for the determination of furosemide and the identification of its main metabolite, chloro-sulphamoyl-anthranilic acid (CSA). For the separation a 25 cm x 4.0 mm I.D. column packed with LiChrosorb RP-18 was used. Detection was carried out by a fluorometer. In order to increase the sensitivity of the method and the stability of furosemide, various eluent mixtures were tested.

The most suitable eluent found was a 40:60 mixture of acetonitrile and a phosphate buffer (pH 2.5).

N-benzyl-4-chloro-5-sulphamoyl-anthranilic acid was used as the internal standard.

As we have found no suitable extraction method described in the literature, we have developed our own method. Its schematic is shown in Fig. 1.

Table I lists the absorption and emission spectra of the compounds present in the sample, while Table II gives the analytical conditions and the average retention times found. Table III presents the evaluation of the reproducibility of furosemide (FD) analysis, Fig. 2. shows a typical chromatogram.

PREPARATION OF THE CALIBRATION CURVE

For the analysis of clinical samples we have prepared a calibration curve consisting of five points (Fig. 3.).

To 0.5 ml serum, taken from healthy patients, 0.5 ml distilled water and 200 ng/ml, 500 ng/ml, 1000 ng/ml, 1500 ng/ml and 2000 ng/ml concentrations of furosemide solution and 1000 ng/ml concentration of internal standard solution was added. Extraction was carried out according to the method explained in Fig.1.

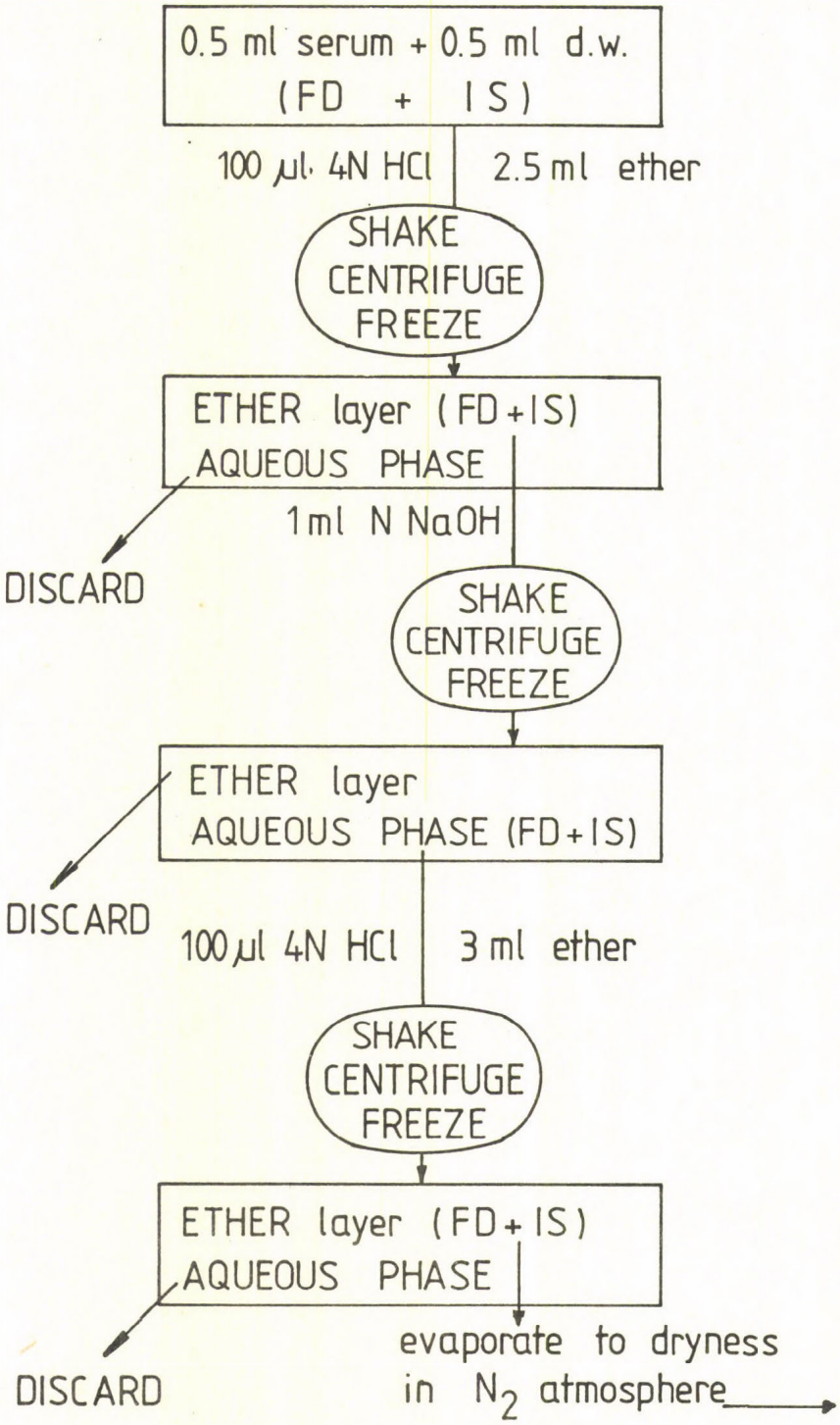
Table I. Absorption and emission spectra of the compounds investigated

Compound	Absorption max. nm	Emission max. nm
Furosemide (FD)	252, 291, 318, 365	406
Chloro-sulphamoyl-anthranilic acid (CSA)	248, 287, 355	399
N-benzyl-4-chloro-5-sulphamoyl-anthranilic acid (internal standard) (I.S.)	324, 370	414

Table II. Analytical conditions and results

Column dimensions	25 cm x 4.0 mm I.D.
Column packing	LiChrosorb RP-18, 10 µm
Mobile phase	40:60 acetonitrile - pH 2.5 phosphate buffer
Flow rate	1.5 ml/min
Excitation wavelength	365 nm
Emission wavelength	406 nm
Temperature	25°C (room temperature)
Retention times* of	
CSA	1'50''; 2'05'' (double peak)
FD	5'17''
I.S.	8'08''

*For the meaning of the symbols of the compounds see Table I.


0.5 ml serum + 0.5 ml d.w.
(FD + IS)
100 µl 4N HCl
2.5 ml ether
SHAKE
CENTRIFUGE
FREEZE
ETHER layer (FD+IS)
AQUEOUS PHASE
1 ml N NaOH
DISCARD
SHAKE
CENTRIFUGE
FREEZE
ETHER layer
AQUEOUS PHASE (FD+IS)
DISCARD
100 µl 4N HCl
3 ml ether
SHAKE
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FREEZE
ETHER layer (FD+IS)
AQUEOUS PHASE
DISCARD
evaporate to dryness
in N_2 atmosphere

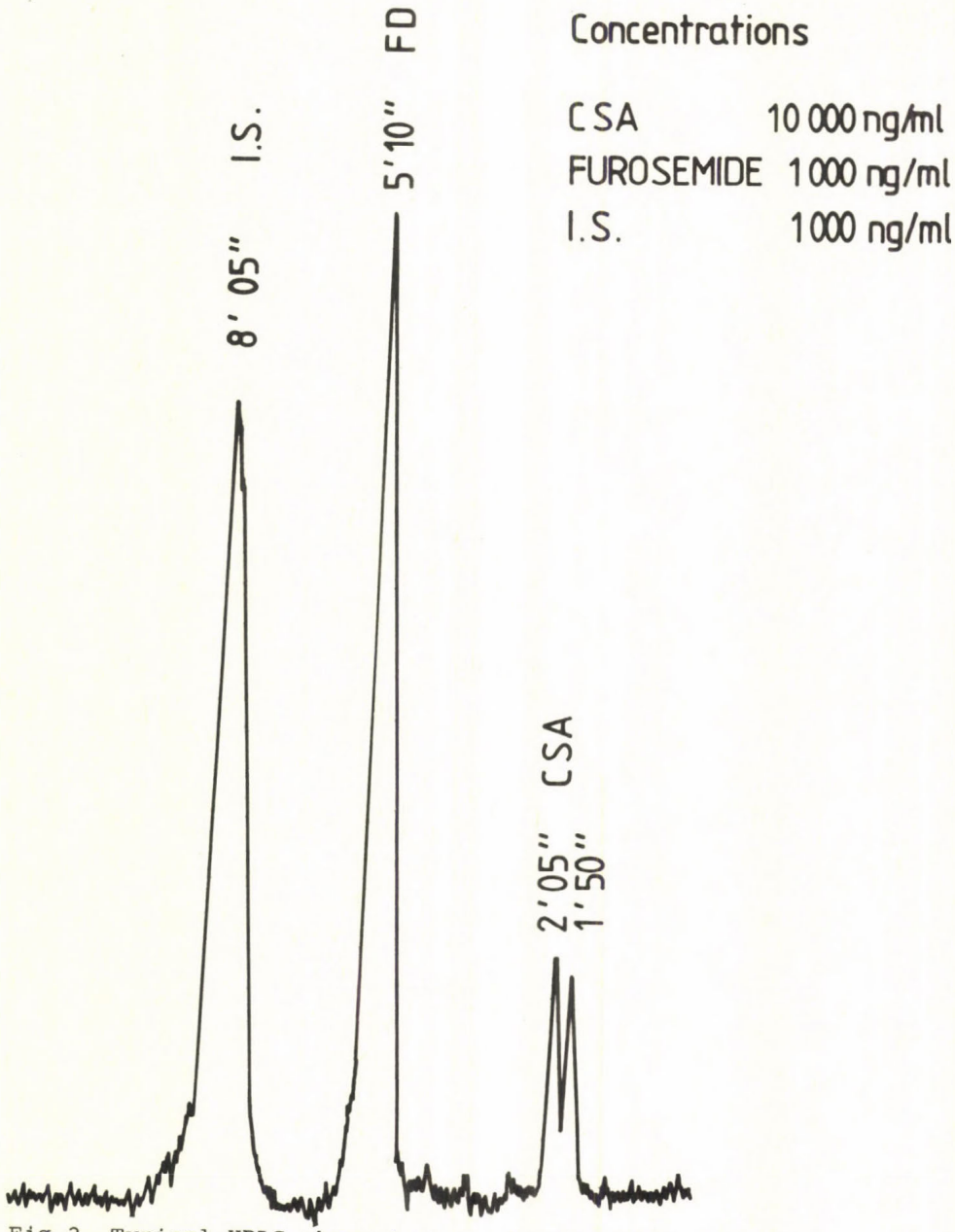

Fig.2. Typical HPLC chromatogram. For conditions see text. Concentration of the individual compounds present in the sample: I.S. = 1000 ng/ml: FD = 1000 ng/ml; CSA = 10,000 ng/ml

Fig.1. Flow diagram of the extraction procedure (FD = furosemide, I.S. = internal standard)

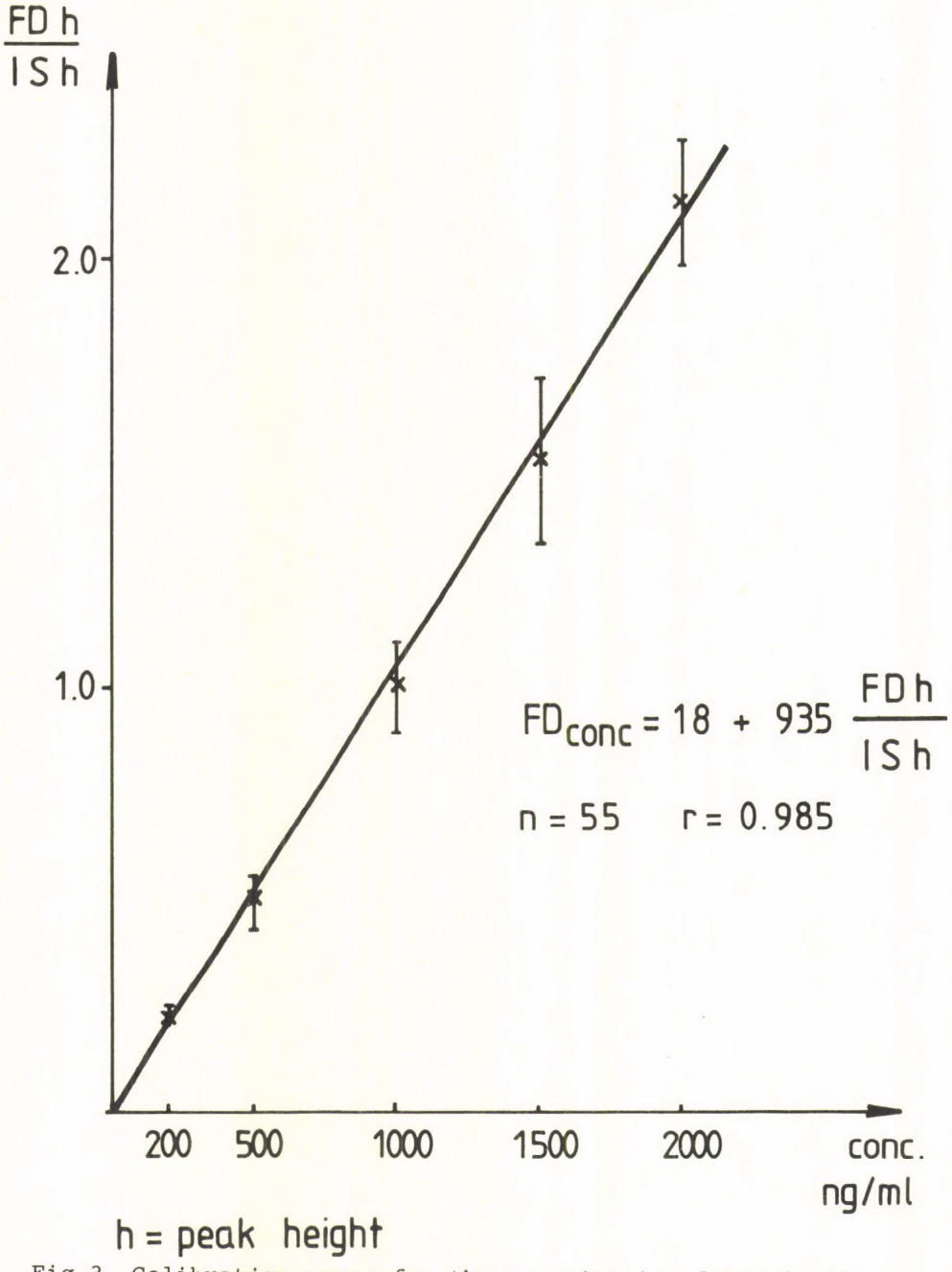

Fig.3. Calibration curve for the quantitative determination of furosemide (FD)
FD_h, IS_h = the peak heights corresponding to furosemide and the internal standard, respectively. The plot corresponds to 55 measurements

Table III. Reproducibility of FD measurement

Concentration of FD added to the serum ng/ml	Concentration ng/ml	mean ± S.D. ng/ml	Rel.st. dev. %	Recovery %
200	161	198 ± 24	12.0	99.0
	214			
	196			
	222			
	196			
500	447	467 ± 17	3.67	93.4
	485			
	459			
	459			
	485			
1000	933	994 ± 54	5.46	99.4
	1064			
	950			
	1029			
	994			
1500	1362	1147 ± 85	7.38	96.4
	1547			
	1512			
	1362			
	1450			
2000	1915	2012 ± 131	6.25	100.6
	2231			
	1906			
	1994			
	2011			

The furosemide content of the extract was determined as described above. The calibration curve is shown in Figure 2. Regression equation (n = 55; r = 0.985)

$$\text{Furosemide conc.} = 18 + 935 \frac{FD_h}{IS_h}$$

where FD_h and IS_h represent the peak heights of furosemide and the internal standard, respectively.
The sensitivity of the method is 50 ng/ml for a signal-to-noise ratio of 2.

ACKNOWLEDGEMENT

The authors thank E. Pálosi for the preparation of chloro-sulphamoyl-anthranilic acid and N-benzyl-4-chloro-5-sulphamoyl-anthranilic acid (used as internal standard) and R. Pálinkás and Zs. Hajnal for technical assistance.

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H. Kalász and L.S. Ettre (Eds)

A NOVEL DESIGN OF A GAS CHROMATOGRAPH FOR FAST CAPILLARY ANALYSIS AND MULTIDIMENSIONAL CHROMATOGRAPHY

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INTRODUCTION

The PU4900 Total Analytical Chromatograph is, as the name suggests, an integrated chromatograph, incorporating in one basic unit a complete analytical system from chromatography through to data handling and report formatting.

The instrument comprises two sections, the chromatography package where chromatography occurs and the electronic package which receives, amplifies and processes the detected signals from the chromatograph. The electronic package has full control of the chromatography via the QWERTY keyboard enabling analytical routines to be set up quickly and efficiently. Each analytical method also contains a programme for full control of integration and report formatting ensuring that the analyst gets the results he wants, when he wants them.

The hardware is capable of expansion by adding satellite chromatograph to the system. This is controlled entirely from the main instrument and data from the satellite is passed to the electronics package for data handling. The satellite can be used either as a stand-alone separation station, up to 15 metres from the main instrument, or can be used in conjunction with the other oven for multi-dimensional, multi-oven work.

The system is capable of complete unattended automation by using the PU4700 microprocessor controlled autojector. Since it is possible to change the analytical method via the sequence table, each sample can be analysed under its own particular conditions.

Expansion of the electronics package is possible and allows simultaneous data collection from three chromatographic sources whether GC or LC, Pye Unicam equipment or other manufacture. Disk and graphics options are available with dual 270 K disk drives enabling storage of raw data, analytical methods and results files for archival purposes or reprocessing at a later date. The graphics option allows on-line display of up to three channels of data collection and postrun display. It is possible to change the integration parameters in the postrun mode to obtain the optimum fit of the baseline for the peaks of interest. The accuracy of the baseline fit can be visually confirmed by expanding the relevant section of the postrun display.

COLUMN OVEN DESIGN

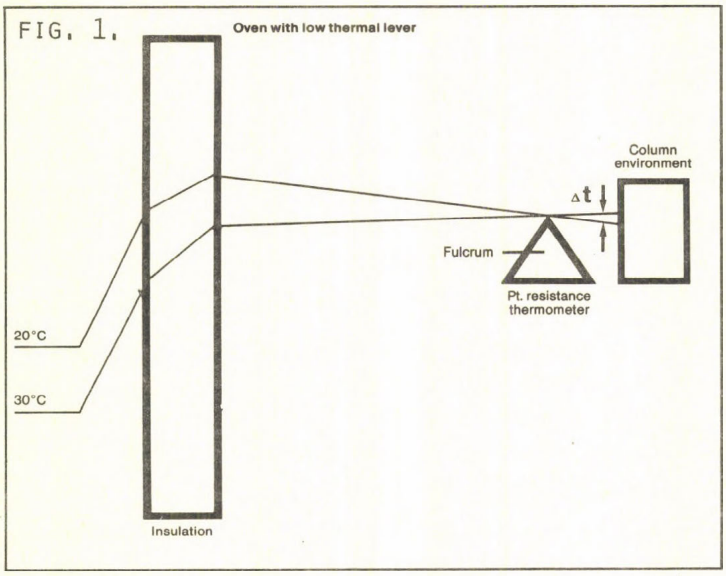
Capillary column chromatography imposes many stringent requirements upon the instrumentation and these requirements have been incorporated into the PU490 during its design and testing stages.

Installation of capillary injectors is possible with the minimum of instrument down time and the extra deep injection port ensures that cold spots in the injector are eliminated.

The range of capillary injection devices, the inlet splitter, the Grob splitless (Universal Injector) and the On-Column Injector are suitable for nearly all applications.

However, it is in the oven design that the biggest advances have been made. The oven has an inner liner which creates a very controlled air flow leading to two zones of very stable temperature. These two zones are the left and right hand column positions. For packed column work, where the large mass of the column makes it less sensitive to fluctuations in oven temperature, the liner can be removed when using large columns.

Despite the oven's small size, accessibility is better than in most chromatographs due to the unique sliding head arrangement whereby the injectors, the columns and the detectors can all be reomoved from the oven simply by sliding them forward. This allows all round 270^{o} of access to the columns, mak-



ing accessory installation as easy as possible. However, the major advantage of the sliding head is that it allows the platinum resistance thermometer (PRT) to be mounted very close to the column. This offers two big advantages. Unlike conventional systems the PRT does not need to be sheathed in steel, to protect it when changing columns, leading to a much more responsive system. It also introduces a low thermal lever (LTL). With the PRT very close to the column, the temperature measured is the actual temperature of the column environment, not the oven temperature some distance away from the column. The LTL is related to the Ambient Rejection Ratio of the oven which is a measure of how efficiently the oven can accommodate changes in ambient temperature and yet still maintain the set temperature. Looking at Figure 1, it can be seen that for an oven with LTL, for a jump in temperature of $10^{\circ}C$ the effect on the column environment, Δt, is much less than an oven without LTL. The effect of this is that, over a 24 hr operating period, retention times will remain more constant despite large changes in the laboratory temperature.

Fast Capillary

Over the past few years the trend in GC has been towards capillary column analysis with its benefits of increased speed and resolution. To derive the maximum advantage from introduction of capillary columns the chromatograph must be designed so that the amplifiers can cope with the very sharp signals resulting from this technique and dead space, resulting in unswept volume within the chromatographic system, is kept to a minimum. Both of these considerations have been taken into account in the design of the PU4900. In changing from packed to capillary columns there is a natural tendency for the chromatographer to fine-tune the chromatographic parameters so that maximum efficiency possible is achieved - a condition that is not always necessary or even desirable if, in achieving it, the analysis time is significantly increased. For many applications, short capillary columns offer adequate resolution with a much reduced analysis time. Short columns are well suited to Quality Control laboratories where a large number of samples need to be analysed in the shortest possible time. Such laboratories are likely to be using packed columns in their routine analysis and will probably resist the change to capillary because such systems often provide more information than is actually required.

An illustration of this was provided in the quality control of essential oils. The customer in question required both to increase the throughput of samples in his laboratory and to exploit the higher efficiency offered by capillary techniques. However, had he changed from the use of packed columns to full length capillary columns, he would have separated many more of the components in the mixture but would not have significantly reduced the analysis time achieved using the existing method. By compromising on column length he achieved the desired reduction in analysis time and obtained results which were superior to those obtained using packed columns. The requirement was for a system which could resolve all components at the 0.1% level and above. One of the analyses regularly carried out at this particular laboratory was that of patchouli oil which can be adulterated with gurjun balsam oil. The incidence of adultera-

FIG. 2 **Pure Patchouli**

Sample	Indonesian Patchouli Oil
Column	7 m WCOT Car 20M
Programme	75°C–230°C at 20°C min^{-1}, 10 min hold at 230°C
Injector Temperature	250°C
Detector	250°C
Carrier	H_2, 2 ml min^{-1}
Detector	F.I.D.
Attenuation	4 x 10
Sample Size	0.5 µl
Injector	Splitter 50:1

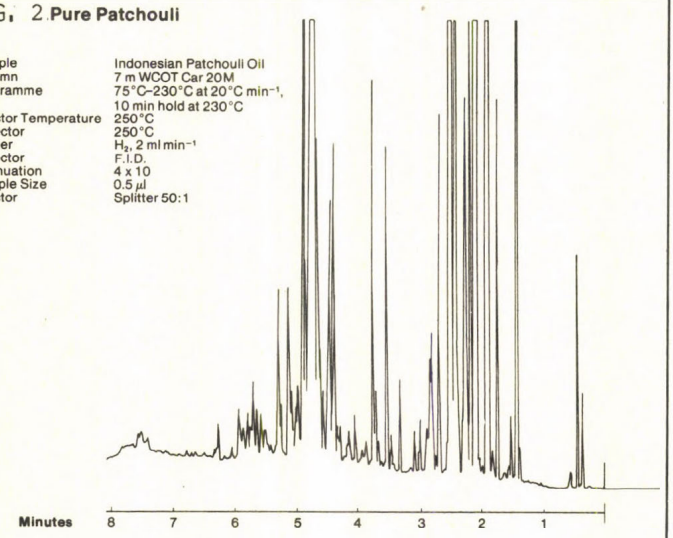
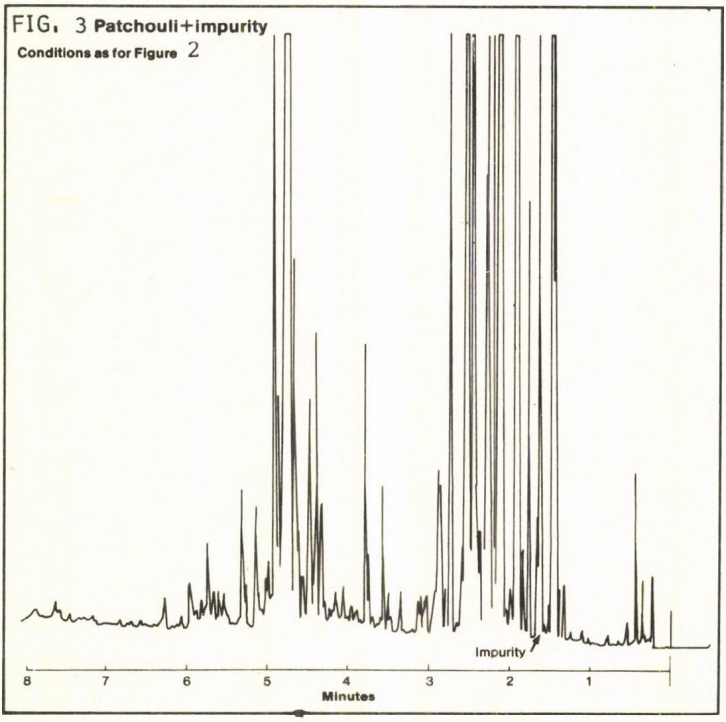


FIG. 3 **Patchouli+impurity**

Conditions as for Figure 2



tion tends to be price related and occurs mostly when the price of patchouli oil is high. It is difficult to detect low concentrations of the gurjun balsam by wet chemical methods because both oils contain large quantities of sesquiterpenes.

Figures 2 and 3 are chromatograms obtained from patchouli oil samples with and without adulterations. The column used was a Carbowax 20 M, 5 m in length. It is interesting that, as the polarity of the stationary phase is decreased, the first contaminant peak elutes earlier and eventually, with a non-polar OV101 column, it is not resolved from the patchouli oil. It was found that using a Carbowax 20 M column 5 m in length adequate resolution could be obtained in 2 minutes comparable to 40 min for a 25 m capillary column and 60 minutes for a packed column.

The technique has also been applied to the analysis of wines for fusel oil content. Fusel oil is the collective name given to the C_3-C_5 alcohols which occur during fermentation in the production of wines and spirits. They are used as a means of characterising wines since, as the wines become fuller and redder so the proportion of fusel oil increases (cf. Figs 4 and 5). This is also true of acetaldehyde which is found in higher concentration in red wines. Screening of fusel oil is, therefore, an important first step in the quality control and classification of wines. As can be seen from Figures 4 and 5 satisfactory chromatograms can be obtained in five minutes using a Carbowax 20 M column 5 m in length.

MULTI-DIMENSIONAL CHROMATOGRAPHY

The three most important forms of column switching are 'back flushing', 'by-passing' and 'heart cutting'.

When the backflushing mode of operation is employed, the higher boiling components in the sample are retained on a pre-column while the components of interest pass on to the analysis column. As the analysis continues on the second column, the first column is flushed backwards to vent with a second supply of carrier gas.

FIG. 4

Sample – Liebfraumilch Wine

Column	5 m x 0.2 mm WCOT Car 20 M
Programme	1 min hold at 60°C then 20°C min^{-1} to 140°C
Injector Temp.	225°C
Detector Temp.	225°C
Carrier	H_2, 2 ml min^{-1}
Detector	F.I.D.
Attenuation	1 x 64
Sample Size	0.5 µl
Injector	Splitter 25:1

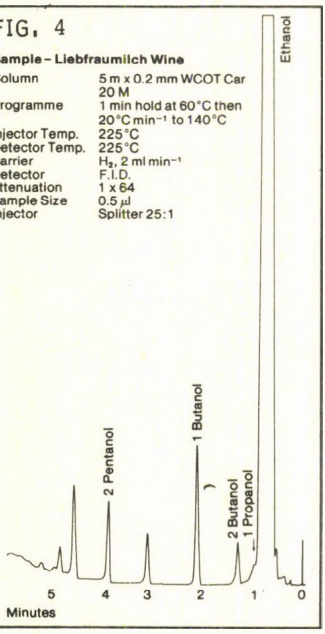


FIG. 5

Sample – Red Wine

Column	5 m x 0.2 mm WCOT Car 20 M
Programme	1 min hold at 60°C then 20°C min^{-1} to 140°C
Injector Temp.	225°C
Detector Temp.	225°C
Carrier	H_2, 2 ml min^{-1}
Detector	F.I.D.
Attenuation	1 x 64
Sample Size	0.5 µl
Injector	Splitter 25:1

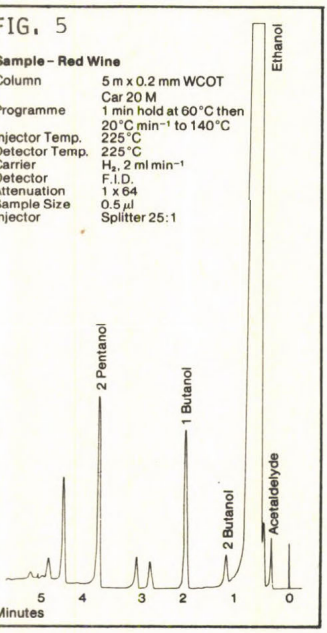
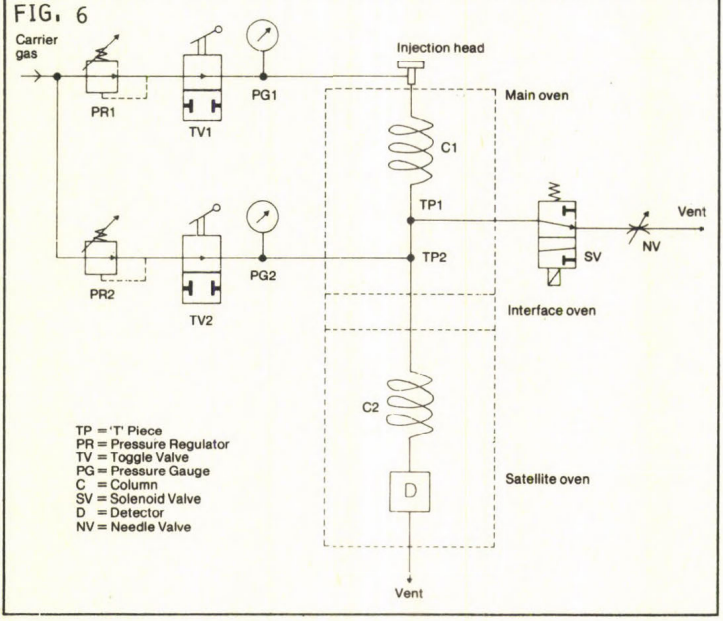


FIG. 6



In the by-pass mode the sample eluting from the first columns in a system can be passed to either of two other columns depending on the components of the mixture and the separation characteristics of the two by-pass columns.

When the component columns of a switching system are housed in separate temperature zones the technique is described as multidimensional chromatography. The design of the PU4900 has taken this requirement into account so that component columns in a chromatographic system can be maintained at different temperatures to suit their separation characteristics.

Switching of the eluent between chromatographic columns during an analysis can be carried out using either pneumatically controlled, low volume, switching valves or by a Deans flow switching technique. The latter method is preferred, offering as it does negligible dead volume and reduced exposure of the sample to metal surfaces on which adsorption might occur. However, should it be required, a valve oven may be mounted on the top of the PU4900 to house the switching valves. The multidimensional system described below uses the Deans technique. The mechanism of the flow switching technique can be explained with reference to Figure 6.

The system is set up as follows:

(a) SV and TV_2 are closed. TV_1 is opened and PR1 adjusted to deliver a suitable flow through C_1 and C_2 to D. The 'mid point' pressure is noted on P.G.2.

(b) TV_2 is opened and PR_2 adjusted to give a pressure reading about 0.5 psig higher than that noted in (a).

The system is now ready for operation.

When SV is opened, the direction of flow is as shown in Figure 7a. When SV is closed, the direction of flow is shown in Figure 7b. In this way a selected portion of the eluent from the first column can be cut to a second column.

FIG. 7a

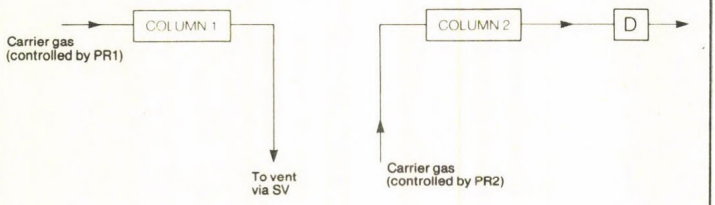
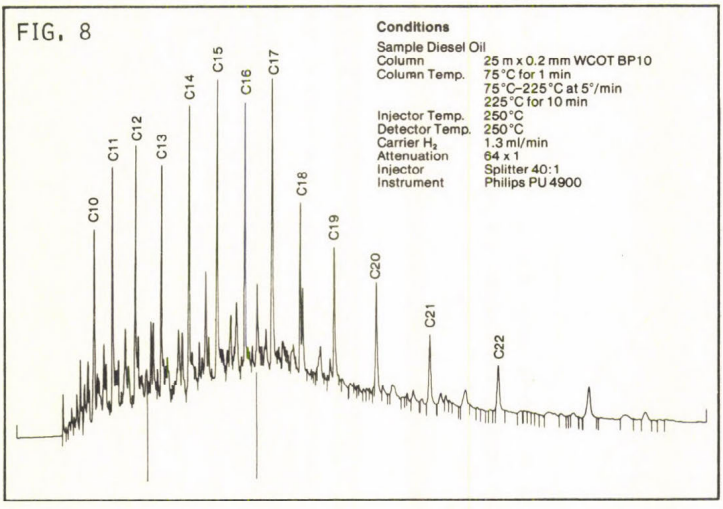


FIG. 7b

FIG. 8



EXAMPLES OF HEART-CUTTING
USING THE PU4900

1. Diesel Oil

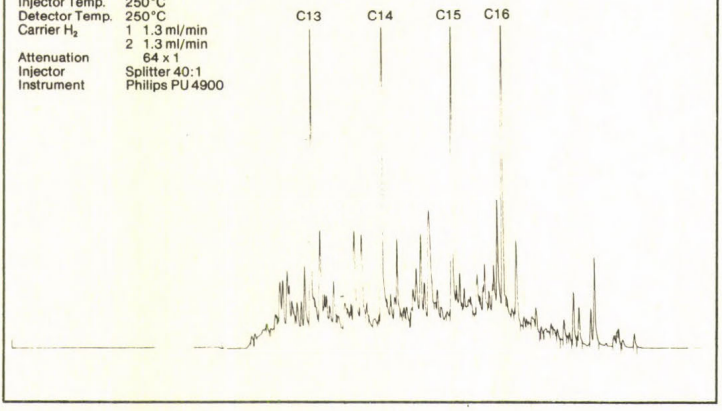
Figure 8 shows a chromatogram of diesel oil obtained using a single capillary column. The second diesel oil chromatogram (Fig. 9) was obtained by passing the eluent cut between C_{13} and C_{16} to a second column where further separation occurs at a slightly lower temperature. It is possible, of course that the separation shown in Figure 8 could be improved by optimising

FIG. 9

Separation of Cut Portion

Conditions
Sample Diesel Oil

Column System	Heart Cut
Columns	1 25 m x 0.2 mm WCOT BP10
	2 25 m x 0.2 mm WCOT FFAP
Column Temps.	1 75°C for 1 min
	75°C–225°C at 5°/min
	225°C for 5 min
	2 100°C for 11 min
	100°C–180°C at 4°/min
	180°C for 5 min
Injector Temp.	250°C
Detector Temp.	250°C
Carrier H_2	1 1.3 ml/min
	2 1.3 ml/min
Attenuation	64 x 1
Injector	Splitter 40:1
Instrument	Philips PU 4900



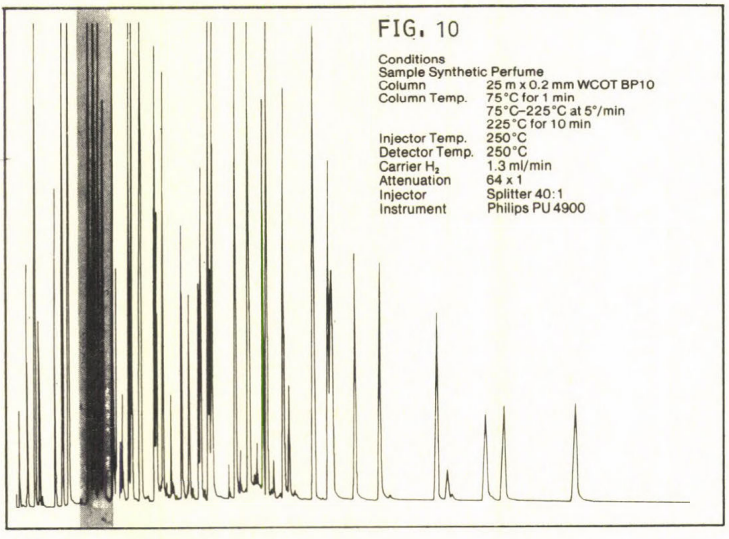


FIG. 10

Conditions
Sample Synthetic Perfume

Column	25 m x 0.2 mm WCOT BP10
Column Temp.	75°C for 1 min
	75°C–225°C at 5°/min
	225°C for 10 min
Injector Temp.	250°C
Detector Temp.	250°C
Carrier H_2	1.3 ml/min
Attenuation	64 x 1
Injector	Splitter 40:1
Instrument	Philips PU 4900

conditions but not in such a marked manner as that achieved in Figure 9. In addition, after the cut has been made, the first column can be rapidly raised in temperature so that the remaining, higher boiling, portion of the samples can be rapidly eluted and the overall analysis time reduced.

2. Synthetic Perfume

Figures 10 and 11 show chromatograms resulting from the analysis of a synthetic perfume with and without heart cutting. Initial separation of the odour-characterising components is carried out on the first column after which these very small concentrations of critical components are switched to the second column where final separation is completed.

3. Anti Knock Additives in Gasoline

Due to recent legislation, lead-based compounds are now being superseded by oxygenated compounds as anti knock additives in gasoline.

Separation of these compounds has been achieved by first carrying out an aqueous extraction of the gasoline. This obviously lengthens the analysis time compared to a direct injection technique and renders quantitation difficult.

FIG. 11
Separation of Cut Portion

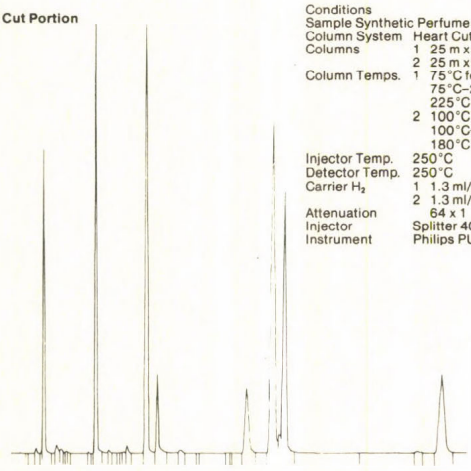


FIG. 12

Conditions	
Sample	Gasoline
Column System	Heart Cut
Column	25 m x 0.2 mm WCOT FFAP,
Column Temp.	35°C for 4.25 min 35°C–150°C at 20°/min 5 min hold at 150°C
Injector Temp.	250°C
Detector Temp.	250°C
Carrier	1.2 ml/min H_2
Detector	F.I.D.
Attenuation	256 x 1
Sample Size	0.25 µl
Injector	Splitter 40:1
Instrument	Philips PU 4900
1st column only	

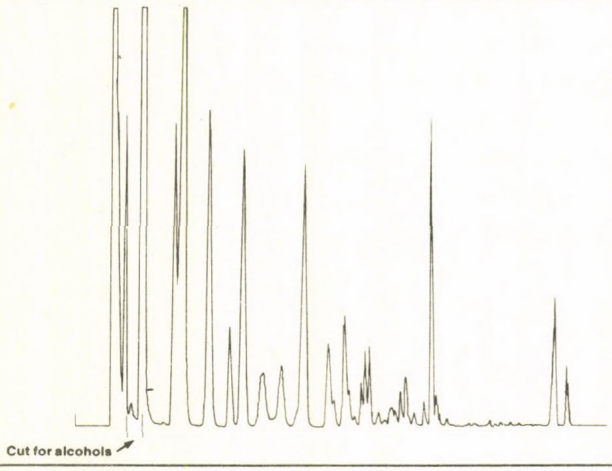


FIG. 13

Conditions	
Sample	Gasoline
Column System	Multi dimensional
Columns	1 25 m x 0.2 mm WCOT FFAP 2 25 m x 0.2 mm WCOT BP10
Column Temp.	1 35°C for 4.25 min 35°C–150°C at 20°/min 5 min hold at 150°C 2 50°C for 4.25 min 35°C–150°C at 20°/min 5 min hold at 150°C
Injector Temp.	250°C
Detector Temp.	250°C
Carrier	1 1.2 ml/min H_2 2 1.5 ml/min H_2
Detector	F.I.D.
Attenuation	256 x 1
Sample Size	0.25 µl
Injector	Splitter 40:1
Instrument	Philips PU 4900

Cut taken for C_3–C_4 alcohols

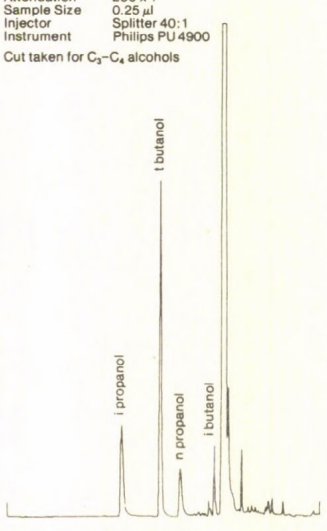
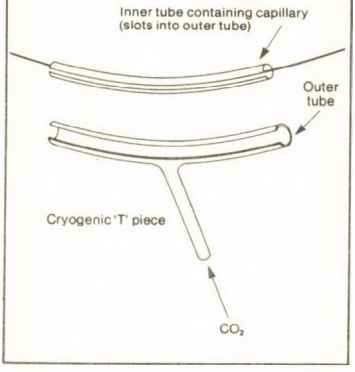


FIG. 14 **Cryogenic trapping device**



It was decided to apply multidimensional chromatography to this analysis since the oxygenated additives (alcohols and ethers) are much more polar than the hydrocarbons of the matrix although they have a similar boiling range. Figure 12 shows a chromatogram of a sample of gasoline eluted from a single FFAP column with the cutting times marked. Figure 13 shows the result of passing this cut portion on to a second (BP10) column. C_3 and C_4 alcohols were well separated from neighbouring hydrocarbon peaks.

CRYOGENIC TRAPPING

During flow switching, due to unavoidable dead space, some diffusion of the peaks does occur, when, for example a selected portion of eluent is passed from the first column to the second column in a heart cut system.

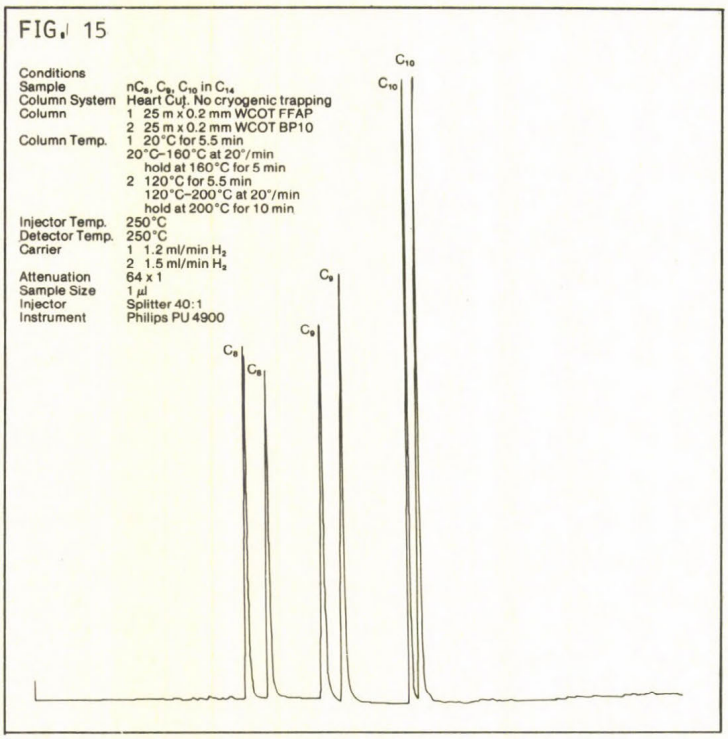
Here, prior to initiating the temperature programme for the second column, the cut components can be 'focussed' by introducing a sub ambient temperature trap at its inlet. This focussing effect is caused by the peaks slowing down in the cryogenic zone and concentrating as a narrow band. When, after focussing, the coolant is switched off and the temperature ballistically raised to its programmed starting point, the components of the mixture are eluted as sharp, Gaussian peaks.

The cryogenic trap consists of a 'T' piece (Fig. 14)the vertical arm of which is an orifice through which coolant (liquid CO_2) is passed. The horizontal arm of the 'T' piece fits snugly round the selected portion of capillary colum because it is bent to form an arc of the same diameter as the column. When the cooling fluid was turned on, with a resistance thermometer secured to the 'T' piece, a temperature of $-60^{o}C$ was recorded.

The efficacy of the trapping device was demonstrated as follows:

1. A solution of normal paraffins (C_8, C_9 and C_{10}) in tetradecane was injected on to the first column of the heart cutting system. After 30 seconds another, identical injection

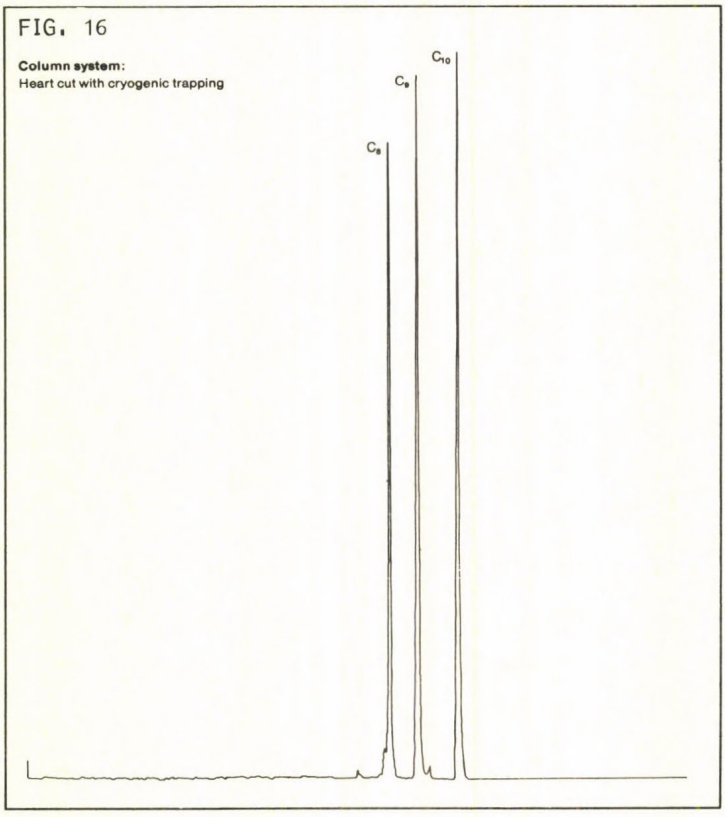
FIG. 15



was made. When the C_{10} from both injections had passed on to the second column the first column was vented to atmosphere and temperature programmed rapidly to remove the C_{14} solvent. The resulting chromatogram showed pairs of peaks, as expected, for C_8, C_9 and C_{10} (Fig. 15).

2. The experiment with "staggered" injections was repeated but with cryogenic trapping at the top of the second column. When all of the C_{10} was eluted to the second column, the cryogenic trapping phase was terminated and the column programmed as in Figure 15. The resulting chromatogram shows single peaks for C_8, C_9 and C_{10} (Fig. 16), indicating that the pairs of peaks had been refocussed in the cryogenic zone.

FIG. 16



SUMMARY

As can be seen from the above data, the PU4900 has been designed as a flexible, sophisticated analysis tool and a high degree of accuracy and repeatability has been achieved. The unique design of the oven has resulted in low thermal gradients giving precise, repeatable control over a wide range of temperature profiles, an ideal basis for high performance capillary chromatography, while the introduction of the satellite and interface ovens, in conjunction with three channel data handling facility, offers unparalleled scope for operation in multi-dimensional chromatographic techniques.

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INVESTIGATION OF THE GAS CHROMATOGRAPHIC CHARACTERISTICS OF FUSED-SILICA CAPILLARY MICRO-PACKED COLUMNS WITH PELLICULAR SORBENTS

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ABSTRACT

Prospects for the GC application of fused-silica capillary micro-packed columns with pellicular sorbents produced for liquid chromatography are shown. These columns are characterized by their high efficiency and relatively small resistance to the carrier gas flow. The inlet pressure is 2-8 times less than for columns with fully-porous particles with the same mean particle diameter. In particular, fused-silica capillary micro-packed columns (6.5 m x 0.38 mm) containing Pellosil HC (30-40 μm) are characterized by an efficiency of 30,000 theoretical plates at a carrier gas (hydrogen) inlet pressure of 4-5 atm. Practical examples of gas chromatographic separations are given.

INTRODUCTION

It has been shown that capillary micro-packed columns (CMPC) are very promising for application in gas chromatography. However, wide practical use of such columns is limited due to the high pneumatic resistance to the carrier gas flow caused by the small diameter of the sorbent particles.

Myers and Giddings /1/ used sorbents with 13-μm particles and, as a result, the inlet column pressure reached 150 atm. The gas chromatographic literature describes micro-packed columns with sorbent particle diameters ranging from 7 to 30-35 μm, the inlet pressure being between 10 to 40 atm. /2-4/.

In capillary micro-packed columns investigated earlier sorbent particles from 5 to 100 μm were used /5-7/. Such columns are characterized by a high efficiency, ease in

utilization and the possibility of application for the solution of a wide range of problems. However, the carrier gas inlet pressure was up to 25 atm.

Therefore, it is of interest to study the possibility of preparing fused-silica capillary micro-packed columns having a low pneumatic resistance. In this respect one may either use relatively short columns operating at a small inlet pressure or substantially increase the column length in order to obtain an enhanced effective separation.

It is known (see e.g., /8/) that the efficiency of columns containing pellicular sorbents depends slightly on the particle diameter which is different from that in the case of columns using conventional fully-porous sorbents where the efficiency significantly depends on the particle diameter. Therefore, when pellicular sorbents are used, the particle size can be raised in order to decrease the pneumatic resistance of the column without an abrupt deterioration of column efficiency.

The purpose of this paper is to study the possibility of using small diameter (less than 40 μm) pellicular sorbents produced for HPLC in fused-silica capillary micro-packed columns. Such sorbents are costly, buth as fused-silica capillary micro-packed columns are miniaturized in size this circumstance is not decisive.

It should be noted that Kirkland /9, 10/ has described the gas chromatographic application of Zipax-type pellicular sorbents (controlled surface porosity sorbents) using 105-150 μm particles in 1.5 mm I.D.columns. Since the internal diameter of fused-silica capillary micro-packed columns is less than 0.4 mm, it is probably reasonable to use smaller particle size in them.

EXPERIMENTAL

Polymer-coated fused-silica capillaries produced in the USSR /11/ and by Scientific Glass Engineering Ltd. Inc. (Australia) were used for column preparation. The length of the columns varied from 40 cm to 10 m. The sorbent layer in

the capillary was fixed at both ends by gas-permeable partitions created at the column ends during its preparation, using some inert granular material and a binding agent /12/.

Pellicular sorbents: Zipax (E.I. du Pont de Nemours Inc., USA), Pellosil HC, 30-40 μm (Reeve Angel, USA), Perisorb A, 30-40 μm (E. Merck, FRG), and Permaphase ETH (E.I. du Pont de Nemours Inc., USA) were used. The columns were packed with these sorbents either directly or after coating the particles with 0.5-1.5 % of some liquid stationary phase.

Fully-porous sorbents: Silasorb 600(LC), 20 μm (Lachema, CSSR), Pragosil, 24-36 μm (Lachema, CSSR), Vydac RP, 10 μm (The Separation Group, USA) were used. These fully-porous sorbents were used to compare the permeability characteristics of the columns.

The investigations were carried out on a standard LKhM-8MD gas chromatograph (Khromatograf, Moscow, USSR).

RESULTS AND DISCUSSION

The results of the investigations of fused-silica capillary micro-packed columns using different sorbents have shown that the columns packed with pellicular sorbents (produced for HPLC) are characterized by the lowest pneumatic resistance. Such columns need an inlet pressure which is 2 to 8 times lower than for columns packed with other sorbents with analogous mean particle size (see Table 1). For the columns packed with pellicular sorbents a high permeability was also observed when they were used in liquid chromatography and it was associated with the stringent spherical shape of the sorbent particles and the narrowness of the fractions /13-14/.

Experimental data on the performance of certain investigated columns are presented in Table 2. From the tabulated data it can be seen that the fused-silica capillary micro-packed columns containing pellicular particles have a fairly high efficiency which is substantially better than for a column with a larger diameter and packed also with a pellicular sorbent.

Table 1. The mean carrier gas (nitrogen) velocity ($\bar{u}$) observed in columns (40 cm x 0.38 mm I.D.; inlet pressure: 6 atm; temperature: 40°C) packed with various sorbents

Sorbent type	Sorbent	$\bar{u}$ cm/sec
Pellicular	Pellosil HC, 30-40 μm	7.6
	Pellosil HC, 30-40 μm + 1 % squalane	8.0
Fully porous	Silasorb 600 (LC) 20 μm	1.0
	Silica gel 30-40 μm	2.0
	Vyolac RP, 10 μm	4.2

Table 2. Chromatographic data for columns packed with pellicular sorbents

Sorbent	Column temperature °C	Chromatographed compound	$HETP_{min}$ mm	C x 10^3 sec
Fused-silica capillary micro-packed columns				
Pellosil HC 30-40 μm	80	n-heptane	0.25	2.6
Pellosil HC, 30-40 μm + 1 % squalane	50	n-hexane	0.21	1.1
		n-heptane	-	0.3
Zipax + 1.5 % 4,4'-azoxy-anisole and 4,4'-azoxy-phenetole (3:2)	98	pseudocumene	0.13	-
1.5 mm I.D. column				
Sorbent with controlled-porosity particles, 105-150 μm	15	n-decane	0.74*	4.5*
		naphthalene	0.67*	

*Calculated from the data given by Kirkland /9/.

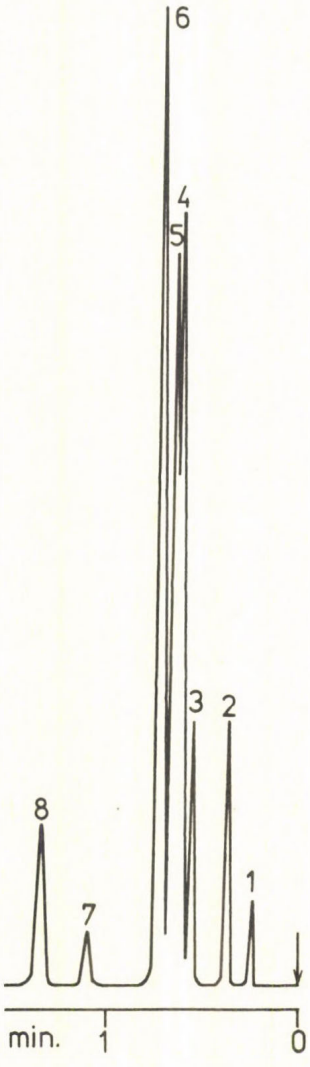

Fig 1. Separation of C_6-C_9 aromatic hydrocarbons.
Column: 49 cm x 0.38 mm I.D.; sorbent: 1.5 % 4,4'-azoxyanisole and 4,4'-azoxyphenetole (3:2) on Zipax; nitrogen pressure: 9.7 atm.; temperature 98°C,
Peaks: 1. benzene
2. toluene
3. ethylbenzene
4. m-xylene
5. p-xylene
6. o-xylene
7. mesitylene
8. pseudocumene

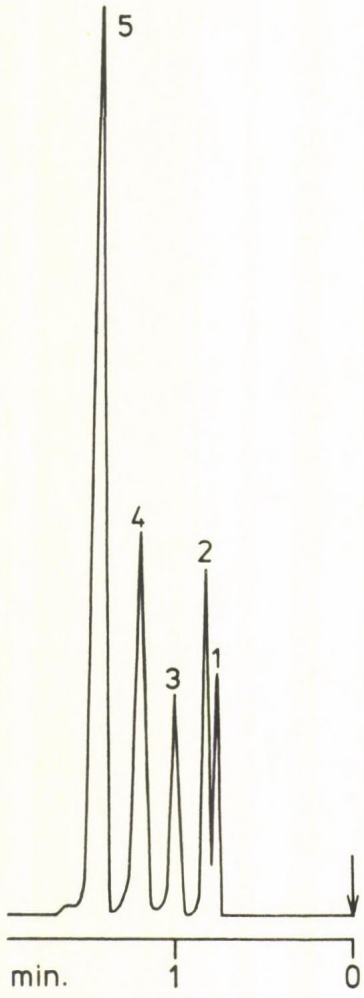

Fig 2. Separation of a mixture of chlorine- and nitrogen-containing compounds.
Column: 1.2 m x 0.25 mm I.D.; sorbent: 1 % Carbowax 20 M on Pellosil HC, 30-40 μm; nitrogen pressure: 4 atm.; temperature: 130°C.
Peaks: 1. chloroform
2. dichlorethane
3. nitromethane
4. 2-nitropropane
5. pyridine

The mass transfer coefficient of the columns under investigation is small, thus allowing the columns to be used at high linear carrier gas velocities without any substantial loss in efficiency.

A column efficiency of up to 40,000 theoretical plates was achieved using columns with Pellosil-type sorbent (column length 8-10 m) at an inlet pressure of about 15 atm.

Examples of the gas chromatographic separation on fused-silica capillary micro-packed columns containing pellicular sorbents (produced for liquid chromatography) are presented in Figs 1-2.

The resulting data show that these columns can be used for the rapid gas chromatographic separation of a wide variety of mixtures. The selectivity of the separation is easily controlled by using sorbents with liquid phases which sharply differ in their polarity (from squalane to tris cyano-ethoxy propane) and which have a certain specific selectivity, such as e.g., liquid crystalline stationary phases. The efficiencies of columns prepared with different liquid stationary phases are fairly consistent.

As a conclusion, in our opinion, due to their merits, fused-silica capillary micro-packed columns may substantially reduce the fields where conventional packed columns are to be applied.

ACKNOWLEDGEMENTS

The authors are grateful to Scientific Glass Engineering Ltd. Pty. (Melbourne, Australia) for supplying the fused-silica capillaries and to E.I. du Pont de Nemours Inc. (Wilmington, DE, USA) for the pellicular sorbents provided for these investigations.

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APPLICATION OF FUSED-SILICA CAPILLARY MICRO-PACKED COLUMNS IN GAS CHROMATOGRAPHY

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SUMMARY

Fused silica capillary micro-packed columns are characterized by their high efficiency, flexibility, mechanical strength and capability of using different sorbents (adsorbents, stationary liquid phases on solid supports, bonded phases, pellicular sorbents, etc.). Based on practical examples these columns are useful in different fields of gas chromatographic separation, especially in fast analysis, separation of gases and low-volatile compounds, and in trace analysis. Columns with an inner diameter varying from 0.25 to 0.40 mm were employed using different types of sorbents for the separation of inorganic gases and low-volatile compounds, various mixtures containing aliphatic, alicyclic and aromatic compounds and mixtures of different oxygen-containing organic substances.

INTRODUCTION

Since 1962 when Halász and Heine /1/ published their paper on capillary packed columns with an inner diameter close to that of open-tubular columns, investigations on such type of columns progressed rather rapidly. This is demonstrated by the papers of Carter /2/, Virus /3/, Berezkin et al /4, 5/, Vigdergauz and Andrejev /6/, Myers and Giddings /7/, Cramers et al. /8, 9/, Bruner et al. /10/, Di Corcia et al. /11, 12/, Kinoshita /13/ and many other investigators (see e.g./14-23/).

Earlier we have suggested fused-silica capillary micro-packed columns for gas chromatography /24/ and a considerable work was devoted to the investigation of their gas chromatographic characteristics /24-27/. These columns, in our opinion,

are better alternatives than the usual stainless steel packed analytical columns. The fused-silica capillary micro-packed columns are flexible, possess an inert, smooth inner wall and a good mechanical strength (see Table I).

The investigations of fused-silica capillary micro-packed columns with an inner diameter of less than 0.40 mm and sorbent particle diameters below 50 μm have shown that such columns are characterized by a high specific efficiency (up to 30,000 theoretical plates per meter), small values of the mass transfer coefficient (of the order of 10^{-4} sec), comparatively large sample capacities, and the possibility of an easy adaptation for both gas-adsorption and gas-liquid chromatography /24-27/.

In comparison with conventional packed columns the fused-silica capillary micro-packed columns are far more efficient and, consequently, lead to a better resolution as well as to a reduction of the analysis time. Being miniaturized in size, these columns need much less amount of the sorbent for column preparation, and a lower carrier gas flow rate, and provide a

Table I. Comparative characteristics of columns prepared from different materials

Characteristics	Fused silica capillary micro-packed columns	Open-tubular columns			Packed columns	
		fused-silica	glass	metal	glass	metal
Mechanical properties	Strong	Strong	Fragile	Strong	Fragile	Strong
Chemical and adsorption activity of the walls	Inert	Inert	Generally inert	Active	Generally inert	Active
Separation efficiency	Effective			Generally effective	Fairly poor	
Analysis speed	Rapid analysis			Medium analysis time	Long analysis time	

closer temperature follow-up in temperature programming (see Table II). It should be mentioned that naturally, the method of fused silica micro-packed columns is somewhat more complicated than the preparation of conventional packed columns.

Compared to open-tubular columns capillary micro-packed columns are characterized by a higher sorption capacity providing a better separation of early eluting compounds (without cooling the column). Applying conventional sampling methods (i.e. methods that do not include any type of sample preconcentration) a larger sample size can be introduced into a capillary micro-packed column (as compared with a conventional open-tubular column) without causing any column overloading. This permits a better accuracy in trace analysis using conventional (easily realizable) sampling methods. Capillary micro-packed columns (including those made of fused silica) present an easier way to use gas-adsorption chromatography as compared with porous-layer open-tubular columns. This is due to the fact that packing a capillary with a given adsorbent can be more easily realized with a better reproducibility than forming a uniform adsorption layer of the same sorbent on the inner surface of the capillary (see Table III). The small particle diameter of the sorbent in capillary micro-packed columns causes an increased resistance to the carrier gas flow, but this is not a severe obstacle for their application /24-27/.

The purpose of the present paper is to continue the study of fused-silica capillary micro-packed columns and to evaluate their possibilities in the solution of various practical analytical problems.

EXPERIMENTAL

Fused-silica capillary micro-packed columns with inner diameters ranging from 0.25 to 0.40 mm and lengths from 10 cm to 2 m were investigated. Fused-silica capillaries with outer polymer coating produced in the USSR, by Scientific Glass Engineering (Australia), and by Orion Analytica (Finland) were used. The capillaries were packed with sorbents having an average particle diameter in the range of 7.5 to 100 μm.

Table II. Comparison of the specific features of fused-silica capillary micro-packed and conventional packed columns

No.	Specific feature	Fused-silica capillary micro-packed columns	Conventional packed columns
1	Efficiency (number of theoretical plates, T.P.)	$\sim 4.10^4$	$\sim 4.10^3$
2	Specific efficiency (T.P./meter)	$\sim 10^4$	$\sim 10^3$
3	Mass transfer term, C, sec	$\sim 10^{-4}$-10^{-5}	$\sim 10^{-2}$-10^{-3}
4	Amount of liquid phase per meter of column length (g/meter)	$\sim 10^{-2}$	~1
5	Sample capacity, µg	~2-5	$\sim 10^2$-10^3
6	Inlet pressure per unit of column length, atm./meter	~1.5-250	~0.5
7	Carrier gas flow rate, cm^3/min	~2-5	~30-60

The following sorbents were used: Silasorb 600 (LC), 7.5 and 20 µm, Silasorb C_{18}, 15 µm (Lachema, CSSR), activated coal SKT, 60-100 µm (USSR), molecular sieve CaA, 60-100 µm (USSR), Pellosil HC, 30-40 µm (Reeve Angel, USA), Zipax RP and Permaphase ETH (E.I. du Pont de Nemours, USA). In some cases Pellosil, Permaphase and Zipax particles were coated with 0.5-2 % of a liquid phase.

The fused-silica capillaries were packed with the sorbents by a dry packing method /28/ under simultaneous action of vibration and gas flow. Stable fixation of the sorbent layer in the column was accomplished with the help of gas-permeable partitions created at the column ends using some granular material and a binder /28/. The fused-silica capillary micro-packed columns with such ends can easily stand an inlet pressure of up to 40 atm. Leak-proof connection of the column

Table III. Comparison of the specific features of fused silica capillary micro-packed and open-tubular columns

No.	Specific features	Fused-silica capillary micro-packed columns	Open-tubular columns
1	GC methods which can be easily and reproducibly realized	Both gas-solid and gas-liquid	Only gas-liquid
2	Efficiency (number of theoretical plates, T.P.)	$\sim 4.10^4$	$\sim 10^5$
3	Specific efficiency (T.P./meter)	$\sim 2.10^4$	$\sim 3.10^3$
4	Mass transfer term C, sec	$\sim 10^{-4}$-10^{-5}	$\sim 10^{-3}$-10^{-4}
5	Amount of liquid phase per meter of the column length (g/meter)	$\sim 10^{-2}$	$\sim 5.10^{-4}$
6	Sample capacity, µg (sampling without preconcentration)	~2-5	~0.2
7	Inlet pressure per unit of column length (atm./meter)	~1.5-250	$\sim 10^{-1}$-10^{-2}

with the sampling and the detection systems was done with the help of rubber septa.

The investigations were carried out on a standard gas chromatograph LKhM-8MD (Khromatograf, Moscow, USSR) which was modified for inlet pressures up to 25 atm. and even higher, and on a high-performance gas chromatograph Micro-mat (Orion Analytica, Finland) operating at an inlet pressure of up to 3 atm. A Gazokhrom 101 micro-syringe (USSR) and a Hamilton gas syringe were used for sample introduction.

RESULTS AND DISCUSSION

For the interpretation of the experimental results we used the equation proposed by Bohemen and Purnell /29/ which considers the influence of the pressure drop when describing the dependence of the height equivalent to a theoretical plate (HETP), H, on the linear gas velocity at column outlet, u_o:

$$H = A + B/u_o + (C_G + C_S j)u_o$$

where A is the term describing the effect of "eddy" diffusion, B is the term considering longitudinal diffusion and the two C terms describe the effect of resistance to mass transfer in the gas (C_G) and stationary (C_S) phase; j is the well-known compressibility correction factor.

Fig. 1 presents the experimental data in the form of H vs. u_o plots for fused-silica capillary micro-packed columns prepared with three different types of sorbents: (a) an adsorbent (Silasorb 600(LC), 7.5 μm), (b) a bonded phase (Silasorb C_{18}, 15 μm) and (c) a pellicular sorbent (Pellosil HC, 30-40 μm) coated with 1 % squalane. From Fig. 1 it follows that the fused-silica capillary micro-packed columns are characterized by a high efficiency for sorbents of different types. For example, as it is evident from plot a (Fig. 1) the minimum value of the HETP, H_{min}, for column b, containing 7.5 μm silica particles equals 0.03 mm which corresponds to an efficiency of 30,000 theoretical plates per meter of column length. This value is about 30 and 10 times respectively higher than the corresponding values for conventional packed columns (with an inner diameter greater than 2 mm) and open-tubular columns. From the practical point of view it means that such a column with a length of only 10 cm can provide as much theoretical plates as a 3-m long conventional packed column.

It should be noted here that the efficiency of a packed column depends to a considerable extent on the packing quality (beside other factors, such as the diameter of the sorbent particles, d_p, the inner diameter of the column d_c, the nature of the sorbent, sorbate and the carrier gas, the temperature,

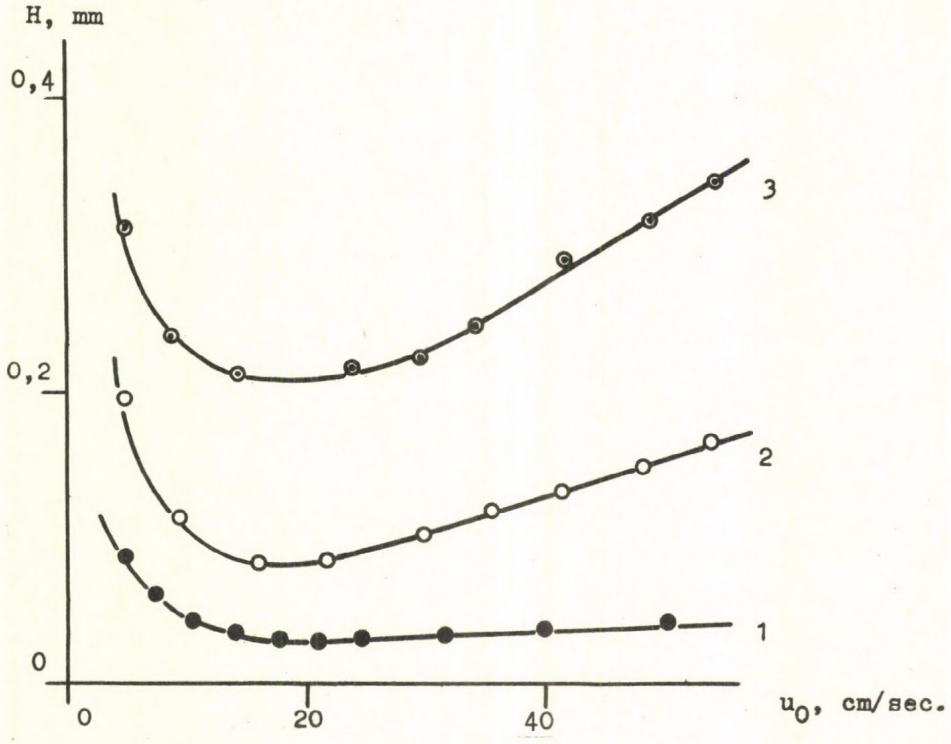

Fig. 1. Dependence of the HETP (H) (measured for hexane) on u_o.
Capillary micro-packed columns and sorbents:
1) 10 cm x 0.38 mm I.D., Silasorb 600(LC), 7.5 µm,
2) 35 cm x 0.38 mm I.D., Silasorb C_{18}, 15 µm,
3) 35 cm x 0.38 mm I.D., Pellosil HC, 30-40 µm.
+ 1 % squalane,
Carrier gas: nitrogen, column temperature 80°C.

Table IV. Reduced HETP values (h)* for fused-silica capillary micro-packed columns**

Column I.D.	Particle diameter (d_p), µm					
mm	7.5	10	15	20	40-50	63-100
0.15	4.1	3.9	3.6	3.3		
0.20	4.2	4.0	3.7	3.5	3.1	
0.25	4.2	4.1	3.9	3.6	3.3	
0.30	4.3	4.2	4.0	3.7	3.4	3.2
0.38	4.5	4.3	4.1	3.8	3.5	3.3

*$h = H_{min}/d_p$. **Sorbent: Silasorb 600(LC); temperature: 75°C; carrier gas: nitrogen; chromatographed compound: n-hexane

etc.). The packing quality can be evaluated by the reduced plate height values, h, ($h = \frac{H_{min}}{d_p}$)/30/. Table IV presents some experimental reduced plate height data for fused-silica capillary micro-packed columns prepared with different fractions of Silasorb 600 (LC). As can be seen the value of h for these columns ranges from 3.1 to 4.5. This, according to Dandeneau and Hawkes /31/, is an indication of good packing quality. Therefore, we may conclude that the method used by us /28/ for the preparation of fused-silica capillary micro-packed columns provides a fairly uniform packing of the small diameter particles in the fused-silica capillaries (d_c<0.5 mm), and consequently, plays a significant role in the enhancement of column efficiency.

A second important property of the fused-silica capillary micro-packed columns which, in our opinion, is very important in gas chromatography, is the very low value of the mass transfer coefficient, C. For example, as it can be seen in Table V, fused silica capillary micro-packed columns containing 7.5 μm silica particles are characterized by a mass transfer term of $C = 4.10^{-5}$sec, whereas for conventional packed columns the value of this term is about 10^{-2}sec /15/ and for open-tubular columns having a moderate film thickness $C \sim 10^{-3}$sec /32/.

The above-mentioned two properties of fused-silica capillary micro-packed columns essentially lead to the following conclusions about their gas chromatographic potentialities. First, the high efficiency of the columns makes possible efficient gas chromatographic separations on a short column. Second, from the practical point of view, low values of the mass transfer coefficient means that high carrier gas velocities can be used practically without any reduction in column efficiency. These two inherent properties of the fused silica capillary micro-packed columns make them particularly suitable means for fast analysis.

As it is known /29/, the overall mass transfer term C consists of two parts: C_G and C_Sj (see eq. 1) contributed by the mobile and the stationary phases respectively. We have estimated these two parts of the mass transfer term for fused-silica capillary micro-packed columns. This was done on the

Table V. Gas chromatographic characteristics of fused-silica capillary micro-packed columns*

Sorbent	d_p	T °C	H_{min} mm	C x 10^4 sec
Silasorb 600(LC) (adsorbent)	7.5	120	0.04	0.4
	20	120	0.10	0.7
	30	120	0.13	1.3
Silasorb C-18 (bonded-phase)	15	80	0.08	2.7
1 % squalance on Pellosil HC	30-40	50	0.17	3.3

*Column I.D. = 0.38 mm; carrier gas: nitrogen.
Symbols: d_p = particle diameter
T = temperature
H_{min} = minimum value of the HETP
C = resistance-to-mass transfer term

Table VI. Estimation of the contribution to the value of the mass-transfer term C*

Chromatography variant	Carrier gas	Cx10^4 sec	C_Gx10^4 sec	$C_S$jx10^4 sec	C_G/C %	C_Sj/C %
Gas-adsorption (GAC)	nitrogen	1.2	1.1	0.15	90	10
	hydrogen	0.75	0.55	0.20	77	23
Gas-liquid (GLC)	nitrogen	2.6	2.0	0.6	75	21
	hydrogen	2.1	1.3	0.8	62	38

*Column: 35 cm x 0.35 mm I.D.
Sorbents: Silasorb 600(LC) 20 µm (for GAC) and 1 % squalane on Pellosil HC 30-40 µm (for GCL).
Chromatographed compound: n-hexane
Temperature: 100°C.

basis of the method proposed by Perrett and Purnell /33/, using hydrogen and nitrogen as the carrier gases. The experimental data are presented in Table VI. From these data it follows that in the case of gas-adsorption chromatography on fused-silica capillary micro-packed columns, the application of hydrogen as the carrier gas instead of nitrogen results in about 40 % decrease in the value of C. It should be noted here

that irrespective of the carrier gas used in gas-adsorption chromatography the C_G term predominates over the C_Sj term. This is, perhaps, due to the fact that the mass exchange on the adsorbent surface takes place rather rapidly and that at high carrier gas velocities the chromatographic zone broadens due to a comparatively slow mass exchange in the gas phase. On the other hand, in the case of gas-liquid chromatography, use of hydrogen instead of nitrogen as the carrier gas results in a decrease in the value of the C term by about 20 %, and the contributions of C_G and C_Sj represent about 60 % and 40 %. This can be explained by the slower mass exchange in the stationary phase as compared with the mass exchange on a solid adsorbent.

From these results it can be concluded that hydrogen should be preferred as the carrier gas when carrying out fast analyses on fused-silica capillary micro-packed columns. It also follows that when hydrogen is used as the carrier gas for fast analysis, a better result can be expected in the case of gas-solid chromatography than in gas-liquid chromatography. It should also be noted here that hydrogen is a low-viscosity gas; therefore, hydrogen can be used as the carrier gas at higher linear velocities at comparatively low inlet pressures. This point is important for fused-silica capillary micro-packed columns characterized by an increased pneumatic resistance.

Based on our present and previous investigations the main advantages of fused-silica capillary micro-packed columns are listed in Table VII.

Some of the investigated columns were used for the separation of various mixtures belonging to different classes of organic and inorganic substances. Thus, a mixture of inorganic gases and methane was separated on a column packed with molecular sieve of the CaA type (63-100 μm) obtained by crushing a coarser fraction with subsequent sieving (Fig. 2).

Fig. 3 illustrates an example of the gas chromatographic separation of a mixture of C_1-C_6 hydrocarbons on fused-silica capillary micro-packed columns containing a modified silica gel. In this case the inlet pressure was 25 atm. For the separation of light hydrocarbons at a lower inlet pressure we used

Table VII. Advantages of fused-silica capillary micro-packed columns

Specific properties of the columns	Corresponding advantages
Flexibility, inertness, mechanical strength, smoothness of the inner surface, etc.	Ease in preparation and use
High specific efficiency Low values of the mass transfer term	Fast analysis
Possibility of easy and reproducible realization of both gas-liquid and gas-solid chromatography, using practically any adsorbent	Analysis of gaseous and highly-volatile compounds
Relatively high sorption capacity	Good separation of early eluting compounds
Relatively high sample capacity	Trace analysis
Miniaturization	Economy in the amount of sorbent and in the carrier gas. Closer follow-up of the temperature in temperature programming. Miniaturization of the column oven.

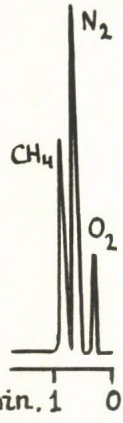

Fig. 2. Chromatogram of a mixture of inorganic gases and methane. Capillary micro-packed column: 60 cm x 0.38 mm I.D.; sorbent: molecular sieve CaA, 60-100 µm; carrier gas: helium; inlet pressure: 5 atm.; temperature: 38°C; thermal conductivity detector. Peaks: 1 oxygen, 2 nitrogen, 3 methane.

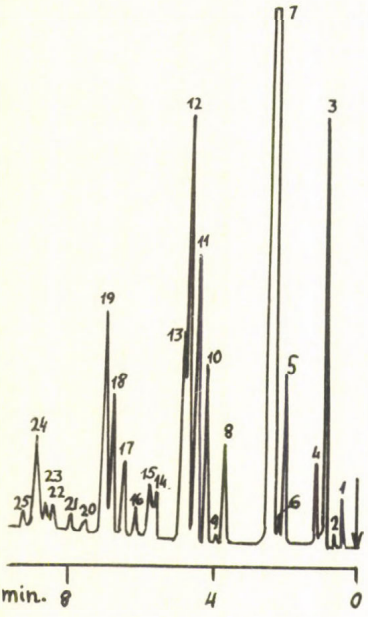

Fig. 3. Chromatogram of C_1-C_6 hydrocarbons. Capillary micro-packed column: 1 m x 0.37 mm I.D.; sorbent: silica gel, 20-25 µm, modified with $Na_2B_4O_7.nH_2O$; initial column temperature 40°C, temperature programming rate 12°/min; carrier gas: nitrogen; inlet pressure: 25 atm. Peaks: 1 methane, 2 ethane, 3 ethylene, 4 propane, 5 isobutane, 6 n-butane, 7 propylene, 8 isopentane, 9 n-pentane, 10 1-butene, 11 trans-2-butene, 12 cis 2-butene, 13 isobutene, 14-16 hexane isomers, 17-19 pentene isomers, 20-25 hexene isomers.

columns prepared with the pellicular sorbent Pellosil HC (30-40 µm), and hydrogen as the carrier gas. A considerably good separation of C_1-C_4 hydrocarbons including the four butene isomers was obtained on a two meter long column, at an inlet pressure of 3 atm. The analysis was carried out on a Micromat gas chromatograph in 3 minutes. (This chromatograph was developed for the special use with fused-silica open-tubular columns).

A rapid and good separation of the C_1-C_2 hydrocarbons including ethylene and acetylene was attained on a fused-silica capillary micro-packed column containing activated carbon (63-100 µm). Here also the inlet pressure did not exceed 3 atm.

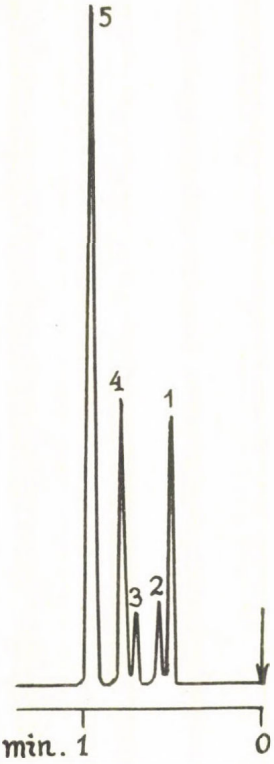

Fig. 4. Chromatogram of oxygen-containing compounds. Capillary micro-packed column: 115 cm x 0.25 mm I.D.; sorbent: 1 % Carbowax 20M on Pellosil HC, 30-40 μm; temperature: 130°C; carrier gas: nitrogen; inlet pressure: 4 atm. 1) diethyl ether, 2) ethyl formate, 3) ethyl acetate, 4) tert. butanol, 5) methyl propyl ketone.

Good separation of mixtures of different classes of compounds at relatively low inlet pressure can also be obtained on fused-silica capillary micro-packed columns containing liquid stationary phases coated on pellicular sorbents such as Pellosil HC or Zipax used as the solid support. For example, Fig. 4 presents a chromatogram of oxygen-containing compounds separated on a column containing Carbowax 20M on Pellosil HC (30-40 μm). Here the inlet pressure was 4 atm.

The relatively high sorption capacity of fused-silica capillary micro-packed columns makes it possible to introduce fairly large samples and to perform rapid trace analysis. For example, traces of C_8 hydrocarbons including m- and p-xylenes were analyzed on a column containing a liquid crystal-

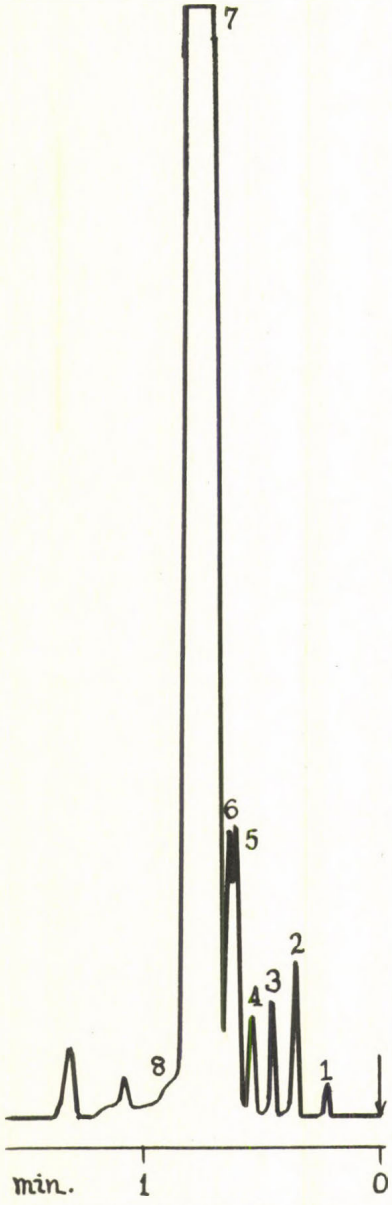

Fig. 5. Determination of trace compounds in o-xylene. Capillary micro-packed column: 49 cm x 0.365 mm I.D.; sorbent: 1.5 % 4,4'-azoxyanisole and 4,4'-azoxyphenetole (3:2) on Zipax; temperature: 98°C; inlet pressure: 9.7 atm.; carrier gas: helium; Peaks: 1 benzene (0.01 %), 2 toluene (0.05 %), 3 n-nonane (0.09 %), 4 ethylbenzene (0.07 %), 5 m-xylene (0.2 %), 6 p-xylene (0.2 %), 7 o-xylene, 8 n-decane

line stationary phase (Fig. 5). The value of H_{min} for this column equals 0.13 mm (for pseudocumene).

In conclusion we may stay that the good resolution power and high speed of separation obtained on fused-silica capillary micro-packed columns shown here and in our previous publications (e.g. /24-27/) demonstrate the analytical potentialities of these columns. One may expect that they will find a wide application in the analytical practice.

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REVERSED-PHASE AND SILICA GEL HPTLC OF AROMATIC ALKOXY ACIDS

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SUMMARY

HPTLC of aromatic alkoxy acids was studied on Kieselgel HF_{254} HPTLC and RP_{18} plates, respectively. The relationship between the R_M values of these compounds and the mobile phase composition was investigated in reversed-phase chromatography; a linearity was found between R_M and the methanol content of the mobile phase. The elution order of the acids and additivity of the ΔR_M values characterizing structural units of the molecules are discussed and compared in silica-gel and in reversed-phase chromatography.

INTRODUCTION

TLC on chemically-bonded reversed-phase layers has an increasing role in the separation of different organic compounds /1-4/. One of the advantages of this method is that the retention data yielded by TLC can be easier converted into column chromatography retention data than in the case of direct-phase silica-gel chromatography /2, 4/.

The subject of this paper is the reversed-phase and silica-gel HPTLC separation of some aromatic carboxy acids containing several alkoxy groups. The R_f and R_M values were studied as a function of the mobile phase composition and the chemical structure of the acids, respectively. The latter relationship in reversed-phase chromatography was compared to the relationship between the R_M values and the chemical structure observed in silica-gel chromatography.

MATERIALS AND METHODS

The following aromatic carboxy acids were studied: benzoic acid, 2-methoxy-, 3-methoxy-benzoic acid, 4-methoxy-benzoic acid, 3,4-dimethoxy-, 3,4,5-trimethoxy-benzoic acid, 3,5-dimethoxy-4-n-butoxy-, 3,5-dimethoxy-4-n-decyloxy-benzoic acid, 4-hydroxy-, and 3-methoxy-4-hydroxy-benzoic acid. In reversed-phase chromatography, the mobile phases consisted of methanol and 1M (pH=2) phosphate buffer. In silica-gel chromatography the plate was equilibrated in the vapour phase with a 1:1 acetic acid:water mixture for 15 minutes before development using a glass tank. (Buffering in vapour phase had been studied in our previous work /5/).

Development was carried out in di-isopropyl ether. The aim of the development in acidic medium was to bring the acids in the undissociated form. In this way differences in retention caused by the different degree of dissociation were eliminated, RP_{18} HPTLC plates (Merck; 10x10 cm) were used as the stationary phase in reversed-phase chromatography and Kieselgel HF_{254} HPTLC (Merck; 10x10 cm) plates in silica-gel chromatography, respectively. Development was carried out at room temperature using a sandwich chamber (CAMAG), without previous saturation. Spots were made visible in 254 nm UV light.

RESULTS AND DISCUSSION

Table 1 lists the R_f and R_M values obtained on RP_{18} and Kieselgel HF plates.

The standard deviation of the R_f values ($\sqrt{\frac{\Sigma \Delta^2}{n(n-1)}}$) calculated from five parallel runs were as follows in the case of benzoic acid: 0.014 in 90% methanol, and 0.007 in both 70% and 80% methanol. Similar standard deviation values were also observed for the other compounds.

In Fig. 1 R_M values of five acids were plotted against the methanol content of the mobile phase (per cent v/v) when chromatographed on the RP_{18} plates. A linear relationship was

Table 1. R_f and R_M values of aromatic alkoxy acids on RP_{18} [mobile phase: 1M (pH = 2) phosphate buffer-methanol mixtures] and Kieselgel HF_{254} (mobile phase: di-isopropyl ether) HPTLC plates

	RP 18								Kiesgel	
	methanol		90% methanol		80% methanol		70% methanol		diisopropyl ether	
	R_f	R_M	R_f	R_M	R_f	R_M	R_f	R_M	R_f	R_M
benzoic	0.94	-1.195	0.88	-0.865	0.82	-0.659	0.70	-0.368	0.70	-0.368
2-methoxy-benzoic	0.95	-1.279	0.91	-1.005	0.87	-0.826	0.75	-0.477	0.17	-0.689
3-methoxy-benzoic	0.92	-1.061	0.88	-0.865	0.80	-0.602	0.65	-0.269	0.56	-0.105
4-methoxy-benzoic	0.92	-1.061	0.87	-0.826	0.79	-0.575	0.64	-0.250	0.52	-0.035
2,4-dimethoxy-benzoic	0.98	-1.690	0.97	-1.510	0.93	-1.123	0.84	-0.720	0.08	1.061
3,4-dimethoxy-benzoic	0.97	-1.510	0.93	-1.123	0.86	-0.788	0.86	-0.501	0.23	0.525
3,4,5-trimethoxy-benzoic	0.97	-1.510	0.93	-1.123	0.85	-0.753	0.73	-0.432	0.23	0.525
3,5-dimethoxy-4-n-butoxy-benzoic	0.87	-0.826	0.80	-0.602	0.62	-0.213	0.38	0.213	0.37	0.231
3,5-dimethoxy-4-n-decyloxy-benzoic	0.71	-0.389	0.36	0.250	0.12	0.865	0.03	1.510	0.43	0.122
4-hydroxy-benzoic	0.97	-1.510	0.95	-1.279	0.95	-1.279	0.86	-0.788	0.45	0.087
3-methoxy-4-hydroxy-benzoic	0.96	-1.380	0.94	-1.195	0.93	-1.123	0.83	-0.689	0.29	0.389

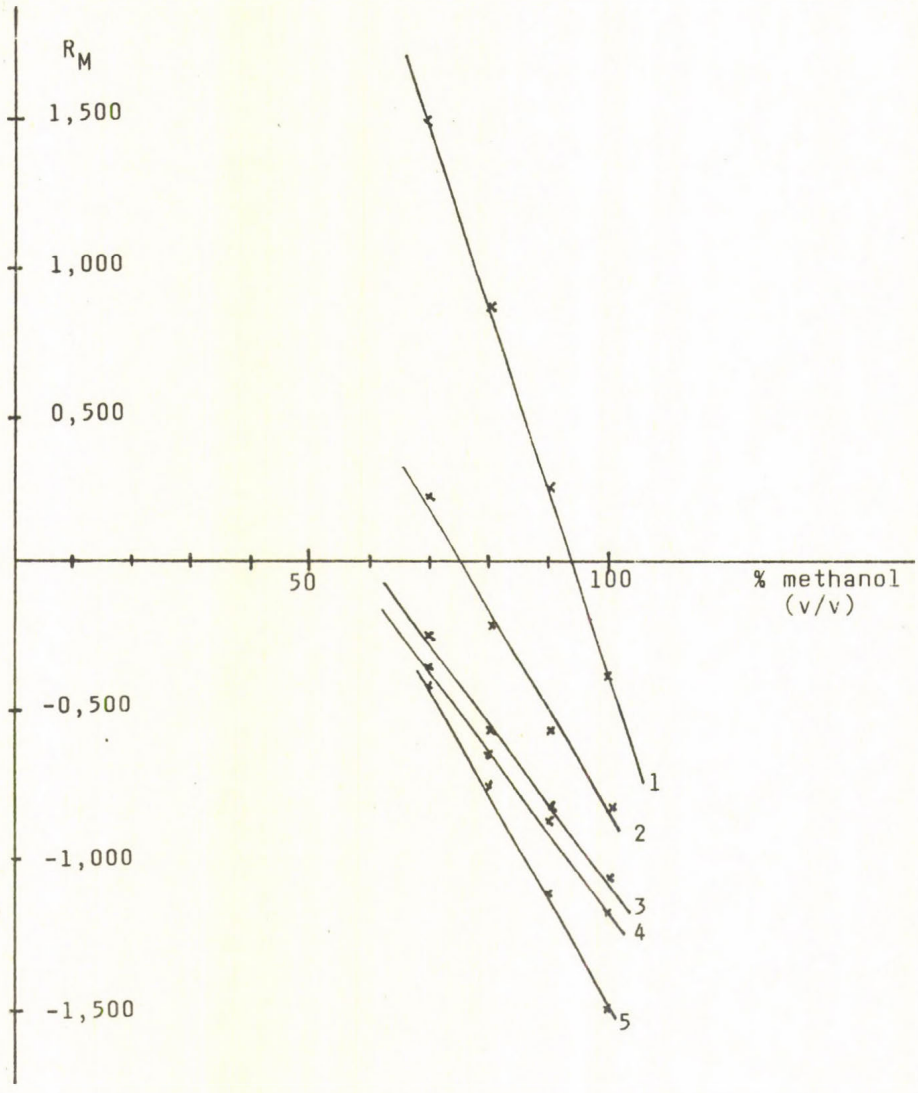

Fig. 1. Relationship between the R_M values of acids and the methanol content of the mobile phase on RP_{18} plates. 1 4-n-decyloxy-3,5-dimethoxy-benzoic acid, 2 4-n-butoxy-3,5-dimethoxy-benzoic acid, 3 4-methoxy-benzoic acid, 4 benzoic acid, 5 3,4,5-trimethoxy-benzoic acid.

observed, according to the equation known from the literature /1, 4/.

Aiming to compare the retention properties in reversed-phase and silica-gel chromatography the relationship between the structure and the R_M values of methoxy acids was studied in both cases. In silica-gel chromatography the ΔR_M values characterising 2, 3- and 4-methoxy-groups (related to the carboxyl group) were calculated as follows:

$$\Delta R_{M(2\text{-methoxy})} = R_{M(2\text{-methoxy-benzoic acid})} - R_{M(\text{benzoic acid})} \text{ etc.}$$

Using these values and the R_M values of benzoic acid the R_M values of di- and trimethoxy acids were calculated and converted into R_f values. The R_f values calculated in this way were compared with the experimentally measured data. Calculated and measured values are listed in Table 2.

Table 2. R_f values of di- and trimethoxy-benzoic acids calculated on the basis of the R_M values and experimentally measured R_f values (silica gel)

Acid	R_f, calculated	R_f, measured
2,4-dimethoxy-benzoic	0.09	0.08
3,4-dimethoxy-benzoic	0.37	0.23
3,4,5-trimethoxy-benzoic	0.24	0.23

It can be seen that - except for 3,4-dimethoxy-benzoic acid - the calculated and measured R_f values show a good agreement when considering the experimental error. In the case of 3,4-dimethoxy-benzoic acid the measured value was lower than the calculated value. A similar phenomenon was found in silica-gel chromatography for 3,4-dichloroaniline /6/. The behaviour of this compound also differed from that of other dichloroanilines regarding the additivity of the R_M values.

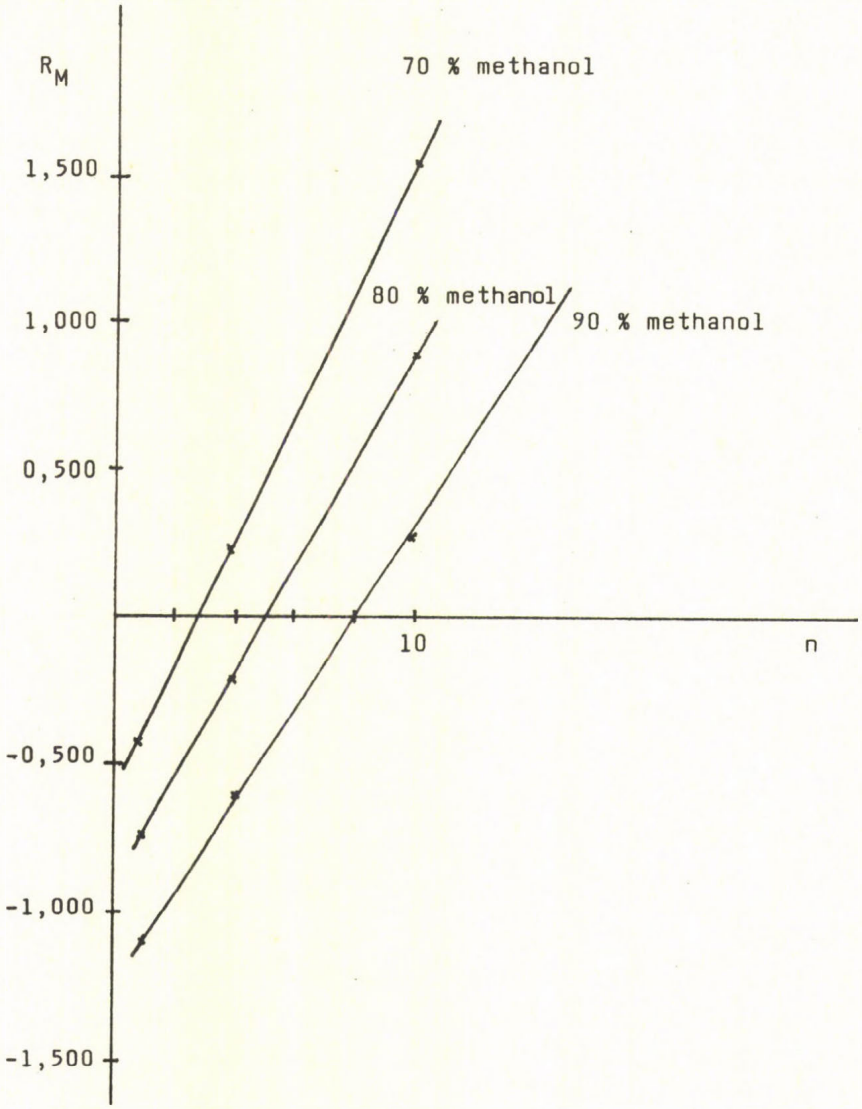

Fig. 2. R_M values plotted against the carbon number of the group substituting in 4-position. Plate: RP_{18}. Mobile phase: mixtures of methanol and 1M (pH=2) phosphate buffer.

Considering the R_f values of acids in reversed-phase TLC, we can see that the elution order observed in silica-gel chromatography did not change unambiguously. While on the RP_{18} plate the R_f values of 3- and 4-methoxy-benzoic acids are close to or are somewhat lower than the value of benzoic acid, the R_f values of 2-methoxy-benzoic acid and those of di- and trimethoxybenzoic acids are higher than the R_f value of benzoic acid. None of the R_f values of di- and trimethoxy benzoic acids can be calculated using the ΔR_M values characterizing the methoxy groups in the monomethoxy acids. This seems to indicate that retention of monomethoxy acids on a reversed-phase adsorbent is more effected by the increase in the carbon atom number than by the appearence of the more polar etheric oxygen atom; the R_f values of monomethoxy acids were lower than that of benzoic acid. In the case of 2-methoxy-benzoic acid, however, retention was reduced by the steric effect. In the case of the di- and trimethoxy acids the hindrance of retention, caused by the increasing polarity of the molecule and the steric effect, results in an increase of the R_f values.

The effect of increasing number of carbon atoms can be seen very well in the case of 4-n-butoxy-3,5-dimethoxy-benzoic and 4-n-decyloxy-3,5-dimethoxy benzoic acids. The R_f value rapidly decreases with increasing carbon number. Fig. 2 plots the R_M values against the carbon number of the group in the 4-position. Using different mobile phases the relationship proved to be linear. This linearity (i.e., the additivity of the ΔR_M values characterizing a $-CH_2$ group) cannot be found in the case of the R_f values measured on silica gel. There, the change of R_f with the carbon number is much slower than in the case of reversed-phase chromatography.

As a conclusion, we can state that it is obvious that in silica-gel chromatography of these acids the properties, number and position of the polar groups are the most important factors determining retention. In the case of reversed-phase chromatography the carbon number is dominating. Steric effects, however, influence the retention in both methods.

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PREADSORBED SALTS TO MODIFY THE RETENTION BEHAVIOUR OF SILICA

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INTRODUCTION

Silica is the most widely used polar stationary phase in chromatography /1/. Its retention behaviour depends on a number of physico-chemical parameters such as the specific surface area, specific pore volume, average pore diameter, and the quantity of the silanol groups on the silica surface /2/. The pH value of the silica surface influences the retention in both gas /3/ and liquid chromatography /4/. It is well known that the ions adsorbed on the sorbents modify their retention behaviour. This may be due to the facts that they decrease the number of available adsorption sites on the sorbent surface, acidify the silica /4/ and specifically interact with the solutes /6/.

The retention strength and selectivity of the support determined experimentally depend considerably on the experimental conditions and on the solutes investigated /7/. With large number of supports and solutes, computer-assisted multivariate techniques offer the unique possibility to simultaneously evaluate all retention data /8, 9/.

Retention strength of the support can be expressed by the retention of a number of solutes. The use of retention spectra is aimed at grouping those supports which share same (possibly unknown) mechanism of retention. However, the information on the spectral properties is usually disturbed by the relative strength of the supports and, for this reason, any classification procedure has to be preceded by the separation of the re-

lative strength of support from the retention mechanism. The technique of spectral mapping complies with these requirements /10/. With the help of this technique it is possible to calculate first the order of retention strengths (in arbitrary units) and then the spectra of supports independently of their retention strength. In the multidimensional spectra supports that exhibit similar retention mechanism form clusters. As the human brain is not constructed to think in multidimensional spaces, the non-linear two-dimensional mapping of spectra highly facilitates the evaluation /11/.

These techniques have been successfully applied to classify eluents in reversed-phase thin-layer chromatography /12/ and to compare the retention characteristics of the same covalently bonded silicas /13, 14/.

The objectives of our work were to study the effect of preabsorbed salts on the TLC behaviour of some triphenylmethane derivatives and to correlate it with the physico-chemical parameters of the salts.

EXPERIMENTAL

The chemical structures of triphenylmethane derivatives are compiled in Table I.

Kieselgel 60H (Merck, Darmstadt, FRG) was applied as control (sorbent 1.). The modified sorbents are listed in Table II. The eluents used were chloroform for compounds 6, 7, 10, 12, 13, 14, 15, 16, 23, 24, 25 and 26 (System I) and carbon tetrachloride for compounds 1, 2, 3, 4, 5, 8, 9, 11, 17, 19, 19, 20, 21 and 22 (System II).

To compare the retention behaviour of the supports without separating the retention strength from selectivity, principal component analysis (PCA) was applied (PCA I for System I and PCA II for System II.).

To elucidate the role of the physico-chemical characteristics of the salts (added quantity = Q; charge of cation = Ch and ion radii of cation = R), correlations were calculated between them and the first two component loadings of PCAs and the

retention strength data. As the exact form of correlation (linear, logarithmic or quadratic) between the variables mentioned above was not previously known, stepwise regression analysis (SRA) was applied to select the independent variables (Q, Ch or R values) that significantly influence the retention behaviour of the supports. SRA was carried out six times taking separately the loadings of first and second, principal components for System I (SRA I and II) and for System II (SRA V and V and VI) as dependent variables. The independent variables were in each case the linear, quadratic and logarithmic forms of the Q, Ch and R values. The number of accepted independent variables was not limited, their partial F values was set to 1.0. To facilitate calculations 100 R_f values were used instead of the R_f values.

RESULTS AND DISCUSSION

The results of PCAs are compiled in Table III. Two background variables explain more than 90% of the total variance in both cases. It means that with the help of these variables the retention of compounds on each support can be predicted at a probability of about 90%.

The two dimensional plots of the first two PCA loadings are shown in Figs 1 and 2 for Systems I and II, respectively.

In System I the retention behaviour of untreated silica differs considerably from that of the treated silicas, however the treated silicas do not form well-defined clusters, which means that neither the type of salts nor their quantity exert an outstanding influence on retention. In Fig. 2 the positions of the supports deviate from those observed in Fig. 1, the untreated silica does not separate from the others and supports 4 and 8 exhibit different retention characters. This deviation between the two plots suggests that the modification effect of preadsorbed salts varies according to the compounds and the applied eluents.

The two-dimensional plots of PCA I and II variables (clustering of compounds according to their retention behaviours)

Table I. Chemical structures of the studied compounds

No. of comp.	R_1	R_2	R_3	R_4
1	-phenyl	-phenyl	-phenyl	-H
2	-phenyl	-phenyl	-phenyl	-OH
3	-phenyl	-phenyl	-phenyl	-2-imidazole
4	-phenyl	-phenyl	-phenyl	-1-triazole
5	-phenyl	-phenyl	-2-pyridine	-OH
6	-phenyl	-phenyl	-3-pyridine	-OH
7	-phenyl	-2,4-dichloro-phenyl	-5-pyrimidine	-OH
8	-phenyl	-phenyl	-4-chloro-phenyl	-OH
9	-phenyl	-4-chloro-phenyl	-2-pyridine	-OH
10	-phenyl	-phenyl	-2-chloro-phenyl	-2-imidazole
11	-phenyl	-phenyl	-2-chloro-phenyl	-OH
12	-phenyl	-4-methoxy-phenyl	-3-pyridine	-OH
13	-phenyl	-phenyl-SO_2-phenyl	-phenyl	-OH
14	-phenyl	-phenyl	-phenyl-SO_2-phenyl	-OH
15	-phenyl	-phenyl-SO_2-phenyl	-phenyl	-2-imidazole
16	-4-methoxy-phenyl	-4-methoxyphenyl	-3-pyridine	-OH
17	$-C_2H_4-$	-H		
18	$-C_2H_4-$	-Cl		
19	$-C_2H_4-$	$-OCH_3$		
20	$-O-CH_2-$	-H		
21	$-O-CH_2-$	$-OCH_3$		

22 $-CH_2-S-CH_2-$ -Cl

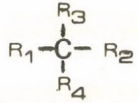
23 -H

24 -Cl

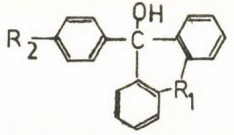
25 -OH

26 $-CO-CH_3$

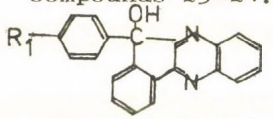
Basic structure for compounds 1-16:



compounds 17-22:



compounds 23-24:



compounds 25-26:

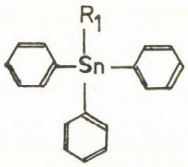


Table II. Modification of silica by preadsorbing of salts

No of silica	Salt added Type	Quantity, mM/g silica
1	0	0
2	NaCl	0.5
3	NaCl	1.0
4	NaCl	2.0
5	LiCl	1.5
6	LiCl	2.0
7	KCl	1.5
8	KCl	2.0
9	$MgCl_2$	1.5
10	$MgCl_2$	2.0
11	$CaCl_2$	2.0

Table III. Results of principal component analysis (PCA)

No	Eigenvalue PCA I	PCA II	Sum of total variance explained % PCA I	PCA II
1	8.90	8.97	80.90	81.51
2	1.49	1.17	94.48	92.11
3	0.32	0.41	97.43	95.85

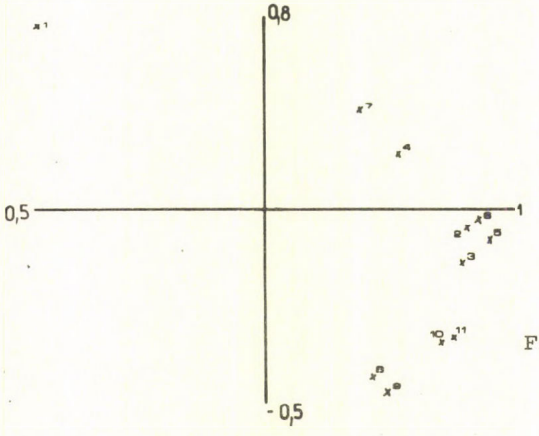


Fig. 1. Two-dimensional plot of PCA I loadings. Numbers indicate supports in Table II.

are shown in Figs 3 and 4 respectively. Compounds 13-14 and 25-26 form two distinct clusters; this means that in System I the substitution of the central carbon atom by Sn and the presence of phenyl-SO_2-phenyl groups (together with free hydroxyl) exert a preponderant influence on retention (Fig. 3).

In System II the retention of free hydroxyl groups by the basic heterocyclic structures influences most markedly the retention behaviour of the compounds (Fig. 4). The retention strengths of the supports calculated by the spectral map technique are listed in Table IV. According to the expectation the preadsorbed salts decrease the retention strength of silica occupying the free silanol groups responsible for the retention.

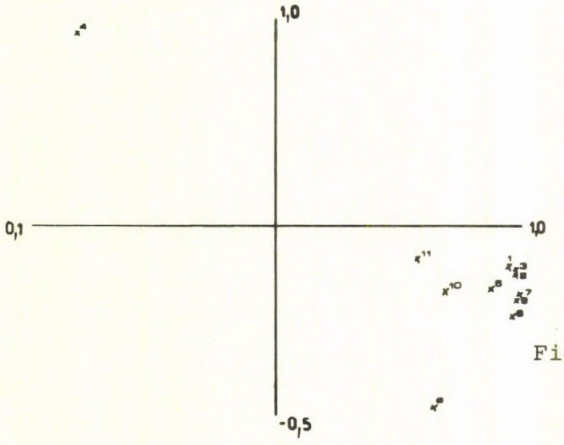


Fig. 2. Two-dimensional plot of PCA II loadings. Number indicate supports in Table II.

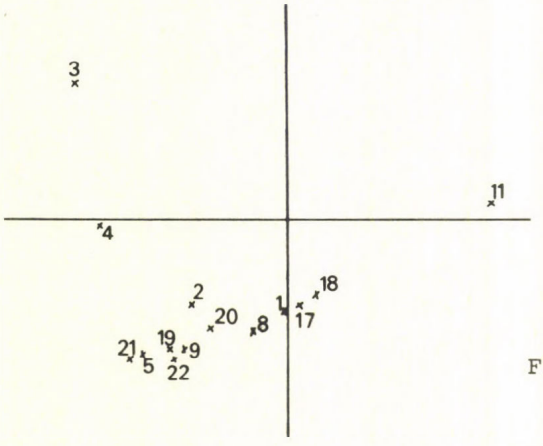


Fig. 3. Two-dimensional plot of PCA I varaiables. Numbers indicate compounds in Table I.

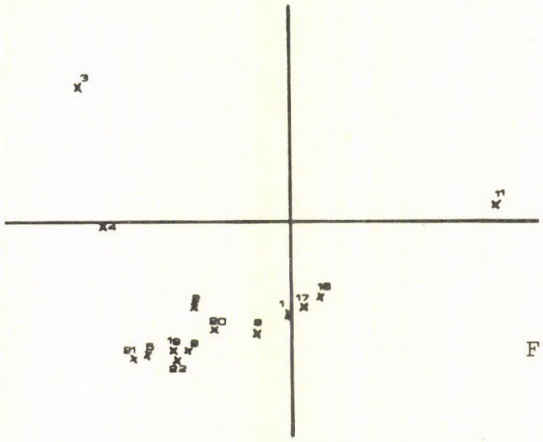


Fig. 4. Two-dimensional plot of PCA II variables. Numbers indicate compounds in Table I.

Table IV. Retention strength of supports

No. of support	System I	System II
1	4.35	8.00
2	3.97	9.26
3	2.99	7.30
4	1.23	1.79
5	4.20	5.49
6	3.89	4.95
7	4.03	6.45
8	1.00	1.00
9	3.05	3.36
10	2.61	2.03
11	1.72	2.13

This inhibitory effect increases with increasing quality of the added salts. Significant linear correlation was found between the retention strength of the two systems (intercept = 1.32; slope = 0.36 ; r_{calc} = 0.828 ; $r_{99\%}$ = 0.735) indicating that the effect of salts in similar in both cases.

The two-dimensional non-linear maps of the spectral characteristics are shown in Figs 5 and 6 for Systems I and II, respectively.

The separation of the retention strength from the overall retention pattern of the supports change considerably their clustering (compare Figs 1-5 and 2-6). No definite clustering was observed on the spectral maps (Figs 5 and 6) showing that neither the quantity nor the quality of the absorbed salts influence decisively the selectivity change.

The results of stepwise regression analysis are compiled in Table V.

In case of SRA III, IV and V the independent variables did not significantly influence the dependent variables, therefore they were omitted from Table V. The loadings of the first principal component of PCA I (SRA I) correlated well (over 99.9% significance level) with the physico-chemical parameters of the

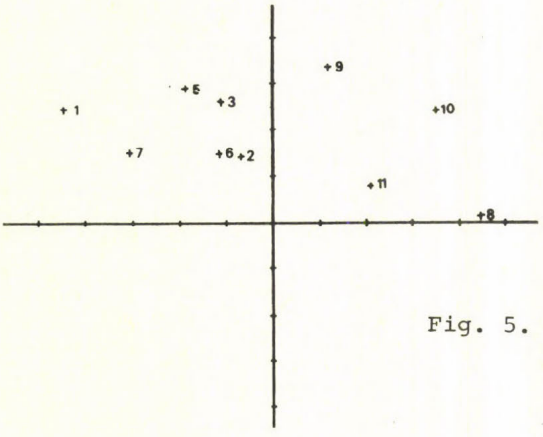


Fig. 5. Two-dimensional non-linear map of spectral characteristics. Numbers indicate supports in Table II.

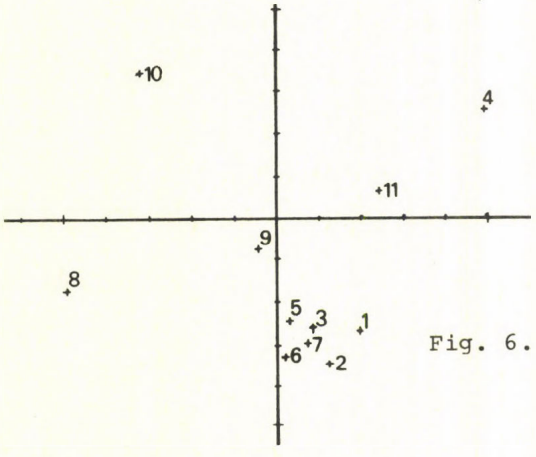


Fig. 6. Two-dimensional non-linear map of spectral characteristics. Numbers indicate supports in Table II.

Table V. Results of stepwise regression analysis (SRA)

n = 11

Parameters	Independent variables	SRA I	SRA II	SRA VI
a		1.25	0.63	0.15
b_1	Ch	-	$5.09.10^5$	
b_2	log Ch	$3.72.10^4$	-	2.87
b_3	Q^2	-	-	-4.37
b_4	R^2	$-1.61.10^4$	-	
b_5	Ch^2	$-1.38.10^4$	-	-2.08
s_1		-	$1.30.10^5$	
s_2		$5.51.10^3$	-	1.20
s_3		-	-	0.88
s_4		$6.03.10^3$	-	
s_5		$4.58.10^3$	-	1.12
r		0.967	0.794	0.924
r^2		0.936	0.630	0.856
$b_1'\%$		-		
$b_2'\%$		60.85		30.41
$b_3'\%$		-		49.10
$b_4'\%$		15.03		
$b_5'\%$		24.12		20.49
t_1		-	3.92	
t_2		6.75		2.39
t_3				4.99
t_4		2.67		
t_5		3.46		1.85
F		34.05	15.27	13.68
$F_{99.9\%}$		18.77		
$F_{99\%}$			10.56	8.45
$t_{99.9\%}$		5.41		
$t_{99\%}$			3.25	3.50
$t_{95\%}$		2.37		2.37

salts. Two of the three independent variables were related to the change of adsorbed cations (logarithmic and quadratic forms) and the normalized slope values (b_i') were higher than the slope of the third variable (quadrate of ion radii). This result indicates that in System I the modifying effect of the salts is mainly governed by the ion change. SRA II supports our previous conclusions. Also in this case the ion change significantly influences the loadings of the second principal component of PCA I, however, the correlation is of linear character.

The observation that no significant correlations were found between the various loadings of PCA II and the physico-chemical parameters of the salt underlines our previous statement that the solutes and eluents have a preponderant influence on the retention behaviour of modified silicas.

Good correlation was found between the retention strength values of system II and the physico-chemical parameters of salt (SRA VI). The normalized slope values indicate that the quantity of adsorbed salts and the ion charge have similar impact on the retention strength.

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THE ADSORPTION PROPERTIES OF GRAPHITIZED THERMAL CARBON BLACK MODIFIED WITH MONOLAYERS OF CROWN-ETHERS

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The molecular interaction monolayers of dibenzo-18-crown-6 ether and its nitro-derivatives deposited on the surface of graphitized thermal carbon black (GTCB) with the molecules of different substances have been experimentally determined by gas chromatography. The dense monolayer capacity of the modifiers was estimated from their size and from experimental data. The modification of the GTCB surface results in a considerable decrease of the Henry constants (K_1) and of the heats of adsorption and in an increase of the selectivity toward polar compounds. The prepared adsorbents are used for concentration and for the chromatographic-mass spectrometric analysis of halocarbons present in the atmosphere.

In the present work the adsorption properties of the monolayers of dibenzo-18-crown-6 and dinitrodibenzo-18-crown-6 ethers deposited on the surface of carbochrom have been studied by gas chromatography.

Carbochrom is a thermal carbon black modified with pyrolytical carbon. The adsorption properties of carbochrom and of GTCB are analogous, but the mechanical strength of carbochrom granules is much greater than that of the granules of GTCB /1, 2/. The properties and application of the relatively

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new compound class - crown ethers - hav been intensively studied /3-5/. Crown ethers are crystalline, readily soluble in many organic solvents and have high thermal stability. This permits their use as modifiers of the carbochrom surface by adsorption from solution. Some physico-chemical properties of crown ethers used in this work are given in Table I. Fig.1 shows their structural formulas.

The modifying substances were deposited on the adsorbent by adsorption from a solution. The dense monolayer capacity, α_m, was determined as follows: 1) from the adsorption isotherm of the modifiers from chloroform on carbochrom at 25°C; 2) from the van der Waals dimensions of the modifier molecules arranged in the plane of the carbochrom surface and 3) from the dependence of the retention volumes of various adsorbates on the amount of the modifying substance on the carbochrom surface. The three methods yielded analogous results ($\alpha_m \approx 0.6$ mg/m^2 /6, 7/).

In order to characterize the adsorption properties of the prepared adsorbents the specific retention volumes ($V_{m,1}$), and the heats of adsorption (q_1) of the tested adsorbates were determined for zero surface coverage.

The chromatographic measurements were performed on a Model Tsvet gas chromatograph equipped with flame-ionization and electron-capture detectors. The prepared adsorbents were packed in small-diameter glass columns. The measurements were carried out under isothermal conditions at ten temperatures.

RESULTS AND DISCUSSION

Table II lists the relative retention volumes of the test substances on the original and modified adsorbents. As it can be seen the modification of carbochrom with dibenzo-18-crown-6 ether monolayers results in an increase of the selectivity toward those substances, which have acceptor properties including isomeric, chloroorganic compounds.

Figs 2-3 show the dependence of ln $V_{m,1}$ on 1/T for some chloroorganic compounds on the original and modified carbochrom. These relationships are linear. The values of the heat

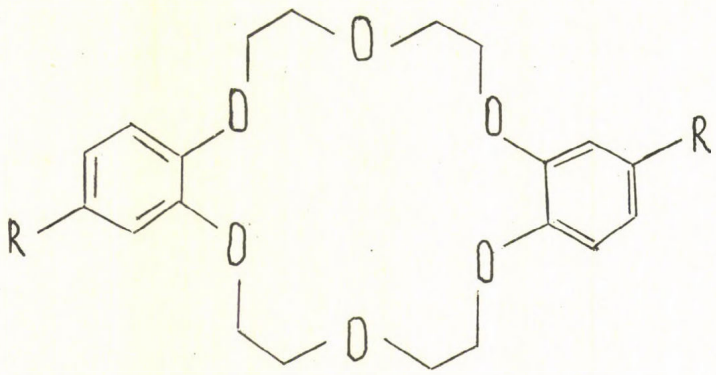
Table I. Selected physico-chemical properties of dibenzo-18-crown-6 and dinitro-dibenzo-18-crown-6 ethers

	$M^{+a)}$	m.p. °C	Decomposition temperature °C
dibenzo-18-crown-6	360	164 /3/	380
dinitro-dibenzo-18-crown-6	450	228-229 /8/	>300

a) From the mass spectrum

Table II. Relative retention volumes $V_{m,1}/V_{m,1(n\text{-}C_6H_{14})}$

Adsorbate	Carbochrom	Carbochrom+ dibenzo-18-crown-6	Carbochrom+ dinitro-dibenzo-18-crown-6
n-Hexane	1	1	1
n-Heptane	5.0	3.4	2.7
n-Octane	-	10.7	7.3
Benzene	0.60	0.56	0.70
Toluene	2.7	1.75	2.14
Ethylbenzene	-	4.0	4.46
Nitrobenzene	17.0	10.0	
Benzonitrile	3.8	8.1	
Aniline	4.6	6.7	
Chloroform	0.16	0.30	
Carbon tetrachloride	0.30	0.50	
Pentanone-2	0.45	1.4	
Pentanol-2	0.45	1.5	



R = NO_2 , H

Fig.1. Structural formulas of the crown ethers.

Table III. Retention volumes ($V_{m,1}$ cm^3/g) of selected chlorinated pesticides

Adsorbate*	Column temperature, °C	$V_{m,1}$	
		carbochrom	carbochrom + dibenzo-18-crown-6
α-HCH	230	82.4	65.6
γ-HCH	230	143.0	102.2
aldrine	230	150.7	111.9
heptachlor	230	180.8	117.8
DDE	285	550.1	212.2

*HCH = hexachlorocyclohexane

Table IV. Heat of adsorption values (q_1, kcal/mole) of selected chlorinated pesticides

Adsorbate	Carbochrom	Carbochrom + dibenzo-18-crown-6
α-HCH	16.6 ± 0.4	11.5 ± 0.3
γ-HCH	16.8 ± 0.4	13.3 ± 0.4
aldrine	15.2 ± 0.3	14.0 ± 0.4
heptachlor	17.6 ± 0.5	15.1 ± 0.4
DDE	18.2 ± 0.5	16.6 ± 0.5

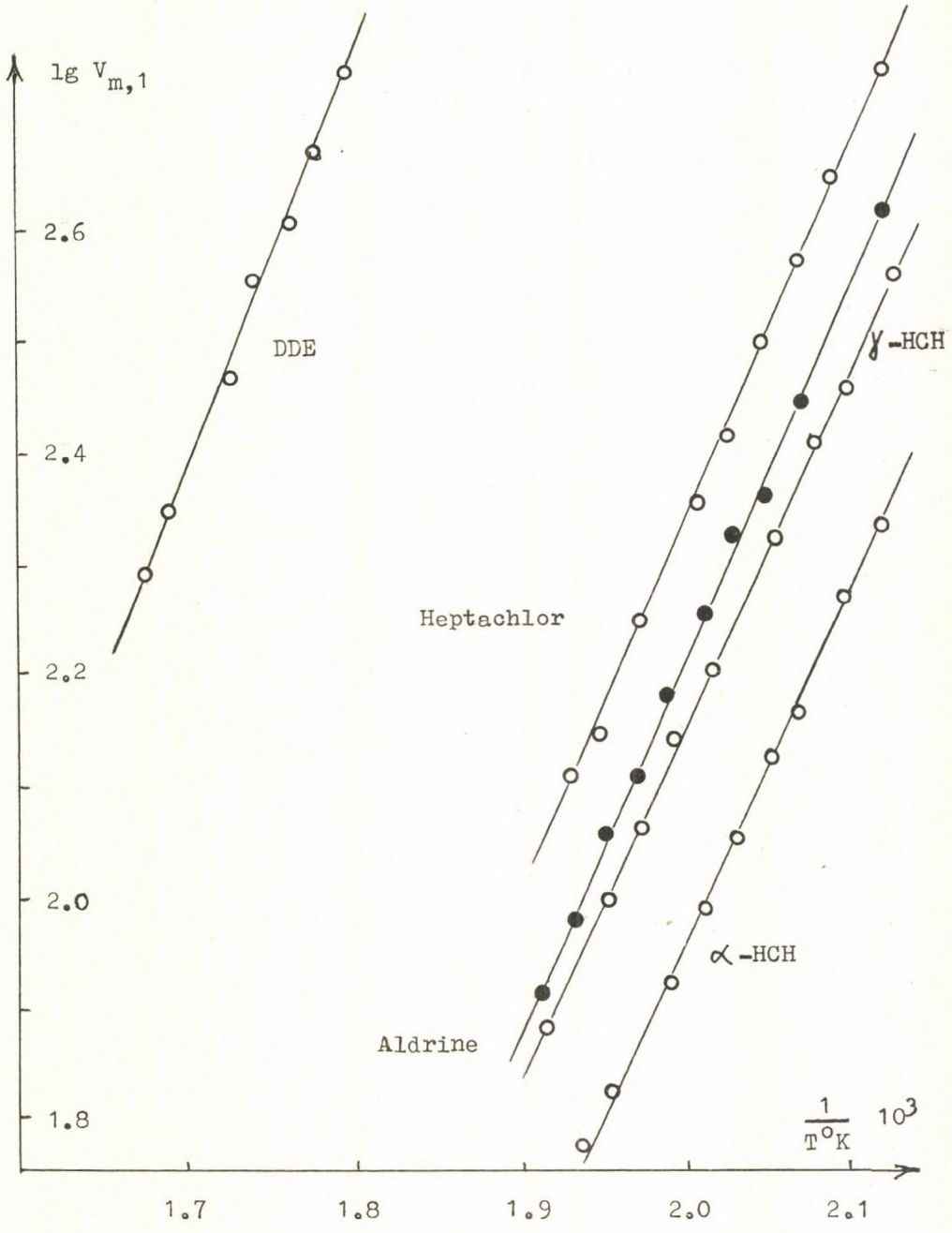


Fig.2. Dependence of log $V_{m,1}$ on 1/T for some chlorohydrocarbons on non-modified carbochrom.

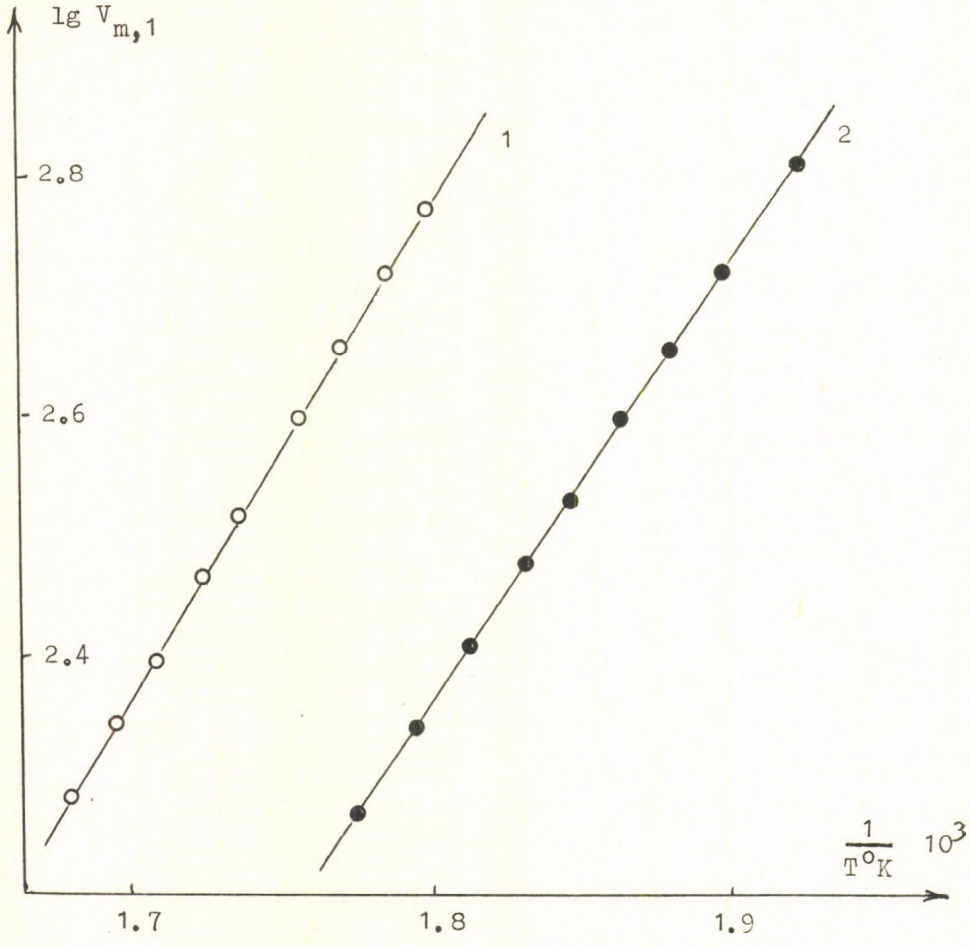


Fig.3. Dependence of log $V_{m,1}$ on 1/T for DDE on the original carbochrom (1) and on carbochrom modified with dibenzo-18-crown-6 (2).

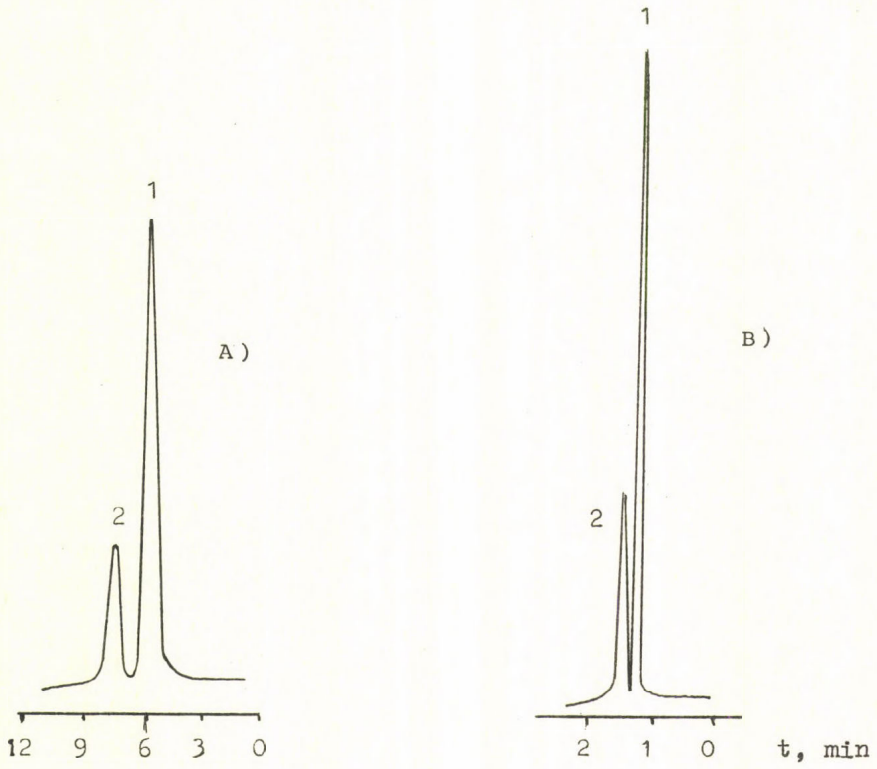


Fig.4. Separation of the exo (1) and endo (2) isomers of 1,2-dimethylbicyclo (2,2,1) heptane.
a) Non-modified carbochrom; column 1 m x 4 mm I.D.; He flow rate 30 cm^3/min; temperature, 140°C.
b) carbochrom modified with dinitro-dibenzo-18-crown-6, glass column: 500 cm x 2.0 mm I.D.; He flow rate, 20 cm^3/min; temperature, 48°C.

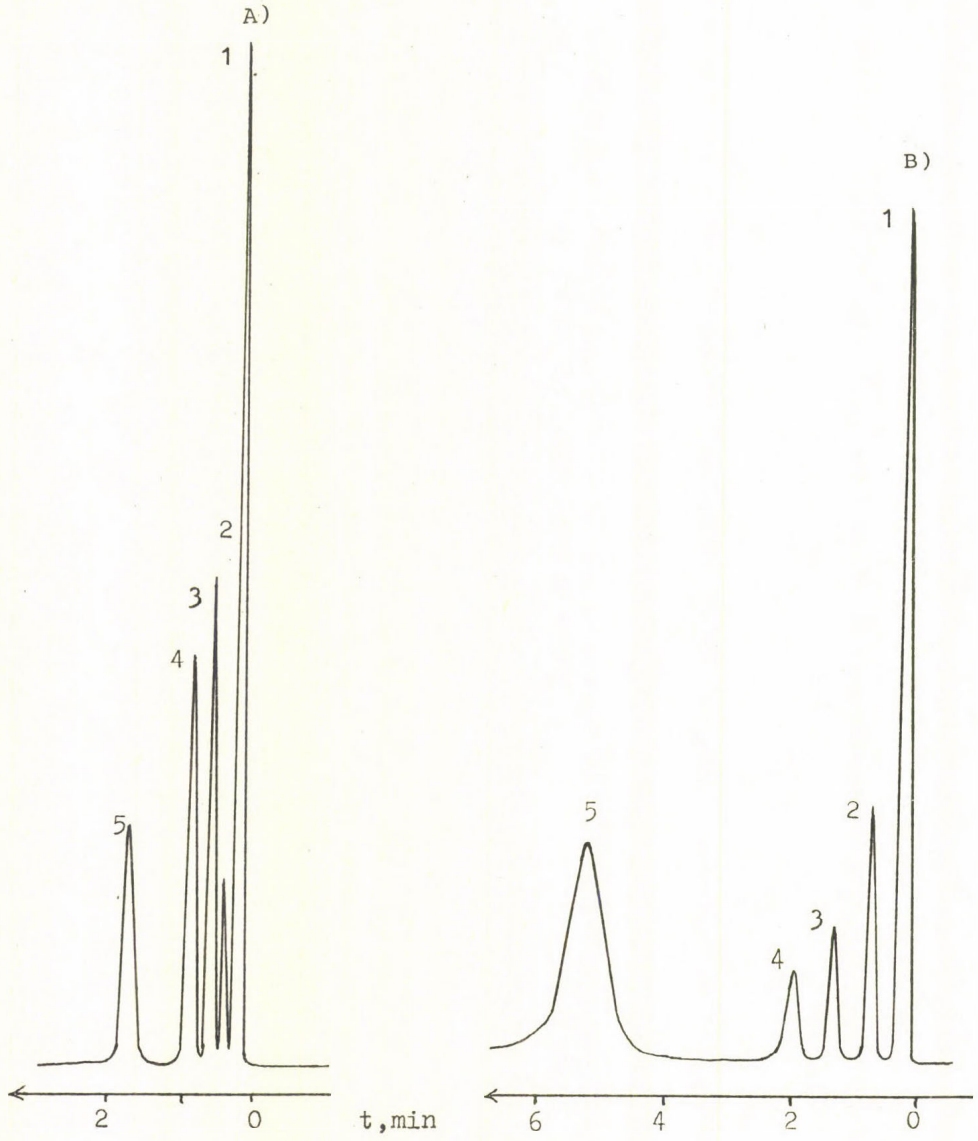


Fig.5. Separation of hydrocarbons on carbochrom modified with dibenzo-18-crown-6 glass column, 500 cm x 1.5 mm I.D.; He flow rate, 15 cm^3/min; temperature, 87°C.
a) 1-benzene; 2-toluene; 3-heptane; 4-ethylbenzene; 5-n-octane; temperature, 70°C.
b) C_7-C_{11} n-alkanes.

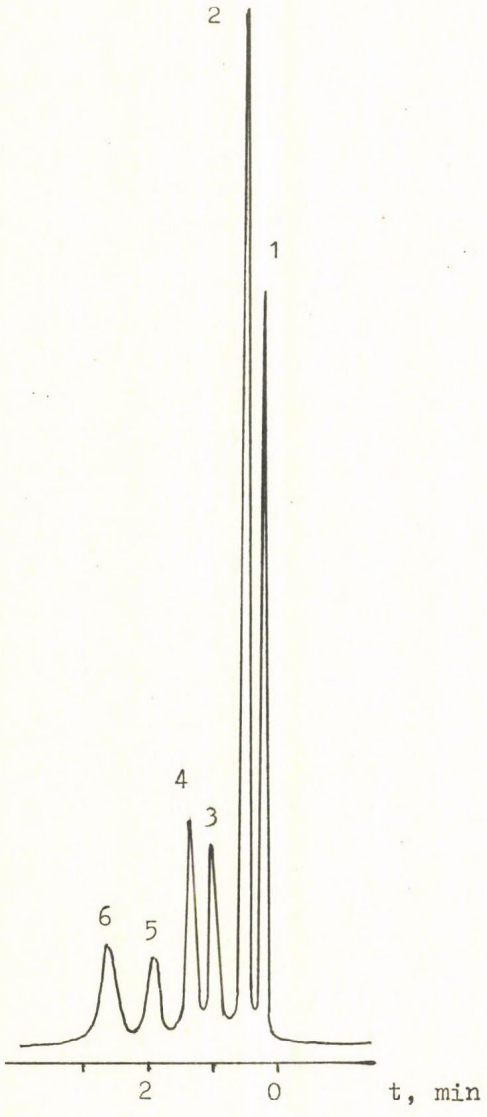


Fig.6. Separation of C_1-C_4 alcohols on carbochrom modified with dibenzo-18-crown-6. Column and conditions as in Fig.5; temperature, 60°C.
1) ethanol; 2) isopropanol; 3) n-propanol; 4) sec-butanol; 5) isobutanol; 6) n-butanol.

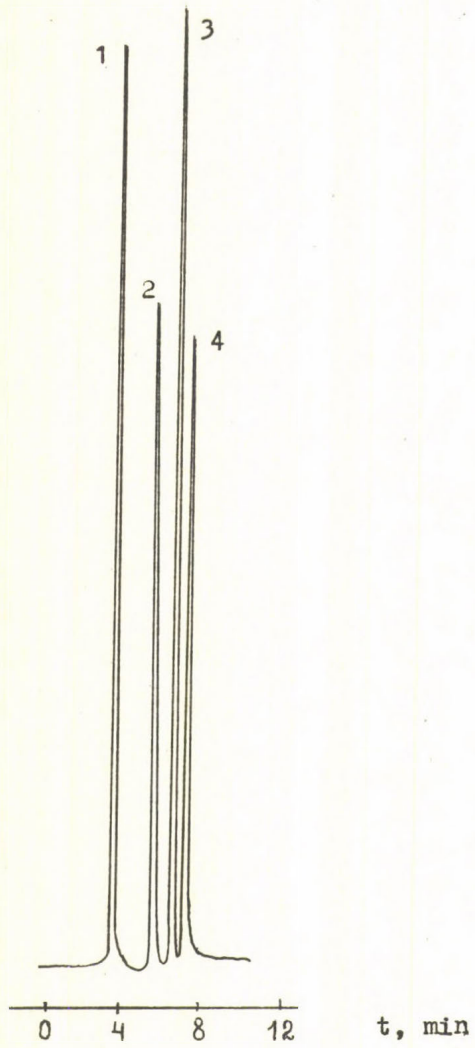


Fig.7. Separation of some chloroorganic compounds on carbochrom modified with dibenzo-18-crown-6; column: 250 cm x 3 mm I.D.; the flow rate, 20 cm^3/min; temperature, 200°C.
1) α-HCH, 2) γ-HCH, 3) aldrine, 4) heptachlor.

of adsorption, q_1, for the adsorbates were calculated from the slope of these linear plots. The retention of chlorinated pesticides on the modified carbochroms are significantly lower than on the original carbochrom (Table III). This results in a decrease in the concentration of the force centres on the coated surface as compared with the uncoated surface. As it can be seen from Table IV the modification of carbochrom surface results in a decrease of the adsorption heats of the chlorinated pesticides and simultaneously in an increase in the selectivity toward isomeric compounds. For example, the heat of adsorption values for α and γ hexachlorocyclohexane (HCH) on the initial carbochrom are paractically equal. On the modified carbochrom the heat of adsorption of the α-isomer is lower than that of the γ-isomer. This is due to the specific interaction of the polar adsorbate molecules with the functional groups of the molecules of the modifying substance.

The prepared adsorbents were used for the concentration and gas chromatographic analysis of halocarbons present in the atmosphere. For the identification of these compounds an electron capture detector and the combination of a gas chromatograph with a mass spectrometer (Nermag R10-10) was used. The weaker energy of dispersion of the adsorbent-adsorbate interaction on the modified adsorbent makes this adsorbent an excellent material for the concentration of organic impurities with subsequent thermal desorption of these compounds at a lower temperature than needed when using the initial carbochrom for the same purpose.

The modified carbochrom can be also used in the analysis of mixture of alkanes, arenes and various polar substances (see Figs 4-7).

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ADSORPTION EQUILIBRIA OF POLAR AROMATIC SOLUTES IN SYSTEMS CONSISTING OF SILICA AND A BINARY MIXTURE OF HEPTANE AND A POLAR MODIFIER

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In liquid chromatography, the optimal range of capacity factors (k') is usually obtained by the use of mixed mobile phase. It is essential to express the relationship between the retention and the composition of the mobile phase by simple equations which permit the quantitative description of the chromatographic system and its optimization. Particularly in gradient elution it is necessary to express the variation of the k' values and the migration of zones during the gradient program /1-3/.

In certain cases a definite molecular mechanism of distribution between the mobile and stationary phase can be assumed; then, the law of mass action can be applied to describe the equilibrium. This approach was first applied to gas-liquid /4/ and liquid-liquid partition systems /5, 6/. For instance, for a partition system consisting of water and an organic phase consisting of a nonpolar diluent and a polar solvent S which forms with the solute Z the solvation complex ZS_n (predominating in the investigated concentration range of polar solvent S) we can write that:

$$Z_w + n\ S_{org} \rightleftharpoons ZS_{n(org)}$$

where the subscripts w and org denote the two immiscible phases.

The equilibrium constant is

$$K = \frac{[ZS_n]_{org}}{[Z]_w [S]^n_{org}} \qquad (1)$$

Thus, for reversed-phase systems (extraction chromatography/7/):

$$k' = \frac{[Z]_w}{[ZS_n]_{org}} \cdot \frac{V_w}{V_{org}} = K \frac{V_w}{V_{org}} [S]^n_{org} \qquad (2)$$

$$\log k' = \text{const} + n \log [S]_{org} \qquad (3)$$

where k' - capacity factor, V - volume.
Note that various concentration units (brackets) can be used since at low solute concentrations they are proportional. Mole fractions are more rational, but volume per cent or molar concentrations are more convenient for practical purposes; naturally, different values are obtained for the constant in eq. 3 for the different concentration units.

An example of such relationship is given in Fig. 1 for two series of hydroxy-benzoylbenzoic acids; the solvation of 2'-hydroxy derivative is weaker due to formation of an internal hydrogen bond so that lower slopes are obtained in comparison to the 4'-hydroxy derivatives /8/.

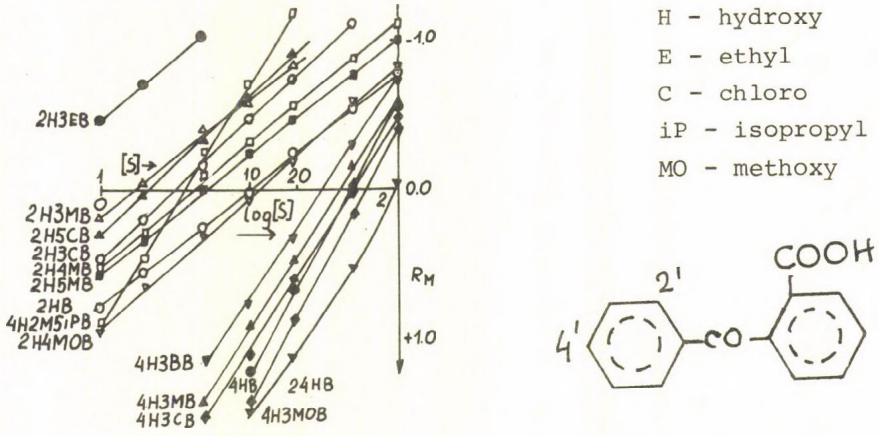
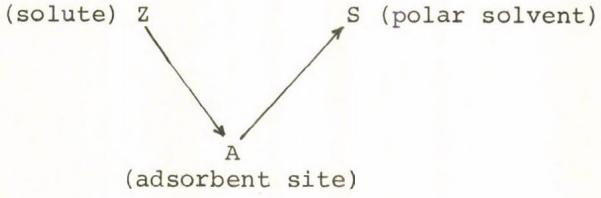


Fig.1. R_M(log k') vs. log(% hexanol) plots of a series of 2'-hydroxy- and 4'-hydroxy-benzoic acids for the system cyclohexane + n-hexanol - aqueous buffer solution (pH 6.0). The slopes of the plots for the 2'-hydroxy series (symbols starting from 2H) are less steep due to the formation of an internal hydrogen bond /8/.

With increasing dilution of the polar solvent S, the solvation number n (slope of the plot) gradually decreases to zero.

Similar linear log-log relationships were obtained in the late 1960s for liquid-solid adsorption systems consisting of silica and a mixture of a nonpolar diluent and a polar modifier. The pattern of molecular interactions in this case was assumed to be different; the consideration of the molecular interactions involved suggested that a displacement mechanism (similar to ion exchange) takes place:



$$A\text{-}S + Z \rightleftharpoons A\text{-}Z + S$$

In this case the application of the law of mass action also leads to a logarithmic relationship /9/:

$$K = \frac{[AZ]\,[S]}{[AS]\,[Z]} \qquad (4)$$

The capacity factor of solute Z is defined as the ratio of concentrations (mole fraction) of the temporarily immobilized solute molecules A-Z and the free molecules Z:

$$k' = \frac{[AZ]}{[Z]} = K\,\frac{[AS]}{[S]} = \frac{\text{const.}}{[S]} \qquad (5)$$

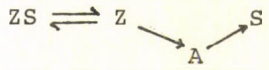
or

$$\log k' = \text{const} - \log [S] \qquad (6)$$

with a unit slope for 1:1 displacement ratio and weak solvation of solute Z by the modifier S.

The competitive solvation of the solute by the polar modifier S was also considered in our first paper in the series dealing with a "simple molecular model of LSC" /9/,

assuming a mixed "displacement + solvation" model:



In the limiting case of strong solvation:

$$A\text{-}S + Z\text{-}S \rightleftharpoons A\text{-}Z + 2S$$

$$K = \frac{[AZ]\,[S]^2}{[AS]\,[ZS]} \tag{7}$$

$$k' = \frac{[AZ]}{[ZS]} = K\,\frac{[AS]}{[S]^2} = \frac{\text{const.}}{[S]^2} \tag{8}$$

$$\log k' = \text{const} - 2 \log [S] \tag{9}$$

Thus, the slope of the log k' vs. log [S] plot cannot serve as an unambiguous criterion of the molecular mechanism of adsorption. In the case of weaker solvation, slopes between 1.0 and 2.0 can be obtained for monofunctional solutes /10, 11/.

The molecular mechanism of adsorption corresponding to eqs. 6 and 9 is graphically represented in Figs 2a and 2b.

Still more complex situation is observed for two- and polyfunctional solutes. For a two-functional solute the slope equal to two can be explained by two mechanisms:

a, Two-point adsorption of non-solvated polar groups (Fig. 2c).

b, One-point attachment of previously solvated polar group while the second group remains solvated after adsorption (solvation effect cancelled, Fig. 2d).

The second mechanism seems to be more probable for silica and chemically bonded polar groups, such as e.g., aminopropyl. Its assumption is not contradictory to the principle of summation of the adsorption energies of the polar groups in Snyder's model /12/: the probability of adsorption of a two-functional solute molecule is greater (and thus the adsorption stronger) because the presence of two or more polar groups increases the probability of adsorption.

The molecular model of adsorption which assumed the formation of definite molecular complexes at the surface and in the bulk solution /9/ was recognized as complementary to a somewhat earlier Snyder's model /13, 14/; this has been characterized as

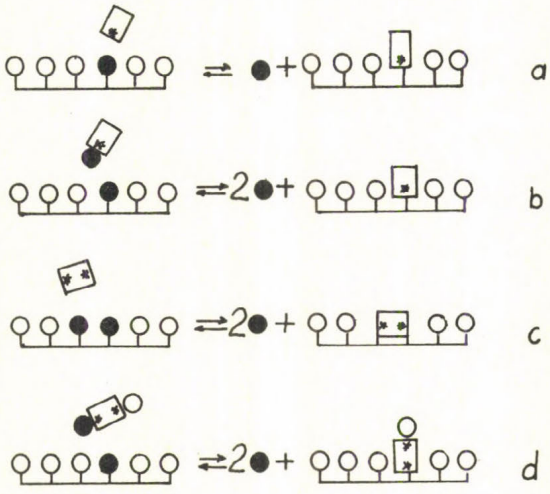


Fig.2. Mechanistic models of competitive adsorption of solute (rectangles) and solvent molecules (circles; filled circles participate in the exchange process). Asterisk denotes the polar group(s) of the solute molecule
a - adsorption of a nonsolvated monofunctional solute molecule (slope = 1);
b - adsorption of a solvated monofunctional solute molecule (slope = 2);
c - two-point adsorption of non-solvated bifunctional solute molecule (slope = 2);
d - single-point adsorption of a solvated bifunctional solute molecule (slope = 2, solvation effect of the second group cancelled).

the Snyder-Soczewiński model.

The simple semiempirical linear equation

$$\log k' = \log k'_S - n \log X_S$$

(concentration of modifier S expressed in mole fraction; for $X_S = 1$ $\log k' = \log k'_S = \text{const.}$) was confirmed by numerous authors; for a review of earlier papers see Ref. /15/.

Several examples of log k' vs. log S plots are given in Figs 3-6.

Recently, the displacement model had been further developed and applied. These are briefly discussed and illustrated below.

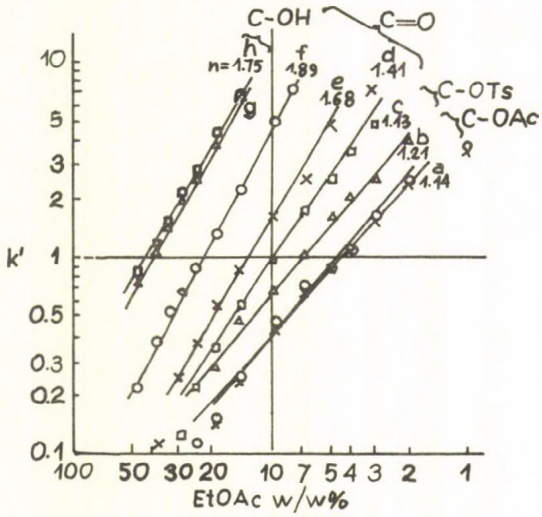


Fig.3. log k' vs. log % EtOAc plot of steroids, silica - hexane + ethyl acetate. HPLC /16/.

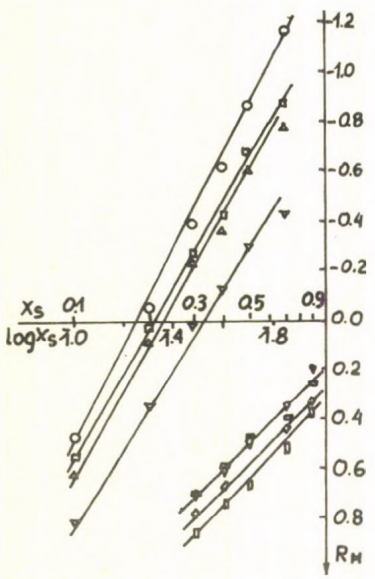


Fig.4. R_M(log k')vs. XEt_2O plots of acyclic and cyclic diastereomers; silica-benzene + diethyl ether. TLC /17/.

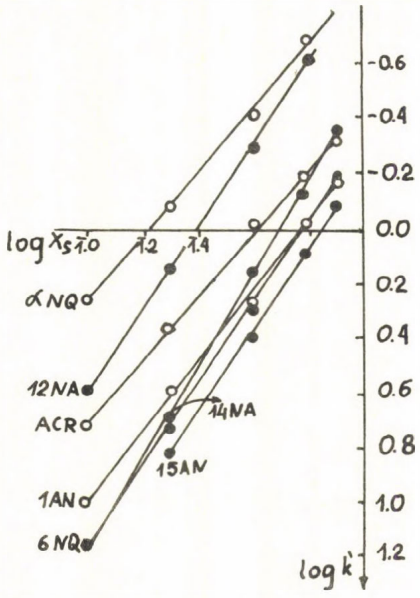


Fig.5. log k' vs. log X_{MeEtCO} plots of quinoline bases; silica - cyclohexane + methyl-ethyl ketone. HPLC /18/.

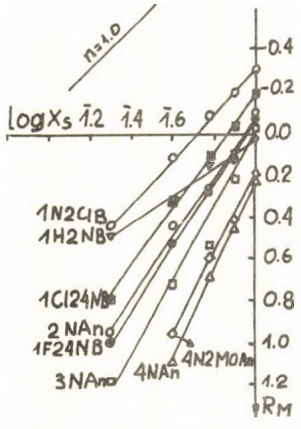


Fig.6. log k' vs. log X_{EtOAc} plot of aromatic nitro compounds; Florisil - cyclohexane + ethyl acetate. HPLC /19/.

Snyder, Glajch and Kirkland /20, 21/ analyzed the molecular mechanism of interactions between the adsorbent surface, solute and mobile phase, considering some complicating effects such as the restricted access and site-competition delocalization. The detailed analysis of the effects permitted the formulation of general principles for the optimization of the eluent composition in the case of ternary or quaternary mobile phases.

Continuing the attempts of Poppe et al. /22/ to formulate a generalized theory of liquid-solid equilibria which would take into account the nonideality of the solution and surface phase, several theories have been published based on statistical thermodynamics and mechanistic model /23-26/.

The simpler equations, following from the displacement model, derived by Poppe et al. /22/ and Ościk /27/ are obtained as limiting cases of the general equation by assuming definite simplifications (e.g., surface homogeneity, constant activity coefficients in the bulk and/or surface phase). The generalized equation is closer to real systems and provides a much better insight into the parameters involved; it should be mentioned, however, that the full experimental verification of the complex multiparameter equation is extremely difficult because it would require the independent determination of the individual parameters.

Therefore, the simple relationship mentioned earlier, in spite of its gross simplifications, retains its practical value and is more easily adapted by the practical chromatographers. A good illustration is the systematic investigation carried out by Hara /28-32/ who determined numerous relationships between log k' and log X_S for steroids and other compounds. The computerized analysis of these data permitted the formulation of a generalized equieluotropic series of mixed solvents /29/. A computer data base for the optimization of HPLC analysis of steroids has been formed /31/. The approach has been extended for ternary eluents /28/(Fig. 7). Nurok /32/ applied these results to optimize TLC analysis, and Papadopoulou-Mourkidou /33, 34/ described systematically the retention behaviour of large groups of pesticides in LSC systems.

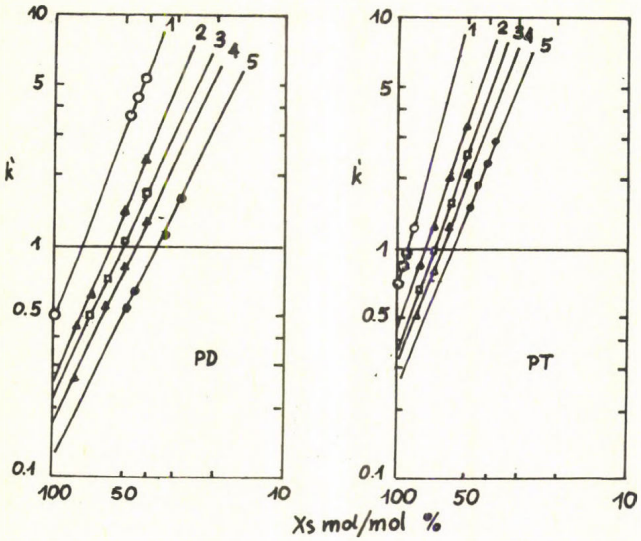


Fig.7. log k' vs. log $X_{\text{ethyl acetate + acetone}}$ plots of panaxadiol and panaxatriol; silica - hexane + ethyl acetate + acetone for various proportions of the two polar modifiers. HPLC /28/.

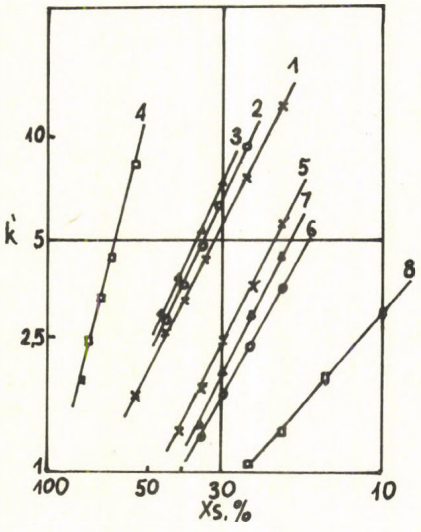


Fig.8. log k' vs. log X_{diox} plots of steroids; aminopropyl silica - hexane + dioxane. HPLC /30/.

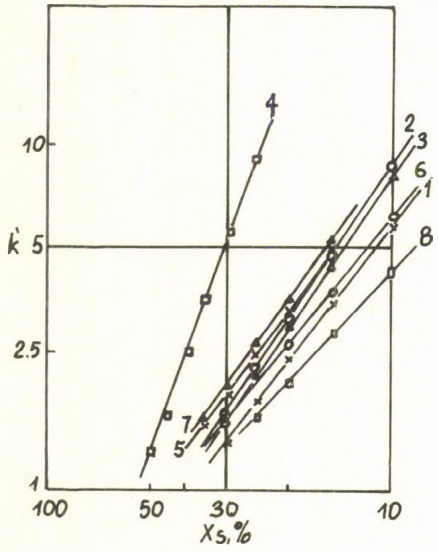


Fig.9. log k' vs. log X_{diox} plots of steroids; cyanopropyl silica - hexane + dioxane. HPLC /30/.

The Snyder-Soczewiński model was at first restricted to inorganic adsorbents such as silica, alumina and Florisil. Recently, the range of adsorbents has been extended to chemically bonded phases. Hara /30/ (Figs 8 and 9) demonstrated linear logarithmic relationship for cyanopropyl and aminopropyl phases; Hurtubise /35/ reported correlation coefficients close to unity for numerous phenols chromatographed on an aminopropyl phase. Similar results were obtained by Snyder /36/ and Hennion /37/.

The range of adsorbents for which the retention model is assumed has recently been extended to nonpolar bonded phases which was rather unexpected because for such systems linear semilogarithmic plots are typical /1, 6/. Murakami /38/ (Fig. 10) reported logarithmic relationships for octadecyl silica and low molecular weight compounds, and Regnier /39/ even for proteins (Fig. 11). The molecular mechanism assumed by Regnier is illustrated in Fig. 12; it is analogous to the pattern assumed in the displacement model (Fig. 2).

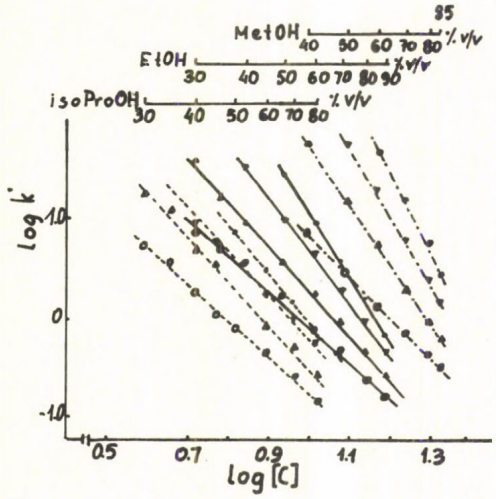


Fig.10. log k' vs. log C_{mod} plots of aromatic compounds; octadecyl silica - water + modifier (methanol, ethanol or isopropanol). HPLC /38/.

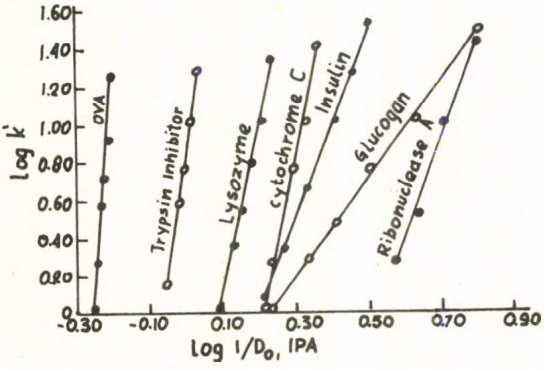


Fig.11. log k' vs. log C_{iPrOH} plots of proteins; octyl silica - water + formic acid + isopropanol. HPLC /39/.

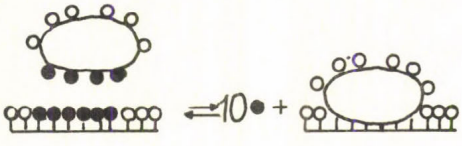


Fig.12. Molecular mechanism of adsorption assumed by Geng and Regnier /39/: displacement + solvation effects (compare to Fig. 2).

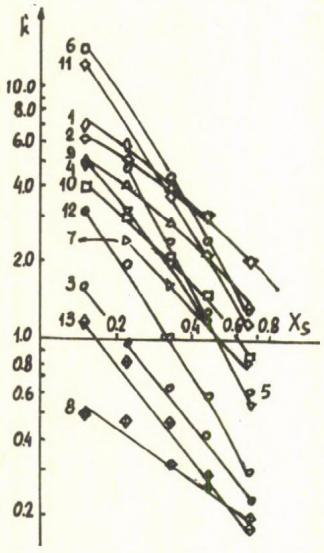


Fig.13. log k' vs. log X_{iPrOH} plots of bi- and trifunctional solutes; silica - heptane + isopropanol. HPLC /40/.

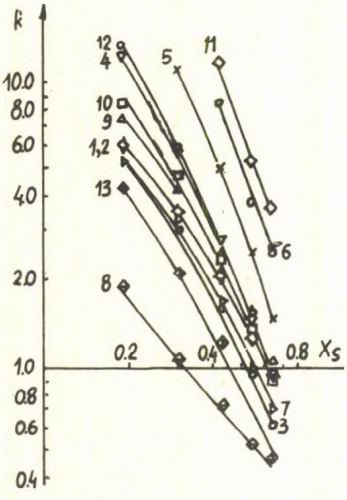


Fig.14. As in Fig.13, silica - heptane + dioxane. HPLC /40/.

The analysis of molecular structure effects is particularly difficult in the case of polyfunctional solutes. Fig. 13 shows the plots of some two- and three-functional organic bases for silica, using heptane solutions of isopropanol as the mobile phase. There are differences in the slopes and even changes in the sequence of the k' values. For dioxane (Fig. 14)

the lines are close together and approximately parallel. An interesting conclusion follows from the correlation of log k' values obtained for the two modifiers (Fig. 15). The solutes are subdivided into two groups. Proton-donor solutes represented by black points have similar selectivities in the two systems. The points of solutes without proton-donor groups fall bellow the correlation line indicating a relatively weaker retention in the dioxane system. The differences can be tentatively interpreted by a peculiar mechanism of adsorption in dioxane systems: the monolayer of dioxane can still adsorb proton-donor solutes due to the outward oxygen atoms so that dioxane molecules form bridges between the silanol groups and the solute molecules /40/. A similar mechanism of bridging the solute molecule to a surface silanol group with a water molecule was assumed by R.P.W. Scott /41/.

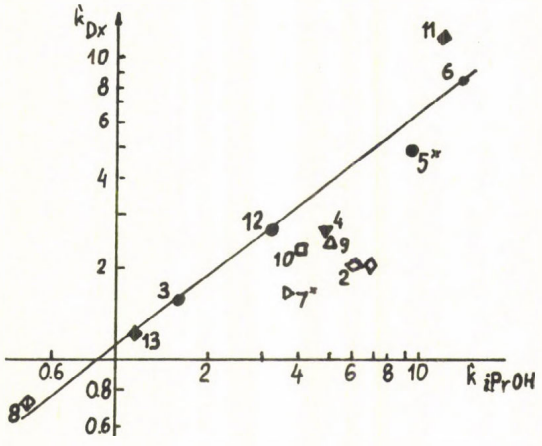


Fig.15. Correlation of log k' values obtained for 30 % dioxane eluent and for 8.33 % isopropanol in heptane (Figs 13 and 14). Black points - compounds containing a proton-donor group (OH or NH_2) /40/.
For notation of solutes 1-13 in Figs 13-15 see Ref. /40/.

The hypothesis requires still further verification on a larger group of model compounds. The confirmation of this mechanism would have some practical consequence for the selectivity of separation of components of different donor-acceptor properties.

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MASS SPECTROMETRIC ANALYSIS OF MALODOROUS AIR POLLUTANTS FROM SEWAGE PLANTS

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ABSTRACT

Thermal desorption capillary column GC-MS in the CI(NH_3) mode is a sensitive and specific method for analyzing malodorous compounds adsorbed on activated carbon used in air purification systems of sewage plants, animal breeding stations, etc. With the use of mass spectrometric profiles the air purification process can be evaluated not only with respect to the malodorous and/or toxic substances it is intended to remove, but also in recognition of the most abundant substances present, which can control its effectiveness.

INTRODUCTION

Sewage plants are usual sources of odor emissions leading in densely populated areas to serious complaints by their neighbours. This is one of that industry's most formidable problems. The main emission sources are the primary settling tanks, the aeration tanks and the thermal sludge treatment plant. Malodors interfere with the wellbeing and therefore - according to a World Health Organization definition - with human health. In extreme cases this results in loss of appetite, nausea, loss of sleep and decreased productivity. Malodors from sewage plants are complex mixtures which are difficult to separate and the malodorous substances (osmogenes) are only partly known.

By anaerobic and aerobic decomposition of carbohydrates and proteins mixtures of hydrocarbons, fatty acids, fatty aldehydes, ketones and especially N- and S-compounds are formed

(see also Table 1). Purification of the malodorous air using activated carbon filters is often necessary. To monitor the air treatment process, particularly, the removal of malodorous and/or toxic components, an effective analytical method is needed.

Our recent GC-MS investigations on odour emissions from municipal sewage plants (refs /1-9/) have shown that the high concentration of non-odorous hydrocarbons usually present in this and similar emissions prevents direct identification and quantification of the osmogenes present in the electron impact (EI) mode and, thus, characterization of a malodour by a GC^2EI-MS odour profile.

Even the extracted ion-current profiles (EICPs) of the osmogenes are not useful as an odour profile due to the high background of hydrocarbon ions. Removal of the hydrocarbons by column chromatography over silica is very difficult, tedious, and losses of volatile osmogenes easily occur. With nitrogen- and sulphur-containing compounds the odour situation can be assessed by capillary column gas chromatography, using N- and S-selective detectors /6, 9/. The disadvantage of this method is that only by using pre-determined GC retention times can one be certain of measuring the characteristic osmogenes. A mass spectrometric method that allows selective detection, identification and quantification of the trace concentrations of osmogenes hidden in the bulk of hydrocarbons would be very welcome. The results reported in this paper show that this problem might be solved by ammonia chemical-ionization mass spectrometry, $CI(NH_3)MS$. In contrast to methane or isobutane CI-MS, the use of ammonia as a reactant gas allows the selective ionization of the basic components of a complex mixture. Aliphatic and aromatic hydrocarbons are usually not ionized /6, 7/.

METHODS

GC-MS profiles were obtained using a Finnigan 4500 COM-GC-MS system. Activated carbon samples from air purification filters were desorbed at 250°C (helium flow rate 10 ml min^{-1}, desorption time 10 min) in the glass capillary injection port,

with the normal injection port liner. Alternatively solvent desorption was employed /2/. The column was a 50 m x 0.25 mm I.D. glass capillary coated with OV-101; the temperature was held during desorption at -196°C, then increased to 200°C at 2°C min^{-1}; the carrier gas was helium at a flow rate of 1 ml min^{-1}. The GC capillary was combined directly with the ion source. Positive CI mass spectra were recorded continually with an Incos data system, using ammonia as reactant gas. The ammonia flow rate was adjusted to an ion source prepressure of 0.3 torr. The ion source temperature was 250°C and the ionization energy 70 eV. N- and S-profiles were similarily obtained using a Carlo Erba Model 793 N/P- and a Model 250 S-selective detector.

RESULTS

Our investigations on the determination of key compounds in the emissions of a municipal sewage plant were carried out in the following way (see Fig. 1). To be able to identify the odorous compounds it was necessary to remove the bulk of the masking non-odorous hydrocarbons (>90%) by column chromatography over silica. The enriched osmogenes in the ethereal eluate were then investigated, after methylation with diazomethane, by COM-GC-MS in the electron-impact mode. The results have already been published /1-5/. At present, it can be assumed that dimethyl sulphide, polysulphides, alkylated and acylated thiophenes, alkylated pyrazines and indole are mainly responsible for the odour emission from a municipal sewage plant. Oxygen compounds, such as lower fatty aldehydes and free fatty acids, are essential for the characteristic odour observed.

Fig. 2 shows the reconstructed ion chromatogram (EI-RIC) of the dichloromethane extract from a sample of activated carbon obtained from a malfunctioning air filter of a sewage plant. n-Alkanes are marked as C_{11}-C_{17} for clarity. This RIC is very similar to analogous chromatograms of non-odorous air samples, except for the peaks identified as dimethyl trisulphide and tetrasulphide. The other peaks are mainly the usual aliphatic and aromatic hydrocarbons and chlorinated hydrocarbons found in polluted air plus phthalates and antioxidants. With the EICP

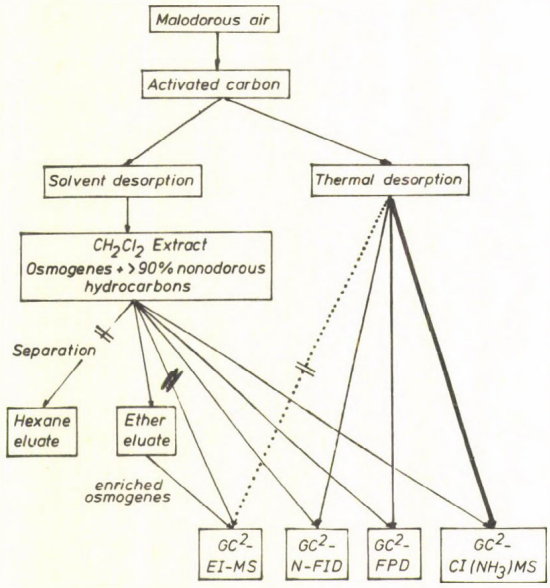

Figure 1. Scheme of analysis

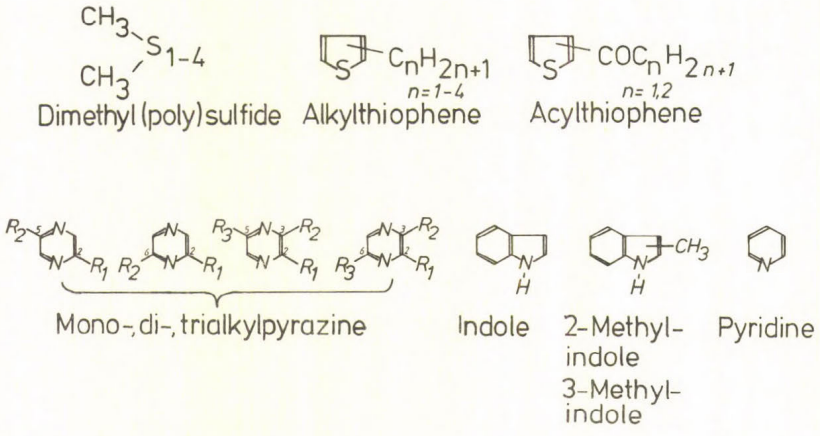

Table 1. Typical malodorous components in emissions from sewage plants

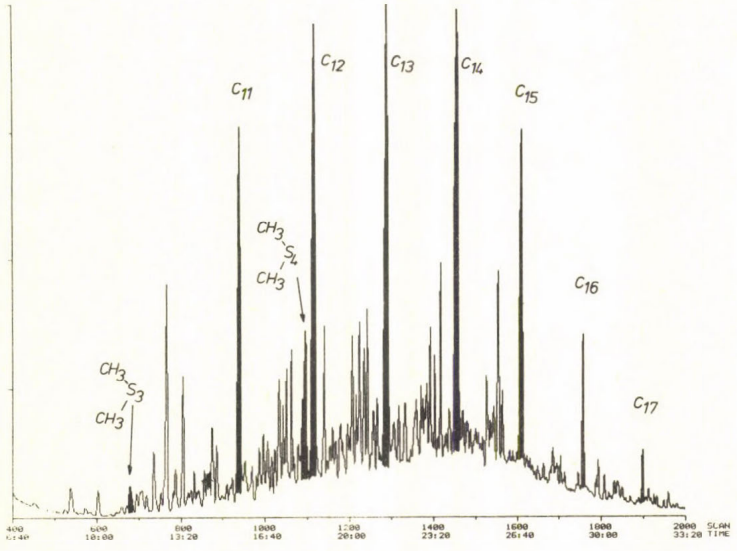

Figure 2. Dichloromethane extract from a sample of activated carbon representing a malfunctioning air filter

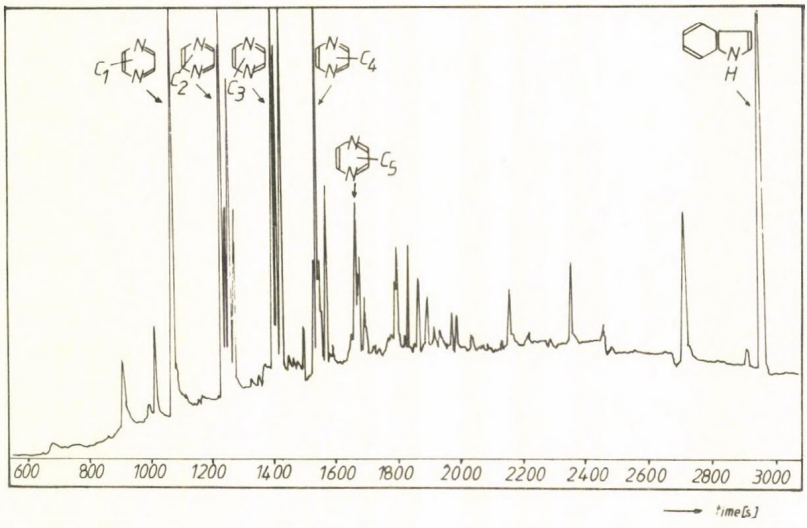

Figure 3. Typical nitrogen odour profile corresponding to Fig. 2

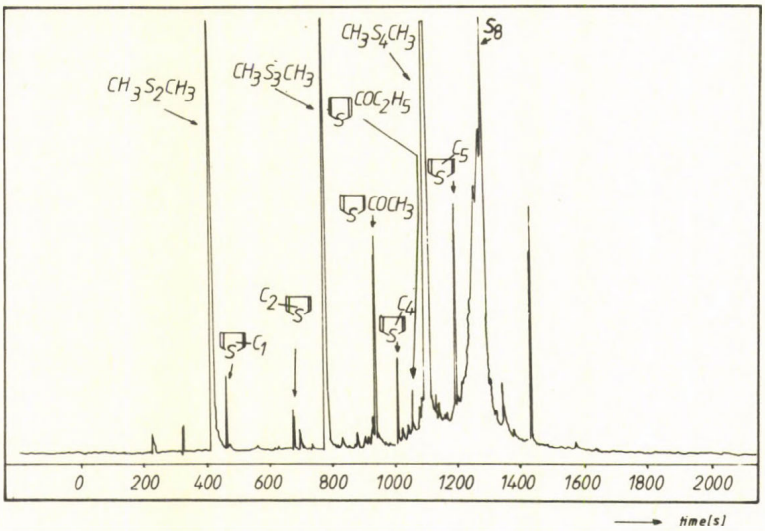

Figure 4. Typical sulphur odour profile corresponding to Fig. 2

method, only a few 2-alkanones can be identified and the other osmogenes are not detectable. Clearly the GC EI-MS profile cannot be used to characterize the osmogenes and/or toxic compounds adsorbed on the carbon filter, and thus its effectivity. Figs 3 and 4 show that by establishing N and S odour profiles the relevant pollutants adsorbed on the carbon, and previously identified by MS, can be characterized because the interfering hydrocarbons are not detected.

A distinct disadvantage is that the oxygen-containing osmogenes, which contribute considerably to the odour situtation, are lost. The problem of measuring the typical key odour compounds by relying on GC retention times only, has already been mentioned.

Reports on CI(NH_3)MS of different classes of organic compounds are still scarce. Ammonia is mostly used to serve as a highly selective reactant gas to ionize preferentially nitrogen compounds. We reported /6, 7/ that the characteristic osmogenes of biogenic origin (see Table 1) were also preferentially ionized in contrast to hydrocarbons. Very intense $(M+H)^+$ and/or $(M+NH_4)^+$ ions are observed and can be used to establish an osmo-

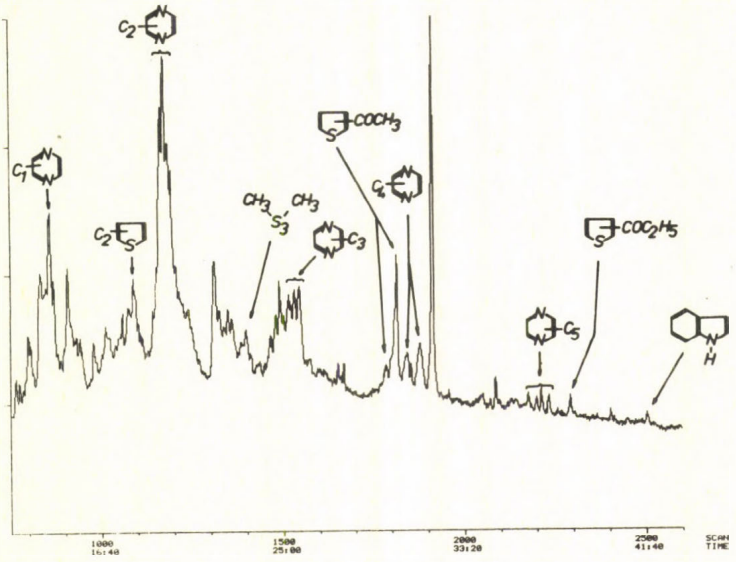

Figure 5. GC-CI(NH_3)-MS profile obtained by thermal desorption of a carbon sample

gene specific odour profile. Fig. 5 shows the GC CI(NH_3)MS profile (RIC) of a typical carbon sample. No peaks corresponding to hydrocarbons can be found. Therefore, this profile, obtained by direct thermal desorption of activated carbon from the filter, can be used advantageously for qualitative and/or quantitative monitoring of the air purification process because it is a real odour profile.

The following questions can be evaluated:

i) are the odorous and/or toxic substances to be removed actually adsorbed on activated carbon and what is their concentration?

ii) what are the components preferentially adsorbed?

iii) what is the adsorption capacity of the air filter depending on certain components under the conditions of air purification employed?

By means of the EICPs the characteristic pollutants can be identified and quantified using their $(M + H)^+$ and/or $(M + NH_4)^+$ ions. This is shown in Fig. 6, a pyrazine plot of a

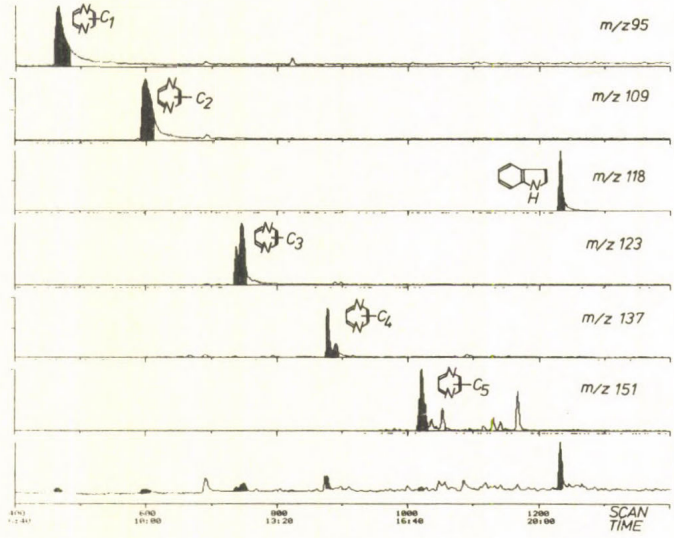

Figure 6. CI(NH_3)-MS extracted ion current profile for alkylated pyrazines and indole

thermal desorbate from an activated carbon filter used for air purification in the thermal sludge treatment building.

Concerning the possibility of low recovery and artifact formation during thermal desorption, we now realize that this is not a serious problem if a very low amount of sample (ca. 1 mg) is desorbed. Also high boilers like indole can be desorbed and thermal artifact formation is usually not observed.

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Budapest, 1986

Chromatography '85
H. Kalász and L.S. Ettre (Eds)

RECENT ADVANCES IN AUTOMATED SAMPLE PREPARATION OF BIOLOGICAL MATERIALS FOR HPLC ANALYSIS

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Before considering the possibilities of automated sample preparation in HPLC analysis, it is necessary to review some fundamental aspects of this facet of the analytical process.

Laboratories involved in the study of biological samples (noteably clinical laboratories and laboratories involved in research into drug metabolism) are faced with increasing demands upon their expertise. Essentially their problems are threefold:

1. An ever-increasing number of samples must be analysed (in order to satisfy regulatory requirements).

2. More complex quantitation and detection requirements must be satisfied because of the proliferation of new drugs and their associated metabolites.

3. More complex isolation and sample preparation requirements must be met as a result of the increasing number of compounds involved, many of which occur at very low levels.

Recent developments in laboratory instrumentation have focussed primarily upon problems relating to sensitivity (i.e. detection) and automation (autosampling and data-handling).

Regardless of the sophistication of modern apparatus, however, little regard has been given to the common situation where samples must be treated by a preparation scheme (involving isolation and concentration) prior to HPLC analysis.

Isolation of the compound of interest and minimisation of interferences prior to chromatography, is often the most

underrated step in the analytical methodology. In spite of this, the efficacy of this step often determines the limits of performance of the overall analytical procedure.

In order for a sample preparation method to be effective it should be:

1. Selective in isolating the compounds of interest.
2. Reproducible and efficient in terms of its ability to recover the compound or compounds being isolated.
3. Rapid.
4. Simple with respect to actual manipulations of the sample.
5. Cost-effective.

Some methods require minimal sample preparation. As an example, one can add a polar organic solvent such as acetonitrile to plasma in order to precipitate proteins, with the subsequent injection of an aliquot of the supernatant into an HPLC system. Such methods offer little in terms of selectivity, and concentration (indeed dilution occurs!). Further, well-developed sample preparation allows for extended HPLC column lifetimes /1/.

Most isolation techniques employ a physical distribution of the compounds between two immiscible phases, most often two immiscible liquids or a liquid and a solid.

Liquid/liquid partitioning

In most biological and clinical applications the phases employed are an aqueous and an organic phase.

In order to maximise the partition coefficient for a given solute, one must optimise several variables within the system (noteably solvent selection, pH and ionic strength /2/).

With such optimisation, liquid/liquid partitioning may be used to good effect in the preparation of biological samples for HPLC.

Problems may be encountered when using liquid/liquid extraction,however, and these may include:

Poor recoveries	due to the incomplete partition of the analyte into the desired phase.
Poor reproducibilities	due to the above, and to adsorption of polar compound to glassware.
Poor sample throughput	due to the labour-intensive nature of liquid/liquid extraction.
Poor laboratory environment	many organic solvents are toxic to a greater or lesser degree.

Liquid/solid interactions

In recent years, numerous sample preparation techniques using a liquid/solid approach have been introduced /3-8/. Examples of products employed for solid phases are charcoal, XAD resins (Rohm and Haas, Philadelphia, Pa.) and cartridges of bonded silica gels such as Sep-Pak (Waters Associates, Milford, Mass.) and Bond-Elut (Analytichem International, Harbor City, Calif.).

Why would one consider a liquid/solid approach toward sample preparation? One reason is that solid surfaces can provide a specific interaction with the compound of interest - a mechanism that differs substantially from solubility, the basis of liquid/liquid partitioning. Solids also provide large surface areas in which these interactions can take place, and the condition of solvent immiscibility is not a consideration in the selection of solvents to provide interchange with the solid surface. Liquid/solid systems thus often provide a separation mechanism which is both more selective and more efficient than that associated with liquid/liquid partitioning.

Liquid/solid interaction is not a new concept. Charcoal, alumina and magnesium silicate, to name a few examples, are naturally occurring sorbents that have been used with varying degrees of success /9/.

A limitation of naturally occurring sorbents is the variability of the solid material itself together with a tendency for non specific adsorption.

The above limitations are largely overcome by the use of bonded-phase sorbents.

Bonded-phase sorbents

A bonded phase can be defined as a chemical moiety covalently bonded to a solid surface. At present, the most widely used and most effective substrate for this bonding is silica gel. Silica gel offers many advantages over other substrates for bonded phase production. The silica gel surface provides a unique bridge so that one can covalently bond nearly any chemical moiety to the surface. Porous silica gels provide large surface areas (approximately 500 m^2/g), which allows for high capacities of the bonded phase moiety. Silica gel is also mechanically stable. Its rigidity allows it to withstand high-pressure differentials associated with high-velocity fluid flow, which allows sample preparation to proceed rapidly.

The isolating attributed to bonded phase sorbents for sample preparation lies in the wide variety of chemical functionalities. The specific chemical interaction allows the chemist to achieve utlraselective chemical isolation which is the overriding goal of sample preparation. Table 1 provides a partial list of some currently available bonded phases.

When compared to liquid/liquid extraction procedures, liquid/solid extraction often offers the benefits of:

Good Recoveries	-	typically 95-100 %
Good reproducibility	-	due to the above and to the absence of glassware
Improved laboratory environment	-	solvent usage is minimised
Improved sample throughput	-	this may be further enhanced by the automation of sample preparation

If we view the fundamentals of a conventional solid phase extraction, it is clear that the procedure is amenable to automation (See Fig. 1).

Table 1 PARTIAL LISTING OF COVALENTLY BONDED PHASES TO SILICA GEL

NON-POLAR PHASES

PHASE	BONDED MOIETY
Methyl (C-1)	Si - CH_3
Ethyl (C-2)	Si - CH_2 - CH_3
Butyl (C-4)	Si - $(CH_2)_3$ - CH_3
Hexyl (C-6)	Si - $(CH_2)_5$ - CH_3
Octyl (C-8)	Si - $(CH_2)_7$ - CH_3
Cyclohexyl (CH)	Si - C_6H_{11}
Phenyl (PH)	Si - Ph
Diphenyl (2PH)	Si - $(Ph)_2$
Octadecyl (C-18)	Si - $(CH_2)_{17}$ - CH_3

POLAR AND WEAK ION EXCHANGE PHASES

PHASE	BONDED MOIETY
Cyanapropyl (CN)	Si - $(CH_2)_3$ CN
Diol (2OH)	Si - $(CH_2)_3$ -O-CH_2-CH(OH)-CH(OH)
Aminopropyl (NH_2)	Si - $(CH_2)_3$ -NH_2
Primary/Secondary Amine (PSA)	Si - $(CH_2)_3$ -NH-$(CH_2)_2$-NH$_2$
Propyl Carboxylic Acid (CBA)	Si - $(CH_2)_3$ - COOH

STRONG ION EXCHANGE PHASES

PHASE	BONDED MOIETY
Propyl Sulfonic Acid (SCX-P)	Si - $(CH_2)_3$-$SO_3^-Na^+$
Benzene Sulfonic Acid (SCX-B)	Si - $(CH_2)_2$Ph $SO_3^-Na^+$
Quaternary Amine (SAX)	Si - $(CH_2)_3$- $N(CH_3)_3^+$ Cl^-

The basic procedure for solid-phase extraction using bonded silica sorbents is as follows:

A sorbent is selected which has the ability to adsorb the analyte out of the sample matrix (together with as few interfering substances as possible). The solution sample matrix is passed through a quantity of the chosen sorbent, and the analytes concentrate on the sorbent bed as they are retained, while other interferences pass through the sorbent and are discarded. If necessary, the sorbent bed may be rinsed with a solvent that elutes other remaining interferences but not the analyte.

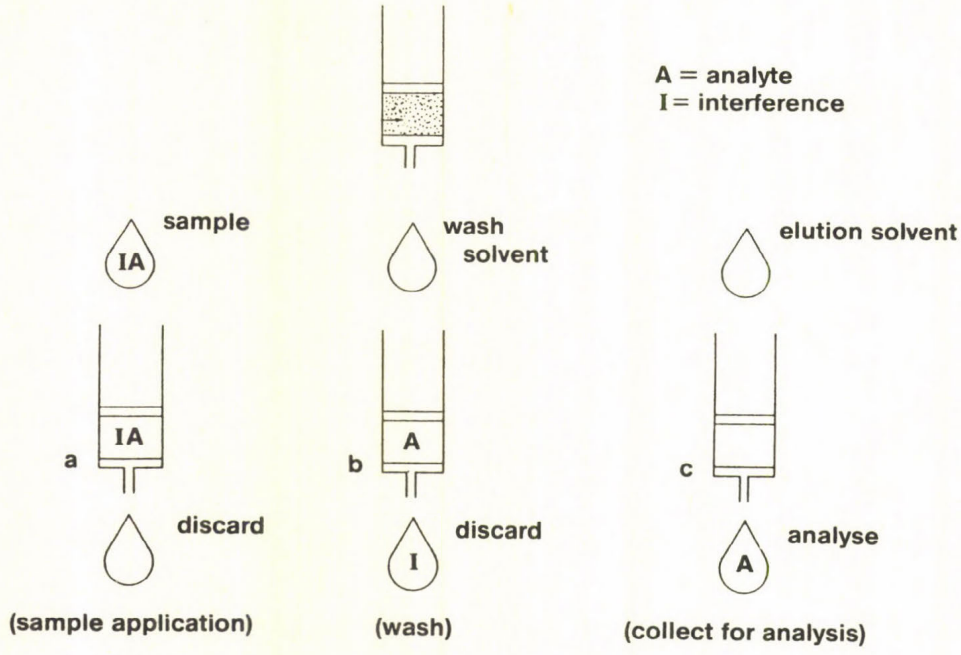
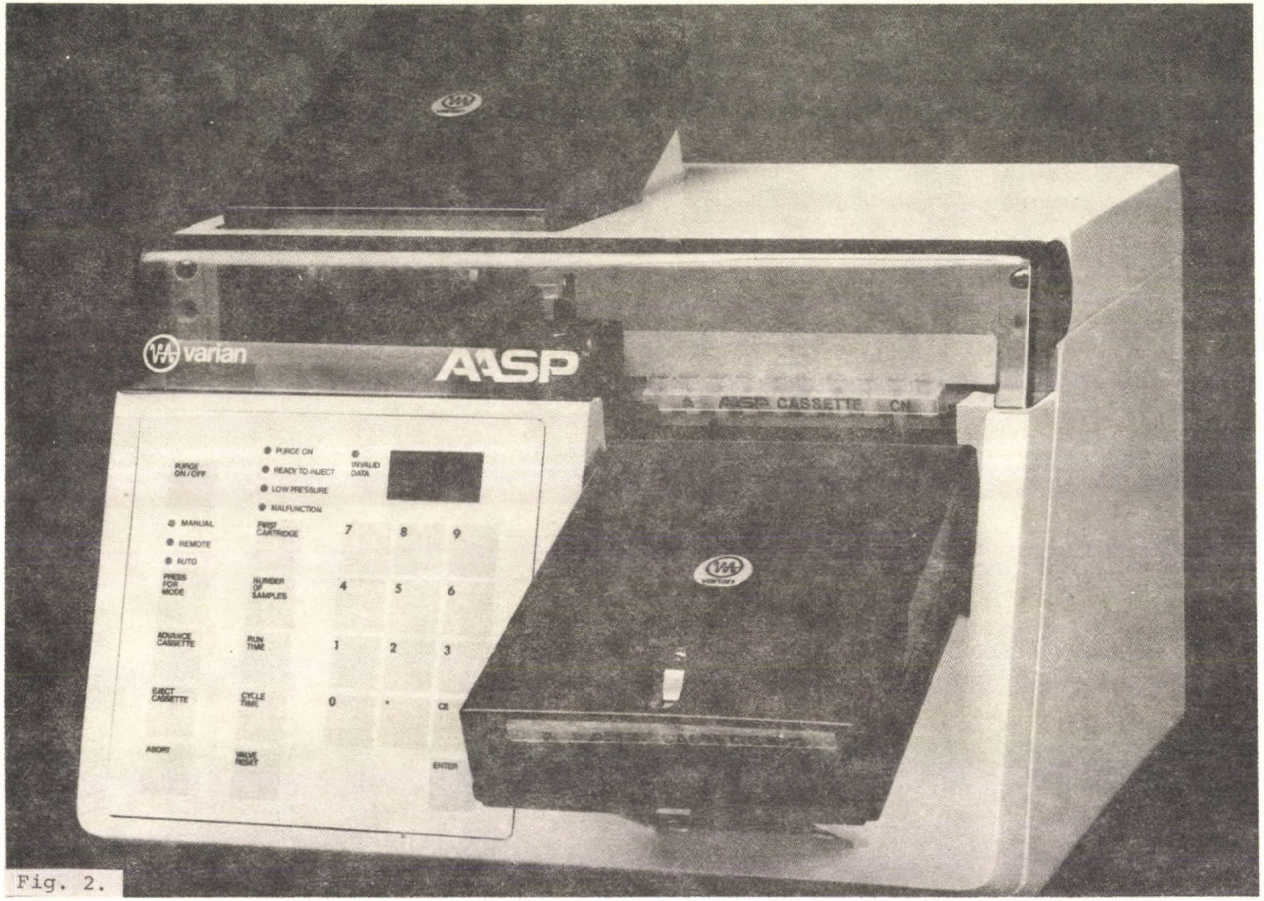


Fig. 1. Bonded phase extraction

Finally, the purified analyte is eluted from the surface with as small a volume as possible of solvent chosen for its eluting properties. This concentrated extract can then be analysed by an instrumental technique such as HPLC.

While bonded silica extraction as a bench technique is extremely useful in its own right, further enhancements of the technique are possible, as will be demonstrated. For example, on-line elution of the sorbent directly into the HPLC analytical column allows complete transfer of analytes from the original sample directly into the chromatograph /10/. This allows for maximum sensitivity of the chromatographic assay, because all of the analyte is detected. The automation of this procedure is achieved by the Varian AASP (Advanced Automated Sample Preparation) (See Fig. 2).

The heart of this system is a sorbent cassette constructed of surgical grade polypropylene and comprising 10 individual sample cartridges (See Fig. 3).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A-SP
A ASP CASSETTE CN
PURGE ON / OFF
MANUAL
REMOTE
AUTO
PRESS POS MODE
ADVANCE CASSETTE
EJECT CASSETTE
ABORT
PURGE ON
READY TO AJECT
LOW PRESSURE
MALFUNCTION
INVALID DATA
FIRST CARTRIDGE
NUMBER OF SAMPLES
RUN TIME
CYCLE TIME
VALVE RESET
7
8
9
4
5
6
1
2
3
0
CE
ENTER

Fig. 2.

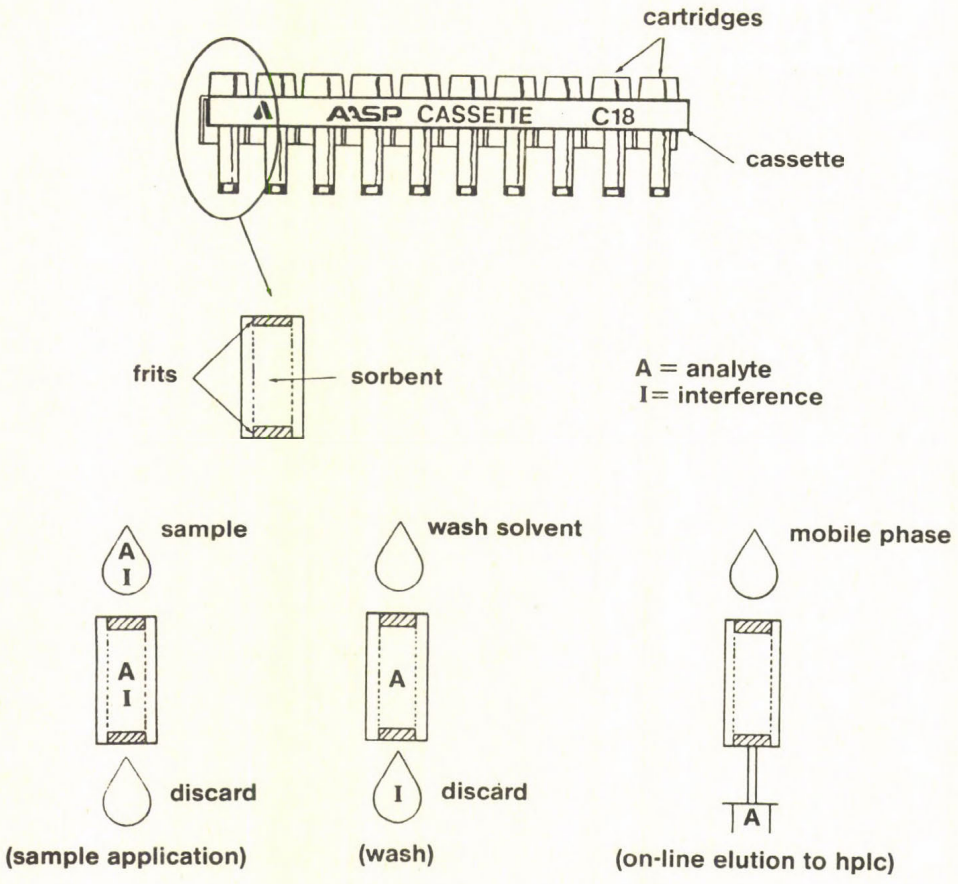

Fig. 3. Advanced automated sample preparation (A.A.S.P.)

Each cartridge contains 40-50 mg of bonded-silica sorbent (quantity depends on sorbent density). The sorbent is held in place by two porous 316 stainless steel frits. This arrangement allows facile application of sample solutions and rinse solvents to the sorbent bed.

Samples are applied to the sorbent cartridges using either a vacuum device or a pressure device.

After sample application and analyte sorption, the cartridges may be rinsed with appropriate solvents to remove interferences retained in the analyte. The cassettes are then placed into the AASP for on-stream elution (Fig. 3).

Having received a cassette (up to 10 cassettes or 100 samples may be loaded) the AASP is able to process samples sequentially moving each cartridge in turn into the mobile phase stream for elution onto the HPLC column.

The AASP moves cartridges into and out of a low dead volume compression chamber which allows each cartridge to withstand up to 6000 psi.

The AASP is easily interfaced to any liquid chromatograph, preferably via an automated 10-port injection valve. Thus mobile phase may be directed from the pump, directly to the column or via an "encapsulated" AASP cartridge to the column (thereby allowing elution of the analyte onto the column).

The use of a 10-port valve facilitates manual injection with no requirement for replumbing.

APPLICATIONS

The potential applications of the AASP in HPLC analysis are very wide-ranging. Automated sample preparation and auto-injection may be undertaken via the AASP, for the HPLC analysis of any solution sample containing analyte which can be retained onto, and eluted off of, a bonded silica sorbent. The cassettes which are used for analyte sorption and extraction are currently available in 12 different bonded silicas, including non-polar, polar and ion-exchange surface moieties. This broad range of surfaces allows for sorption of analytes with widely varying molecular properties from many different matrices.

One example of the utility of the AASP in the analysis of biological samples is seen in the measurement of theophylline from serum.

Theophylline is the most widely administered antiasthmatic drug currently in use. HPLC methods for theophylline assay are numerous, as are sample preparation methods. Sensitivity of detection in HPLC is not a serious problem, since serum therapeutic levels are fairly high (10-20 µg/mL). Theophylline is administered to very small infants, however, and available sample size is limited. It was therefore necessary to develop a method which allowed for handling of very small sample

volumes with high recoveries of the drug. In addition, the large number of analyses performed required a procedure which was rapid and simple. The AASP technique that evolved meets these needs quite satisfactorily.

A cassette containing C-18 (octadecylsilane) bonded silica sorbent was chosen for the extraction procedure. Samples were either 100 µL serum containing 10 µg/mL theophylline or blank serum. The internal standard solution was water containing 20 µg/mL β-hydroxipropyltheophylline. Standards were 10-µL injections into a 20-µL loop of methanol solution containing 100 µg/mL theophylline and 200 µg/mL β-hydroxypropyltheophylline. All solutions were drawn through the cassettes using an AASP Vac-Elut vacuum manifold.

The sample cartridges were first treated with 1 mL of methanol, to wet the sorbent, followed by a 1 mL water rinse, to remove the methanol. The following solutions were then added to the reservoir and drawn through the cartridge in a single step: 200 µl 0.01 M Tris HCl at pH 7.0, 100 µL serum sample (or blank serum), 100 µL internal standard solution (or water), and another 200 µL 0.01 M Tris HCl at pH 7.0. This was followed with a 500 µL water rinse. Total sample preparation time for 20 samples was 10 minutes. The cassettes were then loaded into the AASP for elution.

Chromatographic results are presented in Fig. 4. As Table 2 shows, absolute recoveries for both theophylline and the internal standard are 100 %. Retention time reproducibility is also excellent, being well within acceptable levels of experimental error. It is important to note that the chromatographic peaks represent 1 µg theophylline and 2 µg β-hydroxypropyltheophylline, respectively (100 µL serum sample). Adequate sensitivity exists to easily handle as little as one-tenth (10 µL) the original sample used at these drug levels. It would be extremely difficult to handle this small sample using any technique other than AASP. In addition, the overall procedure is rapid and simple.

In addition to the most straightforward mode of operation as detailed in the above application, the AASP embodies some additional facilities which allow flexibility in sample

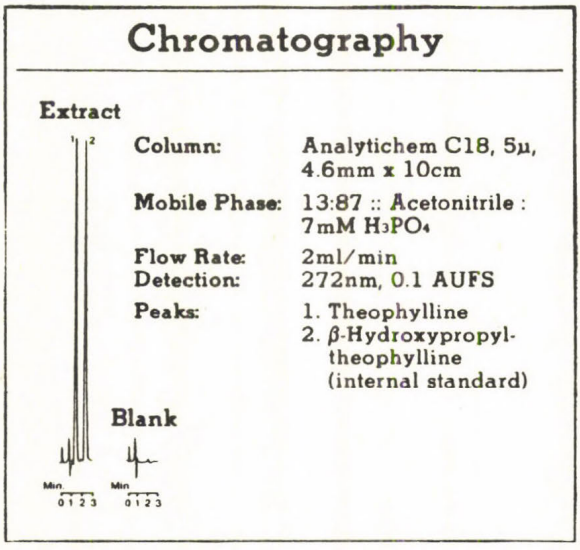

Fig. 4.

Table 2 ABSOLUTE RECOVERY

	Theophylline, 1 µg		Internal standard, 2 µg	
	Standard (n = 6)	Sample (n = 40)	Standard (n = 6)	Sample (n = 40)
Area counts: ($\bar{x}$)	608291	608470	880945	880686
Rel.st.dev.		2.59 %		3.38 %
% recovery: (calc)		100.0 %		100.0 %
Retention time: $\bar{x}$	1.52	1.53	2.47	2.48
rel.st.dev.	0.36 %	0.97 %	1.29 %	1.20 %

preparation/injection. These include:

- use of a solvent other than the HPLC mobile phase for elution of the analyte from the AASP cartridge (the chosen solvent being delivered by an integral "purge pump").
- the possibility of automated pre-column derivatisation (with reagent supplied via the integral purge pump).
- the elimination of late-running chromatographic peaks by trapping these on the sorbent cartridge via a timed valve switch (thus reducing analysis time).

CONCLUSION

If we consider once again the fundamental goals of sample preparation which are -

High Recovery of analyte
Concentration of analyte
No "Memory" from Previous Sample
Ultraselective Clean-up
Automatable Interface to Analytical Instrument
Speed
Economy

- it is clear that all of these requirements may often be satisfied by liquid/solid extractions as performed by the Varian AASP.

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COMPARISON OF THE RPLC RETENTION OF ORGANIC S, SE, AND TE COMPOUNDS

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INTRODUCTION

Organic compounds containing divalent sulphur can very conveniently be separated by reversed-phase HPLC (RPLC), using preferably methanolic eluents. The retention characteristics of thiols, thioethers, multisulphides, disulphides and polysulphides have been described /1/. Very recently we reported on bis-alkylthiopolyselenides /2/. In order to understand the influence on retention of the nature of the chalcogenide atoms, we have synthesized a variety of di-alkylpolyselenides, as well as di-ethylmonotelluride to tetratelluride. In what follows the retention of these, mostly new, compounds will be described and compared with data found for the corresponding polysulphides.

EXPERIMENTAL

The chromatographic apparatus consisted of a Varian 8500 pump, a Rheodyne sampling valve with 20 µl loop, a Knauer spectrophotometric detector in series with a Knauer RI detector, a Varian CDS111L integrator and a Linear strip chart recorder. The column was a Waters 10 cm x 8 mm RadPAK A in a RCM100 compression module. The RCM was kept in an air thermostate.

The polysulphides were prepared by heating about equal amounts (~10 g each) of the respective disulphide and di-n-

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butylamine with twofold excess of S_8, until the latter had dissolved. Maximum temperature was 100°C. The progress of the reaction can easily be followed by injecting samples of the crude mixture into the RPLC system. After cooling, 100 ml CH_2Cl_2 were added. The methylenechloride solution was neutralized with diluted HCl and washed with H_2O. After drying over Na_2SO_4, the CH_2Cl_2 was removed by suction. A very similar procedure has been described by Westlake /3/.

The disulphides were purchased from Aldrich or prepared by the oxidation of the corresponding thiol with $K_3[Fe(CN)_6]$ in alkaline solution. The preparation of dialkylpolyselenides followed a procedure described by Grigsby /4/ for the synthesis of dialkyl-ditelluride. The Te was simply replaced by amorphous Se. Dialkylpoly-tellurides prepared accordingly seem to be very unstable. A freshly prepared solution of diethylpolytelluride shows peaks corresponding to Et_2Te to Et_2Te_5. After a few hours only Et_2Te and some Et_2Te_2 are left.

QUOTATION OF DATA

Raw retention times $t_{ms,\ raw}$ as read from the integrator were corrected for column and extra column dead time and converted to capacity ratios k'. The column dead time was obtained from linearization of n-alkane retention data /1, 5/. Finally, the k' were converted to retention indices I_K. These are RPLC analogues to the well-known GC Kováts indices. If, within the particular chromatographic system in use, the retention of n-alkanes is expressed as

(1) $\quad \ln k' = a + b \times n_c \qquad (n_c$ = carbon atom number)

then the retention of a solute A in the same system is

(2) $\quad I_K(A) = \frac{100}{b} \times [\ln k'(A) - a]$

The retention change caused by a substitution in an alkane is described by the index change

(3) $$\Delta I_K(A) = I_K(A) - 100 \times n_{skeleton}$$

where $n_{skeleton}$ may comprise C, S, Se, Te, O etc., but not H. Comparing I_K data is much more instructive than just using retention times or capacity ratios.

Surface area values were calculated using a computer program developed by Pearlman /6, 7/. The input data are bond distances, bond angles, torsion (dihedral) angles, the connection pattern of the atoms in the molecule and Van der Waals radii. They were taken from the literature /8, 9, 10, 11/. If data for a particular solute were not available, we used approximate values from similar compounds, instead. E.g., the Te-Te bond distance in polytellurides was replaced by the value found in elemental tellurium. All contributing surface area (SA) and total surface area (TSA) values are given in squared Ångströms [$Å^2$].

RESULTS

Figure 1 shows part of a chromatogram of di-n-butyl polysulphide at an ODS phase with 100% MeOH eluent. The typical homologous series pattern is slightly disturbed at the Bu-S_3-Bu position. All di-alkyl trisulphides exhibit higher retention than expected, due to a low permanent dipole moment /1/.

Comparable chromatograms were obtained from various dialkylpolysulphides R-S_n-R with n ranging from methyl (Me) to heptyl (Hep), while -S_n- extended from disulphide to, at least, the heptasulphide.

Figure 2 gives the corresponding retention indices. It is seen that the I_K increases 1. with increasing sulphur rank, 2. with increasing chain length of the terminal groups. Both dependences are linear, if small irregularities for small n_S are neglected.

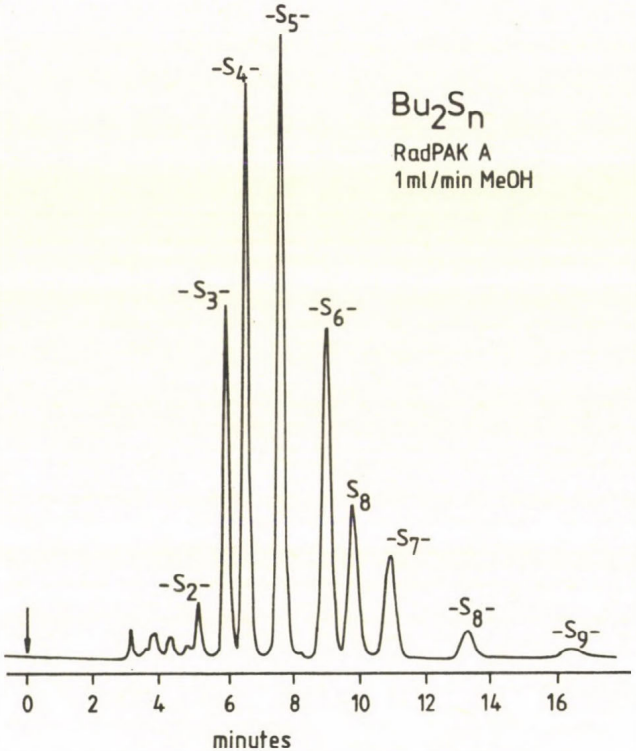

Fig. 1. Chromatogram of di-n-butylpoly-sulphides. Column RadPAK A. Eluent 1m l/min MeOH. Temperature 25°C.

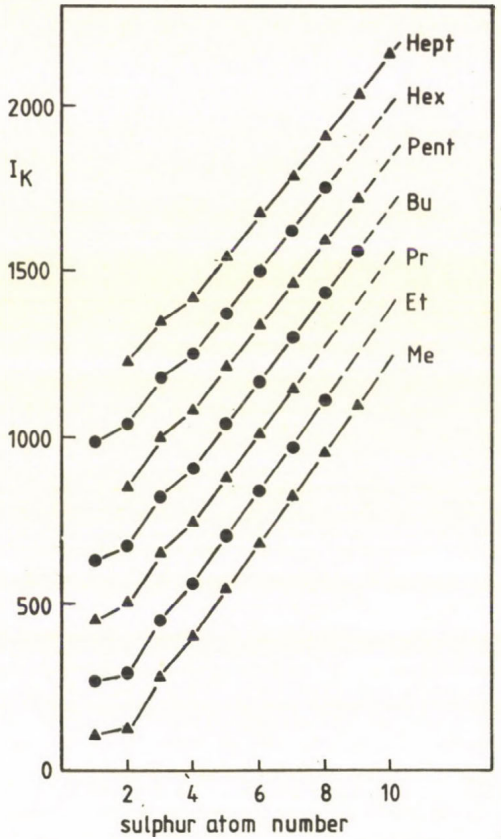

Fig. 2. Retention indices of dialkylpoly-sulphides versus sulphur atom number. Additional parameter: terminal alkyl group. Conditions as in Fig. 1.

Table 1 - Retention indices I_K of various dialkylpolyselenides and some diethylpolytellurides. System as in Fig. 1.

R	Selenium atom number									
	1	2	3	4	5	6	7	8	9	10
Me	173	339	690	849	1021	-	-	-	-	-
Et	318	501	682	836	1003	1165	1327	1483	1640	1815
Pr	513	675	858	1003	1169	1331	1492	-	-	-
Bu	686	846	1019	1159	1319	1482	1639	-	-	-
Pent	872	1018	1189	1323	1480	1639	1791	-	-	-
Oct	1432	1573	1723	1843	1992	2142	2277	-	-	-
	Tellurium atom number									
Et	423	660	835	1046	1249					

The chromatogram of dipropylpolyselenides in Fig. 3. shows clearly that also in this case we have a homologous series. The I_K data for various R and-Se_n- are given in Table 1. Again, retention increases with increasing carbon and selenium chain length. Generally it is observed that with R_2S_n and R_2Se_n, having identical alkyl groups R and chalcogenide chain length n, the respective selenium compound exhibits higher retention.

Attempts to prepare dialkyl polytellurides were less successful. The only compounds we could get in sufficiently high concentrations were diethylmonotelluride to diethyltetratelluride. Their retention is even higher than that of the corresponding selenium compounds (Fig. 4). The data are included in table 1.

DISCUSSION

The above results show that the RPLC retention behaviour of dialkylpolysulphides, - selenides, and - tellurides is similar. A detailed investigation of retentional consequences of

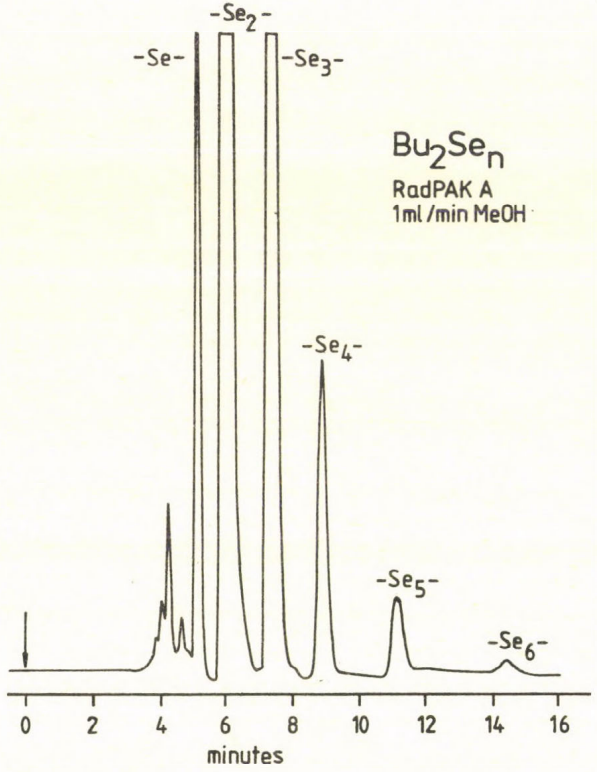

Fig. 3. Chromatogram of di-n-butylpoly-selenides. Conditions as in Fig. 1.

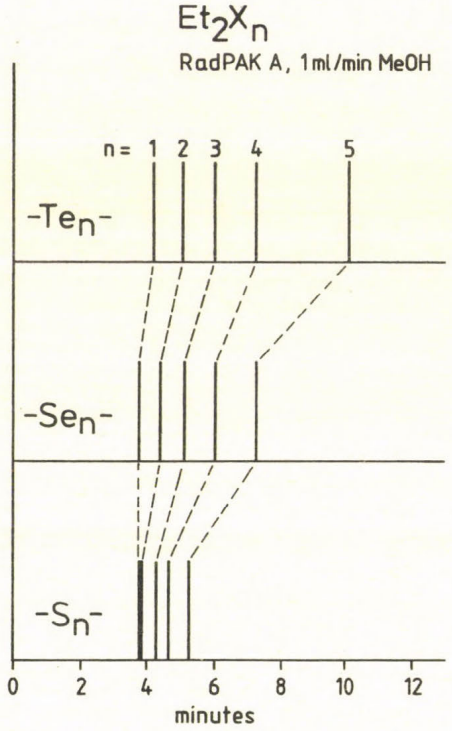

Fig. 4. Comparison of retention times of Et_2S_n, Et_2Se_n and Et_2Te_n. Conditions as in Fig. 1.

this substitution has revealed that in a purely methanolic eluent the solute molecular fragments $-CH_2-S$ are polarized and solvated /1, 12, 13/. The retention of e.g., dibutylpentasulphide

$$CH_3-CH_2-CH_2-CH_2-S-S-S-S-S-CH_2-CH_2-CH_2-CH_3$$

is then determined by three primary effects:

1. the (positive) contribution from the butyl groups
2. the (positive) contribution from the sulphur chain
3. the (negative) contribution from the solvated fragments.

From Fig. 2 and table 1 it is obvious that the whole group of dialkylpolysulphides can be described either as carbon homologues or as sulphur homologues. In the former the S chain length is kept constant and the alkyl groups change from methyl to longer residues. In the latter the terminal groups are kept constant, while the sulphur chain grows. The retention indices of C-homologues follow eq. 4.

$$I_K = A_C + B_C \times n_C \quad (4)$$

For the S-homologues we find

$$I_K = A_S + B_S \times n_S \quad (5)$$

Corresponding equations describe the I_K of dialkylpolyselenides.

It turns out that the A and B are not constant. Fig. 5. shows the A_C and B_C as functions of the number n_S (n_{Se}) of chalcogene atoms in the molecule. A_C increases linearly with $n_S(n_{Se})$. B_C, which represents the index contribution per methylene group decreases with chalcogene atom number in a non-linear way. However, if the $n_S = 2$ or $n_{Se} = 1$ data points are omitted, B_C can be approximated reasonably well as linear functions of n_S and n_{Se}, respectively.

The carbon number dependence of $A_S(A_{Se})$ and $B_S(B_{Se})$ in eq. 5 is given in Fig. 6. Again, we find linear dependence, if the ethyl and methyl compounds are disregarded. It is a well-

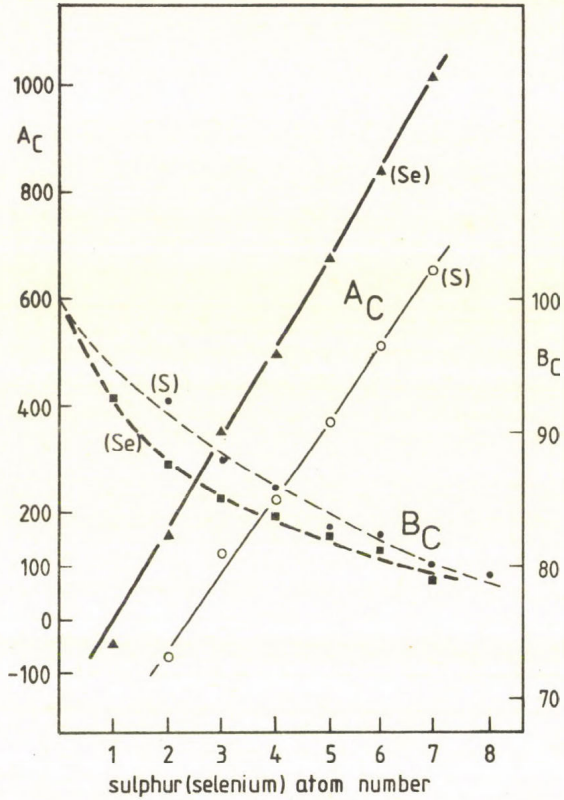

Fig. 5. Parameters A_C and B_C of eq. 4 as function of chalcogene atom number. Circles (A_C) and dots (B_C) for polysulphides, triangles (A_C) and squares (B_C) for polyselenides.

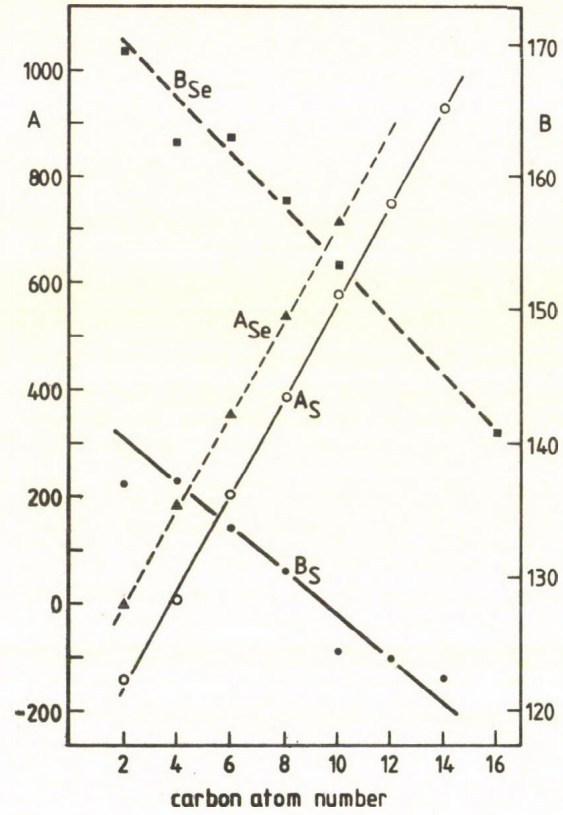

Fig. 6. Parameters $A_{S(Se)}$ and $B_{S(Se)}$ of eq. 5 as function of carbon atom number. Circles A_S, dots B_S, triangles A_{Se}, squares B_{Se}.

known fact that the smallest members of homologous series deviate more strongly from the regular behaviour.

The above mentioned linear or approximately linear dependences of the parameters in eq. 4 and eq. 5 on n_C and $n_S(n_{Se})$, respectively, are expressed in eq. 6 to eq. 9:

(6) $$A_C = a'_C + b'_C \times n_S$$

(7) $$B_C = a''_C + b''_C \times n_S$$

(8) $$A_S = a'_S + b'_S \times n_C$$

(9) $$B_S = a''_S + b''_S \times n_C$$

Corresponding relations exist for the polyselenides and, most probably, for the polytellurides. Then, n_S would have to be replaced by n_{Se} or n_{Te}, respectively.

The quantities in the above equations may be interpreted as follows:

A_C is the intercept of the I_K ./. n_C dependence extrapolated from $n_C > 2$. It consists of a'_C which is twice the (negative) bond index increment $\delta I(CH_2\text{-}S)$, and n_S times the index increment of an S atom in a chain, $b'_C = \delta I^o(S)$.

B_C is the index increase per C atom, consisting of the basic increment per CH_2 group in the presence of 2 "solvation patches", $a''C = \delta I^o(CH_2)$ and the index decrease per S atom, b''_C.

A_S is the intercept of the I_K ./. n_S dependence extrapolated from $n_S > 2$. It comprises twice the negative bond increment, $a'_S = 2 \times \delta I(CH_2)$ and the increment of the CH_2 groups present, $n_C \times b' = n_C \times \delta I^o(CH_2)$.

B_S is the index increase per S atom within the chain. It consists of the basic increment per S atom $a''_S = \delta I^o(S)$ and the decrease of this increment per methylene group present, b''_S. It is trivial to state that $a'_S = a'_C$; $b'_C = a''_S$ and $b'_S = a''C$.

Table 2 - Retention index increment data for dialkylpolychalcogenides. System as in Fig. 1.

	$\delta I^{o}(CH_2)$	$\delta I^{o}(CH_2\text{-}x)$	$\delta I^{o}(X)$	b''_C	b''_S
R_2S_n	+91.0	-167.1	+142.2	-0.75	-0.75
R_2Se_n	+89.1	- 86.5	+172.4	-1	-1
R_2Te_n	+87	- 82.7	+239.0	-1	-1

All pertinent data are listed in table 2. With polysulphides, a'_S = - 335.5 and a'_S = -332.8 was found. The average gives $\delta I(CH_2\text{-}S)$ = -167.1. From a''_S = 143.7 and b'_C = 140.6 we get after averaging $\delta I^{o}(S)$ = 142.2, which is the index contribution of an S atom in a hypothetical pure sulphur chain. For both b'_S and a''_C we found 91.0 = $\delta I^{o}(CH_2)$.

These data hold for both n_C and n_S > 3. If the non-linearities observed with di- and monosulphides are taken into account, $\delta I^{o}(CH_2)$ would eventually reach the alkane value of 100.0.

The above data can be used to express the retention index of dialkylpolysulphides in terms of index increments:

$$(10) \qquad I_K = 2 \times \delta I(CH_2\text{-}S) + n_S \times [\delta I^{o}(S) + b''_S \times n_C] + n_C \times [\delta I^{o}(CH_2) + b''_C \times n_S]$$

For our particular chromatographic system we get

$$(11) \qquad I_K = -334.2 + n_S(142.2 - 0.75 \times n_C) + n_C(91.0 - 0.75 \times n_S)$$

This equation reproduces the experimental data with an accuracy of better than 1%.

The observation that the presence of a chalcogenide chain of increasing length reduces the retention contribution of methylene groups may be explained by two mechanisms, which, however, are still highly speculative.

First, the presence of chalcogene atoms may polarize the C-H bonds within the solute molecule such that the proton-proton interaction energy /14, 15/ between alkyl proton and methyl protons of the surrounding methanole is enhanced. However, such a mechanism would hardly explain the finding, that the n_C dependence of I_K is strictly linear which means that all carbon atoms are influenced to the same extent, independently of the distance from the chalcogene chain.

Second, the presence of the "solvation patches" around the polarized $-CH_2-S-$ or $-CH_2-Se-$ fragments may influence the adjacent eluent MeOH molecules such that their tendency to reject alkyl groups is reduced. If simultaneously the proton-proton interaction is enhanced, the effect could easily propagate along fairly long carbon chains.

Analogous argumentation may be used to explain the n_C dependence of $A_S(A_{Se})$ and $B_S(B_{Se})$. In all, a fully satisfactory explanation of the effects cannot be given at present. It is hoped that further, mainly spectroscopic investigation will lead to a better understanding.

Completely analogous calculations were carried out for selenium chains. The data in table 2 show that the methylene index increment $\delta I^o(CH_2) = 89.1$ is only slightly lower than in the polysulphides. Again, the extrapolation of the B_C versus n_{Se} curve in Fig. 4 yields 100 for zero selenium content.

The selenium atom increment $\delta I^o(Se) = 172.4$ is much higher than the sulphur value. The bond increment $\delta I(CH_2-Se) = -86.5$ is only about half the index loss observed for the CH_2-S arragement. This indicates that the local polar centre around the CH_2-Se bond is distinctly weaker than in the case of CH_2-S, permitting only moderate solvation.

The few data available for tellurium compounds do not allow to perform calculations as extensive as for sulphur and selenium. The I_K equation for the di-ethylpolytelluride is

$$(12) \qquad I_K = 182.5 + 234 \times n_{Te}; \qquad r = .9994$$

If we assume that $\delta I^o(CH_2)$ decreases linearly in going from S through Se to Te, we get a value of 87. The decrease of the

tellurium index increment by the presence of two ethyl groups also can be excepted to be the same as for S and Se, which is about 1 index unit per atom. Then we get

(13) $\delta I(CH_2\text{-}Te) = A_{Te} - 4 \times I^{o}(CH_2) = 182.5 - 348 = -165.5$

or $\delta I(CH_2\text{-}Te) = -82.7$.

This indicates that the CH_2Te arrangement is only slightly less solvated than the CH_2-Se group.

For the index increment of Te atoms $\delta I^{o}(Te)$ we find

(14) $\delta I^{o}(Te) = B_{Te} - 4 \times b''_{Te} = 235 + 4 = 239$

which says that Te atoms in tellurium chains produce 2.4 times as much retention as CH_2 groups in n-alkanes.

From the retention behaviour of homonuclear prcton-free solutes /15/ we know that their retention indices are determined to a very good approximation by only one solute molecular parameter. This is their espective total molecular surface area TSA.

The same can be assumed for the per atom retention index increase in S, Se and Te chains, which means that eq. 15 is expected to hold.

(15) $$\frac{\delta I^{o}\ (\text{Chalcogene 1})}{\delta I^{o}\ (\text{Chalcogene 2})} = \frac{\Delta TSA\ (\text{Chalcogene 1})}{\Delta TSA\ (\text{Chalcogene 2})}$$

The surface area computation was carried out with the diethyl compounds. The regression equations found were

(16) $TSA(Et_2S_n) = 116.06 + 23.44 \times n_S, \qquad r = 0.999999$

(17) $TSA(Et_2Se_n) = 117.20 + 28.09 \times n_{Se}, \qquad r = 1$

(18) $TSA(Et_2Te_n) = 109.92 + 39.54 \times n_{Te}, \qquad r = 1$

(19) $TSA(\text{n-alkanes}) = 33.06 + 20.47 \times n_C, \qquad r = 1 \ (n_C > 3)$

For the ratio in eq. 15 we find

Chalcogene 1 / Chalcogene 2	δI^{o}(Ch.1) / δI^{o}(Ch.2)	ΔTSA(Ch.1) / ΔTSA(Ch.2)
Te/S	1.68	1.69
Te/Se	1.39	1.41
Se/S	1.21	1.20

The agreement is good, which indicates that the retention contributions of S, Se and Te chains are controlled by their respective surface areas. They are distinctly higher than methylene group contributions, due to the lack of any solute-eluent interactions besides purely dispersive forces. Normalized to equal surface areas, S, Se and Te atoms produce an index increase of 6.08 $\pm$ 0.05 units per $\AA^2$, while the methylene group index increase is only 4.89 units per $\AA^2$. These data hold for an ODS/100% MeOH system.

To get an estimate for the free energies corresponding to the above index increase values, we consider two hypothetic chains, one of which is made up from CH_2 groups, the other of proton-free units like S, Se or Te. Both have a surface area of 200 $\AA^2$ (n-octane has 196.82 $\AA^2$). For the $(CH_2)_x$ we calculate I_K = 978 or ln k' = 0.4886, for the $(S)_x$ I_K = 1216 or ln k' = = 0.9601. Assuming a formal phase ratio of ϕ = 0.2 for ODS/MeOH, we find $-\Delta G$ = 1520 cal/mole for $(S)_x$ and $-\Delta G$ = 1240 cal/mole for $(CH_2)_x$; the difference being 280 cal/mole. Using the same ϕ = 0.2, a sorption free energy of -1030 cal/mole is calculated for n-octane, which shows that the effect of the additional proton-proton interaction is quite substantial.

CONCLUSION

The retention index of dialkylpolychalcogenides R_2S_n, R_2Se_n, R_2Te_n can be broken down into index increments corresponding to the alkyl chains, the chalcogenide chains and the methylene-chalcogene arrangement. Once the increments have been

determined, the retention index of any arbitrary member of these compound classes can be predicted with high accuracy. The index increments of the chalcogenes are interrelated via their respective molecular surface area. Normalized to equal surface area, the index increment of CH_2 groups is substantially lower, due to an extra interaction with the eluent methyl group.

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ION CHROMATOGRAPHY OF INORGANIC ANIONS, STUDY OF NATURAL WATER SAMPLES

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In the last years ion chromatography has become the preferred method in the analysis of inorganic species in aqueous solutions. Both the suppressed and nonsuppressed techniques are in wide use. As we have used ion chromatography in the study of natural waters, the investigations have been performed to optimize the analytic conditions.

Column techniques require special packing materials with suitable capacity and particle size, careful optimization of many parameters including column size to decrease the analysis time and assure the good separation capability of the system. In this respect the investigations have provided information on the influence of column length, eluent flow rate and sample volume on the detection limit and on the separation capability of the ion chromatograph. Also the influence of the inlet pressure on the analytical results has been studied.

EXPERIMENTAL

The ion chromatograph IVK-11 (designed and produced by the Special Design Office, Academy of Sciences of the Estonian SSR) was used.

The instrument is equipped with an alternative current conductivity detector at the frequency of 10 kHz. Measurement voltage is 0.5 V. The low cell volume (2 µl) and cell constant (3 cm^{-1} at 18 ^{o}C) offer a detector sensitivity of 3.7×10^{-8} g KCl/ml at 18 ^{o}C. The built-in temperature compensator assures

base line stability at high sensitivities. Minimum detectable conductance change is 10^{-4} μS.

The system has four conductance ranges: 10 and 100 μS, 1 and 10 mS. Full scale zero suppression is possible for all ranges.

The constant-volume reciprocating pump permits a wide choice of eluent flow rates with minimum pump pulse noise.

The anion-exchange resin VARS-2 with a particle diameter of 25-40 μm and a capacity of 12.7 and 38.0 μeq/cm^3 dry material was synthesized in the Institute of Chemistry, Academy of Sciences of the Estonian SSR. In the suppressor columns the cation-exchange resin KY-2(8) was used.

The separation columns used in the experiments had the following dimensions (length x i.d.): 60x3, 100x3, 250x3, 350x3 and 250x4 mm. Two different suppressor columns were used, with the dimensions of 150x6 and 250x6 mm.

The eluent was prepared by dissolving 0.504 g $NaHCO_3$ and 0.508 g Na_2CO_3 in 2 l of bidistilled water.

The samples were introduced by a loop injector equipped with 20, 100 and 200 μl sample loops.

In order to regulate the inlet pressure, a restriction valve has been installed between the separation and the suppressor columns. In other experiments it has been disconnected.

RESULTS AND DISCUSSION

The dependence of the peak height and peak area on the eluent flow rate under the conditions of a constant sample volume and concentration was investigated. The dimensions of the separation columns were 150x3 mm for SO_4^{2-} and 250x3 mm for Cl^-; they were packed with VARS-2 (38 μeq/cm^3). The eluent flow rate was in the range of 1.0-4.0 ml/min. The following sample volumes were used: 20 μl of the 5 mg/l Cl^- solution and 20 μl of the 20 mg/l SO_4^{2-} solution. As the results show (Fig. 1), at an eluent flow rate of 1.5 ml/min, a 0.1 ml/min change in the flow rate causes a 5% change for Cl^- and an 8% change for SO_4^{2-} in the peak area, while its influence on the peak height is

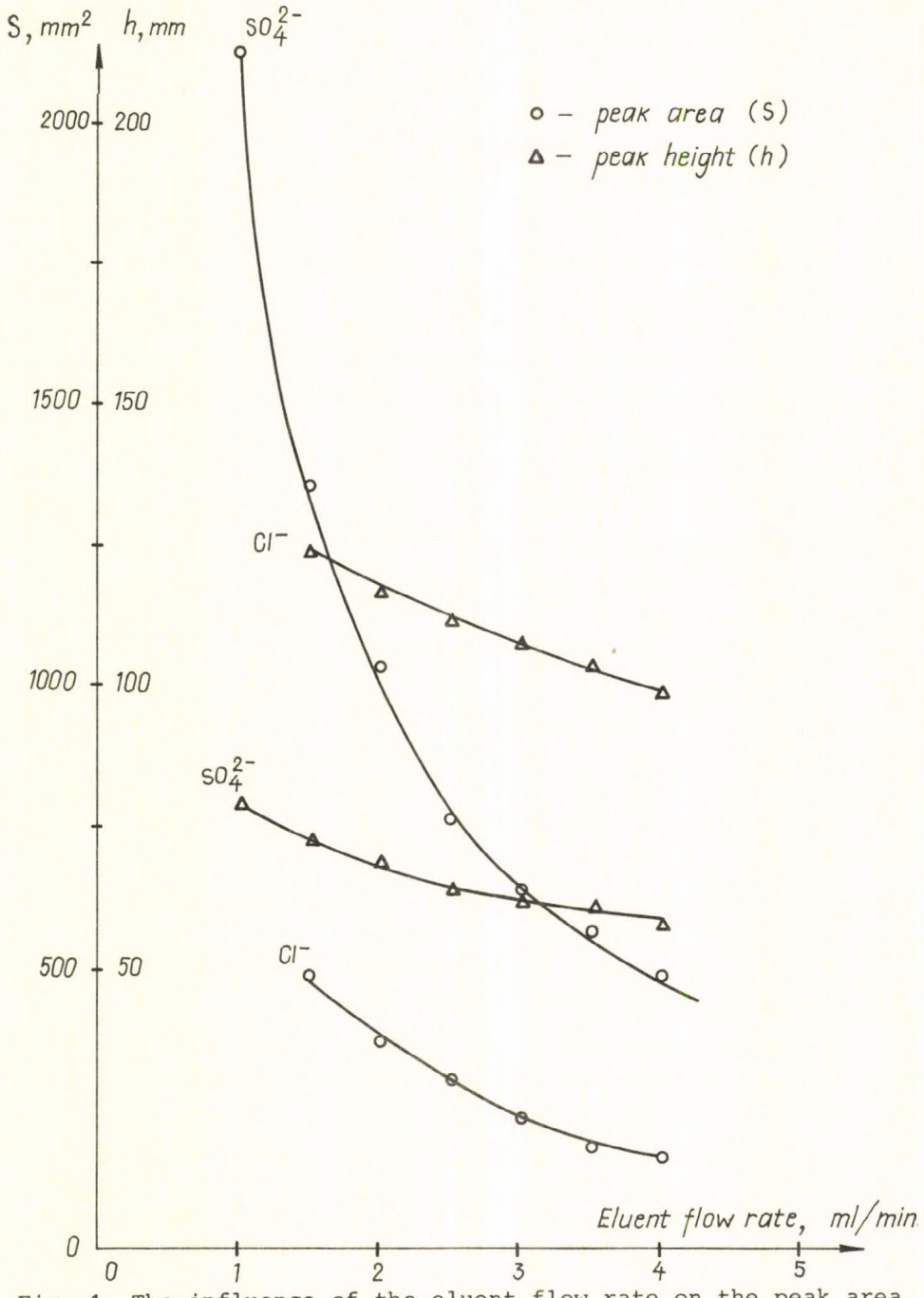

Fig. 1. The influence of the eluent flow rate on the peak area (S) and peak height (h). Sample: 20 µl of a standard solution containing 5 mg/l Cl^- and 20 mg/l SO_4^{2-}

less than 2% for both anions. According to these experiments, the use of the peak height is recommended in order to reduce the possible impact of eluent flow rate instability.

The standard deviation for ten repeat runs of 20 µl each having the concentration of $5x10^{-3}$ N KCl at an eluent flow rate of 2.5 ml/min (separation column: 250x4 mm; capacity: 12.7 µeq/cm^3) was 1.5% for the peak height and for peak area it was 3.5%. The standard deviation for ten repeat runs of 20 µl each having the concentration of $5x10^{-4}$ N KCl at an eluent flow rate of 2.0 ml/min (separation column: 250x3 mm, capacity: 38 µeq/cm^3) was 0.6% for the peak height and 1.9% for peak area. These results confirm the previous recommendation to use the peak height. In literature /1/ the peak height is also considered as the desirable analytical signal.

The ion chromatograph was calibrated at an eluent flow rate of 1.5 ml/min, with a 250x4 mm separation column (capacity 12.7 µeq/cm^3) and a 150x6 mm suppressor column. Samples with Cl^- concentrations from 3.53 to 200 mg/l were injected by a 20 µl loop. A plot of peak height vs. the amount of Cl^- was linear up to 4 µg injected.

The limit of detection (LOD) is characterized as the smallest amount of the analyte that can be detected with reasonable certainty for a given analytical procedure /1/. In our experiments the LOD was taken equal to the analyte amount which gave a system response twice the peak-to-peak noise. The LOD was experimentally measured with a complete chromatographic system under specified conditions.

We have investigated the dependence of the LOD on the experimental conditions.

The results for Cl^- are summarized in Table I. At a constant column inlet pressure the increase in the eluent flow rate also results in an increase in the LOD. At a constant eluent flow rate the increase in the column inlet pressure results in a decrease in the LOD. One of the reasons for this is that the pump pulsation depends on the pressure, decreasing at higher pressures. At a constant column inlet pressure and eluent flow rate the LOD does not significantly depend on the sample volume

Table I. Limit of detection of Cl^- under different experimental conditions*

Column: 250x4 mm; capacity: 12.7 μeq/cm^3; concentration of the standard solution: 3.55 mg/l Cl^-

Sample volume, μl	Eluent flow rate, ml/min	Column inlet pressure, MPa	k'	N	N_{eff}	LOD	
						ppb	pg
20	0.5	4.21	0.42	655	57	3.6	72
20	1.5	4.21	0.44	471	50	4.6	92
20	2.5	4.21	0.43	385	-	6.0	120
20	3.5	4.21	0.55	375	50	7.5	150
20	1.0	1.37	0.34	558	35	6.7	134
20	1.0	2.45	0.36	567	40	5.3	116
20	1.0	5.39	0.40	529	42	5.2	104
20	1.0	7.25	0.40	529	42	4.4	88
20	1.0	0.98	0.28	661	30	5.8	116
100	1.0	0.98	0.24	695	27	1.2	120
200	1.0	0.98	0.23	596	23	0.6	120

*k' = capacity factor;
N = number of theoretical plates;
LOD= limit of detection

in the range of 20-200 μl, at a constant sample concentration (3.55 mg/l).

The capacity factor values have been measured as usual /2/. In our experiments the void time was taken as equal to the retention time of water. The k' values, listed in Table I, show a slight increase with a decrease in the sample volume, and an increase in the eluent flow rate and column inlet pressure.

Our experiments also demonstrated how the separation capability of the chromatographic system and the LODs of different inorganic anions are affected by a change in the column length at constant column diameter, anion exchange resin capacity and suppressor column characteristics. In all experiments 20 μl of a standard solution (4 mg/l F^-, 5 mg/l Cl^-, 10 mg/l NO_3^- and

20 mg/l SO_4^{2-}) was injected. It must be noted that here a change in the eluent flow rate is connected with the change in the column inlet pressure.

As Figs 2-5 show, by reducing the length of the separation column the LOD of different anions decrease. In the case of F^- and Cl^- the negative peak obtained for water may obscure the peaks of these anions. At different eluent flow rates and column lengths the retention time of water changes and the location of its negative peak with regard to the peaks of F^- and Cl^- changes. Therefore, in the case of columns with lengths of 350 and 250 mm, the change in the slope of the LOD vs. eluent flow rate plot at eluent flow rates higher than 2.5 ml/min is caused by the water peak. In the case of a 150 mm long column the negative peak for water causes an increase in the LOD of Cl^- at eluent flow rates lower than 2.5 ml/min. In the case of a 100 mm long column water affects the LOD of Cl^- in the whole range of eluent flow rates. In the case of a 60 mm column the retention time of water is higher than that of Cl^- in the whole range of eluent flow rates. Therefore, the slope of the LOD vs. eluent flow rate plot is constant. The slope of the LOD vs. eluent flow rate plot for F^- is almost constant for 60, 100 and 150 mm long columns as water does not interfere with the F^- peaks under these conditions. The slopes of the LOD vs. eluent flow rate plots are almost constant for NO_3^- and SO_4^{2-} in the whole range of column lengths.

Since generally the LOD increases toward higher eluent flow rates it must be related not with the column lengths, but more probably with the kinetics of the ion exchange process. In conclusion, one may state that under these experimental conditions a 60 mm column represents the best tool for obtaining the lowest LOD: 30 pg for F^-, 40 pg for Cl^-, 240 pg for NO_3^- and 400 pg for SO_4^{2-}. With respect to water elution it must be mentioned that under constant experimental conditions the retention time of water is constant. The standard deviation of the retention time of water was 1.3% for ten repeat runs of 20 µl bidistilled water at an eluent flow rate 2.0 ml/min (separation column: 250x3 mm, capacity 38.0 $\mu eq/cm^3$).

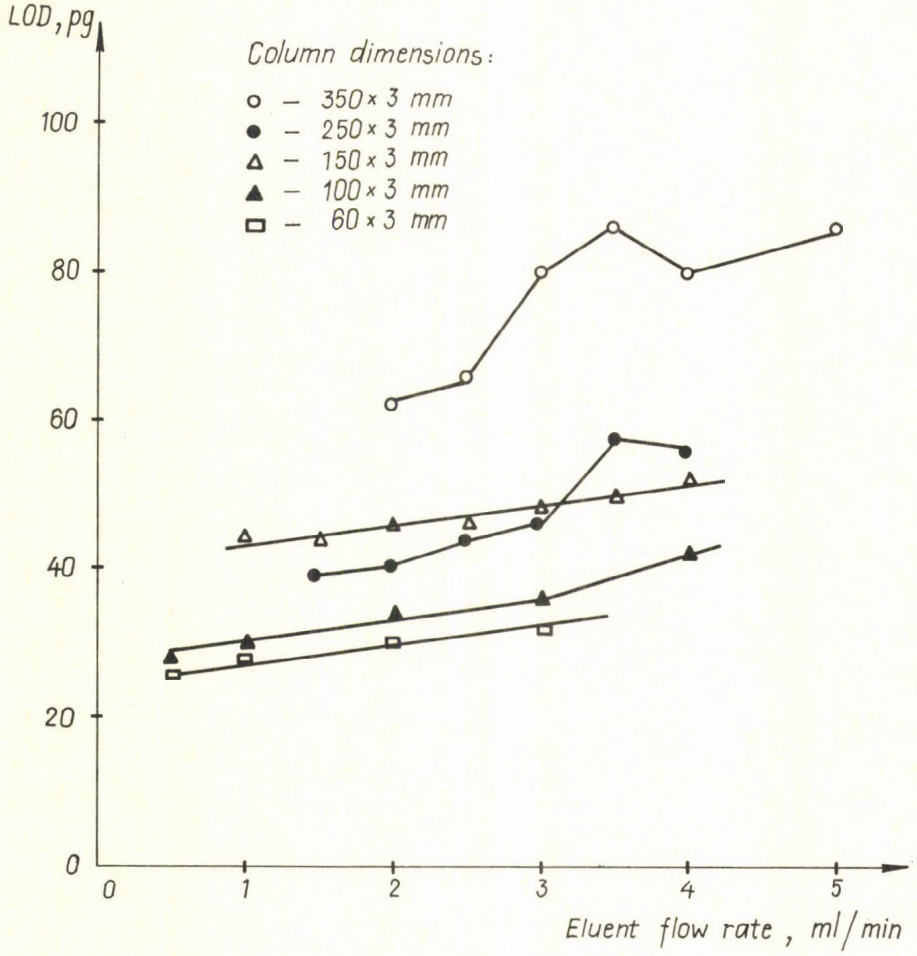

Fig. 2. Limit of detection (LOD) of F^- at different eluent flow rates and column lengths

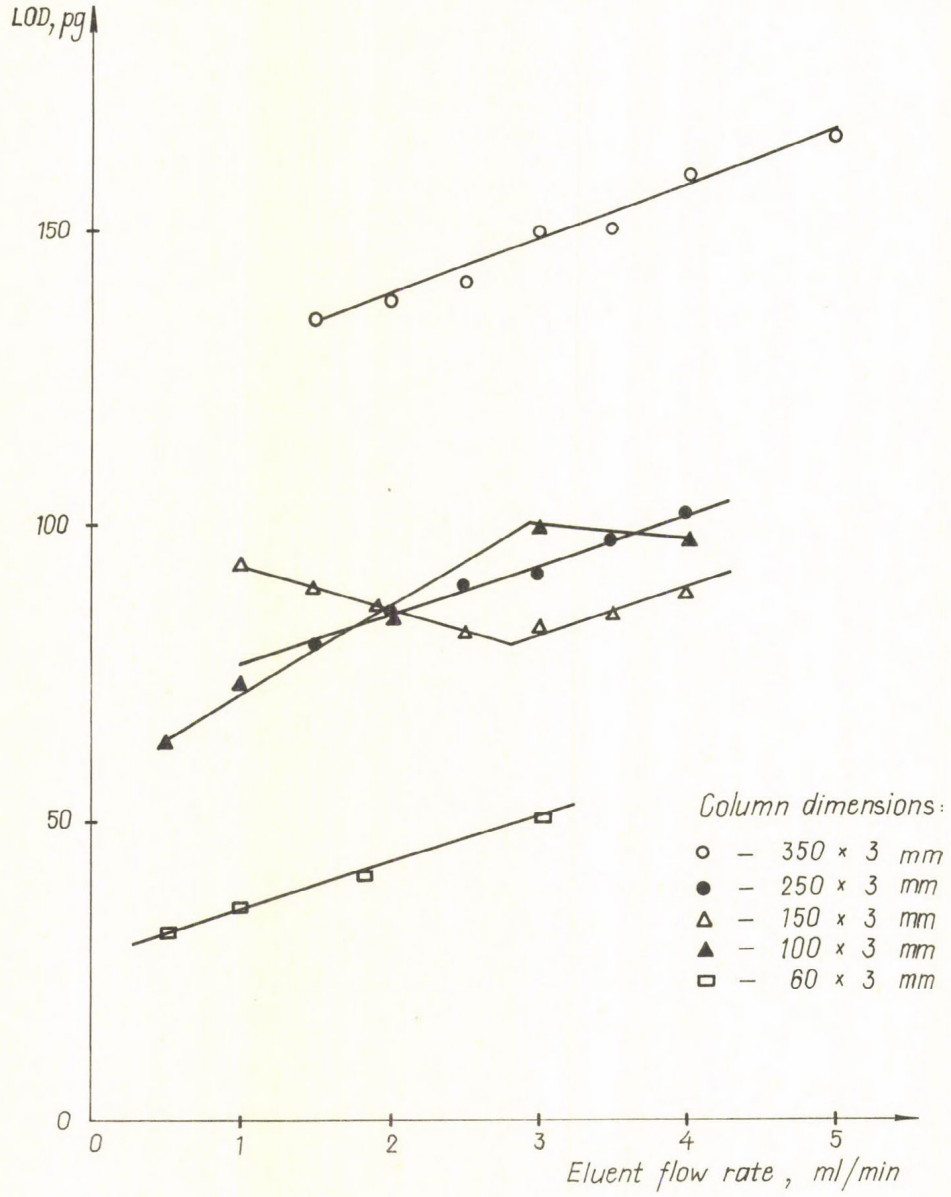

Fig. 3. Limit of detection (LOD) of Cl^- at different eluent flow rates and column lengths

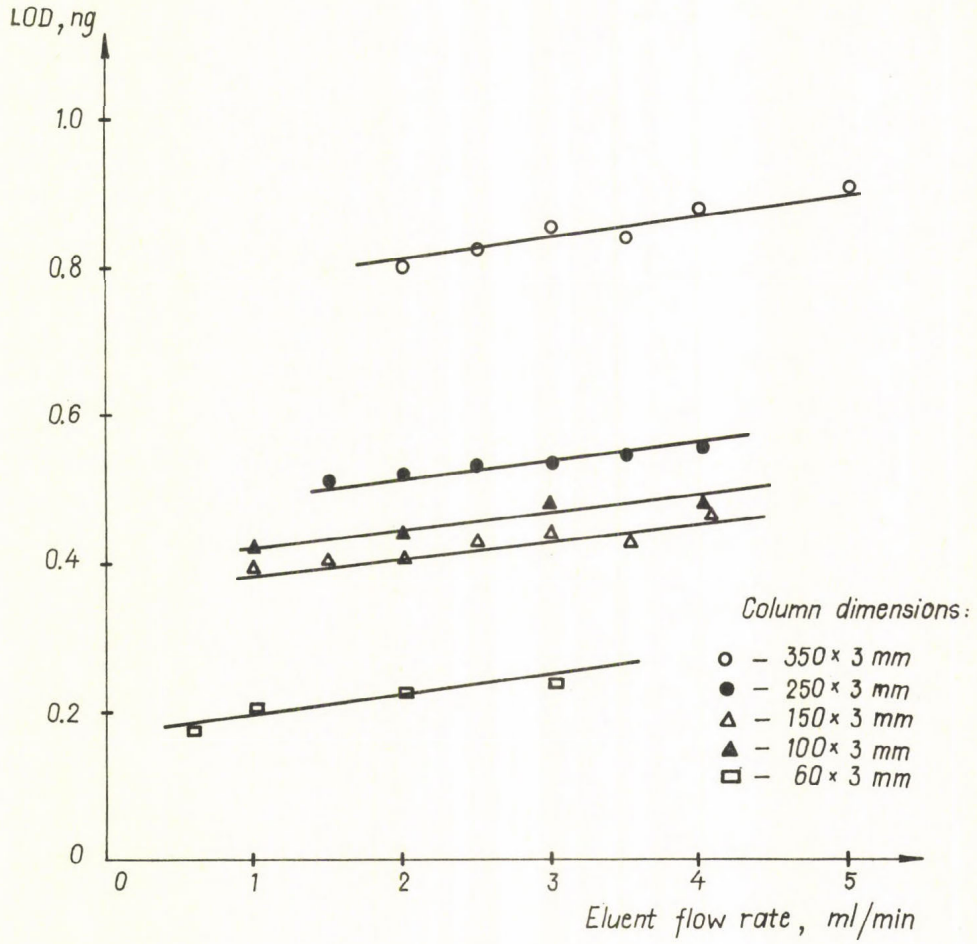

Fig. 4. Limit of detection (LOD) of NO_3^- at different eluent flow rates and column lengths

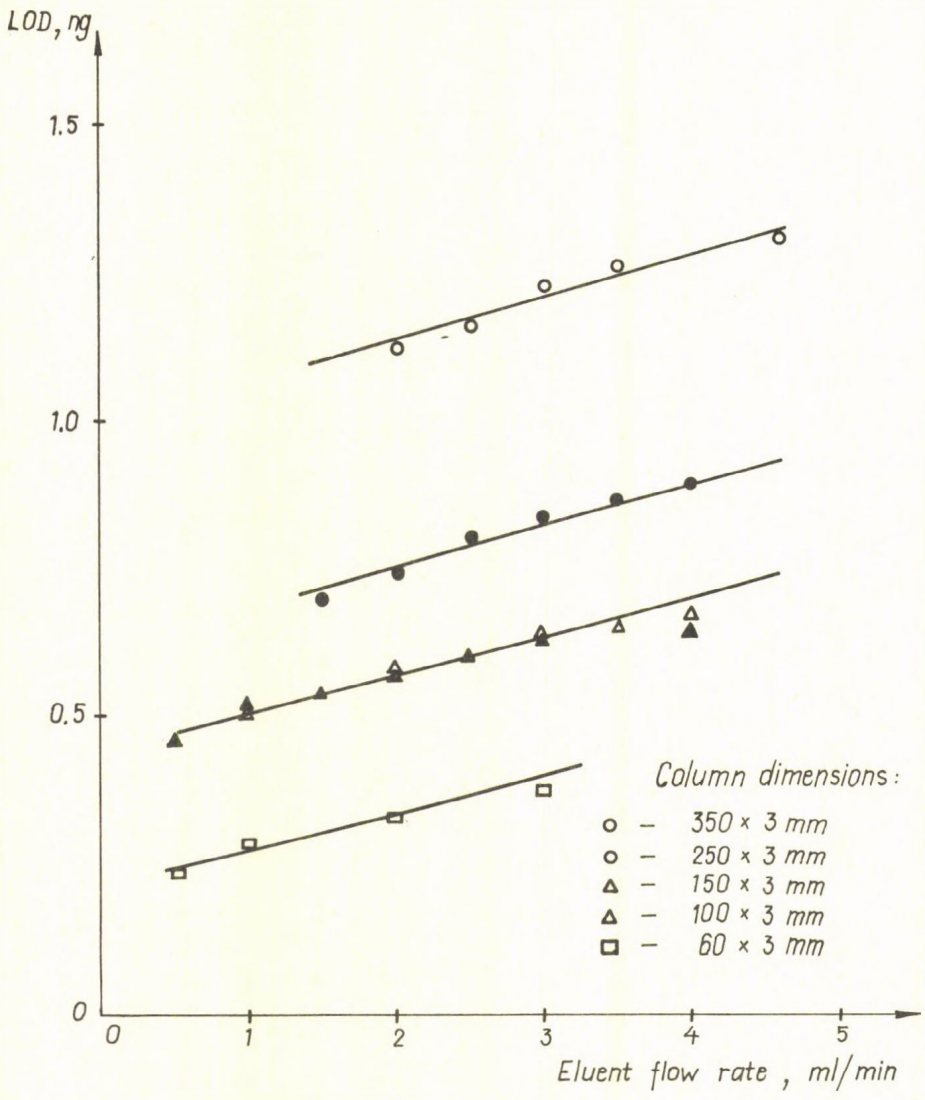

Fig. 5. Limit of detection (LOD) of SO_4^{2-} at different eluent flow rates and column lengths

Table II. Capacity factor (k') values for F^-, Cl^-, NO_3^- and SO_4^{2-}, under different experimental conditions

Column length,* mm	Eluent flow rate, ml/min	k'			
		F^-	Cl^-	NO_3^-	SO_4^{2-}
60	0.5	-0.35	-0.22	0.34	1.19
	1.0	-0.36	-0.23	0.31	1.18
	2.0	-0.35	-0.21	0.34	1.24
	3.0	-0.34	-0.20	0.32	1.25
100	0.5	-0.30	-0.05	0.95	2.22
	1.0	-0.29	-0.05	0.94	2.24
	2.0	-0.29	-0.06	0.89	2.21
	3.0	-0.24	0.01	0.97	2.39
	4.0	-0.28	0.02	1.01	2.48
150	1.0	-0.28	-0.01	0.98	2.83
	2.0	-0.25	0.04	1.00	2.91
	3.0	-0.19	0.11	1.15	3.20
	4.0	-0.14	0.20	1.24	3.45
250	1.5	-0.18	0.28	1.83	4.55
	2.0	-0.15	0.33	1.89	4.81
	3.0	-0.06	0.45	2.05	5.30
	4.0	0.02	0.59	2.26	5.94
350	1.5	-	0.34	-	-
	2.0	-0.15	0.36	1.92	4.83
	3.0	-0.03	0.49	2.17	5.41
	4.0	0.16	0.79	2.75	6.80

*I.D. = 3 mm

Table III. Valves of N, N_{eff} and HETP under different experimental conditions*

Column length, mm	Eluent flow rate, ml/min	N	N_{eff}	HETP, mm
60	0.5	278	82	0.22
	1.0	195	57	0.31
	2.0	128	39	0.47
	3.0	102	31	0.59
100	0.5	216	103	0.46
	1.0	167	80	0.60
	2.0	125	59	0.80
	3.0	104	52	0.96
	4.0	88	44	1.14
150	1.0	236	129	0.64
	2.0	208	115	0.72
	3.0	163	94	0.92
	4.0	136	82	1.10
250	1.5	387	260	0.65
	2.0	340	233	0.74
	3.0	299	212	0.84
	4.0	252	186	0.99
350	2.0	195	134	1.79
	3.0	167	119	2.10
	4.0	157	120	2.23
	5.0	141	105	2.48

*N = number of theoretical plates; N_{eff} = number of effective plates; HETP = height equivalent to one theoretical plate

In order to determine whether the water peak obscures one or another anion peak, it is necessary to analyze standard solutions to measure the individual retention times. By changing the column length or the experimental conditions it is possible to avoid or at least minimize interferences.

Analysis of standards is also necessary to determine the k' value for every anion at different experimental conditions. We have investigated the dependence of k' on column length and eluent flow rate for every anion. The results are given in Table II. The negative k' values show that under the given conditions water elutes later than the given anion. The k' values near zero are the direct indicators of the interference of water. As a rule the k' value depends on the eluent flow rate, increasing with higher flow rates. The fluctuations of the k' values at shorter column lengths are probably connected with the errors in the measurement of the retention times.

Table III presents data on column efficiencies as a function of eluent flow rate. According to these results the number of theoretical plates is the highest for the 250 mm column while HETP is lowest for the 60 mm column. In all cases the 150x6 mm suppressor column was used and 20 µl aliquot of the standard solution were injected.

In conclusion one may say that according to these data it is quite easy to select the experimental conditions for the determination of inorganic anions (F^-, Cl^-, NO_3^-, SO_4^{2-}) in water solutions.

APPLICATIONS

Ion chromatography opens up good possibilities for the solution of various theoretical and practical problems. We have utilized this method while studying the chemical composition of the water in the lake system of north-east Estonia. As a result of regular determination of the ions F^-, Cl^-, NO_3^- and SO_4^{2-} in atmospheric precipitation, ground and surface waters the input mechanism of these anions in the separate lakes and the sources of water pollution could be established.

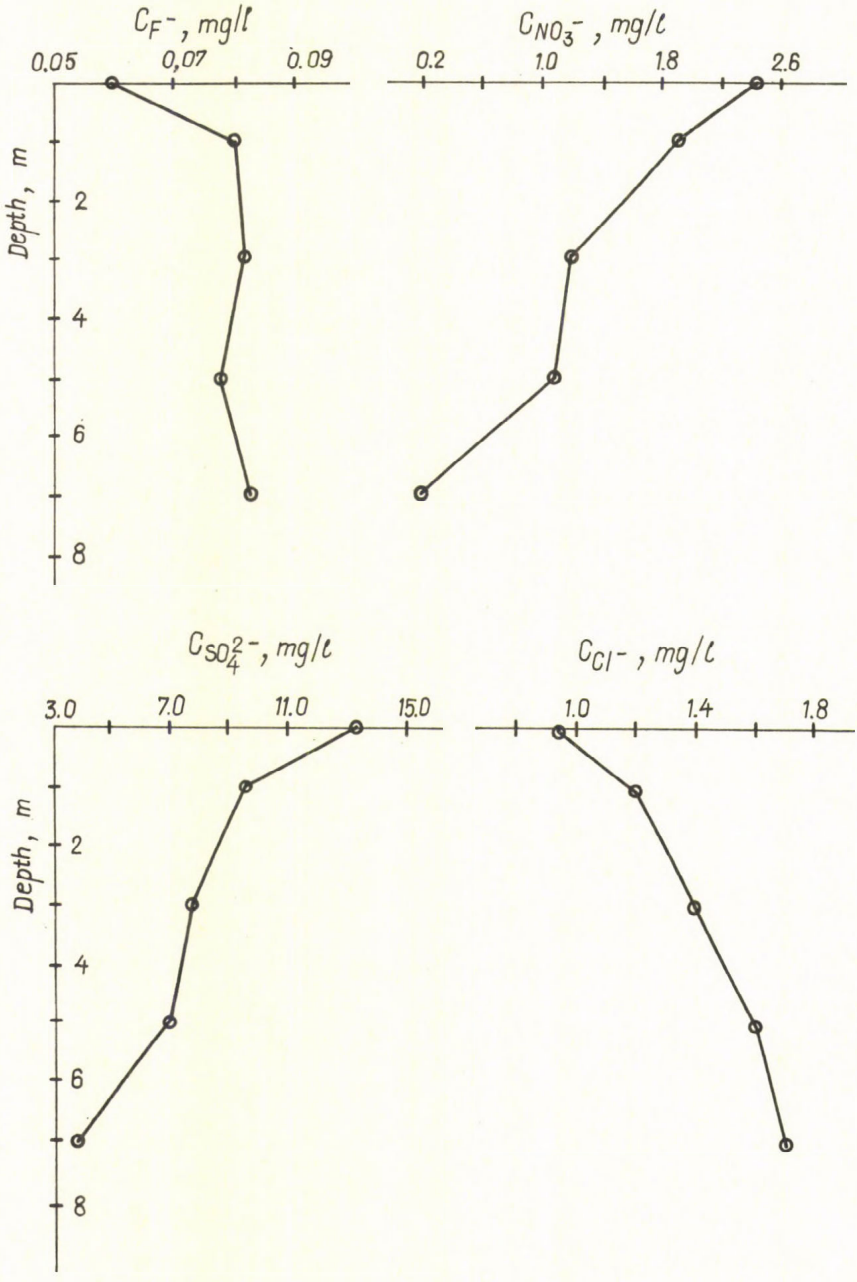

Fig. 6. Variations in the concentration of F^-, Cl^-, NO_3^- and SO_4^{2-} in the vertical profile of a lake without a current (at zero depth line data for snow on the ice cover are given)

The changes in the anion composition of the water during winter time with practically no water exchange with ground waters, was of special interest. The investigations were carried out in a number of deep-water lakes (with a depth up to 7.5 m). Ion chromatographic analysis of their surface water layers revealed that, as a rule, the concentration of F^-, Cl^-, NO_3^- and SO_4^{2-} slightly increases from spring to autumn which corresponds to the input of ions with atmospheric precipitation. The lowest concentrations were determined just before the melting of the ice cover, in March through the beginning of April. Lack of water exchange together with the processes going on in the bottom sediments, causes a considerable decrease in the amount of free oxygen in the bottom layers, which must also influence the ionic composition. The chemical composition data of the vertical profile of one of the typical lakes in this region, shown in Fig. 6 serve as an example to illustrate that in the bottom layers where the O_2 content approaches nearly zero, a sharp decrease can be observed in the NO_3^- and SO_4^{2-} concentrations. This is the result of denitrification during the oxidation of the organogenic bottom sediments under oxygenless conditions. The decrease in the NO_3^- concentration is followed by a reduction in the SO_4^{2-} concentration. These data indicate that the investigated anions do not deposit in the bottom sediments and demonstrate a considerable ability of self-purification of the bottom deposits of the investigated lakes.

Ion chromatography has a promising future in field investigations. The method is indispensable while studying the distribution of trace components in ice cores. Here, the anion concentrations are extremely low, $8\text{-}110\times10^{-6}$% Cl^- and $5\text{-}20\times10^{-7}$% SO_4^{2-} /3/, but their variation provides valuable information about the physico-geographical conditions in the past. As the transportation of ice cores is usually impossible the samples are taken directly on the glacier. Still, keeping the samples in bottles in the form of water is always connected either with sorption of the trace components on the walls of the bottles or with contamination of the sample. It is also difficult to determine the necessary frequency of sampling without knowing the preliminary data. The utilization of the portable and all-

purpose ion chromatograph opened up new possibilities in the development of geochemical glaciology, not even mentioning savings in the material and time which would have been lost with the transportation of samples from the polar regions.

CONCLUSIONS

Inorganic anions were determined in aqueous solutions with help of the ion chromatograph IVK-11, designed and produced by the Special Design Office of the Academy of Sciences of the Estonian SSR.

The limit of detection and separation capability dependence of the system on various experimental conditions were investigated. According to these data it is not difficult to establish the suitable experimental conditions for the analysis of F^-, Cl^-, NO_3^- and SO_4^{2-} ions in aqueous solutions.

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Budapest, 1986

Chromatography '85
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HIGH-PERFORMANCE LIQUID-CHROMATOGRAPHIC METHOD FOR MEASURING THE EPOXIDE HYDROLASE ACTIVITY WITH STYRENE OXIDE SUBSTRATE

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SUMMARY

The assay of microsomal epoxide hydrolase activity with unlabeled styrene oxide substrate has been studied. The method involves a single extraction step followed by adsorption chromatography by high-performance liquid chromatography. The procedure is simple, rapid and well reproducible; it is preferred to radiometric assay, because the disadvantage of the ^{3}H-labeled styrene oxide, (spontaneous decomposition) and the regular repurification can be eliminated.

INTRODUCTION

Epoxide hydrolase (EC 3.3.2.3.) catalyzes formation of vicinal diols from arene and alkene oxides by the addition of water /1/. It also participates in detoxification and activation processes because it can detoxify certain epoxides to dihydrodiols but can also provide precursors for the formation of highly genotoxic agents /2-4/. The majority of assays for epoxide hydrolase activity is radiometric, either in combination with extraction steps /5-7/ or with thin-layer chromatography /8,9/ in order to separate the unmetabolized substrate and the metabolite. Other chromatographic methods are also applied, one involving derivatization before GLC measurement /10/ while the other using methylcholanthrene epoxide substrate, ccmmercially hardly available, in an HPLC assay /11/. A fluorimetric assay serves for the kinetic measurement of epoxide hydrolase activity /12/. One widely used radioactive substrate is ^{3}H-styrene oxide. Its spontaneous decomposition

involves methodological problems, and it has to be repurified from time to time /13, 14/. We have developed a simple, rapid high-performance liquid chromatographic assay with unlabeled styrene oxide substrate by which this problem is eliminated.

MATERIALS AND METHODS

Chemicals

Unlabeled styrene oxide and styrene glycol was obtained from Aldrich-Europe (Beerse, Belgium). /7(n)-^{3}H/ styrene oxide (specific activity 99 mCi/mmol) was purchased from Amersham International (Amersham, UK). All other chemicals used were obtained from Reanal (Budapest, Hungary).

Tissue preparation

Male Wistar rats (140-170 g) were sacrificed by decapitation. A 25 % homogenate of pooled livers was made in 0.15 M KCl, and the 105,000 x g fraction of microsomes was prepared by differential centrifugation. Protein content of microsomes suspended in 0.15 M KCl was measured with Coomassie Brillant Blue G-250 according to Bradford /15/ using bovine serum albumin as the reference, and finally the protein content of the microsomes was adjusted to 20 mg/ml. Microsomes were stored at -80^{o}C until use.

Incubation

Composition of the assay mixture was essentially similar to the one described by Oesch et al. in their radiometric assay /5/. It consisted of 250 µl 0.4 M tris-HCl buffer, pH 8.7, containing 0.1 % Tween 80, microsomes (1.6-5.0 mg protein), styrene oxide (0.25-2.5 µmol) in 50 µl acetonitrile and water to adjust to the final volume of 1.0 ml. Reactions were started by addition of styrene oxide to the assay mixture preincubated in water bath at 37^{o}C for 5 min. After incubation at 37^{o}C for 3-10 min, the reaction was terminated by the addition of 5 ml ice-cold ethyl acetate followed by vigorous shaking. After

phase separation by short centrifugation the ethyl acetate phase was removed and dried with anhydrous Na_2SO_4. A 4.0 ml aliquot was evaporated to dryness by a stream of nitrogen at about 40°C. The residue was dissolved in 100 µl of ethyl acetate and an aliquot was injected for the chromatographic separation. There was no observable glycol formation in blanks with boiled microsomes (denaturation at 100°C for 30 min) within 10 min of incubation time or in zero-time controls with active microsomes.

HPLC analysis

HPLC analysis was carried out with a Labor MIM (Budapest, Hungary) liquid chromatograph consisting of Liquopump 312, a 20-µl injection loop and the 308 variable-wavelength UV detector. A 250 mm x 4.6 mm i.d. Labor MIM silica column (10 µm) was used; the solvent system chloroform - ethyl acetate 1:1 (v/v) with a flow rate of 2.0 cm^3/min was used as the eluent. The temperature of the solvent and column was conditioned with tap water (19°C). The eluent was detected at 258 nm. The retention times were 1.7 min for styrene oxide and 6.2 min for styrene glycol.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of the simple ethyl acetate extraction assay. Peak 1 is unmetabolized styrene oxide. Peak 2 was identified as a contamination of the styrene oxide present in the chemical. Peaks 3, 4 and 6 are of microsomal origin: either boiled or active microsomes were used. Peak 5 is styrene glycol.

Calibration with styrene glycol gave a linear function between concentration and its peak height (Fig. 2). The substrate saturating concentration of epoxide hydrolase was 1 mM styrene oxide (Fig. 3a), and 2.0 mM concentration was routinely used in standard assays. The time course of styrene glycol formation was linear up to 10 min (Fig. 3b) and 6 min incubation time was considered appropriate for routin

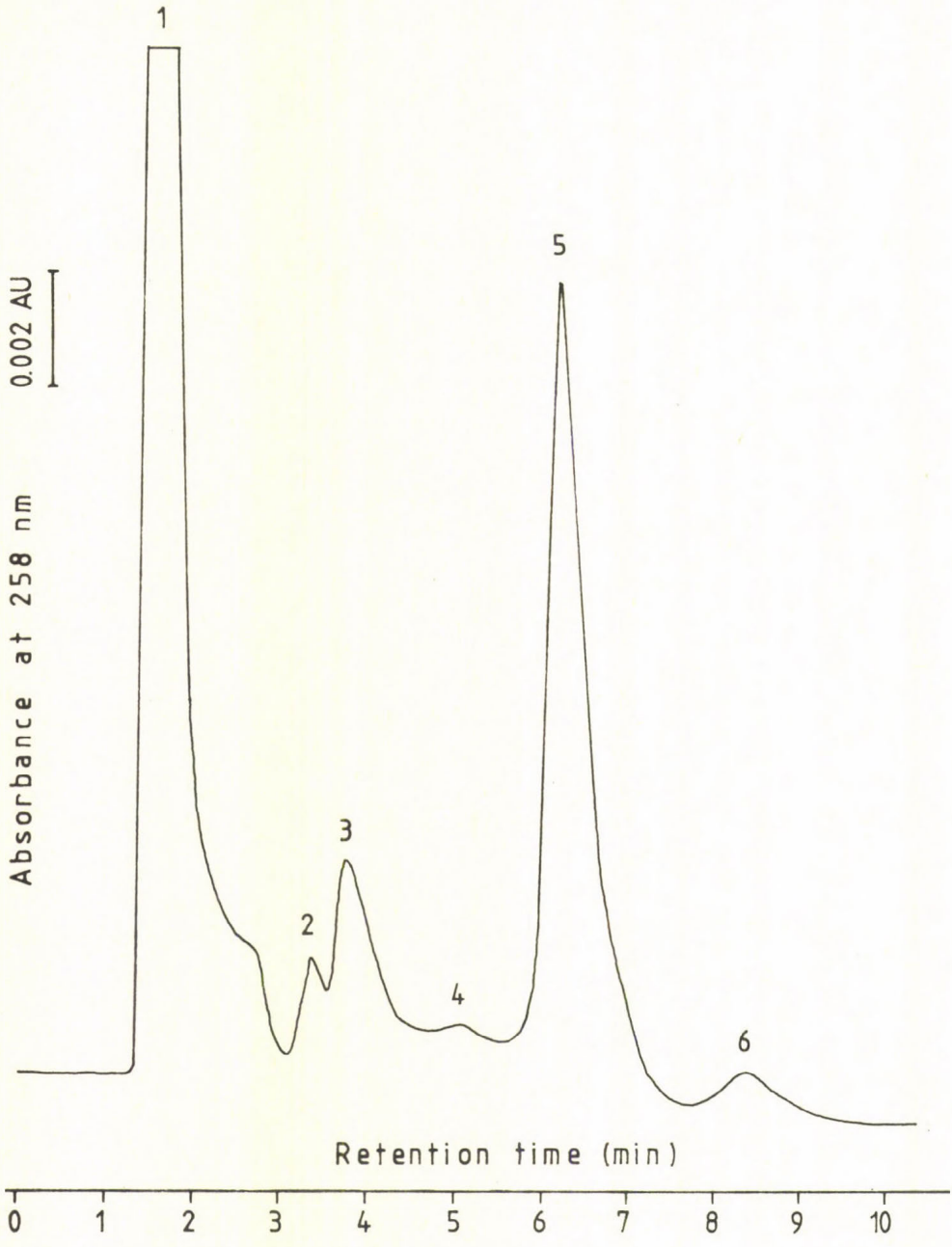


Fig. 1. Chromatogram of the ethyl acetate extract prepared as described in Materials and Methods. 1 - Unmetabolized styrene oxide; 2 - Contamination of the styrene oxide, present in the chemical; 3-4-6 - Peaks of microsomal origin (either inactivated or active microsomes were used); 5 - styrene glycol.

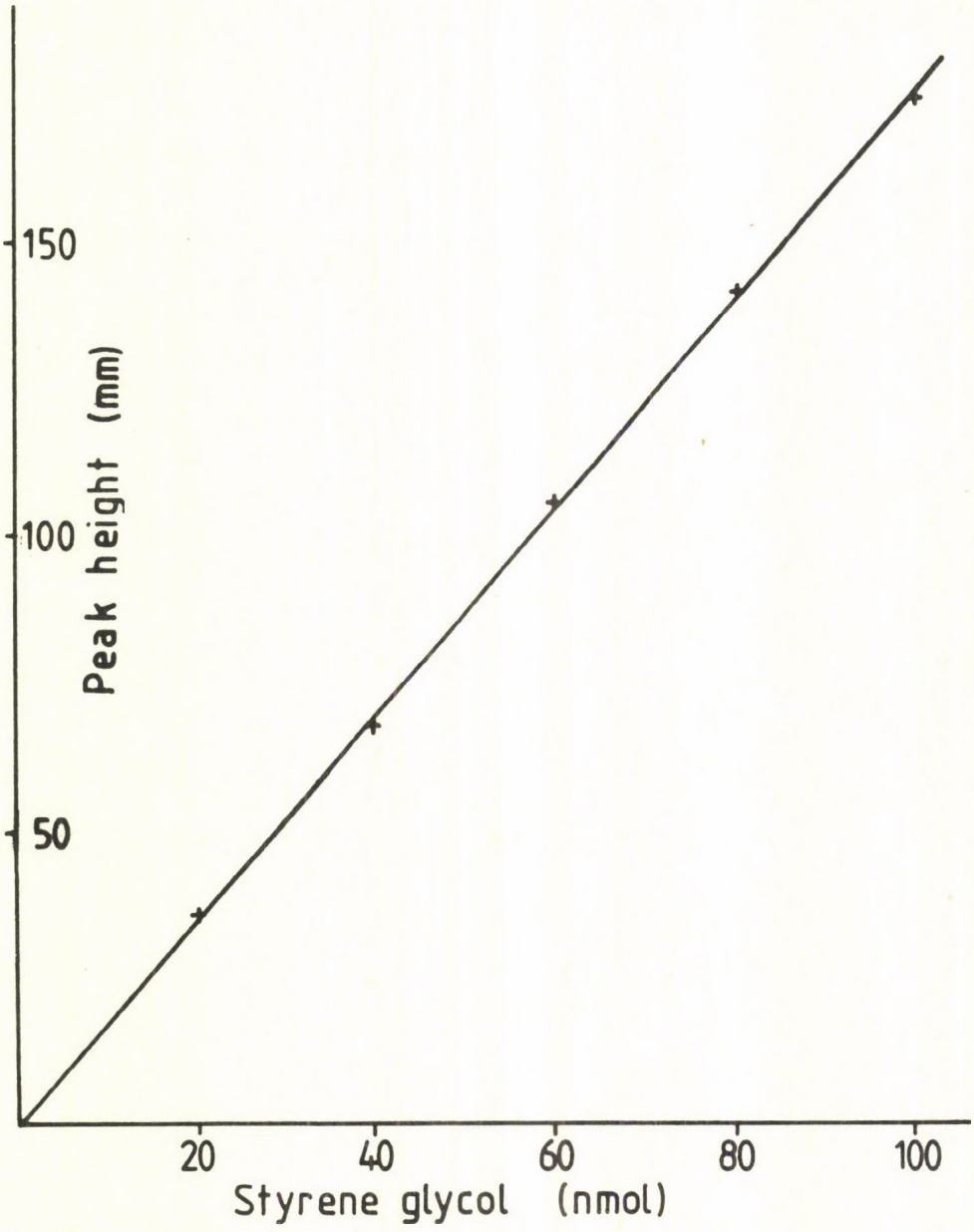


Fig. 2. Standard curve for styrene glycol, representing the relationship between peak height and the quantity of styrene glycol.

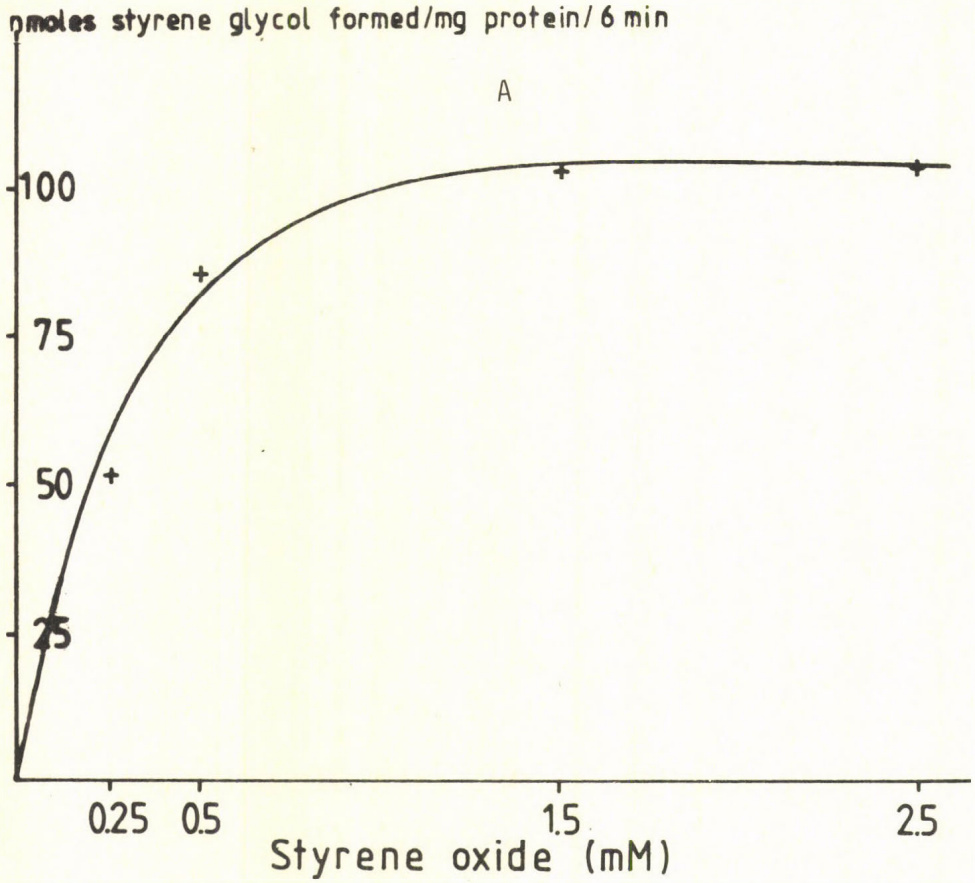
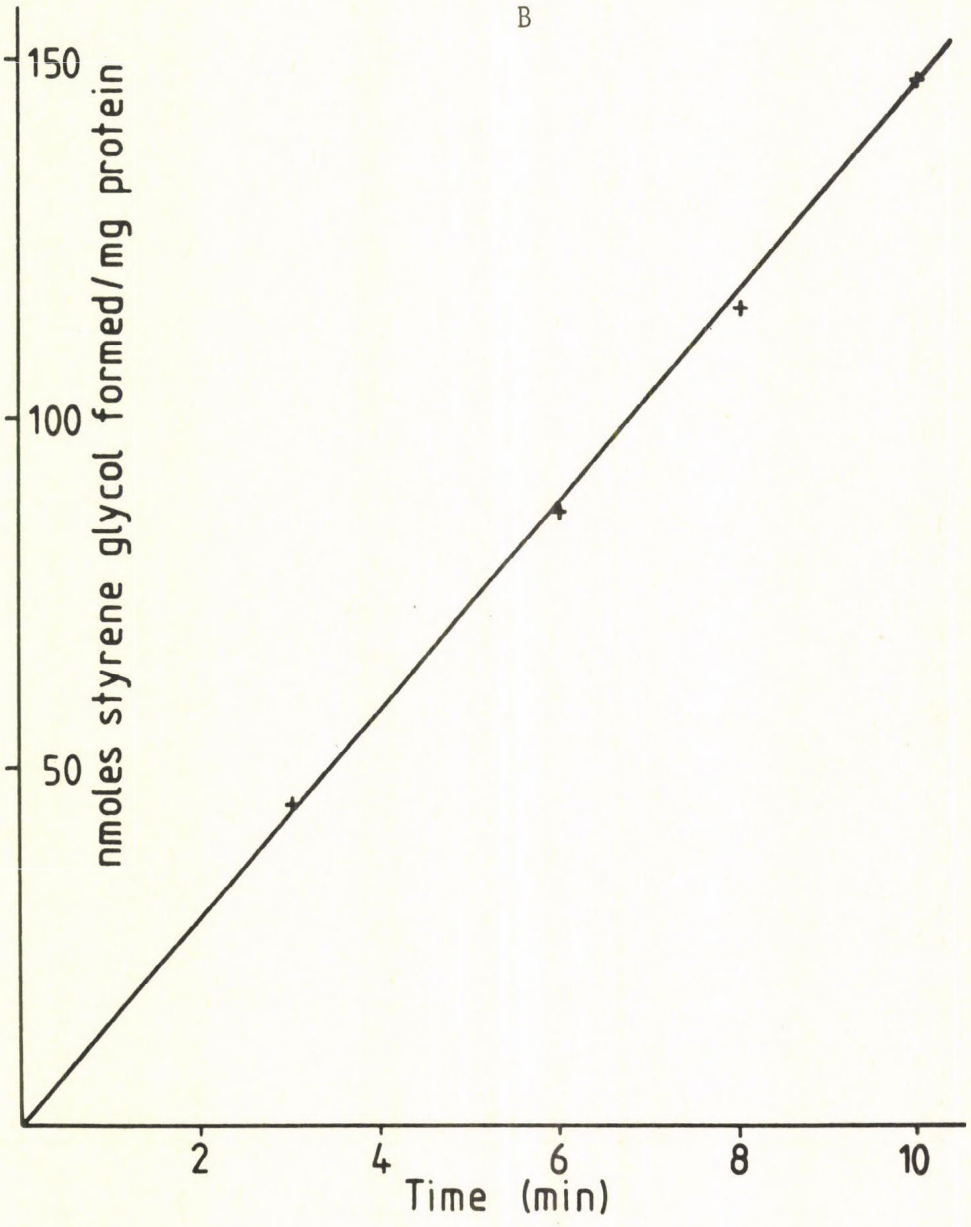


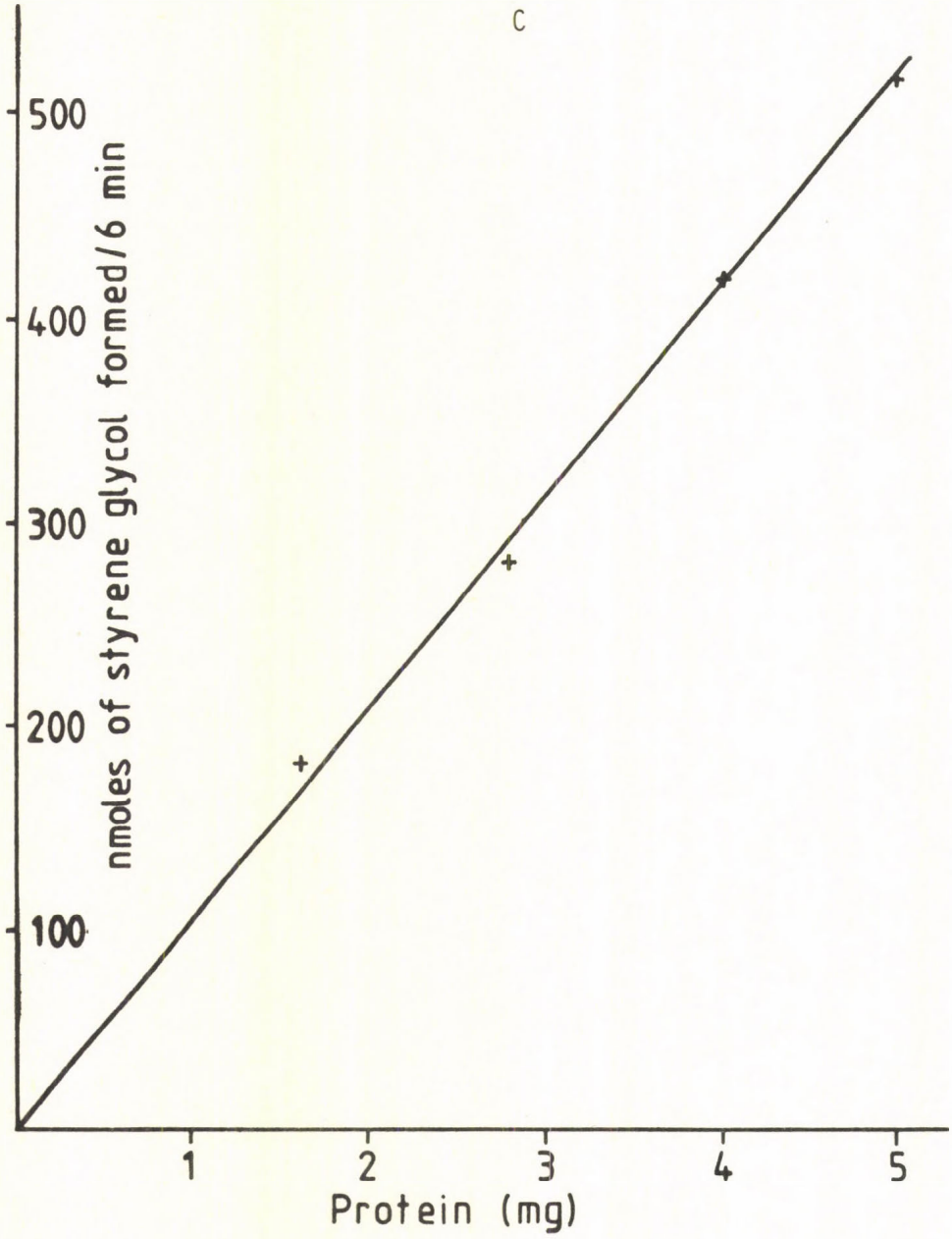
Fig. 3. Styrene glycol formation as a function of (a) styrene oxide concentration, (b) incubation time and (c) microsomal protein content.

determinations. The styrene glycol formation was linear with microsomal protein concentration up to 5 mg/ml incubation mixture (Fig. 3c), and standard assays were set to 3 mg/ml microsomal protein content.

Recovery of styrene glycol in the assay procedure was 85 ± 3 %. Reproducibility of parallel assays was ± 3.7 %.

The standard HPLC assay procedure was performed to check the contamination and unspecific decomposition of the isotope-labeled substrate. Fractions of the eluate were collected in every 30 seconds, evaporated to dryness and put for liquid


B
150
100
50
nmoles styrene glycol formed/mg protein
2
4
6
8
10
Time (min)


C
500
400
300
200
100
nmoles of styrene glycol formed/6 min
1
2
3
4
5
Protein (mg)

scintillation counting. Three ethyl acetate extractable isotope compounds were found together with styrene oxide. The first appeared at a retention time of 4.2 min; its amount was about 5 % of the total radioactivity. The second was (^{3}H)-styrene glycol which was removable from (^{3}H)-styrene oxide by the method of Seidegard et al. /14/. The third compound was slightly more polar than styrene glycol, and it could be completely separated from (^{3}H)-styrene glycol by employing 4:1 (v/v) chloroform-ethyl acetate as the solvent system, at a flow rate of 2.0 cm^3/min. In this separation the retention time was 13.5 min for styrene glycol, and 16 min for the unidentified substance. This substance represented approximately 3 % of the total radioactivity of the "purified" isotope. When incubating the substrate in the buffer, as well as with boiled or enzymatically active microsomes, the amount of this contamination further increased up to 10-20 % of the total radioactivity. This is very large if we consider that the ratio of (^{3}H)-styrene glycol was 12 % to 30 % of the total radioactivity of the extract with 2 to 5 mg microsomal protein/ml, respectively. These methodological problems could be eliminated by the exclusive use of unlabeled substrate and with a single extraction step followed by liquid chromatographic separation of the product.

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TLC AND HPTLC OF ALKYL SUBSTITUTED PHENOXYALKANOIC ACIDS

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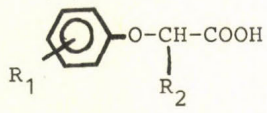
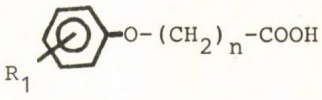
INTRODUCTION

Most of the methods for the thin-layer chromatographic separation of substituted phenoxyalkanoic acids described in the literature deal with the analysis of chlorinated derivatives used as herbicides /1, 2/. Adsorption chromatography on thin silica gel layers is most often used. The aim of our work was to find optimum conditions for the separation of a group of alkyl-substituted phenoxyalkanoic acids (I) involving twenty-seven α- or ω-substituted derivatives:



α-

I



ω-

R_1 = H, methyl, tert. butyl, tert. pentyl, pentadecyl
R_2 = H, methyl, ethyl, n-propyl, n-decyl, n-tetradecyl
n = 1 - 3, 11

MATERIALS AND METHODS

The acids chromatographed were model compounds from our collection, all other chemicals were of current reagent grade purity.

Thin-layer chromatography was carried out using Silufol^{R} ready made sheets (Sklárny Kavalier, Votice, Czechoslovakia), 15x15 cm. The following solvent systems were used:

S_1 = chloroform - ethyl acetate (4:1)

S_2 = 1-butanol - concentrated ammonia (5:1)

S_3 = liquid paraffin/80% acetic acid

The impregnation of the layers was carried out by dipping the sheets into a 10% solution of liquid paraffin in n-hexane. 5 µl of 0.5 - 1% solutions of the acids in ethanol were applied to the origin of the chromatogram. The distance of the solvent front from the starting line after development was 10 cm. High-performance thin-layer chromatography was carried out using HPTLC pre-coated plates RP-2, 8 and 18 F254s (Merck, Darmstadt, FRG), 10 x 10 or 5 x 10 cm. The acids were spotted in the form of ethanolic solutions (5 µl of 0.05 - 0.5% solutions). Mixtures of acetonitrile, methanol, acetone or tetrahydrofuran with water, acetic acid or aqueous ammonia, resp. were used as the mobile phase.

The detection of spots was achieved in both types of chromatography by spraying with a 0.2% ethanolic solution of 2,7-dichlorofluorescein and observation in UV light.

RESULTS AND DISCUSSION

Four types of thin-layer chromatographic systems were used: adsorption chromatography, normal-phase partition chromatography, reversed-phase chromatography and reversed-phase high performance thin-layer chromatography.

Adsorption chromatography

Silica gel was used as the adsorbent and chloroform - ethyl acetate (4:1) as the mobile phase. The migration rate of the phenoxyalkanoic acids was increased with the increasing number of carbon atoms in the alkyl chains on the aromatic ring or with the chain length of the alkanoic acid.

Normal-phase partition chromatography

1-Butanol - concentrated ammonia (5:1) mixture was used as the mobile phase on silica gel layers. This mobile phase was

especially suitable for the separation of the less lipophilic lower phenoxyalkanoic acids.

Reversed-phase partition chromatography

was carried out on silica gel layers impregnated with liquid paraffin. 80% acetic acid was the mobile phase. In this system the acids migrated in a reversed sequence and a remarkable decrease of the R_F values was observed with their increasing chain length.

The separation of the phenoxyalkanoic acids in these three systems is illustrated in Fig. 1.

High-performance reverse phase thin-layer chromatography

RP-2, 8 and 18 sheets were used for reversed-phase thin-layer chromatography differing in the chain length of the alkyl bonded to the silica gel. It can be seen from Figs 2 - 4 that the higher the hydrophobicity of the bonded phase, the smaller the R_F values. The decrease of the R_F values is more significant in the case of higher homologues of the acids. The most efficient analytical separation is obtained on RP-18 layers. Four organic modifiers were used alone or in mixtures with water, aqueous ammonia or acetic acid, resp. as mobile phases: tetrahydrofuran, acetone, methanol and acetonitrile. Schemes of the chromatograms in Figs 5 and 6 show the separation of the acids using some of the mobile phases used. The role of the water, ammonia or acetic acid content in the organic modifier used on the separation efficiency of the mobile phases is seen from Figs 7 through 9. The mobile phases tested can be classified as follows: acetonitrile - acetic acid (10:1) seems to be universal mobile phase for all acids chromatographed yielding a good separation efficiency. Acetonitrile or mixtures of tetrahydrofuran with acetic acid and methanol or acetone with water are suitable mobile phases for acids with lower alkyl chains, whereas mixtures acetone-water (10:1, 5:1) and acetone - ammonia (10:1, 4:1) are suitable mobile phases for the separation of 3-pentadecyl substituted acids.

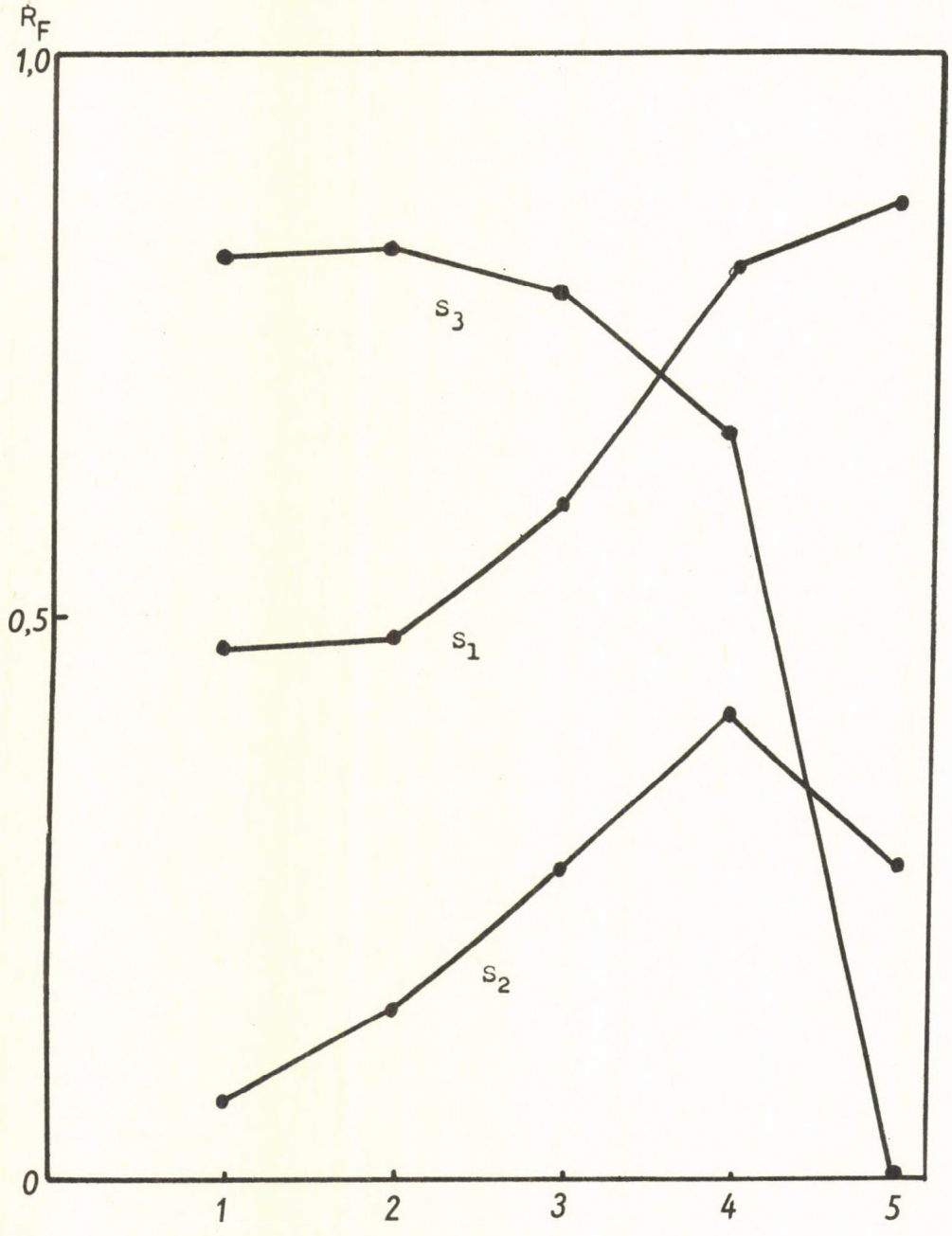


Fig. 1. Chromatogram of alkyl substituted phenoxyacetic acids on silica gel using S_1 - S_3 mobile phases
Substituents: *1* H; *2* p-methyl; *3* p-tert. butyl; *4* 2,4-di-tert. butyl; *5* m-pentadecyl

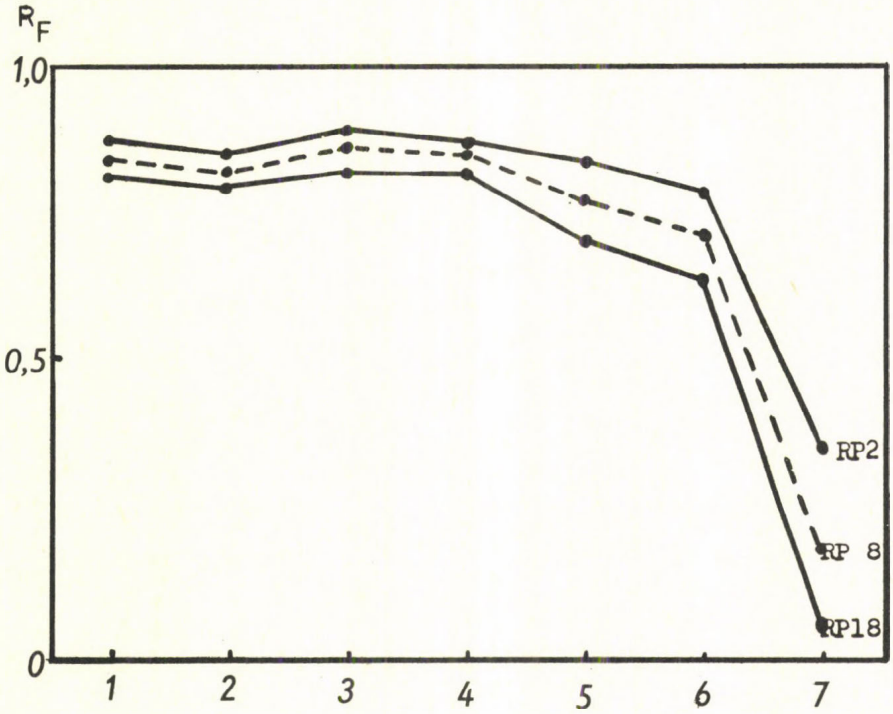


Fig. 2. Influence of the chain length of the alkyl bonded to silica gel on the migration of alkylphenoxyacetic acids using acetonitrile-acetic acid as mobile phase

Substituents:
1 H, *2* 3-methyl, *3* 4-methyl, *4* 4-tert. butyl, *5* 4-tert. pentyl, *6* 2,4-ditert. pentyl, *7* 3-pentadecyl

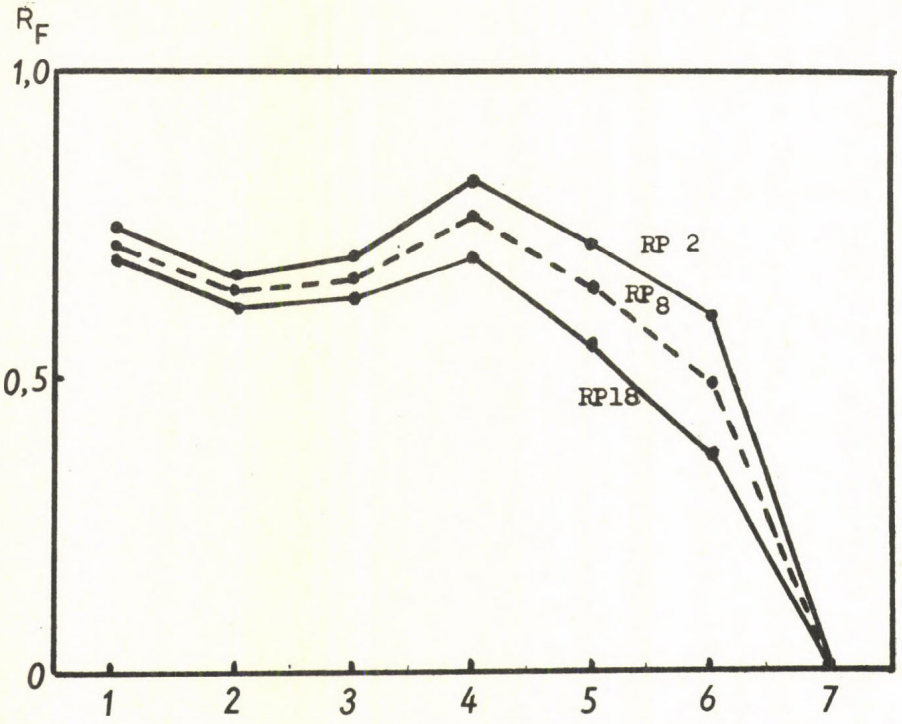


Fig. 3. Influence of the chain length of the alkyl bonded to silica gel on the migration of alkylphenoxyacetic acids using methanol-water (5:1) as mobile phase

Substituents:
1 H, *2* 3-methyl, *3* 4-methyl, *4* 4-tert. butyl, *5* 4-tert. pentyl, *6* 2,4-ditert. pentyl, *7* 3-pentadecyl

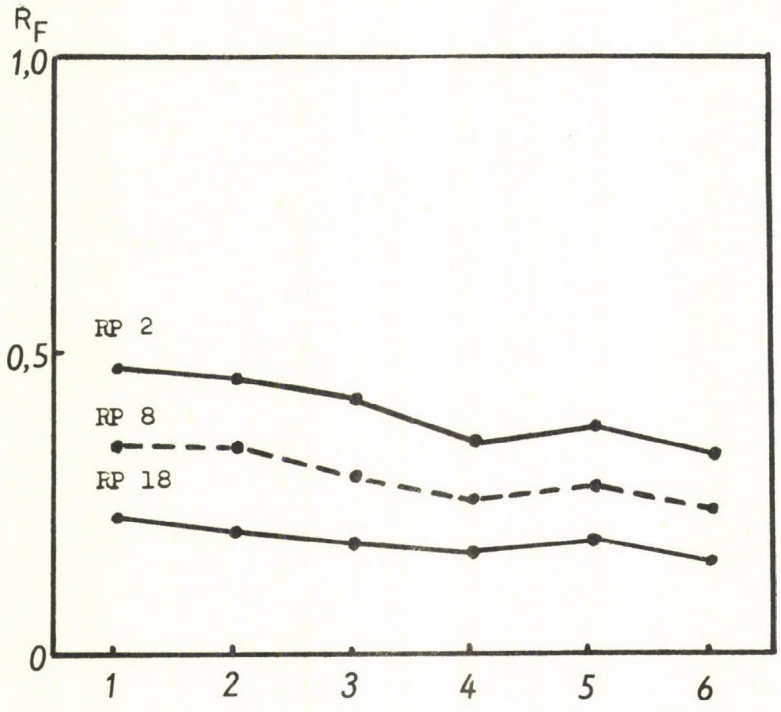


Fig. 4. Influence of the chain length of the alkyl bonded to silica gel on the migration of the 3-pentadecylphenoxy-alkanoic acids using tetrahydrofuran - acetic acid (1:1) as mobile phase

Alkanoic acids:
1 acetic, *2* α-propionic, *3* ω-propionic, *4* α-butyric, *5* α-isobutyric, *6* lauric

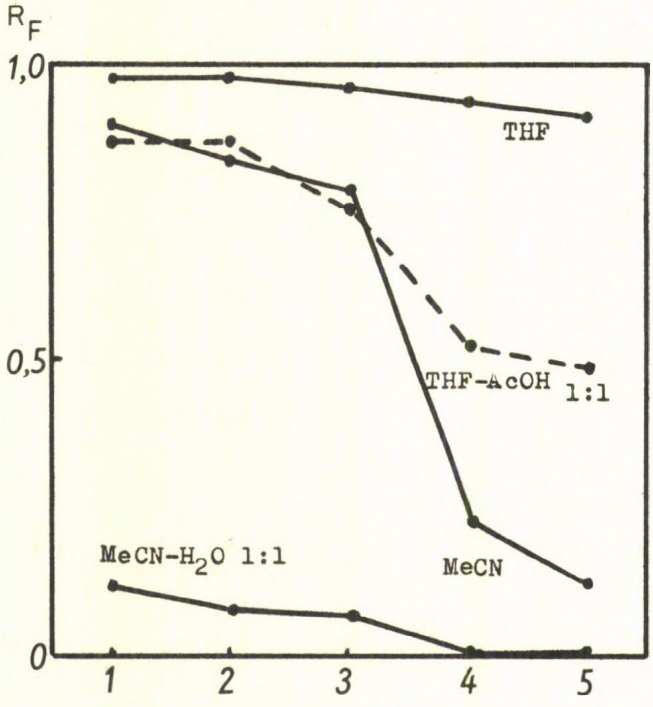


Fig. 5. Separation of 4-methylphenoxyalkanoic acids on RP-8 plates using different mobile phases

Alkanoic acids:
1 acetic, *2* α-propionic, *3* α-butyric, *4* α-lauric, *5* α-palmitic

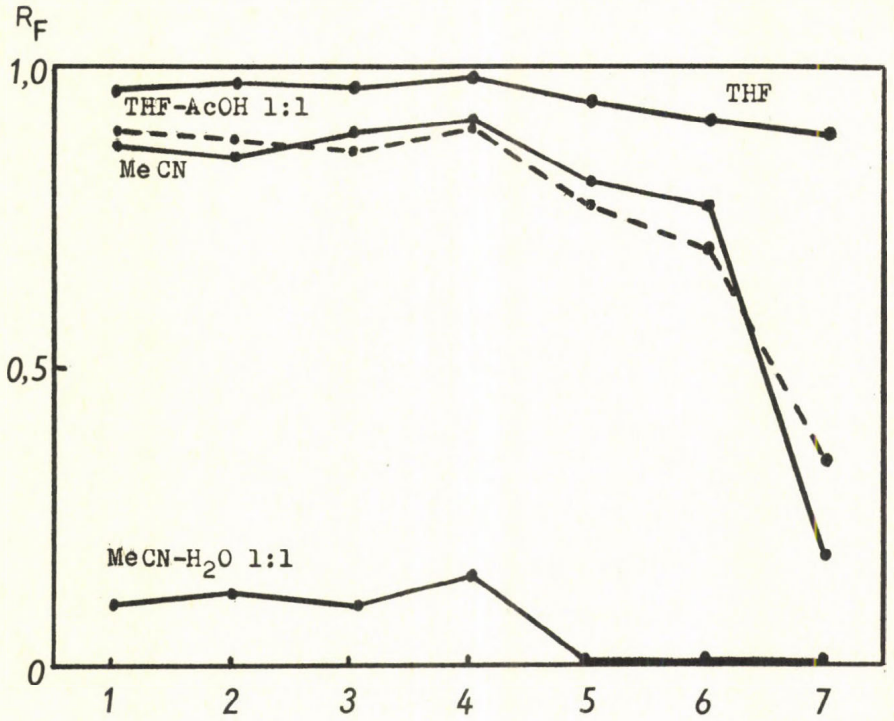


Fig. 6. Separation of alkylphenoxyacetic acids on RP-8 plates using different mobile phases

Substituents:
1 H, *2* 3-methyl, *3* 4-methyl, *4* 4-tert. butyl,
5 4-tert. amyl, *6* 2,4-ditert. pentyl,
7 3-pentadecyl

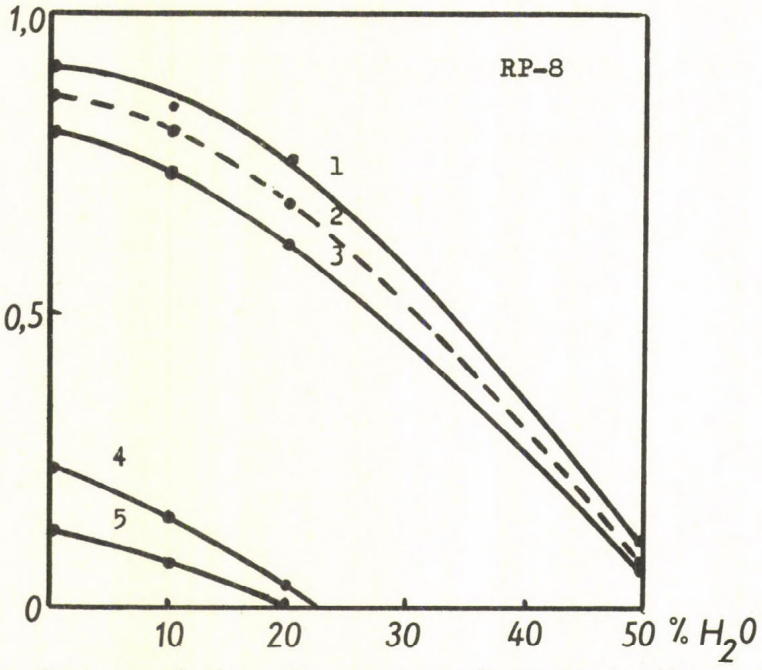
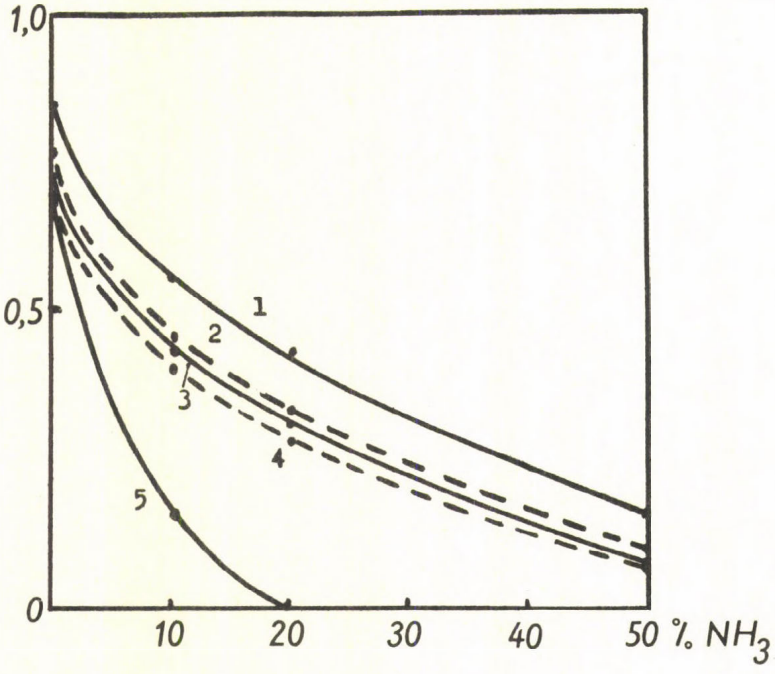


Fig. 7. Influence of the water content in acetonitrile on the separation of 4-methylphenoxyalkanoic acids

Alkanoic acids:
1 acetic, *2* propionic, *3* butyric, *4* lauric, *5* palmitic



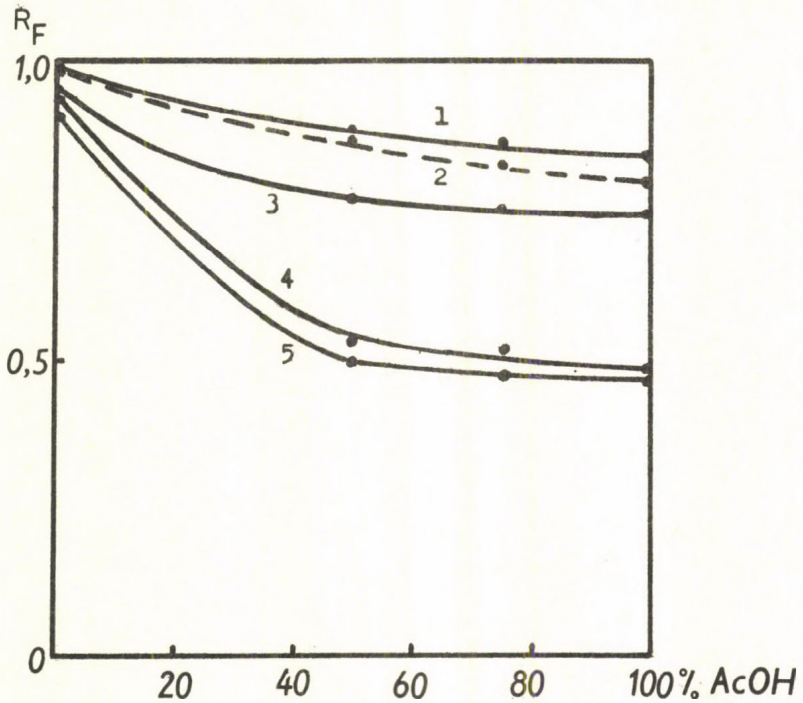


Fig. 9. Influence of the acetic acid content in tetrahydrofuran on the separation of 4-methylphenoxyalkanoic acids on RP-8 plates

Alkanoic acids:
1 acetic, *2* propionic, *3* butyric, *4* lauric, *5* palmitic

←

Fig. 8. Influence of the aqueous ammonia content in acetone on the separation of 3-pentadecylphenoxyalkanoic acids on RP-8 plates

Alkanoic acids:
1 acetic, *2* α-propionic, *3* ω-propionic, *4* α-butyric, *5* α-lauric

CONCLUSIONS

The results obtained with the separation of the series of alkyl substituted phenoxyalkanoic acids by adsorption, normal-phase partition and reversed-phase partition TLC and by reversed-phase HPTLC with chemically bonded phases show that all these types of chromatographic systems are suitable from the analytical point of view. Reversed-phase HPTLC seems to give better results due to the formation of small and sharp spots enabling a sharper analytical differentiation of compounds with smaller R_F differences.

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RELATIONSHIP BETWEEN RETENTION AND ORGANIC SOLVENT MOLARITY IN REVERSED-PHASE HPLC

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EXPERIMENTAL OBSERVATIONS

Capacity factors (k') and molar concentration of the organic component of the mobile phase (C_B) can usually be related by a linear equation such as

$$\log k' = b - p \log C_B \quad (1)$$

where b and p are coefficients (Fig.1).

An analysis of reversed-phase HPLC retention data taken from various publications shows that generally the straight lines corresponding to eq. 1 are arranged fanwise for different members of a given set of solutes. Moreover, a linear correlation exists also between the b and p coefficients for different solutes:

$$b = b_o + b_1 p \quad (2)$$

As a result of this relationship the plots according to eq. 1 extrapolated to large C_B values tend to intercept at one point with the coordinates $\log k' = b_o$, and $\log C_B = -b_1$. To ascertain that such behaviour exists in reversed-phase chromatography we recalculated the retention data for different classes of solutes given in 15 publications by different researchers /1-15/. The results are summarized in Table I. It can be seen that b and p are always correlated, regardless of the class of solute, packing and organic solvent. Our own experimental data given in Table II also support the general character of the discussed phenomenon. By examining the relationship between the cordinates b_o, b_1 on the one hand and the chromatographic con-

Table I. Parameters of eq. (2) calculated from published data /1-15/

Class of solutes	Packing	Organic solvent, concentration range (vol. %)*	b_o	b_1	r**	Ref.
1	2	3	4	5	6	7
Aromatic acids	Hitachi 3011	A, 20...50	-0.486	-1.340	0.997	1
	Chromosorb LC-8	A, 10...70	-0.967	-1.467	0.980	2
N-Alkylbenzamides	Partisil ODS-2	A, 20...40	-0.716	-1.394	0.981	3
		M, 20...40	-0.761	-1.524	0.999	3
Aromatic nitrocompounds	LiChrosorb RP18	A, 60...80	-0.227	-1.394	0.992	4
Barbiturates	Partisil ODS-2	M, 30...50	-0.320	-1.362	0.998	5
		M, 30...50	-1.170	-1.619	0.996	5
N-Substituted phthalimides	LiChrosorb RP18	M, 50...80	-1.726	-1.545	0.947	6
Pyridazinone derivatives		M, 55...80	-1.647	-1.492	0.997	7
Various aromatic and aliphatic compounds	LiChrosorb RP8	A, 0...100	-0.808	-1.391	0.994	8
Aromatic hydrocarbons		M, 20...80	-0.145	-1.352	0.999	8
		E, 20...80	-0.239	-1.160	0.999	8
		T, 30...90	-0.443	-0.975	0.997	8
		P, 10...60	-0.544	-1.050	0.996	8
Isomeric alkylbenzenes	Hypersil SAS	M, 40...50	-0.556	-1.349	0.997	9
Alkylparabenes	μBondapak C18	M, 50...70, 309°	-0.306	-1.353	0.993	10
		M, 50...70, 325°	-0.275	-1.295	0.999	10

(Table I, continued)

1	2	3	4	5	6	7
Aromatic compounds	Zorbax ODS	M, 45...70	-0.235	-1.267	0.996	11
		T, 40...50	-0.541	-0.976	0.998	11
		A, 40...75	-1.306	-1.540	0.998	11
Bile acids	Nucleosil C18	A, 40...70; PB pH 3	-0.921	-1.412	0.972	12
		M, 65...85; PB pH 3	-0.372	-1.397	0.996	12
		A, 35...60; PB pH 7.5; TBA 0.005 M	-0.507	-1.399	0.945	12
		M, 65...85; PB pH 7.5; TBA 0.005M	-1.619	-1.387	0.992	12
Methyl esters of carboxylic acids	Hypersil ODS	M, 10...100	-0.631	-1.387	0.999	13
Alkylbenzenes		M, 60...1000	-1.210	-1.471	0.999	13
		T, 30...80	-0.607	-1.053	0.997	13
Alkanes		M, 60...90	-0.886	-1.416	0.997	13
Phenols	µBondapak C18	M, 50...80	-0.242	-1.440	0.999	14
Substituted N-ethyl-benzamides	Spherisorb ODS-2	M, 40...70	-0.895	-1.867	0.999	15
	µBondapak C18	M, 40...70	-0.388	-1.326	0.999	15
Substituted N-ethyl-benzamides	Nucleosil 7-C_6H_5	M, 40...70	-0.190	-1.304	0.999	15

*A - acetonitrile, M- methanol, E - ethanol, P -propanol, T - tetrahydrofuran, PB - phosphate buffer, TBA - tetrabutylammonium; **r - correlation coefficient

Table II. Parameters of eq. (2) for Zorbax ODS

Class of solutes	Acetonitrile, concentration range (vol. %)	b_o	b_1	r
Alkanes	60...100	-0.953	-1.458	0.999
Alkylbenzenes	50...1000	-0.953	-1.482	0.999
Esters	30...70	-0.837	-1.427	0.999
Ketones	30...70	-0.904	-1.467	0.999
Alcohols	30...70	-0.931	-1.527	0.999
Alkanes, alkylbenzenes, esters, ketones, alcohols	30...1000	-0.873	-1.466	0.999
O-Containing derivatives of cycloheptane	20...70	-0.963	-1.482	0.998
5-Fluoruracil derivatives	5...70	-0.635	-1.165	0.995
1,4-Dihydropyridine derivatives	20...70	-1.388	-1.439	0.983
Peptides	20...50	-1.001	-1.168	0.992
Peptides*		-0.835	-1.127	0.983

*Packing - Silasorb C18 (Lachema, Czechoslovakia)

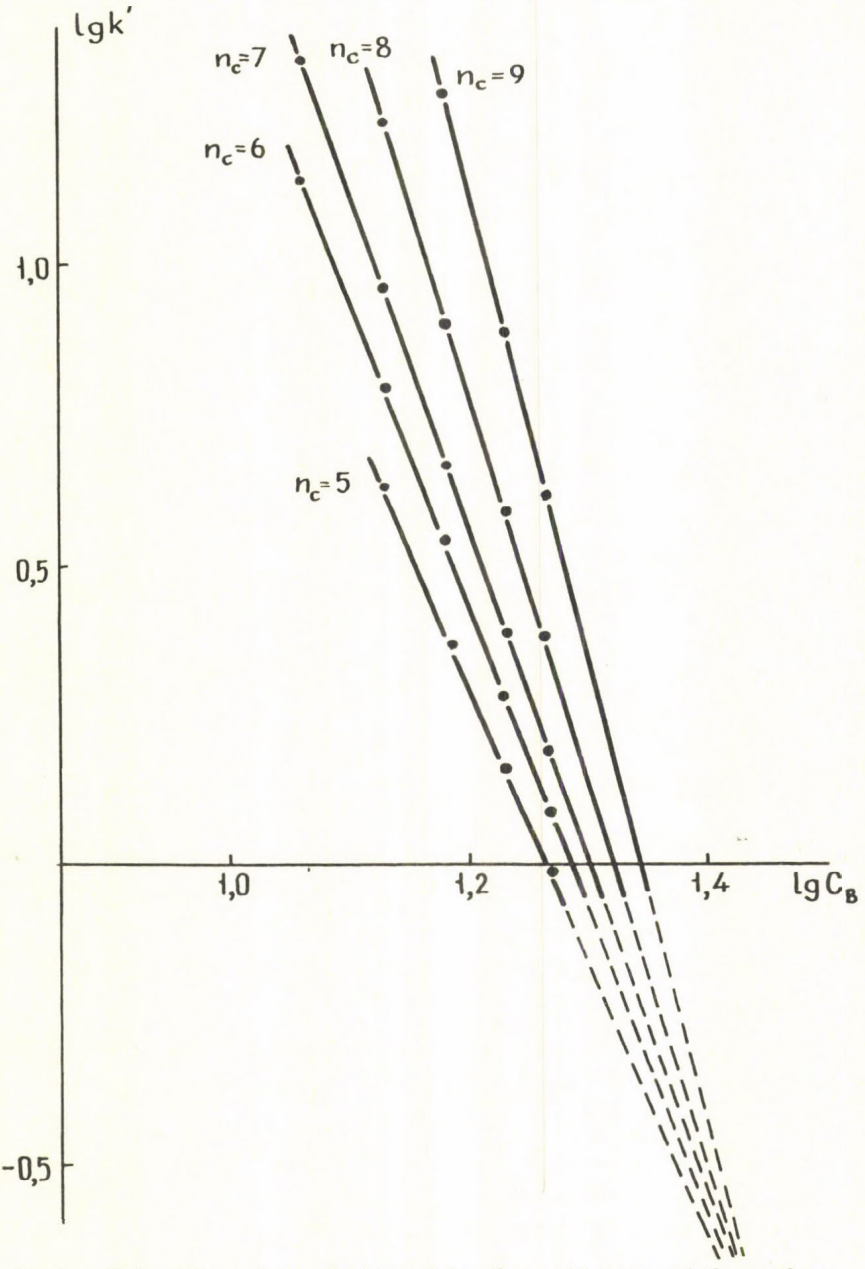

Fig.1. Log k' - log C_B relationship for alkanes with carbon numbers n_c = 5-9.
Packing: Zorbax ODS; mobile phase: acetonitrile-water.

ditions or the types of solutes, on the other hand, the following conclusions can be reached /16/:

- b_1 does not depend on the solute type;
- b_1 decreases with increasing elution strength or hydrophobicity of the organic modifier. In Fig. 2 the hydrophobic surface of the organic modifier molecule expressed as the connectivity index /17/ is compared with the b_1 values;
- it follows from several examples in Table I that b_1 remains constant when water is replaced in the mobile phase by a buffer solution, and even in the presence of ion-pairing agents;
- b_1 remains almost constant for various types of reversed-phase packings such as octadecyl and octyl silicas, phenylsilica and even polystyrene-based Hitachi gel;
- b_o varies from -0.1 to -1.7. No reliable correlation is found between b_o and the type of solutes, packings, and solvents;
- b_o tends to decrease with increasing absolute retaining capability.

THEORETICAL MODEL

The above findings can be interpreted by a model which is deduced from the hydrophobic retention mechanism of reversed-phase chromatography.

The following equilibria are assumed to be dominating in the system:

1. Sorption of solute X on the alkylsilica sites leading to the formation of the adsorbed complex XS:

$$X + S \rightleftharpoons XS \qquad (3)$$

2. Solvation of the solute by organic solvent molecules B:

$$X + nB \rightleftharpoons B_nX \qquad (4)$$

3. Solvation of octadecylsilica by the organic solvent molecules:

$$S + mB \rightleftharpoons B_mS \qquad (5)$$

The hydrophobic surface of the solvates B_mX and B_mS is obviously small if compared with the hydrophobic surfac of non-associated X and S. Therefore it can be assumed that the

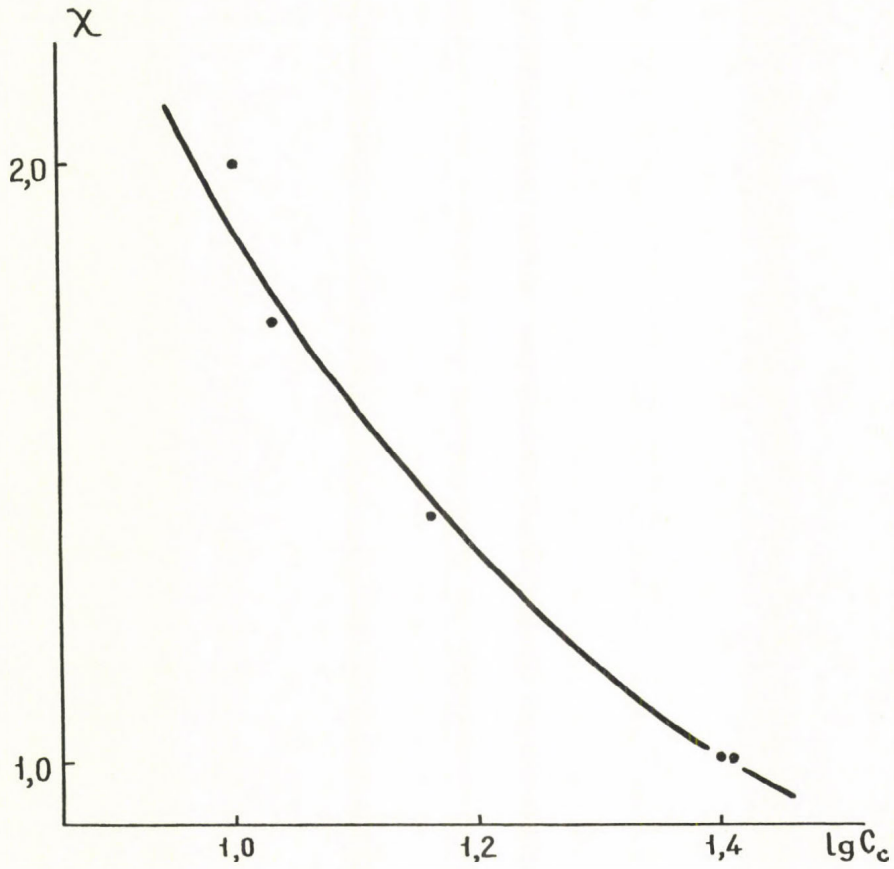

Fig. 2. Relationship between b_1 and the hydrophobic surface area of organic sorbents expressed as the connectivity index χ.

sorptive activity of B_mS and B_mX is small and eq. 3 indeed describes the main process leading to the retention of the solute in the column.

Let us denote the mean concentration for all solute forms in the system as C_{OX} and the total concentration of the stationary phase as C_{OS}. All other subscripts at C correspond to the individual terms of equations (3)-(5).

In conditions typical of reversed-phase HPLC the organic solvent concentration C_B is much higher than the concentration of the solute and that of the active part of the alkylsilica

sorbent. Therefore the equilibrium expressed by eq. 5 can be regarded as being independent of the equilibria expressed by eqs. 3 and 4 and the concentration of the active form of the stationary phase can be expressed as:

$$C_S = C_{OS} - C_{B_mS} \tag{6}$$

The constant K_3 for equilibrium equals:

$$K_3 = \frac{C_{B_mS}}{C_B^m C_S} \tag{7}$$

Substitution eq. 7 into eq. 6 gives

$$C_S = \frac{C_{OS}}{K_3 C_B^m + 1} \tag{8}$$

The constants for the equilibria expressed in eqs. 3 and 4 can be written as:

$$K_1 = \frac{C_{XS}}{C_X C_S} \tag{9}$$

$$K_2 = \frac{C_{B_nX}}{C_X C_B^n} \tag{10}$$

and can be combined with the expression

$$C_S = C_{OX} - C_{XS} - C_{B_nX} \tag{11}$$

and eq. 8. The resulting expression for the mean concentration of the adsorbed solute in the system is:

$$C_{XS} = \frac{K_1 C_{OS} C_{OX}}{K_1 C_{OS} + (K_2 C_B^n + 1)(K_3 C_B^m + 1)} \tag{12}$$

Since the capacity factor is a function of the partition coefficient P ($\log k' = \log P + \log V_S/V_M$) and

$$P = \frac{C_{XS}}{C_{OX} - C_{XS}}$$

the following equation can be deduced from eq. 12:

$$\log P = \log K_1 + \log C_{OS} - \log(K_2 C_B^n + 1) - \log(K_3 C_B^m + 1) \tag{13}$$

It is obvious that equation 13 is not linear against log C_B, and therefore, in view of the proposed model, equation 1 can be regarded only as an approximation. This conclusion is in agreement with some earlier expressed opinions /18, 19/. An analysis of equation 13 shows that if the K_2 values are identical for different solutes their log k' - log C_B plots should be represented by equidistant lines over the whole range of C_B. The observed fanwise location of the lines is possible if solutes with larger K_1 values have also larger K_2 values. In our opinion, such a correlation between K_2 and K_1 appears conceivable, because sorption and solvation of the solute can take place as a result of one and the same hydrophobic effect. Although no strict theoretical relationship is known between K_2 and K_1, it is possible to demonstrate that at certain parameter values equation 13 leads to conclusions which are very similar to those found from experimental data. For example, if

$$K_2 = \beta + \alpha K_1 \tag{14}$$

and n = m = 1, the log P - log C_B lines intercept at a point with coordinates

$$C_{BO} = \frac{1}{\beta} \tag{15}$$

$$P_o = \frac{C_{OS}\beta^2}{(K_3-\beta)} \tag{16}$$

Naturally, the absolute values of C_{BO} and P_o may differ from the corresponding b_1 and b_o values because the latter are found by linear extrapolation of plots which are not strictly linear. Nevertheless, equation 15 appears suitable for predicting all the conlusions made on the basis of experimental data, showing that C_{BO}, which is analogue of b_1 does not depend on any of the experimental parameters except β which is related to the properties of the organic modifier. On the other hand, eq. 16 shows a complex relationship between the parameters of the model, explaining why the differences in b_o were not easily interpretable.

In currently used HPLC columns with the most popular geometry (4.6 mm I.D. x 250 mm) and packing containing 10 % of carbon C_{OS} is about 0.6 moles $\neq$ 1 and V_S/V_m is about 0.15.

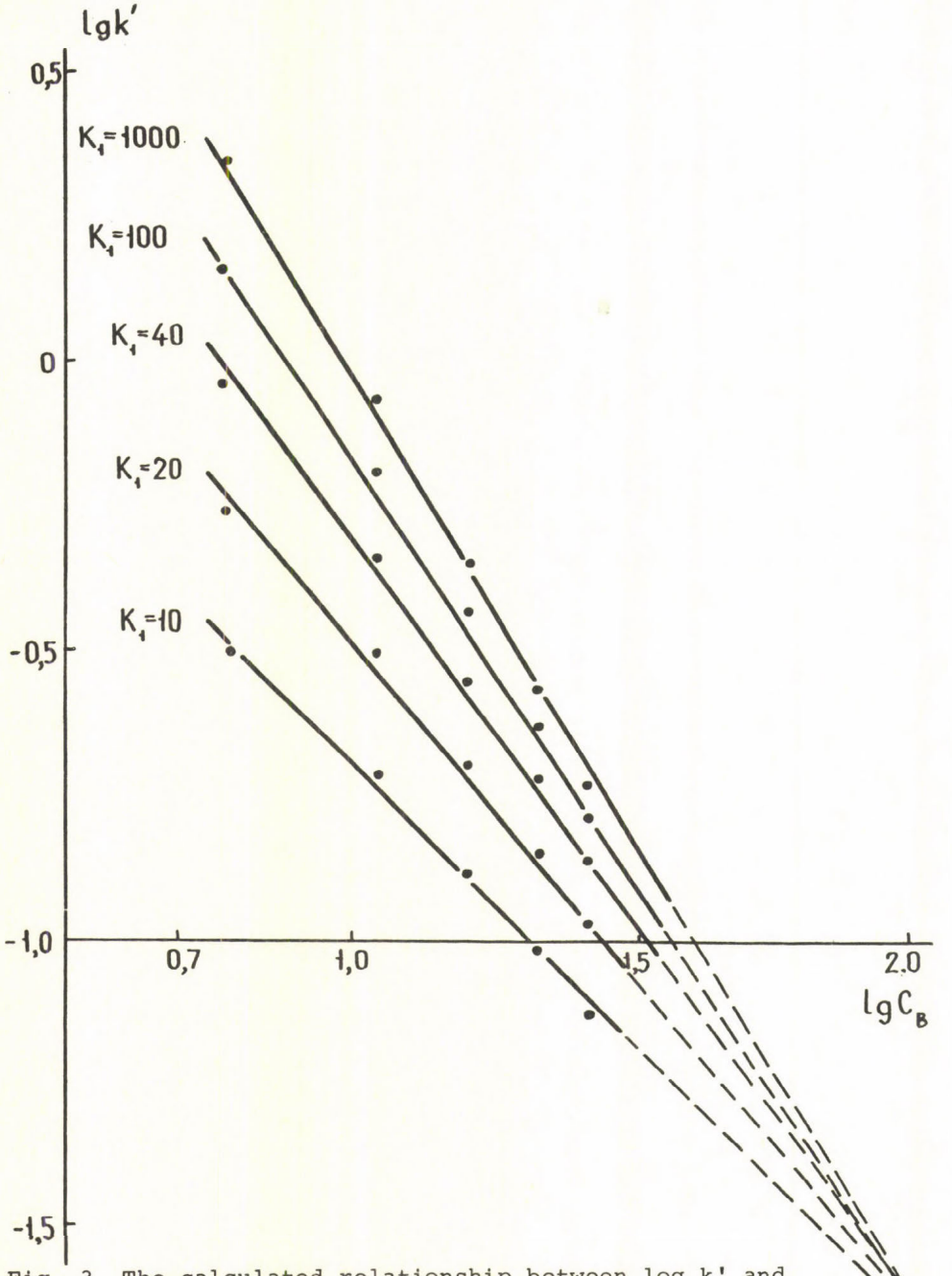

Fig. 3. The calculated relationship between log k' and log $C_B \cdot K_1$ values indicate the corresponding plots; other model parameters are given in the text.

By means of eq. 13, we calculated the log k' values for a series of compounds at different C_B values assuming that $K_3 = 0.25$, $\alpha = 0.00125$, $\beta = 0.001$, $M = n = 1$. Naturally, the calculated lines, which are shown in Fig. 3, are not quite straight. Nevertheless, for the given range of C_B corresponding to 25 to 100 vol. %, the deviations of the calculated points from the straight lines obtained by the least squares method are not large and are close to the typical k' measurement errors. In the C_B range studied the data calculated from eq. 13 can be approximated by eq. 1 with correlation coefficients exceeding 0.995. Therefore, despite the nonlinearity of the model expressed by eq. 13, equation 1 provides a reasonable and valid approximation.

The arrangement of the lines in Fig. 3 is similar to that of the experimental lines given in Fig. 1. The correlation coefficient for eq. 2 calculated from the data used for Fig. 3 equals 0.997.

SUMMARY

The fanwise arrangement of the log k' - log C_B lines is typical for reversed-phase chromatography. On extrapolation the lines tend to intercept at one point. The phenomenon can be explained by the proposed model, taking into account association of the solute and sorbent with organic solvent molecules. For certain sets of parameters the model provides a satisfactory fit with the experimental data.

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H. Kalász and L.S. Ettre (Eds)

COMPUTER CONTROLLED EVALUATION OF THIN-LAYER CHROMATOGRAMS AND ELECTROPHORESIS OBJECTS

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1. Summary

The function of a fully automatic computer controlled system for the evaluation of thin-layer chromatograms and electrophoresis objects is described. It is based on a CAMAG TLC SCANNER II, desktop computer HP 9816 and the appropriate software. [1-9]

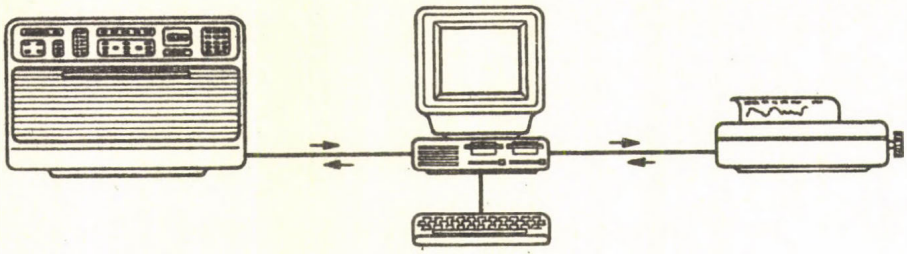
Accuracy, repeatability and reproducibility are discussed. Selected applications employing scanning by reflection and transmission are demonstrated. These relate to HPTLC in pharmaceutical and cosmetic routine quality control and electrophoresis in food analysis.

2. Introduction

One of the important differences between computer controlled evaluation systems and other evaluation techniques is its flexibility by controlling the scanning in conjunction with the evaluation software.

The system currently used for the described examples consists of a CAMAG TLC SCANNER II in combination with a desktop computer HP 9816.

In addition to measuring absorbance or fluorescence the system controls the scanning stage position and optimizes the position of the scanning slit to the fractions in the form of spots or lines.



CAMAG TLC SCANNER II

THE COMPUTER IS CONNECTED BY PLUGGING IN INTERFACE CARD WITH BUILT-IN 12 BIT A/D CONVERTER

COMPUTER HP 9816 WITH KEYBOARD, GRAPHICS SCREEN, 3 1/2 " DUAL FLOPPY DISK DRIVE

MATRIX PRINTER HP 82906 FOR ORDINARY PAPER

The scanner first searches in the Y-direction (separation flow direction) until it detects a signal maximum. When this has been found, the maximum in the X-direction is determined. The actual scan of the track is then conducted with the new slit coordinates, and peak height and peak area are determined and stored. The same procedure is repeated for each fraction of the track.

This type of peak optimization improves the accuracy of the analysis if one of the frequently encountered shortcomings of a separating process occurs such as slanted direction of flow. It does not eliminate errors caused by sample application or grossly distored separation zones.

The scanning curves are displayed, with integration marks and base-line origins and after baseline correction, on the screen. Any graph can be checked visually for plausibility and may be hard copied on the printer if required. Monitoring and correcting operations are normally not performed during the scanning process but postrun after the raw data have been stored on the floppy disc. Complex chromatograms which, because of chromatographic inadequacies, cannot be satisfactorily

evaluated by automatic integration, can be manually integrated on the screen.

The possibility to store complete methods is extremely useful for routine operations. Complete evaluation procedures, including spectra recording and multiwavelength measurement can be stored. They are identified by a short reference code by which they can be called up any time.

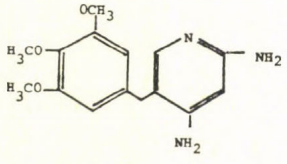
Summary reports for comprehensive documentation can be printed out any time. In this part of the program results can be summarized, e.g. in the case of application of the same sample. If desired, mean and standard deviation of the individual values, concentration and confidence intervalls, are presented.

3. Material and methods

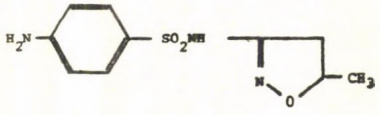
For computer controlled evaluation of all examples the system CAMAG TLC SCANNER II in combination with desktop computer HP 9816, double floppy drive HP 9121D, HP 82906A matrix printer and CAMAG I/D Interface 76681 was used. Quantitative evaluation was conducted with the extended software 85 (76678) for reflection. For electrophoresis evaluation the "transmission" software (76785) was used.

3.1 Sample preparation and chromatographic conditions

Example No. 1: "Antibiotics"



Trimethoprim (TMP) MG: 290,32
5-((3,4,5-trimethoxyphenyl) methyl)-2,4-pirimidindiamine



Sulfamethoxazol (SMZ) MG: 153,31
4-amino-N-(5-methyl 3-isoxazolyl) benzsulfonamide

Sample preparation and chromatographic conditions are described in CAMAG BIBLIOGRAPHY SERVICE (CBS) No. 54.

Example No. 2: "Cosmetic stabilizers"

(Methylparaben and propylparaben)

Sample preparation and chromatographic conditions are described in CAMAG application A-15.2.

3.2 Sample preparation and electrophoretic conditions

Example No. 3: "Proteins of wheat, fractions of gliadines".

Sample preparation for gliadines from different types of wheat and electrofocusing conditions are published [10].

Example No. 4: "Proteins and peptides of cheese".

Sample preparation and electrofucusing conditions are published [11].

Example No. 5: "Proteins of amniotic fluid".

100/ul of amniotic fluid are diluted with a solution of 0.1% carrier ampholyt pH 4-8. 20 µl of the sample are applied on the surface of the gel. Isoelectric focusing was conducted at 3000 volt on a ultra-thin polyacrylamide gel in 8 mol urea.

4. Results

Example No. 1: Fig. 1.0 shows the well separated components trimethoprim (first component) and sulfamethoxazol. The components are baseline separated. The picture is identical with the one on the screen in the integration phase.

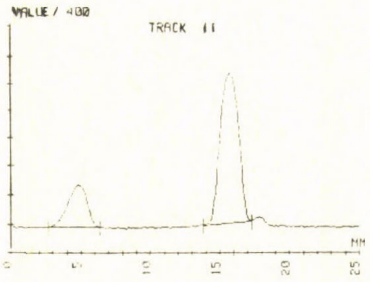


Fig. 1.0 Display of separation of antibiotics

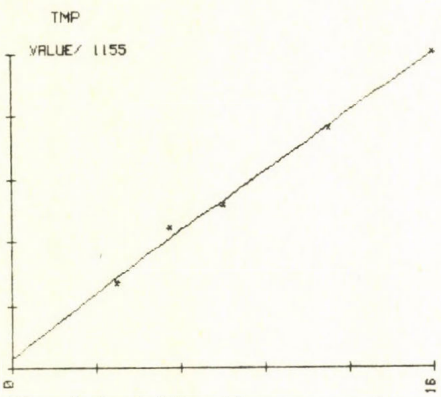


Fig. 1.1 Calibration curve of trimethoprim in the range 40 - 200 ng.

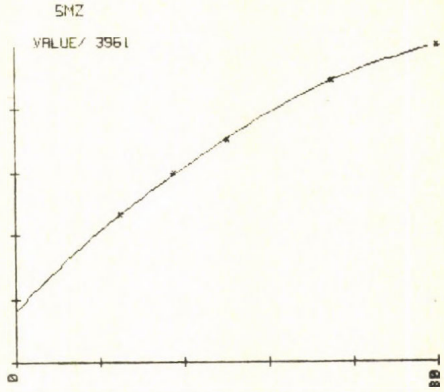


Fig. 1.2 Calibration curve of sulfamethoxazol in the range 190 - 900 ng.

Tolerated deviation from the declared content is ± 10% for the time being, whilst ± 5% is the desired precision. Rel.standard deviation of the HPTLC method described is about 1%.

Tab.1

Sample	Rel.SD	((X) mg/tablet	Declaration	absolute
TMP	3.76	171.45	160	7.15
SMZ	2.05	814.54	800	1.82

Reproducibility of the method

TMP (n = 12), rel. SD = 0.743%
SMZ (n = 12), rel. SD = 0.879%

Repeatability of measurement

TMP (n = 20) = 0.154% rel. SD
SMZ (n = 20) = 0.183% rel. SD

Example No. 2

fig. 2.0 shows the two components of a cosmetic stabilizer, peak No. 1 methylparaben, peak No. 2 propylparaben. The components are baseline resolved. Marks indicate begin and end of each peak as defined by automatic integration.

An example of the identification routine is depicted below. Substances can be identified by measuring spectra of certain fractions and superimposing them (e.g. the equidistant fractions of all unknowns) with the respective fraction of the (identification) standard (figg. 2.2, 2.3). The computer correlates these spectra by calculating the regression coefficient (last column fig.2.4) and states "identity O.K." if a preselected value is reached.

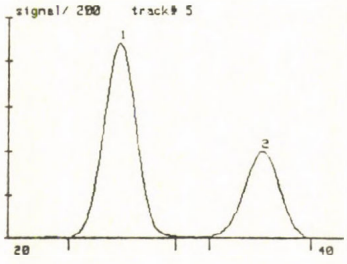


fig. 2.0 Display of separation of cosmetic stabilizer

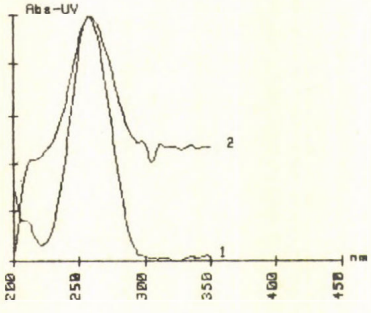


fig. 2.1

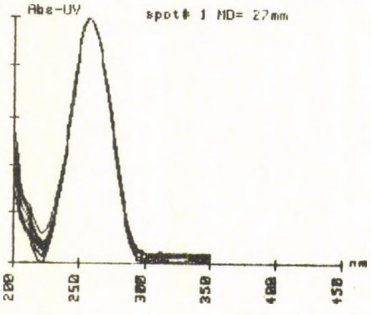


fig. 2.2

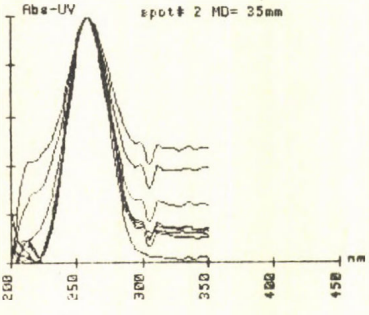


fig. 2.3

spot# 1		spot# 2	
r(2, 1)=	0.998605	r(2, 1)=	0.981795
r(3, 2)=	0.998515	r(3, 2)=	0.991435
r(4, 3)=	0.994435	r(4, 3)=	0.993566
r(5, 4)=	0.994602	r(5, 4)=	0.990320
r(6, 5)=	0.989112	r(6, 5)=	0.992418
r(7, 6)=	0.998205	r(7, 6)=	0.999568
r(8, 6)=	0.985613	r(8, 6)=	0.982304
r(9, 6)=	0.993281	r(9, 6)=	0.994027
r(10, 6)=	0.995672	r(10, 6)=	0.995860

spot# 1 identity O.K.

spot# 2 identity O.K.

fig. 2.4

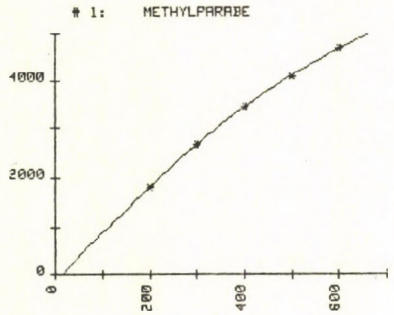


fig.2.5

Calibration curve of methylparaben between 200 and 600 ng.

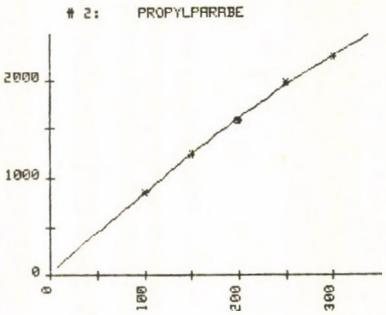


fig. 2.6

Calibration curve of propylparaben between 100 and 300 ng.

The analysis is terminated if the computer is reporting (fig. 2.7) the results as followed (squares). All necessary informations are implemented incl. the confidential interval of each analysis.

```
      # 1        26.6  :  **********
      # 2        35.1  :  **********
EVALUATON MODE   2:  Routine calibration

track#:                                          1234567890
function of track:                               NSSSSSAAAA
sample groups:                                        AAAA

substance # 1      METHYLPARABE
trk# sa  MD[mm]      X    | height   X(calc)  |  area   X(calc)  | ci(H)  ci(A)
  2  st   26.1    200.0      82.7              1824
  3  st   26.4    300.0     118.4              2679
  4  st   26.6    400.0     149.3              3440
  5  st   26.7    500.0     176.4              4082
  6  st   26.9    600.0     200.6              4681
  7  A    26.9              130.8    339.4     2959    336.2     3.8     5.2
  8  A    26.9              130.9    339.7     2989    340.1     3.8     5.2
  9  A    26.9              129.4    335.0     2933    332.8     3.7     5.1
 10  A    26.9              131.7    342.2     2982    339.2     3.8     5.2

substance # 2      PROPYLPARABE
trk# sa  MD[mm]      X    | height   X(calc)  |  area   X(calc)  | ci(H)  ci(A)
  2  st   34.5    100.0      36.0              866
  3  st   34.7    150.0      51.4              1237
  4  st   35.1    200.0      66.0              1583
  5  st   35.2    250.0      79.7              1977
  6  st   35.4    300.0      93.2              2249
  7  A    35.3              65.3     197.8     1599    199.3     1.6     12.8
  8  A    35.3              66.6     202.3     1550    192.4     1.6     12.6
  9  A    35.2              64.6     195.3     1551    192.5     1.6     12.6
 10  A    35.3              66.2     200.9     1566    194.6     1.6     12.7
```

fig. 2.1 spectra of spot No. 1 superimposed with spectra of spot No. 2.

fig. 2.2 spectra of 10 spots on different tracks with equal migration distances (MD = 27 mm spot No. 1)

fig. 2.3 same as (B), migration distance MD = 35, spot No. 2)

fig. 2.4 regression coefficient based on the calculation of the comparison between spectra of standard and analysis.

Example No. 3

Ultra thin layer isoelectric focusing of the fraction of gliadines in diffe rent sorts of wheat to detect false declarations.
The area of significant variations is marked (:) (fig. 3.0)

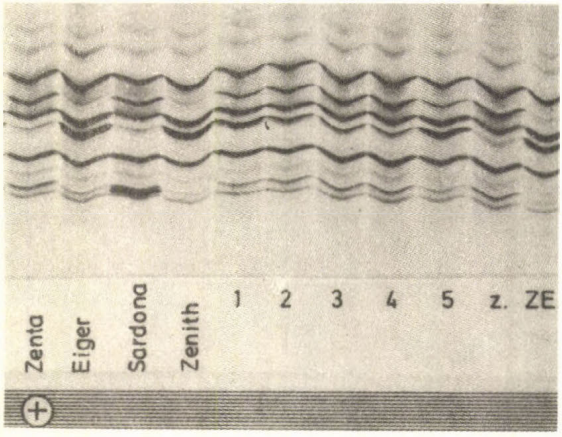


fig. 3.0

Scanning results are depicted in both figures below.
Track No. 1 is depicted in fig. 3.1 and shows a mixture of 50% wheat named Zenith and 50% wheat named Zenta. This mixture is used as standard mixture. The graphic of track No. 2 (fig. 3.2) shows a sample of a client which has verified the pure sort with a cheeper sort.

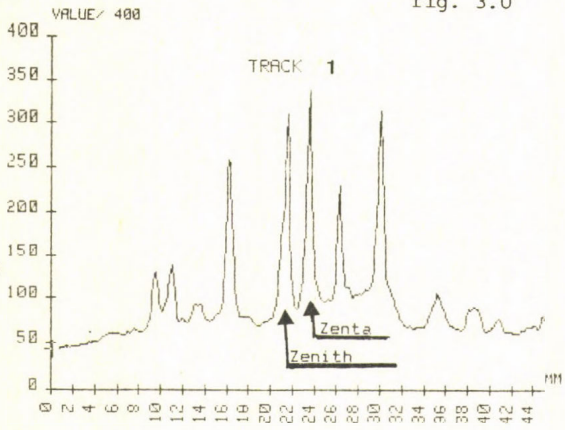


fig. 3.1

The results of the analysis of the samples No.1 to No. 5 are depicted in fig. 3.3.
The calculation was conducted with the method of external standard.

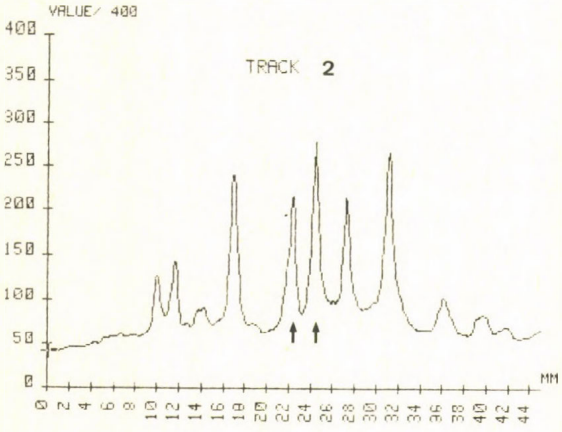


fig. 3.2

EVALUATION BY AREA

ANALYSIS NR	TRACK	Zenith	Zenta
1	2	37.502	48.969
2	3	32.473	53.717
3	4	26.182	55.631
4	5	55.084	53.216

AMOUNT IN : %

fig. 3.3

Example No. 4

In order to demonstrate the correctness of the integration by the computer, an ultra thin layer (0.2 mm gel thickness) isoelectric focusing of cheese proteins is chosen and depicted in fig. 4.0. The very sharp lines of the fraction of the globulins and caseines of track No. 3 (Tilsiter) are scanned.

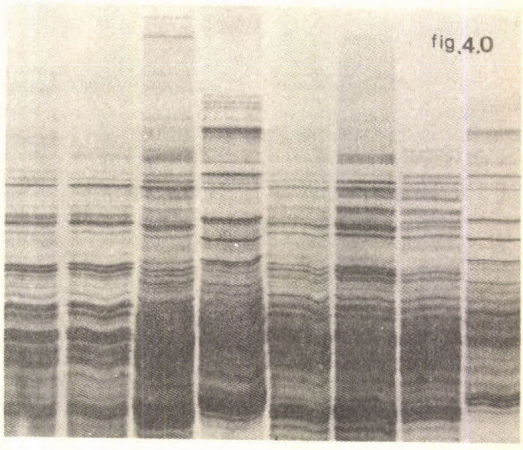


fig. 4.0

The scanning result is depicted in fig. 4.1. The scan was conducted at 580 nm. The integration of the same track is depicted in fig. 4.2.

Under circumstances, considerable errors are possible with regard to the judgement of the correctness of the integration in such a density of marker.

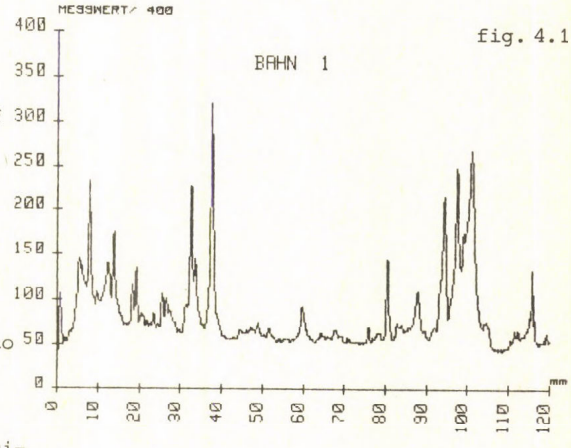


fig. 4.1

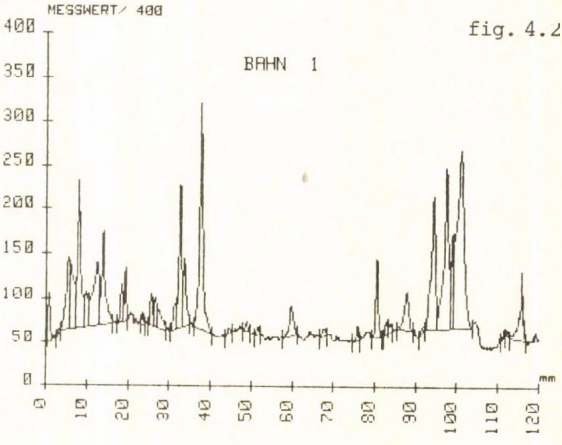


fig. 4.2

fig. 4.3 shows a remedial function of the software called "curve expanding". The access to a section of the graphic is possible. The re-integration in the expanded curve is admissible.

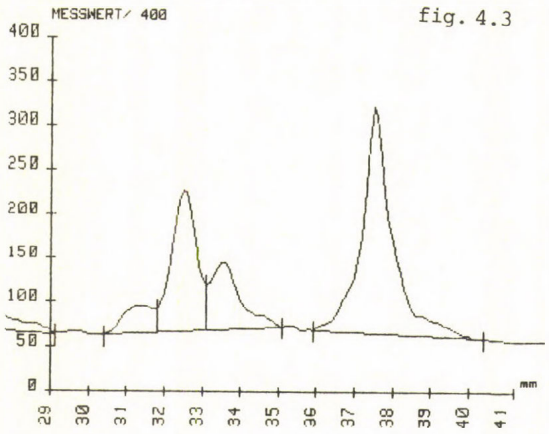


fig. 4.3

Example No. 5

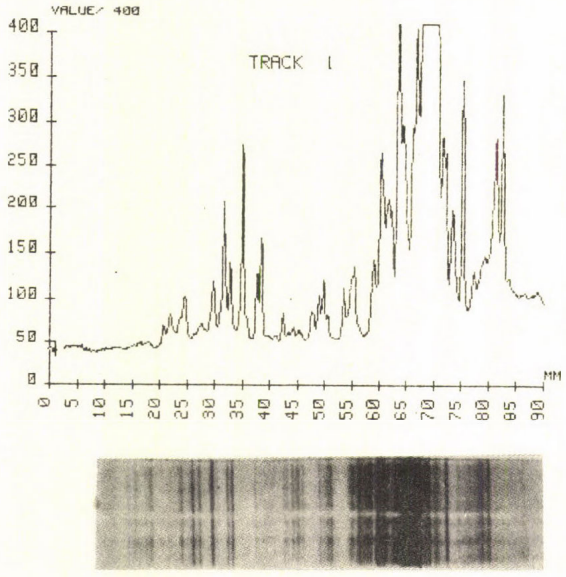


fig. 5.0

Scan of 20/ul untreated amniotic fluid of a foetus with muscle distrophic after ultra thin layer isoelectric focusing (580 nm)

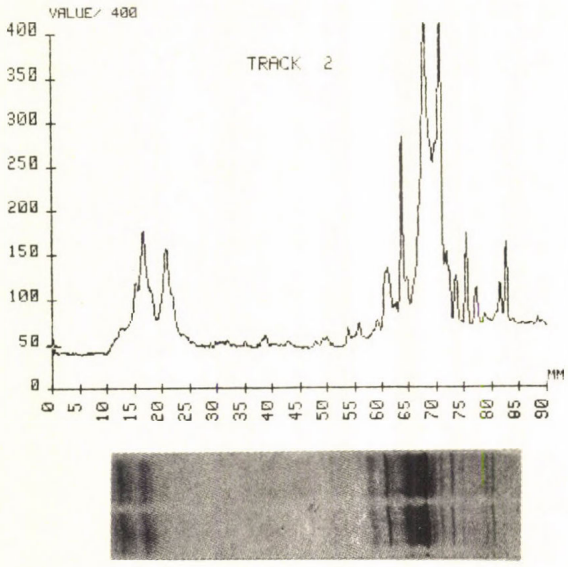


fig. 5.1

Scan of 5 µl human serum after ultra thin layer isoelectric focusing (580 nm)

5. Discussion / Conclusion

Computer controlled evaluation of HPTLC (high performance thin-layer chromatograms) and "high performance" electrophoresis objects (ultra thin-layer isoelectric focusing) is suitable for the quantitative analysis of complex samples of different provenience.

The reproducibility of a method is determined by the errors of all single operations in the procedure. In HPTLC the following steps have to be considered:

VK_V - reproducibility of the sample application (dosage volume)

VK_C - reproducibility of the chromatography

VK_m - reproducibility of the photometric measurement including result computation.

The total error is expressed in the equation:

$$VK^2 = VK_v^2 + VK_c^2 + VK_m^2$$

The measuring error can be determined by repeatedly scanning the same track under identical conditions (repeatability).

All steps in HPTLC and even more so in electrophoresis have to be standardized in order to reduce errors effected by the chemical treatment (staining, destaining, denaturation conditions, staining substance, etc.). Shortcomings in the electrophoretic procedure causing, e.g. waved lines and drifting pH-gradients, etc. can constitute considerable problems for quantification within a reasonable confidence intervall. Computer controlled scanning and data processing is suitable to improve the evaluation of such objects.

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Akadémiai Kiadó
Budapest, 1986

Chromatography '85
H. Kalász and L.S. Ettre (Eds)

ON THE ADDITIVITY OF ISOCRATIC OPLC DATA FOR RETENTION PREDICTION IN MULTISTEP GRADIENT OPLC

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In our earlier work [1,2] we have developed multistep gradient elution for overpressure-layer chromatography (OPLC), and described this technique in detail from the methodological point of view [3]. We have also discussed a number of applications of OPLC [4]. The advantages of OPLC have been described in other publications (see e.g., [5,6]).

Additional advantages arise from multistep gradient development. The possibility of employing practically any number of eluents consecutively during one development, to have n eluent changes, provides great flexibility for the separation of complex mixtures, especially if the components of the sample are spread out on a wide polarity scale. The overrun capability of the instrument can be utilized with any or all of the eluents as required.

We decided to investigate the possibility of calculating migration for multistep gradient elutions from the R_f values of the sample components, determined from independent isocratic measurements, constituting the steps of the multistep gradient run. Our aim was to calculate migrations for multistep gradient analyses and to compare them with the

measured values. The results of our investigations can be used as an aid for planning multistep gradient separations. In the future we also want to estimate multistep gradient migrations from isocratic literature data.

As R_f is defined for the j-th component as:

$$R_{f(j)} = \frac{m(j)}{z} \qquad (1)$$

where $R_{f(j)}$ is the R_f value for the j-th component; m_j is the migration distance of the component j, and z is the migration distance of the front. We have defined [4] a retention factor for multistep gradient runs ($R_f'_{(j)}$, for the j-th component) in an analogous way as:

$$R_f'_{(j)} = \frac{m_j}{\sum_{i=1}^{n} z_i} \qquad (2)$$

where z_i is the distance traveled by the i-th eluent front during the introduction of the i-th eluent, the sum from 1 to n being the total migration distance of the first eluent front. As for multistep gradient cases, $R_f'_{(j)}$ is a function of not only the component j and the eluent system employed, but also of the distances run with each eluent i, we decided to distinguish this R_f value by using the symbol of R_f'.

Chromatography performed in this way is polyzonal, because of two reasons. First, there are the higher order - demixing - fronts usually experienced in OPLC. Second,

additional fronts are induced by changing the eluent at the gradient steps, adding at least n-1 additional fronts in the case of n-step gradient runs.

We also have to consider the other effects of eluent changeover: that the second and later eluents are pumped onto a wet layer having an activity different from that of a dry plate, furthermore that the stationary phase developing on the layer will be different, as it will remember the previous eluents. A problem in the calculations is to find a formula to describe the delay between the times when a later eluent reaches a selected component, i.e., the lag of the gradient caused by migration.

The delay can be calculated by a stepwise method demonstrated in Figure 1. The distance between the later eluent front and the spot is $R_f \times z$. While the eluent front covers this distance the spot migrates a further $R_f \times (R_f \times z)$ etc.

The transition to an infinite number of steps, the sum of this geometrical series, gives the maximum distance the component can travel in the eluent.

$$m_j = z \times \sum_{k=1}^{\infty} R_f^{\,k} = \frac{R_f \times z}{1 - R_f} \qquad (3)$$

The data used for the calculations are given in Table I.

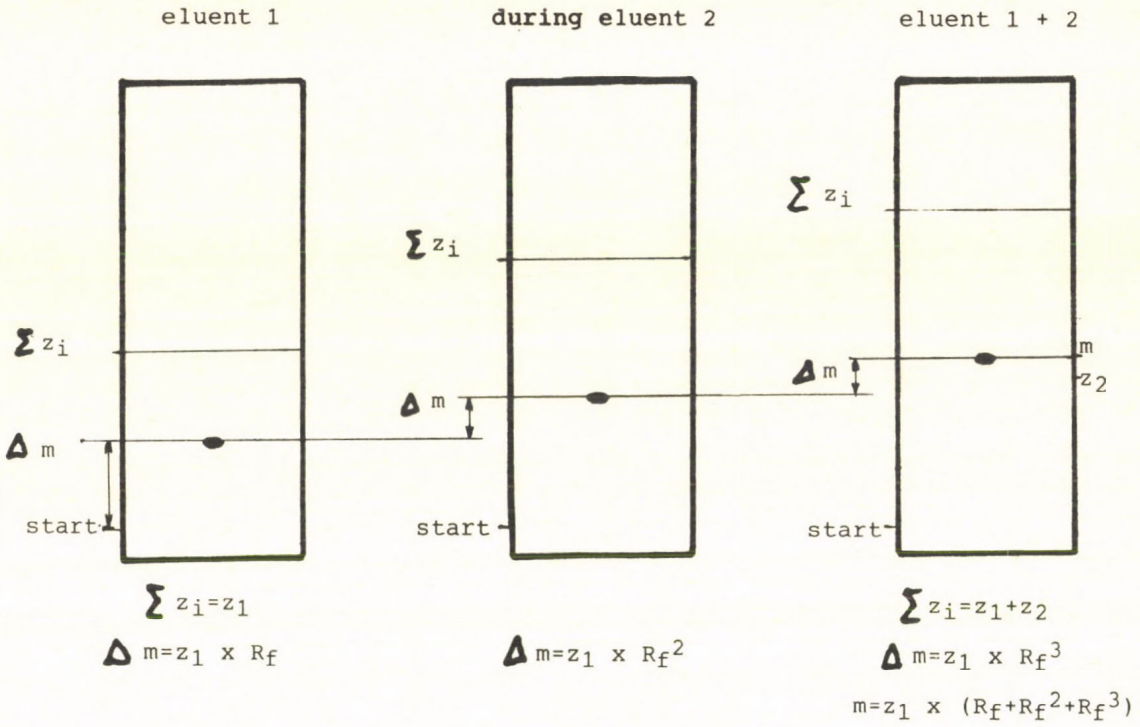

Figure 1. Scheme of stepwise approximation of the distance a component can travel in a given eluent.
m = maximum migration distance
R_f = retention factor in the eluent
z = migration distance of the eluent in question

Table I. Developments and data used

Development	Data	
	measured	calculated
n isocratic runs	n x m_j	$R_{f(i,j)}$
	n x z_i	m_j
multistep	n x z_i	$\sum_{i=1}^{n} z_i$
gradient run	m_j	

The symbols used in Table I. are specified below:
m_j is migration distance for component j, z_i is development distance with eluent i, $R_{f(i,j)}$ is R_f in eluent i for component j. The total front migration is the sum of the individual eluent migrations. Indexes: i is eluent (from 1 to n), j is component (from 1 to p).

The $R_{f(i,j)}$ matrix was calculated from the isocratic runs (R_f with the i-th eluent for the j-th component from the migration of the component for each eluent and component). From the multistep gradient run the development distances z_i, run with each eluent were measured (as no overrun was used the total front migration was also measured).

In our approximation we neglected the diffusion of the

eluent fronts, i.e. we calculated as if the second and further eluents moved along the plate having sharp, straight fronts, perpendicular to the migration direction. Furthermore we also neglected the difference between the effect of an eluent on a dry and a wet plate, (i.e., the difference in activity), and the memory effect of the stationary phase. The dead volumes in the apparatus, described elsewhere [3], were minimized, and the unavoidable dead volumes measured. These were taken into account in the calculations.

Equation 4. was derived, based on the neglections given above, using eq. (3), for determining the migration in multistep gradient development. The data used in this equation can be experimentally determined.

$$m_j = \sum_{i=1}^{n-1} \frac{R_{f(i,j)} \times z_i}{1 - R_{f(i,j)}} + R_{f(n,j)} \times \sum_{i=1}^{n} z_i - \sum_{i=1}^{n-1} \frac{z_i}{1-R_{f(i,j)}} \quad (4)$$

A lipid mixture of biological origin has served as our model sample, in which the polarity of the components spanned a wide range. It was derived from human red blood cells and it contained ten identified components (j=10). For details on sample preparation and the system used see refs. [3,4]. The comparison of the calculated and measured migration distances for the gradient run were used to confirm that the neglections detailed above are suitable for planning.

The confirmation was done on a 3-step gradient OPLC separation (i=3) under the following conditions:

- MERCK HPTLC Si 60 plates, 20 x 10 cm
- membrane pressure 14 bar
- starting eluent pressure: 20 bar
- eluent flow rate: 0.1 ml/min

The composition of the eluents used and the development distances are listed in Table II., where C stands for chloroform, M for methanol, W for water and KCl for 0.25 % KCl in water.

Table II. Eluents and development distances

Eluent #	1.	2.	3.
composition	C:M 95:5	C:M:W 65:25:3	C:M:KCl 55:36:9
development distance	38 mm	100 mm	27 mm

Staining was carried out by immersion in orcinol-sulfuric acid reagent and heating at 105°C for 2 minutes.

The analyses were performed on 21 parallels, distributed on three plates. We determined $m_{i,j}$, for ten components of the sample in each eluent. The control multistep gradient run was also performed on 21 parallels.

The regression of the calculated and the measured data was examined, using relationships described by Massart et al. [7]. The averages of the 21 parallels were used in the first calculation to determine the deviation of the regression

curve from the ideal match (slope = 1, intercept = 0).

21 parallels were used for two purposes:

- to estimate the individual isocratic $R_{f(j)}$ values as precisely as possible,
- to minimize the non systematic experimental errors in the multistep gradient runs.

The systematic error of the method can be seen in Fig. 2. showing the regression line calculated from the averages of 21 parallels both ways, supporting the acceptability of our neglections and the validity of our model. As shown by Fig. 2. the fit is very good, and thus eq. (4) can be used for such calculations.

In the case of planning new multistep gradient separations isocratic $R_{f(j)}$ values are either available from the literature, or have to be experimentally determined. We have examined how multistep gradient separations can be planned if literature data is not available (as in our case), using one series of isocratic runs. In this case the random errors are not eliminated by averaging the parallel experiments. This was simulated by selecting randomly selecting the $R_{f(i,j)}$ values for the calculations. The random selection and the calculation were performed several times, and correlated with the randomly selected gradient migrations. The three with the largest residual variations are shown in Figures 3., 4. and 5. As can be seen from these figures one series of experiments is sufficient for the practice.

The data of Table III. shows numerically the good fit of

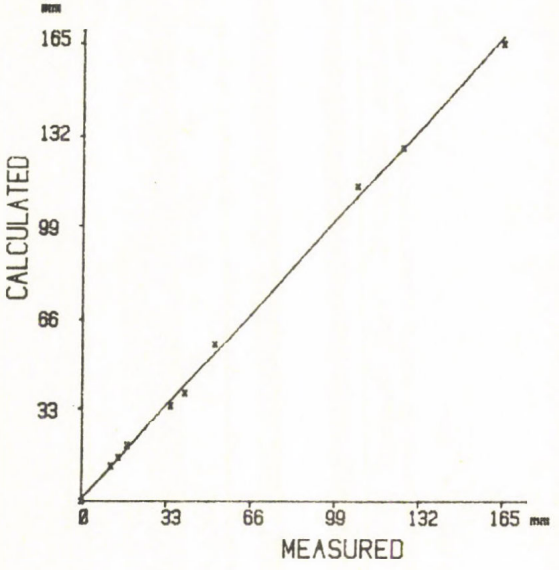

Figure 2. Regression analysis of the calculated and measured multistep gradient development migrations from the average of 21 parallels. (Row 1 of Table III.)

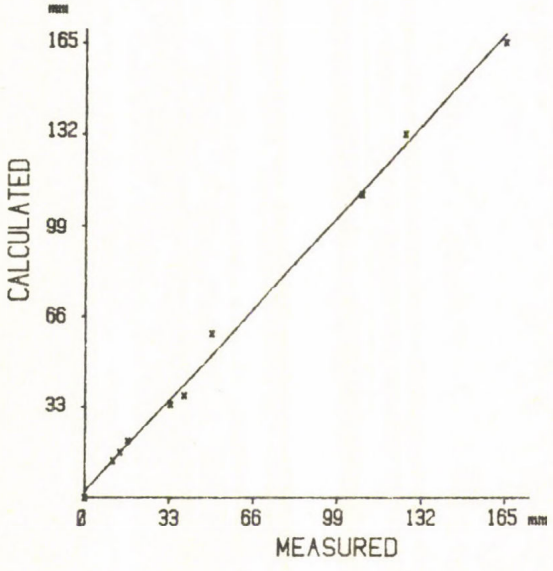

Figure 3. Regression of the calculated and measured multi-step gradient development migrations from random selection 1. (Row 2 of Table III.)

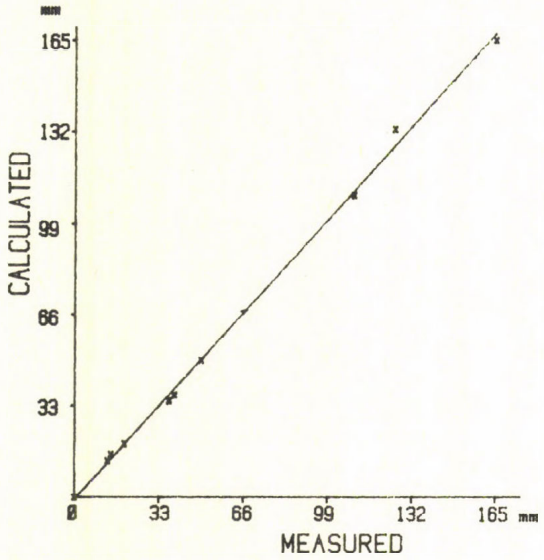

Figure 4. Regression of the calculated and measured multi-step gradient development migrations from random selection 2. (Row 3 of Table III.)

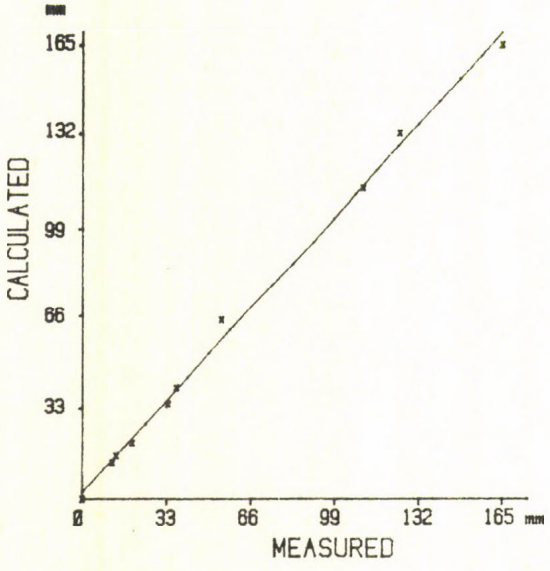

Figure 5. Regression of the calculated and measured multi-step gradient development migrations from random selection 3. (Row 4 of Table III.)

the calculations based on the average and the randomly selected R_f values.

Table III. Regression data

Calculation	slope	intercept	s(resid.)
average of 21	1.009	1.109	2.45
1. random	1.005	2.184	3.74
2. random	1.019	-0.675	2.66
3. random	1.013	2.297	3.55

In conclusion, the regression curve shown in Figure 2. and the first row of Table III. support that eq. (4) adequately describes the multistep gradient migration. This means that our hypotheses and simplifications are acceptable. The regression curves and the numerical data (Figures 3-5. and rows 2-4. of Table 3.) of the randomly selected experimental results show that multistep gradient separation can be planned by our method.

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SOME PROBLEMS OF OPTIMIZATION IN PREPARATIVE LIQUID CHROMATOGRAPHY

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SUMMARY

Equilibrium sandwich chamber for continuous thin-layer chromatography was used to study some problems of the optimization of preparative liquid chromatography. The effect of eluent concentration, sample volume and its concentration on the separation yield was investigated and compared with liquid column chromatography. The dynamic model of migration of the zones of a coloured test mixture and of the extract of the fruits of some Umbelliferae plants was determined.

INTRODUCTION

Thin-layer chromatography (TLC) is frequently used as a pilot technique for liquid column chromatography /1/. A number of authors (e.g. /2, 3/) reported a close analogy between the two techniques. Especially good correlations of the R_M vs. log k' values are obtained for an equilibrium sandwich TLC chamber with glass distributer /4/ constructed by Soczewinski as a modification of the Brenner-Niederwieser chamber /5-7/. The design of the chamber permits the use of continuous elution, spotting the sample behind the solvent front under equilibrium conditions, an easy change of the eluent during the elution process and zonal application of the sample from the edge of the adsorbent layer (frontal chromatography stage). The last feature of the chamber is especially advantageous for the introduction of large sample volumes for separations in the milligram scale as well as in the preliminary optimization of solvent-adsorbent systems for preparative column liquid chroma-

tography. The observed deviations from full analogy between TLC and HPLC are caused by differences in efficiency, solvent flow rates, adsorbent activities and adsorbent-solvent weight/volume ratios; in overloaded systems these differences have particular importance. In TLC relatively slow solvent flows (30-60 min for 20 cm length) lead to an increased spreading of the zones; on the other hand, it is presumably advantageous for mutual displacement effects. The possibility of observing the migration and separation of zones during the elution process in the horizontal sandwich chamber has been utilized for the investigation of the overloaded systems from the formation of the starting band; the effect of the sample volume and concentration, as well as of the qualitative and quantitative composition of the eluent on the dimension of the starting zone and the migration of zones has been investigated. The investigations had also a practical application for the optimization of the chromatographic systems for the preparative separation and isolation of biologically active components from the fruits of some plants belonging to the Umbelliferae family.

EXPERIMENTAL

Silica gel Si 60 and alumina Type E (E.Merck, Darmstadt, FRG) were used as the adsorbents. 0.5 mm layers of the adsorbents on 200 x 100 x 1.3 mm glass plates were prepared using a Jobling-Quickfit spreader, and dried and stored in the standard way. The plates were prepared before sampling and development as described in our earlier paper /8/. In this way the parallel migration of the marker and the zones could be observed and recorded on a transparent foil, thus obtaining a dynamic picture of the migration and separation of the zones. In column experiments a Lobar A type glass column (E.Merck, Darmstadt, FRG) was used. The eluent was delivered from a home-made gas pump equipped with antidiffusion plates and yielding 1.8 - 3.8 ml/min at pressures of 0.4 - 0.6 MPa. The chromatograms were recorded on a TZ 4100 recorder (Czechoslovakia) using a Spekol photometer (Zeiss-Jena) with a flow cell (at 490 or 620 nm) or a home-made UV-detector operated at 254 nm

wavelength. The sample was injected from a calibrated syringe by the stop-flow method. In the TLC experiments binary mixtures were employed as the eluents, consisting of n-heptane and the following solvent modifiers: methyl-ethyl ketone, ethyl acetate, diisopropyl ether or dichloromethane; we also employed ternary mixtures of acetonitrile (1.5 and 3 % v/v) or diisopropyl ether (2-3 % v/v) in a 70:30 (v/v) dichloromethane - n-heptane solution. The ternary eluents were used in liquid column chromatography. In preliminary experiments some coloured substances such as azo- and anthraquinone dyes and aromatic nitro derivatives were used as the model test compounds; in addition, the extracts of Archangelica off. were separated by both column and preparative TLC methods.

RESULTS AND DISCUSSION

For the optimization of the adsorbent - eluent systems used for preparative separations the data of analytical TLC were utilized, usually in the form of the plots of R_F (or R_M) vs. the concentration of the polar modifier in binary solvents (C_{mod}). Such a relationship is shown in Fig. 1; it can be seen that solutes 2, 4 and 5 can be easily separated using silica and 10 % or 20 % (v/v) methyl-ethyl ketone in heptane. For a 10 % solution of methyl-ethyl ketone the respective relative retention values are: $\alpha_{5,4} = 3.29$ and $\alpha_{4,2} = 5.46$. Higher concentrations of ketone are already not suitable (too weak retention). On the other hand, the peak pairs of 1 + 2 and 3 + 4 are not separated in this system.

In order to specify the sample capacity, zonal chromatography was carried out for the selected system, applying increasing sample volumes of known concentration (cf. Ref. /8/, Fig. 2). The limiting "overloading" is determined by the width of the starting band and the spreading of the zones which, in turn, depend on the volume of the sample, its concentration and on the polarity of the solvent of the sample. The preliminary equilibration of the system (application on the wet layer) eliminates the effect of solvent demixing, especially for complex eluents (cf. Fig. 2).

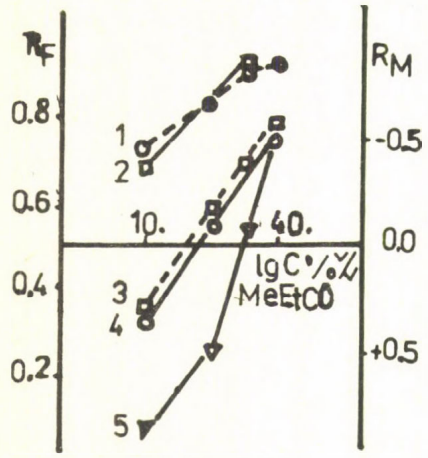

Fig.1. R_M vs. log C_{mod} plots of coloured test substances. Adsorbent: silica gel. Mobile phase: methyl-ethyl ketone (MeEtCO) - heptane. Compounds: 1. N,N-dimethylamino-azo-benzene; 2. 2-hydroxynaphthalene-1-azo-(4'-chlorobenzene); 3. 2-nitroaniline; 4. 1-aminoanthraquinone; 5. 2-amino-5-nitrotoluene.

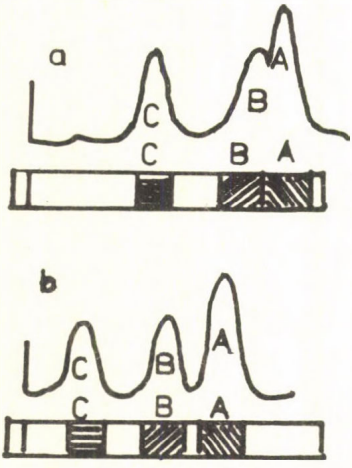

Fig.2. The demixing effect of the eluent (1:1 dichloromethane - heptane). Development conditions: a - nonequilibrated; b - equilibrated. The concentration profiles of the dyes recorded on the ERI 65 densitometer and their zonal chromatograms are: A: 4-N,N-dimethylamino-azo-(4'-chloro-benzene), B: Fatty green, C: 2-hydroxy-naphthalene-1-azo-(3'-methoxybenzene).

Fig. 3 compares the behaviour of the zones of the three coloured compounds in elution with 10 % and 20 % solutions of methyl-ethyl ketone in heptane; 0.8 cm^3 of a 0.01 M solution was applied ($8 \cdot 10^{-6}$ M of each compound, i.e., ca. 6 mg). It follows from the figure that for a 10 % ketone solution the sample size can be increased to 1.5 cm^3 of a 0.01 M solution, which corresponds to 10-15 mg of each component per one gram of the adsorbent. A more polar (20 %) eluent causes faster migration of the zones (higher R_F values, weaker retention) which are narrower; the duration of the development is decreased at the cost of a decrease in the limiting sample size. The situation is similar for alumina used as the adsorbent (Fig. 3 c, d), however, due to its smaller specific surface area the limiting capacity of the system is lower. Nevertheless, alumina can be used for the isolation of solutes capable of stronger specific interactions with its surface sites.

Our earlier paper /8/ illustrated that it is advantageous for the separation to form a narrow starting band; the effect of dilution of the sample on the dimension of the starting band and its migration is illustrated in Fig. 4, in which the behaviour of three dyes under various application conditions and development with 1 + 1 dichloromethane-heptane eluent is represented.

The application of larger sample volume (Fig. 4b) - the sample diluted with the eluent - caused considerable widening of the starting band and of the resulting separated zones. However, dilution of the sample with heptane (Fig. 4c) was favourable: the starting band was narrower. Some decrease in the eluent strength was observed, since introduction of 1 cm^3 sample containing 0.9 cm^3 of heptane caused the dilution of the eluent and formation of a gradient of the mobile phase (the interstitial volume for a 75 x 100 x 0.5 mm layer is ca. 2 cm^3). The effect is less significant in column chromatography: the interstitial volume of the Lobar A column is ca. 14 cm^3; Figs 5a-c show the corresponding chromatograms.

In thin-layer chromatography the dilution of the sample with a non-polar solvent is sometimes impossible because of the possibility of precipitation of the sample components.

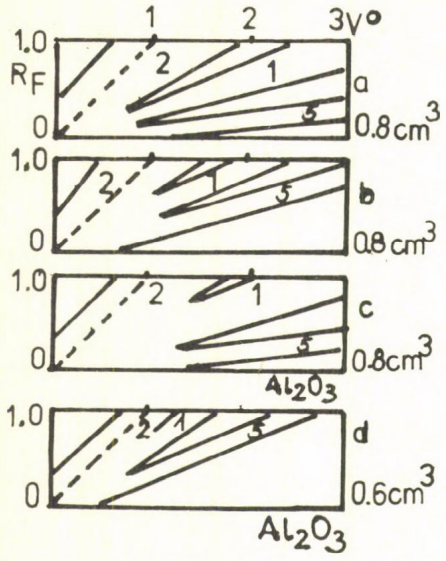

Fig.3. The concentration effect of the polar solvent in the eluent on the formation and migration of the zones of the test substances; for notation see Fig. 1. Adsorbent: (a) and (b) silica gel; (c) and (d) alumina. Mobile phase: methyl-ethyl ketone - heptane; (a) and (c) 10:90; (b) and (d) 20:80.

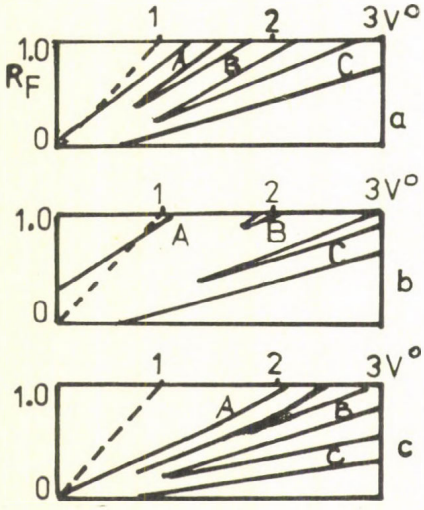

Fig.4. Effect of sample dilution on the formation and migration of zones of the dyes; for notation see Fig.2. Sample: (a) 0.1 ml of a 0.02 M solution of each dye; (b) 1 ml of a 0.002 M solution of each dye, dilution with the eluent; (c) 1 ml of a 0.002 M solution of each dye, dilution with n-heptane.

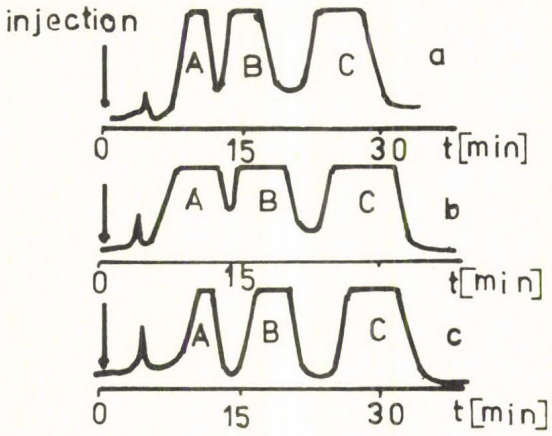

Fig.5. Effect of sample dilution on column separation. Lobar A column. Eluent: 1:1 dichloromethane-heptane. Flow rate 3 ml·min^{-1}, pressure 0.6 MPa. Detection at 490 and 620 nm. Figs a, b, c-sample as in Fig.4.

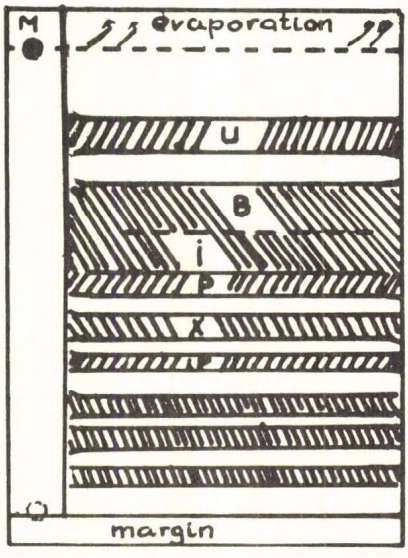

Fig.6

Fig.6. Zonal TLC chromatogram of an extract of Archangelica off. fruits on silica gel. Eluent: 2.5:70:27.5 diisopropyl ether - dichloromethane - n-heptane. Compounds: U-umbeliprenin; B-bergaptene: I-imperatorin; P-pimpinellin; X-xanthotoxin; iP-isopimpinellin.

However, the sandwich chamber permits the focusing of the starting band to a narrow zone /9, 10/ by elution with a strong, volatile eluent under the small cover plate. The preconcentrated narrow zone formed beyond the cover plate due to evaporation of the solvent is then developed with a suitable eluent. This modification of zonal application and elution in the sandwich chamber was applied for the separation and isolation in the milligram range of some biologically active furocoumarins and coumarins from the fruit of Archangelica off. A wide starting band formed after the application of 1 cm^3 of a 0.5 % solution was preconcentrated by elution with acetone and then developed in a continuous elution (one and a half of the interstitial volume) with a weakly polar ternary eluent (2.5:70:27.5 diisopropyl ether - dichloromethane - n-heptane) (Fig. 6).

The composition of the eluent had been chosen after analyzing the relationship between the R_M values and the logarithm of the concentration of diisopropyl ether in dichloromethane for standard furocoumarins samples (Fig. 7). The optimum range of R_F values corresponded to 2.5 % diisopropyl ether. For preparative chromatography dichloromethane was diluted with heptane to form a 70 % solution. As shown by Perry /11/ and Soczewinski /12/ shorter development distance combined with the use of weaker eluent and continuous elution with several interstitial volumes of the mobile phase improves the separation and detection and decreases zone spreading. It can be seen from Fig. 6 which represents the chromatogram of the extract of Archangelica off. fruits that the minor compounds are well separated while bergaptene and imperatorin (40 % content in the extract) form a single, partly separated zone (differences in fluorescence at the boundary of the two zones). The whole combined zone of these two compounds was removed from the plate and their extracts identified by parallel chromatography with the standards. In this way pure components and mixed binary fractions were obtained.

Another system chosen from the TLC data (1.5:70:28.5 acetonitrile - dichloromethane - heptane) was applied for column chromatography using a Lobar A column (Fig. 8a). The

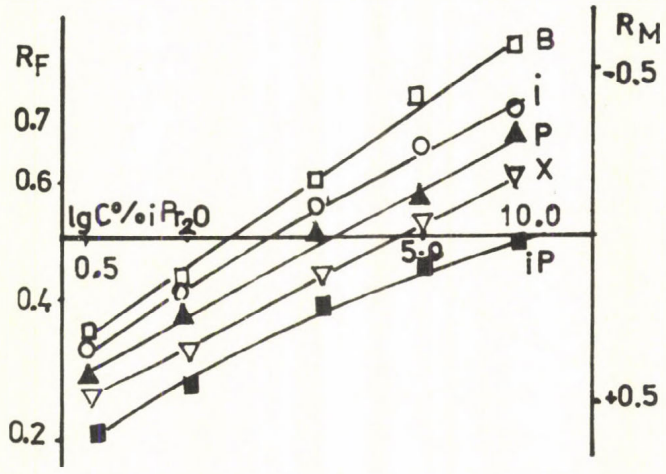

Fig.7. R_M vs. log C_{mod} plots of standards of furocoumarins; I-imperatorin, B-bergaptene, X-xanthotoxin, iP-isopimpinellin, P-pimpinellin, system: isopropyl ether + CH_2CL_2/silica gel.

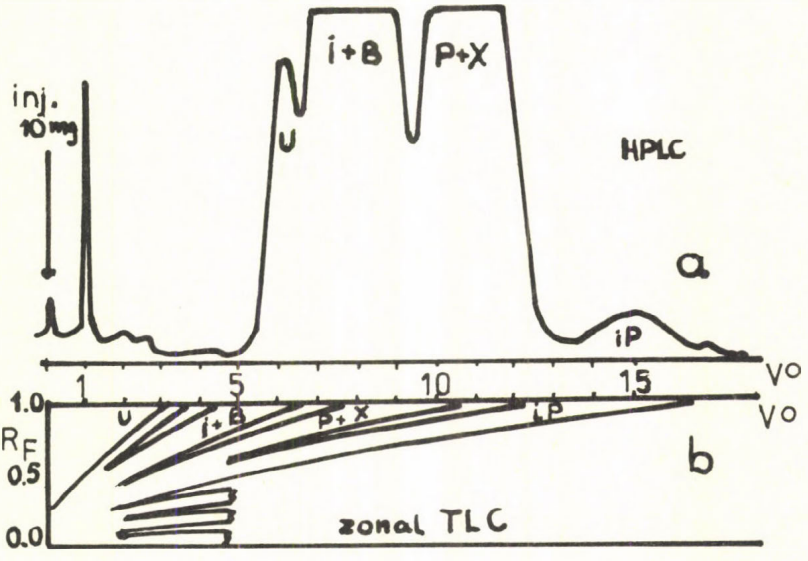

Fig. 8. Comparison of zonal TLC chromatogram of the coumarin fraction and the peaks obtained in column separation: (a) - Lobar A column: 0.6 MPa; 3.2 ml/min; (b) - silica gel plate 200 x 200 x 0.5 mm. Eluent: 1:5:70:28.5 acetonitrile dichloromethane - heptane.

peaks were compared to a zonal TLC chromatogram (Fig. 8b) in which the interstitial volumes are expressed as multiples of the column void volumes. The TLC zones observed under UV light (366 nm) were recorded on a transparent foil for four interstitial volumes and their further movement was extrapolated to the void volume axis (determined by the migration of azulane used as the marker) (Fig. 8b). The dynamic diagram of zone migration is similar to the column data. Partial separation of imperatorin, bergaptene and pimpinellin was obtained: however, collection of narrower fractions of the eluate permitted the isolation in milligram quantities of pure components, due to their mutual displacement.

The results obtained indicate that the analogy of TLC and column chromatography data is quite good, also for preparative, overloaded systems. The equilibrium sandwich chamber which permits the use of continuous elution, equilibration of the system and observation of band formation during the development process was found to be especially useful for the optimization of adsorbent-solvent systems for preparative chromatography.

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EVALUATION OF THE SEPARATION OBTAINED IN THIN-LAYER CHROMATOGRAPHIC SYSTEMS USING BINARY AND TERNARY MOBILE PHASES

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Adsorption thin-layer chromatography (TLC) is widely used for the separation of homogenous mixtures. Because of its simplicity and the relatively low apparatus cost it may serve also as a pilot technique for column liquid chromatography and for investigating certain theoretical considerations of the chromatographic process.

Until recently, a series of chromatographic systems was investigated either by thin-layer chromatography or column chromatography. A better possibility for the optimization of the separation conditions is the application of mixed mobile phases. By the appropriate choice of the mobile phase composition the chromatographic process can be optimized with respect to analysis time and resolution.

In the last years chromatographic systems with ternary mobile mobile phase became widely used. Although the chromatographic process becomes more complicated when using a ternary mobile phase, the investigation of such systems represents an important step in the development and improvement of the chromatographic technique. Their use permits the more precise quantitive evaluation of the effects related to the energetic heterogeneity of the adsorbent surface, the mobile phase and the surface phase and permitting an improvement in the separation of solutes especially those which differ only slightly in their capacity ratio (k') values /1/.

This paper represents an attempt toward the application of a simple graphic method for the evaluation and comparison

of separation in chromatographic systems containing binary and ternary mobile phases. The main purpose of our investigations was to establish the optimum mobile phase composition for the chromatographic separation of inactive substances.

The investigations were carried out by adsorption thin-layer chromatography. The measurements were carried out at 20 $\pm$ 1°C. Silica gel Merck 60 G and neutral aluminium oxide Merck 60 T were used as the adsorbents. The following solvents were used as the mobile phase: carbon tetrachloride, benzene, dioxane, acetone, methylethyl ketone, chloroform, methanol and their binary and ternary mixtures. The solvents were dried on wide-porous silica gel calcinated at 180°C for seven days prior to the chromatographic measurements. Mixtures of polynuclear aromatic cyclic hydrocarbons were used as the sample in the form of their 3 % solution in benzene or acetone.

The selection of the solvents was carried on the basis of the classification of Pimentel and McClellan /2/ according to their ability for hydrogen bond formation. Chromatographic measurements were carried out using two pure /3/ solvents, more and less polar, and their mixtures with a concentration of 0.1, 0.3, 0.5, 0.7 and 0.9 molar fraction. Ternary mobile phases were prepared in the following way: two less polar solvents were mixed in 1:1 proportion and a third, more polar solvent was added to this mixture in 0.1, 0.3, 0.5, 0.7 and 0.9 mole fraction. All chromatograms were developed at a distance of 16 cm. The results obtained are presented graphically, by plotting the ΔR_F values against the mobile phase composition. The resolution of two chromatographed substances is defined by the following equation /5/:

$$R_s = \frac{2(L_1 - L_2)}{(w_1 + w_2)} \qquad (1)$$

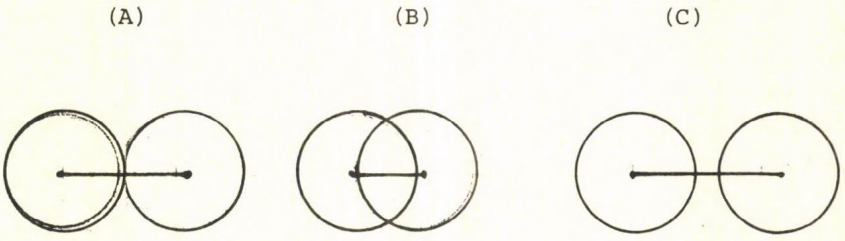
where

$$R_{F_1} = \frac{L_1}{L}, \quad R_{F_2} = \frac{L_2}{L} \qquad (2)$$

R_{F_1}, R_{F_2} represent the R_F value of chromatographed substance 1 and 2 in a given mobile phase; L is the migration length of the solvent front; L_1 and L_2 are the migration length of substance 1 and 2 respectively; and w_1 and w_2 are the widths of the chromatographic spots.

The chromatographed substances were spotted with a calibrated micropipette and the analytical conditions were selected so that the diameter of the developed spots shall be as small as possible. Therefore, we could assume that the widths of the formed spots are $w_1 = w_2 = w = 0.7$ cm = const. The mobile phase migration distance was always 16 cm. Substances from groups N and AB of the classifiation of Pimentel and McClellan were selected as sample components /2/.

The R_s values can be determined visually, directly from the chromatograms without any calculation. The separation of a substance pair graphic method can be presented in the following way:



This illustration makes it obvious that the minimum acceptable R_s value is equal to unity and the optimum values are in the range of 1-2. In the case of higher R_s values the chromatographic separation is indeed satisfactory but the analysis time is too long.

Eq. 1 can be modified in the following way:

$$R_s = \frac{2(L_1 - L_2)}{(w_1 + w_2)} = \frac{2L(\frac{L_1}{L} - \frac{L_2}{L})}{w} = \frac{L(R_{F1} - R_{F2})}{w}$$

If $R_{F1} - R_{F2} = \Delta R_{F(1,2)}$, then

$$\Delta R_{F(1,2)} = \frac{R_s \cdot w}{L} \qquad (3)$$

If $w_1 = w_2 = w = 0.7$ cm, and **L** = 16 cm, then, assuming that $R_{s(min)} = 1.0$, we **obtain:**

$$\Delta R_{F(1,2)min.} = \frac{1 \cdot 0.7}{16} = 0.04$$

$$\Delta R_{F(1,2)opt.} = \frac{2 \cdot 0.7}{16} \approx 0.1$$

On the basis of this calculation the mobile phase composition corresponding to the optimum separation range can be easily established graphically, as shown in Figure 1. Here the mobile phase compositions between A and B correspond to the optimum separation range.

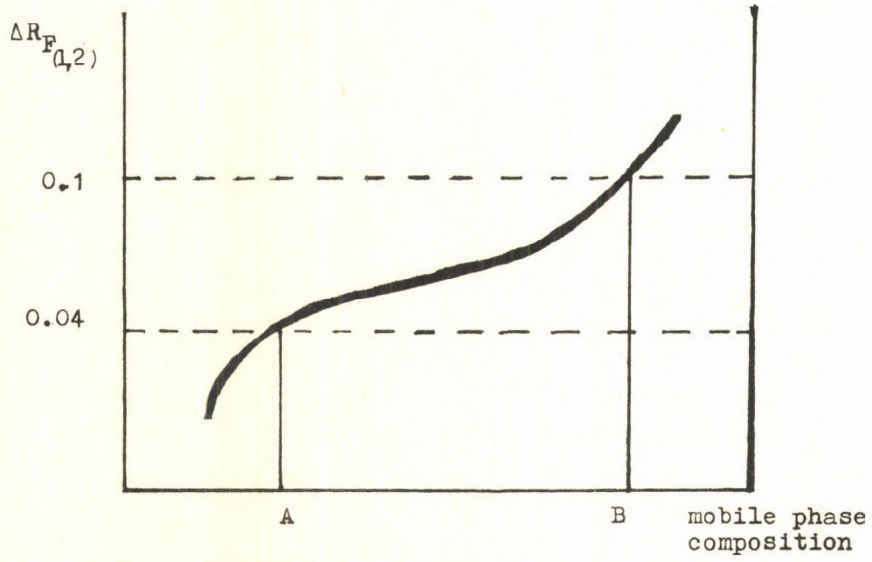


Fig. 1. Establishment of the mobile phase composition corresponding to the optimum separation range

Based on our measurements, Figs 2-10 present the plots illustrating the dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition on two adsorbents, utilizing diferent binary and ternary mobile phases.

In the case of binary mobile phase systems such as e.g. carbon tetrachloride-benzene or carbon tetrachloride-chloroform, it was not possible to achieve the desired separation range (ΔR_F = 0.04-0.1): either the resolution was insufficient (Figs 2, 3) or the $\Delta R_{F(1,2)}$ value was too large, about 0.4, indicating long analysis times (Figs 4, 5).

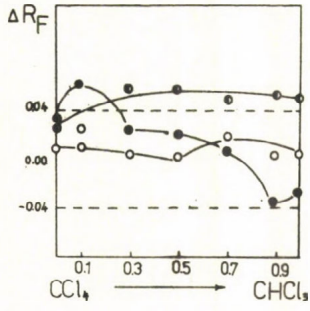


Fig. 2. The dependence of $R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-chloroform binary mobile phase. Adsorbent: SiO_2 60 G. Substance pairs: ● naphthalene-anthracene, o anthracene-chrysene, ◐ pyrene-fluoranthene.

Addition of a third, active component to the mobile phase results in a well-defined concentration range corresponding to the ΔR_F range of 0.04-0.1. This concentration range corresponds to 0.3-0.5 mole fraction of the third component (methyl ethyl ketone or dioxane) in 1:1 carbon tetrachloride-chloroform or carbon tetrachloride-benzene (Figs 6, 7), and to 0.3-0.7 mole fraction of the third component (acetone, dioxane or methyl ethyl ketone) in 1:1 carbon tetrachloride-chloroform or carbon tetrachloride-benzene systems (Figs 8, 9, 10).

The best separations of polynuclear aromatic hydrocarbon pairs were obtained on silica gel in systems containing methyl ethyl ketone, carbon tetrachloride and benzene, and on alumina in systems containing acetone, carbon tetrachloride and chloroform. Thus, an increase of the interactions between the active

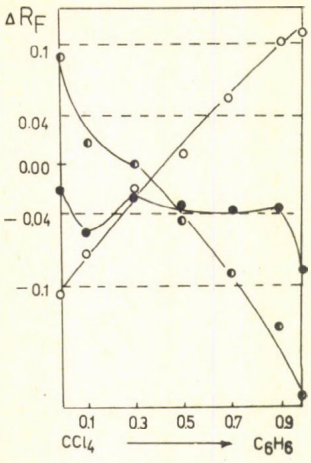


Fig.3. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-benzene binary mobile phase. Adsorbent : Al_2O_3 60 T. Substance pairs: ● naphthalene-anthracene, o anthracene-chrysene, ◐ pyrene-fluorenone.

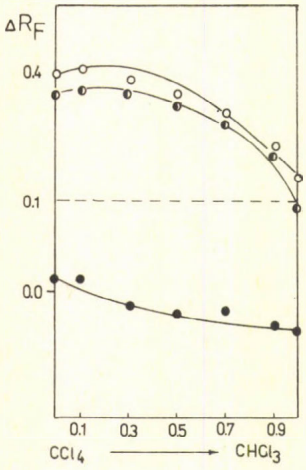


Fig.4. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-chloroform binary mobile phase. Adsorbent: SiO_2 60 G. Substance pairs: ● anthracene-pyrene, o anthracene-fluorenone, ◐ pyrene-fluorenone.

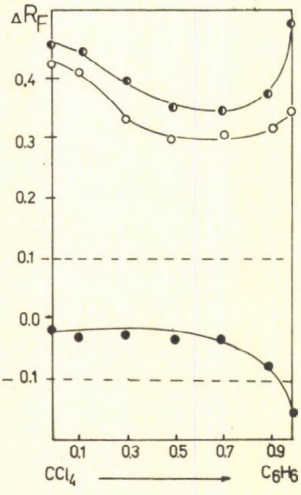


Fig.5. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-benzene binary mobile phase. Adsorbent: SiO_2 60 G. Substance pairs: ● anthracene-pyrene, o anthracene-fluorenone, ◐ pyrene-fluorenone.

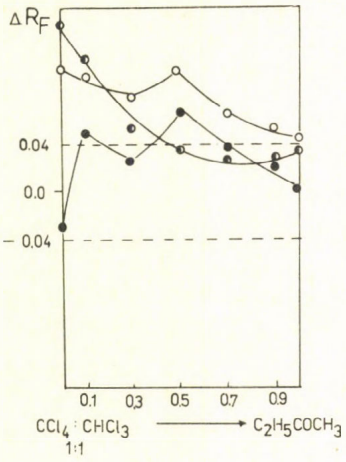


Fig.6. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-chloroform-methyl ethyl ketone ternary mobile phase. Adsorbent: SiO_2 60 G. Substance pairs: ● naphthalene-anthracene, o anthracene-chrysene, ◐ pyrene-fluoranthene

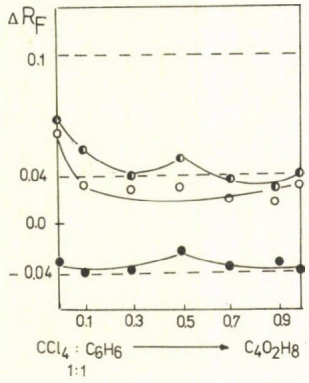


Fig. 7. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-benzene-dioxane ternary mobile phase. Adsorbent: Al_2O_3 60 T neutral. Substance pairs: ● anthracene-pyrene, o anthracene-fluorenone, ◐ pyrene-fluorenone.

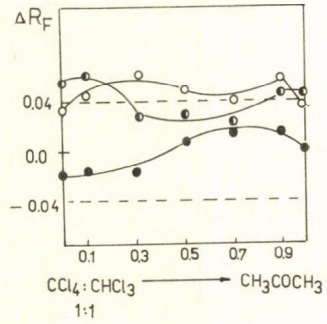


Fig. 8. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-chloroform-acetone ternary mobile phase. Adsorbent: Al_2O_3 60 T neutral. Substance pairs: ● anthracene-pyrene, o nathracene-fluorenone, ◐ pyrene-fluorenone.

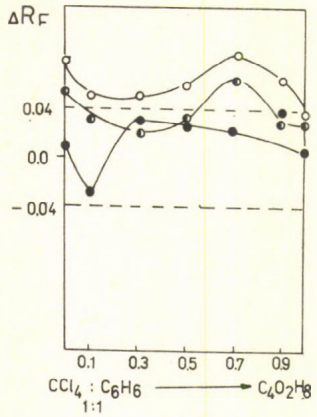


Fig.9. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-benzene-dioxane ternary mobile phase. Adsorbent: SiO_2 60 G. Substance pairs: ●naphthalene-anthracene, o anthracene-chrysene, ◐ pyrene-fluoranthene.

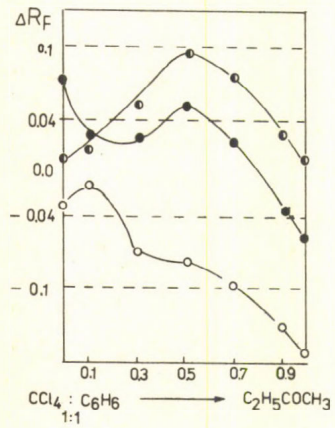


Fig.10. The dependence of $\Delta R_{F(1,2)}$ on the mobile phase composition for carbon tetrachloride-benzene-methyl ethyl ketone ternary mobile phase. Adsorbent: SiO_2 60 G. Substance pairs: ● naphthalene-anthracene, o anthracene-chrysene, ◐ pyrene-fluoranthene.

mobile phase component and the adsorbent, and the localization of this component was again confirmed /6/.

The results presented here suggest that ternary mobile phases can in some instances be useful for chromatographic separations.

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Budapest, 1986

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A LOW-COST COMPUTING INTEGRATOR SYSTEM FOR HPLC

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Data-processing system comprises an important supplementary part of modern liquid chromatographs, by means of which a substantially greater amount of more exact information can be obtained from the chromatograms. The advantages of the use of such systems are as follows:

1. The characteristic retention data can be determined more exactly than by any other method.
2. The different components can be evaluated quantitatively.
3. Overlapping peaks can be evaluated separately, by means of an appropriate software.
4. The chromatogram can be modified, e.g. a baseline shift caused by gradient elution can be corrected or solvent peaks can be eliminated.
5. Chromatograms can be stored in digital form and can be evaluated a number of times using various appropriate programs.

The integrator systems generally used are rather expensive. Our aim was to develop a cheap and simple data processing system based on a handheld computer.

INTRODUCTION

Data processing includes the calculation of peak areas, the determination of peak retention times, and the calculation of peak area ratios, as percent values.

Our aims demanded a system satisfying the following requirements.

1. The chromatogram to be evaluated should be storable in digital form for both short and long periods.
2. It should be possible to evaluate a chromatogram a number of times, from different aspects, without repeating of the measurement.
3. The baseline correction must be automatic.
4. The areas of overlapping peaks should be calculated with the lowest error, and these printed areas should be marked.
5. The evalution should extend either to the whole chromatogram or to a given section of it.
6. A result list should be given, which includes the chromatographic conditions.
7. The result list should be storable for a short period and should be repeatable a number of times.
8. The data-processing system should be cheap and applicable for other purposes, after minor modifications.

For the connection betwen the computer and the detector an interface (UNIDATA) satisfying the following requirements must be developed:

The task of the UNIDATA is to digitalize the analog signal arriving from the HPLC detector, and to forward the new signal to the HP-IL loop with the aid of a converter.

1. The sampling speed of the analog signal should be 2 or 4 samples per sec at 12-bit resolution.
2. The memory of the UNIDATA should simultaneously store 7200 data for a short period and these data should be reuseable several times (a running time of 60 or 30 min, respectively).
3. The UNIDATA should be startable by hand or automatically.
4. The measured data on the appropriate sample should be given during the measurement, and the number of samplings at the end of the read-out.
5. The UNIDATA should be stoppable or resettable during measurements.

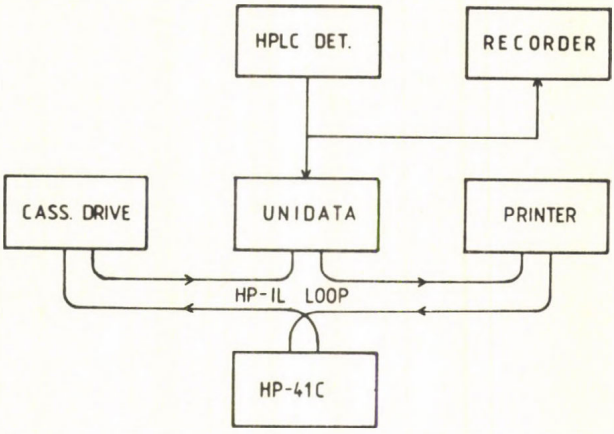

Fig. 1. The connection between the units of the evaluation system

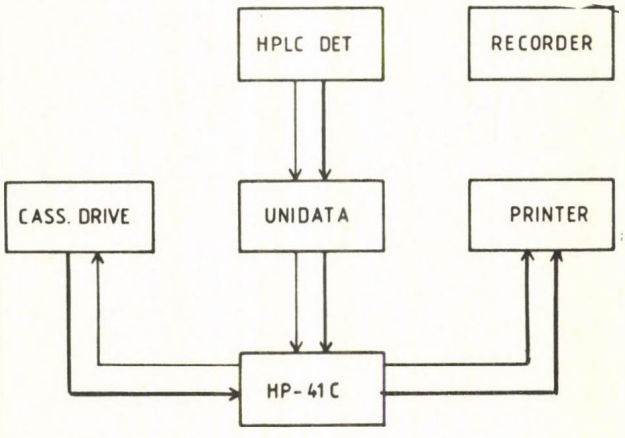

Fig. 2. Possibilites of data flow in the system.
——— : data storage
——— : direct data processing
▬▬▬ : subsequently data processing

6. The sampling signal should be shown with 4-digit "accuracy" on the UNIDATA output.

APPARATUS

Our data-processing system consisted of a Hewlett-Packard HP - 41 handheld computer coupled with an HP 82161 A digital cassette drive and an HP 82143 A thermal printer through an HP 82160 A interface loop (Figs 1 and 2).

The used UV detector was a Knauer variable-wavelength monitor coupled with a UNIDATA interface constructed in our laboratory.

THE DATA-PROCESSING; ALGORITHM OPERATION OF THE PROGRAM

The requirements for data-processing can be met only by an "adaptive" program especially in on-line operation.

Such a program analyzes the slope of the curve from point to point and, on the basis of given parameter, the time scale and logical conditions determine which part of the curve is under evaluation. The block diagram of the program is shown in Fig. 3.

The curve can be divided into the following sections (Fig. 4).

1. Standard baseline
2. Baseline shift or jump in positive direction
3. Baseline shift or jump in negative direction
4. Isolated peak
5. Overlapping peaks
6. Negative peak
7. Section not to be evaluated

Depending on the above sections of the curve, the evaluation may have four different phases:

A. No integration; only the time is calculated from the serial number of the sample and the speed, or the baseline is followed (Sections 1, 2, 3 and 6).

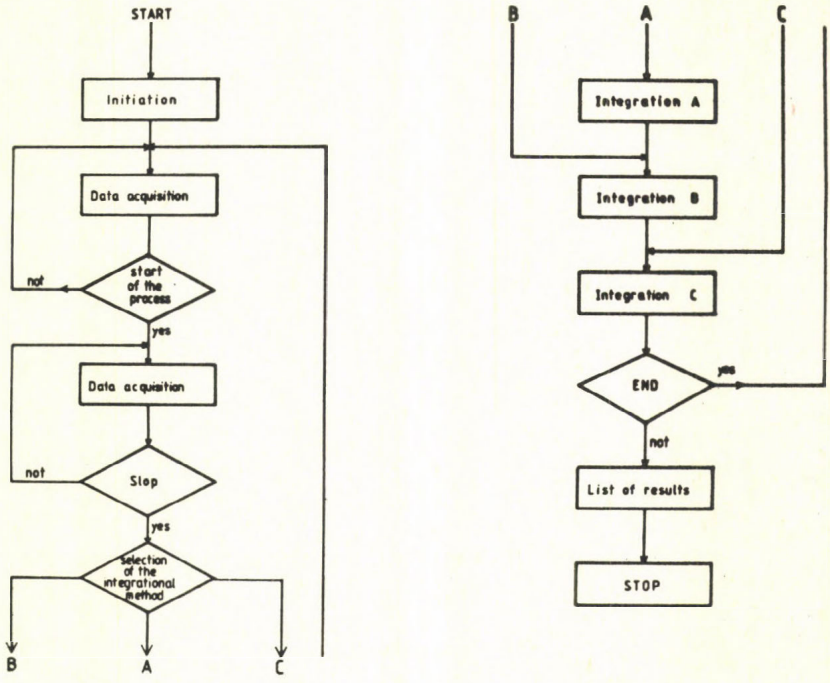

Fig. 3. The block diagram of the program

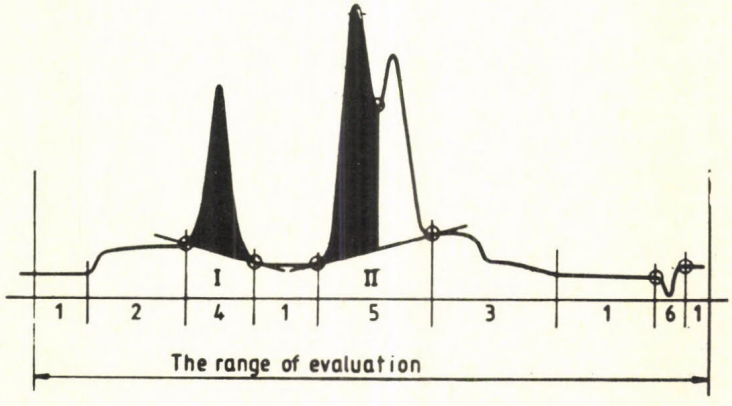

Fig. 4. The main parts of the diagram, with corrections

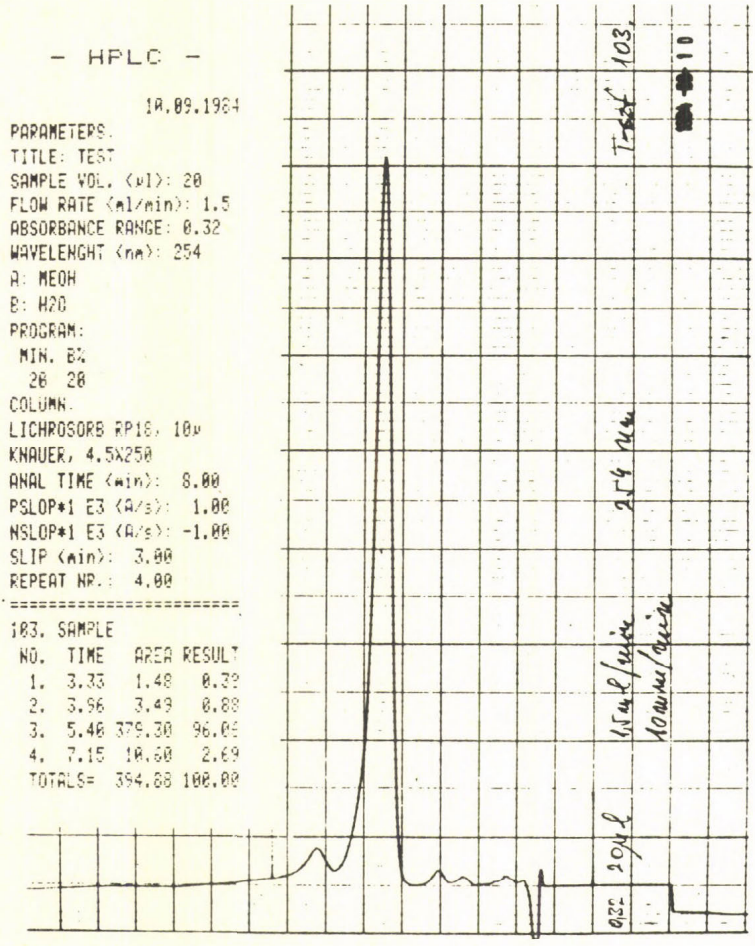

Fig. 5. Analytical HPLC chromatogram of 8-arginine-vasopressin

B. Integration, calculation and storage of retention times where the slope changes positive to negative (baseline correction (section I), and storage of the pure peak area (section 5)).

C. Integration, calculation of retention times, baseline correction, separation of peaks (section II) and storage of the pure peak area (section 5).

D. Printing of the result list, including the chromatographic conditions and the parameters of evaluation (section 7).

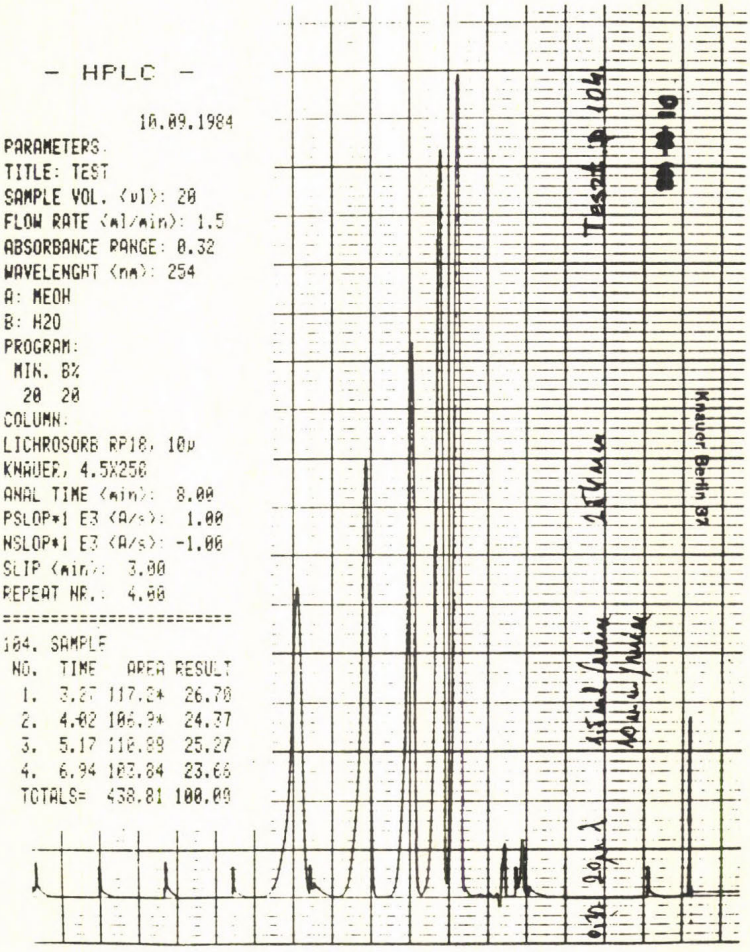

Fig. 6. Analytical HPLC chromatogram of the test mixture (methyl, ethyl, propyl, butyl, pentyl benzoates)

RESULTS

A low-cost, simple computing integrator system was developed on the basis of a Hewlett-Packard 41 CV handheld computer. Appropriate programs have been produced for the evaluation of liquid chromatograms.

These programs could be used for the following purposes:

a. the measurement of exact retention time values;

b. the determination of different peak area rations;

c. the correction of the chromatograms.

Whereas the price of the equipment commercially available is 10,000-20,000 US $ our system costs approximately 1200 $; nevertheless, it possesses all the important characteristics of the more expensive data-processing systems.

A further advantage of the new system (based on the modular construction) is that it is also applicable for essentially different tasks.

Some examples of the use of this system are presented in Figs 5 and 6.

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PRELIMINARY EXPERIENCE CONCERNING THE APPLICABILITY OF THE HIGH-SPEED SPECTROPHOTOMETRIC HPLC DETECTOR IN TOXICOLOGICAL SCREENING

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Systematic toxicological analysis is a most important part of forensic toxicology. Among the methods, used in toxicological screening, most valuable are those which can combine high detectability with high identification potentials. However, there is a lack of such methods; therefore, the combinations of various techniques are used.

An advent of the high-speed spectrophotometric detector for HPLC created a new quality in regard to identification potentials. This detector may assure a combination of two methods of identification (i.e., chromatographic mobility and UV spectrum) in one single analytical run. The method appeared very useful for screening for drugs and their metabolites, and for the analysis of unresolved HPLC peaks (1-6). The purpose of this paper is to present our first experience concerning the use of such a detector for the identification of drugs in pure form and extracted from a biological matrix.

MATERIAL AND METHODS

The investigations were carried out on methanolic solutions of amitriptyline, caffeine, chlordiazepoxide, codeine, methaqualone and strychnine. Extracts of plasma and liver homogenate, spiked with the same drugs, were also analyzed. The

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following isolation methods were applied: ultrafiltration, enzyme digestion with ultrafiltration, RP-18 column extraction, Extrelut column extraction, and chloroform extraction.

The samples were analyzed in a liquid chromatographic system consisting of a Waters HPLC M-45 Pump, a Nucleosil RP C18 column (25 cm x 4.6 mm) and a Hewlett-Packard Model 1040 A diode-array detector with an HP 82901 double-disc drive, an HP 7470 X-Y plotter and an HP-85 computer. A mixture of acetonitrile and phosphate buffer (200:340, pH = 2.8) was used as the mobile phase at a flow rate of 1 ml/min.

RESULTS

Using the HP 1040 A detector, the chromatogram can be monitored at chosen wavelengths and processed by several ways when the analysis is completed. Some examples are shown in the figures, presenting the results obtained from the analysis of the same drug mixture. The multi-signal plot (Fig. 1) may be useful for the optimization of the method and the selection of the best single-wavelength detection. The three-dimensional plot (Fig. 2) gives the insight on the UV spectra of separated peaks, whereas the isoabsorbance plot (Fig. 3) presents a "birds eye view" of the chromatogram. UV analysis of a peak which contains more than one substance may be very useful for identification; Fig. 4 shows an unresolved peak of caffeine and strychnine. UV analysis of various parts of this peak revealed remarkable differences in the UV spectra. These differences were noted when the reference absorbances were mutually subtracted (Fig. 5).

The spectrophotometric detector may be particularly useful for the assessment of the effect of a biological matrix. Figs 6 and 7 show the 3-D chromatograms of methaqualone in liver ultrafiltrate, taken at two different sensitivities, enabling the observation of the matrix peak and the drug itself. The subtraction of the background absorbance is very important for identification: this is illustrated on Fig. 8, showing the chromatogram of an RP C-18 extract of liver containing amitriptyline. The UV spectrum of the peak is uncharacteristic when

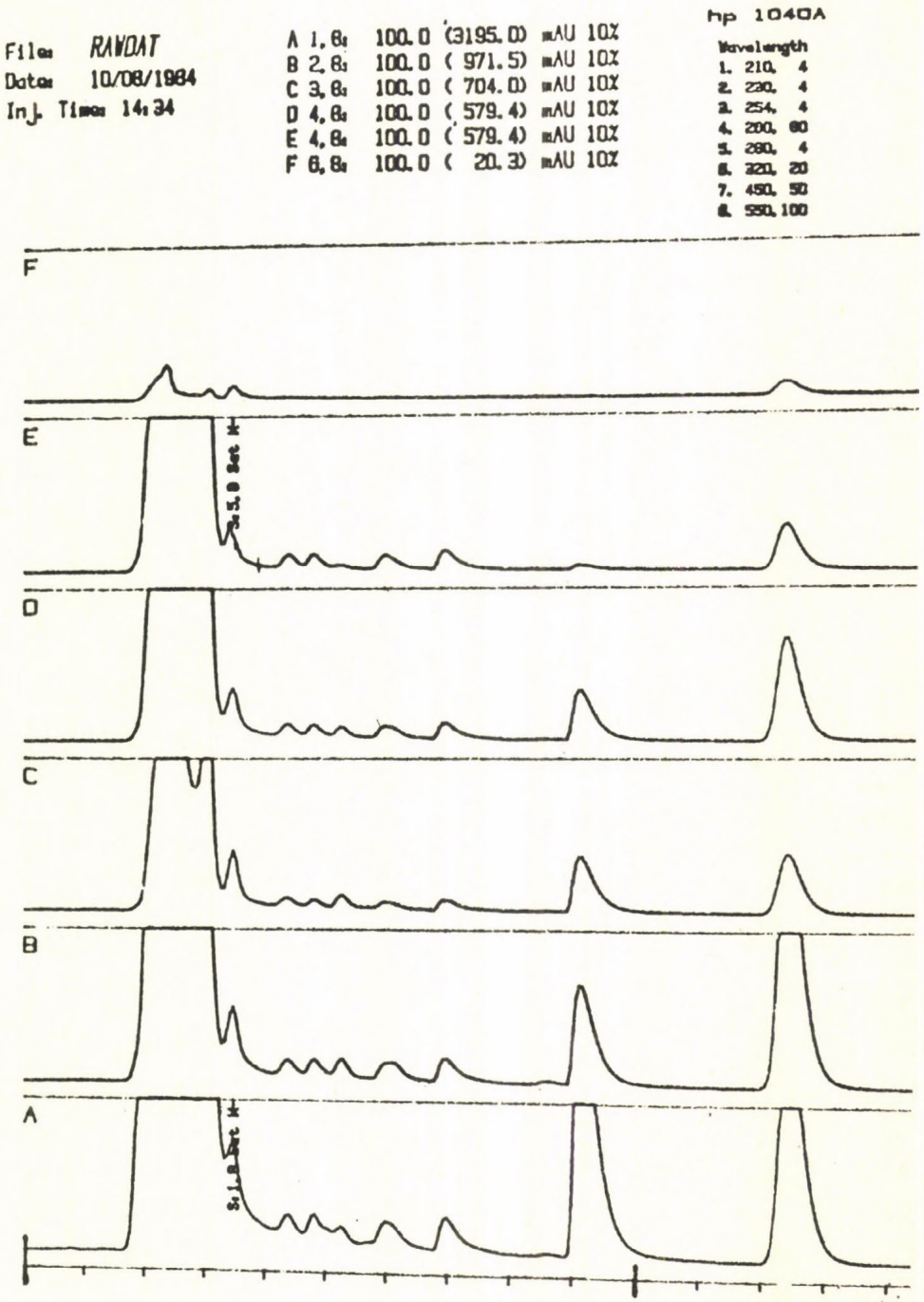

Fig. 1. Multi-signal plot of a drug mixture. Wavelengths (nm): A- 210, B- 230, C- 254, D- 260, E- 280, F- 320.

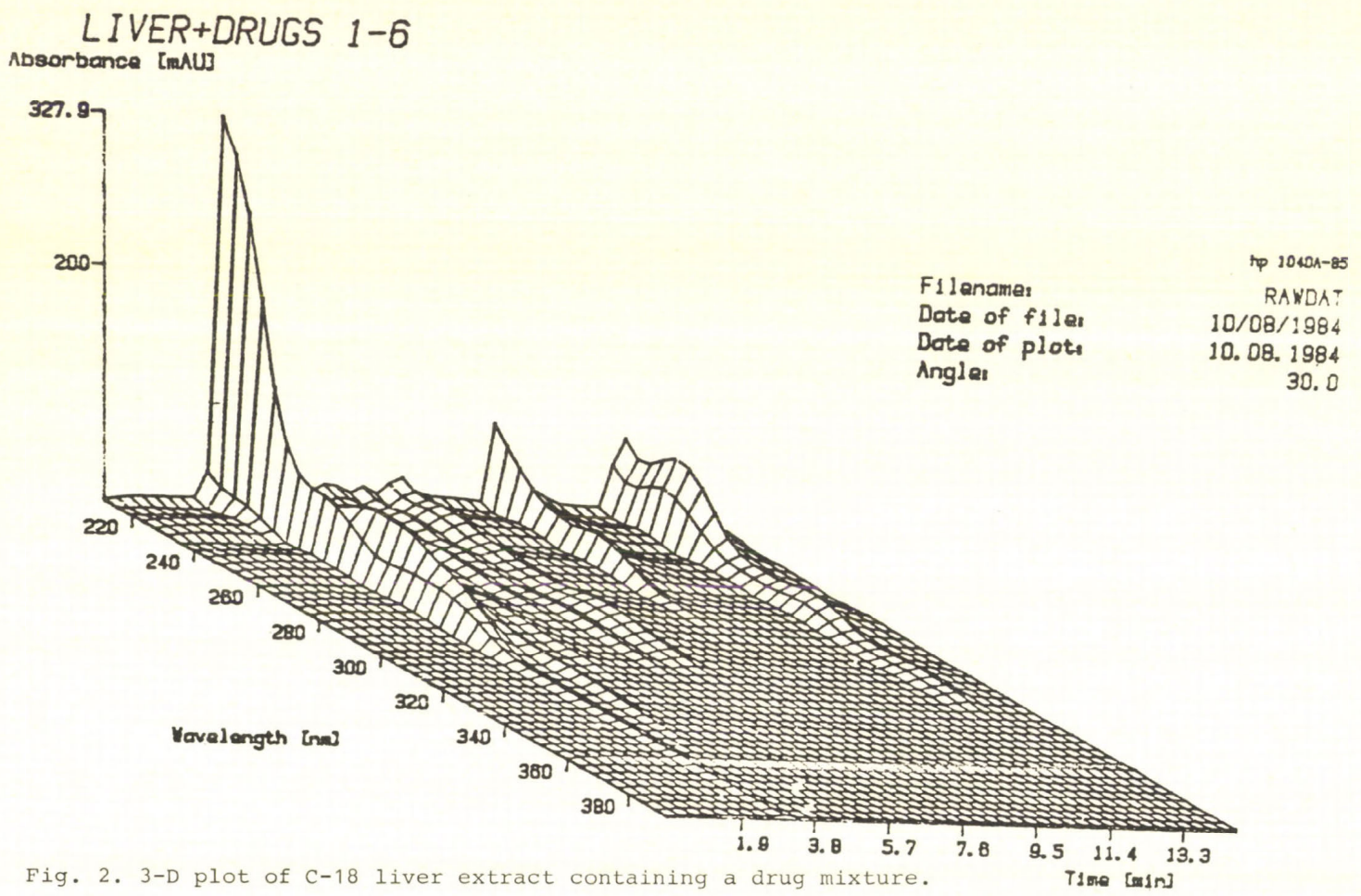

Fig. 2. 3-D plot of C-18 liver extract containing a drug mixture.

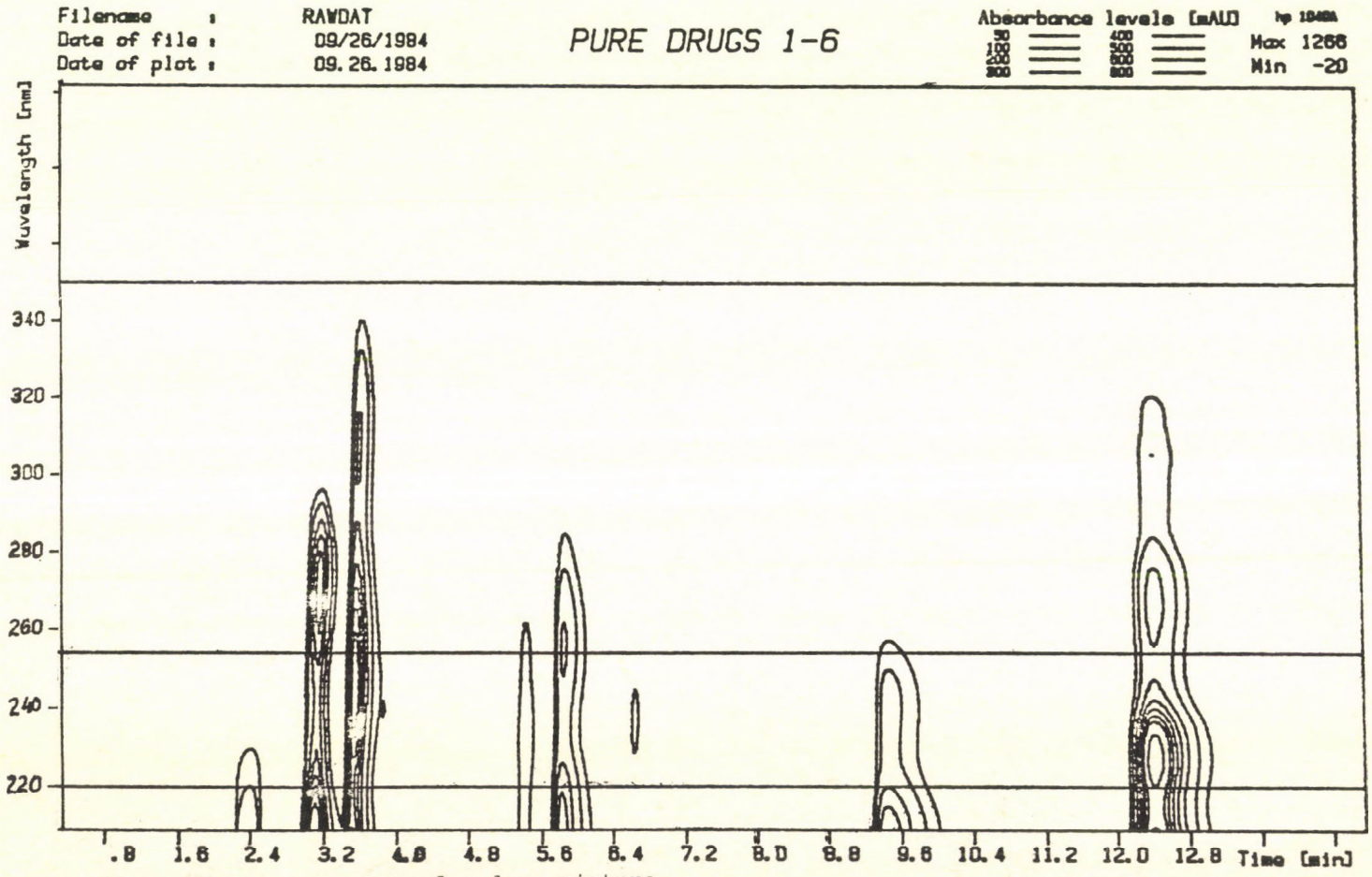

Fig. 3. Isoabsorbance plot of a drug mixture.

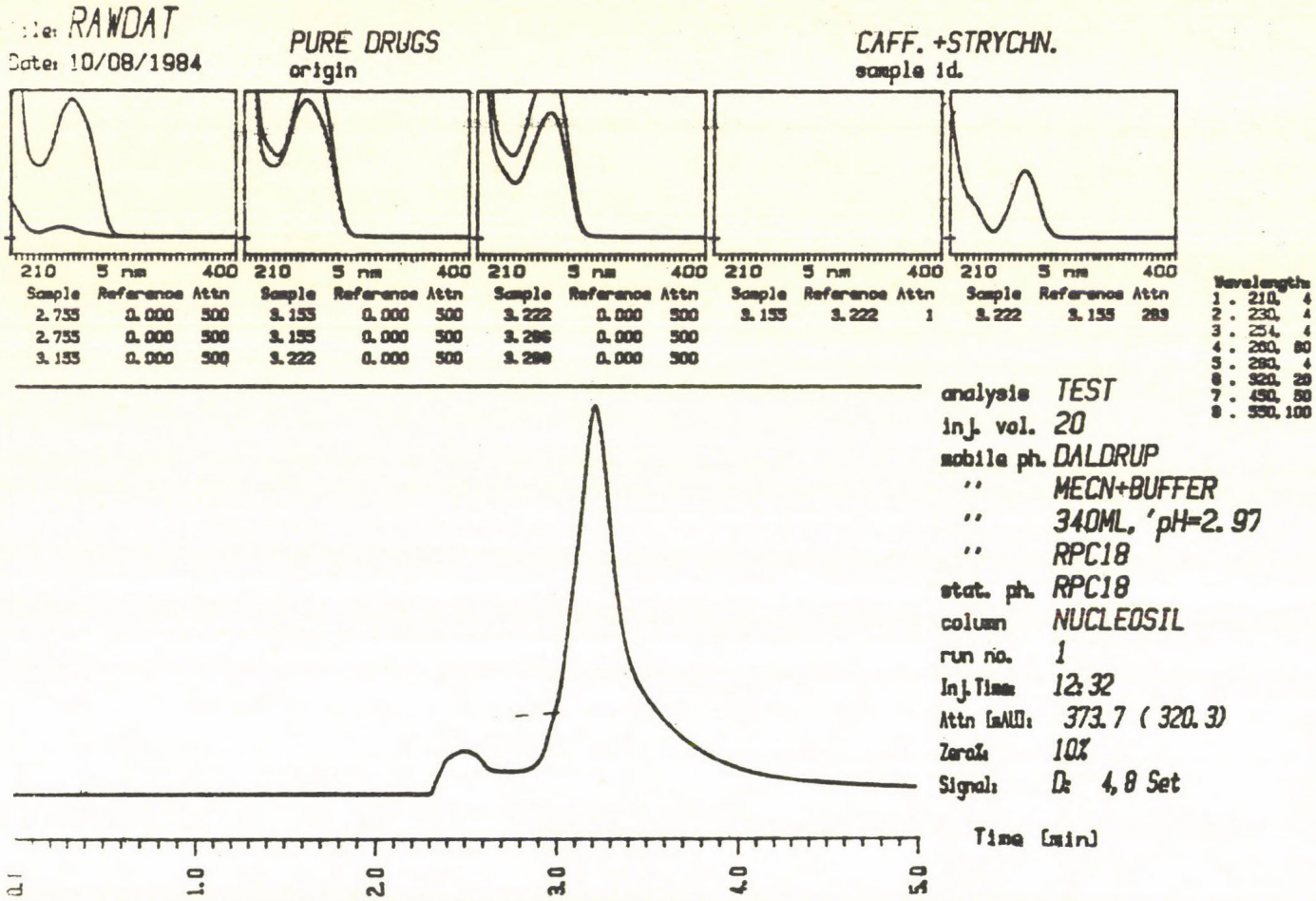

Fig. 4. Unresolved peak of caffeine and strychnine.

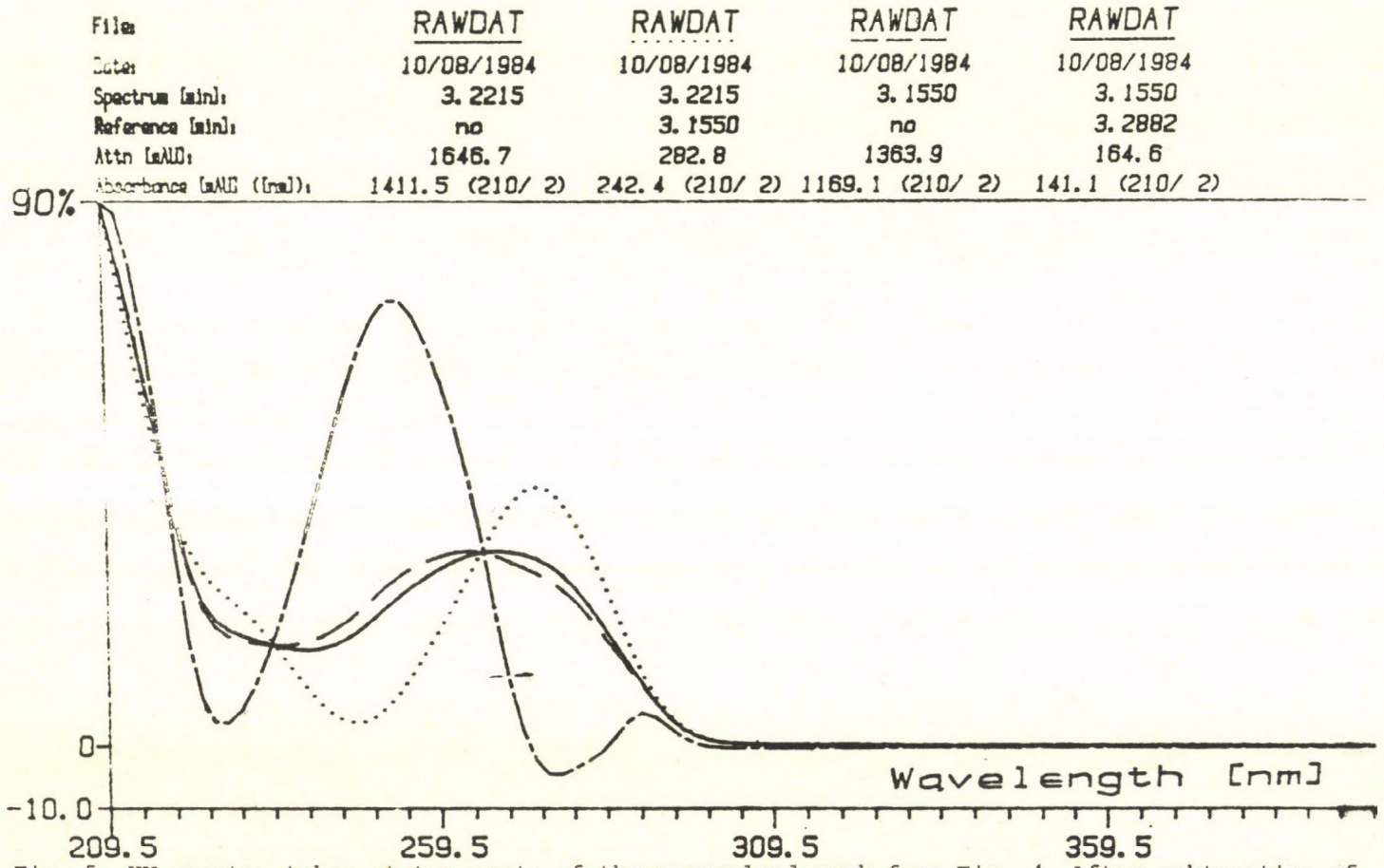

Fig. 5. UV spectra taken at two parts of the unresolved peak from Fig. 4. After subtraction of the reference background the spectra of strychnine and caffeine are clearly visible.

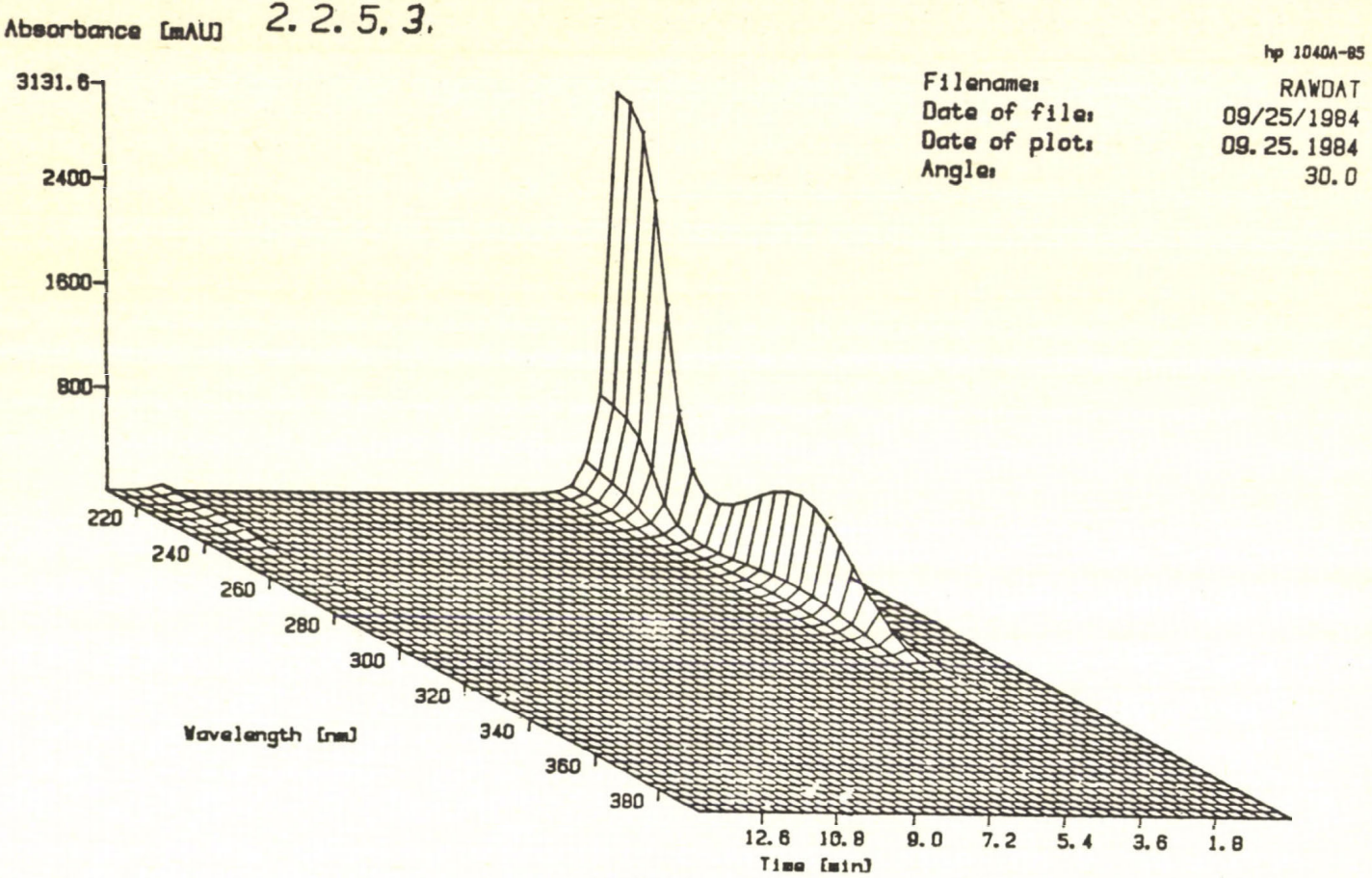

Fig. 6. Liver ultrafiltrate containing methaqualone. Automatic sensitivity scaling, showing a large matrix peak

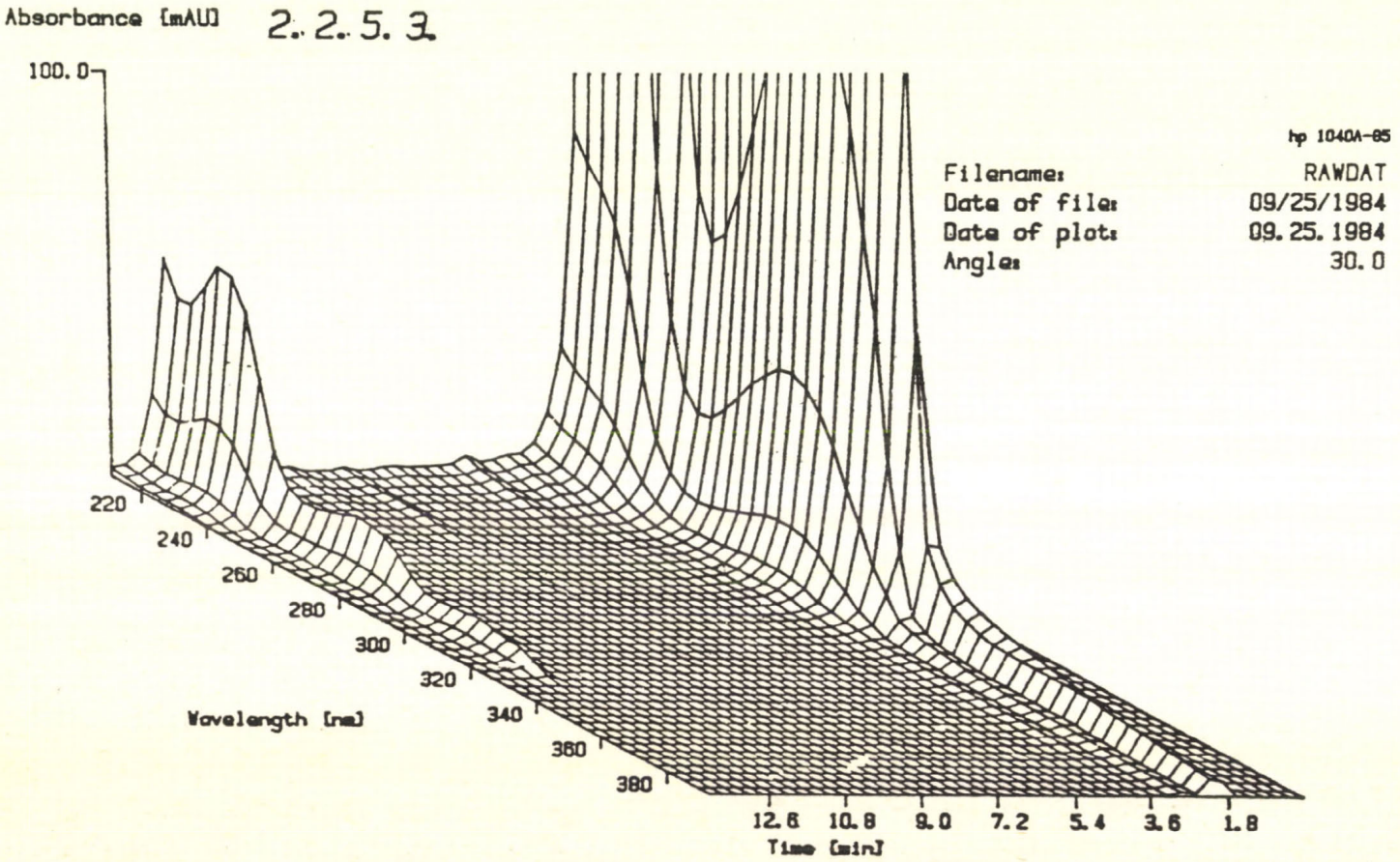

Fig. 7. The same chromatogram as in Fig. 6, plotted at a much higher sensitivity. The peak and spectrum of methaqualone is evident.

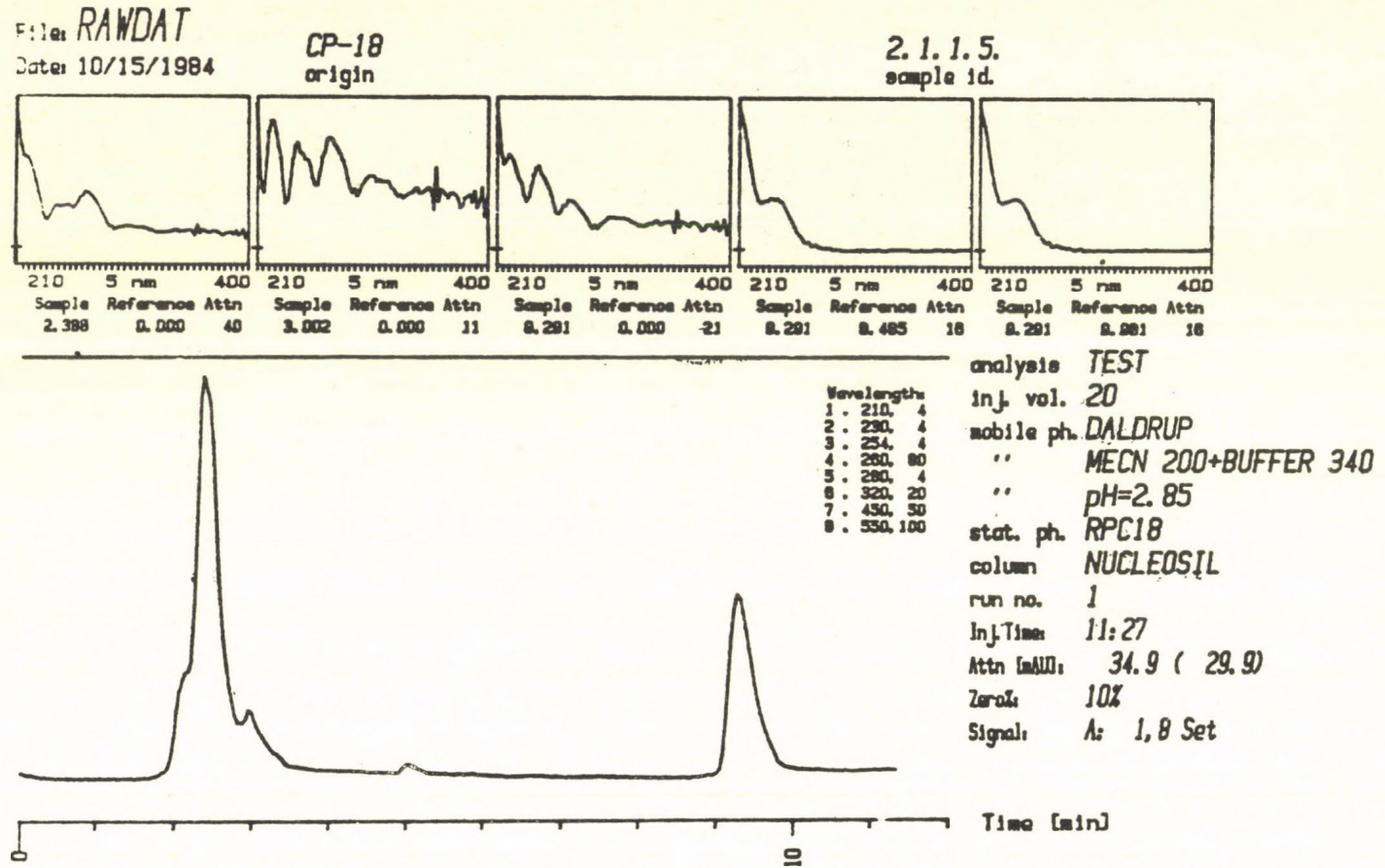

Fig. 8. Liver C-18 extract containing amitriptyline. Signal+spectra plot. Spectrum of amitriptyline is visible after subtraction of the background (spectra 4 and 5).

the background absorbance is not subtracted. After substraction, a clear spectrum of amitriptyline appeared.

CONCLUSION

HPLC with high-speed spectrophotometric detection is potentially a very powerful identification method. Full possibilities of the method, however, may be obtained only when a reliable identification system based on the retention behaviour (similar to the retention index system in GC) will be developed.

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DABSYL CHLORIDE AS A DERIVATIZING REAGENT FOR THE CHROMATOGRAPHIC ANALYSIS OF PHENOLIC ACIDS AND ALCOHOLS

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Derivatization often provides a much higher sensitivity in chromatography. One of the well-known derivatizing reagents is dansyl chloride originally proposed by Gray and Hartley /1/. This compound has found wide application in the chromatographic analysis of amino acids /2-5/, nucleic acids /6/ and amines/7/. Dansyl derivatives of amino acids fluoresce in the ultraviolet light at 360 nm. Using a fluorescence detector, dansyl-derivatives of amino acids and by-products can be separated and determined by HPLC, even at very low concentrations /8/.

Difficulties in obtaining highly pure solvents showing no absorbance in the UV range limit the possibility of the application of HPLC of dansyl derivatives. In 1975 Lin and Chang /9/ have proposed a new chromogenic derivatizing reagent, 4-dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl chloride) which has broader analytical applications and today available from most of the international supply houses.

The disclosed method for the preparation of dabsyl-Cl /10-12/ and the availability of dabsyl-amino acid standards /13/ was helpful in the preparation of the derivatives of compounds including various functional groups /14, 15/.

The present studies were undertaken to investigate the possibilities of the analysis of amino acids containing the specified groups: alcoholic OH (serine, threonine and hydroxyproline) and phenolic OH (tyrosine).

The preparation of dabsyl-derivatives of alcohols was also studied because of the frequent application of alcohols

as the mobile phase in TLC and HPLC. An excess amount of dabsyl-chloride reacts with methanol which is often a component of the mobile phase. The appearance of an unidentified peak during the application of dabsyl-chloride for amine analysis /16, 17/ induced us to carry out these studies.

Dabsyl-chloride also reacts with phenolic acids giving coloured, easily detectable compounds. This permits the determination of the reactivity of the phenolic groups of amino acids and phenols. An additional reason for the present investigations was the fact that these compounds belong to a biologically important group which are formed as a result of the chemical and biochemical transformation of lignin and the products of its decomposition /18/.

EXPERIMENTAL

Dabsyl derivatives of alcohols and phenolic acids were obtained using the method described in our earlier paper /12/. The chromatographic investigations were performed using the buffered paper method /19/. Paper strips (Whatman No 4) were impregnated with pH 2-8 buffers and eluted with diethyl ether. Glass chambers were saturated with water and diethyl ether vapours. The paper strips contained 0.5 g of buffer solution per 1 g of dry paper, resulting in a ratio of about 2 for the volumes of diethyl ether vs. the aqueous buffer.

Thin-layer chromatography was carried out using sandwich tanks with a glass distributor /16, 17/. Kieselgel G Si 60 (E.Merck, Darmstadt, FRG) was used as the adsorbent with a mixture of n-heptane and diisopropyl ether used as the mobile phase (normal phase) while ODS Silica gel (Z.O.Ch. Poland) and a mixture of water and methanol was used as the mobile phase in reversed-phase TLC.

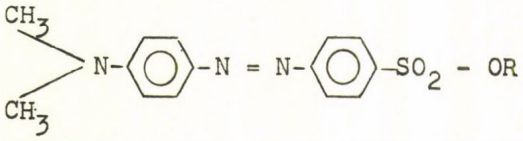
Column liquid chromatography was carried out using a Type 302 liquid chromatograph (Institute of Physical Chemistry of the Polish Academy of Sciences, Warsaw); it consisted of a syringe pump, a UV detector (fixed wavelength at 254 nm) and a four-port injection valve. A 250 x 4 mm i.d. stainless steel column (Z.O.Ch. Lublin, Poland), packed with 10 µm LiChrosorb

Si 60 (E.Merck, Darmstadt, FRG) was used for the measurement of the capacity factors.

RESULTS AND DISCUSSION

Dabsyl derivatives of aliphatic alcohols

Dabsyl derivatives of alcohols obtained in the reaction of dabsyl chloride with the appropriate alcohol can be presented by the following general formula:



(I)

where R is the alkyl chain of the alcohol.

The homologous series of dabsyl derivatives of aliphatic alcohols represents an interesting group of organic compounds in the respect of the structure effects.

According to the theory of adsorption chromatography for normal-phase system (polar adsorbents) and reported experimental data /20/ organic compounds differing only in the chain length should not show significant differences in the values of R_F and the capacity factor (k'). We have expected that the studied compounds will not be separated in typical adsorption systems.

Fig. 1 shows the correlation between the R_M value and the logarithm of the mole fraction (X_S) of diisopropyl ether in the binary mobile phase. According to the figure each dabsyl-alcohol gives a separate line corresponding to the equation of

$$R_M = f(\log X_S). \qquad (1)$$

This means that dabsyl-alcohols can be separated in the adsorption system.

The experimental results presented in Fig. 1 are described by the well-known equation derived by Soczewiński /21-23/:

$$R_M = R_{MS} - n \log X_S \qquad (2)$$

where R_{MS} is the R_M value for the pure modifier ($X_S = 1$), $R_M = \log \frac{1 - R_F}{R_F}$, and n is the slope of the plot corresponding

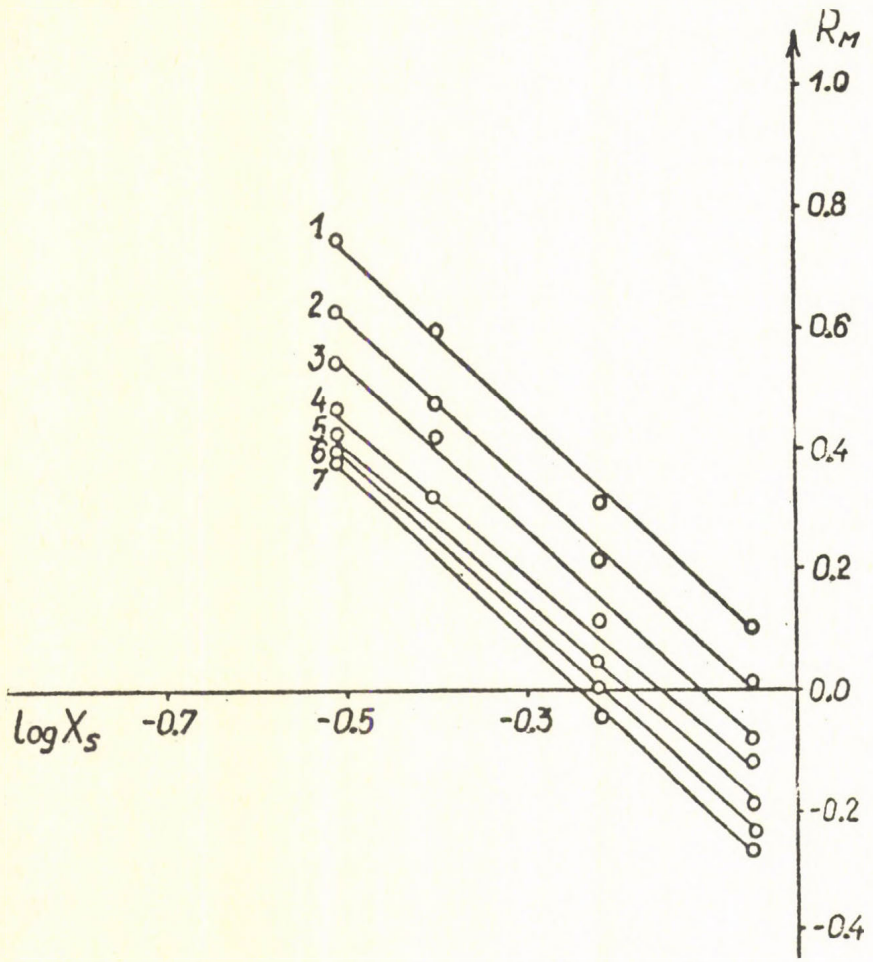


Fig. 1. Relationship of R_M vs. log X_S for some dabsyl-alcohols. Polar solvent: diisopropyl ether. Diluting solvent: n-heptane. 1, dabsyl-methanol; 2, dabsyl-ethanol; 3, dabsyl-propanol; 4, dabsyl-butanol; 5, dabsyl-pentanol; 6, dabsyl-hexanol; 7, dabsyl-heptanol.

to eq 2, and X_S is the mole fraction of the stronger solvent in the binary mobile phase. It can be seen from Fig. 1 that the separation of lower dabsyl-alcohols is easy. Soczewiński /16/ suggested that the dabsyl derivatives undergo adsorption by the $-SO_2$ group (see the general formula). Therefore, it can be assumed that the individual $R_F(R_M)$ values of the dabsyl-alcohols are caused by steric hindrance at the $-SO_2$ group, resulting in the hydrocarbon chain of each alcohol.

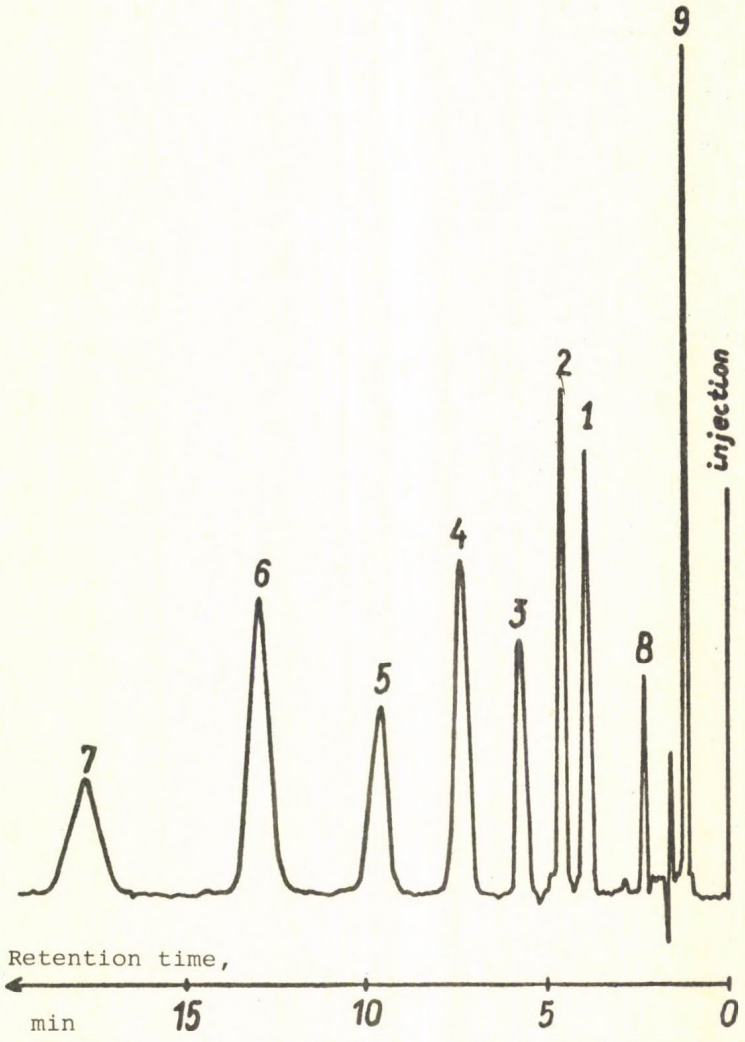


Fig. 2. HPLC chromatogram of seven dabsyl-alcohols. Stationary phase: Silica gel ODS, 10 µm particles. Mobile phase: water-methanol. Column: 250 x 4 mm, i.d. Detection at 254 nm. Peaks: 1, dabsyl-methanol; 2, dabsyl-ethanol; 3, dabsyl-propanol; 4, dabsyl-butanol; 5, dabsyl-pentanol; 6, dabsyl-hexanol; 7, dabsyl-heptanol; 8, dabsyl-NH_2; 9, dabsyl-chloride.

The longer hydrocarbon chain hinders the access of the $-SO_2$ group to the adsorbent surface. As a consequence adsorption of the higher dabsyl-alcohols is decreased, the R_F values are higher and dabsyl-alcohols can be separated. For higher homologues the differences in selectivity become smaller.

Dabsyl derivatives of alcohols can also be separated in reversed-phase systems. Fig. 2 shows such a chromatogram for seven dabsyl-alcohols.

It should be emphasized that the use of a photometric detector working in the visible region would increase the sensitivity of detection. The molar absorption coefficient for the dabsyl derivative of methanol is equal to 31,900 at λ_{max} = 430 nm, but at 254 nm λ it is considerably lower and equals 9,900.

Theoretical considerations /24/ and experimental results /25, 26/ have shown that in reversed-phase systems log k' is a linear function of the volume concentration of the modifier in the binary mobile phase:

$$\log k' = \log k'_W - n \varnothing \qquad (3)$$

where subscript W denotes pure water and $\varnothing$ is the volume fraction of the modifier.
It is well known that

$$\log k' = R_M \qquad (4)$$

Therefore eq. 3 can be rewritten as:

$$R_M = R_{M(W)} - n \varnothing \qquad (5)$$

Fig. 3 presents R_M vs. $\varnothing$ plots. It follows from the figure that the experimental results agree with eq. 5.

The different slopes of the R_M vs. $\varnothing$ plots of dabsyl-alcohols result in the fanwise spreading of the plots, converging at higher methanol concentrations. Thus, the slope for dabsyl-methanol is equal to 4.25 and for dabsyl-heptanol to 7.84.

This confirms the earlier conclusions /27, 28/ that the hydrophobic interactions (and adsorption) between the non-polar surface area of the solute molecules and the stationary phase covered by an inert hydrocarbonaceous ligand increase with the elongation of the alkyl chain.

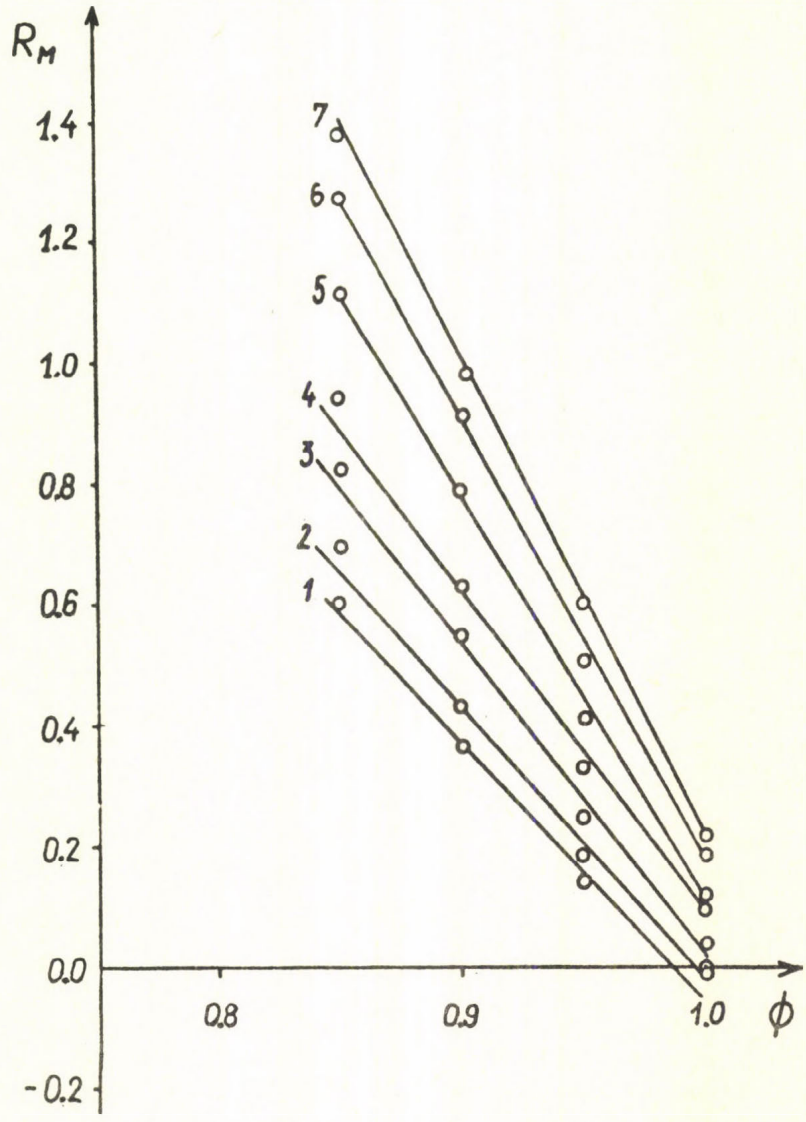
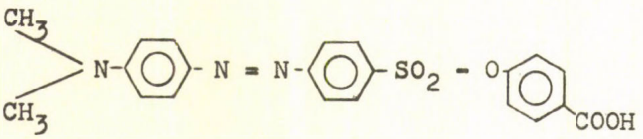


Fig. 3. Relationship of R_M vs. volume fraction of methanol for some dabsyl-alcohols. Mobile peaks: water-methanol. Stationary phase: HPTLC-Fertigplatten, RP-18. Notation of compounds the same as in Fig. 1.

Dabsyl derivatives of phenolic acids

Dabsyl derivatives of phenolic acids, obtained in the reaction of dabsyl chloride with the appropriate phenolic acid, can be presented by the following general formula:



(II)

The yield of the dabsylation reaction of phenolic acids is high; for the dabsyl derivative of vanillic acid it is equal to 87 % at a 3:1 molar ratio of dabsyl-Cl : vanillic acid. It should be emphasized that the dabsyl derivative of vanillic acid was quantitatively determined after extraction. During this process certain loss of the derivative could take place.

In order to establish the optimal extraction conditions of dabsyl phenolic acids from the reaction medium chromatography was carried out using the buffered paper method in a citrate buffer/diethyl ether system (Fig. 4). The chromatographic results show that the excess dabsyl-chloride should be extracted from the reaction medium at pH = 9.0 with diethyl ether. At these conditions dabsyl-phenolic acids are practically not extracted by the organic phase. The aqueous phase is then acidified to pH = 4.0 and the derivatives extracted with diethyl ether.

Fig. 5 shows the spectra of some dabsyl-phenolic acids in the range of 220-520 nm. The dabsyl-phenolic acids have considerably higher molar absorption coefficients in the visible region ($29{,}000 < \Sigma < 31{,}000$ at λ_{max} = 430 nm) than in ultraviolet permitting the detection of the dabsyl-phenolic acids in the visible region.

Fig. 6 shows the chromatogram of three dabsyl derivatives of phenolic acids using the normal-phase system of methylene chloride + acetic acid/silica gel.

The analysed phenolic acids occur as metabolites of the transformation of lignin which is the substrate of humus. Thus this chromatographic system can find practical applications.

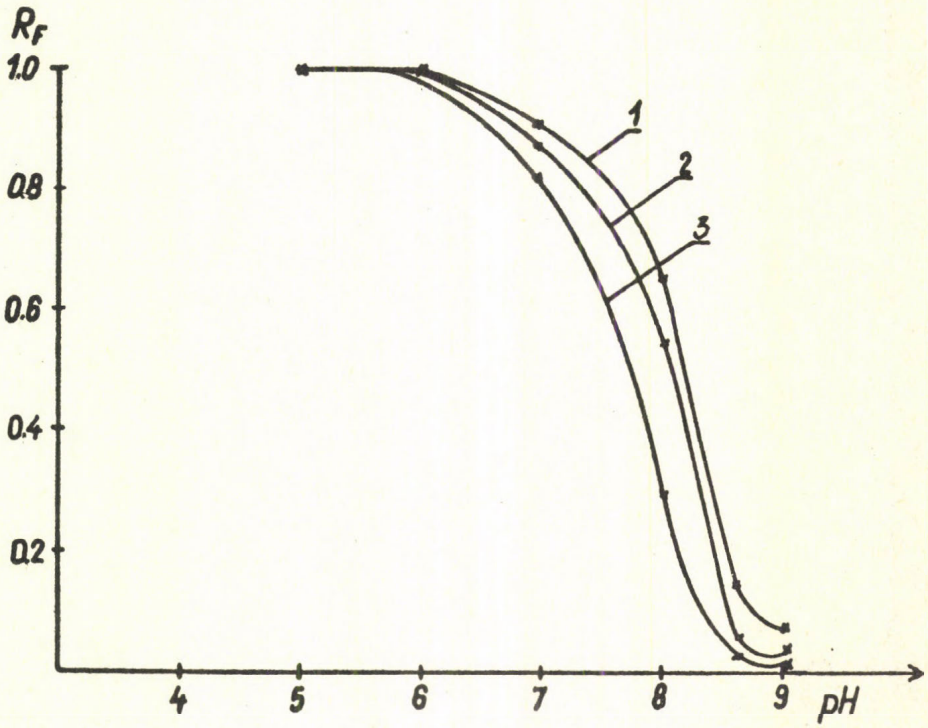


Fig. 4. Relationship between the R_M values and the pH of the aqueous phase for dabsyl derivatives of the phenolic acids in a diethyl ether-citrate buffer system. 1, dabsyl-p-hydroxybenzoic acid; 2, dabsyl-vanillic acid; 3, dabsyl-syringic acid.

The minimum detectable quantity (MDQ) of dabsyl-phenolic acids, calculated from the chromatogram in Fig. 6 is equal to 53 ng (detection at 254 nm). The MDQ could be about three times lower with detection in the 420-440 nm region.

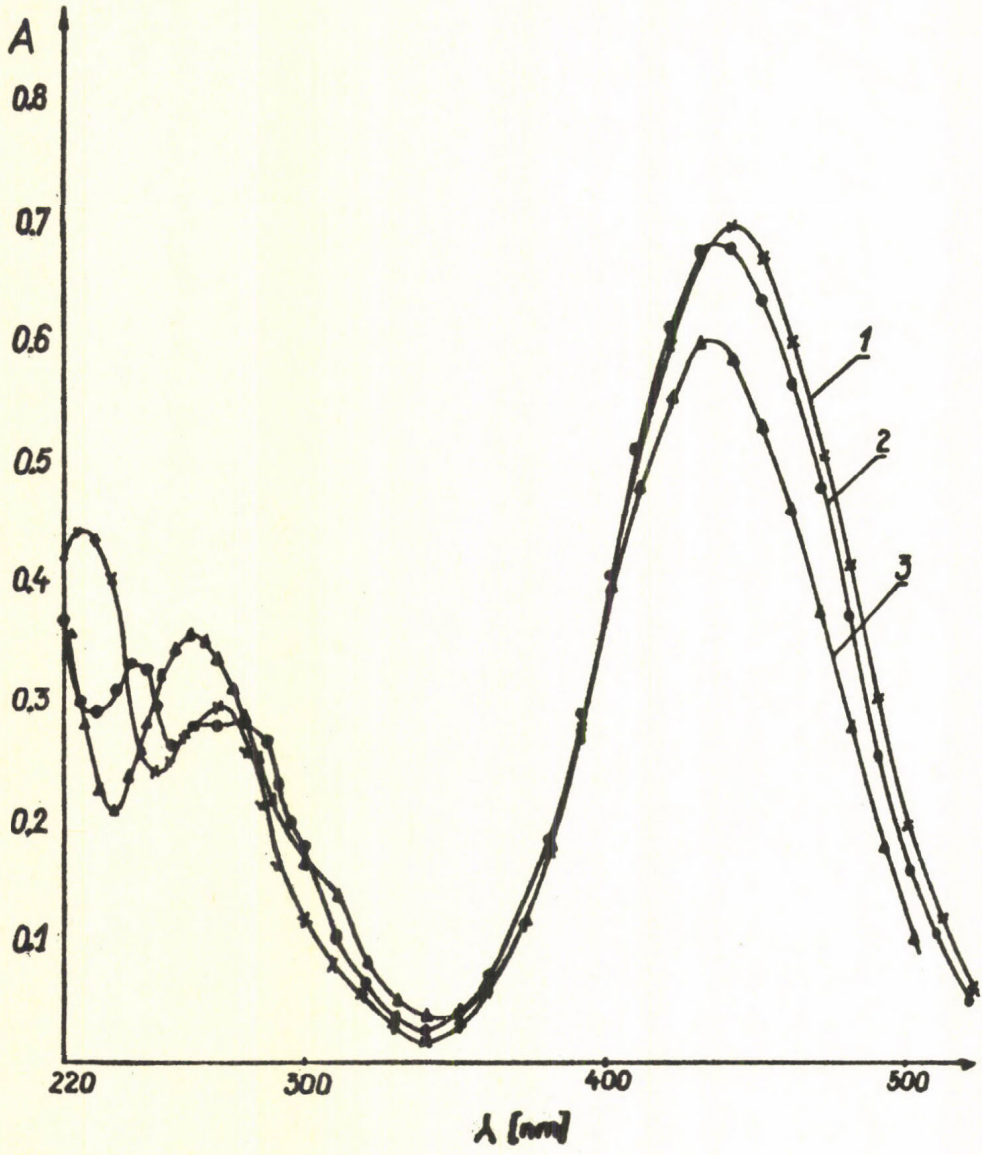


Fig. 5. Absorption spectrum of dabsyl-phenolic acids in diethyl ether. Concentration of each phenolic acid was 10 mg/l. Notation of the compounds the same as in Fig. 4.

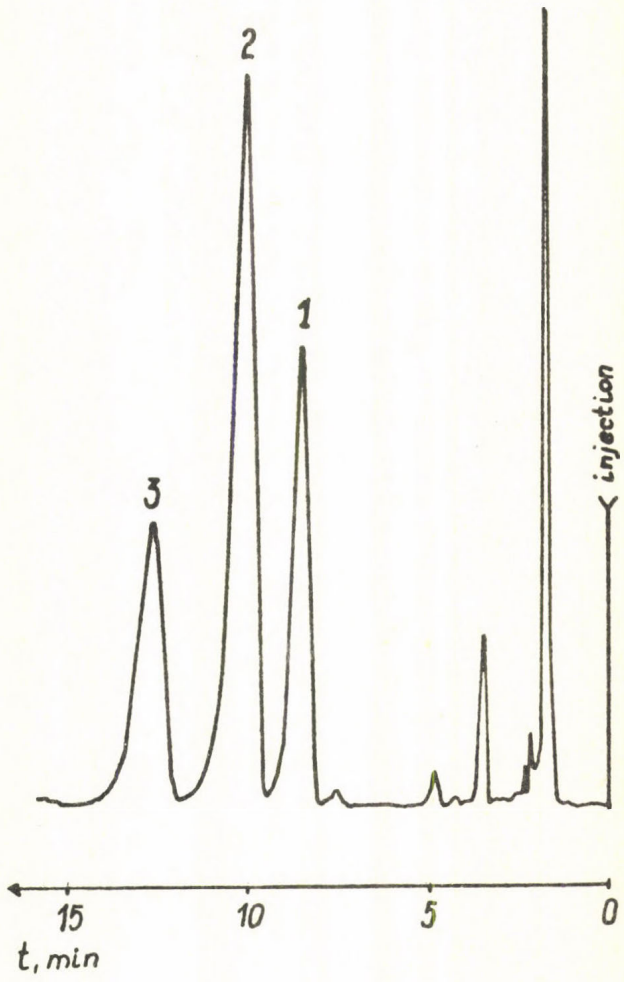


Fig. 6. Separation of dabsyl-derivatives of 1, p-hydroxybenzoic acid; 2, vanillic acid; 3, syringic acid. Column: 250 x 4 mm i.d. LiChrosorb Si 60, 10 µm particles; mobile phase: 95:5 cv/v methylene chloride-acetic acid; flow rate 1.2 cm^3/min; detection at 254 nm.

ACKNOWLEDGEMENTS

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REDOX CHEMILUMINESCENCE DETECTOR (RCD): SUCCESSES IN GAS CHROMATOGRAPHY AND PROSPECTS FOR LIQUID CHROMATOGRAPHY

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Summary

New chromatographic detectors can be based on redox reactions coupled with measurement of chemiluminescence. Measurements involve the catalyzed post-column reduction of nitrogen dioxide or nitric acid by analytes that can readily be dehydrogenated or oxidized, followed by downstream detection of the formed nitric oxide by reaction with ozone. The redox chemiluminescence detector (RCD) responds to ammonia, hydrogen sulfide, carbon disulfide, sulfur dioxide, hydrogen peroxide, hydrogen, carbon monoxide, alcohols, aldehydes, ketones, acids, amines, olefins, aromatic compounds, sulfides and thiols. Sensitivity of the RCD is comparable with that of flame ionization detectors. The RCD is insensitive to solvents such as alkanes, carbon dioxide, most chlorinated hydrocarbons, water and tetrahydrofuran. Prospects for its application to high performance liquid chromatography and supercritical fluid chromatography are discussed.

Introduction

Redox chemiluminescence detectors (RCD) for gas chromatography produce a signal when an eluent undergoes a post-column oxidation reaction, yielding nitric oxide, which subsequently produces chemiluminescence upon reaction with ozone. Although certain other chromatographic detectors are based on the measurement of chemiluminescence produced from various reactions (1-11), the RCD we have described recently (12-

14) derives NO from nitrogen dioxide or nitric acid upon the catalytic oxidation or dehydrogenation of eluents from a chromatographic column.

Details of the detector operation and theory (12,13) and selectivity (14) have been given previously. Only a brief description of the operation of the detector is presented here. Effluent from a gas chromatographic column is mixed with an excess of nitrogen dioxide at a final concentration of approximately 100 ppm and admitted to a heated reaction zone that contains a catalyst containing a metal such as gold. Analytes which react with NO_2 in the reaction zone to yield NO are detected upon subsequent reaction with ozone. This final reaction produces chemiluminescence that is sensitively measured by photon counting techniques.

The RCD responds sensitively, but with marked selectivity, to many compounds including hydrogen, carbon monoxide, carbon disulfide, hydrogen sulfide, sulfur dioxide, ammonia, hydrogen peroxide, formic acid, formaldehyde and phosgene. Its selectivity extends to several classes of organic compounds including alcohols, aldehydes, ketones, acids, amines, olefins, aromatic compounds, sulfides and thiols. For these compounds the sensitivity of the RCD is similar to the response to methane of a flame ionization detector. The RCD is insensitive to alkanes, carbon dioxide, most chlorinated hydrocarbons, water and tetrahydrofuran.

We have demonstrated that the selectivity of the RCD in gas chromatography can be controlled and varied by judicious choice of experimental parameters (14). Two details concerning the selectivity of the RCD are important to note. The response characteristics change drastically as a function of the temperature of the catalyst bed. For example, at 420 °C a good response (indicative of the formation of NO) is observed for alkanes, whereas at 360 °C no discernable response is observed when a gold catalyst is used. Also, different catalyst materials affect the selectivity of the RCD. For

example, even alkanes respond sensitively when a palladium catalyst is operated at 250 °C, while at the same temperature alkanes are inert in contact with NO_2 sorbed on gold.

RCD Applied to HPLC and SFC

The insensitivity of the RCD to several common solvents suggests that it may be a useful detector for high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). In addition, we have studied some redox reactions of organic compounds in the condensed phase using NO_2 as the oxidant in the presence of various catalysts. The results of these experiments suggest that a useful detector for HPLC and SFC can be developed.

In condensed phase experiments, upon which development of an HPLC detector will be based, we have demonstrated that NO_2 participates in redox reactions with a variety of organic compounds. We have observed the oxidation of primary aliphatic alcohols at temperatures between 80 and 200 °C using NO_2 as the oxidant. Aldehydes and esters are products observed in these reactions. The olefinic alcohol, geraniol, yields citral, without oxidation of the double bond. Aromatic alcohols are oxidized easily and yield aldehydes and ketones.

Although some of these reactions occur relatively rapidly without a metal catalyst, certain catalytic effects have been observed. In contrast to the high activity of gold in gas phase reactions at elevated temperatures, it is only slightly active as a catalyst at lower temperatures in the condensed phase reactions that we have studied. Some homogeneous as well as heterogeneous catalysts have been examined. Nickel salts have only a small effect, but copper salts show a large effect on the rate of alcohol oxidation by NO_2, even at 80 to 100 °C.

A few practical problems remain to be solved in the development of an RCD detector for use with HPLC and SFC. If NO is formed in a redox reaction, it must still be transferred to the chemiluminescence detector in the gas phase. In principle, the column-detector interface should be much simpler than, for example, in liquid chromatography/mass spectrometry, because NO is a well behaved gas and it will not be necessary to transfer non-volatile analytes to the detector. We are investigating several means of efficiently removing gaseous NO from liquid eluents and reaction products for subsequent detection in an RCD. Although we believe that these problems are all surmountable in applications of the detector to HPLC, they may be even easier to solve when an RCD is used as an SFC detector.

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