JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

HPLC
TLC
Capillary Electrophoresis
Supercritical Fluid Techniques
Membrane Technology
Field-Flow Fractionation
Preparative & Analytical Separations

OLUME 20 NUMBER 11 1997

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

June 1997

Aims and Scope. The journal publishes an outstanding selection of critical, peer-reviewed papers dealing with analytical, preparative and process-scale liquid chromatography of all types and related technologies such as TLC; capillary electrophoresis: supercritical fluid extraction and chromatography; membrane separation technology; field-flow techniques; and others. As new separation technologies are introduced, they will also be included in the journal. On a regular basis, special topical issues are devoted to specific technologies and applications. Book reviews, software reviews and a calendar of meetings, symposia and expositions are also included.

Identification Statement. *Journal of Liquid Chromatography & Related Technologies* (ISSN: 1082-6076) is published semimonthly except monthly in May, August, October, and December for the institutional rate of $1,750.00 and the individual rate of $875.00 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Periodicals postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography & Related Technologies*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage		
				Surface	Airmail to Europe	Airmail to Asia
20	20	$1,750.00	$875.00	$75.00	$110.00	$130.00

Individual professionals' and student orders must be prepaid by personal check or may be charged to MasterCard, VISA, or American Express. Please mail payment with your order to: Marcel Dekker Journals, P.O. Box 5017, Monticello, New York 12701-5176.

CODEN: JLCTFC 20(11) i–iv, 1651–1828 (1997)
ISSN: 1082-6076

Printed in the U.S.A.

Subscribe Today!

Use the cards below to subscribe to the *Journal of Liquid Chromatography & Related Technologies* or to recommend the journal to your library for acquisition.

Order Form

Journal of Liquid Chromatography & Related Technologies

Please enter my subscription to Vol. 20, 20 Numbers, 1997 at the ☐ institutional rate of $1750.00; ☐ individual rate of $875.00 *Individual subscriptions must be prepaid in American currency by personal check or credit card. Please add $3.75 per issue (number) for shipping outside the U.S. For airmail to Europe, add $5.50 per issue; to Asia, add $6.50 per issue. Canadian customers please add 7% GST.*

____ Please send me a pro forma invoice.
____ Check enclosed made payable to Marcel Dekker, Inc.
____ Charge my: ☐ MasterCard ☐ Visa ☐ American Express
____ Please bill my company: P.O. No. _____

Card No._____ Exp. Date_____

Signature_____

Name_____

Address_____

City/State/Zip_____

Does your library subscribe to the ***Journal of Liquid Chromatography & Related Technologies***? Just complete this card and submit it to your librarian or department head.

Attention: Librarian/Department Head: I have examined the ***Journal of Liquid Chromatography & Related Technologies*** and would like to recommend the journal for acquisition.

Signature_____ Date_____

Name_____ Department _____

Journal of Liquid Chromatography & Related Technologies
Volume 20, 20 Numbers, 1997: $1750.00
ISSN: 1082—6076 CODEN: JLCTFC

Sample copy and pro forma invoice available upon request.

Please contact the Promotion Department at: **Marcel Dekker, Inc.**
270 Madison Avenue
New York, NY 10016
(212) 696-9000 phone
(212) 685-4540 fax

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography & Related Technologies* are selectively indexed or abstracted in:

■ Abstracts Journal of the Institute of Scientific and Technical Information of the Russian Academy of Sciences ■ Alerts ■ Aluminium Industry Abstracts ■ Analytical Abstracts ■ ASCA ■ Berichte Pathologie ■ CAB Abstracts ■ Cambridge Scientific Abstracts ■ Chemical Abstracts ■ Chemical Reactions Documentation Service ■ Current Awareness in Biological Sciences ■ Current Contents/Life Sciences ■ Current Contents/Physical and Chemical Sciences ■ Current Opinion ■ Engineered Materials Abstracts ■ Engineering Index ■ Excerpta Medica ■ Metals Abstracts ■ Reference Update ■ Saltykov-Shchedrin State Public Library ■ Science Citation Index ■ Tobacco Abstracts

Manuscript Preparation and Submission. See end of issue.

This journal is also available on CD-ROM through ADONIS™ beginning with the 1991 volume year. For information contact: ADONIS, Marketing Services, P.O. Box 17005, 1001 JA Amsterdam, The Netherlands, Tel: +31-20-626-2629, Fax: +31-20-626-1437.

The journals of Marcel Dekker, Inc. are available in microform from: University Microfilms, Inc., 300 North Zeeb Road, Ann Arbor, Michigan 48106-1346, Telephone: 800-521-0600; Fax: (313) 761-1203.

Contributions to this journal are published free of charge.

Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1651-1666 (1997)

CONTROLLING THE PORE STRUCTURE OF ZIRCONIA FOR CHROMATOGRAPHIC APPLICATIONS

R.A. Shalliker, G.K. Douglas

Centre for Instrumental and Developmental Chemistry
Queensland University of Technology
Gardens Point, Brisbane, Qld 4001

ABSTRACT

This paper describes the preparation of zirconia for chromatography. Size exclusion chromatography was used to evaluate the pore structure particularly as it applies to chromatographic applications. Using sodium chloride impregnation techniques during calcination, pore diameters, surface areas and pore volumes could be controlled to prepare zirconia stationary phases that had improved chromatographic characteristics. Without sodium chloride, poor quality pore structures were obtained.

INTRODUCTION

As a relatively new stationary phase material, zirconia has undergone development that has illustrated many of its potential applications. Studies have shown that complex elution mechanisms may be present but, because of the chemical stability of the support, a wide range of mobile phase modifiers may be added to control the chromatographic process. Studies by Blackwell and Carr illustrated the usefulness of the support in the analysis of Lewis bases[1,2] and compiled an extensive eluotropic series, illustrating the control of

the separation process. The development of the eluotropic series served as an important step in analyzing proteins on zirconia supports.[3] Methods that control the highly heterogeneous surface of zirconia, and still maintain the novel selectivity afforded by the surface, have received considerable attention. Pre-adsorption of fluoride[4,5] or phosphate[6,7] are two processes involving surface modification that have allowed the controlled chromatographic use of these materials. In particular, these modified zirconias were shown to be useful for the separation of proteins. McNeff and coworkers[8] prepared anionic exchange zirconias with polyethyleneimine (PEI) surface modifications. Ghaemi and Wall[9] reported the first use of zirconia as a reversed phase material in LC using suitable surfactants in the mobile phase. Reversed phase zirconias have also been prepared by Rigney and coworkers[10] and Hanggi and Marks[11] using polybutadiene (PBD). Webber, Carr and Funkenbusch[12,13] described a technique for the preparation of carbon vapour deposited (CVD) zirconia supports where as much as 97% of the surface could be coated. Trudinger, Muller and Unger[14] reported the first successful derivatisation of zirconia with n-octadecylsilane. These phases exhibited similar behaviour to conventional silica C_{18} columns for polycyclic test solutes. Yu and El Rassi[15] also studied the behaviour of n-octadecyl derivatised reversed phase zirconia, comparing the stability of monomeric and polymeric coatings.

While these studies have been important in establishing the usefulness of zirconia as a chromatographic stationary phase, an important aspect of zirconia for chromatographic applications is, however, the optimization of specifically designed surfaces with suitable pore structures. The porous nature of the chromatographic support plays a very important role in the efficiency of the separation process and, ideally, the pores should allow rapid and unhindered equilibration of both solute and mobile phase molecules. Lorenzano-Porras and coworkers[16,17] studied the relationship between pore structure and diffusion tortuosity of zirconia colloidal aggregates and compared the differences in pore structure between two different colloidal aggregation processes. They found that classical methods of surface analysis were insufficient to evaluate the porous structure of zirconia and reported that NMR spin lattice relaxation and NMR self diffusion experiments were not compromised by pore restrictions, as are classical nitrogen sorption and mercury porosimetry. They concluded that in order to evaluate fully the pore structure of chromatographic packings, complimentary techniques of analysis are an invaluable asset.

Using nitrogen sorption experiments to investigate the pore structure of zirconia prepared by the polymerisation-induced colloid aggregation (PICA) method, the authors found that calcination at 700°C for three hours, followed by further calcination at 900°C for three hours, produced pore sizes in the range 35.0-55.0 nm. Nitrogen desorption measurements indicated that 70% of

the pore volume lay behind throats that were only 10.0-25.0 nm in diameter. The authors found large differences in pore sizes depending on whether nitrogen sorption, Hg porosimetry or NMR spin lattice relaxation (NMR-SLR) was used for their determinations. They attributed these differences to pore constrictions on the zirconia and to the assumptions of cylindrical pore shapes. Lorenzano-Porras and co-workers have also shown that the pore size and porosity of zirconia was dependent upon the colloidal aggregation process used. In a recent publication by Dunlap et. al.,[18] a comparison was made between the pore structures of a PICA prepared zirconia and an oil emulsion prepared zirconia using a size exclusion method of analysis.

The chromatographic method of analysis offered the advantage of providing pore analysis results that directly reflected the actual chromatographically available surface for solute molecules during an elution process. They found that both methods of synthesis yielded zirconias with greatly varied pore structures. Mercera and coworkers[19] presented a study on zirconia as a support for catalysis with respect to the effect of calcination temperature on the pore structure. Zirconia was precipitated from zirconyl chloride in ammonia at pH 10.0. The particles were large and irregular, requiring grinding to a finer size of approximately 100 μm. Their results showed that this method of preparation produced zirconia with a surface area of 111 m^2/g when calcined at 450°C. As the temperature of calcination was increased, the surface area decreased by as much as 100 fold at 850°C. They attributed the decrease in surface area to crystallite growth accompanying phase transformations and inter-crystallite sintering, which in turn increased the pore size. Zirconia, that was thermally treated below 650°C, was reported to contain micropores, and this led to an over estimation of the BET surface area. Zirconias, calcined at temperatures above 750°C, were free from micropores. No maximum pore size was recorded for the uncalcined zirconia, indicating that the pores were less than 1.5 nm, whereas calcination at 850°C produced zirconia with a most frequent pore radius of 10.29 nm. Pore size distributions of all zirconia samples were unimodal. Recent studies in our laboratory also illustrate similar results for zirconias prepared for chromatography.[20]

Despite these recent studies on porous zirconia, few researchers have attempted to optimize the surface area, pore size and pore volume for specific separation problems. Krebs and Heinz[21] described a method of preparing macroporous silica using salt impregnation during calcination. Their technique primarily involved calcining narrow pore diameter silica in the presence of salts to enlarge the pores. Despite their success in preparing macroporous silica gel, few studies have since followed where pore structures could be optimized using their methods. Perhaps the bimodal pore size distributions of such silica gels as reported by Novák and Belek may have hindered its

development.[22] In the current study we illustrate how the pore volume, surface area and pore diameter of zirconia can be optimized during the calcination process by the inclusion of sodium chloride. In particular, this study is important for applications in bio-molecule separations where larger pore diameters are required for the separation of high molecular weight proteins.

EXPERIMENTAL

The surfactants, Span 80, Brij 35, and Tween 85 were supplied by the Sigma Chemical Company Inc (St. Louis, MO. USA). Sodium metasilicate pentahydrate was obtained from BDH Chemicals Ltd (Poole, England). Zirconyl chloride (99%), urea and hexamethylenetetramine were supplied by the Aldrich Chemical Company Inc (Milwaukee Wis. USA). Polystyrene standards with molecular weights $1.8x10^6$, $8.5x10^5$, $4.5x10^5$, $1.85x10^5$, $8.7x10^4$, $4..4x10^4$, $28x10^4$, $1.02x10^4$, $3.355x10^3$,and $1.35x10^3$ were obtained from Shodex. HPLC grade methanol was obtained from BDH Chemicals (Poole, England) and AR grade dichloromethane was supplied by Ajax Chemicals (N.S.W., Australia). All chromatographic solvents were filtered through a 0.45 μm Millipore filter prior to use.

The preparation of zirconia was described in a previous publication.[20] The particle size of the amorphous zirconia prior to calcination measured using a Jeol 35JSM electron microscope was approximately 2 - 3 μm.

Sodium chloride impregnation of the zirconia microspheres was achieved by stirring a slurry of zirconia with the desired quantity of sodium chloride in water (4% w/v with respect to the zirconia). This slurry was stirred with heating (50°C-60°C) until all water evaporated (usually 36 hours). The resulting paste was dried overnight at 110°C and then mixed thoroughly using a mortar and pestle. The zirconia-sodium chloride mixture was then calcined in air using the appropriate calcination conditions. In all cases, the calcination temperatures involved a heating rate of 350°C h^{-1} to the temperature X-30°C, afterwhich, the heating rate was 60°C h^{-1} to the final temperature X°C. All zirconias were removed from the furnace immediately at the end of the calcination program and allowed to cool rapidly to room temperature. The sodium chloride was washed from the zirconia using copious quantities of water followed by washings in methanol and drying at 110°C.

Chromatographic columns were prepared in 5 cm x 0.46 cm stainless steel column blanks fitted with 0.5 μm stainless steel end frits. A Haskel air driven fluid pump (Haskel Engineering and Supply Co. Burbank, CA.USA.) was used as a packing pump and columns were packed in a downward slurry using a

methanol packing solvent, a methanol slurry solvent, and a dichloromethane displacement solvent. Columns were packed until the flowrate became constant.

Surface areas, pore volumes and pore size distributions were measured using size exclusion chromatography according to the method of Halász and Martin.[23] Chromatographic analysis was achieved using a Varian 5000 chromatographic system fitted with a 10 μL Rheodyne injection port and a variable wavelength UV detector set at 254 nm. Size exclusion experiments were performed by injecting polystyrene standards of various molecular weights (0.5 mg/mL) into a dichloromethane mobile phase. For accuracy, flowrates were approximately 0.2 mL min^{-1}. The exact flowrate during the elution of each polystyrene standard was recorded during elution and elution volumes were corrected for actual flowrates. Column efficiencies were measured using benzene in 100% dichloromethane. All chromatographic experiments were carried out at 20°C ± 1°C and repeated in duplicate.

For ease of discussion, the stationary phases will be referred to using abbreviations such as Zr810NaCl(1:2), where Zr refers to a zirconia stationary phase, 810 refers to the temperature of calcination, and NaCl indicates that calcination was carried out in the presence of sodium chloride with a ratio of 1:2 zirconia:sodium chloride. Hence, Zr700, refers to a stationary phase of zirconia calcined at 700°C without salt, and Zr600NaCl(1:1) refers to zirconia calcined at 600°C in a 1:1 mixture of zirconia:sodium chloride.

RESULTS

The pore dimensions, determined in the present study, may be evaluated by comparing the results of two previous studies in which zirconias were extensively studied using both nitrogen-sorption experiments and size exclusion chromatograph.[20,24] The zirconia analyzed in previous studies will be referred to throughout this study. The largest difference in surface area measurements for four zirconias using the two techniques was 14% and the greatest difference in pore diameter was 30%, which was for a zirconia containing a high degree of micropores where the average pore size using nitrogen sorption BET measurements was 5.7 nm compared to 7.9 nm using the size exclusion method.[20,24]

Nitrogen adsorption/desorption isotherms for the reference zirconias may be obtained from reference[20] and size exclusion curves for each of these reference zirconias are presented in reference.[24]

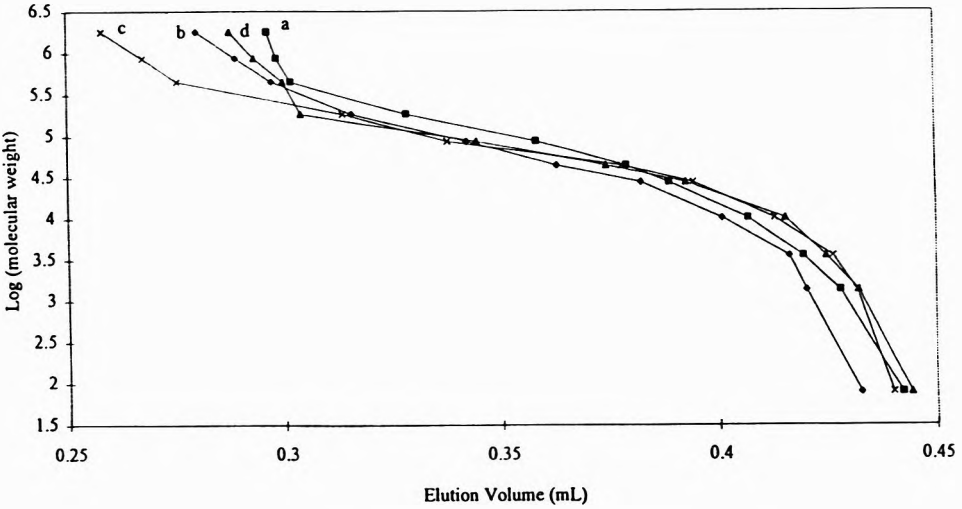


Figure 1. Size exclusion curves of polystyrene standards on the zirconias calcined with various concentrations of sodium chloride. (a) Zr810NaCl(4:1), (b) Zr810NaCl(2:1), (c) Zr810NaCl(1:1), (d) Zr810NaCl(1:2). Conditions, mobile phase was 100% dichloromethane at a flowrate of 0.2 mLmin^{-1}. Detection at 254 nm.

The Effect of Salt Concentration

The size exclusion elution behaviour of polystyrene standards with narrow molecular weight distributions was examined in a dichloromethane mobile phase on each of the zirconia stationary phases. The size exclusion curves for the zirconias calcined at 810°C with various salt concentrations are given in Figure 1. Pore size distributions were calculated from the size exclusion data for each of the stationary phases. The mean pore diameter (P_d) and the standard deviation (log σ) for each of the stationary phases was calculated by plotting the sum of residues versus the log of the pore diameter on probability paper.[23] The probability plots (not shown) for each of the zirconia samples resulted in a scatter of points along a straight line with some variation at the extremes, indicating the pore size distribution was approximately normal. The mean pore diameter determined from the probability plots are presented in Table 1, together with the standard deviation. Specific surface areas (S_a) were determined using the Guassian mean pore diameter according to the method of Halász and Martin[23] and these results are given in Table 1. The specific pore volumes (V_p) and column efficiencies are also included in Table 1.

Table 1

The Pore Dimensions of Zirconias Calcined with Various Concentrations of Sodium Chloride

Zirconia	P_d (nm)	Logσ	V_p (mL)	S_a (m^2g^{-1})	H(m)
Zr810NaCl (4:1)	34.0	0.423	0.1004	12.2	8.1×10^{-5}
Zr810NaCl (2:1)	42.0	0.396	0.310	12.4	8.1×10^{-5}
Zr810NaCl (1:1)	45.0	0.400	0.1324	11.8	7.5×10^{-5}
Zr810NaCl (1:2)	35.5	0.381	0.1136	12.8	8.5×10^{-5}

The size exclusion curves illustrated in Figure 1 indicate that all stationary phases produced classical size exclusion curves, as opposed to the exclusion curves previously observed for zirconia reference standards calcined without sodium chloride.[24] Each of the exclusion limits and inclusion limits of the zirconias in Figure 1 were similar, although slight differences were apparent as illustrated by the pore diameters in Table 1. Despite the constant calcination temperature, as the quantity of sodium chloride increased the mean pore diameter increased, except for the sample that had the highest quantity of sodium chloride (Zr810NaCl(1:2)), which showed a pore diameter similar to the zirconia prepared using the lowest concentration of sodium chloride (Zr810NaCl(4:1)). At this point, we cannot offer an explanation for the inconsistency, except to say that possibly, the high quantity of salt inhibited the salt diffusion into the pores. The surface areas of each sample remained approximately constant despite the differences in the pore diameters. However, a significant difference between each sample was the increase in pore volume as the concentration of sodium chloride increased up to a ratio of 1:1 zirconia:sodium chloride. The pore volume decreased for the highest salt concentration (1:2 zirconia:sodium chloride), which would be expected because of the smaller pore diameter. The most efficient stationary phase was the Zr810NaCl(1:1), which was consistent with the highest pore volume, indicating that mass transfer was less restricted by narrow necked pores.

The Effect of Calcination Temperature

A range of zirconia stationary phases, that each contained a 1:1 zirconia:sodium chloride mixture at calcination, were tested using size exclusion chromatography. Each zirconia was calcined at either 600°C, 700°C or 810°C. Size exclusion curves for each of these supports are illustrated in

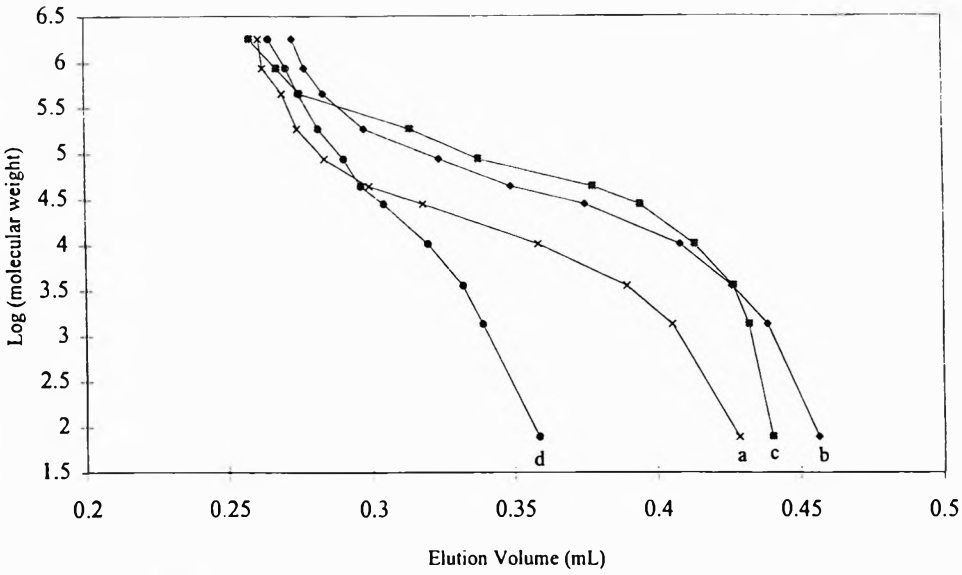


Figure 2. Size exclusion curves of polystyrene standards on the zirconias calcined at various temperatures with a 1:1 ratio of zirconia:sodium chloride. (a) Zr600NaCl(1:1), (b) Zr700NaCl(1:1), (c) Zr810NaCl(1:1), (d) Zr700. Conditions as in Figure 1.

Table 2

TheEffect of Calcination Temperature on the Pore Dimensions of Zirconia

Zirconia	P_d (nm)	Logσ	V_p (mL)	S_a (m^2g^{-1})	H(m)
Zr600NaCl (1:1)	19.0	0.387	0.1159	24.4	6.8×10^{-5}
Zr700NaCl (1:1)	28.1	0.378	0.1343	19.2	6.4×10^{-5}
Zr810NaCl (1:1)	45.0	0.400	0.1324	11.8	7.5×10^{-5}
Zr700	20.0	0.567	0.0511	10.2	1.0×10^{-4}

Figure 2. Pore size distributions were measured using the procedure outlined above. Pore diameters were calculated from the probability plots described above and are presented in Table 2. Table 2 also includes surface areas. pore volumes and column efficiencies calculated for each of the stationary phases. Cumulative pore size frequency plots are shown in Figure 3.

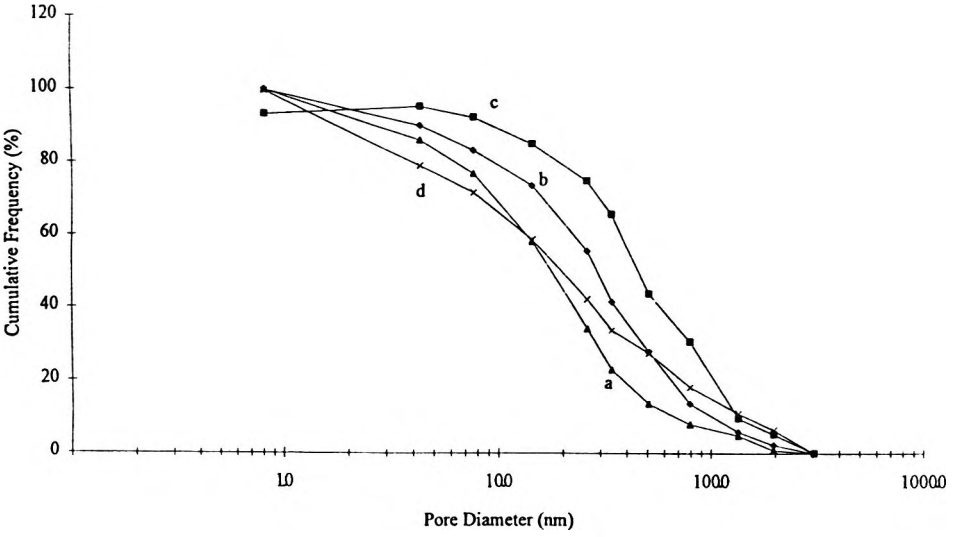


Figure 3. Cumulative frequency plots of zirconias calcined at various temperatures with a 1:1 ratio of zirconia:sodium chloride. (a) Zr600NaCl(1:1), (b) Zr700NaCl(1:1), (c) Zr810NaCl(1:1), (d) Zr700. Conditions as in Figure 1.

The size exclusion curves in Figure 2 show that the exclusion limit of each stationary phase increased as the temperature of calcination increased. Hence, the mean pore diameter increased as the calcination temperature increased and this can be observed by inspection of the cumulative frequency plots in Figure 3 and the results presented in Table 2. Such a result is not unexpected as the pore diameter has previously been shown to increase as calcination temperature increases.[19,20] The cumulative frequency plots in Figure 3 best illustrates the decrease in the percentage of smaller pores as the temperature of calcination increases.

Pore volume has been shown to decrease with increasing calcination temperature.[19,25] The pore volumes of our zirconia reference standards[20,24] calcined without the sodium chloride were consistent with these results. However, in the current study where sodium chloride was included during the calcination process, the pore volume increased between 600°C and 700°C, and decreased slightly at 810°C. However, the pore volume of the zirconia calcined at 810°C (0.1324 mL) was still much greater than the pore volume of the zirconia calcined at 600°C (0.1159 mL).

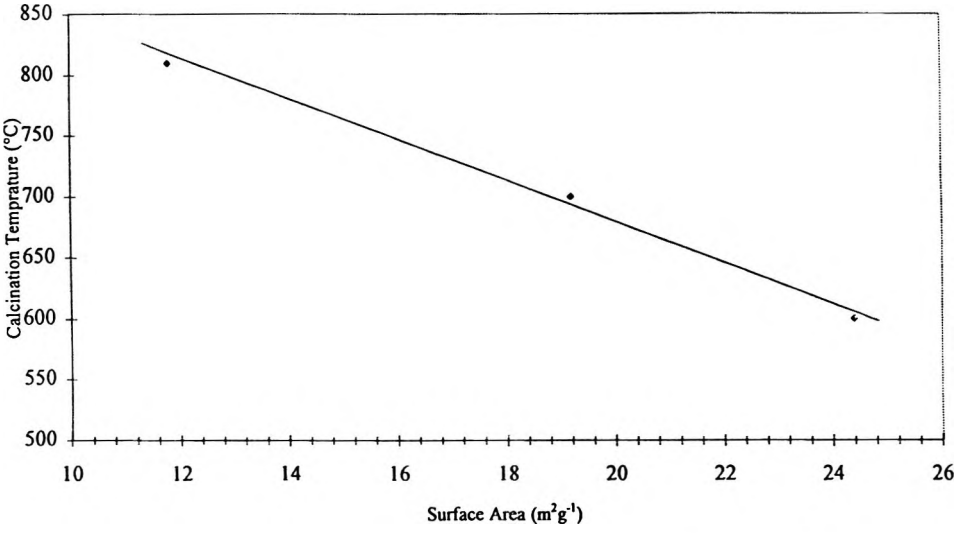


Figure 4. Plot of surface area versus calcination temperature for zirconias calcined in a 1:1 mixture of zirconia and sodium chloride.

The surface area of the zirconias decreased as the temperature of calcination increased, as illustrated in Table 2 and Figure 4. The linear relationship between surface area and temperature of calcination is somewhat fortuitous.

For comparison, a sample of zirconia was calcined at 700°C in the absence of sodium chloride. The size exclusion curve is illustrated in Figure 2 alongside the size exclusion curves of the salt impregnated zirconias. The pore dimensions of this zirconia are given in Table 2 and Figure 3 displays the cumulative frequency plot. These results clearly illustrate the significant part the sodium chloride plays during the calcination. Firstly, the salt-free zirconia yielded a size exclusion curve that had indistinct exclusion and inclusion limits with a very low pore volume [see Table 2 - less than half of that of the zirconia calcined with sodium chloride at the same temperature]. The mean pore diameter of the Zr700 was also smaller than the mean pore diameter of Zr700NaCl(1:1) and similar to the mean pore diameter of the Zr600NaCl(1:1). Significantly, the standard deviation of the mean pore diameter was higher than the standard deviations of the salt impregnated zirconias, indicating a greater distribution.

Table 3

The Effect of the Calcination Time of the Pore Dimensions of Zirconia

Zirconia	P_d (nm)	Logσ	V_p (mL)	S_a (m^2g^{-1})	H(m)
Zr700NaCl (1h)	28.1	0.378	0.1343	19.2	6.4×10^{-5}
Zr700NaCl (5 h)	31.6	0.468	0.1178	14.9	5.1×10^{-5}

The cumulative frequency plots in Figure 3 illustrate that the percentage of small pores is much greater when sodium chloride is not present during calcination for a given calcination temperature. Additionally the surface area of the Zr700 stationary phase was almost half that of the Zr700NaCl (1:1) stationary phase (Table 2).

Examination of the column efficiencies of each stationary phase provides some valuable information. The most efficient material was the Zr700NaCl(1:1) stationary phase, despite the higher surface area and smaller mean pore diameter of the Zr600NaCl(1:1) stationary phase. This is most likely due to a small yet discernible contribution from pores that may have partially closed necks not yet eliminated from the Zr600NaCl(1:1) stationary phase. The lower efficiency of the Zr810NaCl(1:1) stationary phase would be expected due to a much larger pore size and lower surface area. Clearly, calcination in the presence of sodium chloride greatly improved stationary phase mass transfer, as the least efficient column was the Zr700 stationary phase as shown in Table 2.

The Effect of Calcination Time

Two zirconia samples, each containing a 1:1 mixture of zirconia:sodium chloride were calcined at 700°C for a period of 1 hour and 5 hours respectively. The resulting size exclusion curves of these materials are illustrated in Figure 5. Pore size distribution plots for both materials are given in Figure 6. Pore volumes, surface areas, mean pore diameters and column efficiencies are given in Table 3.

Both materials produced almost identical size exclusion behaviour, with only a slight increase in pore diameter being observed with the increased calcination time. However, the pore volume and surface area decreased as the calcination duration increased.

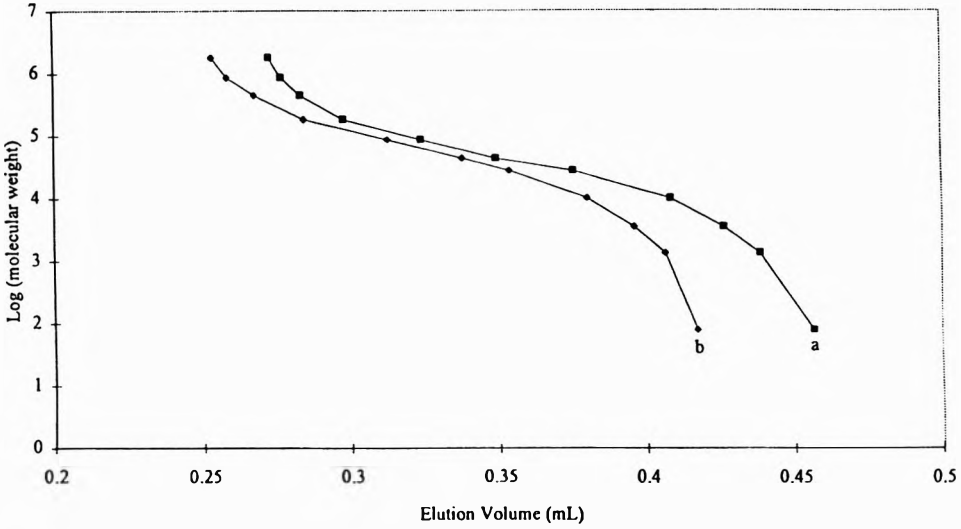


Figure 5. Size exclusion curves of polystyrene standards on the zirconias calcined for (a) 1 hour and (b) 5 hours at 700°C with a 1:1 ratio of zirconia:sodium chloride. Conditions as in Figure 1.

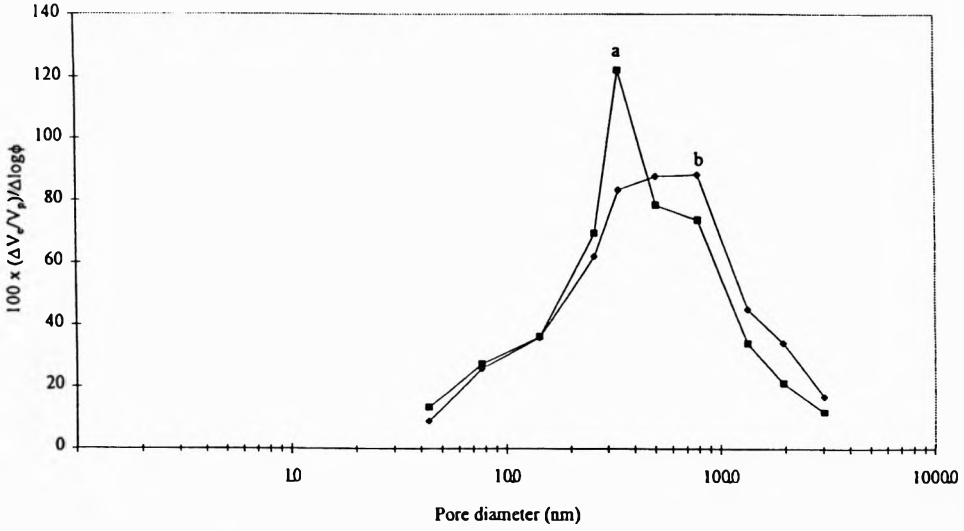


Figure 6. Pore size distribution plots of (a) Zr700NaCl(1h) and (b) Zr700NaCl(5h).

Comparison between plate heights on stationary phases with different calcination times, show that increasing the duration of the thermal treatment improves mass transfer, despite a reduction in the surface area and pore volume. This fact, together with the increase in pore diameter, would suggest that narrow pore openings are still being broken even after one hour of calcination.

Further studies would be required to optimise the duration of thermal treatment.

DISCUSSION

Zirconia chromatographic supports will become more widespread if the pore structure can be optimized to produce ideal chromatographic behaviour for a given separation problem. This means that the porous structure should be designed to maximize the surface area, pore volume and allow precise control over desired pore diameters.

In a previous publication,[20] we showed that the reference sol-gel zirconia, calcined without the sodium chloride, produced nitrogen sorption isotherms indicative of 'ink bottle' shaped pores (type H2 isotherms). Such surfaces offer inefficient chromatography due to the slow diffusion of solute and solvent molecules into and out from the pores.

Calcination of the same zirconia in the presence of sodium chloride produced type H1 isotherms indicating that the necks of the pores were broken leaving cylindrical pores. Such pores are more favorable chromatographic surfaces as diffusion becomes a more efficient process. In the present study, we have illustrated how complete control over the porous structure of the zirconia may be obtained by consideration of the factors outlined.

Column efficiency measurements support our data, which suggest that the inclusion of sodium chloride in various quantities and judicious selection of calcination conditions allows the pore structure of the zirconia to be controlled. Although nitrogen sorption experiments have not been undertaken on the current materials, when compared to our reference zirconias,[20,24] the results indicate that these materials would probably contain pores with type H1 hysteresis loops.

Certainly, the size exclusion curves have been significantly improved by the inclusion of the salt during calcination. The pore diameters, surface areas and pore volumes can be optimized in such a manner that zirconias could be

prepared for a given separation problem and this could be particularly useful in the analysis of proteins and macromolecules where larger pore sizes are desirable.

CONCLUSION

Increasing the salt concentration increases the pore diameter and specific pore volume, to a limiting degree, while surfaces areas remain constant.

For a constant concentration of salt, increasing the calcination temperature increases the pore diameter, and decreases the surface area in a linear relationship.

Specific pore volumes of salt impregnated zirconias do not decrease rapidly in the same manner as for zirconias treated without sodium chloride.

Increasing the duration of the heat treatment, marginally increases the mean pore diameter and decreases the specific pore volume and surface areas, but increases the column efficiency.

ACKNOWLEDGMENTS

The authors express their gratitude for support from the Centre for Instrumental and Developmental Chemistry, School of Chemistry, Queensland University of Technology.

One of the authors (RAS) would like to gratefully acknowledge the receipt of a Queensland University of Technology Postdoctoral Research Fellowship.

REFERENCES

1. J. A. Blackwell, P. W. Carr, Anal. Chem., **64**, 853 (1992).

2. J. A. Blackwell, P. W. Carr, Anal. Chem., **64**, 863 (1992).

3. J. A. Blackwell, P. W. Carr, J. Chromatogr., **596**, 43 (1992).

4. J. A. Blackwell, P. W. Carr, J. Chromatogr., **549**, 43 (1991).

5. J. A. Blackwell, P. W. Carr, J. Chromatogr., **549**, 59 (1991).

6. W. A. Schafer, P. W. Carr, J. Chromatogr., **587**, 149 (1991).

7. W. A. Schafer, P. W. Carr, E. F. Funkenbusch, K. A. Parson, J. Chromatogr., **587**, 137 (1991).

8. C. McNef, Q. Zhao, P. W. Carr, J. Chromatogr., **684**, 201 (1994).

9. Y. Ghaemi, R. A. Wall, J. Chromatogr., **174**, 51 (1979).

10. M. P. Rigney, T. P. Weber, P. W. Carr, J. Chromatcgr., **484**, 273 (1989).

11. D. A. Hanggi, N. R. Marks, LC-GC, **11**, 131 (1993).

12. T. P. Weber, P. W. Carr, Anal. Chem., **62,** 2620 (1990).

13. T. P. Weber, P. W. Carr, E. F. Funkenbusch, J. Chromatogr., **519** 31, (1990).

14. U. Trudinger, G. Muller, K. K. Unger, J. Chromatogr., **535**, 111 (1990).

15. J. Yu, Z. El Rassi, J. Chromatogr., 631, 91 (1993).

16. C. F. Lorenzano-Porras, P. W.Carr, A.V. McCormick, J. Colloid. Int. Sci., **164**, 1 (1994).

17. C. F. Lorenzano-Porras, M. J. Annen, M. C. Flickinger, P. W. Carr, A. V. McCormick, J. Colloid. Int. Sci., **170**, 299 (1995).

18. C. J. Dunlap, P. W. Carr, A. V. McCormick, Chromatographia, **42**, 273 (1996).

19. P. D. L. Mercera, J. G. Van Ommen, E. B. M. Doesburg, A. J. Burggraaf, J. R. H. Ross, Appl. Catal., **57**, 127 (1990).

20. R. A. Shalliker, G. K. Douglas, P. R. Comino, P. E. Kavanagh, (submitted for publication).

21. K. F. Krebs, H. Heinz, German Patent, P 20 42 910.1, 2 March 1972.

22. I. Novák, D. Berek, J. Chromatogr., **665**, 33 (1994).

23. I. Halász, K. Martin, Agnew. Chem. Int. Ed. Eng., **17**, 901 (1978).

24. R. A. Shalliker, G. K. Douglas, L. Rintoul, P. R Comino, P. E. Kavanagh (submitted for publication).

25. J. Nawrocki, M. P. Rigney, A. McCormick, P. W. Carr, J. Chromatogr., **657**, 229 (1993).

Received September 9, 1996
Accepted November 27, 1996
Manuscript 4288

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1667–1687 (1997)

CHARACTERISATION OF WIDE-PORE REVERSED PHASE COLUMNS FOR BIOPOLYMER SEPARATIONS. II. MULTIPARAMETRIC EVALUATION

G.Rippel[1], Gy.Bacsur[1], Á.Bede[2], Á.Sándi[2], L.Szepesy[2]

[1] Department of Agricultural Chemical Technology
[2] Department of Chemical Technology
Technical University of Budapest
Szt.Gellért tér 4.
H-1521. Budapest. Hungary

ABSTRACT

Nine commercially available silica based, wide-pore columns for protein separation were tested and compared using some low molecular weight aromatic test compounds recommended in the literature. From the retention values obtained under identical mobile phase conditions the strength of stationary phases could have been well characterised by spectral mapping analysis (SMA). However, inconsistency with experience was observed with respect to the polar-neutral and polar-ionic nature of packings. After equalising the mobile phase conditions, the confounding effect of the different column strength could have been eliminated and the selectivity differences amongst the stationary phases could have been revealed.

From the retention of the most relevant test compounds selected from the biplots obtained by SMA a set of single characteristics were constructed, providing a simple evaluating scheme applicable also for stationary phases of widely different type.

INTRODUCTION

In the past few years, numerous methods have been described in the literature to characterise RP-HPLC columns.[1-3] Attention was solely devoted to narrow-pore (NP) stationary phases, probably the most commonly used type of reversed phase packing materials. In spite of great efforts in column characterisation, none of the proposed testing procedures has been generally accepted. The existing methods usually involve isocratic measurements of previously selected compounds with various chemical properties.[1-4,6,10] Computed from the isocratic retention times, retention factor (k') and selectivity (α) are the two basic input data for column characterization.

However, these standard testing procedures are not applicable for wide-pore (WP) columns that are primarily developed for reversed phase separation of biopolymers. A WP column has weaker hydrophobic character than the corresponding NP media due to the lower surface area. Therefore, isocratic runs with the proposed mobile phase strength do not give meaningful data since most of the test components are eluted at or near to the hold-up time of column, i.e. without significant retention. To characterise these specific RP columns, lower mobile phase elution strength and multivariate evaluation of chromatographic data is required.

The endeavour to find chemicals that exclusively measure one certain physico-chemical property of the stationary phase, i.e. hydrophobic strength, hydrophobic selectivity, steric selectivity, acidic and basic activity constitutes the subject of many papers.[1-7,10-11] Multiparametric methods have been successfully applied to reveal the capability of a certain column to engage in different interactions during the chromatographic process (for a review see[5]). They can be used to classify both columns and substances and to furnish qualitative information about retention mechanism.

For this purpose, one of the most powerful multivariate statistical technique is *principal component analysis* (PCA).[6-8] Strongly related to factor analysis (FA), it is applied to uncover hidden or underlying nature of the database. To set up the starting database, chromatographic data are arranged in matrix format so that rows represent HPLC columns while columns of the

matrix represent test substances. The mathematical process aims to find the lowest number of fundamental factors required to account for the greatest possible variance. By combining the original variables into few artificial variable called principal component or factor, PCA sorts out the significantly different test substances and reduces the number of compounds needed for characterization. The projection of the subsequently calculated "factor scores", i.e. the share of each column from a certain abstract factor can provide a good visualisation of column differences.

Spectral mapping analysis (SMA) developed by Massart and co-workers[9] employs also PCA as redundancy extraction method. However, the input database is a matrix derived from double-centering of the original data. This scaling technique allows the user to project columns and substances on the same multidimensional space. One can attribute chemical meaning to compounds lying far from each other and thereby obtain characterization of columns.

Both in PCA and SMA, the weakest point of the data evaluation is to find out the certain chemical interaction or property a test substance is indicating.

It is also possible to classify columns on the basis of chromatographic data without embarking on interpreting the often ambiguous factor structures extracted. Cluster analysis (CA) offers a quick view of column similarity/dissimilarity.[17] On the icicle plot (or dendrogram) presentation the columns are arranged according to their similarity distances computed. Several distance measures and linkage rules can be selected. To obtain a set of clusters, the dendrogram is cut at a certain level of similarity resulting in grouping of columns into specific sets.

We have previously reported the characterization of nine prepacked wide-pore columns, using six different test mixtures and single parametric methods.[12] The results indicated that amino acids, peptides or proteins as test compounds can not reveal the physico-chemical differences of the stationary phases examined. Interestingly, they led to nearly uniform characterization, mainly in order of column hydrophobicity. To achieve sufficient discrimination we extended our small molecule test group and included several substances recommended earlier for NP column characterization.

For the interpretation of chromatographic data and for evaluating the nine WP, stationary phases spectral mapping analysis (SMA) was carried out. Under identical mobile phase conditions only the strength of the stationary phases could have been characterised. After equalising the conditions for different column strength, also, the selectivity differences could have been

revealed. From the retention values obtained under balanced conditions for some specific compounds selected by SMA, single characteristics were computed, which provided a flexible scheme for comparing and evaluating stationary phases of widely different type.

MATERIALS

The test components used for characterization - toluene (T), ethylbenzol (EB), methyl p-hydroxybenzoate (ME), nitrobenzol (NB), aniline (A), N,N-dimethyl-aniline (DA) and phenol (P) - were all of analytical-reagent grade and were obtained from different sources. For chromatography, a Merck-Hitachi (Merck, Darmstadt, Germany) fully automated system was used consisting of an L-4250-UV/VIS detector, L-6200 programmable pumps and a Rheodyne injector (Cotati, CA, USA) with a 10 μl loop. System control, data acquisition and evaluation were performed with HPLC Manager D-6000 software (Merck) running on an IBM386-compatible computer. All the columns investigated were prepacked with silica based, wide-pore (300Å) reversed phase stationary phases. Further characteristics are listed in Table 1.

METHODS

Measurements were carried out under isocratic conditions with acetonitrile-water mixtures. No buffer or salt was added, since it was shown that additives of this kind can mask the actual quality of the stationary phase, i.e. moderate the effect of surface silanols.[13] It was also demonstrated that the silanol effect can not be completely suppressed at pHs applicable on silica based RP phases. This effect can influence significantly the peak shape of basic compounds even in buffered eluents.[9] However, application of such additives under real conditions should be considered just on the basis of characterisation obtained for a column as it is. Consequently, no pH control and no masking additive was applied here.

According to our earlier practice[12] the composition of the eluent was always adapted to the test compounds, i.e.: it was varied so as to obtain 4-5 retention values in the range $-1 < \ln k < 3$ for all the components on all the columns investigated. This region is not only advisable for isocratic separations but it is optimal for stationary phase tests.[1] The hold-up time was measured with aqueous solution of sodium nitrite. All measurements were repeated at least twice and the average values were used for calculations.

Table 1

Characteristics of the Columns

Column Name	Manufacturer	Ligand Type	Dimensions (mmxmm i.d.)	Particle Size (μm)	Symbol
AQUAPORE OD-300	Applied Biosystems (San Jose, CA, US)	C18	100 x 4.6	7.0	A-C18
AQUAPORE BUTYL	Applied Biosystems (San Jose, CA, US)	C4	100 x 4.6	7.0	A-C4
SYNCHROPAK RP-PC18	Synchrom (Linden, IN, USA)	C18	100 x 4.6	6.5	S-C18
SYNCHROPAK RP-C4	Synchrom (Linden, IN, USA)	C4	250 x 4.1	6.5	S-C4L
SYNCHROPAK C4	Synchrom (Linden, IN, USA)	C4	100 x 4.6	6.5	S-C4
ZORBAX SB 300 C8	Rockland Technol. (Newport, DE, USA)	C8	150 x 4.6	5.0	Z-C8
ZORBAX SB 300 C3	Rockland Technol. (Newport, DE, USA)	C3	150 x 4.6	5.0	Z-C3
ZORBAX SB 300 CN	Rockland Technol. (Newport, DE, USA)	CN	150 x 4.6	5.0	Z-CN
ZORBAX SB 300 TRIF	Rockland Technol. (Newport, DE, USA)	trifluoro-acetamid	150 x 4.6	5.0	Z-TFA

The retention data obtained on different columns can be arranged in a data matrix. The rows of this matrix represent objects (here, the stationary phases), the columns are the variables (here, the test solutes) describing the objects.

Accordingly, a complete classification of the objects are given in a hyperspace having as many dimensions as many test components were applied. Clearly, graphical interpretation of such multivariate data tables is impossible, and thus, visual evaluation being more useful than any numerical representation is also infeasible.

For revealing the underlying structure of database principal component analysis (PCA) and related techniques have been proposed. These methods reduce the dimensions of the hyperspace by defining new variables called principal components (PC) along which the objects can be represented in a plane having significantly lower dimensions than the raw data while reserving all or most of the variation in the original data set..

PCs are calculated as weighted sum of the original variables. The corresponding weights are called the loadings of variables on the PCs. The objects are characterised by their values on these new variables. These values are called scores. For displaying the results of PCA both the loadings and scores are applied. Prior to extraction of PCs, the data are generally pre-treated. These mathematical operations transform the raw data to a different set, thus, PCA can reveal different aspects of the original data structure.[15]

Spectral mapping analysis (SMA) is a technique of PCA which was proposed for characterisation of stationary phases.[9] In SMA log double centering is applied as pre-treatment i.e. a logarithmic transformation followed by double centering. The graphical display of the SMA results (the so called biplot) is of central importance. It is a joint graphical representation of the objects and the variables by means of the scores and loadings on two selected factors; i.e. the biplot superimposes the scores plot for the objects and loadings plot for the variables. The position of objects and variables with respect to the origin of graph indicates contrast. Points located far away from (close to) the centre of plot have high (low) specificity or high (low) contrast.

On such a display the relationships or interactions between objects and/or variables can be easily identified. The closer two variables or objects on the plot, are the more similar behaviour these test compounds or columns exhibit. The proximity of a variable and an object being in the same direction means attraction, i.e. the high affinity of that test compounds for the corresponding column. This affinity can be related to specific feature(s) of the stationary phases and, thus, it can be used for characterisation. On the contrary, variables and objects located in the opposite direction repel each other, which are also typical for them.

The characteristic variables can be selected according to contrasts. Test compounds with high contrast located far from one another are the most dissimilar and are therefore the most specific. These variables can be considered poles and can be used for constructing bipolar axes. If the number of poles is less than that of the variables (which is generally held true), the original set of test compounds can be reduced[9] which makes it possible to use much simpler characterisation techniques, e.g. ternary diagrams.

RESULTS AND DISCUSSION

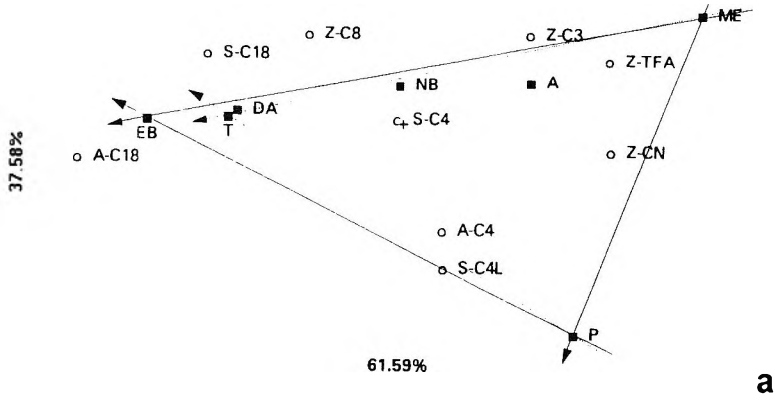
A rough estimate of retentive strength of the stationary phases could have been established immediately from the retention measurements. The order of columns obtained conformed to the hydrophobicity of ligands. It prompted that the characteristic playing dominant role on retention process is the hydrophobicty of columns determined mainly by the ligand type. It also involved that any characterisation with respect to hydrophobicity must reflect, at least approximately, that of the ligands.

In the preceding part of this paper[12] the stationary phases were characterised on the basis of the relative hydrophobicity (x^*) of the test components. (x^* is the composition of that eluent in which the retention factor equals 1, i.e. the component is distributed equally between the stationary and the mobile phase.). Earlier we found that x^* is a sensitive indicator of the nature of the phase system (the stationary and mobile phase), therefore, it is applicable for characterisation of the stationary phases.[16] The relative order concerning the strength of columns could have been established very well with x^* but some differences in selectivity was also indicated which could not have been identified unanimously.[12]

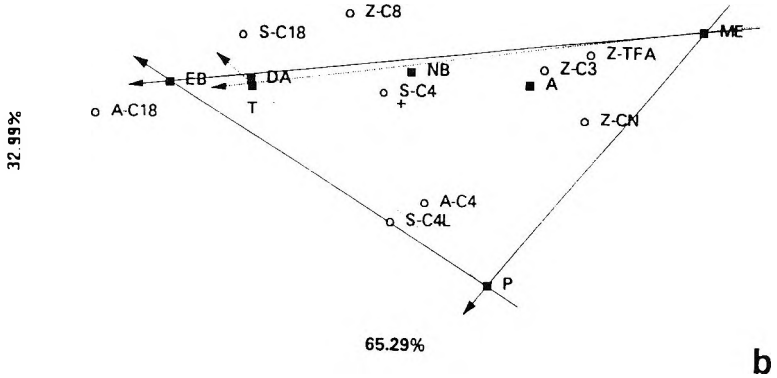
"Traditionally", columns are characterised in isocratic mode on the basis of retention factors of some test compounds measured under identical eluent conditions, i.e. in the same mobile phase. When conditions are properly selected all or most of the test components are significantly retained on all columns. Under such conditions, the variation of retention values obtained can be related to the differences between the stationary phases. When nominally identical columns are to be compared the reference conditions can be easily selected and even varied, some deviations from the methods recommended do not alter significantly the characterisation.

However, the strength of stationary phases investigated here vary in wider range which limited seriously the selection of such reference eluent composition. When all columns were taken into consideration a very narrow range (approx. 30 - 40 % ACN) was only applicable. This range corresponds to the requirements outlined above within which three compositions (30%, 35%, 40%) were selected for the characterisation of the stationary phases.

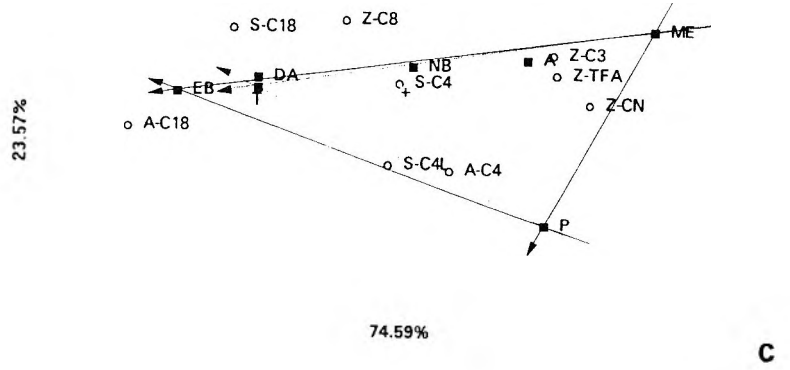
The retention factors obtained at the specified compositions were subjected to SMA subsequently. The biplots obtained are shown on Figure 1a-c. In each case, the first two principal components account for more than 98% of the variance in the data table.


37.58%
Z-C8
S-C18
Z-C3
ME
Z-TFA
NB
A
DA
EB
T
S-C4
A-C18
Z-CN
A-C4
S-C4L
P
61.59%

a


32.99%
Z-C8
S-C18
ME
Z-TFA
EB
DA
NB
Z-C3
A
S-C4
A-C18
T
Z-CN
A-C4
S-C4L
P
65.29%

b


23.57%
S-C18
Z-C8
ME
NB
A
Z-C3
DA
EB
S-C4
Z-TFA
T
Z-CN
A-C18
S-C4L
A-C4
P
74.59%

c

It means, the objects and the variables can be described by these two PCs without meaningful loss of information. As seen, the most specific (dissimilar) variables are EB, P and ME, thus they can be considered poles and were selected to construct bipolar axes.

The EB-ME axis seems to represent a kind of polar-apolar axis. Clearly, the spread of variables (test compounds) between the two extrema reflects their polarity. It is seen that DA is very similar to T, i.e. the basic character of amino group is fairly hindered by the methyl substitutions. This kind of behaviour of DA is well known.[1,13] It seems, under conditions applied here T and DA are equivalent with respect to characterisation.

The order of stationary phases obtained by orthogonal projection of the respective points on the EB-ME axis corresponds well to that expected from the hydrophobicity of ligands, as denoted above. The extreme position of A-C18 indicates its profound strength with respect to the other columns. The S-C18 is somewhat weaker, however, the two octadecyl columns are located near to the most hydrophobic test compounds which indicates their large hydrophobic strength. The other columns are bunched to two groups. Indeed, within each group the average retention of test compounds were approximately the same, only selectivity differences could have been experienced. The first group consists of the butyl phases and Z-C8. Note, the octyl column exhibits lower hydrophobicity than would be expected from the ligand type. The second group comprises the three weakest columns. Note, here the "weakness" of stationary phases is resulted from different effects. The strength of columns decreases as the hydrophobicity of ligand decreases (Z-C3) or as its polarity increases (Z-CN, Z-TFA). The order obtained along this axis reflects correctly the strength of stationary phases experienced.

When poles are properly selected, the corresponding ratio of retention factors can be reproduced by orthogonal projection of objects (stationary phases) upon a bipolar axis formed by two test compounds. In this meaning the EB-P axis is incorrect, since it shows the retention of P relative to EB was significantly higher on Z-CN than on the butyl columns (A-C4 and S-C4L). However, the experimental values obtained for these columns were in the same range at all concentration investigated.

Figure 1 (left). SMA biplots obtained from k retention factors measured in 30% (a), 35% (b) and 40% (c) acetonitrile. (For details and explanation see text.)

The correlation between the calculated and the experimental values can be improved significantly by selecting T as pole instead of EB (dashed axes on the biplots). Note, this alteration does not affect the characteristics concerning the polar-apolar nature of stationary phases outlined above, since the hydrophobicity axis (T-ME) remains practically the same as was before (EB-ME).

The property indicated by the third pole selected (P) must be different from that represented by the former ones. It is very likely the polar-ionic (acid-base) character of the stationary phases. The relatively high affinity of A-C4, S-C4L and Z-CN towards P can be attributed to this kind of feature. This interpretation is supported by the behaviour of benzoic acid also used for characterisation. Retention appropriate for evaluation could have been obtained only on A-C18, A-C4, S-C4L and Z-CN for this compound, which corresponds to the postulation given. On other columns no retention was achieved, consequently, benzoic acid was excluded from the evaluation. Since the first step of SMA is a logarithmic transformation, zero retention factors can not be included. So as to keep this compound for evaluation, a "small value" of retention should have been used instead of zero. Arbitrarily selected "small values" may, however, distort significantly the result of analysis just because of the logarithmic transformation.

The relatively high affinity of P towards Z-CN can be attributed to the strong dipole interaction induced by the cyano groups. But this interpretation can not be true for the butyl phases. In these instances another kind of interaction (e.g. acid-base) induced by the uncovered part of silica support must be presumed. The position of stationary phases on the biplot clearly shows this difference. The butyl columns are located relatively far from the polar-neutral pole (ME) but they are close to the polar-ionic pole (P). Z-CN is rather closer to ME but the closeness to P seems to be also significant. It is very likely that on this column both interactions contribute to the whole retention process.

The retention behaviour of benzoic acid on these columns can be explained similarly. However, the position of A-C18 on the biplots does not indicate any kind of polar or ionic activity, therefore, it can not explain the retention behaviour of this compound. In this instance it can be attributed, rather, to the large hydrophobicity of this stationary phase. As seen, quite different column characteristics can result in the same retention phenomena which can not be revealed by single parametric evaluation, i.e. by examining the absolute or relative retention values obtained. For the decomposition and identification of the underlying effects multivariate techniques are needed, e.g. PCA or related techniques such as SMA.

Besides the diversity indicated above a common feature of these four columns can be found, namely, they all have negative scores on the second PC, i.e. they all are located under the origo (marked with + on biplots). The retention of acidic compounds seems to be proportional rather to the distance from the EB-ME axis and not simply to the affinity towards some selected poles.

The bigger is the angular distance between an object and a variable, the more they "repel" each other. In this meaning Z-C8 and, to a lesser extent, S-C18 and S-C4 exhibit lack of affinity towards P. However, this repulsion is not associated with attraction to any of the basic compounds, thus this characteristic (P-affinity) can be considered "one dimensional". In this meaning, the EB-ME axis represents a "two dimensional" characteristic. It seems as if the deficiency of one of the features (e.g. hydrophobicity) was associated with the excess of the other (polarity).

However, this interpretation seems to be somehow oversimplified. The position of Z-C3, Z-CN and Z-TFA indicates the low strength of these columns, but the effects from it's results is not revealed clearly by the biplots. The polarity of silica-based RP stationary phases can be the consequence of the ligand polarity and/or the accessibility of the polar support. The latter effect can be pronounced even for apolar ligands, especially for shorter ones, or, irrespective of size, at lower ligand density. In addition, depending on the manufacturing and ligand bonding chemistry the silica surface can induce polar-neutral and/or polar ionic interactions.[3] Under practical conditions the result of these factors is the same, namely, an overall decrease of hydrophobicity, but from a viewpoint of characterisation these effects should be exactly identified.

A further problem of the characterisation scheme given is that the composition of the mobile phase affects the results. The spread of points on the biplots decreases as the organic content increases which can be attributed to the overall decrease of retention. The biplots are equally scaled for better comparison. It means, at higher organic content only larger differences can be revealed, but this problem can be overcome by the proper selection of the mobile phase strength. The bigger problem is that the relative position of the stationary phases also changes with the acetonitrile concentration. At 30% ACN S-C4L exhibits higher affinity towards P than does A-C4 which indicates larger polarity for S-C4L. But this order is highly dependent on the mobile phase conditions, and, as it is seen, it is reverted at 40% (cf. Figure 1a and Figure 1c). It is the consequence of the non-uniform behaviour of this compound on the different columns.

The retention of P decreases much steeper on S-C4L than on A-C4 as the organic content of eluent increases. The relative characteristics of the weakest columns are also different from plot to plot. The position of these columns changes not only on the P-ME axis but also on the T-ME axis as the concentration of acetonitrile varies.

The eluent composition must be varied even within one test if the strength of stationary phases to be compared differ too much or if the different kind of characteristics to be evaluated necessitate it. In these instances the strength of mobile phase applied depends on the average retention of test compounds[9] or it follows different recommendations given in the literature.[17] However, the above results indicate that the selection of mobile phase conditions for characterisation of stationary phases of widely different type is not a trivial task. Most of the test methods published in the literature are designed and optimised for stronger, alkyl type stationary phases (C18 and C8). These schemes can not be applied on columns of different type without some modifications, but, as was shown, different selection of mobile phase conditions could result in different classifications.

Here we postulate that the uncertainty of the above characterisation is resulted from the confounding of strength and selectivity of the stationary phases. A practically relevant characterisation should concentrate also on the selectivity of stationary phases and not only on their strength, as do most of the test methods. A C18 phase is trivially stronger than a C8 or C4, no evaluation is really needed. An overall decrease of retention could be expected on the latter ones. Theoretically, the differences in strength of stationary phases can always be compensated by adjusting the strength of the eluent, i.e. by varying its organic content. However, selectivity differences can only be counteracted by changing the type of the eluent, i.e. replacing the organic constituent with another or adding further modifier(s), e.g. using ternary eluents. The quite frustrating result of these modifications is that the complete method development process (optimisation and validation) must be repeated. Consequently, it is very useful and advisable to reveal these characteristics in advance.

So, as to test selectivity differences, the eluent conditions must be selected according to the strength of stationary phases, i.e. these kind of differences must be balanced by the mobile phase. Under such conditions the variation of retention behaviour of test compounds reflect only the selectivity differences amongst the columns.

For a precise selection of the eluent conditions it is better to test the behaviour of only one appropriate test component than using the average retention of a complete test mixture. Accordingly, the characterisation of stationary phases is to be performed in mobile phases providing definite retention for this compound. For reference value we selected unit retention ($k = 1$). Note, it means the use of x^* for defining test conditions (cf. definition of x^* given above.). Under this conditions the strength of the mobile and stationary phase is equal with respect to the indicator, and the differences of column strength are also compensated for this compound

By comparing our present and earlier results[12,14,16] toluene (T) seems to be a good choice. The retention behaviour of this compound is governed mainly by hydrophobic interaction with the stationary phase, the effect of polar or ionic interaction can be considered negligible. Identical retention conditions can be maintained for T even on most stationary phases designed for hydrophobic interaction chromatography[14] which means widespread applicability indeed.

The biplot obtained under mobile phase conditions equalised for unit retention of T is shown on Figure 2a. The most dissimilar compounds are DA, ME and P, which prompts that they should be selected as poles. Since the first two PCs account for only 94% of the variance in the data table, the third PC must be also taken into consideration. Figure 2b shows the result of projection onto the plane defined by PC1 and PC3.

As it is seen, the position of test components and also that of the stationary phases changed significantly compared to Figure 1a-c. Since the characteristics indicated by the poles selected are practically the same as were given for Figure 1a-c, the attraction and repulsion of objects (stationary phases) can be interpreted similarly, but the features represented by the poles relate here to the selectivity of columns, i.e. to the differences which can not be compensated by adjusting the strength of mobile phase.

Taking into account the similarity of DA and T indicated by the biplots above, DA can be considered an indicator of hydrophobicity. Accordingly, the DA-ME axis is a kind of polar-apolar axis. The order of columns obtained by orthogonal projection on it corresponds well to that of the ligands, however, it is not exactly the same as on Figure 1a-c. It indicates that higher value of some characteristics (e.g. hydrophobicity) does not imply inevitably higher selectivity. For example, the hydrophobic selectivity of butyl phases is higher than that of Z-C8 as indicated by the attraction towards the presumed hydrophobic pole (DA), while the hydrophobic strength of these phases is nearly equal (see Figure 1a-c).

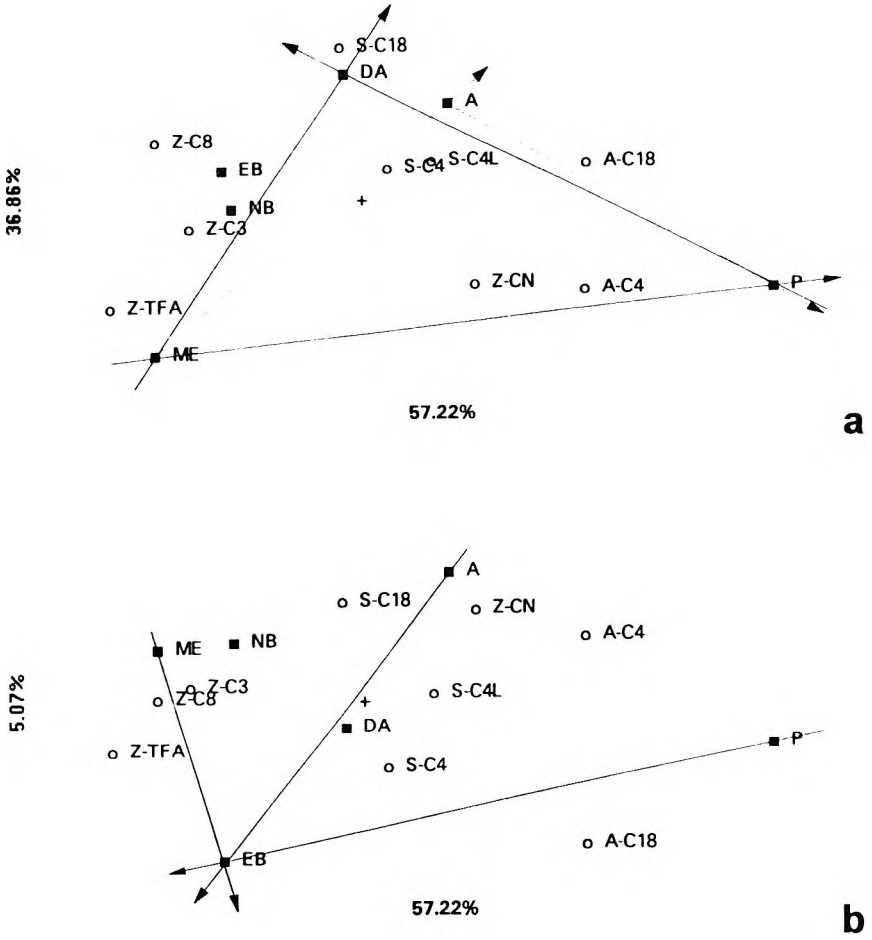


Figure 2. SMA biplots obtained under mobile phase compositions where k retention factor of toluene equals 1.(a) PC1 vs. PC2; (b) PC1 vs. PC3. (For details and explanation see text.)

The examination of the spread of stationary phases immediately reveals, that it is governed rather by their origin than by the ligand type. Columns from different sources are located at different regions on the biplot. The Zorbax columns are between the most hydrophobic (EB) and the polar-neutral (ME) test compounds, the Synchropak columns show the highest affinity towards the basic ones (A, DA) and the Aquopore columns are the nearest to the acidic pole (P). The only exception is Z-CN, which is shifted towards P, but this attraction can be attributed to the specific nature of ligand, as was indicated above.

This arrangement has nothing in common with the ligand type, rather it can be attributed to the different manufacturing and/or ligand binding chemistry of the columns.

The dissimilarity of nominally identical stationary phases from different sources is frequently experienced, and it is explained mainly by the unlike production process. Since this process is generally quite complex, many factors affect the final quality of stationary phases simultaneously. Consequently, similar selectivity differences can be expected for identical types of columns even from the same source, as it is shown by S-C4 and S-C4L. As seen, these differences can be revealed and identified under properly selected mobile phase conditions.

Note, these selectivity differences are less pronounced or less characteristic when conditions are not balanced for strength of the stationary phases. For example, Figure 1 indicates that the increased retention of acidic compounds on A-C18 results only from the large hydrophobicity of this column, but Figure 2 shows that some kind of polar interaction(s) also contribute. It is also seen that the differentiation between weaker columns is more relevant if the characterisation scheme is not dominated by the strength of columns.

Since the first two PCs explain only 94% of total variance, the spread of points along the third PC, i.e. the distance of objects and variables from the plane defined by the PC1 and PC2, must be also taken into consideration,. As it is seen on Figure 2b, EB and A are farest from this plane, the former is below and the latter is above. It prompts that these compounds should be also selected for poles. The closeness of the points in Figure 2a indicates that DA could be replaced with A (dashed axes on Figure 2a), but EB is a new pole. Correspondingly, three new axes must be formed, as shown on Figure 2b. It means, the complete representation of objects can be given in a tetrahedron instead of a triangle.

In most publications data are explained according to only the first two PCs. Further PCs are usually not used for interpretation or are completely neglected even if they have significant contributions. Here the third PC must be significant, since without it, i.e. only on Figure 2a, the position of EB can not be interpreted. The rather small contribution of this PC indicates that the feature represented by EB (hydrophobic selectivity) is less important with respect to present characterisation, the selectivity differences of stationary phases can be attributed mainly to polar interactions.

The inclusion of EB as indicator of hydrophobic selectivity involves the correction of interpretation given for DA, since this compound was used for the same purpose above. The intermediate position of DA between EB and A indicates that its retention is affected not only by hydrophobic interaction but by polar interaction too, originating from its basic character (see Figure 2b). Under non-balanced conditions this polarity is of secondary importance, but when the condition is equalised for different column strength, this feature becomes dominant. It means A and/or DA indicates the affinity of stationary phases towards basic compounds.

It is also seen that better selection of the basic indicator(s) is needed. The biplots show that the retention of A and DA does not result from a single effect, but their basic nature is suppressed, or at least confounded, by other stronger interaction(s). Since basic substances have primary importance in the pharmaceutical industry, the affinity or neutrality of stationary phases towards basic compounds should be clearly identified. This kind of affinity is generally attributed to the silanol activity of columns and it is often regarded as "polarity". However, the above results indicate that this classification is oversimplified, more exact distinction of possible interactions is needed.

Summarising the results obtained from the biplots, it seems, at least four different types of test compounds - hydrophobic, polar-neutral, acidic and basic- should be used for complete evaluation of the stationary phases. If the number of test compounds can be significantly reduced, more simple characterisation techniques providing the same information (e.g. ternary diagrams) can be used instead of SMA involving rather complex mathematical operations.[1,9] Although the calculations of these schemes are much simpler, they have the same shortage. Adding or removing stationary phases and/or test compounds is not a trivial task when multivariate techniques are used for characterisation. Modifications of this kind can significantly alter the results, consequently, after each changes the complete data set must be recalculated and re-evaluated . It is also true for testing column ageing in regular use.

After selecting the appropriate test components from the biplots obtained by SMA, the stationary phases are characterised according to their relative positions along the bipolar axes. However, these values can be derived directly from the retention data by calculating the corresponding relative retention factors, which provides a much simpler characterisation scheme. The strength (hydrophobicity) of columns can be tested by the relative hydrophobicity of T (x^*_T), i.e. by the mobile phase composition in which T is eluted with unit retention. This composition is calculated from the retention profile of T.[12,14,16]

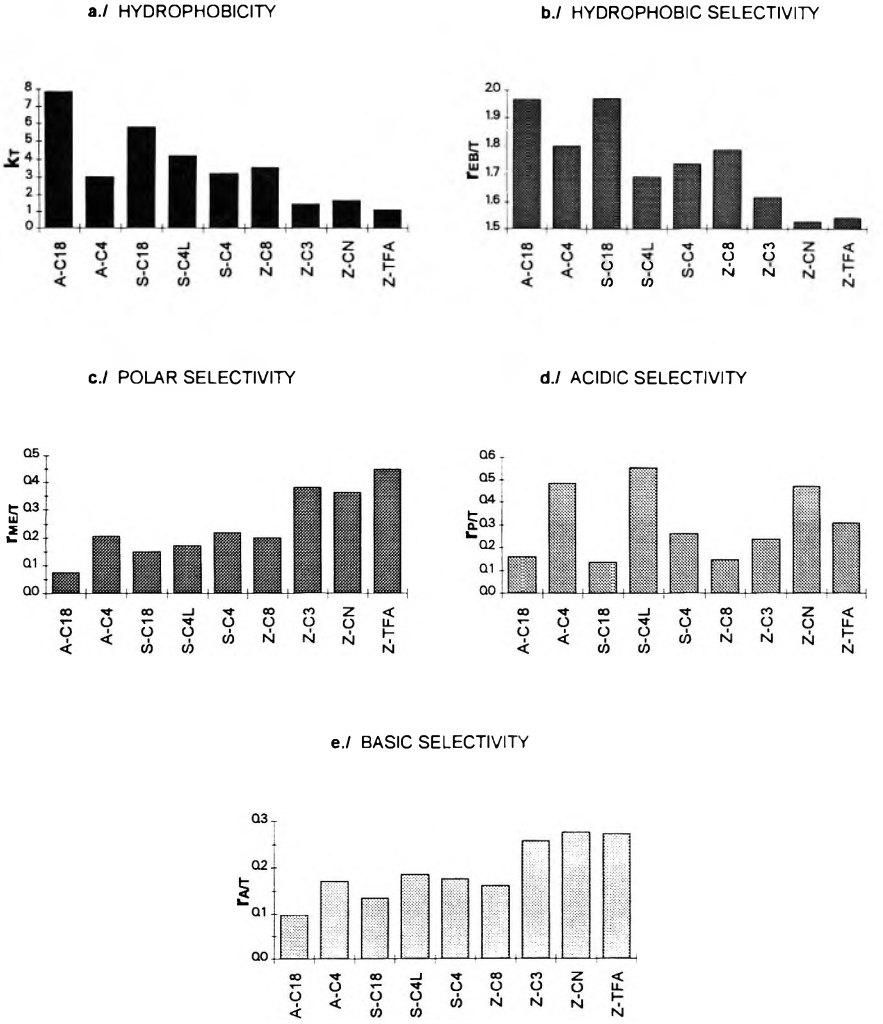


Figure 3. Single characteristics calculated from retention data obtained in 30% acetonitrile. (a) Retention factors of toluene; (b) Relative retentions of EB/T; (c) Relative retentions of ME/T; (d) Relative retentions of P/T; (e) Relative retentions of A/T.

Selectivity differences can be evaluated on the basis of retention of other test compounds. Confounding can be avoided, or at least minimised, if the measurements are carried out in mobile phases corresponding to x^*_T instead of using identical eluent on all columns.

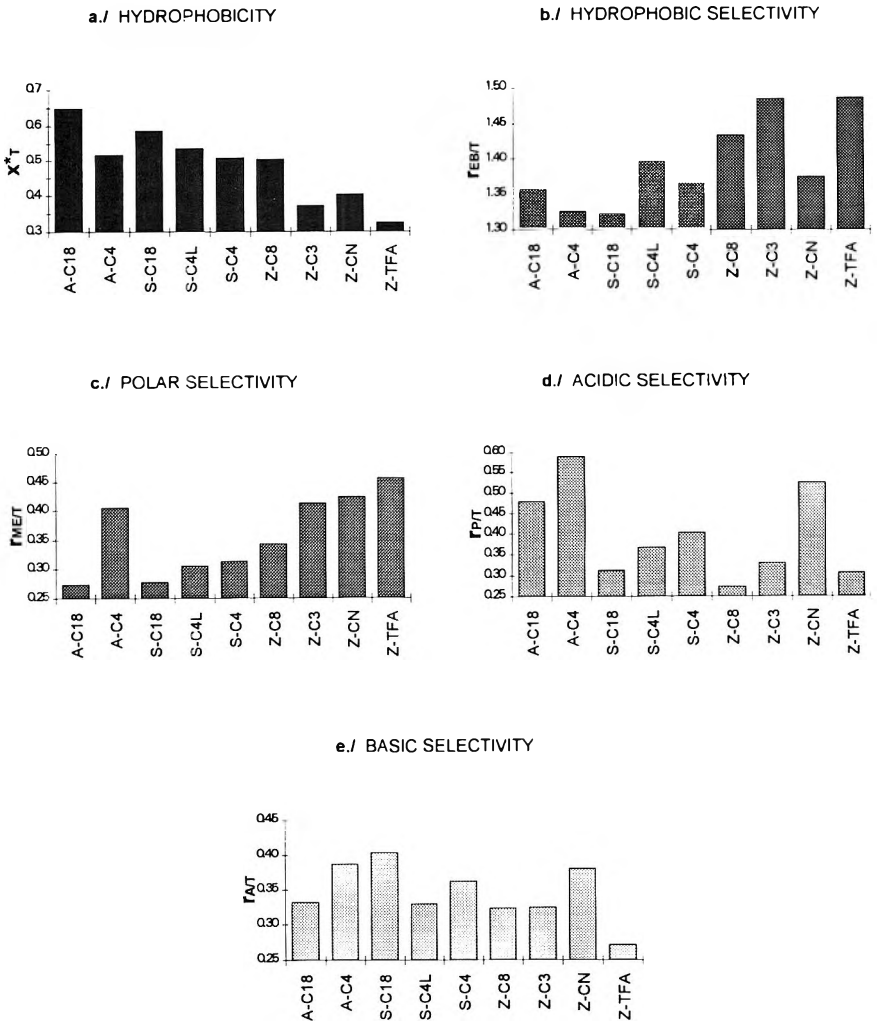


Figure 4. Single characteristics calculated from mobile phase compositions where k retention factor of toluene equals 1. (a) Retention factors of toluene; (b) Relative retentions of EB/T; (c) Relative retentions of ME/T; (d) Relative retentions of P/T; (e) Relative retentions of A/T.

The two approaches should give the same results for identical or very similar RP phases. In these instances, the retention behaviour of T does not change significantly from column to column; thus, hydrophobicity can be related directly to its retention and selectivity differences can be evaluated by

the retentions of other types of test compounds relative to T. In our case, the two methods result in dissimilar characterisations. Figures 3a-e show the characteristics calculated from retention values measured in 30% ACN. These characteristics were computed for all compounds found to be relevant with respect to characterisation.

This means some of the bipolar axes were neglected (e.g. P-ME), but some new ones, considered meaningful, were constructed. The retention of P relative to T ($r_{P/T}$) indicates the affinity of stationary phases towards polar-acidic compounds. $r_{A/T}$ relates to affinity towards polar-basic compounds. $r_{EB/T}$ measures the hydrophobic selectivity.

The same characteristics obtained under mobile phase conditions equalised for column strength are shown on Figure 4a-e. Here the hydrophobicity of stationary phases are described by the relative hydrophobicity of T (x^*_T). Under such conditions all the relative retention values used for characterisation equal to the absolute retention factors of test compounds ($k_T = 1$), however, the same notation was used on the figures for better comparison.

Note, Figure 3a-e and Figure 4a-e do not contain more information than Figure 1a and Figure 2a-b, they are only one-dimensional representations of the corresponding bipolar axes. However, the construction and interpretation of these scales are more straightforward than that of SMA biplots. In addition, this procedure is very convenient to use. Inserting a new column, or deleting an old one, does not affect the characterisation of others, similarities and differences remain relevant and characteristic. It can be easily improved further by applying new test compounds. In addition, the method can be used even for a single column, e.g., when the stability of a column is tested during regular use.

The results should be evaluated according to the task to be solved. During method development the separation may be further improved by changing to a stationary phase having different strength and/or selectivity. However, when a column in regular use must be exchanged, the most similar stationary phase is needed. The characteristics to be taken into account depend always on the features of the sample components. For example, the acidic or basic selectivity has no relevance if the mixture to be separated contains no compound of this type. On the other hand, for complex mixtures all characteristics should be taken into consideration. In this instances the use of biplots instead of single scales are more advantageous for selecting similar or different columns, since on biplots all the attractions and rejections towards different kind of test compounds are shown on the same plot.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support given by the Hungarian Academy of Sciences under grants OTKA No. F7634/1993 and OTKA No. 014977/1995.

REFERENCES

1. H.Engelhardt, H.Löw, W.Götzinger, J.Chromatogr., **544**, 371-379 (1991).

2. A. P.Goldberg, Anal. Chem., **54**, 342-345 (1982).

3. N.Tanaka, K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosoya, M. Araki, J. Chromatogr. Sci., **27**, 721-728 (1989).

4. H. A.Claessens, E. A.Vermeer, C. A. Cramers, LC-GC Int. **6(11)**, 692-700 (1993).

5. R. M.Smith, **Retention and Selectivity in Liquid Chromatography**, Journal of Chromatography Library, Vol. 57, Elsevier Sciences, 1995, pp.403-449.

6. S. J.Schmitz, H. Zwanziger, H. Engelhardt, J. Chromatogr., **544**, 381-391 (1991).

7. M. Righezza, J. R.Chrétien, J.Chromatogr., **544**, 393-411 (1991).

8 M. F.Delaney, A. N.Papas, M. J.Walters, J.Chromatogr., **410**, 31-41 (1987).

9. T. Hamoir, F. C. Sánchez, B. Bourguignon, D. L.Massart, J. Chromatogr. Sci., **32**, 488-498 (1994).

10. L. C.Sander, J. Chromatogr. Sci., **26**, 380-387 (1988).

11. L. C.Sander, S. A.Wise, J. Chromatogr., **656**, 335-351 (1993).

12. A. Bede, G. Rippel, L. Szepesy, H. A.Claessens, J.Chromatogr., **728**, 179-189 (1996).

13. H. Engelhardt, M. Jungheim, Chromatographia, **29**, 59-68 (1990).

14. G. Rippel, E. Alattyani, L. Szepesy, J. Chromatogr., **668**, 301-312 (1994), **678**, 380 (1994).

15. F. C.Sánchez, P. J.Lewi, D. L.Massart, Chem.Intell.Lab.Syst., **25**, 157-177 (1994).

16. G. Rippel, L. Szepesy, J. Chromatogr., **664**, 27-32 (1994).

17. B. A.Olsen, G. R.Sullivan, J. Chromatogr., **692**, 147-159 (1995).

Received May 23, 1996
Accepted September 10, 1996
Manuscript 4186

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1689–1701 (1997)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION AND SPECTROSCOPIC CHARACTERIZATION OF METABOLITES FROM THE BILE OF RATS RECEIVING RAPAMYCIN (SIROLIMUS) INTRAVENOUSLY

C. Paul Wang,*† Heng-Keang Lim, Kelvin W. Chan, JoAnn Scatina, Samuel F. Sisenwine

Division of Drug Metabolism
Wyeth-Ayerst Research
CN 8000
Princeton, New Jersey 08543

ABSTRACT

Ten major metabolites of rapamycin (M2, M3, M8, M9, M10, M11, M13, M14, M15, and M16) were isolated from pooled bile of intravenously dosed rats. Metabolites were extracted from the bile with ethyl acetate prior to isolation by HPLC using a Supelcosil SPLC-18, μm, 10 x 250 mm column. The mobile phase was a methanol/ammonium acetate linear gradient system. The isolated metabolites were characterized by negative ion FAB MS, ion-spray MS and ion-spray MS/MS analyses.

Metabolite M2 is oxygenated in the southern portion of rapamycin and the macrolide ring is opened. M3 is a structural isomer of rapamycin where the lactone ring is opened. M10 is oxygenated in the southern portion of rapamycin and the macrolide ring is intact. M13 is a monohydroxylation and demethylation metabolite and both biotransformations occurred at the southern portion. M8, M9, and M11 are monohydroxylation and demethylation metabolites. M14 and M15 are dihydroxylation metabolites. M16 is mainly a dihydroxylation metabolite.

INTRODUCTION

Rapamycin,[3S[3R*[S*(1R*,3S*,4S*)],6S*,7E,9S*,10S*,12S*,14R*,15E, 17E,19E,21R*,23R*,26S*,27S*,34aR*]]-9, 10, 12, 13, 14, 21, 22, 23, 24, 25, 26, 27, 32, 33, 34a-Hexadecahydro-9,27-dihydroxy-3-[2-(4-hydroxy-3-methoxy cyclohexyl)-1-methylethyl]-10,21-dimethoxy-6, 8, 12, 14, 20, 26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine-1, 5, 11, 28, 29 (4H,6H,31H)-pentone, (Figure 1), an antitumor and antifungal agent isolated from the fungus *Streptomyces* hygroscopicus,[1,2,3] has been found to have potent immunosuppressive activity while exhibiting little toxicity in primates.[4,5] Rapamycin is currently undergoing clinical trials as an immunosuppressive agent. The drug demonstrates a synergistic effect when co-administered with cyclosporine.[6,7]

We have recently reported the isolation and characterization of two major *in vitro* degradation products following incubation of rapamycin at 37°C in rat bile or ammonium acetate (pH 8.0).[8] Degradation product A was a macrolide ring-opened hydrolysis product of rapamycin where the ester bond linking C23 and C25 has been hydrolyzed. Degradation product B was a ring-opened isomer of rapamycin.

In another report, three major metabolites have been isolated and characterized from the plasma of rats receiving rapamycin orally.[9] M2 is monohydroxylated in the southern portion of rapamycin and the macrolide ring is opened. M3 is the degradation product B and M5 is O-demethylated on the C41 moiety. This 41-O-demethyl rapamycin was one of the metabolites isolated by Christians et. al. from in vitro metabolism of rapamycin in microsomes from human liver and rat small intestinal microsomes.[10] The other was a hydroxylated metabolite but the position of hydroxylation was not determined.

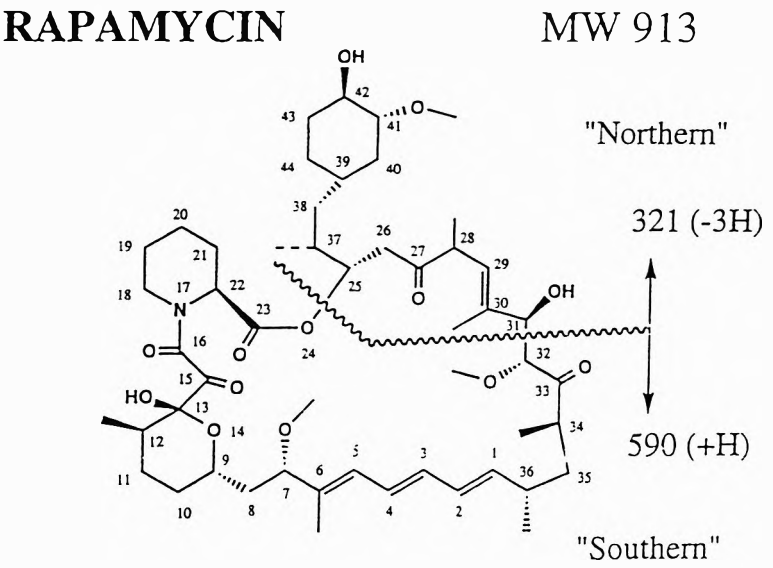


Figure 1. Chemical structure of rapamycin and its major fragmentation pathway.

However, the identification of *in vivo* biliary metabolites of rapamycin has not yet been reported. The present study was therefore conducted to isolate rapamycin metabolites from the bile of rats using a semi-preparative HPLC system and followed by structural characterization using mass spectrometry.

EXPERIMENTAL

Materials

Rapamycin was obtained from Wyeth-Ayerst Research, Rouses Point, New York. It was formulated in a vehicle consisting of 6% (v/v) ethanol, 5.6% (w/v) Polysorbate 80, 51.7% (w/v) PEG 300 and 36.7% (v/v) water at a concentration of 1.45 mg/mL for the doses used in the study. Ammonium acetate (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ). All solvents used in the study were HPLC grade.

Animals and Treatment

Twenty-nine Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottdale, PA), weighing between 330 and 385 g were surgically prepared with implanted bile duct and jugular vein cannulas. Animals were not fasted overnight and received electrolyte supplemented water. Prior to dosing, control bile samples were collected. The animals were dosed slowly via the indwelling jugular vein cannula or tail vein when the jugular vein did not remain patent. The animals received rapamycin at a dose of 1.45 mg/kg. Bile was collected over dry ice at intervals of 0-4, 4-8, 8-12 and 12-24 hr after dosing. The samples were frozen at -20°C until analysis.

Instrumentation

The HPLC system consisted of a Waters 600E system controller and pump (Waters Associates, Milford, MA), a Waters 490E programmable multi-wavelength detector, a Waters U-6K manual injector and a Hewlett-Packard 3390A integrator. Separation of metabolites was achieved using Supelcosil SPLC-18, 5 μm, 10 x 250 mm column (Supelco, Bellefonte, PA). Negative electrospray ionization (ESI) single and multiple-stage mass spectra were acquired with a Sciex TAGA 6000E mass spectrometer upgraded to an API III and equipped with a home made ion sprayer.[11] FAB mass spectra were acquired using a Kratos MS 50 mass spectrometer equipped with a FAB ion source and operated in the negative ion mode.

Isolation of Rapamycin Metabolites in Rat Bile

The bile (0 to 4 hr), which contained the largest number and quantity of metabolites, was used for the isolation of metabolites. A total of 122 mL of bile was extracted with ethyl acetate. The ethyl acetate extracts were taken to dryness under nitrogen. The residues were dissolved in methanol/water (65/35) prior to injection onto the HPLC system. A Supelco semipreparative SPLC-18, 25 cm x 10 mm, 5 μm column was used for isolation of metabolites. The mobile phase gradient profile is described in Table 1. Detection was by UV absorbance at 276 nm. The HPLC eluates corresponding to the ten major metabolite peaks were collected manually. Methanol in the eluates was removed with a Savant AS-160 at room temperature under manual mode. The remaining aqueous residues were lyophilized to dryness. Metabolite fractions were then frozen at -80°C until analysis.

Table 1

Stepwise Gradient System Used for the Isolation of Rapamycin in Metabolites[a]

Time (min)	Flow Rate (mL/min)	Percent Methanol	Percent 0.05 M Ammonium Acetate
0.0	2.0	62	38
8.0	2.0	84	16
95	2.0	84	16
100	2.0	62	38

[a]Linear gradient was used between each time point; column was Supelco SPLC-18, 250 x 10.0 mm, 5 μm.

Structural Elucidation of Metabolites by Mass Spectrometry

The isolated metabolites were subjected to either FAB MS and/or ion spray MS and MS/MS analyses. Due to limited quantities, only M2, M3, M10 and M13 were analyzed by LC/MS/MS. In the FAB experiments, the primary beam was 1 mA of 7kV Xenon atoms. Resolution of the instrument was adjusted to 2000 (10% valley). Triethanolamine was employed as the matrix for the samples. Each sample was dissolved in methylene chloride, and an aliquot of the solution was mixed with the matrix on a copper probe tip before insertion into the mass spectrometer for analysis.

For infusion ESI/MS and MS/MS analyses, the sample was initially dissolved in either dichloromethane or absolute ethanol and an aliquot was removed for analysis. The organic solvent was then evaporated to dryness under nitrogen at room temperature. Prior to dissolving in 20% 4 mM ammonium acetate in methanol, the solution was infused at 4 μL/min into the mass spectrometer using a Harvard Syringe Pump. The mass spectrometer was calibrated in positive ion mode up to 2000 daltons with PPGs (polypropylene glycols) and switched to negative polarity prior to analysis. Final optimization of sprayer position and mass spectrometer conditions for maximum sensitivity was carried out by infusing with rapamycin standard (1 mg/mL). The sample was sprayed into the mass spectrometer at -3.2 kV and desolvation was carried out at declustering potential of 66 volts. Full scan ESI mass spectra were acquired by scanning Q1 at unit resolution from m/z 300 to 1000 at step size of 0.2 and dwell time of 0.57 seconds. Product ion mass spectra were obtained by setting Q1 to transmit deprotonated molecular anion $(M-H)^-$ at unit resolution

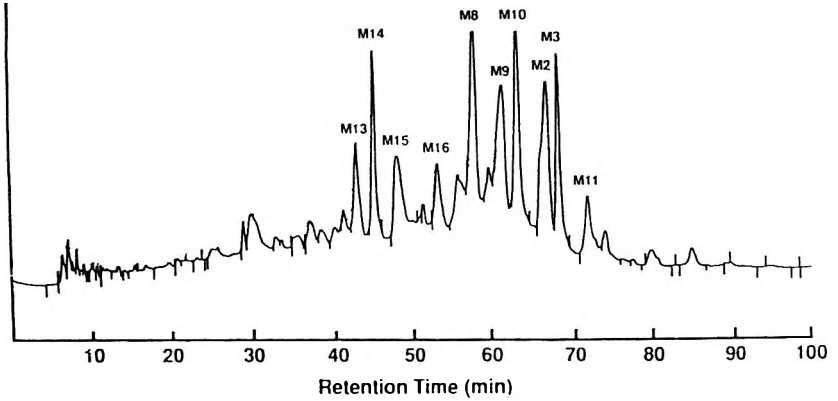


Figure 2. Semipreparative HPLC chromatogram showing separation of rapamycin metabolites labelled as M13, M14, M15, M16, M8, M9, M10, M2, M3 and M11 from analysis of pooled 0-4 hour post-dose bile from rats administered rapamycin intravenously at a dose of 1.45 mg/kg.

Table 2

Summary of MS Analysis of the Biliary Metabolites of Rapamycin in Rates

Metabolite Number	Mw[a]	Possible Metabolic Transformation[b]
M2	929	monohydroxylation
M3	913	ring opened isomer
M8	915	demethylation and monohydroxylation
M9	915	demethylation and monohydroxylation
M10	929	monohydroxylation
M11	915	demethylation and monohydroxylation
M13	915	demethylation and monohydroxylation
M14	945	dihydroxylation
M15	945	dihydroxylation
M16[c]	945	dihydroxylation
	931	hydrolysis product of rapamycin
	915	demethylationand monohydroxylation

[a]MW based on nominal monoisotopic mass.
[b]epoxidation across a double bond can be substituted for hydroxylation.
[c]this metabolite was contaminated with two other metabolites of Mws = 931 and 915.

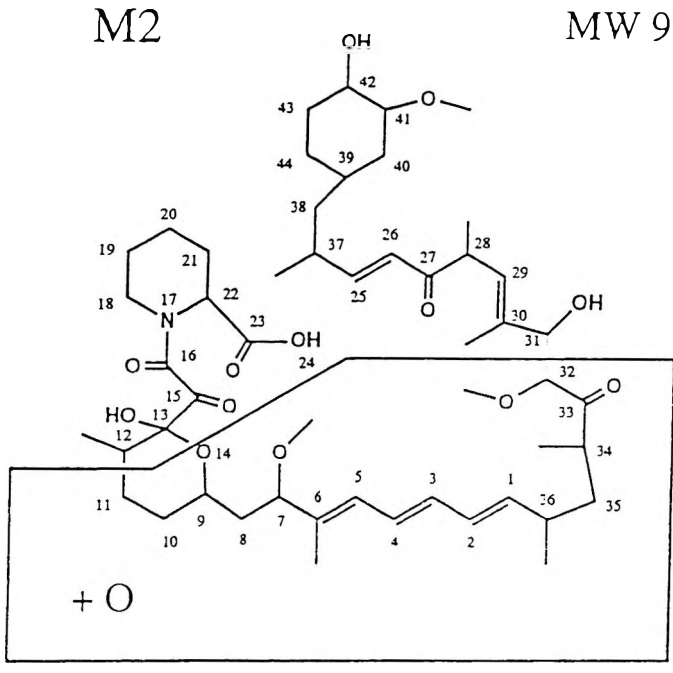


Figure 3. Proposed site of metabolic transformation of rapamycin to metabolite M2.

to Q2. The molecular anion was subjected to collision-activated dissociation (CAD) at collision energies of 50-53 eV and at collision pressure of 5 x 10^{12} atoms of argon/cm^3. The product ions were mass analyzed by scanning Q3 at unit resolution from m/z 40 to 1000 at step size of 1 and dwell time of 2.08 seconds. In each case, the mass spectrum was a sum of 10 scans.

RESULTS

Identification of Rapamycin Metabolites in Rat Bile

The major metabolites that were isolated by semipreparative HPLC system are labeled as M13, M14, M15, M16, M8, M9, M10, M2, M3 and M11 as shown in Figure 2. The notation of M2 and M3 is consistent with that used previously to identify metabolites in rat plasma.[9] The molecular weights (MW) and possible metabolic transformation of these metabolites were obtained from their mass spectra and summarized in Table 2.

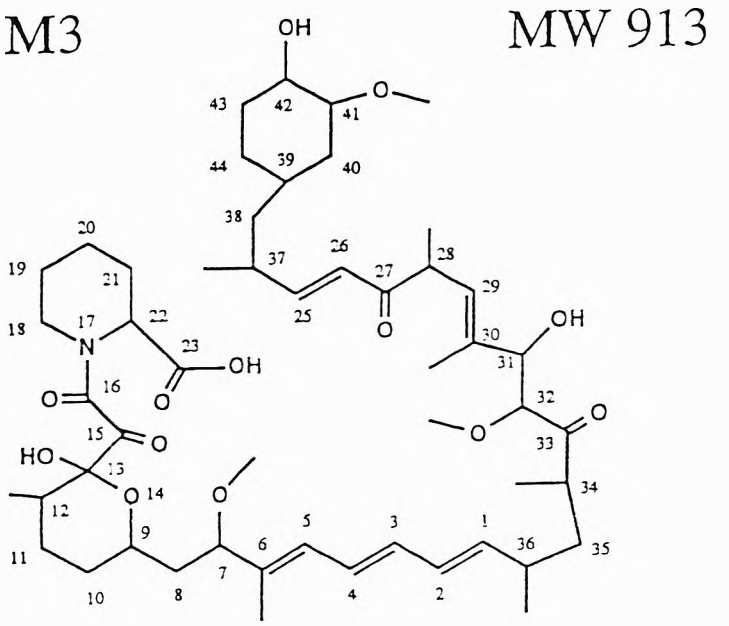


Figure 4. Proposed structure of M3.

Negative FAB mass spectrometric analysis was used to determine whether the macrolide ring is intact or open. For example, the observation of a deprotonated molecular anion $(M-H)^-$ is an indication of opening of the macrolide ring whereas a compound with an intact macrolide ring gave a molecular anion by capturing an electron (M^-).[12] Further insight into the possible sites of metabolic transformation was provided by tandem mass spectrometry (MS/MS) analysis. The product ion mass spectrum of rapamycin shows two complimentary diagnostic product ions at m/z 321 and 590 which correspond to the "northern" and "southern" portions of the molecule (Figure 1), respectively. The latter two product ions are useful for monitoring biotransformation of the rapamycin molecule by the mass shift technique.[13]

M2: The full scan mass spectrum of M2 shows the presence of a low abundance deprotonated molecular anion at m/z 928.8 which is 16 daltons more than rapamycin. This suggests a biotransformation by either aliphatic hydroxylation or epoxidation. The site of biotransformation can be located from the product ion mass spectrum which shows the complimentary fragment ions at m/z 321 and 606. The fragment ion at m/z 321 suggests that the "northern" portion of rapamycin is unchanged, and biotransformation at the

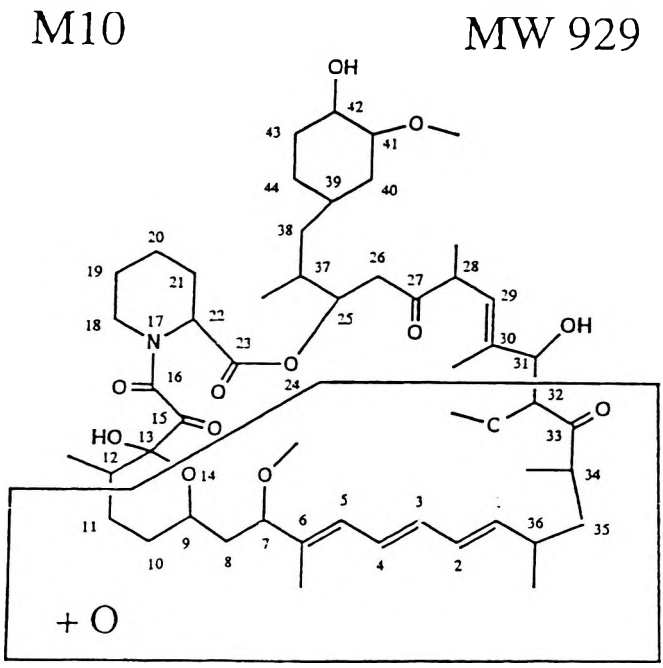


Figure 5. Proposed site of metabolic transformation of rapamycin to metabolite M10.

"southern" portion of rapamycin is inferred from the ion at m/z 606, which is 16 daltons higher than the corresponding fragment ion at m/z 590 from the parent compound. Furthermore, the base peak at m/z 240 indicates that the change is unlikely on carbons 12 to 23 since m/z 240 product ion corresponds to that portion of rapamycin molecule as shown in Figure 1.

The negative ion FAB mass spectrum obtained from this metabolite shows a $[M-H]^-$ at m/z 928.5, suggesting that it is in the macrolide ring opened form. The proposed site of metabolic transformation of M2 is depicted in Figure 3.

M3: This metabolite has the same deprotonated molecular anion (m/z 912.8) as rapamycin in the full scan ion spray mass spectrum. That this metabolite is isomeric with rapamycin is indicated by the similarity of its product ion mass spectrum to that of rapamycin. M3 has been identified and reported as a degradation product of rapamycin.[8] Figure 4 shows the structure of M3.

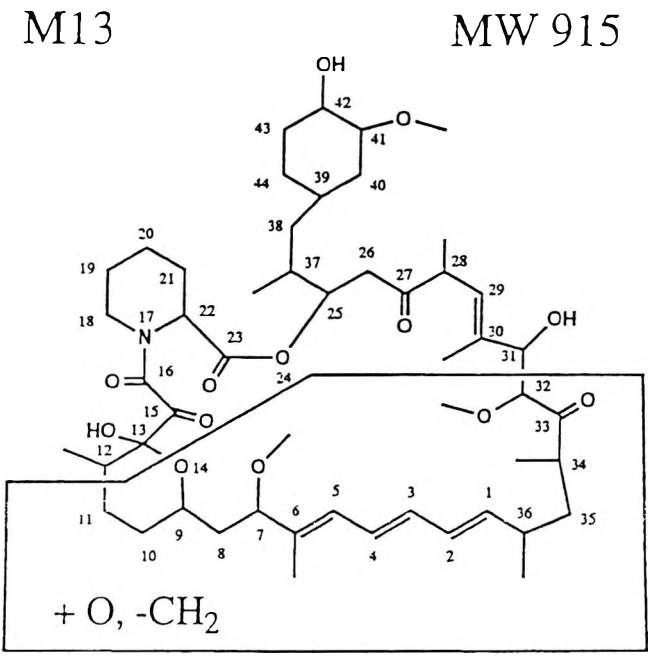


Figure 6. Proposed site of metabolic transformation of rapamycin to metabolite M13.

M10: The mass spectra of this metabolite are similar to those of M2 and, therefore, very likely to be produced from either aliphatic hydroxylation or epoxidation. Since the retention time of this metabolite is different from that of M2, this metabolite is likely to be isomeric with M2. Unlike M2 and M3, the M (m/z 929.5) is observed in the FAB mass spectrum, indicating that the macrolide ring is intact. Figure 5 shows the proposed structure of M10.

M13: The full scan ion spray mass spectrum contains a low abundance molecular anion at m/z 914.6 which suggests that this metabolite is likely to be a product of both hydroxylation/or epoxidation and demethylation. This speculation is corroborated by the complimentary product ions at m/z 321 and 592 which indicate that the "northern" portion of rapamycin is intact.

That both biotransformations must have occurred at the "southern" portion of rapamycin is deduced from the product ion at m/z 592. The abundant product ion at m/z 240 precludes any metabolic change occurring on carbons 12 to 23.

Demethylation is likely to occur at either of the two metabolic soft spots; for example, the methoxy group on carbon 7 or 32. There was not sufficient amount of sample for FAB MS experiment to determine if the macrolide ring is opened or intact. A proposed, closed ring structure of M13 is depicted in Figure 6.

DISCUSSION

A semipreparative HPLC procedure using a C-18 column has been successfully developed for the isolation of ten major biliary metabolites of rapamycin in rats (M13, M14, M15, M16, M8, M9, M10, M2, M3 and M11). Baseline separation of these metabolites was achieved with a reverse phase gradient system.

The major metabolic transformations of rapamycin in rats are aliphatic hydroxylation and O-demethylation. Ten major metabolites were isolated in the current study. Tandem mass spectral data were obtained on four samples and FAB MS data on three metabolites. These data have enabled us to propose possible structures for M2, M3, M10 and M13 as shown in Figures 3-6. Metabolites M2 and M3 are the major metabolites in rat plasma.[9] However, the other major plasma metabolite M5 (O-demethylated in the C41 moiety) and rapamycin were not found in rat bile at significant amounts in the current study. The pattern of metabolism of rapamycin is similar to that of FK506, another immunosuppressive agent, whose major metabolites are also produced via demethylation and oxygenation.[14]

ACKNOWLEDGMENT

We would like to acknowledge Professor Jack D. Henion at Cornell University for use of the Sciex TAGA 6000E for acquisition of the data. We wish to thank Mr. Joseph Cantone at Wyeth-Ayerst Research for acquiring the FAB mass spectra of the metabolites.

REFERENCES

1. S. N. Sehgal, H. Baker, C. Vezina. "Rapamycin (AY-22989), A New Antifungal Antibiotic. Fermentation, Isolation and Characterization," J. Antibiot., **28**, 729-32 (1975).

2. C. Vezina, A. Kudelski, S. N. Sehgal, "Rapamycin (AY-22989), A New Antifungal Antibiotic I. Taxonomy of the Producing Streptomycete and Isolation of the Active Principle," J. Antibiot., **28**, 721-726 (1975).

3. C. P. Eng, S. N. Sehgal, C. Vezina, "Activity of Rapamycin (AY-22989) Against Transplanted Tumors," J. Antibiot., **37**, 1231-1237 (1984).

4. R. Y. Calne, S. Lim, A. Somaan, D. St. J. Collier, S. G. Pollard, D. J. G. White, "Rapamycin for Immunosuppression in Organ Allografting," The Lancet, 227 (1989).

5. S. N. Sehgal, J. Y. Chang, "Rapamycin: A New Immunosuppressive Macrolide," Transplant. Immunol. Lett., 7, 12-14 (1990).

6. P. M. Kimball, R. H. Kerman, D. B. Kahan, "Production of Synergistic but Non-identical Mechanisms of Immunosuppression by Rapamycin and Cyclosporine," Transplantation, **51**, 486-490 (1991).

7. B. D. Kahan, S. Gibbons, N. Tejpal, S. M. Stepkowski, and T. C. Chou, "Synergistic Interactions of Cyclosporine and Rapamycin to Inhibit Immune Performances of Normal Human Peripheral Blood Lymphocytes In Vitro," Transplantation, **51**, 232-239 (1991).

8. C. P. Wang, K. W. Chan, R. A. Schiksnis, J. A. Scatina, S. F. Sisenwine, "High Performance Liquid Chromatographic Isolation, Spectroscopic Characterization and Immunosuppressive Activities of Two Rapamycin Degradation Products," J. Liq. Chrom., **17 (16)**, 3383-3392 (1994).

9. C. P. Wang, H. K. Lim, K. W. Chan, J. A. Scatina, S. F. Sisenwine, "High Performance Liquid Chromatographic Isolation and Spectroscopic Characterization of Three Major Metabolites from the Plasma of Rats Receiving Rapamycin (Sirolimus) Orally, J. Liq. Chrom., **18 (13)**, 2559-2568 (1995).

10. U. Christians, M. Sattler, H. M. Schiebel, C. Kruse, H. H. Radeke, A. Linck, K.-FR. Sewing, "Isolation of Two Immunosuppressive Metabolites After In Vitro Metabolism of Rapamycin," Drug Metab. Dispos., **20**, 186-191 (1992).

11. A. P. Bruins, T. R. Covey, J. D. Henion, "Ion Spray Interface for Combined Liquid Chromatography/Atmospheric Pressure Ionization Mass Spectrometry," Anal. Chem., **59**, 2642-46 (1987).

12. J. Cantone, K. Chan, "Mass Spectrometric Characteristics of the Immunosuppressive Agent Rapamycin," Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics. 1881-1882 (1992).

13. F. P. Abramson, "Mass Spectrometry in Pharmacology," in **Biomedical Applications of Mass Spectrometry,** C. H. Suelter, J. D. Watson, editors., John Wiley & Sons, New York, 289-347 (1990).

14. K. Iwasaki, T. Shiraga, K. Nagase, Z. Tozuka, K. Noda, S. Sakuma, T. Fujitsu, K. Shimatani, A. Sato, M. Fujioka, "Isolation, Identification, and Biological Activities of Oxidative Metabolites of FK506, A Potent Immunosuppressive Macrolide Lactone," Drug Metab. Dispos., **21 (6)**, 971-977 (1993).

† This work was presented at the Fourth International ISSX Meeting, Seattle, WA, August 1995.

Received July 30, 1996
Accepted August 27, 1996
Manuscript 4252

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1703-1715 (1997)

ESTIMATION OF THE RP-HPLC LIPOPHILICITY PARAMETERS LOG K', AND LOG K_W, A COMPARISON WITH THE HYDROPHOBICITY INDEX φ_0[†]

M. L. C. Montanari, C. A. Montanari,
D. Piló-Veloso, Q. B. Cass[20,]*

Departamento de Química/ICEx
Universidade Federal de Minas Gerai
Belo Horizonte, 31270-901,MG, Brazil

Departamento de Química
Universidade Federal de São Carlos
Caixa Postal 676
São Carlos, 13565-905, SP, Brazil.

ABSTRACT

Chromatographic retention parameters k' and k_w were determined by RP-HPLC for a set of standard non-congeneric compounds and the correlation with log $P_{o/w}$ established. Then, log $P_{o/w}$ was calculated for a series of antimicrobial hydrazides, as a test set. A comparison of the chromatographic parameters log k' and log k_w with the hydrophobicity index φ_0 were also made. It seems that for the study compounds, log k_w describes the partitioning better than the parameter φ_0.

INTRODUCTION

Chromatographic retention parameters have been used in quantitative structure-retention relationships (QSRRs) as numerical descriptors of physico-chemical parameters. Many such descriptors have been described - log k', log k'_w, φ_0.[1-5] Due to the advantages of the chromatographic technique over the classical "shake-flask" method, attempts have been made to correlate the chromatographic retention parameters with log $P_{o/w}$.[6,7] Therefore, the literature is full of examples of these relationships.[4,8] However, many of them rely upon determination of retention time ignoring the models employed.[9,10]

As far as the octanol/water model is concerned, the RP-HPLC is believed to mimic not only the biological membrane but also the "shake-flask".[1, 4] However, there is no agreement regarding which stationary phase should be used. Also, the use of either the isocratic or the polycratic mobile phase constitutes a problem. Thus, columns ranging from C_4 to C_{18}, including polymeric and, more recently, immobilised artificial membrane have been used.[1] The mobile phase has been used usually with methanol as the modifier in the polycratic method.[8,11]

Despite the disagreement, chromatographic retention parameters have been correlated to log P through a "Colander type equation":[13]

$$\log k' = a \log P + b \qquad (1)$$

Provided that a equals 1 and b equals zero, equation (1) could be regarded as the descriptor of the partitioning mechanism itself. However, this is far from being the case, and much of the data in the literature, including some reported in this paper, deviate substantially from the ideal behaviour.

It is important to notice that the retention process that operates in the RP-HPLC is very complex and although hydrogen bonding and solvophobic interactions seem to play the predominant role, other interactions[14-16] do not allow a well behaved relationship between log k' and log P.

Minick et al.[8] have shown that the coefficients of equation (1) are satisfied when a small portion of octanol is added into the mobile phase with n-decilamine. These reagents act as a silanol suppresser.

The use of water alone, as a mobile phase, would be desired for the measurement of retention time used as a hydrophobic index. However, this causes very long retention time. Also, the retention mechanism with

chemically bonded stationary phases, changes with high water content in the mobile phase. Thus, the use of a co-solvent is needed. The use of a co-solvent diminishes the distinction between the two phases because the solute "drags" not only water molecules into the stationary phase, but also the co-solvent.[17] In trying to overcome these problems, polycratic measurements have been used. The capacity factor k_w can be obtained by extrapolating the values of k' measured to the value of 0% organic modifier in the mobile phase.

$$\log k' = \log k'_w - S\varphi \quad (2)$$

where φ is the volume fraction of organic modifier.

The relationship is not always linear:

$$\log k' = a\varphi + b \quad (3)$$

It can sometimes be described as a relationship of quadratic dependence of φ:[12]

$$\log k' = a\varphi^2 + b\varphi + c \quad (4)$$

However, the linear portion has been used, mainly when methanol is the modifier agent.

Log k' can not be used as a universal scale because log k' is dependent on chromatographic conditions, i.e., it depends on the mobile and stationary phases used. K_w has been suggested as a better descriptor than k', as it is independent of any effect of the organic modifier and relies on the stationary phase alone.[18] However, one of major problems with bonded stationary phases is that, although nowadays they show good reproducibility by being prepared by the same manufacturer, big differences in behaviour are observed with bonded stationary phases prepared from different makers. This is evident if one takes into consideration the differences of the silica used as support in the preparation of those phases.[18]

Bearing this in mind, Valks and Sligel[2] introduced a new chromatographic hydrophobicity index, φ_0, based on the slope and intercept of the log k' versus organic modifier concentration plot. The parameter φ_0 is set up by taking log k' = 0 instead of $\varphi = 0$. Thus, this parameter is independent of the RP column type and length, flow-rate and also the mobile phase composition.

As an on-going program in lipophilicity, a series of antimicrobial hydrazides was studied. Log k', log k_w and φ_0 were determined and their correlation with log P was examined. The attempt to apply this new parameter φ_0 to our work was based on the fact that this parameter, as it is claimed,[2] could be used as an universal scale while k' and k_w cannot.

MATERIALS AND METHODS

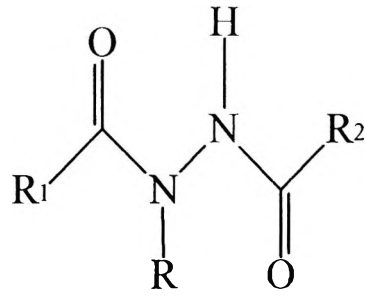
The HPLC system consisted of a Shimadzu LC-10AD pump, a SPD-6AV UV detector operated at 210 or 254 nm, and a LC-R6A chromatopac recorder. A Waters 510 pump with a LC-481 UV detector and a Shimadzu CR-4A recorder were also used. A Rheodyne 7125 injector fitted with a 20 μL loop was used in both cases. The column used was a LiChrosorb RP-8, 5m, 25x0.4 cm id, obtained from Merck (Darmstard, Germany).

The retention time of sodium nitrate, detected at 210 nm, was used as the column dead-time. Methanol was also used for the determination of the dead-time. The solvents used were of Merck HPLC-grade. They were filtered through a nylon membrane (4.7 Å) and degassed prior to use. The mobile phase consisted of methanol: 0.1M ammonium acetate buffer, pH = 4.6 (65:35v/v) for the isocractic measurements (system I). When octanol was used it was added at 0.25%v/v (system II). For the polycratic measurements the composition of the mobile phase was varied from 75% to 25% methanol in the 0.1M ammonium acetate buffer, pH_{app} = 4.6. The samples were run in triplicate and the capacity factor, k', were calculated as usual:

$$k' = t_r\text{-}t_0/t_0.$$

All test compounds were newly synthesised and their purity checked by HPLC. Provided that the retention times of the test compounds are allocated correctly this procedure is not necessary.

The compounds used as standard for the scale of hydrophobicity were: (I) acetanilide, (II) benzaldehyde, (III) benzophenone, (IV) pyridine, (V) toluene, (VI) formamide, (VII) benzyl alcohol, (VIII) acetophenone, (IX) 2-naftol, (X) ethyleneglicol, (XI) quinoline, (XII) ethyl acetate and (XIII) octanol. Measured octanol-water partition coefficients were obtained from Leo et al.[6] Partition coefficients of the study hydrazides, Figure 1, were calculated by employing the Hansch/Leo. approach.[6]



Hydrazides	R	R^1	R^2
1	$R = C_6H_5$	$R^1 = CH_3$	$R^2 = CH_3$
2	$R = C_6H_5$	$R^1 = CH_2CH_3$	$R^2 = CH_2CH_3$
3	$R = C_6H_5$	$R^1 = C_6H_5$	$R^2 = C_6H_5$
4	$R = C_6H_5$	$R^1 = O_2NC_6H_4$	$R^2 = O_2NC_6H_4$
5	$R = C_6H_5$	$R^1 = BrC_6H_4$	$R^2 = BrC_6H_4$
6	$R = C_6H_5$	$R^1 = MeOC_6H_4$	$R^2 = MeOC_6H_4$
7	$R = C_6H_5$	$R^1 = ClC_6H_4$	$R^2 = ClC_6H_4$
8	R = H	$R^1 = C_6H_5$	$R^2 = C_6H_5$
9	R = H	$R^1 = MeOC_6H_4$	$R^2 = MeOC_6H_4$
10	R = H	$R^1 = O_2NC_6H_4$	$R^2 = O_2NC_6H_4$
11	R = H	$R^1 = MeC_6H_4$	$R^2 = C_6H_5$
12	R = H	$R^1 = MeOC_6H_4$,	$R^2 = C_6H_5$
13	$R = CH_3$	$R^1 = O_2NC_6H_4$	$R^1 = O_2NC_6H_4$

Figure 1. Structures of study hydrazides 1-13.

RESULTS AND DISCUSSION

Table 1 shows log k' values obtained with the standard compounds used in the calibration scale. The correlation between log k' versus log $P_{o/w}$ for those dissimilar compounds (donors, acceptors and amphiprotics) using system I resulted in equation 5:

$$\log k' = 0.242(\pm 0.08)\log P_{o/w} - 0.452(\pm 0.16) \quad (5)$$
$$(n = 13,\ r = 0.902,\ r^2 = 0.814,\ s = 0.191,\ F = 48.14,\ r^2_{cv} = 0.720)$$

When the proton donors (except pyridine) compounds IV, IX, X and XIII were excluded equation 6 was obtained:

$$\log k' = 0.336(\pm 0.04)\log P_{o/w} - 0.556(\pm 0.7) \quad (6)$$
$$(n = 9,\ r = 0.992,\ r^2 = 0.984,\ s = 0.060,\ F = 453.26,\ r^2_{cv} = 0.950)$$

Table 1

Log K' and Log $P_{o/w}$ for the Standard Compounds Composing the Chromatographic Scale

	Compounds	Classif.	log k'(i)	log k'(ii)	log $P_{o/w}$
I.	Acetanilide	m, f	-0.24	-0.274	1.16
II.	Benzaldehyde	a	-0.018	-0.148	1.48
III.	Benzophenone	a	0.573	0.407	3.18
IV.	Pyridine	a	-0.054	---	0.65
V.	Toluene	m, f	0.415	0.069	2.80
VI.	Formamide	m, f	-0.991	-1.172	-1.51
VII.	Benzyl Alcohol	d, f	-0.272	-0.516	1.10
VIII.	Acetophenone	a	-0.053	-0.046	1.58
IX.	2-Naftol	d, f	0.014	---	2.70
X.	Ethyleneglicol	d, ff	-0.799	---	-1.79
XI.	Quinoline	a	0.128	0.162	2.03
XII	Ethyl Acetate	a	-0.332	-0.253	0.73
XIII.	Octanol	d, f	-0.067	---	3.15

(i) System with no added octanol: (ii) system with octanol (see text for explanation); (iii) Data from reference [6]. a = acceptor, d = donor, m = mixed, f = amphiprotic, ff = double amphiprotic. (-) not measured

Using system II, the correlation observed was that shown in equation 7:

$$\log k' = 0.321(\pm 0.09)\log P_{o/w} - 0.644(\pm 0.16) \quad (7)$$
$$(n = 9,\ r = 0.955,\ r^2 = 0.911,\ s = 0.144,\ F = 72.68,\ r^2_{cv} = 0.865)$$

The weakening in the statistical significance of equation 7, when compared to equation 6, can probably be explained according to Beezer's[19] remark that the amount of octanol added to the mobile phase was too high.

Equations 6 and 7 have similar coefficients which suggests that the same mechanism operates in both systems. Equation 6 was then used to predict the log $P_{o/w}$ for the hydrazides showed in Fig.1.

Table 2 shows log k' obtained for the study compounds and equation 8 shows the regression analysis:

Table 2

Experimental Log K' and Calculated Log $P_{o/w}$ for Some Study Hydrizides

Compounds	log k' (I)	log k' (II)	log P (III)
1	-0.47	-0.58	-0.52
2	-0.18	-0.29	0.48
3	0.09	0.13	3.68
4	0.23	---	3.12
5	0.85	0.82	5.40
6	0.39	0.26	3.64
7	0.83	0.75	5.10
8	-0.35	-0.61	1.92
9	-0.24	-0.45	1.88
10	-0.86	-0.97	1.36

See Figure 1 for structures. (i) System with no octanol; (ii) system with octanol (see text for explanation); (iii) Data calculated as from reference 6.

$$\log k' = 0.252(\pm 0.12)\log P_{o/w} - 0.627(\pm 0.37) \quad (8)$$
$$(n = 10,\ r = 0.871,\ r^2 = 0.759,\ s = 0.290,\ F = 25.14,\ r^2_{cv} = 0.608)$$

It is noticeable that equation (8) also has similar regression coefficients, which contribute to a good inference to the "Collander's type equation".

Excluding compounds 1 and 2 (Figure 1), the equation (8) improves to (9):

$$\log k' = 0.380(\pm 0.11)\log P_{o/w} - 1.122(\pm 0.38) \quad (9)$$
$$(n = 8,\ r = 0.962,\ r^2 = 0.925,\ s = 0.173,\ F = 74.93,\ r^2_{cv} = 0.863)$$

When system II was used equation 10 came out:

$$\log k' = 0.421(\pm 0.07)\log P_{o/w} - 1.394(\pm 0.27) \quad (10)$$
$$n = 7,\ r = 0.988,\ r^2 = 0.976,\ s = 0.114,\ F = 213.52,\ r^2_{cv} = 0.952)$$

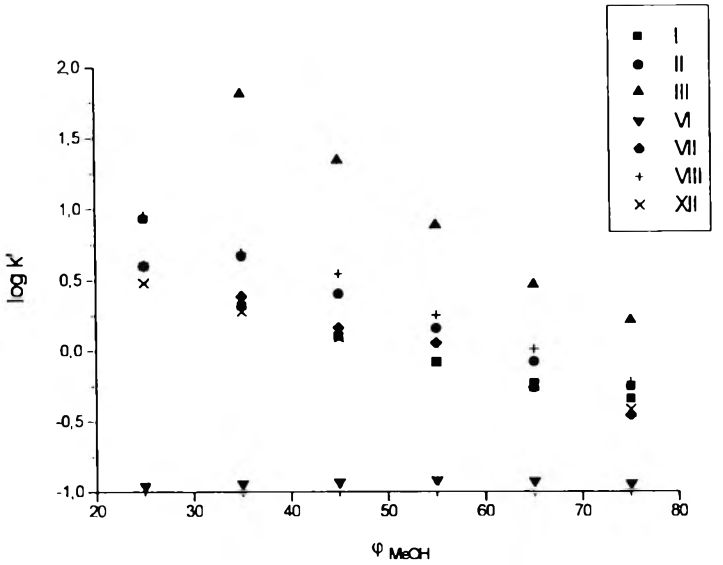


Figure 2. log k′ versus $P_{o/w}$ for the standards compounds.

Table 3

Log K' Versus φ_{Meoh} and Log K_w Obtained Through the Equation: Log K' = -Sφ_{Meoh} + Log K_w, for Scaling Standards Compounds

Compounds	φ_{MEOH} (%)/log k'						log k_W
	25	35	45	55	65	75	
I.	0.599	0.322	0.100	-0.079	-0.231	-0.340	0.995
II.	0.936	0.671	0.405	0.158	-0.077	-0.257	1.514
III.	---	1.810	1.343	0.883	0.464	0.210	3.185
VI.	-0.957	-0.942	-0.931	-0.916	-0.920	-0.939	-0.990
VII.	0.606	0.390	0.166	0.058	-0.258	-0.458	1.137
VIII.	0.959	0.694	0.546	0.256	0.011	-0.223	1.552
XII.	0.482	0.280	0.098	-0.082	-0.253	-0.414	0.913

See text for structures. (-) Peak too broad to be measured.

It is noteworthy that the use of octanol (system II) in this case results in a better correlation than the one obtained with system I, as can been seen by equation 10.

Based on these observations, it was concluded that either system I or II could be used to evaluate log $P_{o/w}$ for the amphiprotic compounds studied. However, one should notice that the retention mechanism operating on these systems is not a pure partitioning one, as shown by the coefficients in all equations given above.[14,15]

Because of this, log k'_w was then obtained for the following compounds: (I) acetanilide, (II) benzaldehyde, (III) benzophenone, (VI) formamide, (VII) benzyl alcohol, (VIII) acetophenone and (XII) ethyl acetate. Table 3 shows log k_w for seven standards and Figure 2 shows the results.

All regression equations obtained showed correlation coefficients in the range 0.988-0.999 and F = 162-2947. However, formamide had a different slope which means that the retention mechanism operating for this solute was also different. Thus, this solute was excluded from the calibration scale. Figure 3 shows the plot log k_w versus log $P_{o/w}$ and equation (11) is shown below.

$$\log k_w = 0.974(\pm 0.18)\log P_{o/w} + 0.054(\pm 0.31) \quad (11)$$
$$(n = 6,\ r = 0.991,\ r^2 = 0.982,\ s = 0.123,\ F = 230.18,\ r^2_{cv} = 0.932)$$

From the above equation, the log k_w and log P_{app} have been estimated for some of the hydrazides given in Fig. 1. Table 4 shows these results. All the regression coefficients are in the range of 0.996-1.000, F = 386-5640.

The above results agree with the assumption that k_w is independent of the effects of the modifier, yet it is not free from the effects of the stationary phase used.

Proceeding in this way, the new chromatographic hydrophobicity index,[2] φ_0, was examined for the same standards as derived in equation (11). However, the correlation obtained was not better than the one obtained using log k_w, as can be seen by equation (12).

$$\varphi_{0,MeOH,298,4.6} = 11.38(\pm 4.75)\log P_{o/w} + 42.93(\pm 8.21) \quad (12)$$
$$(n = 6,\ r = 0.958,\ r^2 = 0.918,\ s = 3.289,\ F = 44.21,\ r^2_{cv} = 0.498)$$

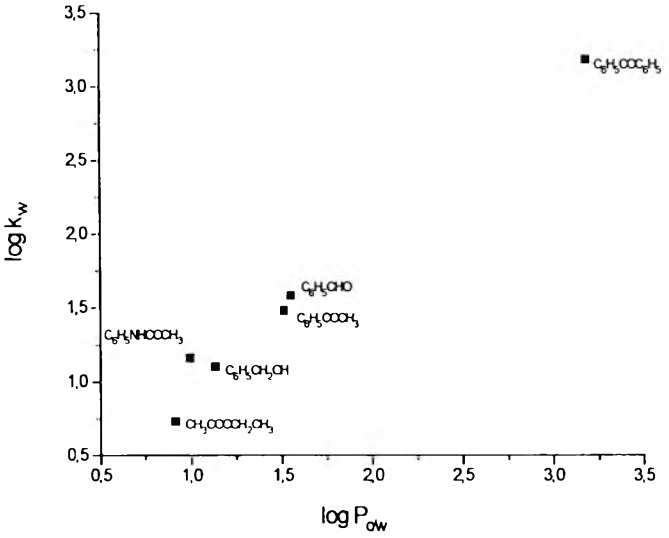


Figure 3. log k_w versus log $P_{o/w}$ for some standard compounds.

Table 4

Log K' and Log P_{app} for Some Study Hydrazides

Compounds	φ_{MEOH} (%)/log k'						log k_W	log P_{app}
	25	35	45	55	65	75		
3.	0.599	0.322	0.100	-0.079	-0.231	-0.340	0.995	3.319
4.	0.935	0.671	0.405	0.158	-0.077	-0.257	1.514	4.182
9.	---	1.810	1.343	0.883	0.464	0.210	3.185	1.552
10.	-0.957	-0.942	-0.931	-0.916	-0.920	-0.939	-0.990	2.073
11.	0.606	0.390	0.166	0.058	-0.258	-0.458	1.137	1.877
12.	0.959	0.694	0.546	0.256	0.011	-0.223	1.552	1.599

See Figure 1 for Structures.

In this case, log P could not be calculated as accurately as with equation 11. We believe this result suggests that it is inappropriate to model partition systems for structurally unrelated compounds, through φ_0. Nevertheless, we have tried the same calculations for the study hydrazides and, equation 13 shows the results.

$$\varphi_{0\text{-MeOH.298,4 6}} = 6.15(\pm 2.39)\log P_{app} + 44.74(\pm 6.26) \quad (13)$$
$(n = 6, r = 0.964, r^2 = 0.929, s = 2.053, F = 51.92, r^2_{cv} = 0.670)$

From the above equation, it seems that φ_0 fits better for these structurally related compounds. Yet, it seems that equation (11) describes these results better than those due to equations (12) and (13).

CONCLUSIONS

Equations 12 and 13 cannot be used as a model for obtaining partition coefficients. Both equations (12 and 13) were produced by chance correlation with a poor cross-validation, which is not the case described by equation 11.

Nevertheless, it is evident from the above that there is an important paradigm to be established. Log k' has led to log k_w, but k_w does not take into account all the shortcomings of chromatographic measurement. This is mainly due to the fact that problems with column manufacture, for instance, affect k_w. Moreover, it involves a free range concentration of the organic modifier, since it is dependent on whether one chooses the linear or parabolic portion of the mutual partitioning behaviour between the mobile and stationary phases. The "dragging' of solvent molecules also plays an important role. This happens mainly when the modifier is methanol.

However, it is clear that k', due to experimental conditions, has a bearing on the way k_w is determined. This means that k' is necessary to describe k_w but not sufficient to describe log P. In spite of this, it seems that until a better method is suggested, k_w shall remain the best approximation of log P.[16]

ACKNOWLEDGMENT

The authors are indebted to CNPq, CAPES and PRPq-UFMG for the grants.

REFERENCES

1. R. Kaliszan, J. Chromatography A, **656**, 417 (1993).

2. K. Valks, P. Sligel, J. Chromatography A, **631**, 49 (1993).

3. L. C. Tan, P. W. Carr, J. Chromatography A, **656**, 521 (1993).

4. D.P. Nowotnik, T. Feld, A. D. Nunn, J. Chromatography A, **630**, 105 (1993).

5. H. Mei-Ming, J. G. Dorsey, J. Chromatography A, **631**, 63 (1993).

6. A. Leo, C. Hansch, D. Elkins, Chemical Reviews, **71**, 525 (1971).

7. A. J. Leo, Chemical Reviews, **93**, 1281 (1993).

8. D. J. Minick, J. H. Frenz, M. A. Patrick, D. A. Brent, J. Med. Chem., **31**, 1923 (1988).

9. E. Tomlinson, J. Chromatography A, **113**, 1 (1975).

10. J. Dearden, Environ. Health Perspect., **61**, 203, (1985).

11. T. Braumann, J. Chromatography, **373**, 191 (1986).

12. J. G. Dorsey, M. G. Khaledi, J. Chromatography A, **656**, 485 (1993).

13. R. Collander, Acta Chem. Scand., **5**, 774 (1951).

14. W. Melander, J. Stoveken, C. Horvath, J. Chromatography A, **19**, 35 (1980).

15. R. P. W. Scott, C. F. Simpson, J. Chromatography A, **197**, 11 (1980).

16. R. Kaliszan, in **Quantiative Structure-Chromatographic Retention Relationships**, Chemical Analysis, Vol. 93, John Wiley & Sons, New York, 1987.

17. R. S. Tsai, W. Fan, N. El Tayar, P. A. Carrupt, B. Testa, L. B. Kier, J. Am. Chem. Soc., **115**, 9632 (1993).

18. R. P. W. Scott, **Silica Gel and Bonded Phases, Their Production, Properties and Use in LC,** R. P. W. Scott, C. F. Simpson eds., John Wiley & Sons, 1993.

19. A. E. Beezer, Chemical Laboratory, University of Kent at Canterbury, personnal communication.

† Work presented at the International Symposium on Lipophilicity and Toxicology, Lausanne, Switzerland, 21-24 March, 1995.

Received May 22, 1996
Accepted June 19, 1996
Manuscript 4188

J. LIQ. CHROM. & REL. TECHNOL., 20(11) 1717-1739 (1997)

PEPTIC PEPTIDE MAPPING BY HPLC, ON LINE WITH PHOTODIODE ARRAY DETECTION, OF A HEMOGLOBIN HYDROLYSATE PRODUCED AT PILOT-PLANT SCALE FROM AN ULTRAFILTRATION PROCESS

Qiuyu Zhao, Patricia Molina, Jean Marie Piot

Laboratoire de Génie Protéique et Cellulaire
Pôle Sciences et Technologies
Université de La Rochelle
Avenue Marillac
17042 La Rochelle, France

ABSTRACT

The analysis of a peptic bovine hemoglobin hydrolysate, produced at pilot plant scale, was carried out using two techniques: SE-HPLC and RP-HPLC. Analysis of amino acid composition, second-order derivative spectrometry and FAB mass spectrometry of isolated peptides allowed us to determine the exact positions of these peptides in the sequence of bovine hemoglobin. This, consequently, gave rise to a peptidic map of the hydrolysate. It also revealed, at the same time, some biologically active peptides in the hydrolysate. This information should find use in the potential future application of enzymatic bovine hemoglobin hydrolysate.

INTRODUCTION

Enzymatic hydrolysis of proteins frequently destroys their functionality. In some cases, however, proteolysis is useful for improving their functional properties, since it gives rise to peptide mixtures which generate many potential applications such as culture media,[1] in stimulating fermentation,[2] nutritional therapy,[3] and research focused on finding peptides possessing biological activity.[4] In this area enzymatic hydrolyses of casein,[5] soya proteins,[6] gluten,[7] tuna myoglobin,[8] and bovine hemoglobin[9] were investigated.

Concerning hemoglobin, extensive research has been carried out investigating some endogenous substances to their opioid activity or affinity for opioid receptors.[10-13] Indeed, many fragments of hemoglobin were isolated from various organs exihibiting potential biological activity.[14,15] These results indicate that hemoglobin could be a precursor of many biologically active peptides.

The peptic hydrolysis of bovine hemoglobin, which is a source of high annual industrial waste, was carried out in an ultrafiltration reactor at pilot plant scale.[9] This experimental set up allowed us to obtain a well-defined and very reproducible peptic hydrolysate from bovine blood hemoglobin.[16]

Some biologically active peptides such as opioid peptides,[17] bradykinin potentialisating peptide,[18] and bacteria growth stimulating peptide[19] were also isolated. This hydrolysate has also been shown to have growth stimulating properties in fermentation.[20]

In this study, we isolated and identified all of the peptides present in the hydrolysate. Peptide fractionation and purification were performed by using reverse-phase (RP) HPLC to complement the size exclusion (SE) HPLC separation and the amino acid composition of each peptide analysed. The masses of some of the peptides were confirmed by fast atom bombardment mass spectrometry (FABMS). At the same time, a second-order derivative spectrometry technique[21] was used to determine the presence of aromatic amino acids in the peptide. This was particularly useful for peptides containing tryptophan, since these are destroyed during amino acid analysis.

The identification and location of the different peptides in the known bovine hermoglobin structure were carried out; this resulted in a peptidic map of this hydrolysate. Such a peptide map provides important information for potential future applications.

MATERIALS AND METHODS

All common chemicals and solvents were of analytical grade from commercial sources. Acetonitrile was of HPLC grade. Water was obtained from a Waters (Saint-Quentin, France) Milli-Q system. The amino acids standard kit H was from Pierce Chemical Co. Porcine pepsin was purchased from Sigma Chemicals. All aqueous HPLC eluents were degassed with helium (Air Liquide, La Rochelle, France) during analysis.

Peptic Hemoglobin Hydrolysis

Decolorized bovine hemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor, followed by decolorization with magnesia, desalting and atomization as previously described.[9] The nitrogen content, determined by the Kjeldahl method, allowed evalution of the quantity of peptide in the hydrolysate greater than 90% (N X 6.25).

HPLC System

The liquid chromatographic system consisted of a Waters 600E automated gradient controller-pump module, a Waters WISP 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and analyze chromatcgraphic data.

SE-HPLC

The elutions were performed with a TSK G2000SWG column (300 mm x 21.9 mm i.d.) with 7 mM ammonium acetate. Hydrolysate powder samples (400 mg) were dissolved in 2 mL of the same solution and filtered through 0.22 μm filters before being applied to the column. The flow rate was 5 mL/min. Fractions were collected and freeze dried immediately.

RP-HPLC

Analysis of the peptide fractions eluted from the TSK G2000SWG were carried out using a Delta Pak C-18 column (300 mm X 3.9 mm i.d.). The mobile phase comprised 10 mM ammonium acetate buffer pH 6.0 as eluent A

and acetonitrile as eluent B. The flow rate was 1.5 mL/min. Samples were dissolved in buffer A, filtered through 0.22 μm filters and then injected. The gradient applied was 0-40% B over an 80 min period for fractions F I, F II and F III, 0-20% B over 40 min then 20-40% B over 20 min for fractions F IV, F V, F VI and F VII. On-line, instantaneous UV absorbance spectral scans were performed between 200 nm and 300 nm at a rate of one spectrum/second. The resolution was 1.2 nm. Chromatographic analysis was completed using Millennium software.

Amino Acid Analysis

Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved by constant-boiling of the peptides with HCl (6N) which contained 1% phenol, for 24 h. at 110 °C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids were performed on a Waters RP-Picotag column (150 mm x 3.9 mm i.d.) according to Bidlingmeyer et al.[22] The detection wavelength was 254 nm and the flow rate 1 mL/min.

Fast Atom Bombardment Mass Spectrometry

A Kratos MS 50 RF high resolution mass spectrometer, equipped with a DS 90 (DGDG/30) data system, was used. The mass spectrometer was operated at an 8-keV accelerating potential. An Ion Tech Model B 11 NF saddle field fast atom source energized with the B 50 current-regulated power supply was used with xenon as the bombarding atom (operating condition: 7.3 kV, 1.2 mA). Peptides were dissolved in water (250 mg in 50 mL) and 1 mL of the solution was loaded onto the copper tip with thioglycerol as a matrix. In this case, the source housing was not heated. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Caesium iodide was the calibration standard.

RESULTS AND DISCUSSION

SE-HPLC

According to the HPLC gel filtration conditions described above, 30 mg/150μL of bovine hemoglobin hydrolysate could be loaded per run. The elution profile is shown in Figure 1. In this figure, seven fractions (F I to F

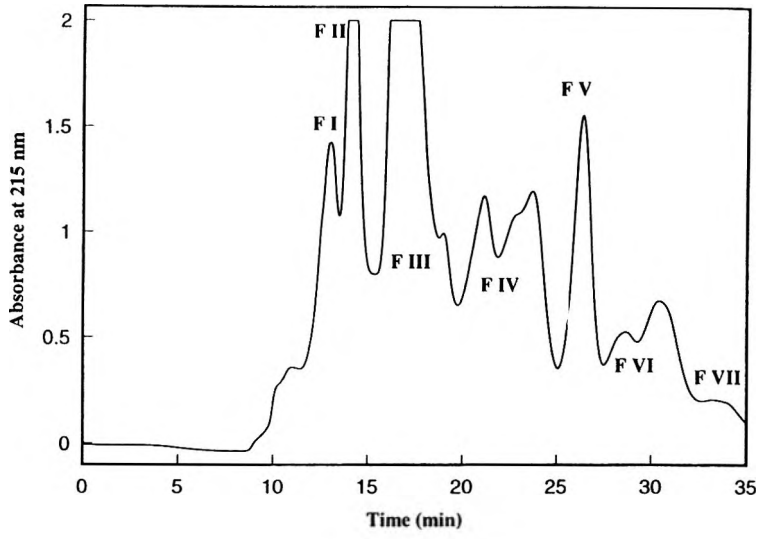


Figure 1. Elution profile of total peptic hydrolysate on TSK G2000 SWG column (300 mm x 21.5 mm i.d.) with 7 mM ammonium acetate . Flow rate is 6mL/min. Quantity injected for each run was 30 mg/150 µL.

VII) can be seen. It appears, from this profile, that the resolution of the sample components could be achieved in less than 35 min. with a similar efficiency as observed in the analytical separation.[23] Such separations result in easy collection of any fraction. More than seventy elutions were performed and, in each case, the same elution pattern was found. This led us to purify the largest quantity of material available for further RP-HPLC analysis or experimental application.

Reversed-Phase HPLC

Each fraction eluted from TSK G2000 SWG column was analysed by RP-HPLC with a Delta Pak C18 column. In order to improve optimal separation conditions of peptides having very different hydrophobicities, the gradients (acetonitrile / ammonium acetate) applied were altered for each fraction. This indicated that FI to FIV were relatively hydrophilic in comparison to FV to FVII. This may also be an important factor in the separation of hydrolysates by SE-HPLC. Indeed, many factors, such as hydrophobicity and electric charge, can affect the behavior of the peptides during size exclusion chromatography.[24] The chromatograms issued from the RP-HPLC separation are presented in

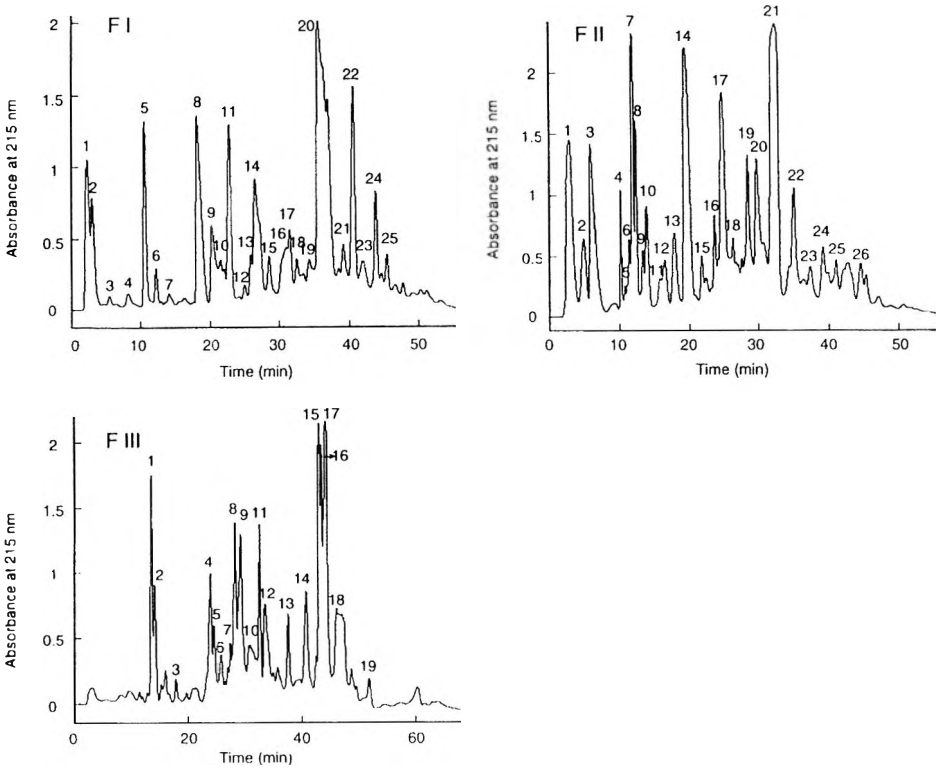


Figure 2. RP-HPLC on Delta Pak -C18 column (300 mm X 3.9 mm i. d.) of peptide fractions F I to III eluted from TSK G2000 SWG column. The mobile phase comprised 10 mM amonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 1.5 mL/min. Injected quantity was 1.5 mg / 100 μL. The gradient applied was 0-40% B in 80 min .

Figure 2 and Figure 3. From these profiles, an excellent resolution of the peptide mixtures for each fraction was obtained. Therefore, the individual peaks were collected separately and they were labelled according to the process of isolation. Each peak was checked for its purity by RP-HPLC. In total, 131 peptides were collected and freezed-dried before amino acid analysis.

Second-Order Derivative Spectrometry

Before amino acid analysis, the secondary derivative spectrum of each peptide was obtained. As previously reported,[21] HPLC, coupled with a

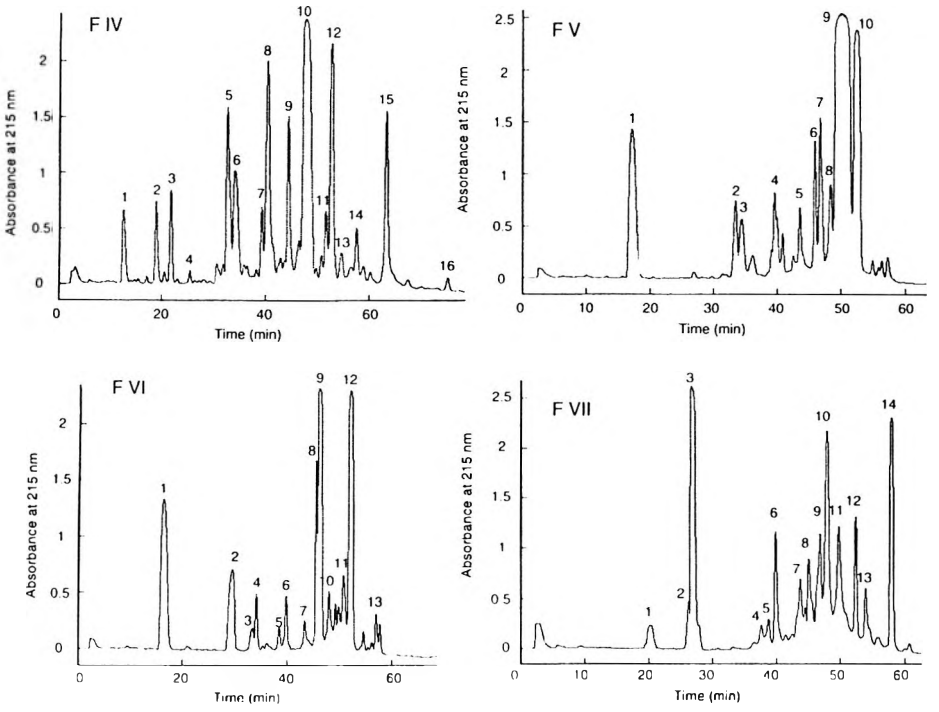


Figure 3. RP-HPLC on Delta Pak -C18 column (300 mm X 3.9 mm i. d.) of peptidic fractions (F IV to VII) eluted from TSK G2000 SWG column. The mobile phase comprised 10 mM amonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 1.5 mL/min. Injected quantity was 1.5 mg / 100 µL. The gradient applied was 0-20% B in 40 min then 20 to 40% in 20 min for IV to VII.

photodiode array detector, allowed us to obtain the UV spectrum of each peak, instantaneously, during the chromatographic separation. The second-order derivative spectrum obtained from the UV spectrum of each peak was used for the detection of aromatic amino acids in the peptide. Figure 4 presents the second-order derivative spectra of peptides F I-8, F V-1, F II-19 and F V-10. The presence of Phe, Tyr, Trp in F I-8, F V-1, F II-19, respectively, were indicated by minima at 258.5 nm, 283.5 nm, and 289.5 nm. F V-10 contained three aromatic amino acids as was previously found.[21] Therefore, the second-order derivative spectrum of each peptide was studied and the presence/absence of aromatic amino acids in each peptide determined; these results are shown in Table 1. This was very useful for the peptides containing tryptophan, since it is destroyed during amino acid analysis and was also an important marker in the subsequent sequence analysis of each peptide.

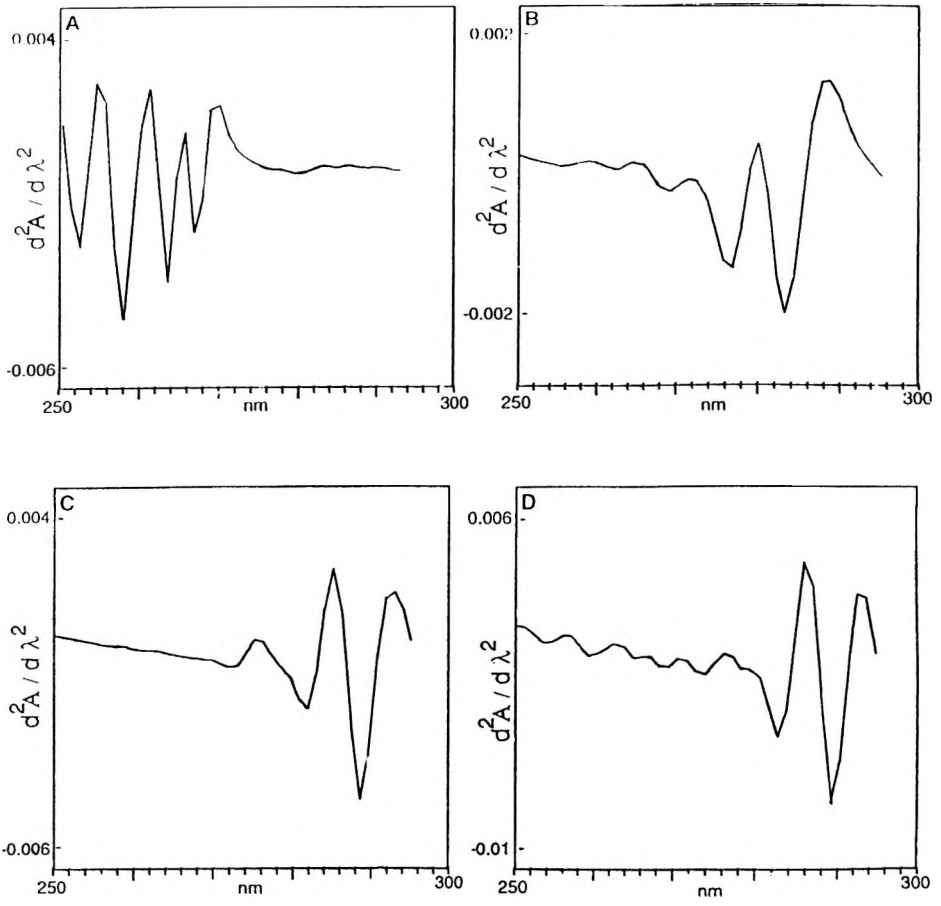


Figure 4. Second-order derivative spectra of peptides F I-8 (a), F V-1 (b), F II-19 (c) and F V-10 (d). The presence of Phe, Tyr, Trp in F I-8, F V-1, F II-19, respectively, was indicated by the absorbance minima at 258.5, 283.5, and 289.5 nm. F V-10 contained three aromatic amino acids.

Amino Acid Analysis

All isolated peptides were analyzed on the Waters Pico-Tag column after converting amino acids into their PTC (phenylthyocarbamyl) derivatives. For example, Figure 5 shows the chromatographic profile of peptide F V-1. In comparison with that of standards, this led us to determine the composition of this peptide as: Ser (1), Arg (1), Thr (1), Tyr (1) and Lys (1). With regard to

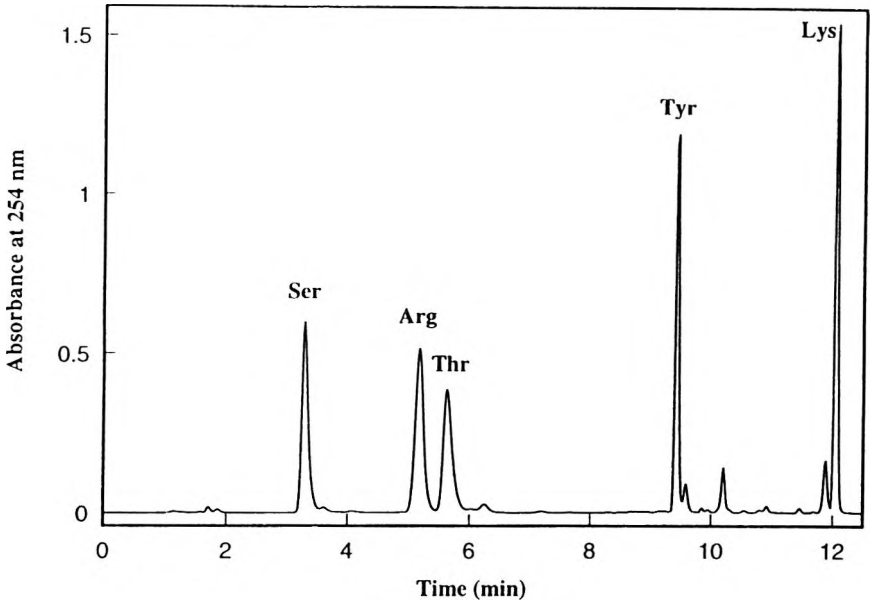


Figure 5. Elution profile of PTC amino acid of peptide F V-5 obtained by HPLC with Waters picotag column. The amino acid composition of this peptide was deduced as: Ser (1), Arg (1) Thr (1), Tyr (1) Lys (1).

the known sequence of bovine hemoglobin, this peptide was determined as fragment 137-141 of the α chain of bovine hemoglobin. In the same way, all the peptides were localized and represented in Table 1. A peptidic map of this hydrolysate could then be constructed as shown in Figure 6 and Figure 7. It was observed that some peptides, issued from different fractions of the SE-HPLC separation, had an identical sequence (F I-9 = F II-11; F V-1 = F VI-1; FVI-3 = F VII-1, etc). These results were also verified by a UV-spectral comparison technique.[25] From this, we observed that the SE-HPLC was only a preliminary separation, and it was possible that the same peptide was present in different fractions. It was also noticed that some peptides issued from the same zone of α or β chains were eluted in the same fraction from the SE-HPLC separation by a TSK G2000 SWG column. For example: many peptides from fraction I or Fraction II were localised in the zone 65-85 of α chain of hemoglobin. The two major peaks from fraction V-9 and V-10 were issued from the region 31-40 of β chain of hemoglobin. It was also observed that almost all of the peptides from fraction V, fraction VI and fraction VII contained aromatic amino acids. These phenomena were also found in the

Table 1

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
I	I-1	3-6 β	Thr-Ala-Glu-Glu	
	I-2	126-128 β	Leu-Gln-Ala-Asp	
	I-3	20-21 β	Asp-Glu; Glu-Asn (100-101 β); Asn-Glu (119-120 β)	
	I-4	2-5 β	Leu-Thr-Ala-Glu	
	I-5	96-101 β	His-Val-Asp-Pro-Glu-Asn	
	I-6	45-51 β	Gly-Asp-Leu-Ser-Thr-Ala-Asp	
	I-7	2-6 β	Leu-Thr-Ala-Glu-Glu	
	I-8	127-130 β	Ala-Asp-Phe-Gln	Phe
	I-9	1-6 β	Met-Leu-Thr-Ala-Glu-Glu	
	I-10	82-85 α	Glu-Leu-Ser-Asp	
	I-11	93-102 β	Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Phe	Phe
	I-12	125-128 β	Leu-Gln-Ala-Asp	
	I-13	42-47 β	Glu-Ser-Phe-Gly-Asp-Leu	Phe
	I-14	74-80 α	Asp-Asp-Leu-Pro-Gly-Ala-Leu.	
		73-79 α	Leu-Asp-Asp-Leu-Pro-Gly-Ala	
	I-15	71-75 α	Glu-His-Leu-Asp-Asp	
	I-16	68-78 α	Lys-Ala-Val-Glu-His-Leu- Asp-Asp-Leu-Pro-Gly	
	I-17	93-100 β	Asp-Lys-Leu- His-Val-Asp-Pro-Glu	
	I-18	42-48 β	Glu-Ser-Phe-Gly-Asp-Leu-Ser	Phe
	I-19	72-80 α	His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
	I-20	41-47 β	Phe-Glu-Ser-Phe-Gly-Asp-Leu	Phe
	I-21	100-105 β	Glu-Asn-Phe-Lys-Leu-Leu	Phe
	I-22	65-78 α	Ala-Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly	
		67-80 α	Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
	I-23	67-82 α	Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu-Ser-Glu	
	I-24	66-80 α	Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
	I-25	68-80 α	Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
II	II-1	48-52 β	Ser-Thr-Ala-Asp-Ala	
	II-2	45-49 β	Gly-Asp-Leu-Ser-Thr	
	II-3	80-82 α	Leu-Ser-Glu, Ser-Glu-Leu (81-83 α), Glu-Leu-Ser (82-84 α)	
	II-4	95-101 β	Leu-His-Val-Asp-Pro-Glu-Asn	
	II-5	27-29 α	Glu-Ala-Leu, Ala-Leu-Glu (28-30 α)	
	II-6	125-131 β	Leu-Gln-Ala-Asp-Phe-Gln-Lys	Phe
	II-7	22-27 α	Ala-Glu-Tyr-Gly-Ala-Glu, Glu-Tyr-Gly-Ala-Glu-Ala (23-28 α)	Tyr
	II-8	22-28 α	Ala-Glu-Tyr-Gly-Ala-Glu-Ala	Tyr
	II-9	22-27 β	Val-Gly-Gly-Glu-Ala-Leu	
	II-10	126-130 β	Gln-Ala-Asp-Phe-Gln	Phe
	II-11	1-6 β	Met-Leu-Thr-Ala-Glu-Glu	
	II-12	81-85 α	Ser-Glu-Leu-Ser-Asp	
	II-13	2-7 β	Leu-Thr-Ala-Glu-Glu-Lys	
	II-14	24-29 α	Tyr-Gly-Ala-Glu-Ala-Leu	Tyr
	II-15	73-79 α	Leu- Asp-Asp-Leu-Pro-Gly-Ala	
	II-16	89-94 β	Glu-Leu-His-Cys-Asp-Lys	
	II-17	41-44 β	Phe-Glu-Ser-Phe; Phe-Phe-Glu-Ser (40-43β)	Phe
	II-18	125-128 β	Leu-Gln-Ala-Asp	
	II-19	14-21 β	Trp-Ser-Lys-Val-His-Val-Asp-Glu	Trp
	II-20	42-47 β	Glu-Ser-Phe-Gly-Asp-Leu	Phe
	II-21	14-28 β	Trp-Ser-Lys-Val-His-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly	Trp
	II-22	67-80 α	Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
		66-79 α	Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala	
		65-78 α	Ala-Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly	
	II-23	110-120 α	Ala-Ser-His-Leu-Pro-Ser-Asp-Phe-Thr-Pro	
	II-24	109-120 α	Leu-Ala-Ser-His-Leu-Pro-Ser-Asp-Phe-Thr-Pro	
	II-25	124-128 β	Val-Leu-Gln-Ala-Asp	
	II-26	124-129 β	Val-Leu-Gln-Ala-Asp-Phe	

(continued)

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
III	III-1	97-99 α	Asn-Phe-Lys; Asp-Lys-Phe (126-128 α)	Phe
	III-2	84-87 β	Phe-Ala-Ala-Leu	Phe
	III-3	30-32 α	Glu-Arg-Met	
	III-4	134-136 α	Thr-Val-Leu; Val-Leu-Thr (135-137 α); Val-Thr-Leu(107-109; α); Leu-Val-Thr (106-108, α); The-Leu-Val (105-107 α)	
	III-5	48-56 α	Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys	
	III-6	24-34 α	Tyr-Gly-Ala-Glu-Ala-Leu-Glu-Arg-Met-Phe-Leu	Tyr, Phe
	III-7	124-128 β	Val-Leu-Gln-Ala-Asp	
	III-8	123-138 β	Pro-Val-Leu-Gln-Ala-Asp-Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn	Phe
	III-9	127-134 α	Lys-Phe-Leu-Ala-Asn-Val-Ser-Thr	Phe
	III-10	105-107 α	Leu-Leu-Val	
	III-11	33-35 α	Phe-Leu-Ser; Leu-Ser-Phe (34-36 α)	Phe
	III-12	130-140 α	Ala-Asn-Val-Ser-Thr-Val-Leu-Thr-Ser-Lys-Tyr	Tyr
	III-13	129-134 α	Leu-Ala-Asn-Val-Ser-Thr	
	III-14	113-122 α	Leu-Pro-Ser-Asp-Phe-Thr-Pro-Ala-Val-His	Phe
		(112-121) α	His-Leu-Pro-Ser-Asp-Phe-Thr-Pro-Ala-Val	Phe
	III-15	93-107 β	Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Lys-Leu-Leu-Gly-Asn-Val	Phe
	III-16	84-98 α	Ser-Asp-Leu-His-Ala-His-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe	Phe
	III-17	110-125 α	Ala-Ser-His-Leu-Pro-Ser-Asp-Phe-Thr-Pro-Ala-Val-His-Ala-Ser-Leu	Phe
	III-18	101-106 β	Asn-Phe-Lys-Leu-Leu-Gly, Phe-Lys-Leu-Leu-Gly-Asn (102-107 β)	Phe
	III-19	33-34 α	Phe-Leu; Phe-Leu; (128-129 α)	Phe
IV	IV-1	27-29 β	Leu-Gly-Arg; Gly-Arg-Leu (28-30 β); Gly-Arg-Leu-Leu (28-31 β)	
	IV-2	80-83 β	Leu-Lys-Gly-Thr	

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
IV (cont.)	IV-3	114-120 β	Ala-Arg-Asn-Phe-Gly-Asn-Glu	Phe
	IV-4	84-90 α	Ser-Asp-Leu-His-Ala-His-Lys	
	IV-5	7-13 β	Lys-Ala-Ala-Val-Thr-Ala-Phe	Phe
	IV-6	47-64 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys-Val-Ala-Ala	
	IV-7	6-15 α	Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly	Trp
	IV-8	2-20 α	Leu-Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His	Trp
	IV-9	88-98 α	Ala-His-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe	Phe
	IV-10	8-29 β	Ala-Ala-Val-Thr-Ala-Phe-Trp-Ser-Lys-Val-His-Val-Asp-Glu-Val-Gly-Gly-Gly-Glu-Ala-Leu-Gly-Arg	Phe, Trp
	IV-11	103-109 β	Lys-Leu-Leu-Gly-Asn-Val-Leu	
	IV-12	99-106 α	Lys-Leu-Leu-Ser-His-Ser-Leu-Leu	
	IV-13	40-51 α	Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly	Phe, Tyr
	IV-14	40-56 α	Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys	Phe, Tyr
	IV-15	102-109 β	Phe-Lys-Leu-Leu-Gly-Asn-Val-Leu	Phe
	IV-16	81-97 β	Lys-Gly-The-Phe-Ala-Ala-Leu-Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val	Phe
V	V-1	137-141 α	Thr-Ser-Lys-Tyr-Arg	Tyr
	V-2	47-61 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys	
	V-3	47-65 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys-Val-Ala-Ala-Ala	
	V-4	12-23 α	Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu	Trp
	V-5	2-20 α	Leu-Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His	Trp
	V-6	130-145 β	Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	V-7	34-46 α	Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe	Phe, Tyr

(continued)

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
V (cont.)	V-8	47-58 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His	
	V-9	32-40 β	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	Phe, Tyr, Trp
	V-10	31-40 β	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	Phe, Tyr, Trp
VI	VI-1	137-141 α	Thr-Ser-Lys-Tyr-Arg	Tyr
	VI-2	140-145 β	Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-3	135-141 α	Val-Leu-Thr-Ser-Lys-Tyr-Arg	Tyr
	VI-4	139-145 β	Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-5	130-145 β	Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-6	131-145 β	Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-7	14-23 α	Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu	Trp
	VI-8	129-145 β	Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Phe, Tyr
	VI-9	37-46 α	Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe	Phe, Tyr
	VI-10	61-70 β	Ala-His-Gly-Lys-Lys-Val-Leu-Asp-Ser-Phe	Phe
	VI-11	61-74 β	Ala-His-Gly-Lys-Lys-Val-Leu-Asp-Ser-Phe-Ser-Asp-Gly-Met	Phe
	VI-12	33-45 α	Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His	Phe, Tyr
	VI-13	128-145 β	Phe-Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Phe, Tyr
VII	VII-1	135-141 α	Val-Leu-Thr-Ser-Lys-Tyr-Arg	Tyr
	VII-2	138-141 α	Ser-Lys-Tyr-Arg	Tyr
	VII-3	140-145 β	Leu-Ala-His-Arg-Tyr-His	Tyr
	VII-4	13-18 α	Ala-Trp-Gly-Lys-Val-Gly	Trp
	VII-5	12-24 α	Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu-Tyr	Tyr, Trp

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
VII (cont.)	VII-6	7-24 α	Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu-Tyr	Tyr, Trp
	VII-7	24-34 β	Gly-Glu-Ala-Leu-Gly-Arg-Leu-Leu-Val-Val-Tyr	Tyr
	VII-8	130-145 β	Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VII-9	40-61α	Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys	Tyr, Phe
	VII-10	64-76 β	Lys-Lys-Val-Leu-Asp-Ser-Phe-Ser-Asp-Gly-Met-Lys-His	Phe
	VII-11	64-77 β	Lys-Lys-Val-Leu-Asp-Ser-Phe-Ser-Asp-Gly-Met-Lys-His-Leu	Phe
	VII-12	32-53 α	Met-Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His	Phe, Tyr
	VII-13	129-145 β	Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Phe, Tyr
	VII-14	36-53 α	Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His	Phe, Tyr

peptic hydrolysate of myoglobin.[8] This may be a result of the interaction between molecules to be separated. In fact, the mobile phase we used in this separation had a lower ionic concentration (7 mM ammonium acetate); therefore, the Van der Waals force may have induced these interactions between the molecules and, therefore, became the overriding factor in the resolution of SE-HPLC. This factor may be used as a principal rule in the SE-HPLC separation. Since many peptides of peptic bovine hemoglobin hydrolysate contain aromatic amino acids, the quantitative determination of these peptides, if necessary, was simplified.[26]

It was also observed that some peptides appeared to be broken down into other peptides (F I-9 to F I-1; F II-21 to F II-19, etc). This phenomenon has been previously described for peptic myoglobin hydrolysate.[8] The location of

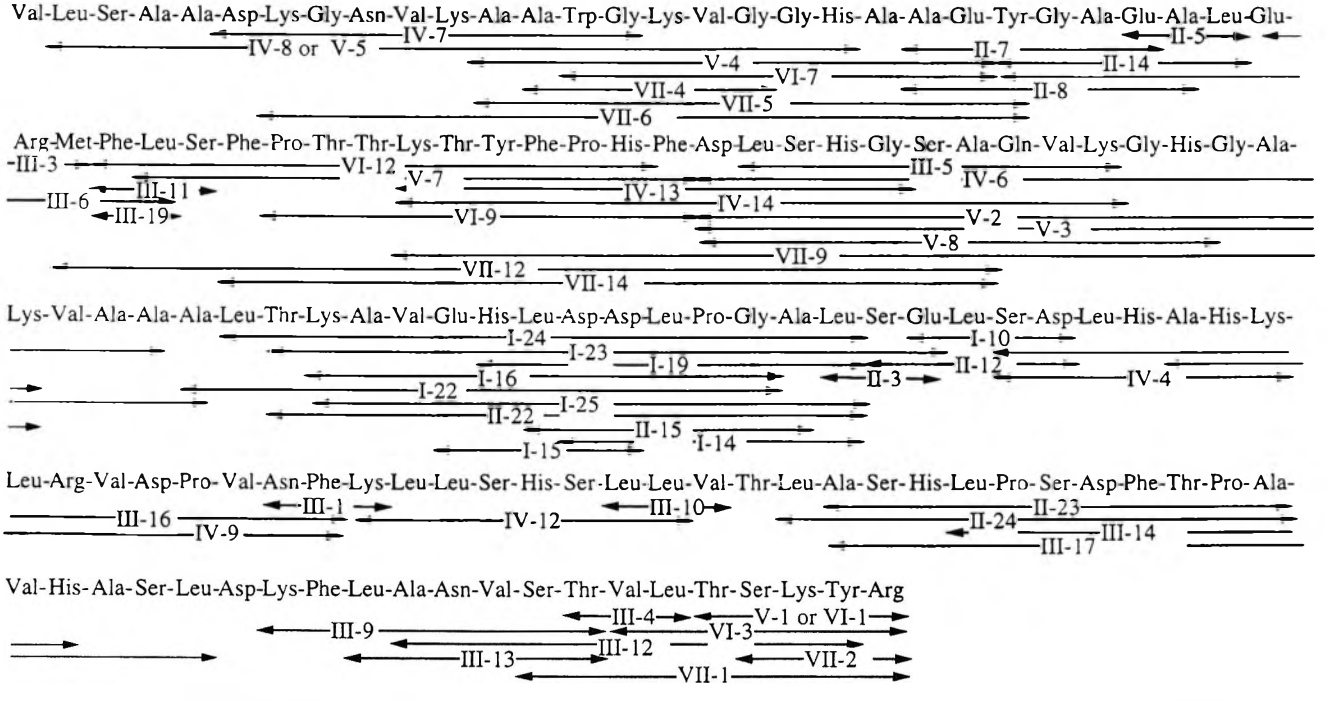


Figure 6. The localization of the peptides isolated from peptic bovine hemoglobin hydrolysate in the α chain of bovine hemoglobin.

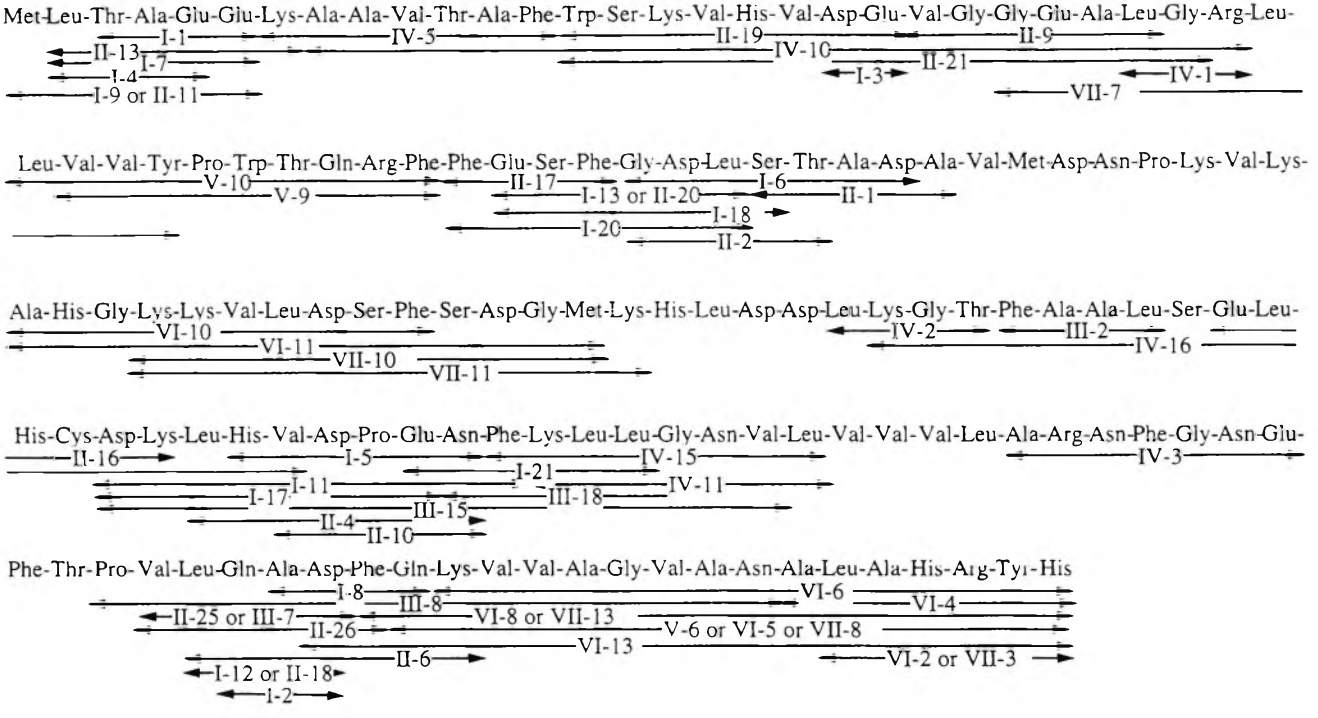


Figure 7. The localization of the peptides isolated from peptic bovine hemoglobin hydrolysate in the β chain of bovine hemoglobin.

these peptides in the α and β chains of bovine hemoglobin confirmed the lower specificity of pepsin. The hydrolysis mechanism of pepsin in an ultrafiltration reactor, however, may be different to that in a batch preparation. We have previously reported that a batch peptic hydrolysis of hemoglobin could constitute a multisubstrate system.[27] The complete evolution of peptides during the hydrolysis should result in a defined order of peptic hydrolysis of hemoglobin, even though pepsin is a rather non-specific enzyme. This means that, for a certain degree of hydrolysis, the composition of the hydrolysate should be defined. This hydrolysis should give rise to the reproducibility of such a hydrolysate. In an ultrafiltration reactor, this hydrolysis mechanism may be modified by the ultrafiltration process. In other words, the variation of the concentration of the substrates relies not only on the hydrolysis process, but also on the ultrafiltration process. However, the reproducible production of such a hydrolysate has been clearly demonstrated.[16]

Mass Spectrometry

In order to confirm the results obtained by amino acid analysis, some peptides were also analysed by mass spectrometry. Figure 8 shows the mass spectrometry profile of peptide F III-16. The molecular weight deduced from $(M+H)^+$ was 1747 daltons. This is in excellent agreement with the MW calculated from the amino acid composition. Regarding the MW of the peptides in the hydrolysate, the peptides MW varied from about 200 to 2000 Daltons (i. e. 2 to 21 amino acids). Moreover, more than 50% of the peptides identified in the bovine hemoglobin hydrolysate were composed of less than 8 amino acids. This may be an advandage for nutritional properties. It is well known that the end products of digestion, i.e., short oligopeptides, are not absorbed in a similar way to that of free amino acids.[28] It has previously been demonstrated that enteric infusions containing small peptides (di- or tri-peptides) were more efficiently assimilated than corresponding free amino acid mixtures and displayed enhanced nutritional value.[29]

Bioactive Peptide Analysis

In relation to the the bioactive peptides, we have already reported some bioactive peptides isolated from this hemoglobin hydrolysate, such as opioid peptides for F V-9 and F V-10[17], bradykinin potentialisating peptide for F III-13[18] and bacteria growth stimulating peptide F II-1[19]. Additionally, it can be seen that peptides F V-1 and F VI-1 have a common sequence with neo-kyotorphin, which was first studied by H. Takagi et al.[30] Its biological

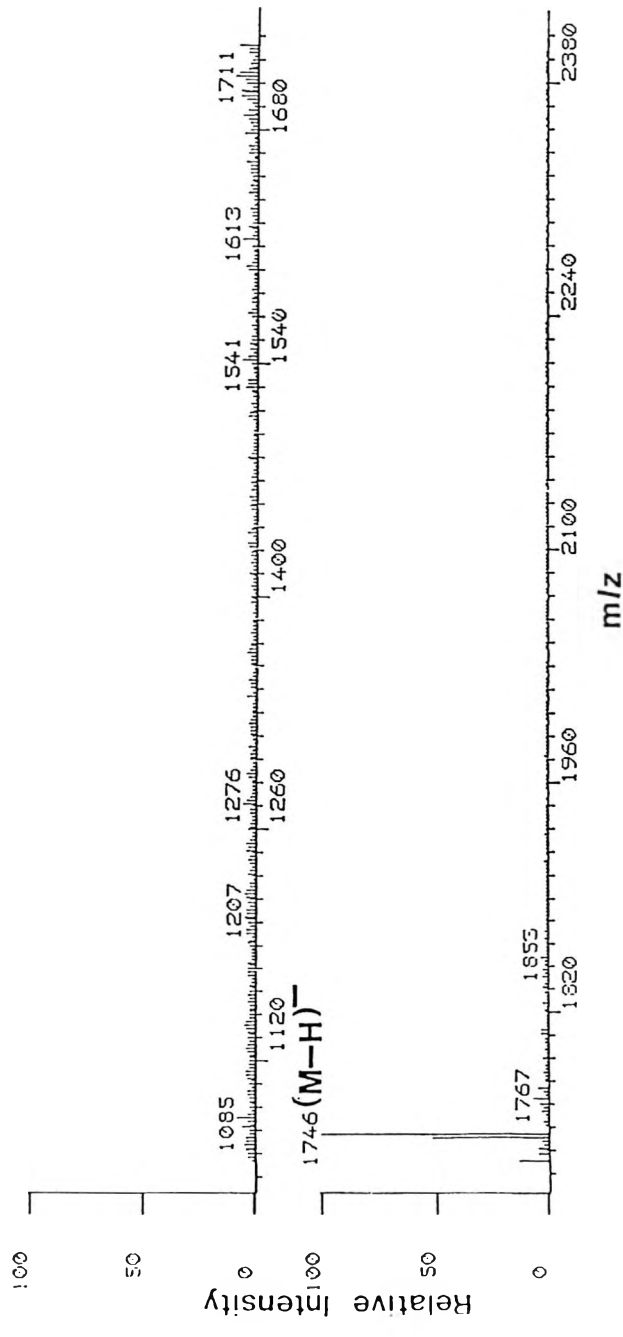


Figure 8. FAB mass spectrometry peptide F III-16. The abundant ion at 1746, designated as a molecular cation $(M+H)^-$, suggested that F III-16 has a molecular weight of 1747.

Table 2

Biological Activity of Some Endogenous Hemoglobin Fragments and Related Peptides Isolated from Peptic Bovine Hemoglobin Hydrolysate

Peptides from Hydrolysate		Endogenous Hemoglobin Fragments			
Peptide	**Localization**	**Position**	**Source**	**Biol. Activity**	**Ref.**
F III-17	110-125 α	110-124 α	Bovine hypo-thalamus	Coronaro-constrictory in vitro	33
F V-5	2-20 α	1-21 α	Bovine hypo-thalamus	Coronaro-constrictory in vitro	33
F V-7 F VI-12	34-46 α 33-45 α	33-46 α	Pig brain	ACTH-releasing in vitro	34
F VII-1, VI-3	135-141 α	137-141 α	Bovine brain	Analgesic in vivo	30
F VII-2	138-141 α				
F VI-13	128-145 β	133-145 β	Bovine hypo-thalamus	Coronaro-consrictory	33
F VI-8	129-145 β				
F V-6, VI-5	130-145β				

activities such as analgesic, antihibernatic in vivo and ion current regulation in vitro,[30-32] have been investigated. F III-17 has been investigated for its function of potential hydrophobic carrier.[35] It was also observed that some of our peptides have almost identical sequences as those of bioactive peptides of bovine hemoglobin found in vivo (Table 2). Therefore, this indicated that this enzymatic hydrolysate contained many bioactive peptides which can confer an important potential value to this hydrolysate for many applications. Moreover, some of these peptides were the same, or similar to, that found in vivo, signifying that peptic hydrolysis could represent an important physiological hydrolysis model. In other words, these active fragments of hemoglobin found

in vivo may be issued from a peptic-like hydrolysis of hemoglobin during a physiological process. This hydrolysis model has already been proposed for the investigation of the production of certain bioactive peptides.[36]

In conclusion, a peptic bovine hemoglobin hydrolysate, produced at pilot-plant scale, was analyzed fully by SE-HPLC and RP-HPLC. This analysis was completed with second-order derivative spectrometry, amino acid composition analysis, and mass spectrometry, which gave rise to the complete peptidic composition of this hydrolysate. It revealed, at the same time, the presence of some bioactive peptides and some potentially bioactive peptides. These important findings will be of use in potential applications of this enzymatic hydrolysate.

REFERENCES

1. D. Dive, F. Tonon, P. A. Trinel, D. Ochin, Protistologica, **22(3)**, 271-277 (1986).

2. B. Tchorbanov, G. Lazarova, Biotechnol. Appl. Biochem., **10**, 301-304 (1988).

3. D. B. A. Silk, G. H. Grimble, R. G. Rees, Proc. Nutr. Soc., **44**, 63-72 (1985).

4. A. Hasegawa, H. Yamashita, S. Kondo, T. Giyota, H. Hayashi, H. Yoshizaki, A. Murakami, M. Shiratsuchi, T. Mori, Biochem. Biophys. Res. Commun., **150**, 1230-1236 (1988).

5. J. P. Pelissier, Sci. Aliments., **4**, 1-35 (1984).

6. R. C. Gunther, J. Am. Oil Chemists Soc., **56**, 345-349 (1979).

7. W. A. Klee, C. Zioudrou, R. A. Streaty, **Endorphins in Mental Health Research**, E. Usdin, W. E. Bunney, N. S. Kline, Macmillan, New York, 1978, pp 209-218.

8. C. Lecoeur, Q.Y. Zhao, I. Garreau, F. Sannier, M. Maurice, P. Durand, J. M. Piot, J. Liq. Chromatogr., **18**, 2353-2371 (1995).

9. J. M. Piot, D. Guillochon, P. Charet, D. Thomas, "Brevet N° 8404004", France (1984).

10. V. Brantl, C. Gramsch, F. Lottspeich, R. Mertz, K. H. Jaeger, A. Herz, Eur. J. Pharmacol., **125**, 309-310 (1986).

11. E. L. Glamsta, B. Meyerson, J. Silberring, L. Terenius, F. Nyberg, Biochem. Biophys. Res. Commun., **184**, 1060-1066 (1992).

12. E. L. Glamsta, A. Marklund, U. Hellman, C. Wernstedt, L. Terenius, F. Nyberg, Regul. Peptides., **34**, 169-179 (1991).

13. A. A. Karelin, M. M. Philippova, V. T. Ivanov, Peptides, **10**, 693-697 (1995).

14. A. A. Karelin, M. M. Philippova, E. V. Karelina, V. T. Ivanov, Biochem. Biophys. Res. Commun., **202**, 410-415 (1994).

15. I. Aubes-Dufau, J. Capdevielle, J. L. Seris, D. Combes, FEBS Lett., **364**, 115-119 (1995).

16. J. M. Piot, D. Guillochon, D. Leconte, D. Thomas, J. Chem. Technol. Biotechnol., **42**, 147-156 (1988).

17. J. M. Piot, Q. Y. Zhao, D. Guillochon, G. Ricart, D. Thomas, Biochem. Biophys. Res. Commun., **189**,101-110 (1992).

18. J. M. Piot, Q. Y. Zhao, D. Guillochon, G. Ricart, D. Thomas, FEBS Lett., **299(1)** 75-79 (1992).

19. Q. Y. Zhao, J. M. Piot, V. Gautier, G. Cottenceau, Appl. Microbiol. Biotechnol., **45**, 778-784 (1996).

20. D. Dive, J. M. Piot, F. Sannier, D. Guillochon, P. Charet, S. Lutrat, Enzyme Microb. Technol., **11**, 165-172 (1989).

21. Q. Y. Zhao, I. Garreau, F. Sannier, J. M. Piot, J. Liq. Chromatogr., **18**, 1077-1092 (1995).

22. B. A. Bidlingmeyer, S. A. Cohen, T. L. Tarvin, J. R. Napier, W. S. Hancock, J. Chromatogr., **336**, 93-104 (1984).

23. J. M. Piot, D. Guillochon, Q. Y. Zhao, G. Ricart, B. Fournet, D. Thomas, J. Chromatogr., **481**, 221-231 (1989).

24. C. T. Mant, R. S. Hodges, J. Liq. Chromatogr., **12**, 139-172 (1989).

25. Q. Y. Zhao, F. Sannier, G. Ricart, J. M. Piot, J. Liq. Chromatogr., **18**, 93-103 (1995).

26. Q. Y. Zhao, F. Sannier, I. Garreau, C Le Coeur, J. M. Piot, J. Chromatogr., A, **723**, 35-41 (1996).

27. Q. Y. Zhao, F. Sannier, J. M. Piot, Biochim. Biophys. Acta., **1295**, 73-80 (1996).

28. J. M. Rouanet, J. L. Zambonino Infante, B. Caporiccio, C. Pejoan, Nutr. Metab., **34**, 175-182 (1990).

29. G. K. Grimble, P. P. Keohane, B. E. Higgins, M. V. Kaminski, D. B. A. Silk, Clin. Sci., **71**, 65-69 (1986).

30. H. Takagi, H. Shiomi, K. Fukui, K. Hayashi, Y. Riso, K. Kitagawa, Life Sci., **31**, 1733-1736 (1982).

31. B. V. Vaskovsky, V. T. Ivanov, I. L. Mikhaleva, **Peptides Chemistry, Structure and Biology**, J. E. Rivier, G. R. Marshall, eds., Leiden ESCOM, 1990, pp. 302-304.

32. Y. X. Zhu, K. I. Hsi, Z. G. Chen, FEBS Lett., **208**, 253-257 (1986).

33. N. Barkhudaryan, J. Kellermann, A. Galoyan, F. Lottspeich, FEBS Lett., **329**, 215-218 (1993).

34. A. V. Schally, W. Y. Huang, T. W. Redding, Biochem. Biophys. Res. Commun., **82**, 582-588 (1978).

35. N. Cempel, J. M. Aubry, J.M. Piot, D. Guillochon, Biotechnol. Appl. Biochem., **21**, 287-294 (1995).

36. R. E. Carraway, S. P. Mitra, G. F. Ferris, Endocrinology, **119**, 1519-1526 (1986)

Received September 28, 1996
Accepted October 30, 1996
Manuscript 4293

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1741–1756 (1997)

A NEW APPROACH TO STUDY BENZODIAZEPINE SEPARATION AND THE DIFFERENCES BETWEEN A METHANOL/WATER AND ACETONITRILE/WATER MIXTURE ON COLUMN EFFICIENCY IN LIQUID CHROMATOGRAPHY

Y. Guillaume,* E. J. Cavalli, E. Peyrin, C. Guinchard

Laboratoire de Chimie Analytique
Faculté de Médécine Pharmacie
Place Saint-Jacques 25030
Besançon Cedex, France

ABSTRACT

A chemometric methodology was used to study column efficiency and the separation of 10 benzodiazepines in reversed phase liquid chromatography. New simple mathematical models and the organic modifier (OM) organization of ACN in the water, explained differences on column efficiency observed when ACN is chosen instead of CH_3OH. A new response function, which takes into account the separation quality and the analysis time, was proposed for the separation optimization. The result, a mobile phase ACN/water (60/40)(V/V), with a flow rate = 1.00 mL/min and a column temperature = 47°C were optimum values for a rapid chromatographic separation.

INTRODUCTION

Several authors have developed chemometric methods for the study of column efficiency and separation optimization of compounds in reversed phase liquid chromatography (RPLC). The chromatographic response functions optimize, simultaneously, the separation quality and the analysis time.[1-5] Yau and Kirkland,[6] presented an improved algorithm for the characterization of band broadening of skewed (but well-resolved) peaks. Dondi and Gianferra[7] used sequential methods to optimize a mixture of natural secoiridoid compounds in HPLC. Chaminade et al[8] used the cubic spline interpolation algorithm for the selection of ternary mobile phase in HPLC. Larew and Olsen[9] proposed a comparison of theory based and empirical modelling for the prediction of chromatographic behavior in the ion pairing separation of benzodiazepines derived pharmaceutical compounds. Guillaume et al[10] described a rapid method suitable for routine analysis of a mixture of sulbactam and tazobactam in human serum. Guillaume and Guinchard[11] proposed a process to study, simultaneously, the effect of mobile phase composition, its flow rate and column temperature, on both column efficiency and the benzodiazepine separation in a methanol/water mixture. This paper discusses, using experimental data, improvements in peak efficiency for changing the organic modifier from methanol to acetonitrile. A new optimization process which obtains an efficient separation in a minimum analysis time is also proposed.

EXPERIMENTAL

Reagents

Acetonitrile was an analytically determined HPLC grade. Bromazepam, nitrazepam, flunitrazepam, clobazam, lorazepam, oxazepam, tofisopam, chlordiazepoxide, clorazepate dipotassic, and diazepam were obtained from Hoffmann La Roche (Basel, Switzerland). Naphtalene obtained from Merck (Nogent-sur-Marne, France) was used to determine the column efficiency. These were diluted to a concentration of 10-80 mg/mL.

Apparatus

The HPLC system consisted of a Waters HPLC pump (Saint Quentin, Yvelines, France), an Interchim Rheodyne injection valve Model 7125 (Montlucon, France) fitted with a 20 µL sample loop, a Merck L4000 variable

wavelength UV spectrophotometer detector, and a Merck D2500 chromato-integrator (Nogent-sur-Marne, France). A Waters column (Nova pak C_{18}, 5µm, 150 mm x 3.9 mm I.D) was used at controlled temperature in an Interchim oven TM N 701 (Montluçon, France). Overall temperature control was maintained within ± 1°C with a variation from 25°C to 50°C. The detection wavelength was 254 nm. The flow-rate used varied from 0.6 to 1.6 mL/min. The mobile phase was an ACN/water mixture with percentage of ACN varying from 40 to 80 %.

Chemometric Methodology

The chemometric approach is based on factorial designs. Two-level factorial designs give a fitting of a first order (linear) model to the data.[12] If the effects of each of the three factors do not vary linearly, a design which requires 13 experiments to detect curvature in the response can be used. Thus, the Box and Benhken design[13-15] was developed, specifically, to enable a second order response surface to be fitted to the data, as it provides sufficient information for the fitting of a quadratic model to a data set. Such models are amenable to regression analysis. For three factors this takes the form of:

$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad (1)$$

where y is the response or dependent variable, and x_1, x_2, x_3 are the logarithms respectively of, percentage of ACN θ (%) in the ACN/water mixture, mobile phase flow rate F (mL/min) and column temperature T(°C). x_1, x_2, and x_3 were coded to have a variation range from -1 to +1. The a terms represent the parameters of the model.

Simplex Optimization

To optimize the mathematical model (y) given by the experimental design, a simplex method was used. The y value was calculated for m sets of starting conditions where m was given by the number of factors to be optimized plus 1. Therefore, in this case m was 4. The point, corresponding to the lowest value of y, was then reflected in relation to the surface defined by the three other points to give a fifth set of starting conditions. Once again, the point with the lowest y was reflected and the process repeated, sequentially, until the same mobile phase composition, flow rate and column temperature continued to be selected.

Table 1

Estimates of Regression Parameters for the Two Regression Models

Independent Variables	Parameter Terms	(a) N/L	$Ln(k'_{diazepam})$
Intercept	a_0	+ 130.23	+2.87
x_1	a_1	+ 12.31	- 1.73
x_2	a_2	+ 9.41	+ 0.03
x_3	a_3	+ 2.31	- 0.15
x_1^2	a_{11}	- 10.25	+ 0.04
x_2^2	a_{22}	- 16.52	+ 0.00
x_3^2	a_{33}	- 13.62	- 0.03
x_1x_2	a_{12}	- 7.40	+ 0.01
x_1x_3	a_{13}	- 15.40	+ 0.06
x_2x_3	a_{23}	+ 19.00	+ 0.02

(a) inverse of the height to a theoretical plate (mm^{-1})

RESULTS AND DISCUSSION

Column Plate Height

The experimental H were calculated from the chromatograms. All experiments were repeated three times. The coefficient of variation of the H values was less than 3 % in most cases, indicating a high reproductibility and good stability for the chromatographic system. Using the experimental design, the column efficiency represented by the height to a theoretical plate (H) was modelled by the two order polynomial (Eq.1) where y is equal to 1/H. From the full regression model (Table 1) a student T-test was used to provide the basis for the decision as to whether or not the model coefficients were significant. Results of the student T-test show that no variables can be excluded from the model. This generated model was assessed statistically using a Fischer Snedecor test (F-test) and a coefficient of multiple determination R^2. These criteria were respectively equal to 180 and 0.971. These values show a good validity for the model. Experimental and calculated values of the H are summarized in Table 2. These values were chosen in the parameter space, but not among the 13 experiments given by the experimental design, to show that the H-model was within the range. The variation of H versus column temperature and mobile phase flow rate was similar to those obtained in recent works.[11,16]

Table 2

Calculated and Experimental H Values (H_{cal} and H_{exp}) for Different Values of F (mL/min), T(°C), and θ(%)

F(mL/min)	T(°C)	θ(%)	H_{cal} (10^3 mm)	H_{exp} (10^3 mm)
0.8	25	45	11.01	11.40
1.1	35	45	8.36	8.42
1.4	35	55	7.86	7.98
0.8	45	55	8.88	8.93
0.8	35	55	8.17	8.39
1.4	45	55	7.51	7.75
1.1	25	55	9.17	8.99
0.8	45	75	9.09	9.14
1.4	45	75	8.12	8.22
1.1	45	45	7.98	8.02
0.8	25	65	7.92	8.00
0.8	35	65	7.79	7.85
1.1	25	75	7.96	8.02
1.4	45	65	7.71	7.83
1.4	25	55	10.33	10.21
1.1	35	55	7.66	7.99
0.8	35	45	9.19	9.43
1.4	25	65	9.44	9.34
1.4	45	75	8.12	8.44
1.4	25	55	10.33	10.12
1.1	45	75	8.15	8.65
1.1	45	65	7.81	7.92
0.8	25	55	8.85	8.75
1.1	45	55	7.71	7.84
1.4	25	45	12.58	12.81
1.4	25	75	9.09	9.14
1.4	45	65	7.71	7.95
0.8	45	45	9.48	9.65
1.1	25	45	11.60	12.00
1.4	35	45	8.44	8.66
0.8	45	65	8.83	9.00

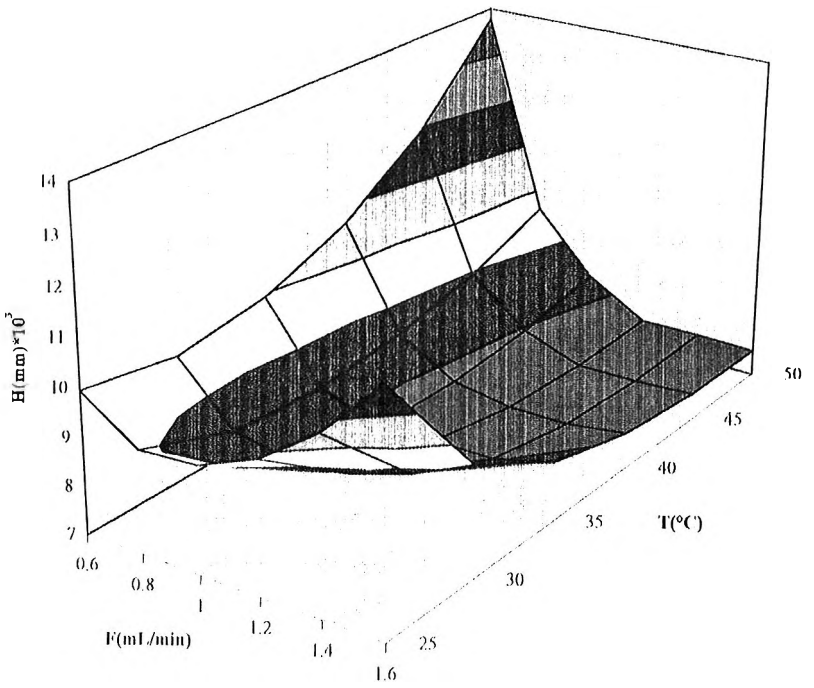


Figure 1. Response surface of the height to a theoretical plate (H) versus temperature/flow rate (percentage of ACN kept at 53 %).

For a constant percentage of ACN in the ACN/water mixture, the surface S presented a minimum (maximum column efficiency) for a couple, column temperature/flow rate (Figure 1). Equation 1, shows that for a constant flow rate (or temperature) the variation of H versus the ACN percentage presents a minimum (maximum column efficiency). The response surfaces generated for the H model are given in Figures 2 and 3. This variation, as in the case of the binary CH_3OH/H_2O mixture,[11,16] can be explained by the decrease in solvent polarity and viscosity with an increase in organic modifier (OM) percentage. In addition, in these experiments there was a specific organization of ACN molecules in the ACN/water mixture. In this mixture, ACN is organized in agregates or loosely defined clusters.[17-19]

For the low region of θ, when θ increased, the number and size of the clusters increased. The non polar solute such as a naphtalene molecule, was embedded in the clusters which increased its solubility.

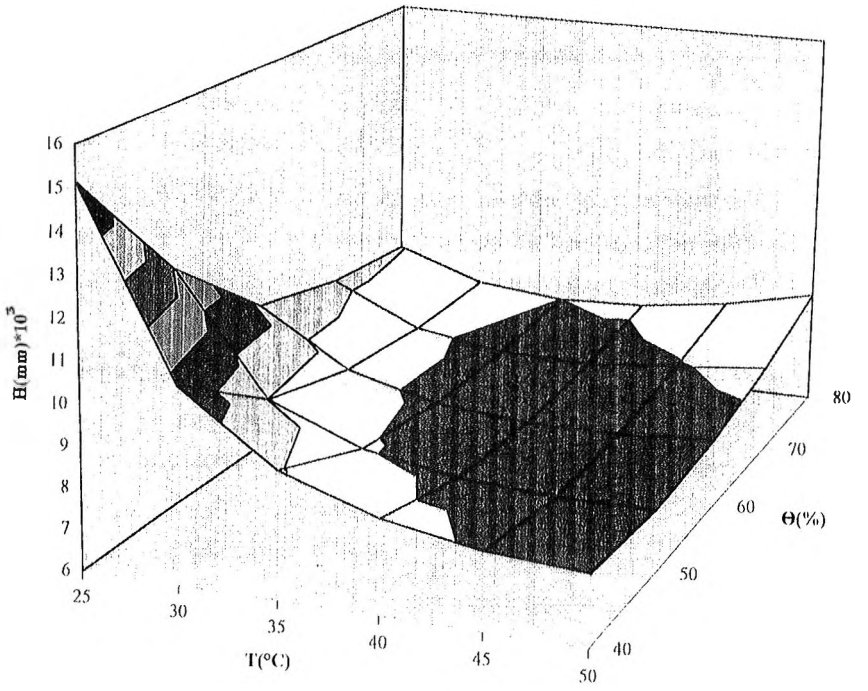


Figure 2. Response surface of the height to a theroetical plate (H) versus temperature/percentage of ACN (flow rate kept at 1.40 mL/min).

Therefore, the mass transfer of the solute in both the stationary and mobile phases increased, by decreasing solvent polarity and its viscosity (OM = CH_3OH or ACN) and increasing the molecule solubility (more specifically for OM = ACN). The factor peak band broadening due to mass transfer decreased. Thus, the plate height decreased. But, the rapid decrease in the capacity factor with an increase of θ necessitated a higher plate number. Thus, beyond an optimal mobile phase composition, this second effect supplanted the increase in the column efficiency referred to above and the plate number decreased. The optimum value of the column efficiency was determined using the simplex optimization method. The results obtained were θ = 53 % of ACN, F = 1.40 mL/min and T = 45°C. The corresponding calculated H value was 0.0075 mm. The experimental value of H obtained for this condition was 0.0080 mm. The theoretical result was also good. In a previous paper,[11] the column efficiency was studied in a CH_3OH/water mixture. The optimum column efficiency in this mixture was calculated as being equal to 0.0138 mm.

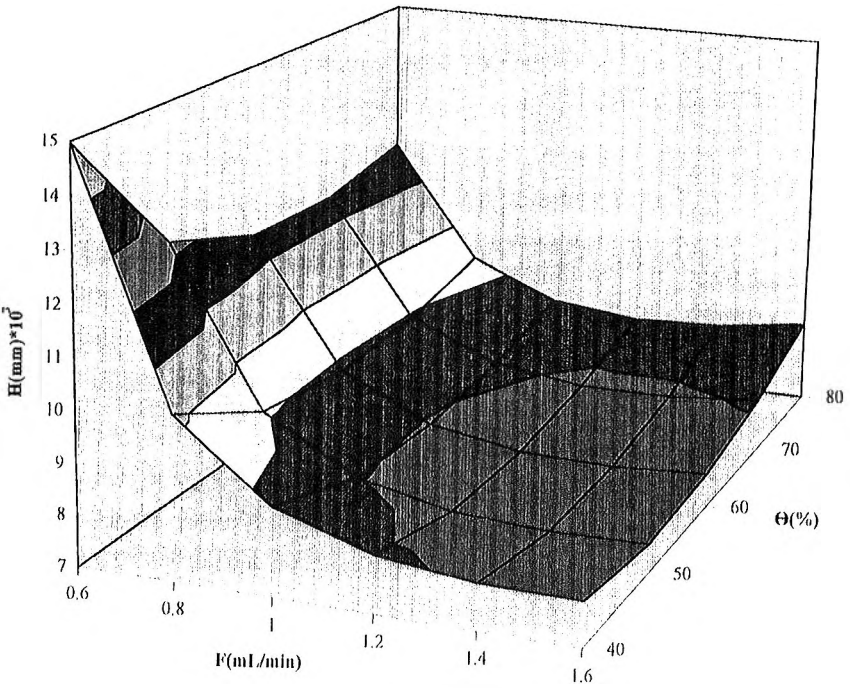


Figure 3. Response surface of the height to a theoretical plate (H) versus flow rate/percentage of ACN (column temperature kept at 45° C).

In the optimum conditions, column efficiency is approximately two times poorer than with acetonitrile. Table 3 shows the calculated and experimental H values for different percentages of organic modifier (OM) in the OM/water mixture (OM = ACN or OM = CH_3OH). The values were found to be markedly dependent on the organic component in the eluent. In CH_3OH/Water, the plate height was $\geq$ 0.013mm corresponding to a plate number N $\leq$ 11540 and in ACN/Water H $\geq$ 0.007mm corresponded to N $\leq$ 21430. From the data in Table 3, it was shown that for a given θ-value, in the ACN/water mixture, the column was two or five times more efficient than with methanol. For example, if F = 1.6 mL/min, T = 50°C and θ = 70% in the OM/Water mixture, the experimental H value was 0.00821 mm for OM = ACN and 0.01875 mm for OM = CH_3OH. Another work[20] has reported that the column efficiency was poorer than with ACN. However, the authors did not systematically note the experimental column efficiency in a large variation range of percentages of OM in the mixture OM/Water, flow rate, and column temperature.

Table 3

Calculated and Experimental H (10^3 mm) Values for Different Percentages of Organic Modifier OM in the Mixture OM/Water Mixture (OM = ACN or OM = CH_3OH)

		Acetonitrile		Methanol	
	0(%)	H_{cal}	H_{exp}	H_{cal}	H_{exp}
A	45	11.01	11.40	51.00	49.89
	50	9.63	9.54	34.81	34.78
	55	8.85	8.75	29.90	29.82
	60	8.30	8.21	28.78	28.72
	65	7.92	8.00	29.80	29.75
	70	7.66	7.70	33.00	32.97
B	45	8.30	8.20	22.78	22.54
	50	7.92	7.82	20.30	20.00
	55	7.69	7.75	19.83	19.78
	60	7.58	7.66	20.53	20.72
	65	7.54	7.65	22.45	22.68
	70	7.57	7.68	25.84	26.01
C	45	7.52	7.53	14.42	14.27
	50	7.53	7.57	13.82	14.01
	55	7.62	7.55	13.99	14.10
	60	7.77	7.83	14.76	14.85
	65	8.00	8.10	16.17	16.25
	70	8.30	8.21	18.43	18.75

A. F = 0.8 mL/min; T = 25°C
B. F = 1.0 mL/min; T = 38°C
C. F = 1.6 mL/min; T = 50°C

A new equation was developed relating H to the nature of OM and its percentage in the mixture OM/water. For a given couple, temperature/flow rate, the 1/H model [Eq.1] can be rearranged to obtain the analytical equation relating 1/H to the percentage of OM:

$$1/H_{OM} = \alpha_1 + \alpha_2 Ln\theta + \alpha_3 (Ln\theta)^2 \qquad (2)$$

Table 4

Values of ξ_1, ξ_2,ξ_3, of Equation 4 ($V(\theta)$ in 10^{-1} mm^{-1}) for Three Values of Column Temperature/Flow Rate

F(mL/min)	T(°C)	ζ_1	ζ_2	ζ_3	r^2*
0.8	25	96.10	-50.81	7.19	0.98
1.0	38	115.89	-57.97	7.74	0.97
1.6	50	114.88	-55.22	6.99	0.98

* Correlation coefficients of the fits.

where α_1, α_2, α_3 were constants and H_{OM} is the height equivalent to a theoretical plate determined on naphtalene in the OM/Water mixture. Therefore the following can be written:

$$1/H_{ACN} - 1/H_{CH3OH} = V(\theta) \quad (3)$$

$$V(\theta) = \xi_1 + \xi_2 Ln\theta + \xi_3 (Ln\theta)^2 \quad (4)$$

Values of the constants ξ_1, ξ_2, ξ_3 (determined using experimental data from Table 3) and correlation coefficients for the fits are given in Table 4. In each case, these constants were of the same signs and similar ranges. In the interval [45%.80%], the polynomial $V(\theta)$ was always greater than zero indicating that $H_{ACN} \leq H_{OM}$. This conclusion can be equally supported by the fact that the methanol solution is dominated by competitive hydrogen bonding and the availability of free "methanol" for solute solvatation decreases rapidly with an increasing fraction of water.

However, ACN solution chemistry is governed by clusters of ACN where the naphtalene molecule is preferentially solvated. ACN molecules increase solute naphtalene solubility in an aqueous solution and consequently its transfer between the two phases (mobile and stationary).

Separation Optimization

This separation was studied in a CH_3OH/water mixture.[11] The separation analysis time was determined as being equal to 18 min.

It is of interest to decrease this value and to obtain an efficient separation in a minimum analysis time. To reduce the time a new response function ζ is proposed. ζ is defined as:

$$\begin{aligned} \zeta &= \text{Min}(R_{ij}) && \text{if } \text{Min}(R_{ij}) \leq R_l \\ \zeta &= R_l + 1/t_a && \text{if not} \end{aligned} \qquad (5)$$

where $\text{Min}(R_{ij})$ = the resolution for the worst separated pair of peaks on the chromatogram.

R_l is the limit resolution accepted. If the separation is to be presented to industrial process control engineers, the objective might be to optimize the analysis time and not be to demanding for the value of the resolution of the worst separated pair of peaks. In our application, R_l was 0.8.

Therefore, if the resolution for the worst separated pair of peaks is inferior to the chosen limit resolution, then the ζ-function is equal to the resolution. If not, separation conditions were obtained and then the analysis time t_a intervened in the form 1/ta. Thus, the ζ-function was maximal when both efficient separation conditions and a minimal analysis time were obtained. The resolution between two peaks is given by the well known equation:

$$R_{ij} = \frac{\sqrt{N}}{4}\left(\frac{k'}{1+k'}\right)\left(\frac{\alpha-1}{\alpha}\right) \qquad (6)$$

where L is the column length, N is the column plate number = L/H, α is the separation factor given by the ratio of the capacity factor k' for the two solutes between which resolution is being calculated. The coefficient of variation of the k' values was less than 2%. Using the experimental design, ln(k') for each of the ten compounds and the column dead time t_o were modelled by a two order polynomial.

All the correlation coefficients were higher than 0.991. The student T-test confirmed that, for each compound, the k' value was not mobile phase flow rate dependent On the contrary t_o depended entirely on it. Obviously, the t_o model was found to be the same if ACN was used instead of CH_3OH (polynomial coefficients of the t_o model are given in Ref. 11). The analysis time t_a was given by the retention time of the last compound on the chromatogram (diazepam)

$$t_a = t_o\,(1 + \exp(\text{Ln}k'_{\text{diazepam}})) \qquad (7)$$

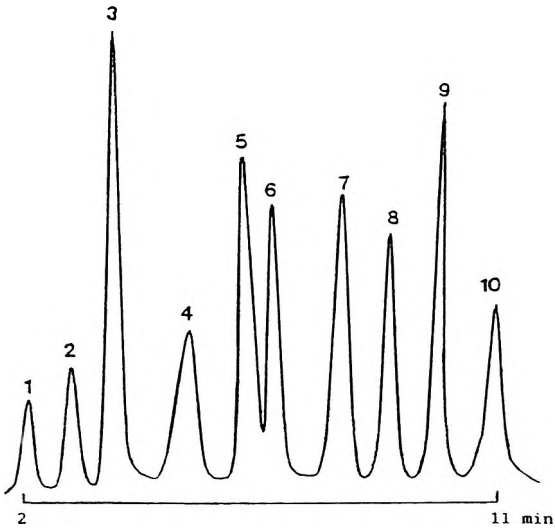


Figure 4. Benzodiazepine chromatogram in the conditions: ACN/water (60/40)(V/V), F = 1.00 mL/min. T = 47°C. Number above peaks refers to the 10 compounds: see experimental reagents.

Table 5

Comparison of Calculated $(t_a)_c$ and Experimental $(t_a)_{exp}$ Analysis Time (in min) for Different Experimental Conditions

θ(%)	F(mL/min)	T(°C)	$(t_a)_c$	$(t_a)_{exp}$
55	1.4	35	13.65	13.50
55	0.8	45	20.69	20.30
75	0.8	45	5.69	5.60
75	1.1	25	4.66	4.60
65	1.4	45	5.90	6.01
65	1.4	25	6.77	6.60
45	1.4	45	31.49	31.30
45	1.4	25	42.00	41.00

For example, the corresponding polynomial coefficients of $Ln(k'_{diazepam})$ are given in Table 1. Experimental and calculated values of t_a for different values of the three factors are given in Table 5. Computer simulations have begun to play an increasing role in the optimization separations.[21-25] The utility

Table 6

Result of the Simplex Process for the Optimization of the ξ-Function

θ(%)	F(mL/min)	T(°C)	ζ
80.00	1.60	25	0.0101
75.85	1.57	32	0.0129
72.48	1.65	45	0.0581
70.44	1.60	30	0.0542
78.12	1.75	40	0.0121
74.65	1.23	42	0.0226
69.75	1.00	46	0.2081
65.45	1.45	25	0.0201
69.63	1.32	35	0.1998
66.00	1.20	48	0.3888
77.45	1.19	38	0.0123
68.12	1.33	50	0.3423
64.13	1.00	44	0.3951
67.14	1.20	46	0.3884
65.47	0.80	50	0.5411
55.22	0.90	48	0.8532
54.96	0.85	48	0.8534
50.01	0.80	37	0.8273
45.05	0.70	34	0.8134
52.41	0.75	42	0.8346
54.23	0.84	48	0.8496
50.74	0.92	50	0.8411
56.12	1.02	47	0.8694
59.56	1.00	45	0.8864
59.95	1.01	47	0.8920

of the ζ-method, is that it takes into account the analysis time t_a and the simultaneous variation of column efficiency with the three factors, mobile phase composition, its flow rate, and the column temperature. The experimental design reduced the number of experiments to be carried out. Therefore, knowing the variation of H, k', α and t_a with the mobile phase composition, its flow-rate and column temperature, the ζ-values can be

calculated for different values of the three factors. ζ-reached its maximum for θ = 60%, F = 1.00mL/min, T = 47°C (values determined using the simplex method (Table 6). The chromatogram with these conditions is given in Figure 4. The analysis time was 11 min.

CONCLUSION

The use of an ACN/water instead of a CH_3OH/water mixture improved column efficiency. The results are corroborated by simple new equations relating the column efficiency to both the nature of the organic modifier and its percentage in the OM/water mixture, and by the organization of the ACN molecules in the H_2O molecules. In addition, the separation of these compounds and the analysis time were both optimized with a new response function developed in our laboratory. The results showed that the analysis time was reduced by 50 % when ACN was chosen instead of CH_3OH.

AKNOWLEDGMENT

The authors thank V. David Maupoil for her help.

REFERENCES

1. S. L. Morgan, S. N. Deming, Chromatographia, **112**, 267 (1975).

2. H. J. G. Debets, B. L. Bajema, D. A. Doornbos, Anal. Chem. Acta, **15**, 131 (1983).

3. H. J. G. Debets, J. W. Weyland, D. A. Doornbos, Anal. Chem. Acta, **150**, 259 (1983).

4. M. W. Watson, P. W. Carr, Anal Chem., **51**, 1835 (1979).

5. J. L. Glajch, J. J. Kirkland, K. M. Squire, J. M. J. Minor, J. Chromatogr., **199**, 57 (1980).

6. W. Yau, J. J. Kirkland, J Chromatogr., **556**, 111 (1991).

7. F. Dondi, T. Gianferra, J Chromatogr., **485**, 631 (1989).

8. P. Chaminade, A. Baillet, D. Ferrier, J. Chomatogr., **672**, 67 (1994).

9. L. A. Larew, B. A. Olsen, J. Chromatogr., **672**, 183 (1995).

10. Y. Guillaume E. Peyrin, C. Guinchard, J Chromatogr., **665**, 363 (1995).

11. Y. Guillaume, C. Guinchard, J. Liq. Chromatogr., **17(17),** 1443, (1994).

12. A. F. Fell, T. A. G. Noctor, J. E. Manna, B. J. Clark, J. Chromatogr., **434**, 377 (1988).

13. G E. P. Box, D. W. Benhken, Technometrics **2**,455 (1960).

14. G. E. P. Box, K. B. Wilson, J. Royal. Stat. Soc. B., **13**, 1 (1951).

15. G. E. P. Box, W. G. Hunter, S. J. Hunter, **Statistics For Experiments**, Wiley, New York, 1978, Part III, Ch 9-13.

16. Y. Guillaume, C. Guinchard, J. Chromatogr. Sci., **33**, 204 (1995).

17. A. M. Stalcup, D. E. Martire, S. A. Wise, J. Chromatogr., **442**, 1 (1988).

18. A. Lowenschuss, N. Yellin, Spectrochim. Acta, **31A**, 207 (1975).

19. K. L. Rowlen, J. M. Harris, Anal. Chem., **63**, 964 (1991).

20. R. M. Smith, D. R. Garside, J Chromatogr., **407**, 19 (1987).

21. Q. S. Wang, G. Ru-Yu, W. Heng-Yan, J. High Resol. Chromatogr., **13**, 173 (1990).

22. S. G. Lisseter, Lab. Microcomput. **9**, 109 (1990).

23. R. Matsuda, Y.Hayashi, T. Suzuki, J. Chromatogr., **585**, 187 (1991).

24. Y. Hayashi, R. Matsuda, Anal Chim Acta., **222**, 313 (1989).

25. R. Matsuda, Y. Hayashi, M. Ishibashi, Y. Takeda, Anal. Chem. Acta., **222**, 301 (1989).

Received January 1996
Accepted April 24, 1996
Manuscript 4066

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1757–1772 (1997)

DETERMINATION OF ABAMECTIN IN SOME FOREST MATRICES BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

K. M. S. Sundaram, J. Curry

Natural Resources Canada
Canadian Forest Service
1219 Queen Street East
P.O. Box 490
Sault Ste. Marie, ON, P6A 5M7, Canada

ABSTRACT

A liquid chromatographic method was developed and validated for the determination of abamectin B_{1a} residues in some terrestrial (balsam fir and oak foliage, sandy and clay loam soils and leaf litter) and aquatic (stream water and sediment) forest matrices. The processed foliage, soil, litter, and sediment were fortified with abamectin and extracted with ethyl acetate. The fortified steam water was extracted with dichloromethane. Aliquots of crude extracts were cleaned with Florisil® column chromatography and the purified extracts were derivatized using 1-methylimidazole and trifluoroacetic anhydride. The derivatized abamectin was analysed by reverse phase liquid chromatography, with a fluorescence detector set at 232 nm excitation and 461 nm emission wavelengths. A Spherisorb® ODS2, 5μm, 250 × 4 mm column was used. The samples were run isocratically using methanol-water as the mobile phase. Mean recoveries for the analyte ranged from 83.0 to 93.0%, with a coefficient of variation from 6.3 to 12.4%. Limits of detection and

limits of quantitation for solid matrices ranged from 0.10 to 0.20 and from 0.30 to 0.60 ng/g, respectively, and for stream water the corresponding values were 0.003 and 0.009 ng/mL. The procedure provides a reliable and sensitive method for determining abamectin B_{1a} residues in forest matrices.

INTRODUCTION

Environmental and ecological concerns regarding the use of synthetic chemical pesticides coupled with increasing public pressure have resulted in the use of microbials to combat several defoliating lepidopterous pests in Canadian forestry. However, apart from the bacterium, *Bacillus thuringiensis (B.t.)*, the control options available at present, for the forest defoliators, are very limited. Avermectin B_1, a mixture of two homologous components [avermectin B_{1a} ($\geq 80\%$) and avermectin B_{1b} ($\leq 20\%$)], (Figure 1), is a natural macrocyclic lactone produced by the soil microorganism actinomycete, *Streptomyces avermitilis*.[1] The major component B_{1a} differs from the minor component B_{1b} by a single methylene group. B_{1a} contains a secondary butyl substituent at the C-25 position, whereas B_{1b} has an isopropyl substituent at that carbon. Because it is not economical to separate the two avermectin components on a large scale, the mixture is marketed commercially as abamectin.[2] Abamectin is found to be toxic to different species of insects and mites.[3] It has high activity against a broad range of lepidopterous larvae including the spruce budworm (*Choristoneura sp.*) and gypsy moth [*Lymantria dispar* (L.)], the two leading destructive defoliators of Canadian forests.[4] Field application rates to control various lepidoptera were estimated to be in the order of 15 g/ha.[2] It is a very low dosage compared to the synthetic insecticides used before.

Aerial application of insecticides is a favorable method used in forestry to cover vast areas of infested forests. Abamectin has many desirable properties to become a choice insecticide in forestry, *viz.*, its broad spectrum of activity at low dosage levels, its natural origin compared to the synthetic neurotoxic pesticides, and short environmental persistence. If it is chosen as a candidate material to control insect pests in forestry and sprayed aerially, different matrices such as foliage, soil, litter, surface water, sediment, etc. would be exposed and contaminated by the sprayed chemical. Exposed matrices have to be analyzed to determine the initial deposition and persistence of the material in order to assess its biological effectiveness, fate and mobility patterns, and overall environmental safety. Until now, no attempt has been made to analyze the abamectin residues in forest matrices and no published method has been reported for them. Analysis of abamectin and its dihydroderivative (ivermectin) at residue levels is an involved process and a number of high performance liquid chromatographic (HPLC) methods using UV or fluorescence detection have been reported for

agricultural products, animal tissues, milk, plasma, feces, wine, and other biological matrices.[2,5-18] The principal steps involved in these methods are the solvent extraction of the marker analyte (B_{1a} for abamectin) using suitable solvent or solvent mixtures, followed by partition and cleanup steps, derivatization, and eventual quantification by HPLC. Forest matrices, such as conifer needles, soil, litter, and sediment, are complex in nature and are associated with coextractive impurities, such as lipids, pigments, proteins, carbohydrates, etc., compared to agricultural and biological samples. Residue isolation and cleanup techniques must be such that they give high percentage recovery of the target analyte while simultaneously minimizing interferences, which may contribute to high background in the analysis. With this objective in mind, we have developed a reliable and sensitive HPLC method using fluorescence detection to isolate and quantify abamectin in forest matrices using B_{1a} as the marker for the analyte. Details of the method are reported in this paper.

MATERIALS AND METHODS

Analytical Standards

Abamectin standard containing 0.893% B_{1a} (w/w) and 0.044% B_{1b} (w/w) components dissolved in glycerol formal was kindly supplied by Ms. Boutin-Muma of Agriculture Canada, Ottawa, ON. Standard stock solution of B_{1a} containing 25 μg/mL was prepared by transferring exactly 140 mg of the standard glycerol formal solution (containing 1250 μg of avermectin B_{1a}) to a 50.0-mL amber volumetric flask and diluted to the mark with acetonitrile. This standard stock solution also contained 61.6 μg or 1.23 μg/mL B_{1b}. Intermediate standard solution (1 μg/mL) was prepared by transferring 2.0 mL avermectin B_{1a} stock solution to a 50.0-mL amber volumetric flask and diluting to the mark with acetonitrile. Working standards (0.5 to 25 ng/mL) for instrument calibration and fortification of substrates were prepared by the serial dilution of the intermediate standard solution using acetonitrile. All standards were kept in sealed amber volumetric flasks and stored at -10°C when not in use. Avermectin solutions were quite stable for six weeks under these conditions and fresh standards were prepared afterwards.

Reagents

Acetone (AT), acetonitrile (ACN), dichloromethane (DCM), ethyl acetate (EA), hexane (HX), methanol (MT), and tetrahydrofuran (THF) were HPLC grade obtained from VWR Canlab (Mississauga, ON); water was purified with a Millipore Milli-Q® water purification system (Millipore Corp., Milford, MA); trifluoroacetic anhydride (TFAA) and 1-methylimidazole (MIZ) were from Fisher Scientific

(Unionville, ON) and Aldrich Chem. Co. (Milwaukee, WI), respectively, Florisil® [PR grade, activated, solvent (MT and ether) washed and dried at 150°C for 24 h and stored in sealed bottles] was from Floridin Co. (Pittsburgh, PA); Nuchar® SN charcoal (acid washed and dried), silated Pyrex brand glass wool, ammonium hydroxide (30%), sodium sulphate (Na_2SO_4, anhydrous), and Whatman CF-11® cellulose powder were supplied by Fisher Scientific; and the HPLC mobile phase used in the study was prepared by mixing in the ratio of 95:5 (v/v), MT and water, filtering (0.45-μm Acrodisc® filter) and degassing the mixture prior to use.

Apparatus

A chopper (Hobart) for the initial maceration of foliage, litter, and soil, and a Kendall Mixer (Fisher) for the extraction of analyte from different matrices, were used. Centrifugations were achieved with a bench top Centra-8R® centrifuge from Damon, MA (USA) and filtrations were done using Acrodisc®-3 PTFE membrane filters. The solvents were evaporated using a Flash-Evaporator from Buchler Instruments (Fort Lee, NJ) and a Meyer N-Evap® (Organomation). Purification of the extracts were done using Florisil, PrepSep®-N_2 (Fisher) and Nuchar minicolumn chromatography. Solvent extraction and partition of the analyte from different solid matrices and subsequent derivatization after necessary cleanups were all done in Teflon® (VWR Canlab) wares (separatory funnels and centrifuge tubes) to minimize loss of the analyte by adsorption. HPLC analyses were performed by a computer controlled Hewlett-Packard (HP) 1090M liquid chromatograph fitted with an automatic sampler, a variable volume auto-injector, a binary solvent delivery system with helium degasser and a dual-syringe metering pump assembly to give consistent flows. An HP 1064 A fluorescence detector equipped with a 5-μL flow-cell and variable excitation and emission wavelengths, both scannable from 190 to 800 nm, was used to detect the abamectin derivative. The excitation and emission wavelengths were optimized and set at 232 and 461 nm, respectively. The computer work station (HP-9000/310), operated by the HP-7995R software, processed the peak area of each chromatogram obtained. The pre- and analytical columns used were HP-Spherisorb ODS2, 5 μm, 20 × 4 mm and 5 μm, 250 × 4 mm, respectively. They were thermostated at 25°C in the HPLC column compartment. A 100-μL portion of the derivatized extract was injected into the HPLC column and eluted using the mobile phase. The samples were run isocratically at 1.5 mL/min. Under these conditions, the larger B_{1a} component of abamectin eluted at 9.5 ± 0.2 min as a sharp, narrow peak and the smaller B_{1b} component eluted at 8.3 ± 0.5 min. However in the present study, because of its consistency in response, the larger B_{1a} component was taken as the standard to validate the method for abamectin from different forest matrices.

Forest Matrices

Red oak (*Quercus rubra* L.) and balsam fir [*Abies balsamea* (L.) Mill.] foliage were collected from a mixed forest north of Sault Ste. Marie (ON, Canada). Forest litter [pH 5.4; organic matter (OM) > 88%] and soils (sandy loam, pH 5.6, OM 5.4%, sand 56%, clay 6% and silt 38%; clay loam, pH 5.1, OM 11.7%, sand 38%, clay 16% and silt 46%) were collected from forested areas around Timmins (ON, Canada). Water [pH 6.1; alkalinity and hardness (mg/L of $CaCO_3$) 16.2 and 14.3, respectively; turbidity 0.31 JTU] was collected in 1-L Teflon bottles from a slow-flowing stream near Searchmont (ON, Canada) and stored at 4°C in the dark. Sediment (sand 78%, silt 19%, clay 3%, OM 1.4%) was also collected from the stream by gently lowering a capped glass jar to the stream bottom, scooping the sediment after removing the cap, and closing the jar after sufficient amount was collected. Standard procedures required for the collection of various matrices, their transportation, storage, and processing prior to fortification with the analyte are described elsewhere[19] and were followed in this study.

Extraction Procedure

Foliage

Five-gram aliquots of processed[19] fir needles and oak foliage were taken in separate 50-mL Teflon centrifuge tubes and mixed with 5 g Na_2SO_4. Each sample was fortified in quintuplicate with abamectin standard ranging from 0.60 to 5.0 ng/g. The tubes were vortexed for 5 min to mix the analyte uniformly with the matrix and allowed to equilibrate for 30 min. Twenty mL of EA was added to each sample and shaken for 30 min in a Kendall mixer to extract the material. After centrifugation at 3000 rpm for 5 min, the supernatant was filtered through a glass funnel using the EA washed Whatman #4 filter paper into a 250-mL round bottom flask (RBF). The residue in the tube was reextracted twice using 20 mL of the extractant each time and filtered through the same funnel into the RBF. The residue was then transferred carefully to the filter paper. The centrifuge tube and the residue on the filter were then rinsed with 3 x 10 mL of EA and the rinses were collected in the same RBF. The residue was discarded.

The pooled filtrate and rinses were flash evaporated to dryness at 30°C and under low pressure in a rotary evaporator. The residue was dissolved in 20% AT in HX and transferred quantitatively to a graduated Teflon tube, evaporated under nitrogen atmosphere and the volume adjusted to 1.0 mL for column cleanup. Care was taken to rinse the RBF with AT-HX mixture thoroughly, so that no analyte was lost through irreversible adsorption.

Soil and litter

The extraction procedure used for these matrices was similar except that 10.0-g aliquots of the soil samples fortified at 0.30 to 2.50 ng/g were used and the volume of the extractant was adjusted accordingly. The fortification level in litter was the same as in foliage. Care was taken to mix the Na_2SO_4 thoroughly with the matrix to avoid clumping and the resultant slurry after extraction was centrifuged at 5000 rpm for 10 min to completely settle the soil and litter particulates. The extract was further treated as in foliage.

Sediment

The sediment was initially filtered through a Buchner funnel (43 mm ID) using Whatman #4 filter paper under moderate vacuum to remove most of the absorbed water. Ten-g aliquots, mixed thoroughly with an equal amount of Na_2SO_4, were used for fortification (0.30 to 2.50 ng/g) and subsequent extraction studies. The sample was further treated, as above, in soil and litter.

Water

One hundred-mL aliquots of filtered (0.45 μm Acrodisc) stream water, in 250-mL Teflon separatory funnels, were fortified in quintuplicate with abamectin standard ranging from 0.010 to 0.250 ng/mL. Ten mL of 5% aqueous sodium chloride was added to each water sample to minimize emulsion formation and they were extracted with 4 x 30 mL of DCM. The lower organic phase was drained through a column of Na_2SO_4 (3 cm diam. x 3 cm length) into a 250-mL RBF and the column was rinsed with 3 x 10 mL of the extractant. The combined extracts and the column rinses of each sample were then flash evaporated to dryness and the residue was further treated as in foliage.

Chromatographic Column Cleanup

The chromatographic minicolumn (disposable pasteur pipet, 15 cm x 0.8 cm ID) was packed from bottom to top with a small wad of silated glasswool, 5 cm of activated Florisil and 1 cm of Na_2SO_4. After prewashing the column successively with 5 mL of AT and 5 mL of HX, 1.0 mL of the crude extract was loaded onto the column. It was first eluted with 10 mL of 20% AT in HX followed by 15 mL of AT, without allowing the column to run dry. The initial 10 mL of the eluent was discarded. The eluted abamectin in the 15 mL AT fraction was then collected in a 50-mL Teflon centrifuge tube. It was evaporated under nitrogen to dryness and reconstituted in 0.6 mL of ACN for derivatization.

Derivatization of Samples and Standards

The cleaned-up samples of matrices (each in 0.6 mL of ACN) and 0.6-mL aliquots of each abamectin standard used in the preparation of calibration curve, were treated separately with 0.10 mL of MIZ, vortexed gently and cooled in an ice bath for 10 min. Afterwards, 0.3 mL of cooled, fresh TFAA in ACN was added, vortexed gently and incubated at 30°C for 10 min. The derivatized abamectin was then filtered through a 0.45-μm filter (Acrodisc 3 PTFE, 3 mm) and analyzed by HPLC by injecting 100-μL volumes in quadruplicate. Derivatized standard concentrations ranged from 0.5 to 25 ng/mL and the amount injected ranged from 0.05 to 2.5 ng. In a typical working day *ca* three foliage or soil samples could be extracted and analyzed. The derivatives formed were stable for about 12 d at -20°C (in the dark), after which the material gradually degraded and the RT shifted from 9.5 ± 0.2 min to 5.6 ± 0.3 min. Total breakdown occurred after 45 d and the addition of aqueous ammonia was found to facilitate the rapid degradation. The structures of abamectin and the fluorescent derivative formed in the presence of MIZ and TFAA are given in Figure 1.

Calibration Curve Preparation

Calibration samples of abamectin in ACN were prepared to cover the range from 0.50 to 25 ng/mL. After derivatization, 100-μL aliquots were injected (0.05 to 2.5 ng of abamectin) in quadruplicate and a calibration curve was constructed by plotting the average peak area counts against amount of abamectin in ng (Figure 2). The area counts of the four injections agreed within 1.7%, showing good repeatability. A linear regression line fitted over the 50-fold concentration range gave a regression equation of $y = 4.531 \times c$ (y = the peak area count and c = the amount of abamectin injected in ng). The coefficient of determination was 0.9996, thus indicating the excellent linearity in the response of FD to the analyte over the range of concentrations studied. Abamectin concentrations in the fortified forest matrices were computed from the calibration curve using the measured area counts of the derivatized extracts.

RESULTS AND DISCUSSION

Validation of Method

The quantification procedure and conditions (column, mobile phase, flow rate, wavelength, column temperature and pressure, etc.) reported in this paper were

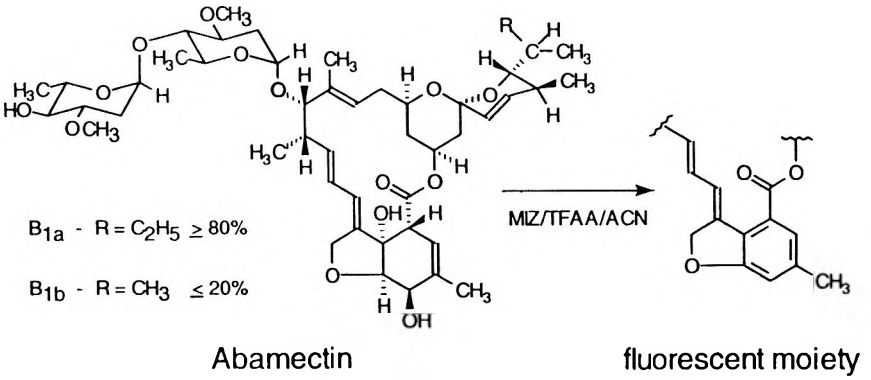


Figure 1. Structure of abamectin and its fluorescent derivative.

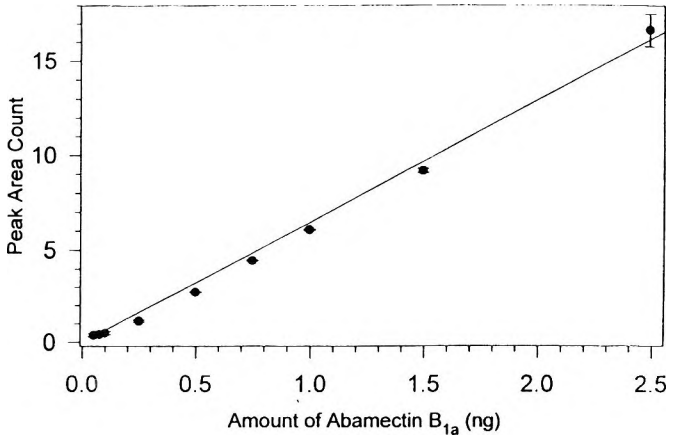


Figure 2. Abamectin standard curve. Amount vs. peak area count.

selected by trial and error and using the earlier publications[3,5-18] as a guideline. They were optimized to avoid interferences from the matrix constituents with the active ingredient, *i.e.*, derivatized abamectin. Repeatability of the experiment was verified by injecting 100 μL of different derivatized abamectin standards in quadruplicate onto the Spherisorb ODS2 column and measuring the peak area. The peak area for the four injections of the same analyte concentration were very similar, deviating only about 1.7% from the mean. The linearity of the detector response was checked

and confirmed by injecting increasing amounts of the derivatized analyte (100-μL quantities) over a concentration range of 0.50 to 25 ng/mL (0.05 to 2.5 ng) and preparing a calibration curve with a correlation coefficient $R^2 = 0.9996$. Different excitation and emission wavelengths were scanned, ranging from 200 to 400 nm for excitation and 400 to 600 nm for emission. The optimum wavelengths chosen for the excitation and emission were 232 and 461 nm, respectively, and this combination produced the best fluorogenic response for the derivatized analyte. Attempts to alter either the reported mobile phase composition (5% water in MT) or to substitute MT with ACN or THF resulted in deficient resolution and stationary phase clogging, consequently leading to tailing of chromatographic peaks.

No silylation of glassware (especially the RBF and the mini-chromatographic columns) was done, as required;[8] however the results obtained from recovery studies (Tables 1 and 2) were good and were within the limits of acceptance (80 - 100% of the expected value), presumably due to the use of Teflon ware in the extraction, partition and derivatization steps. Nevertheless, adequate care was necessary in many other areas to obtain good chromatographic profile and recovery levels. The optimum solvent volume (0.6 mL of ACN) used to dissolve the abamectin residue prior to derivatization, appropriate residue concentration required for the complete derivatization, absence of moisture in the reaction mixture, impurity free Na_2SO_4 and Florisil (for purification see ref. 20), as well as the activity level of the latter, were essential for the success of the study. HPLC column overloading, after frequent injections, resulted in clogging of the stationary phase, and consequent build-up of back pressure did occur occasionally, resulting in peak asymmetry. This was avoided by frequent changing of the guard column and reversing the analytical column and flushing it with the mobile phase.

Many extracting solvents were tried for quantitative recovery of the analyte from fortified environmental samples. HX and other nonpolar aliphatic hydrocarbons were unsuitable because of the poor recoveries of abamectin from fortified water samples. Among the chlorinated aliphatics, DCM gave consistently good recoveries and was chosen as the preferred extractant. Use of MT and AT as extracting solvents for solid matrices brought with them considerable amounts of coextractive impurities, necessitating extensive, time-consuming, and costly cleanups, which ultimately resulted in low analyte recoveries. Trial studies showed that EA was appropriate and suitable to extract abamectin from fortified solid matrices. Similarly, different chromatographic column packings (PrepSep-N_2, Nuchar-cellulose and activated Florisil) were tried along with pure and different combinations of eluting solvent systems (MT, ACN, THF, AT, HX, EA, and mixtures of these in different ratios). Among them, the use of Florisil minicolumn, pre-rinsed successively with AT then HX and elution of the analyte first with AT/HX mixture to remove lipids and other coextractives and later with AT, gave

Table 1

Recovery and Intra-Assay Precision in the Analysis of Abamectin (B_{1a} Component) from Fortified Terrestrial Matrices (N = 5)

Matrix	Fortification Value (ng/g)	Average Recovery (% of Fortified Value)	S D (±)	CV (%)
	0.60	78.2	9.8	12.5
	1.00	82.8	7.1	8.6
Fir needles	2.00	85.0	4.1	4.8
	3.00	85.4	4.8	5.6
	5.00	83.7	7.3	8.7
	mean	83.0	6.6	8.0
	0.60	72.6	9.8	13.5
	1.00	88.2	7.7	8.7
Oak foliage	2.00	83.7	9.6	11.5
	3.00	87.4	3.1	3.6
	5.00	86.7	6.0	6.9
	mean	83.7	7.2	8.8
	0.60	89.4	19.5	21.8
	1.00	80.0	12.2	15.3
Litter	2.00	85.6	10.0	11.7
	3.00	85.3	8.3	9.7
	5.00	85.7	3.0	3.5
	mean	85.2	10.6	12.4
	0.30	86.5	11.2	13.0
	0.50	84.4	6.6	7.8
Soil	1.00	88.7	3.1	3.5
(clay loam)	1.50	87.3	5.4	6.2
	2.50	86.7	2.6	3.0
	mean	86.7	5.8	6.7
	0.30	75.0	5.2	6.9
	0.50	89.5	3.7	4.1
Soil	1.00	86.0	7.2	8.4
(sandy loam)	1.50	89.9	6.1	6.8
	2.50	93.2	4.9	5.3
	mean	86.7	5.4	6.3

Table 2

Recovery and Intra-Assay Precision in the Analysis of Abamectin (B_{1a} Component) from Fortified Aquatic Matrices (N = 5)

Matrix	Fortification Value	Average Recovery (% of Fortified Value)	S D (±)	CV (%)
	0.30 ng/g	95.6	16.8	17.6
	0.50	88.3	13.7	15.5
Sediment	1.00	86.5	5.4	6.2
(stream)	1.50	86.0	6.2	7.2
	2.50	91.7	3.3	3.6
	mean	89.6	9.1	10.0
	0.010 ng/mL	76.8	10.2	13.3
	0.050	99.9	6.8	7.8
Water	0.100	96.9	4.7	4.1
(stream)	0.150	95.9	7.6	7.9
	0.250	95.6	6.1	6.4
	mean	93.0	7.1	7.9

cleaner eluates and acceptable recoveries of the material. The use of commercially available PrepSep-N_2 columns were found to be satisfactory, except for their high cost compared to the home-made Florisil columns. The Nuchar-cellulose columns were unsatisfactory due to strong adsorption of the analyte onto the column packing.

Limits of Detection and Quantitation

The limits of detection (LOD) of abamectin in different forest matrices were determined as 3 × SD at the lowest fortification level for a particular substrate. The limits of quantitation (LOQ) were expressed arbitrarily as 3 × LOD. The LOD and LOQ values obtained for the different substrates in this study are given below:

	LOD*	LOQ*
Water	0.003	0.009
Foliage	0.20	0.60
Litter	0.20	0.60
Soil	0.10	0.30
Sediment	0.10	0.30

* water, ng/mL; others, ng/g; injection volume 100 μL.

Abamectin Recovery

The recovery of abamectin from various terrestrial matrices fortified at different concentration levels (foliage and litter, 0.60 to 5.00 ng/g; and soils 0.30 to 2.50 ng/g) is given in Table 1. The recovery for aquatic substrates (fortified: sediment 0.30 to 2.50 ng/g and water 0.010 to 0.250 ng/mL) is given in Table 2. The mean percentage recovery for each sample with its standard deviation (SD) and average coefficient of variation (CV) were derived from multiple injections of quintuplicate samples. Generally, as seen in Tables 1 and 2, the recoveries varied from the expected values according to the complexity of the matrix and to some extent, with the level of fortification. The recovery levels were relatively low in foliage and high in water, and often, but not always, the recoveries were better at higher fortification levels.

The mean percent recoveries of fir and oak foliage were 83.0 and 83.7, respectively, with the percent means of the measured values differing from the fortified concentrations by 17.0 and 16.3, respectively. Mean recovery values (%) for litter, clay loam and sandy loam soils, sediment, and stream water were 85.2, 86.7, 86.7, 89.6, and 93.0, respectively (Tables 1 and 2). The percent means of the measured values differed from the fortified concentrations for these matrices by 14.8, 13.3, 13.3, 10.4, and 7.0, respectively. The good percent recoveries of abamectin obtained from the fortified samples of known concentrations show that the accuracy of the method was satisfactory. The inter-assay precision, showing the reproducibility of the abamectin recoveries between each replicate sample, as indicated by the corresponding SD and CV (%) values, varied according to the fortification level. The trend observed was that the precision was low (high SD and CV) at low fortification levels and vice versa. For example, for litter the SD (±) and CV (%) at 0.60 ng/g fortification level were 19.5 and 21.8, respectively, whereas at 5.0 ng/g, the corresponding values were only 3.0 and 3.5 (Table 1). However, the intra-assay precision reported in terms of the mean values of the SD (±) and CV (%) obtained respectively within each substrate, *viz.*, fir needles (6.6 and 8.0), oak foliage (7.2 and 8.8), litter (10.6 and 12.4), clay loam soil (5.8 and 6.7), sandy loam soil (5.4 and 6.3), sediment (9.1 and 10.0), and water (7.1 and 7.9) were rather low indicating good precision of the reported method (Tables 1 and 2).

Chromatograms

A typical chromatogram of the reagent blank and abamectin standard obtained by injecting 5.0 ng in 100 μL onto the HPLC is shown in Figure 3a. The peaks with RT (average) 9.5 ± 0.2 min for B_{1a} (major peak) and 8.3 ± 0.5 min for B_{1b} (minor peak) were symmetrical, with the baseline separated and well removed from the solvent front. Deviation in RTs for each injection was observed, however it was not

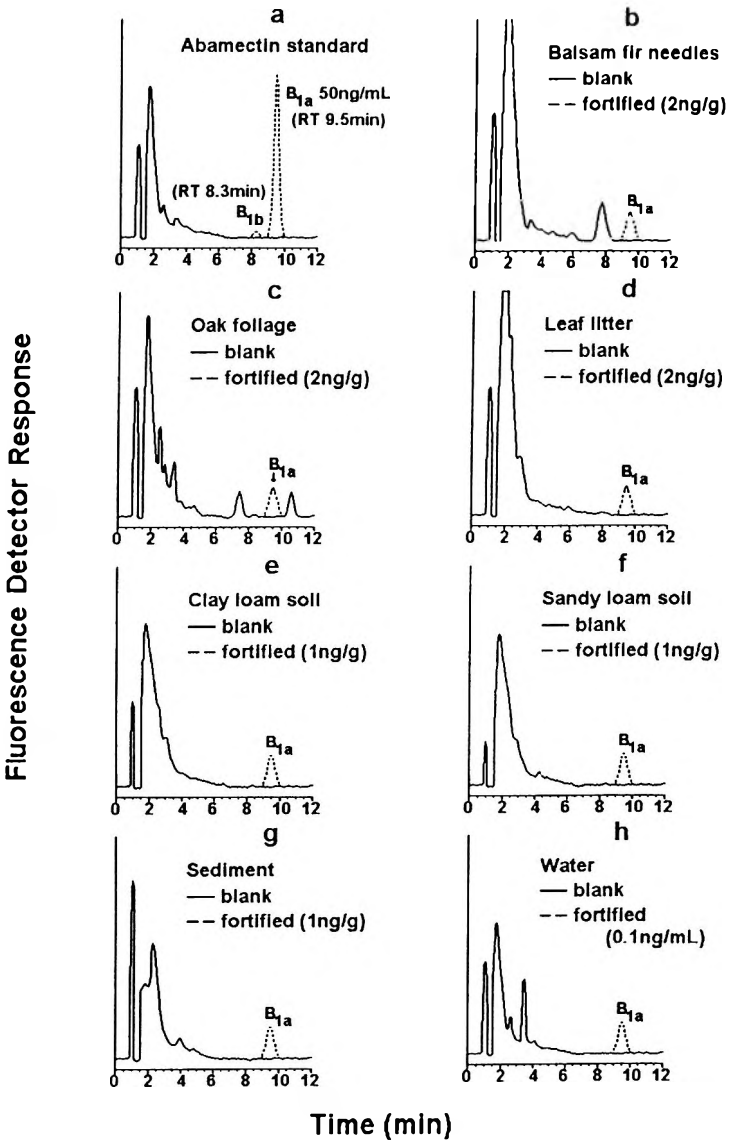


Figure 3. Typical chromatograms of fortified and blank forest substrates (abamectin peak shown as dotted line over blank chromatogram).

significant, as can be ascertained from the recorded SD values. Figure 3b is a typical chromatogram obtained for conifer needles, blank and fortified at 2.00 ng/g and injecting 100-μL volumes (abamectin B_{1a} is shown by dotted line). The B_{1a} analyte peak was narrow and separated well without any interference from the peaks derived from the endogenous materials present in the needle. Similar patterns were also observed in the chromatograms obtained for blank and fortified samples of oak foliage (Figure 3c), leaf litter (Figure 3d), forest soils (fortified at 1.00 ng/g; Figures 3e and 3f), and stream sediment (Figure 3g). The optimal analytical and HPLC conditions used in the study were adequate to quantify the abamectin B_{1a} analyte from forest foliage, soil, litter, and sediment. The chromatograms (blank and fortified at 0.100 ng/mL) from the water extracts (Figure 3h) were cleaner, without the many extraneous peaks found in the other matrices. The total chromatographic analysis time was 20 min. Based on the analysis of 56 fortified samples, comprising different sample types (including water), the average analysis time per sample was found to be about 2.5 h.

CONCLUSIONS

Within the framework of method validation, and using parameters such as consistency in the recovery of the analyte from fortified forest matrices reflecting accuracy, satisfactory intra-assay precision defined in terms of SD, and inter-assay precision reflecting reproducibility, the good recoveries obtained for all the matrices at different fortification levels, clearly show that the method is applicable to determine abamectin residues from a wide range of forest matrices. The method is reliable, robust, and sensitive and will be a useful tool for the routine determination of abamectin concentrations; however, it is somewhat time-consuming. Nevertheless, to ensure good results, it is essential that the various steps reported in this method should be carefully followed. With necessary modifications, this method could find wide applicability in examining the initial deposition and the fate of abamectin in diverse forest matrices. The throughput of the assay is primarily dependent upon the complexity of the matrix and the chromatographic separation. For example, when no column cleanup is required (typically for the analysis of standards), the throughput is limited only by the derivatization step and speed of the autosampler.

ACKNOWLEDGMENT

The authors sincerely thank Bernadette Boutin-Muma, Laboratory Services Division, Agriculture Canada, Ottawa, ON for providing the abamectin standard used in this study.

REFERENCES

1. R. W. Burg, B. M. Miller, E. E. Baker, J. Birnbaum, S. A. Currie, R. Hartmar, Y-L. Kong, R. L. Monaghan, G. Olson, I. Putter, J. B. Tunac, H. Wallick, E. O. Stapley, R. Oiwa, S. Omura, Antimicrob. Agents Chemother., **15**, 361-367 (1979).

2. J. A. Cobin, N. A. Johnson, J. AOAC Int., **79(5)**, 1158-1161 (1996).

3. T. Wehner, J. Lasota, R. Demchak, "Abamectin," in **Comprehensive Analytical Profiles of Important Pesticides**, J. Sherma, T. Cairns, eds., CRC Press, Boca Raton, FL 33431, USA, pp. 73-106 (1993).

4. R. Martineau, **Insects Harmful to Forest Trees**, Canadian Forest Service, Science and Sustainable Development Directorate, Natural Resources Canada, Ottawa, ON, Canada K1A 0E4, 1984, 261 pp.

5. I. Norlander, H. Johnson, Food Addit. Contam., 7, 79-82 (1990).

6. J. Vuik, J. Agric. Food Chem., **39**, 303-305 (1991).

7. S. V. Prabhu, R. J. Varsolona, T. A. Wehner, R. S. Eagan, P. C. Tway, J. Agric. Food Chem., **40**, 622-625 (1992).

8. P. J. Kijak, J. AOAC Int., **75(4)**, 747-750 (1992).

9. F. J. Schenck, S. A. Barker, A. R. Long, J. AOAC Int., **75(4)**, 655-658 (1992).

10. J. Markus, J. Sherma, J. AOAC Int., **75(4)**, 757-767 (1992).

11. C. D. C. Salisbury, J. AOAC Int., **76**, 1149-1151 (1993).

12. M. Roth, G. Rae, A. S. McGill, K. W. Young, J. Agric. Food Chem., **41**, 2434-2436 (1993).

13. J. M. Degroodt, B. Wyhowski De Bukanski, S. Srebrnik, J. Liq. Chrom., **17(6)**, 1419-1426 (1994).

14. F. J. Schenck, J. Liq. Chrom., **18(2)**, 349-362 (1995).

15. J. A. Cobin, N. A. Johnson, J. AOAC Int., **78(2)**, 419-423 (1995).

16. L. D. Payne, M. B. Hicks, T. A. Wehner, J. Agric. Food Chem., **43**, 1233-1237 (1995).

17. M. W. Brooks, P. C. Uden, Pestic. Sci., **43**, 141-146 (1995).

18. J. Li, C. Qian, J. AOAC Int., **79(5)**, 1062-1067 (1996).

19. K. M. S. Sundaram, "Sampling Forestry Materials for Chemical Accountability Studies", in **Forest Insect Pests in Canada**, J. A. Armstrong, W. G. H. Ives, eds., Natural Resources Canada, Canadian Forest Service, Science and Sustainable Development Directorate, Ottawa, ON, Canada K1A 0E4, 1995, pp. 485-492.

20. K. M. S. Sundaram, J. Environ. Sci. Health, **B25(3)**, 357-378 (1990).

Received October 28, 1996
Accepted December 16, 1996
Manuscript 4313

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1773–1787 (1997)

OPTIMIZATION OF A REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION USING AN ION-PAIR REAGENT FOR THE DETERMINATION OF CARBOXYLIC ACIDS IN PLANT MATERIALS

Christopher F. Harrington,*† David J. Roberts, Graham Nickless

University of Bristol
School of Chemistry
Cantocks Close
Bristol BS8 1TS, United Kingdom.

ABSTRACT

A method was developed to separate a number of mono-, di- and tri- carboxylic acids present in plant materials, using a C_{18} column and a simple eluent containing a phosphate buffer and tetra-n-butylammonium phosphate, an ion-pairing agent. The effects of the ion-pairing agent, pH, presence or absence of organic modifier and the concentration of the buffer, on the chromatographic response function (CRF), were investigated using a two level factorial experimental design. The results indicated that a number of main, two and three factor effects were important. The largest effect was due to the interaction of pH with ion-pairing concentration and was further investigated using univariate experiments. The mobile phase developed consisted of 10 mM tetra-n-butylammonium phosphate and 20

mM potassium dihydrogen orthophosphate at pH 3.3. Formic, glutamic, quinic, succinic, malic and tartaric acids were extracted from 3 different grass ecotypes, the extracts were cleaned-up using a C_{18} Sep-Pak® cartridge and then identified using the developed procedure.

INTRODUCTION

The analysis of short chain carboxylic acids such as citric, malic and tartaric has been widely reported in the literature because of their significance in the food and beverage industry,[1-4] medicine[5] and the transport of metals in plants.[6] These acids have also been shown to be important in the regulation of metals within metal tolerant plants, which are able to grow on soils containing elevated levels of metals.[7] A number of different biochemical and physiological adaptations have been postulated to account for this metal tolerance. For instance, one of the suggested zinc-tolerance mechanisms involves cellular chelation by malic acid, followed by transport to the vacuole for storage.[8] This mechanism has been investigated in various plant species by growing the plants in metal doped nutrient solutions followed by analysis of the organic acids present.[8-10] Methods used for the analysis of the acids in tolerant plants include titration,[8] enzymatic analysis[9] and gas chromatography,[10] all of which have a number of drawbacks. The first two methods are used for single acids only and the latter involves a derivatization step, which can lead to low and/or variable recoveries and multiple peaks.[5]

The determination of carboxylic acids by high performance liquid chromatography (HPLC) does not require their derivatization and has been reviewed by Schwarzenbach.[11] who identified four approaches: ion exchange, ion exclusion, ion-pair and ion suppression. The Krebs cycle acids have been separated by ion exclusion chromatography using a cation exchange column and dilute hydrochloric acid as the eluent.[12] Ion suppression was used to determine the organic acids in apple and cranberry juice, using two C_{18} columns in tandem and a potassium dihydrogen phosphate pH 2.4 buffer, with UV detection at 214 nm.[2] The analysis of the tomato xylem organic acids (malic, malonic, maleic, citric and fumaric acids) by ion suppression chromatography has been reported.[6] An ion-pairing method was used to separate citric acid cycle intermediates found in orange juice, urine and rat liver mitochondria. The method used a C_{18} column (150 x 4.6 mm i.d., 5 μm) and an eluent composed of 20 mM tetrabutylammonium hydroxide and 20 mM sodium sulphate at pH 7.0.[13]

The objective of HPLC optimization is the achievement of the desired separation with a minimum of time, effort and quantity of reagents.[14,15] There are a number of efficient optimization methods, which can be divided into two approaches.

The simultaneous approach (used in the present work) collects data according to a pre-defined experimental design, and aims to model chromatographic parameters by interpolation or extrapolation. Sequential approaches are based on search algorithms, where the response from previous separations are used to direct the search.

The ion-suppression and ion-exclusion methods described above, rely on the use of eluents with pH less than 2.5, which lead to a reduction in column life time. Ion-pair separations are carried out with a pH greater than the pKa values of the acids of interest and is usually in the range 3 to 7, hence deterioration of the column is reduced. The present work describes experiments carried out to develop a separation that could be used to simultaneously determine a number of acids present in plant material, without the need for derivatization, or the use of a mobile phase pH < 2.5.

MATERIALS AND METHODS

Reagents and Solvents

Tetra-n-butylammonium phosphate (Reagent grade), potassium dihydrogen orthophosphate (Analar grade) and acetonitrile (HPLC grade), all from BDH (Poole, UK). 1000 mg mL^{-1} stock standards of each of the following acids; quinic, malic, tartaric, succinic, citric, glutamic, maleic, glutaric, oxalic and fumaric (BDH, Poole, UK) were prepared in deionized water and stored frozen. The necessary mixtures and single acid standards were prepared from the stock solutions as necessary.

Chromatography Eluents

The eluents described in Table I were prepared in deionized water and filtered through a 0.45 μm pore size type HA filter (Millipore, Bedford, MA). The pH was adjusted using phosphoric acid or 2 M sodium hydroxide solution (BDH, Poole, UK), acetonitrile was added when necessary. The eluents were pumped through the columns for one hour prior to injection of the analyte.

Table 1

Mobile Phase Compositions and Responses For the Factorial Experiments

Expt. No.	Modifier % v/v		TBAP mM		pH		Buffer mM		Response CRF
	Level	Value	Level	Value	Level	Value	Level	Value	
1	--	0	--	1	--	3	--	20	43
2	--	0	+	10	--	3	--	20	31
3	--	0	--	1	+	5	--	20	20
4	--	0	--	1	--	3	+	200	23
5	--	0	--	1	+	5	+	200	0
6	--	0	+	10	--	3	+	200	26
7	--	0	+	10	+	5	--	20	18
8	--	0	+	10	+	5	+	200	38
9	+	5	--	1	--	3	--	20	21
10	+	5	+	10	--	3	--	20	23
11	+	5	--	1	+	5	--	20	21
12	+	5	--	1	--	3	+	200	18
13	+	5	--	1	+	5	+	200	7
14	+	5	+	10	--	3	+	200	10
15	+	5	+	10	+	5	--	20	21
16	+	5	+	10	+	5	+	200	21

TBAP = tetra-n-butylammonium phosphate concentration; Buffer = potassium dihydrogen ortho - phosphate concentration, Modifier = concentration of acetonitrile and CRF = chormatographic response function.

The time for an unretained peak to elute from the column (t_0) was determined as the first baseline disturbance after injection of deionized water. In the factorial experiments with a flow rate of 0.8 mL min^{-1}, t_0 was 2.8 minutes.

Equipment

A model 6000 Waters pump (Waters Assoc., Milford, MA) and a Rheodyne sample injector (Rheodyne model 7125, Contati, CA) fitted with a 20 μL sample loop. Detection at 210 nm was facillitated using a Pye-Unicam LC3 UV detector.

Two columns were used, in series, for the factorial experiments, namely a Waters μBondapak ODS 300 x 4.6 mm i.d., 5μm (Waters Assoc., Milford, MA) and an Alltech ODS 250 x 4.6 mm i.d., 10μm. The second column was removed for the univariate experiments.

Ion-Suppression Method

The starting point for this work was the Association of Official Analytical Chemists method developed to determine citric and malic acids found in apple and cranberry juice cocktail.[2] The method uses two C_{18} columns in tandem, a mobile phase consisting of a phosphate buffer at pH 2.4 and UV detection at 214nm. This approach was applied to the separation of a standard mixture of carboxylic acids, containing, 200 mg L^{-1} succinic, malic, tartaric, citric, quinic and 100 mg L^{-1} fumaric in deionized water. Peak identification was carried out by comparison of retention times with standards of the same concentration.

Factorial Experiment

Factorial experimental design and experimentation has been comprehensively described by a number of authors including: Box *et al,*[17] Morgan *et al.*[18] and Sundberg.[19] In the present work a two level full factorial experimental design was used to establish which eluent components have an influence on the separation of the acids, and to determine their interdependence. In such a design each variable or factor is investigated at two levels denoted by + or - (see Fig. 1). With four factors, 2^4 experiments (16 in total) have to be performed to calculate the magnitude of effects due to the factors themselves, and interactions between factors. The numerical values for the effect of one variable are obtained by subtracting the response at the minus level from another experiment at the plus level. The magnitude of the mean effect of one variable is then equal to the mean of these response differences. The interaction effect between two variables is calculated as the difference between those response differences where the variable is at a high level (+) and those where the same variable is at a low level (-). A tabular method has been developed to calculate these values.[16]

The factors chosen for investigation were: the concentration of phosphate buffer, eluent pH, concentration of ion-pairing reagent and inclusion of organic modifier. These factors and the values to use for the upper and lower levels of each were established from the literature.[11-13]

The practical limitations to the levels used were: (1) that the buffer concentration be sufficiently high to maintain a constant pH during the chromatographic run, and (2) the pH be less than 5.5 so that no problems with high mobile phase absorption at 210 nm would be encountered. The experiments described in Table 1 were used to analyse a standard mixture randomly and in triplicate.

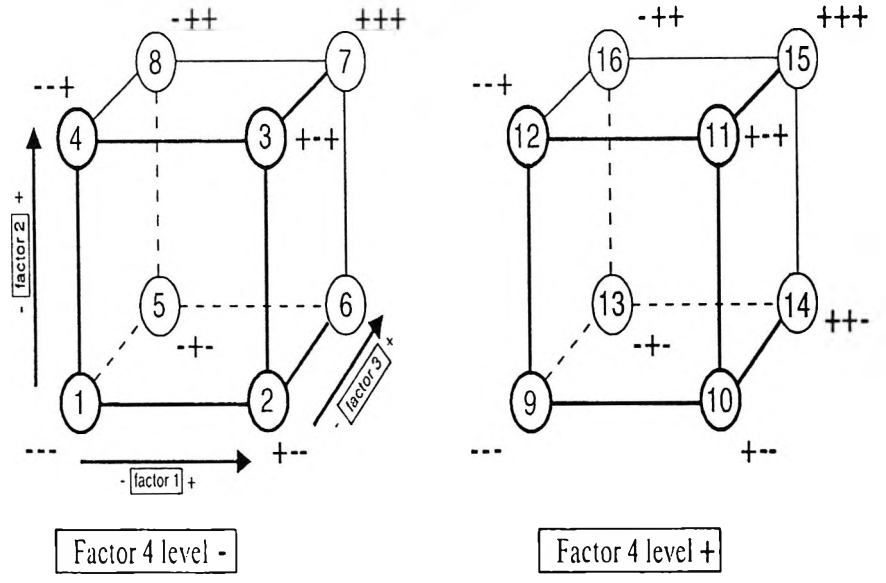


Figure 1. Schematic representation of a full 2-level factorial design for 4 variables. The number in each circle represents the experiment number.

Measurement of Response

A necessary condition for successful experimental optimization is a means of ordering the experiments numerically in relation to the quality of the separation. The choice of quality criteria is problematic because the separation requirements are often unclear and difficult to express quantitatively. The chromatographic response function (CRF) was first proposed by Morgan and Deming[20] as a measure of separation quality. Berridge[14] extended the concept to include more information concerning the quality and time taken for the separation. In the present work the approach of Berridge is used to measure the quality of each chromatogram and it is this measure that is optimized. The specific form used is given in Eqn. 1.

$$CRF = \sum_{i=1}^{L} R_i + L^x - a\,|T_m - T_L| - b(T_0 - T_1) \qquad (1)$$

R_i is the resolution between the peaks and limited to a maximum value of 2. L is the total number of peaks appearing in the chromatogram and is weighted

with an exponent x. The difference between an acceptable analysis time, T_m, and the time for the last peak to elute, T_L, is weighted by a factor a. The final term reduces the CRF value if the first peak, T_1, elutes prior to a specified minimum time T_0, and b is another weighting factor.

In the present work the weighting factors were set at a = 0.5, b = 0.5 and x = 2.0 because the maximum number of resolved peaks was the goal of the experiment, whereas analysis time was only a secondary consideration.

Extraction and Clean Up of the Carboxylic Acids

The extraction of the short chain carboxylic acids from the shoots of different species of grass was achieved using the method of Philips and Jennings[21] with slight modifications, such that the final evaporation under reduced pressure was carried out twice to reduce the carry over of formic acid.

A clean-up procedure was developed using a C_{18} solid phase extraction cartridge (Sep-Pak® Waters Assoc., Milford, MA) as follows: 20 mL extract shaken for 10 minutes, filtered through Whatman 542 paper (Whatman International Ltd., Maidstone, UK) and passed through the Sep-Pak®. The cartridge was pre-wetted with 2-3 mL methanol, flushed with 5 mL deionized water, 4 mL of extract was then passed through the column and the eluent retained.

This was repeated until all of the 20 mL extract had been passed through the column. The collected isolates were pooled, evaporated to dryness at 50 °C under reduced pressure and redissolved in 10 mL of the mobile phase.

RESULTS AND DISCUSSION

Initial Screening Experiments

The separation achieved for standards of malic, citric and fumaric acids was the same as that reported by Coppola and Starr.[2] However, when six acids representative of those found in plant material were analysed, co-elution of a number of components of the mixture occurred. This is shown in Fig. 2. Further experimentation[22] indicated that with the inclusion of an ion-pairing agent resolution of the analytes may be possible.

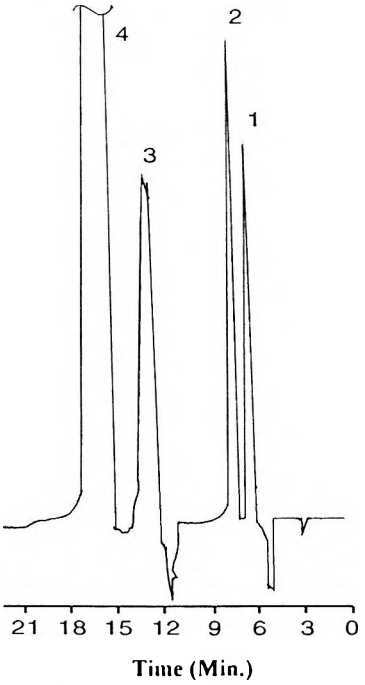


Figure 2. Separation of malic, citric, tartaric, quinic, succinic, and fumaric acids using the conditions of Coppola and Starr.[2] Eluent: 200 mM potassium dihydrogen orthophosphate, pH 2.4, with phosphoric acid. Column: Waters μBondapak ODS followed by Anachem ODS. Detection: UV at 210 nm. Flow rate: 1.0 mL $min.^{-1}$. Elution order: 1 = tartaric and quinic; 2 = malic; 3 = citric and succinic; 4 = fumaric.

Factorial Experiments

The CRF values for each run were determined and the mean values, shown in Table 1, were used to calculate the main and interactive effects as described elsewhere.[16] A positive value indicates that the separation measured is better at the higher variable level, whereas a negative value indicates the opposite. It can be seen from Table 2, that the presence of acetonitrile as an organic modifier (A), the pH of the eluent (P) and the concentration of the buffer (B) have relatively large numeric values and are, thereby, of importance. All three of these values are negative, indicating that the separation is better at the lower level of these factors. The only positive value is for the concentration of ion-pairing agent.

Table 2

Calculated Variable Effects and Interactions of the Factors Showing the Variation in CRF Value Caused by the Change in Eluent Composition

Variable	Calculated Estimate
Main Effect	
Modifier (A)	-7.1
Concentration of TBAP (T)	4.4
pH (P)	-6.1
Buffer concentration (B)	-6.9
Two-Factor Effects	
A & T	-2.4
A & P	5.6
A & B	-0.6
T & P	8.1
T & B	7.4
P & B	3.4
Three-Factor Effects	
A & T & P	-3.1
A & T & B	-6.4
A & P & B	-2.9
T & P & B	6.1
Four-Factor Effects	
A & T & P & B	-0.1

The two and three factor interactions T.P, T.B, A.T.B and T.P.B give large values whereas the four factor effect is negligible, the largest factor effect was for the interaction between ion-pairing reagent and pH. Therefore, the effect of pH on the separation was investigated univariately, at constant buffer and ion-pairing concentration.

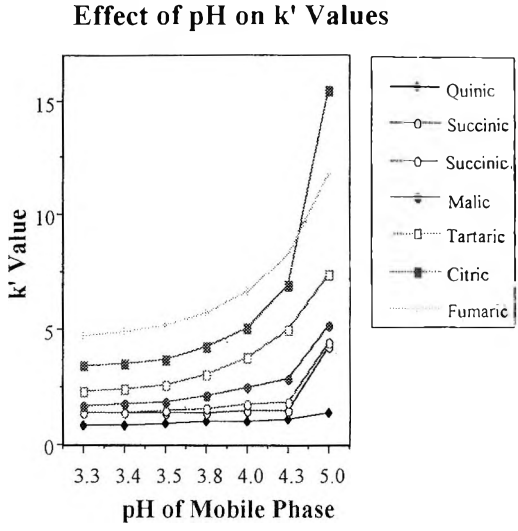


Figure 3. Effect of pH on the capacity factor, k′, of various carboxylic acids. The acids are identified in the legend.

Effect of pH on Separation

Initial screening experiments indicated that it was possible to remove the second column to improve the peak shape, without affecting the resolution.[22] Therefore, the univariate experiments were carried out using only the Waters μBondapak column.

The values and signs of the variable terms (Table 2) suggest that the most promising conditions at which to vary the pH would be: high level ion-pair reagent (10 mM), low level of buffer (20 mM) and absence of acetonitrile. The pH of this mobile phase was adjusted to 3.3, 3.4, 3.5, 3.8, 4.0, 4.3 and 5.0 using phosphoric acid or 2 M sodium hydroxide, depending on the desired pH. Each of these eluents was randomly chosen and the standard mixture of six acids was analyzed.

The results are summarized in Fig. 3, which shows the variation of capacity factor, k′, with pH. This graph can be interpreted by consideration of the pKa values for each acid. The largest change in k′ for every acid except quinic, occurs between pH 4.3 and 5.0. This is because the pKa_2 values are such that each acid looses a second proton between these pH values and so can

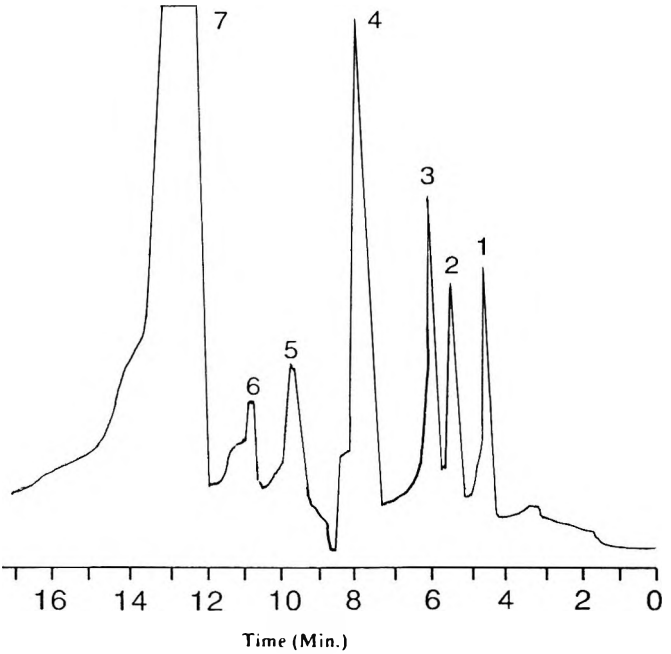


Figure 4. Separation of seven acids using the optimized conditions (all 200 mg L^{-1} except fumaric which was 100 mg mL^{-1}). Eluent: 10 mM tetra-n-butylammonium phosphate, 20 mM potassium dihydrogen orthophosphate, pH 3.3. Column: Waters μBondapak ODS. Detection: UV at 210 nm. Flow rate: 1.0 mL $min.^{-1}$. Elution order: 1 = quinic; 2 = succinic; 3 = malic; 4 = tartaric; 5 = citric; 6 = malonic; and 7 = fumaric.

interact more strongly with the ion-pair reagent. This interaction markedly increases their retention time. Two peaks were observed for succinic acid at pH's greater than 3.8, which could be because of the presence of multiply charged species (pKa_1 4.21 and pKa_2 5.64). Multiple peaks have been reported in ion-pair separations when inappropriate pH conditions are used and occurs because the variably ionized acid groups interact differently with the ion-pair reagent.[15] The lines for tartaric and fumaric are both similar in shape, which is a reflection of their virtually identical pKa values (pKa_1 3.04 and 3.03; pKa_2 4.37 and 4.37 respectively).

All six acids were separated satisfactorily after the optimization procedure, as shown in Fig. 4. This eluent composition was chosen for the analysis of plant extracts and represents a compromise, because the separation

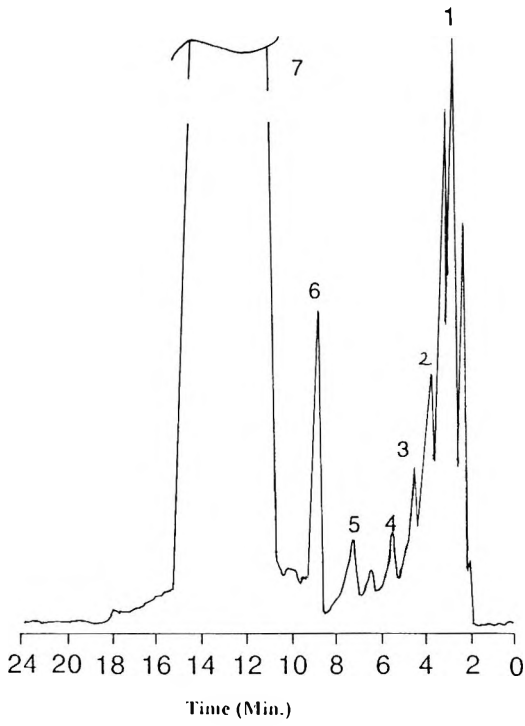


Figure 5. Separation of carboxylic acids extracted from copper-tolerant Parys *Agrostis tenuis*. Eluent: 10 mM tetra-n-butylammonium phosphate, 20 mM potassium dihdrogen orthophosphate, pH 3.3. Column: Waters μBondapak ODS. Detection: UV at 210 nm. Flow rate: 1.0 mL min^{-1} Elution order: 1 = formic; 2 = glutamic; 3 = quinic; 4 = succinic; 5 = malic; 6 = tartaric; and 7 = partially fumaric plus citric.

mechanism is a combination of ion suppression and ion-pairing depending on the pKa values for each acid. The use of a pH greater than the highest pKa value for the acids in the system, may have improved the separation, but was not possible due to the high absorption of this mobile phase at 210 nm.

A number of acid standards were run prior to the analysis of plant extracts, so as to determine retention times. Using the eluent developed above, glutamic, quinic, succinic, malic, tartaric, citric, malonic and fumaric acids could be separated. Maleic, glutaric and oxalic could be partially resolved from other acids.

Quantitation

Linear response was in the range 5.0 to 40.0 mg L^{-1} for each acid except fumaric,which was 2.5 to 20.0 mg L^{-1}. These were determined using standards made up in the mobile phase and containing 15.0 mg L^{-1} glutamic acid as internal standard. The curves for all the acids (except citric) had linear correlation coefficients between 0.990 and 0.950. The coefficient for citric acid was 0.850, which was due to peak splitting at concentrations greater than 10.0 mg L^{-1}.

Analysis of Plant Material

Copper tolerant Parys *Agrostis tenuis*, zinc/cadmium tolerant Merlin *Festuca rubra* and metal non-tolerant Cascade *Festuca rubra* were analyzed using the conditions described above and the chromatogram for *Agrostis tenuis* is shown in Figure 5. Co-injection of a number of different acid standards was used to identify the following acids as present in the extract: formic, glutamic, quinic, succinic, malic and tartaric. Elution of a large unidentified peak between 11 and 15 minutes interfered with the identification of citric and fumaric acids. Using the methodology outlined in this paper, we were able to show an increase in the concentration of malic acid in zinc-tolerant Merlin *Festuca rubra* on exposure to zinc but not copper, which did not occur to the same extent in a non-tolerant population.[23]

CONCLUSIONS

A simple separation procedure for the carboxylic acids present in plant material was investigated and optimized using factorial experiments. The optimized mobile phase consisted of 20 mM sodium dihydrogen orthophosphate and 10 mM tetra-n-butylammonium phosphate at pH 3.3 and could be used to separate glutamic, quinic, succinic, malic, tartaric, citric, malonic, and fumaric acids, which commonly occur in plant materials. This procedure could also be used for the analysis of carboxylic acids in other sample matrices.

ACKNOWLEDGMENT

The work was supported financially by the Science and Engineering Research Council (UK).

REFERENCES

† Current address Laboratory of the Government Chemist, Queens Road, Teddington, Middlesex, TW11 0LY, United Kingdom.

1. D. Tusseau, C. Benoit, J. Chromatogr., **395**, 323-333 (1987).

2. E. D. Coppola, M. S. Starr, J. Assoc. Off. Anal. Chem., **69**, 594-597 (1986).

3. K. I. Tomlins, D. M. Baker, I. J. McDowell, Chromatographia, **29**, 557-561 (1990).

4. R. M. Marie, M. Calull, R. M. Manchrobas, F. Borrull, F. X. Ruis, Chromatographia, **29**, 54-58 (1990).

5. H. M. Liebich, Anal. Chim. Acta, **236**, 121-130 (1990).

6. M. H. M. N. Senden, A. J. G. M. Van der Meer, J. Limborgh, H. T. H. Wolterbeck, Plant and Soil, **142**, 81-89 (1992).

7. J. Antonovics, A. D. Bradshaw, R. G. Turner, Adv. in Ecol. Res., 7, 1-85 (1971).

8. W. Mathys, Physiol. Plant., **40**, 130-136 (1977).

9. D. L. Godbold, W. J. Horst, J. C. Collins, D. A. Thurman, H. Marschner, J. Plant Physiol., **116**, 59-69 (1984).

10. D. A. Thurman, J. L. Rankin, New Phytol., **91**, 629-635 (1982).

11. R. Schwarzenbach, J. Chromatogr., **251**, 339-358 (1982).

12. V. T. Turkelson, M. Richards, Anal. Chem., **50**, 1420-1423 (1978).

13. J. F. Keefer, S. M. Schuster, J. Chromatogr., **383**, 297-305 (1986).

14. J. C. Berridge, J. Chromatogr., **244**, 1-14 (1982).

15. S. Ahuja, **Chemical Analysis**, a Series of Monographs on Analytical Chemistry and its Applications, edited by J. D. Winefordner, John Wiley and Sons, New York, 1989.

16. F. Yates, **The Design and Analysis of Factorial Experiments**, Imperial Bureau of Soil Science, Harpenden, UK, 1937.

17. G. E. P. Box, W. G. Hunter, J. S. Hunter, **Statistics for Experimenters**, John Wiley, New York, 1978.

18. E. Morgan, K. W. Burton, P. A. Church, Chem. and Intell. Lab. Sys., **5**, 283-302 (1989).

19. R. Sundberg, Chem. and Intell. Lab. Sys., **24**, 1-17 (1994).

20. S. L. Morgan, S. N. Deming, J. Chromatogr., **112** 267- 285 (1975).

21. R. D. Philips, D. H. Jennings, New Phytol., **77**, 333-339 (1976).

22. C. F. Harrington, PhD. Thesis, University of Bristol, UK, 1994.

23. C. F. Harrington, D. J. Roberts, G. Nickless, Can. J. Bot., **74**, 1742-1752 (1996).

Received October 10, 1996
Accepted November 4, 1996
Manuscript 4302

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1789–1796 (1997)

AN HPLC METHOD FOR THE MEASUREMENT OF POLYAMINES AND LIPIDIC AMINES BINDING TO DNA

G. A. Karikas,[1] V. Constantinou-Kokotou,[2] G. Kokotos[1,*]

[1]Laboratory of Organic Chemistry
Department of Chemistry
University of Athens
Panepistimiopolis
Athens 15771, Greece

[2]Laboratory of Chemistry
Agricultural University of Athens
Iera Odos 75
Athens 11855, Greece

ABSTRACT

An HPLC method is employed to measure the binding of polyamines and lipidic amines to DNA. DNA interaction is expressed as the % DNA peak size exclusion. The natural polyamine, spermine, has clearly demonstrated the highest ability for binding. Among the synthetic lipidic amines tested 1,2-hexadecanediamine has been found to be the most active.

INTRODUCTION

Natural polyamines such as spermine, spermidine, putrescine, are widely distributed in biological systems and have been shown to be critical for cell growth and carcinogenesis.[1,2] It is well known that the association of cationic polyamines with negatively charged DNA induces significant structural changes in DNA in cell-free systems.[3] Spermidine and spermine can cause DNA to condense and aggregate[4] and induce both B-to-Z and B-to-A transitions in certain DNA sequences.[5,6] The hypothesis that structural transitions and condensation in specific DNA sequences caused by polyamines may be related to nucleosome formation and the condensation of DNA into chromatin is gaining experimental support.

Measurements of polyamine-DNA interactions might yield therapeutically active compounds. The ability of organic molecules to bind to nucleic acids by various mechanisms such as intercalation and ionic interaction have previously been studied by a variety of techniques, including equilibrium dialysis,[7,8] absorption spectroscopy,[9,10] and fluorescence spectroscopy.[7,11] Additionally as the DNA or RNA interaction is best described as a dynamic equilibrium, an HPLC method based on DNA or RNA size exclusion was introduced.[12] This method was modified by several investigators and used for testing intercalating agents,[13] other small organic molecules,[14] and crude plant extracts.[15]

Although a variety of methods for HPLC analysis of polyamines have been reported,[16] the interaction of polyamines with DNA has not been studied by HPLC up to now. Natural polyamines (spermine, spermidine, putrescine, cadaverine) together with a number of lipidic diamines and amino alcohols have been studied by DNA size exclusion and the results are presented in this paper. The lipidic diamines and amino alcohols tested, have been previously synthesized[17,18] and preliminary screening has shown interesting biological activities (unpublished data).

MATERIALS AND METHODS

Materials

Calf thymus DNA, activated type XV was purchased from Sigma Chemical Co. Doxorubicin hydrochloride USP (adriamycin HCl) was gifted from Pharmacia. HPLC (MeOH) grade solvents were obtained from Lab Scan. Thrice-deionized water was obtained from a Millipore purification system.

Mobile phase was degassed with ultrasonic prior to use. All solvents and solutions for HPLC analysis were filtered through 0.22 μm Millipore membrane filters before injection. DNA and test samples were dissolved in deionized water.

Apparatus and Chromatography

A Hewlett-Packard (Palo Alto, CA, USA) model 1050 HPLC, isocratic pump and a Rheodyne injector Model 7125 fitted with a 20 μL loop were used. A line source detector at 254 nm was used in the validation experiments. The signals were recorded on a Hewlett-Packard Model HP3395 integrator. The column was a 4 x 250 mm, 5 μm octadecylsilane column (Lichrospher RP-18). The column was equilibrated with a H_2O : MeOH, 80 : 20 mixture. Test samples and DNA solutions were then introduced in a ratio 1 : 1 (v / v) into the sample loop (20 μL) without incubation. Flow rate was maintained at 1 mL/min and the free DNA eluted in approximately 1 min. After the appearance of DNA peak, the column was washed with MeOH (100 %) for 20 min to elute the sample mixture. DNA binding is expressed as a % DNA peak exclusion.

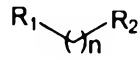
All tested compounds were measured at three different concentrations (0.25, 0.10, 0.05 mg/mL) and tested in triplicate.

RESULTS AND DISCUSSION

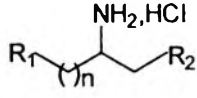
The structures of the compounds tested are shown in Figure 1. % DNA peak size exclusion are summarised in Table 1. An example of complete (100 %) peak exclusion is demonstrated in Figure 2.

All the compounds containing two or more amino groups (**1-6**, **9-11**), except compound **14**, exhibited a complete DNA binding (100 % DNA peak exclusion) at a concentration of 0.25 mg/mL (Table 1). Monovalent molecules (one aminogroup) such as hexadecanamine (**7**), the classical intercalating agent doxorubicin (**15**) and the aminoalcohols **8**, **12**, **13** demonstrated a weaker effect on DNA, ranging from 13 ± 2 % up to 75 ± 4 %.

At the concentration of 0.1 mg/mL the effect caused by the monovalent molecules has substantially decreased (0 - 19 ± 2 %), while divalent or polyvalent molecules retained their potency (85 ± 7 - 100 %). At even lower concentration (0.1 mg/mL) spermine (a tetravalent molecule) exhibited a



	n	R_1	R_2
1	2	NH_2.HCl	NH_2.HCl
2	3	NH_2.HCl	NH_2.HCl
3	4	NH_2.HCl	NH_2.HCl
4	5	NH_2.HCl	NH_2.HCl
5	3	NH_2.HCl	$NH(CH_2)_4NH_2$.2HCl
6	3	NH_2.HCl	$NH(CH_2)_4NH(CH_2)_3NH_2$.3HCl
7	16	H	NH_2.HCl
8	2	NH_2.HCl	OH



	n	R_1	R_2
9	14	H	NH_2.HCl
10	12	H	NH_2.HCl
11	14	H	CH_2NH_2.HCl
12	14	H	OH
13	16	H	OH
14	1	C_6H_5	NH_2.HCl

Figure 1. Structures of compounds tested.

complete interaction with DNA (100 %). The trivalent molecule spermidine (**5**) and the divalent molecules **1**, **2**, **3**, **4**, **9**, **10**, **11** showed weaker ability for binding (75 ± 9, 28 ± 2, 65 ± 7, 60 ± 4, 58 ± 5, 23 ± 3, 8 ± 3, 0 % respectively). Spermine showed a measurable peak exclusion (35 ± 3 %) even at the concentration of 0.01 mg/mL.

Table 1

Effect of Compounds Tested on the DNA Peak Size

	Compound	DNA Peak Exclusion[a,b] (%) [c]	[d]	[e]
1	1,2-ethanediamine dihydrochloride	28 ± 2	100	100
2	1,3-propanediamine dihydrochloride	65 ± 7	100	100
3	putrescine dihydrochloride	60 ± 4	100	100
4	cadaverine dihydrochloride	58 ± 5	100	100
5	spermidine trihydrochloride	75 ± 9	100	100
6	spermine tetrahydrochloride	100	100	100
7	hexadecanamine hydrochloride	0	0	13 ± 2
8	ethanolamine hydrochloride	0	19 ± 2	34 ±2
9	1,2-hexadecanediamine dihydrochloride	23 ± 3	100	100
10	1,2-tetradecanediamine dihydrochloride	8 ± 3	100	100
11	1,3-heptadecanediamine dihydrochloride	0	85 ± 7	100
12	2-amino-hexadecanol hydrochloride	0	10 ± 2	65 ± 7
13	2-amino-octadecanol hydrochloride	0	10 ± 3	75 ± 4
14	2-amino-3-phenyl-propanamine dihydrochloride	10 ± 2	15 ± 1	34 ± 2
15	doxorubicin hydrochloride	0	16 ± 4	47 ± 3

[a] Concentration of DNA 0.1 mg/mL.
[b] Mean ± standard deviation based on n=3.
[c] Concentration of compound tested 0.05 mg/mL.
[d] Concentration of compound tested 0.10 mg/mL.
[e] Concentration of compound tested 0.25 mg/mL.

It seems that the ability of binding is determined by the number of amino groups. The potency of the phenomenon, which has to be attributed to ionic interactions, is decreased when the number of amino groups decreases. These observations are in full agreement with recent NMR studies which showed that spermine binded to t-RNA more strongly than spermidine.[19]

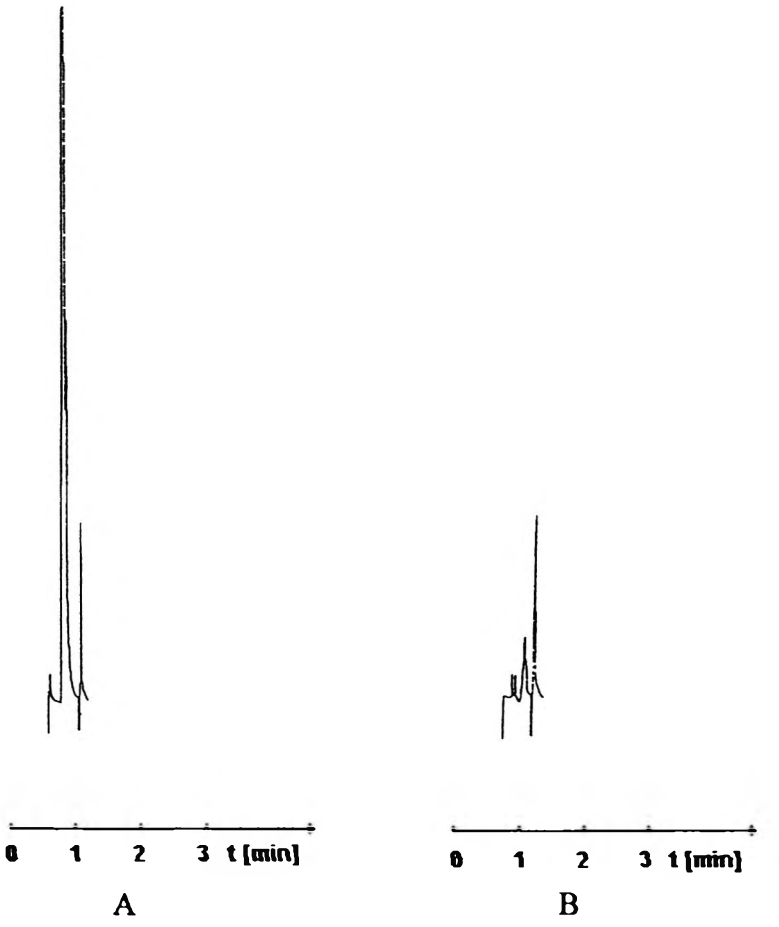


Figure 2. Chromatograms of DNA (0.1 mg/mL) (A) and DNA (0.1 mg/mL) + 1,3-heptadecanediamine dihydrochloride (0.25 mg/mL) (B). Chromatographic conditions: column, octadecylsilane Lichrospher RP 18 (4 x 250 mm, id 5 μm); eluent, water-methanol 80:20 (v/v); flow rate, 1.0 mL/min; injection volume, 20 μL; detector UV set at 254 nm; temperature, 25 °C

Among the divalent molecules, the presence of a long chain resulted in weaker effects, as concluded by comparison of compounds **9**, **10** to **1** and **11** to **2**. Comparing compounds **1-4**, the system of 1,3-diamino groups showed the best result indicating that this is the optimum distance between the two amino groups.

CONCLUSIONS

The binding of natural polyamines and other related synthetic cationic compounds to DNA was studied for the first time by an HPLC method. Compounds containing two or more amino groups exhibited stronger effect than the classical intercalator doxorubicin. The ratio DNA : ligand determines the potency of the observed peak exclusion. The described chromatographic method could serve as a valuable pre-screen antitumor *in vitro* assay, since it possesses simplicity, reproducibility, considerable reliability and it is of low cost.

REFERENCES

1. L. J. Marton, A. E. Pegg, Ann. Rev. Pharmacol. Toxicol., **35**, 55-91 (1995).

2. A. Sjoerdsma, P. J. Schechter, Clin. Pharmacol. Ther., **35**, 287-300 (1994).

3. B. G. Feuerstein, L. D. Williams, H. S. Basu, L. J. Marton, J. Cell Biochem., **46**, 37-47 (1991)

4. L. C. Gosule, J. A. Schellman, J. Mol. Biol., **121**, 311-326 (1978).

5. M. Behe, G. Felsenfeld, Proc. Nat. Acad. Sci. USA, **78**, 1619-1623 (1981).

6. S. Jain, G. Zon, M. Sundarlingam, Biochemistry, **28**, 2360-2364 (1989).

7. J. M. Pezzuto, P. P. Lau, Y. Luh, P. D. Moore, G. N. Wogan, S. M. Hecht, Proc. Nat. Acad. Sci. USA, 77, 1427-1431 (1980).

8. W. R. Wilson, B. C. Baguley, L. P. G. Wakelin, M. J. Waring, Mol. Pharmacol., **20**, 404-414 (1981).

9. E.J. Gabbay, R. E. Scofield, C. S. Baxter, J. Amer. Chem. Soc., **95**, 7850-7857 (1973).

10. J. M. Pezzuto, S. K. Antosiak, W. M. Messmer, M. B. Slaytor, G. R. Honig, Chem. Biol. Interact., **43**, 323-339 (1983).

11. B. C. Baguley, E. M. Falkenhaug, Nucleic Acid Res., **5**, 161-171 (1978).

12. J. P. Hummel, W. J. Dreyer, Biochim. Biophys. Acta, **63**, 530-539 (1962).

13. H. J. Pezzuto, C. T. Che, D. D. McPherson, J. Zhu, A. Topcu, C. A. J. Erdelmeirer, G. A. Cordell, J. Nat. Prod., **54**, 1522- 1530 (1991).

14. K. H. Schulpis, G. A. Karikas, G. Kokotos, J. Eur. Pediat., submitted.

15. M. P. Gupta, A. Monge, G. A. Karikas, A. Lopez de Cerain, P. N. Solis, E. de Leon, M. Trujillo, O. Suarez, F. Wilson, G. Montenegro, Y. Noriega, A. I. Santana, M. Correa, C. Sanchez, Int. J. Pharmacognosy, **34**, 19-27 (1996).

16. H. M. H van Eijk, D. R. Rooyakkers, N. Deutz, J. Chromatogr. A, **730**, 115-120 (1996) and references cited therein.

17. G. Kokotos, V. Constantinou-Kokotou, E. del Olmo Fernandez, I. Toth, W. A. Gibbons, Liebigs Ann. Chem., 961-964 (1992).

18. G. Kokotos, J. M. Padron, C. Noula, W. A. Gibbons, V. Martin, Tetrahedron: Asymmetry, **7**, 857-866 (1996).

19. B. Frydman, W. M. Westler, K. Samejima, J. Org. Chem., **61**, 2588-2589 (1996).

Received October 20, 1996
Accepted November 15, 1996
Manuscript 4307

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1797–1808 (1997)

SIMULTANEOUS DETERMINATION OF ANTIOXIDANTS AND PRESERVATIVES IN COSMETICS AND PHARMACEUTICAL PREPARATIONS BY REVERSED-PHASE HPLC

L. Gagliardi,[†] D. De Orsi,[†] L. Manna,[‡,*] D. Tonelli[§]

[†] Laboratorio di Chimica del Farmaco
Istituto Superiore di Sanità
Rome, Italy
[‡] Dipartimento di Chimica e Tecnologia delle Sostanze Biologicamente
Attive Università "La Sapienza"
Rome, Italy
[§] Dipartimento di Chimica Fisica ed Inorganica
Università di Bologna
Viale del Risorgimento 4
40136 Bologna, Italy

ABSTRACT

A high performance liquid chromatographic method for the simultaneous determination of multiple additives in o/w cosmetic and pharmaceutical formulations was developed by using a RP-8 Select B column, a linear gradient elution and UV detection. A very simple extraction procedure was required. The separation obtained for nine antioxidants and seven preservatives was very good under the chromatographic conditions used. Their analysis was carried out in commercial samples and satisfactory results were obtained both for the recovery and the coefficient of variation.

INTRODUCTION

Many chemicals are permitted as additives to protect food, pharmaceuticals, cosmetics, drugs and other materials from the effects of oxidation and/or bacterial contamination. Antioxidants are added to products containing fats or oils to prevent rancidification. They can be naturally occurring compounds, especially present in the vegetable products, or synthetic molecules based on phenolic structures with varying degrees of hydroxylation and side-chain substitutions.

Antioxidants are molecules that react readily with oxygen and, as a result, act as inhibitors being oxidized themselves. They can be present single or as combinations, and are permitted at a maximum concentration range from 0.01 to 0.3%. It has been demonstrated that some compounds, like ascorbyl palmitate may act as synergists: either it enhances the effect of a phenolic antioxidant or it forms complexes with traces of copper and iron, and hence deactivates their prooxidant effect.

Preservatives, particularly esters of 4-hydroxybenzoic acid, commonly known as parabens, are widely added to food., drugs and cosmetics[1]. Because of the critical role of antioxidants and preservatives in the improvement of shelf-life of a product, various methods of analysis have been developed for their quantitative determination including TLC,[2,3] GC,[4,5] HPLC,[6-12] and, recently, capillary electrophoresis.[12]

We describe here a simple analytical procedure for the simultaneous determination of 16 additives, 9 antioxidants and 7 preservatives, in o/w cosmetic or pharmaceutical formulations, which involves a one-step extraction or a dilution of the sample and analysis by reversed phase HPLC.

EXPERIMENTAL

Standards and Reagents

All reagents used were of analytical-reagent grade and used without further purification. Acetonitrile and methanol was of HPLC grade. Water was deionized and doubly distilled from glass apparatus. All solvents and solutions for HPLC analysis were filtered through a Millipore filter (pore size 0.45 μm) and vacuum degassed by sonication before use. Commercially available antioxidant and preservative agents used in this study are listed in Table 1.

Table 1

List of the Antioxidants and Preservatives Studied

Chemical Name	Abbreviation	Supplier
n-propyl gallate	PG	Fluka
octyl gallate	OG	Fluka
dodecyl gallate	DG	Fluka
3-tert-butyl-4-hydroxy anisole	BHA	Fluka
2,5-di-tert-butyl-4-hydroxy toluene	BHT	Fluka
2-tert-butyl hydroquinone	BHQ	Acros
2,5-di-tert-butyl hydroquinone	DBHQ	Acros
ascorbyl palmitate	AP	Fluka
tocopheryl acetate	TA	Fluka
methyl-4-hydroxy benzoate	MP	Formenti
ethyl-4-hydroxy benzoate	EP	Formenti
propyl-4-hydroxy benzoate	PP	Formenti
benzyl-4-hydroxy benzoate	BeP	Formenti
butyl-4-hydroxy benzoate	BP	Formenti
sorbic acid	SA	Fluka
2,4,4'-trichloro-2'-hydroxydiphenyl ether (triclosan)	TC	Ciba

Apparatus

Chromatography was performed on a Varian 9012 liquid chromatograph equipped with two pumps, a 10 μL sample loop, a Hewlett Packard 1050 photodiode-array detector, and a personal computer Vectra HP 486.

The analytical column was of stainless-steel (250 mm x 4.0 mm I.D.) packed with 5 μm SelectB RP-8 (Merck,Darmstadt, Germany).

HPLC Conditions

The composition of mobile phase was acetonitrile (A), methanol (B), water containing 10^{-3} M perchloric acid (C). The following conditions of linear gradient elution were used: t=0 , 35% A, 10% B, 55% C; t=30, 20% A, 65% B, 15% C; t=35, 70% A, 30% B.

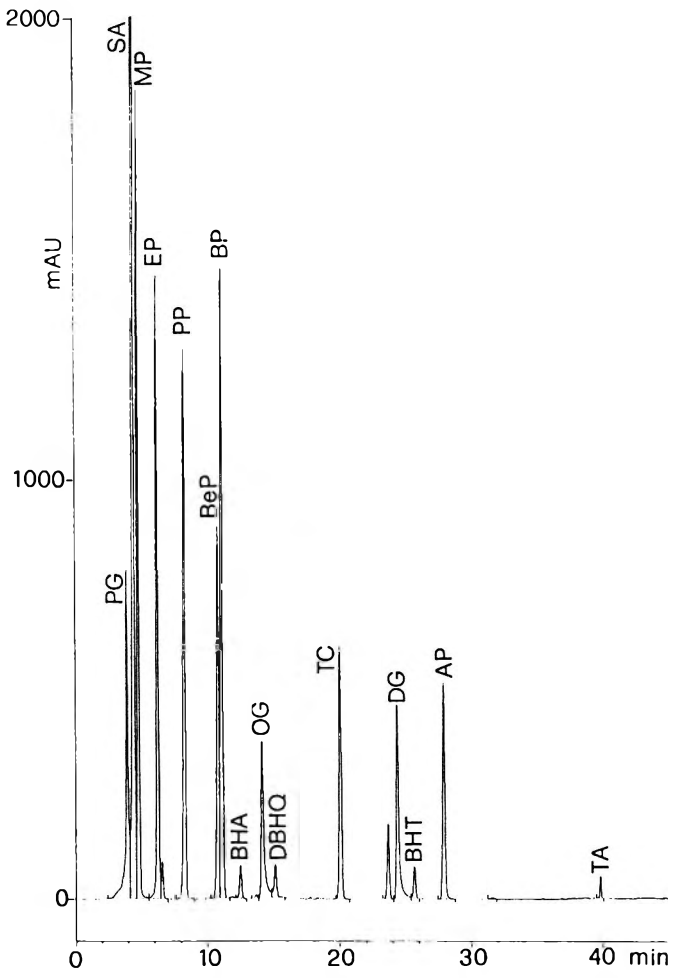


Figure 1. Typical chromatogram obtained at 254 nm for a standard solution containing 1 mg mL^{-1} of all the antioxidants and preservatives studied with the exception of SA, (0.4 mg mL^{-1}).

At the end of the elution, the initial mobile phase was passed through the column for 10 min to allow a good re-equilibration of the chromatographic system. Flow-rate was 1.0 $mL \cdot min^{-1}$ and detection was performed at 254, 263, and 280 nm. The temperature of the column was maintained at 25 °C.

Table 2

Analytical Parameters

Compound	Retention Time (min)	Detection Wavelength (nm)	Detection Limit (ng Injected)
PG	4.02	280	10
SA	4.43	263	5
MP	4.91	254	10
EP	6.37	254	10
BHQ	6.71	280	20
PP	8.46	254	10
BeP	10.81	254	10
BP	11.03	254	10
BHA	12.74	280	15
OG	14.39	280	10
DBHQ	15.43	280	15
TC	19.98	280	10
DG	24.62	280	10
BHT	25.90	280	20
AP	28.25	254	30
TA	39.79	280	20

Calibration Standard Solutions

Stock solutions were prepared by dissolving the appropriate amounts of the standard additives in a solvent consisting of A and B in the ratio 7:3. A set of working standard solutions was prepared by diluting aliquots of the stock solutions to give concentrations ranging from 5.0 to 1000 $\mu g \cdot mL^{-1}$ for each compound studied. The calibration graphs were constructed by plotting the peak areas obtained at the optimum wavelength of detection versus the amounts (μg) injected.

Sample Preparation

Eleven oil-rich products (five pharmaceutical specialties and six cosmetic products) were analyzed by the following procedure. About 2 g of the o/w emulsions was dissolved by sonication with 8 mL of a mixture methanol-acetonitrile (1:1, v/v), transferred to a 10 mL volumetric flask and brought to

Table 3

Calibration Curves: Linear Regression of the Amount Injected (x) Versus the Peak Area (y)

Compound	Intercept[a]	Slope[a]	R^2
PG	(-0.11 ± 0.02)E2	(2.04 ± 0.01)E3	0.9996
SA	(-2.22 ± 0.23)E2	(10.33 ± 0.15)E3	0.9997
MP	(0.10 ± 0.03)E2	(1.64 ± 0.02)E3	0.9995
EP	(0.71 ± 0.04)E2	(1.52 ± 0.03)E3	0.9997
BHQ	(-0.04 ± 0.01)E2	(0.70 ± 0.02)E3	0.9998
PP	(0.15 ± 0.02)E2	(1.43 ± 0.02)E3	0.9987
BeP	(-0.06 ± 0.02)E2	(1.08 ± 0.04)E3	0.9996
BP	(0.58 ± 0.09)E2	(1.57 ± 0.11)E3	0.9998
BHA	(0.02 ± 0.02)E2	(0.69 ± 0.11)E3	0.9999
OG	(-0.14 ± 0.05)E2	(1.20 ± 0.01)E3	0.9984
DBHQ	(0.07 ± 0.04)E2	(0.74 ± 0.07)E3	0.9989
TC	(0.11 ± 0.04)E2	(1.33 ± 0.03)E3	0.9992
DG	(-0.01 ± 0.02)E2	(1.39 ± 0.01)E3	0.9993
BHT	(0.09 ± 0.04)E2	(0.55 ± 0.01)E3	0.9981
AP	(0.01 ± 0.02)E2	(0.095 ± 0.001)E3	0.9980
TA	(-0.03 ± 0.01)E2	(0.14 ± 0.01)E3	0.9999

[a] mean value ± standard deviation at 95% confidence interval (t=3.18; n=5).

volume. After centrifugation at 3000 rpm for 5 min, the supernatant was filtered through a 0.45 μm filter. In the case of lipid fusions (lipsticks) about 2g of product was treated with 10 mL of N,N dimethylformamide, left under reflux for 10 min, and then filtered through a 0.45 μm filter.

RESULTS AND DISCUSSION

Figure 1 shows the chromatogram of a standard solution of the 16 additives studied at the concentration of 1mg · mL^{-1} with the exception of SA (0.4 mg · mL^{-1}). The chromatogram was recorded at 254 nm. In order to obtain maximun sensitivity the detection wavelength could be selected according to the values reported in Table 2, in dependence on the molar absorptivity of the compound concerned.

Table 4

Analysis of Pharmaceutical Formulations*

Formulation	Additive	Amount Declared (%)	Found (%)	C. V.
	AP	4.0 E-1	3.7 E-1	1.3 E-2
	TA	2.5 E-1	2.4 E-1	0.83 E-2
A	BHA	1.5 E-1	1.3 E-1	0.46 E-2
	MP	2.0 E-1	1.9 E-1	0.51 E-2
	PP	2.0 E-1	1.9 E-1	0.46 E-2
	BHT	0.15 E-1	0.16 E-1	0.43 E-3
B	BHA	0.15 E-1	0.14 E-1	0.42 E-3
	MP	1.8 E-1	1.7 E-1	0.59 E-2
	PP	0.20 E-1	0.19 E-1	0.79 E-3
	BHT	1.0 E-1	0.95 E-1	0.28 E-2
C	MP	1.5 E-1	1.4 E-1	0.53 E-2
	PP	1.0 E-1	0.94 E-1	0.35 E-2
	BHA	0.30 E-1	0.27 E-1	0.95 E-3
D	MP	1.0 E-1	1.0 E-1	0.34 E-2
	EP	1.0 E-1	0.96 E-1	0.40 E-2
	BHT	0.70 E-1	0.66 E-1	1.9 E-3
E	MP	1.0 E-1	1.0 E-1	0.38 E-2
	PP	0.5 E-1	0.49 E-1	1.7 E-3

*mean of five determinations.

The chosen elution conditions allowed a very good separation of all the agents taken into account. Retention characteristics are summarized in Table 2. The retention times were reproducible under the experimental conditions used, the coefficient of variation (C.V.) ranging from 1.7 to 2.4 for within-day and from 2.6 to 4.1% for between-day studies.

The photodiode-array detector allowed the estimation of the peak purity factors. These values were calculated over the range 190-600nm and resulted > 99.5% for all the agents studied, thus confirming the good resolution achieved.

Table 5

Analysis of Cosmetic Formulations*

Formulations	Additive	Found (%)	C.V.
F	BHA	1.1 E-1	0.54 E-2
	BHT	1.0 E-1	0.49 E-2
G	BHA	0.45 E-1	0.19 E-2
	BHT	0.10 E-1	0.45 E-3
	MP	1.5 E-1	0.70 E-2
	PP	1.5 E-1	0.79 E-2
H	BHA	0.25 E-1	1.2 E-3
	TA	5.0 E-1	2.3 E-2
	MP	0.7 E-1	0.29 E-2
	PP	0.5 E-1	0.25 E-2
I	BHA	0.15 E1	0.72 E-3
	TA	0.5 E-1	2.5 E-3
	MP	1.0 E-1	0.39 E-2
	PP	0.5 E-1	2.0 E-3
L	TA	1.2 E-1	0.54 E-2
	EP	0.50 E-1	2.0 E-3
	MP	1.0 E-1	0.43 E-2
	PP	0.5 E-1	1.8 E-3
M	TA	1.0 E-1	0.49 E-2
	TC	3.0 E-1	1.5 E-2

* mean of five determinations.

The calibration graphs were constructed from five consecutive injections over the covered range of concentration, as indicated in the experimental section. The least square regression fit showed good linearity, passing through the origin. The data obtained for the calibration lines are shown in Table 3. The detection limits, calculated as a signal-to-noise ratio of 3:1, are reported in Table 2.

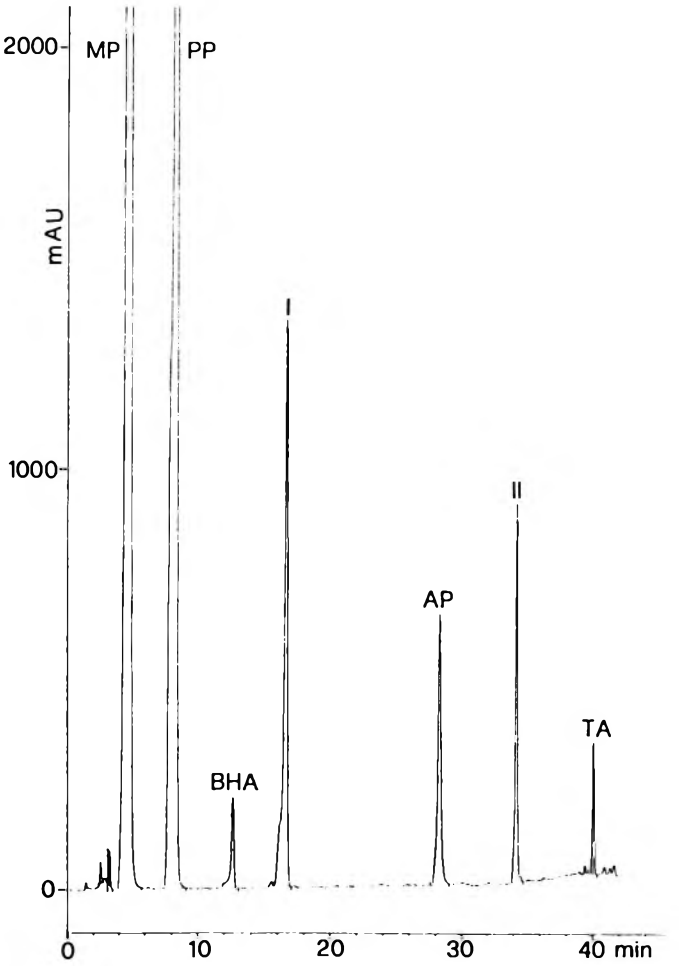


Figure 2. Chromatogram obtained at 254 nm for the pharmaceutical formulation A (see Table 4). Peak I and II correspond to dexamethasone valerate and pyridoxine tripalmitate, respectively.

Recovery tests were carried out to evaluate the reproducibility and accuracy of the proposed method. An oil-water emulsion and a lipstick, prepared in our laboratory so that their composition was similar to that of the formulations found on the market, were spiked with 0.05% (w/w) of all the

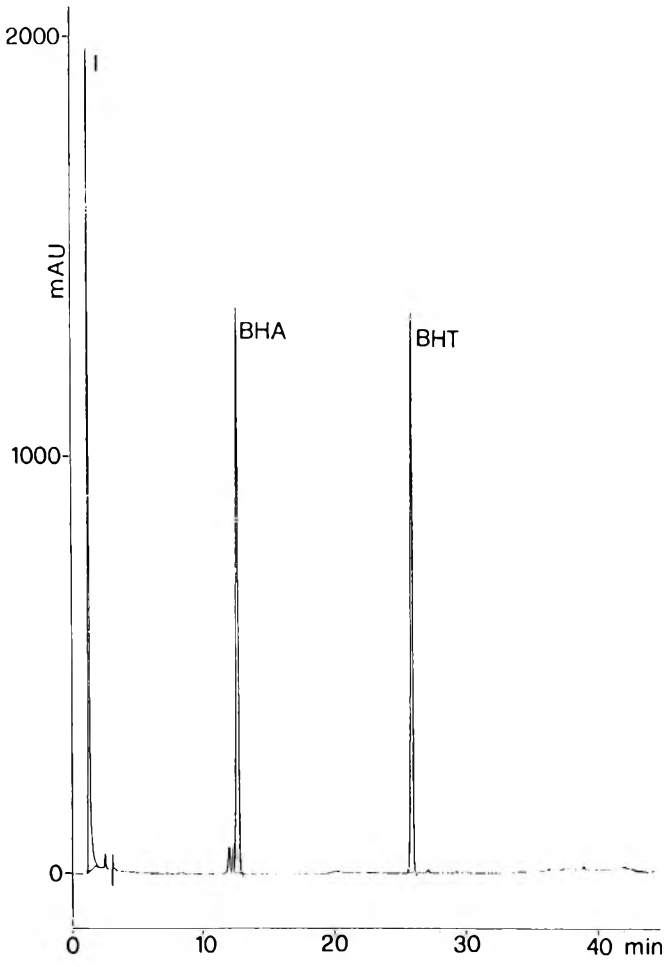


Figure 3. Chromatogram obtained at 280 nm for the lipstick E (see Table 5). Peak I corresponds to the dye C.I.15850.

antioxidants and preservatives studied and subjected to the described analytical procedure. The mean recoveries (n = 4) for all the compounds ranged from 94.5 to 99.6% with a C.V. < 3.7%. Therefore good recovery and precision were observed.

The content of the agents under study in 11 commercially available pharmaceutical and cosmetic formulations was determined in triplicate by using the proposed method. The samples contained combinations of the antioxidants and preservatives which were identified by comparing the retention times and the purity values of the peaks observed, with those obtained from the standard solutions. The assay results are shown in Tables 4 and 5. The quantities found for the pharmaceutical formulations were in conformity with the values claimed by the manufacturer. Typical chromatoghrams (for the cream A and the lipstick F) are shown in Figures 2 and 3. In Figure 2 the peaks named I and II correspond to the active principles dexamethasone valerate and the additive pyridoxine tripalmitate, respectively. In Figure 3 the peak named I correspond to a water-soluble dye (C.I. 15850) present in the composition of the lipstick, which is practically unretained by the column. From both chromatograms we can also adfirm that interferences from the excipients were not observed.

The analytical results obtained lead to the conclusion that the developed method can be successfully adopted for an accurate determination of the sixteen additives considered in oil-rich cosmetics and pharmaceuticals.

REFERENCES

1. S. C. Rastogi, A. Schouten, N. De Kruijf, J. W. Weijland, Contact Dermatitis, **32**, 28-30 (1995).

2. C. H. Van Peteghem, D. A. Dekeyser, J. Assoc. Off. Anal. Chem., **64**, 1331-1335 (1981).

3. A. Dooms-Goossens, J. Pharm. Belg., **32**, 213-228 (1977).

4. D. M. Wyatt, J. Am. Oil Chem. Soc., **58**, 917-920 (1981).

5. D. A. Kline, L. J. Frank Jr,T. Fazio, J. Assoc. Off. Anal. Chem., **61**, 513-519 (1978).

6. B. D. Page, J. Assoc. Off. Anal. Chem., **66**, 727-745 (1983).

7. T. S. Vicente, E. H. Waysek, W. M. Cort, J. Am. Oil Chem. Soc., **62** 745-747 (1985).

8. L. Gagliardi, A. Amato, G. Cavazzutti, E. Gattavecchia, D. Tonelli, J. Chromatogr., **325**, 353-358 (1985).

9. S. Yamamoto, M. Kanda, M. Yokouchi, S. Tahara, J. Chromatogr., **370,** 179-187 (1986).

10. L. Gagliardi, G. Cavazzutti, L. Turchetto, F. Manna, D. Tonelli, J. Chromatogr., **508,** 252-258 (1990).

11. J. M. Irache, F. A. Vega, I. Ezpeleta, Pharm. Acta Helv., **67**, 152-155 (1992).

12. R. Boussenadji, M. Porthault, A. Berthod, J. Pharm. Biomed. Anal., **11**, 71-78 (1993).

13. C. A. Hall III, A. Zhu, M. G. Zeece, J. Agric. Food Chem., **42**, 919-921 (1994).

Received August 16, 1996
Accepted December 2, 1996
Manuscript 4271

J. LIQ. CHROM & REL. TECHNOL., 20(11), 1809–1814 (1997)

THE BOOK CORNER

CAPILLARY ELECTROPHORESIS PROCEDURES MANUAL, A LABORATORY USER'S AID FOR QUICK STARTS, edited by E. Jakim and L. W. Jakim, a joint publication of Applied Science Communications, Peace Dale, RI and Elsevier Science B.V., Amsterdam, 295 pp., 1996. Price: $175.00.

Capillary electrophoresis literature over the last few years has changed from mainly basic theory and instrumentation to applications, which is normal for such a high resolution technique. As a result, there are, at times, many procedures for the separation of the same group of compounds. This is especially true when separating mixtures of amino acids. The manual simplifies the search for the best available procedure, since a listing of many procedures is given which allows the analyst to pick and choose the one that fits his needs.

The manual is divided into many sections. The first one is quick tips for (a) obtaining reproducible migration time and quantitative results; (b) preparing and knowing your buffers; and (c) capillary conditioning and cleaning. This section is followed by abbreviations, then the separation procedures which are divided into pharmaceutical; clinical; proteins, amino acids and peptides; nucleic acid, "tides" and "sides"; pesticides; forensic/explosives; natural products; and metabolites; miscellaneous and inorganic ions. The final section deals with equipment. The separation procedure page includes what is being separated, a reference from which the method is cited, and experimental conditions, such as buffer (type, concentration, and pH), capillary dimensions, applied voltage, current, polarity, temperature, detection mode, sample size injected and injection mode, and instrument used. A pherogram is given for each procedure.

The manual is comprehensive, filled with nearly 290 procedures covering about 1000 compounds. It is organized in a very simple and straightforward manner, with an index to quickly locate the compounds of interest. This manual is highly recommended as a useful reference.

CHROMATOGRAPHIC ANALYSIS OF PHARMACEUTICALS, edited by J. A. Adamovics, Chromatographic Science Series, Volume 74, Jack Cazes, Editor, Marcel Dekker, Inc., New York, NY, 544 pp., 1996. Price: $165.00.

The first edition of *Chromatographic Analysis of Pharmaceuticals* was published in 1990. This second edition updates and expands coverage of the topics in the first edition.

The overall organization of the first edition — a series of chapters on regulatory considerations, sample treatment (manual/robotic), and chromatographic methods (TLC, GC, HPLC), followed by an applications section — has been maintained. To provide a more coherent structure to this edition, the robotics and sample treatment chapters have been consolidated, as have the chapters on gas chromatography and headspace analysis. This edition includes two new chapters, on capillary electrophoresis, and supercritical fluid chromatography. These new chapters discuss the hardware behind the technique, followed by their respective approaches to methods development, along with numerous examples. All the chapters have been updated with relevant information on proteinaceous pharmaceuticals. The applications chapter has been updated to include chromatographic methods from the *Chinese Pharmacopoeia* and updates from *U.S. Pharmacopeia 23* and from the *British and European Pharmacopoeias*. Methods developed by instrument and column manufacturers are also included in an extensive table, as are up-to-date references from the chromatographic literature.

The chapters on CE and SFC are comprehensive enough for such a book. These two techniques, especially CE, have resulted in a substantial increase in the number of capillary electrophoresis (CE) applications in the pharmaceutical industry over the last 5 years. Capillary electrophoresis has been utilized in the quantitation of drug-related impurities, stability studies, chiral analysis, stereoisomeric separations, and formulation analysis. Continued interest in the research and development of biotechnology-derived products has promoted the widespread use of CE to monitor the synthetic and purification processes, in addition to the analysis of these therapeutic entities in formulations.

This second edition should appeal to chemists and biochemists in pharmaceutics and biotechnology who are responsible for analysis of pharmaceuticals. As in the first edition, this book focuses on analysis of bulk and formulated drug products, and not on analysis of drugs in biological fluids. The book is well written and illustrated, and is free of typographical errors. It is recommended to all those interested in pharmaceutical analysis.

Table of Contents

Chapter 1. Regulatory Considerations for the Chromatographer, J. A. Adamovics (1).

Chapter 2. Sample Pretreatment, J. A. Adamovics (23).

Chapter 3. Planar Chromatography, J. A. Adamovics, J. C. Eschbach (57).

Chapter 4. Gas Chromatography, J. A. Adamovics, J. C. Eschbach (79).

Chapter 5. High Performance Liquid Chromatography, J. A. Adamovics, D. L. Farb (135).

Chapter 6. Capillary Electrophoresis, S. R. Rabel, J. F. Stobaugh (209).

Chapter 7. Supercritical Fluid Chromatography of Bulk and Formulated Pharmaceuticals, J. T. Stewart, N. K. Jagota (239).

Chapter 8. Applications, J. A. Adamovics (273).

CAPILLARY ELECTROPHORESIS IN ANALYTICAL BIOTECHNOLOGY, edited by P. G. Righetti, CRC Series in Analytical Biotechnology, edited by W. S. Hancock, CRC Press, Boca Raton, FL, 551 pp., 1996. Price: $145.00,

and

CAPILLARY ELECTROPHORETIC SEPARATIONS OF DRUGS, edited by A. S. Cohen, S. Terabe, Z. Deyl, reprinted from Journal of Chromatography A, Volume 735, Elsevier Science B.V., Amsterdam, 447 pp., 1996. Price: $281.25.

These two books were recently received, dealing with application of capillary electrophoresis. The first one deals with "CE in Analytical Biotechnology," while the second one is entitled "CE Separations of Drugs." These two books tell us that CE, which was introduced in 1967 by S. Hjerten, and later modernized and simplified to its present form by J. Jorgenson, have moved from the theoretical and development stage to the applications stage. This means that CE is not only maturing but it is acceptable in different fields as an analytical tool. Dr. Pier Giorgio Righetti has done an excellent and

commendable job in editing a comprehensive book on CE in analytical biotechnology, definitely a growing field to which CE is well suited. The book is devoted to proteins, peptides, and techniques especially useful in the area of recombinant DNA products. Emphasis is also placed on glycoproteins.

Because of the growing role of the glycosylation process in CE, a comprehensive chapter on the subject acts as a book within a book. The book is made up of 12 chapters dealing with basic aspects of CE and their application to analytical biotechnology. The chapters are well written and the flow of the book is excellent.

The second book is devoted to the separation of drugs and is edited by a well known group of scientists. The book is a reproduction of the Journal of Chromatograph A, Volume 735. The volume is divided into (a) reviews (nine reviews, 191 pages); (b) research papers (7 papers, 80 pages); (c) chiral separations (8 papers, 70 pages); and (d) macromolecular drugs (9 papers, 90 pages).

This volume mostly follows a Journal format and is not really designed to be a book. This does not mean that this volume is not valuable; it is of use to those interested in drug separations. The price of this book ($281.25) is high compared to the price of the first book ($145.00) which is very reasonable and well worth the cost.

CE IN ANALYTICAL BIOTECHNOLOGY

Table of Contents

Chapter 1. Surface Modifications of Silica Walls: A Review of Different Methodologies, M. Chiari, M. Nesi, P. G. Righetti (1).

Chapter 2. Buffers, Electrolytes, and Additives for Capillary Electrophoresis, S. E. Moring (37).

Chapter 3. Injection Methods in Capillary Electrophoresis, J. D. Olechno J. A. Nolan (61).

Chapter 4. Consecutive Protein Digestion and Peptide Derivatization Employing an On-Line Analyte Concentrator to Map Proteins Using Capillary Electrophoresis, N. A. Guzman (101).

Chapter 5. Capillary Electrophoresis Interfaced with Mass Spectrometry: Electrospray Ionization and Continuous Flow Fast Atom Bombardment, K. B. Tomer, C. E. Parker, L. J. Deterding (123).

Chapter 6. Micellar Electrokinetic Chromatography in the Analysis of Amino Acids and Peptides, N. Matsubara, S. Terabe (155).

Chapter 7. Analysis of rDNA-Derived Proteins and their Post-Translational Modifications, K. Ganzler, N. W. Warne, W. S. Hancock (183).

Chapter 8. Capillary Zone Electrophoresis for the Analysis of Peptides, M. Castagnola, I. Messana, D. V. Rossetti (239).

Chapter 9. Analysis of Glycoproteins, Oligo- and Monosaccharides, C. Chiesa, R. A. O'Neill, C. G. Horvath, P. J. Oefner (277).

Chapter 10. Capillary Electrophoresis of DNA, P. G. Righetti, C. Gelfi (431).

Chapter 11. Principles of Size-Based Separations in Polymer Solutions, J.-L. Viovy, C. Heller (477).

Chapter 12. Isoelectric Focusing in Capillaries, P. G. Righetti, C. Gelfi, M. Chiari (509).

CAPILLARY ELECTROPHORETIC SEPARATIONS OF DRUGS

Table of Contents

Chapter 1. Foreword, A. Cohen, S. Terabe, Z. Deyl.

Chapter 2. Micellar Electrokinetic Chromatography. Perspectives in Drug Analysis, H. Nishi, S. Terabe.

Chapter 3. Capillary Electrophoretic Analyses in Drugs in Body Fluids: Sample Pretreatment and Methods for Direct Injection of Biofluids, D. K. Lloyd.

Chapter 4. Determination of Drug-Related Impurities by Capillary Electrophoresis, K. D. Altria.

Chapter 5. Enantiomer Separation of Drugs by Electrokinetic Chromatography, H. Nishi.

Chapter 6. Identification of Chiral Drug Isomers by Capillary Electrophoresis, S. Fanali.

Chapter 7. Capillary Electrophoresis of Cardiovascular Drugs, N. T. Nguyen, R. W. Siegler.

Chapter 8. Capillary Electrophoresis of Diuretics, M.-L. Riekkola, J. H. Jumppanen.

Chapter 9. Capillary Isoelectric Focusing as a Tool in the Examination of Antibodies, Peptides and Proteins of Pharmaceutical Interest, X. Liu, Z. Sosic and I. S. Krull.

Chapter 10. Capillary Gel Electrophoresis and Antisense Therapeutics. Analysis of DNA Analogs, L. A. DeDionisio, D. H. Lloyd.

Research Papers

Techniques, Optimization and Comparison of CE with Chromatography (7 papers).

Chiral Separations (8 papers).

Macromolecular Drugs (3 papers).

Miscellaneous Applications (6 papers).

Books reviewed by
Haleem J. Issaq, Ph.D.,
Editor, The Book Corner

J. LIQ CHROM. & REL. TECHNOL., 20(11), 1815 (1997)

ANNOUNCEMENT

A. A. BENEDETTI-PICHLER AWARDEE WILL BE PRESENTED TO PROFESSOR JOHN G. DORSEY

by the

AMERICAN MICROCHEMICAL SOCIETY

at the Eastern Analytical Symposium, Somerset, New Jersey November 20, 1997

Professor Dorsey has made outstanding contributions to analytical chemistry and to an understanding of chromatographic retention mechanisms. He has made seminal contributions to flow analysis, and to the estimation of biological and environmental partitioning processes. The Foley-Dorsey equation is now the recognized standard for calculation of the number of theoretical plates that measure the resolving power of a separation. The paper in which this equation was first published, Anal. Chem., 55, 730-737 (1983), has been cited in numerous papers since its publication.

John G. Dorsey is Professor and Chairman of the Department of Chemistry at Florida State University. His research interests are in the areas of fundamental liquid chromatography; capillary electrophoresis; analytical applications of micelles and organized media; flow-injection analysis; and old Bordeaux wines.

He has about 95 publications in these areas, serves on the Editorial Boards of five journals, and is an Associate Editor of Journal of High Resolution Chromatography. Since 1990, he has been the senior author for the biannual Fundamental Review of Liquid Chromatography in Analytical Chemistry.

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1817 (1997)

EDUCATION ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

A Two-Day In-House Training Course

The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware module and system problems.

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection and Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration and Quantitation
- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on GPC, and has taught at Rutgers University. He is currently Visiting Scholar at Florida Atlantic University.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097. Tel.: (954) 570-9446; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1819–1828 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

AUGUST 2 - 9: 39th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency Denver, Denver, Colorado. Contact: Patricia Sulik, Rocky Mountain Instrumental Labs, 456 S. Link Lane, Ft. Colleins, CO, USA. (303) 530-1169; Email: gebrown@usgs.gov.

AUGUST 11 - 13: 10th International Symposium on Polymer Analysis and Characterization (ISPAC-10), University of Toronto, Canada. Contact: Prof. S. T. Balke, Dept. of Chem. Engg & Appl. Chem., Univ. of Toronto, Toronto, Ont., Canada, M5S 1A4. Tel/FAX: (416) 978-7495; Email: balke@ecf.toronto.edu.

SEPTEMBER 2 - 5: 12th International Bioanalytical Forum, Univ. of Surrey, Guildford, UK, sponsored by the Chromatographic Society (U.K.). Contact: Dr. E. Reid, 72 The Chase, Guildford GU2 5UL, U.K. Tel/FAX: (0) 1483-565324; Email: D.Stevenson@surrey.ac.uk.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

SEPTEMBER 14 - 17: International Ion Chromatography Symposium, Westin Hotel, Santa Clara, California. Contact: Janet Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052-0493, USA. Tel: (508) 359-8777; FAX: (508) 359-8778; Email: century@ixl.net.

SEPTEMBER 14 - 19: ACS Int'l Symposium on Systems Approach to Service Life Prediction of Organic Coatings, Breckenridge, Colorado. Contact: J. Martin, NIST, Bldg. 226, Rm B-350, Gaithersburg, MD 20899, USA. Email: jmartin@nist.gov.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

SEPTEMBER 22 - 25: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Fairmont Hotel, San Francisco, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 30 - OCTOBER 2: 53rd Southwest ACS Regional Meeting, Tulsa, Oklahoma. Contact: F. B. Growcock, Amoco Corp., E&PT, P. O. Box 3385, Tulsa, OK 74012, USA. Tel: (918) 660-4224; Email: fgrowcock@amoco.com.

OCTOBER 5 - 8: Conference on Formulations & Drug Delivery, La Jolla, California, sponsored by the ACS Div. of Biochem. Technol. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286; FAX: (202) 872- 6013; Email: miscmtgs@acs.org.

OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France. (Training course given in French) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 23: Sensors Expo: Conference on Exposition of Sensors, Detroit, Michigan. Contact: Expocon Mgmt. Assoc., 3363 Reef Rd, P. O. Box 915, Fairfield, CT 06430-0915, USA. Tel: (203) 256-4700; Email: sensors@expo.com.

OCTOBER 21 - 23: Biotechnica Hannover '97: Int'l. Trade Fair for Biotechnology, Hannover, Germany. Contact: D. Hyland, Hannover Fairs USA, Inc., 103 Carnegie Center, Princeton, NJ 08540, USA. Tel: (609) 987-1202; Email: dhyland@hfusa.com.

OCTOBER 21 - 24: 152nd Fall Technical Meeting & Rubber Expo'97, Cleveland, Ohio, sponsored by ACS Div. of Rubber Chem. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 25 - 30: 24th Annual Conference of the Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Providence, Rhode Island. Contact: ACS Div. of Anal. Chem., Tel: (301) 846-4797; FAX: (301) 694-6860; Internet: http://FACSS.org/info.html.

OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Doubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. FAX: (301) 898-5596.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 2 - 6: AAPS Annual Meeting & Expo, Boston, Mass. Contact: AAPS, 1650 King Street, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000.

NOVEMBER 2 - 6: 11th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Klyczynski, Electrosysthesis Co., 72 Ward Rd., Lancaster, NY 14086, USA.

NOVEMBER 6 - 8: 2nd North American Research Conference on Emulsion Polymers/Polymer Colloids, Hilton Head Is., SC. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561.

NOVEMBER 7 - 8: 6th Conference on Current Trends in Computational Chemistry, Jackson, Miss. Contact: J. Leszcynski, Jackson State Univ., Chem. Dept., 1400 J. R. Lynch St., Jackson, MS 39217, USA. Tel: 601) 973-3482; Email: jersy@iris5.jusms.edu.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: AIChE Annual Meeting, Westin Bonaventure/Omni Los Angeles, Los Angeles, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

NOVEMBER 20 - 21: New Horizons in Chemistry Symposium, Los Angeles. Contact: J. May, Tel: (213) 740-5962; Email: jessy@methyl.usc.edu.

1998

FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721. Email: reglmtgs@acs.org.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353. Email: reglmtgs@acs.org.

MAY 26 - 29: VIHth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34; FAX: (32) 9/264.81.99; Email: carlos.vanpeteghem@rug.ac.be.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography & Extraction, Adams Mark Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; Email: Janetbarr@aol.com.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

DECEMBER 14 - 19: 2000 Int'l Chemical Congress of the Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th St., NW, Washington, DC 20036, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: pacific@acs.org.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 25 - 31: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2003

MARCH 23 - 28: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 12: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 18: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 2: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 10 - 15: 232nd ACS National Meeting, San Francisco, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2007

MARCH 25 - 30: 233rd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

The **Journal of Liquid Chromatography & Related Technologies** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in the Liquid Chromatography Calendar, we will need to know:

a) Name of the meeting or symposium,
b) Sponsoring organization,
c) When and where it will be held, and
d) Whom to contact for additional details.

Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography & Related Technologies, P. O. Box 970210, Coconut Creek, FL 33097, USA.**

INSTRUCTIONS TO AUTHORS

The *Journal of Liquid Chromatography & Related Technologies* is published in the English language for the rapid communication of research results in liquid chromatography and its related sciences and technologies.

Directions for Submission

One complete original manuscript and two (2) clear copies, with figures, must be submitted for peer review. After all required revisions have been completed, and the final manuscript has been accepted, the author will be asked to provide, if possible, a 3½" or 5¼" PC-Compatible computer diskette containing the complete manuscript. Microsoft Word, Word for Windows, WordPerfect, WordPerfect for Windows and ASCII are preferred formats. Text, including tables, and figures, if in electronic format, should be saved in separate files on the diskette. Label the diskette with the corresponding author's last name, the title of the manuscript and the file number assigned to the manuscript.

Submission of a manuscript on diskette, in a suitable format, will significantly expedite its publication.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography & Related Technologies
P. O. Box 970210
Coconut Creek, FL 33097

Reprints

Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript

NOTE: Failure to adhere to the following guidelines will delay publication of a manuscript.

1. The preferred dimensions of the printed area of a page are
   6" (15.2 cm) width by 8.5" (21.6 cm) height.
   Use Times Roman 12 point font, if possible.

The general organization of the manuscript should be:

Title
Author(s)' names and full addresses
Abstract
Text Discussion
References

2. **Title & Authors**: The entire title should be in bold-face capital letters and centered within the width of the printed area, located 2 inches (5.1 cm) from the top of the page. This should be followed by 2 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN WATER BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce, H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract**: The heading **ABSTRACT** should be typed boldface, capitalized and centered, 2 lines below the addresses. This should be followed by a single-spaced, concise abstract. Allow 2 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion**: Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGMENTS

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed boldface, in capital letters, centered.

Secondary headings, if any, should be placed flush with the left margin, and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The **first line of each paragraph** within the body of the text should be indented a half inch.

6. **Acknowledgments**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference.

Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES**, in boldface type, should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used. **Hand-drawn characters are not acceptable.**

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on **good quality white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). International paper, size A4 is also acceptable. The typing area of the first page, including the title and authors, should be 6" (15.2 cm) wide by 8.5" (21.6 cm) height.

2. All text should be **typed single-spaced**.

3. It is essential to use **dark black** typewriter or printer ribbon so **that clean, clear, solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed as part of the text, but in such a way as to separate them from the text by a 2-line space above and below the table. Tables should be inserted in the text as close to the point of reference as possible. **A table may not be longer than one page**. If a table is larger than one page, it should be divided into more than one table. The word **Table** (followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures** (drawings, graphs, etc.) should be professionally drawn in black India ink on separate sheets of white paper, and should be placed at the end of the text. They should not be inserted into the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). Photographs should be professionally prepared, black and white, *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the word **Figure** and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The reference list should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.

Manuscripts which require correction of English usage will be returned to the author for major revision.

Achieve accurate and precise quantitative analysis with...

Generously illustrated with 160 richly detailed diagrams that clarify the construction of the detectors, facilitating understanding of their performance and function!

Chromatographic Detectors

Design, Function, and Operation

(Chromatographic Science Series/73)

RAYMOND P. W. SCOTT
Georgetown University, Washington, D.C., and Birbeck College, University of London, United Kingdom

July, 1996 / 536 pages, illustrated / $150.00

Written by a seasoned chromatographer with more than 40 years' experience in the field, this reference comprehensively covers the design, construction, and operation of gas chromatography (GC), liquid chromatography (LC), and thin-layer chromatography (TLC) detectors—*all in one convenient, up-to-date source.*

Emphasizing the essential use of common specifications to describe all detectors, allowing easy comparison of their attributes, this practical guide

- discusses the properties of chromatography detectors and the best way to measure their efficacy
- reviews factors that impair the column resolution *before* solutes reach the detector
- describes and explains the relatives merits of the more popular detectors, including the most recent commercially available models as well as lesser-known devices
- explores the exclusive number of TLC detectors available, including automatic scanning devices
- surveys those chromatograph/spectrometer tandem systems that have been satisfactorily developed and details their interfacing, function, and areas of application
- and more!

Chromatographic Detectors serves as an important on-the-job tool for chromatographers; analytical, physical, pharmaceutical, oil, petroleum, clinical, forensic, environmental, and synthetic chemists and biochemists; biotechnologists; and upper-level undergraduate, graduate, and continuing-education students in these disciplines.

Contents

Detector Properties and Specifications
An Introduction to Chromatography Detectors
Detector Specifications
Data Acquisition and Processing

Gas Chromatography Detectors
Gas Chromatography Detectors: Their Evolution and General Properties
The Flame Ionization Detector and Its Extensions
The Argon Ionization Family of Detectors
The Katherometer and Some of the Less Well-Known Detectors

Liquid Chromatography Detectors
Introduction to LC Detectors
The Fluorescence and Other Light Processing Detectors
The Electrical Conductivity Detector and the Electrochemical Detector
The Refractive Index Detector and Associated Detectors
Multifunctional Detectors and Transport Detectors
Chiral Detectors
The Radioactivity Detector and Some Lesser-Known Detectors
Detection in Thin-Layer Chromatography

General Detector Techniques
Spectroscopic Detectors and Tandem Systems
Practical Detector Techniques
Quantitative Analysis

Appendixes

ISBN: 0—8247—9779—5

This book is printed on acid-free paper.

Marcel Dekker, Inc. 270 Madison Avenue, New York, NY 10016 • (212) 696-9000
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland • Tel. 061-261-8482

Establish efficient strategies for the practical application of liquid chromatography in the field of oligomer characterization with...

LIQUID CHROMATOGRAPHY OF OLIGOMERS

(Chromatographic Science Series/72)

CONSTANTIN V. UGLEA
Institute of Biological Research, Ministry of Research and Technology, Iasi, Romania, and the Medical and Pharmaceutical University, Iasi, Romania

June, 1996 / 360 pages, illustrated / $150.00

This **state-of-the-art** reference details the principles and mechanisms of, and the equipment and optimal working conditions for, the liquid-chromatographic separation of well-defined oligomeric species and fractions with narrow molecular weight distribution.

Provides a complete description of the applications and possible performance of liquid chromatography in the field of oligomer separation!

Liquid Chromatography of Oligomers

- elucidates theoretical concepts such as the mechanism of retention and the thermodynamic explanation of oligomer separation
- discusses the definition and history of chromatography
- covers both simple and complex forms of chromatographic instrumentation
- delineates procedures used for the fractionation of oligomeric mixtures
- lists the main producers of chromatographic devices
- describes the chemical nonhomogeneity of polymers and oligomers
- presents the nomenclature of oligomers
- examines gel-permeation chromatography
- and more!

Containing over **1475** citations to key literature sources, ***Liquid Chromatography of Oligomers*** is an ideal reference for analytical chemists, biochemists, chromatographers, laboratory technicians, and upper-level undergraduate and graduate students in these disciplines.

Contents

Definition, History, and Nomenclature

Molecular Nonhomogeneity of Synthetic Oligomers

Liquid Chromatography

Gel Permeation Chromatography

ISBN: 0—8247—9720—5

This book is printed on acid-free paper.

Marcel Dekker, Inc.

270 Madison Avenue
New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812
CH-4001 Basel, Switzerland
Tel. 061-261-8482

Analyze bulk and formulated drug products with the ***new edition*** *of...*

Chromatographic Analysis of Pharmaceuticals

SECOND EDITION

(Chromatographic Science Series/74)

edited by **JOHN A. ADAMOVICS**
Cytogen Corporation, Princeton, New Jersey

October, 1996 / 544 pages, illustrated / $165.00

Praise for the first edition...

"There is no doubt that this book should have wide appeal to all those involved in pharmaceutica analysis. Over 1300 drugs and related substances are considered....**a veritable mine of information.**"—***Analyst***

"...an **excellent** reference for those who are involved in the chromatographic analysis of pharmaceutical compounds and their formulation."
—***Journal of Pharmaceutical Sciences***

"...**recommended** as a primary source of information for drug analysis in different drug forms."
—***Journal of Chromatography***

"...a **valuable** reference source for practicing chromatographers in the pharmaceutical industry."
—***Microchemical Journal***

"...provides a **comprehensive** overview of the chromatographic methods of analysis for pharmaceuticals."
—***Analytical Chemistry***

"...has a place as a **quick reference tool** anc as a general introduction to chromatography in pharmaceutics."
—***LC-GC***

"...contains **a wealth of information** on the main chromatographic techniques and numerous examples on the application of these methods in the drug industry."
—***Journal of Labelled Compounds and Radiopharmaceuticals***

Updated and revised throughout, the ***Second Edition*** of ***Chromatographic Analysis of Pharmaceuticals*** explores the chromatographic methods used for the measurement of drugs, impurities, and excipients in pharmaceutical preparations—such as tablets, ointments, and injectables.

Contains a 148-page table listing the chromatographic data of over 1300 drugs and related substances—including sample matrix analyzed, sample handling procedures, column packings, mobile phase, mode of detection, and more!

Maintaining the features that made the first edition so popular, ***Chromatographic Analysis of Pharmaceuticals, Second Edition*** now offers

- *new chapters* on capillary electrophoresis and supercritical fluid chromatography
- *up-to-the-minute* information on proteinaceous pharmaceuticals
- *new coverage* of chromatographic methods from the Chinese Pharmacopoeia
- *updated data* from *US Pharmacopeia 23* and from the British and European Pharmacopoeias
- the *latest methods* developed by instrument and column manufacturers
- over 2100 key literature citations, including *recent references* from the chromatographic literature up to 1996

Written in a clear, easy-to-read style, ***Chromatographic Analysis of Pharmaceuticals, Second Edition*** serves as a timesaving resource for analytical, bioanalytical, pharmaceutical, organic, clinical, physical, quality control, and process chemists and biochemists; chromatographers; pharmaceutical scientists; and professional seminars and graduate-level courses in these disciplines.

Contents

Regulatory Considerations for the Chromatographer, *John A. Adamovics*

Sample Treatment, *John A. Adamovics*

Planar Chromatography,
John A. Adamovics and James C. Eschbach

Gas Chromatography,
John A. Adamovics and James C. Eschbach

High-Performance Liquid Chromatography,
John A. Adamovics and David Farb

Capillary Electrophoresis,
Shelley R. Rabel and John F. Stobaugh

Supercritical Fluid Chromatography of Bulk and Formulated Pharmaceuticals,
James T. Stewart and Nirdosh K. Jagota

Applications, *John A. Adamovics*

ISBN: 0—8247—9776—0

This book is printed on acid-free paper.

Marcel Dekker, Inc.

270 Madison Avenue, New York, NY 10016 • (212) 696-9000
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland • Tel. 061-261-8432

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

Volume 20, Number 11, 1997

CONTENTS

Controlling the Pore Structure of Zirconia for Chromatographic Applications **1651**
R. A. Shalliker and G. K. Douglas

Characterization of Wide-Pore Reversed Phase Columns for Biopolymer Separations. II. Multiparametric Evaluation **1667**
G. Rippel, Gy. Bacsur, Á. Bede, Á. Sándi, and L. Szepesy

High Performance Liquid Chromatographic Isolation and Spectroscopic Characterization of Metabolites from the Bile of Rats Receiving Rapamycin (Sirolimus) Intravenously **1689**
C. P. Wang, H.-K. Lim, K. W. Chan, J. Scatina, and S. F. Sisenwine

Estimation of the RP-HPLC Lipophilicity Parameters Log K', and Log K_w, a Comparison with the Hydrophobicity Index φ_0 ..... **1703**
M. L. C. Montanari, C. A. Montanari, D. Piló-Veloso, and Q. B. Cass

Peptic Peptide Mapping by HPLC, On Line with Photodiode Array Detection, of a Hemoglobin Hydrolysate Produced at Pilot-Plant Scale from an Ultrafiltration Process **1717**
Q. Zhao, P. Molina, and J. M. Piot

A New Approach to Study Benzodiazepine Separation and the Differences Between a Methanol/Water and Acetonitrile/Water Mixture on Column Efficiency in Liquid Chromatography **1741**
Y. Guillaume, E. J. Cavalli, E. Peyrin, and C. Guinchard

(continued on inside back cover)

MARCEL DEKKER, INC. NEW YORK • BASEL • HONG KONG
Contributions to this journal are published free of charge

J. LIQ. CHROM. & REL. TECHNOL., 20(11), (1997)

Contents Continued

Determination of Abamectin in Some Forest Matrices by Liquid Chromatography with Fluorescence Detection 1757
K. M. S. Sundaram and J. Curry

Optimization of a Reversed-Phase High Performance Liquid Chromatography Separation Using an Ion-Pair Reagent for the Determination of Carboxylic Acids in Plant Materials 1773
C. F. Harrington, D. J. Roberts, and G. Nickless

An HPLC Method for the Measurement of Polyamines and Lipidic Amines Binding to DNA 1789
G. A. Karikas, V. Constantinou-Kokotou, and G. Kokotos

Simultaneous Determination of Antioxidants and Preservatives in Cosmetics and Pharmaceutical Preparations by Reversed-Phase HPLC 1797
L. Gagliardi, D. De Orsi, L. Manna, D. Tonelli

The Book Corner 1809

Announcements 1815

Liquid Chromatography Calendar 1819