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CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Current Awareness In Biological Sciences (CABS), Current Contents/Life Sciences, Current Contents/Physical, Chemical & Earth Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Referativnyi Zhurnal, Research Alert and Science Citation Index)

REGULAR PAPERS

Column Liquid Chromatography

Simultaneous optimization of pH and organic modifier content of the mobile phase for the separation of chlorophenols using a Doehlert design
by B. Bourguignon (Jette, Belgium), F. Marcenac (Saint-Fons, France) and H. R. Keller, P. F. de Aguiar and D. L. Massart (Jette, Belgium) (Received September 21st, 1992) . . . 171

Use of computer simulations in the development of gradient and isocratic high-performance liquid chromatography methods for analysis of drug compounds and synthetic intermediates
by L. Wrisley (Rouses Point, NY, USA) (Received September 22nd, 1992) . . . 191

High-performance liquid chromatographic determination of polyamines in selected vegetables with postcolumn fluorimetric derivatization
by H. Ohta, Y. Takeda, K.-I. Yoza and Y. Nogata (Hiroshima, Japan) (Received September 18, 1992) . . . 199

Partitioning of ecdysteroids using temperature-induced phase separation
by P. A. Alred and F. Tjerneld (Lund, Sweden) and R. F. Modlin (Huntsville, AL, USA) (Received September 1st, 1992) 205

Liquid chromatography–electrospray ionization-mass spectrometry of cyanobacterial toxins
by G. K. Poon and L. J. Griggs (Sutton, UK) and C. Edwards, K. A. Beattie and G. A. Codd (Dundee, UK) (Received September 16th, 1992) . . . 215

Determination of paraquat and diquat by liquid chromatography–thermospray mass spectrometry
by M. Yoshida, T. Watabiki, T. Tokiyasu and N. Ishida (Moriguchi, Japan) (Received August 12th, 1992) . . . 235

Temperature dependence of the anion-exchange chromatographic behaviour of cyclic phosphate oligomers and their anion-exchange separation
by G. Kura and E. Kitamura (Fukuoka, Japan) and Y. Baba (Kobe, Japan) (Received September 22nd, 1992) . . . 241

Gas Chromatography

Quantitative structure–retention relationship approach to prediction of linear solvation energy relationship coefficients. I. H-Bond acceptor capability of gas chromatography stationary phases in McReynolds data set
by D. J. Ballantine, Jr. (DeKalb, IL, USA) (Received September 15th, 1992) . . . 247

Preconcentration of volatile components of foods: optimization of the steam distillation–solvent extraction at normal pressure
by G. P. Blanch, J. Tabera, M. Herraiz and G. Reglero (Madrid, Spain) (Received August 4th, 1992) . . . 261

Separation and identification of free amino acid enantiomers in peat by capillary gas chromatography
by A. V. Kunnas and T.-P. Jauhianen (Joensuu, Finland) (Received September 4th, 1992) . . . 269

Supercritical Fluid Chromatography

Packed-column supercritical fluid chromatography of omeprazole and related compounds. Selection of column support with triethylamine- and methanol-modified carbon dioxide as the mobile phase
by O. Gyllenhaal and J. Vessman (Mölndal, Sweden) (Received September 7th, 1992) . . . 275

Electrophoresis

Simulated quantitative and qualitative isotachophoretic indices of 73 amino acids and peptides in the pH range 6.4–10
by T. Hirokawa and Y. Kiso (Higashi-Hiroshima, Japan) and B. Gaš, I. Zusková and J. Vacík (Prague, Czechoslovakia) (Received July 24th, 1992) . . . 283

Separation of polymyxins by micellar electrokinetic capillary chromatography
by H. K. Kristensen and S. H. Hansen (Copenhagen, Denmark) (Received September 15th, 1992) . . . 309

(Continued overleaf)

DISCUSSION

High-performance liquid chromatographic determination of urinary 2,5-hexanedione
by A. Sturaro, G. Parvoli and L. Doretti (Padova, Italy) (Received August 31st, 1992) 316

BOOK REVIEWS

Ion chromatography —principles and applications (Journal of Chromatography Library, Vol. 46) (by P. R. Haddad and P. E. Jackson) reviewed by P. Hajos (Lausanne, Switzerland) 319

Laboratory experiments in liquid chromatography (by W. V. Willis), reviewed by H. Irth (Leiden, Netherlands) 321

Advances in applied lipid research (edited by F. B. Padley), reviewed by W. W. Christie (Ayr, UK) 322

AUTHOR INDEX 323

Journal of Chromatography, 628 (1993) 171–189
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CHROM. 24 635

Simultaneous optimization of pH and organic modifier content of the mobile phase for the separation of chlorophenols using a Doehlert design

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ABSTRACT

By optimizing the pH and volume fraction of organic modifier, the complete resolution of an isocratic separation of a mixture of phenol and thirteen of the nineteen chlorophenol isomers and of a mixture of the three tetrachlorophenols and pentachlorophenol was achieved. The effectiveness of the Doehlert design in optimizing both experimental parameters was investigated. A quadratic model was applied. For mixtures of a small number of compounds, a retention boundary map is proposed to determine limits of the concentration of organic modifier so as to elute compounds within a reasonable analysis time. The resulting three-dimensional graph of the minimum resolution as a function of the experimental parameters allows the direct visual evaluation of the ruggedness of the optimum conditions that are attainable in the selected parameter space and with a given stationary phase.

INTRODUCTION

Chlorophenols are used extensively as fungicides, herbicides, algicides, insecticides, ovicides, pharmaceuticals, dyes, as preservatives for wood, glue, paint, vegetable fibres and leather and as intermediates in chemical syntheses [1]. About 200 000 tons of chlorophenols are manufactured annually, while some additional chlorophenols are formed by the reaction of chlorinated water supplies with phenol in the environment or through the degradation of chlorinated pesticides [2].

Procedures for the separation and determination of the chlorophenols, as such or after derivatization, include gas chromatography–mass spectrometry, thin-layer chromatography, gas–liquid chromatography and high-performance liquid chromatography (HPLC). A great number of HPLC methods have been reported, which for the greater part do not allow quantification as the peaks are incompletely resolved. Most often the pH of the mobile phase and/or concentration(s) of organic modifier are applied as the experimental parameters. Only a few representative examples are mentioned to give an idea of what has already been achieved. Nair *et*

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al. [3] and Sha and Stanley [4] used isocratic elution for the separation of thirteen and nineteen isomers, respectively, of phenol. Paleologou *et al.* [2] reported an isocratic separation of the members of each category of chlorophenols (*e.g.*, mono-, di-) and a gradient elution method to separate fifteen of the nineteen chlorophenols on a β-cyclodextrin-bonded column. Ugland *et al.* [5] resolved most congeners in a mixture of phenol and eighteen chlorophenols using a linear gradient with a mobile phase of pH 4.

These separations were obtained by trial-and-error, which involves several well known disadvantages. The most evident disadvantage is the often long development time that is required to select experimental conditions that are not necessarily the optimum ones. The first application of a systematic approach to the optimization of the mobile phase composition for the separation of phenols was described by Ong *et al.* [6]. He applied a simplex method combined with overlapping resolution mapping and successfully improved the separation of eleven substituted phenols with a quaternary mobile phase (methanol–acetonitrile–tetrahydrofuran–water).

In this paper the systematic optimization of the isocratic separation of a mixture of phenol and fourteen of the nineteen chlorophenol isomers and of a mixture of three tetrachlorophenols and pentachlorophenol by applying a Doehlert design is described. The former mixture contains phenol and mono-, di- and trichlorophenols, with the exception of 3,4,5-trichlorophenol. As far as we know, the complete resolution of a reversed-phase isocratic separation of such a complex mixture of chlorophenols has not been reported previously. Lores *et al.* [7] resolved a mixture of ten mono- and dichlorophenols by applying a ternary mobile phase in the isocratic mode. We achieved the complete resolution of an isocratic separation of a mixture of phenol and thirteen of the nineteen chlorophenol isomers.

EXPERIMENTAL APPROACH

It was decided to optimize the pH of the mobile phase and the volume fraction of organic modifier. Preliminary experiments indicated that volume fractions of methanol higher than 75% were required to elute tetra- and pentachlorophenols within a reasonable analysis time. Such a volume fraction of methanol is already very close to the solubility limit of the buffer, which in practice imposes the highest possible limit of organic modifier content, and a few percent more of methanol would certainly cause precipitation problems. For that reason, it was decided to apply a modifier with a higher solvent strength, namely acetonitrile. With volume fractions of modifier of at most 61% this allows the same solvent strength to be obtained as with 75% of methanol without problems of buffer solubility.

The chlorophenols are more acidic than phenol and the acidity of the phenol function increases with increasing chlorination of the benzene ring. Therefore, the pH of the mobile phase is a powerful parameter for optimizing selectivity [2,3,8]. The concentration of modifier also has an effect on the selectivity, although a smaller effect than pH. Both parameters should be optimized simultaneously in view of the dependence of both pH [9] and pK_a values [10] on the modifier content of the mobile phase. Because of this dependence, the optimum pH depends on the modifier concentrations (and *vice versa*).

In interpretive optimization methods, a model is needed to describe the retention as a function of the parameters to be optimized. The relationship between retention ($\log k'$) and volume fraction of organic modifier has been shown to be linear over a limited range of capacity factors (*e.g.*, $1 < k' < 10$) and can be described by the following equation [11,12]:

$$\ln k' = \ln k'_0 - S\,\varphi \qquad (1)$$

where k'_0 is the extrapolated retention of the solute in the aqueous phase, S is the slope and φ is the volume fraction of organic modifier. Over larger ranges of mobile phase composition, the following quadratic equation, proposed by Schoenmakers [13], provides a better approximation:

$$\log k' = A\,\varphi^2 + B\,\varphi + \log k'_0 \qquad (2)$$

The relationship between retention and pH is sigmoid, as has already been shown for the chlorophenols in several studies [14,15]. Two very different approaches to pH optimization have been described. That proposed by Dolan *et al.* [16] uses linear models in a restricted pH range whereas the other [17] uses non-linear models (in both the statistical and chemical sense). Both approaches are very interesting and allow useful results, but may have some disadvantages. The former has the advantage of being very simple, but works in a restricted range. Often this will be sufficient or even necessary, but occasionally it would be preferable to be able to work in a larger range. This approach was successfully applied in the systematic optimization of a mixture of acids with, however, a pH range of 1 [16] or 2 pH units [17]. Even in the latter range one risks considerable deviations from linearity. Consider, for instance, Fig. 1. In regions A and C a linear relationship perhaps may be assumed. When selecting a larger region of pH , such as A + B (see further), the model of Horvath *et al.* [18] was shown to be appropriate in the optimization of pH in a reversed-phase liquid chromatographic separation of weak organic acids by a window diagram method [19]. This model has also been applied in combination with eqn. 1 [20] and computer simulation software (Drylab I/mp) was described for predicting HPLC separation as a function of both pH and solvent strength [21]. However, to obtain reliable predictions it is recommended that the pH region is restricted.

The second approach has the advantages and disadvantages of requiring a physical model with nine coefficients. Such non-linear models were recently proposed by Schoenmakers and co-workers [22,23] and require a considerable amount of experimental data. Moreover, a non-linear physical model is not easy to construct and is limited to the particular situation(s) for which it was constructed. We wondered whether a mid-way approach between the two would be useful.

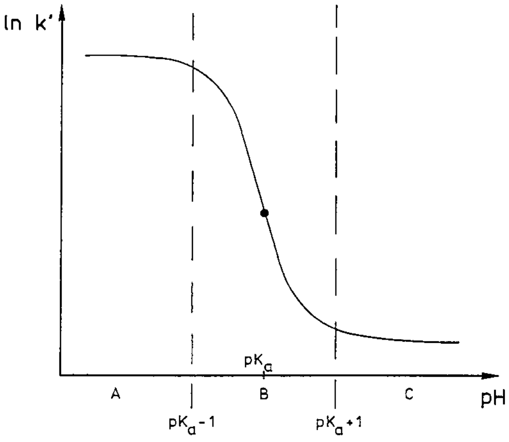

Fig. 1. Evolution of retention (ln *k'*) as a function of pH for an acidic compound.

In experimental design, it is usual to apply quadratic models to optimization. This does not necessarily mean that one assumes the actual relationship to be quadratic, but rather that it is a relatively smooth curved (hyper)plane, which can be approached by a quadratic plane. In the present instance, it is known that the model is not quadratic, but one can hope that in many instances the quadratic curve approaches reality sufficiently closely to allow good prediction. It is hoped that by making the equation quadratic instead of linear, a larger pH range would be feasible. The utility of the following model for the simultaneous optimization of the two experimental parameters will be established:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x \quad (3)$$

where x_1 and x_2 are the experimental parameters.

It is expected that over moderately broad pH ranges, such as regions A+B or B+C in Fig. 1, eqn. [3] may be an improvement over linear approximations when only a limited number of experiments are performed. The strategy we proposed to investigate can be considered as an extension of that described by Dolan *et al.* [16]. Eqn. 3 is fitted by the Doehlert design, which was introduced into the optimization of HPLC methods by Hu and Massart [24]. They showed this design to have a higher efficiency (expressed by number of coefficients/number of experiments to be carried out) than other designs. A Doehlert design with two factors forms a centred hexagon (Fig. 2) where each point represents an experiment. Seven experiments are to be carried out for a model with two factors.

A feature of the Doehlert design is that the number of levels for each experimental parameter is not the same. A Doehlert design with two parameters uses three levels for one parameter and five levels for the other. One should use the design so that the parameter with the most complex relationship is modelled with the largest number of levels [24]. Therefore, the highest number of levels (= 5) is used to model the pH. Once the experimental parameters

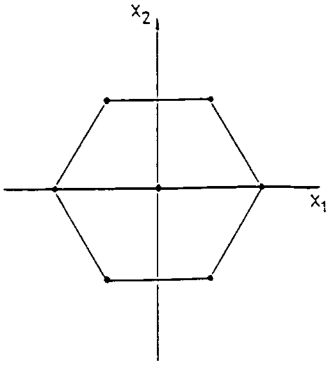

Fig. 2. The Doehlert design.

have been selected, the parameter space has to be defined by fixing boundary values. For the volume fraction of acetonitrile, the highest concentration should be selected so that the peak of the first eluted compound is still separated from the solvent peak. The lower limit should be selected so that the analysis time will not exceed a certain time.

Different approaches have been proposed in the literature, such as selected isocratic experiments or, most often, gradient runs. Berridge [25] carried out an initial gradient separation in order to estimate the likely range of solvent strength required in the simplex optimization of a reversed-phase separation. A similar approach was followed by De Smet *et al.* [26] who combined an initial gradient separation with a small number of isocratic experiments in a normal-phase separation of sulphonamides. Preliminary isocratic separations combined with low-wavelength detection termed "sequential isocratic step" (SIS) chromatography have been applied successfully in ion-pair separations [27].

A retention boundary map is proposed to establish in a reasonable time an optimum region of concentrations of organic modifier. Conventionally with binary mobile phases the organic modifier content is selected to give capacity factors in the range $1 < k' < 10$ for all compounds, as only a very small gain in resolution is obtained with k' values > 10. By applying eqn. 1 and capacity factors measured at two different solvent strengths, percentages of acetonitrile that lead to capacity factors between 1 and 10 are calculated. In this way, a region of acceptable concentrations of organic modifier is defined for each compound at the upper and lower limit of pH. Both regions are connected to constrain an area in the plane of pH *versus* organic modifier content of experimental conditions that should lead to the desired retention times for one compound. This is, of course, an approximation but it allows an area to be established with sufficient accuracy and with a minimum of experimental effort. By overlapping of the areas of each compound one can select one or more areas where for all compounds of interest or at least for most of them the postulated requirements are fulfilled (Fig. 3) .

One should not confuse the retention boundary map with overlapping mapping techniques as described by Glajch and Kirkland [28]. The aim of constructing a retention boundary map is to select a region of concentrations of organic modifier so that the capacity factors of all compounds are within a certain range. When optimizing selectivity it is recommended to select pH limits so that for all compounds both ionized and non-ionized forms are chromatographed.

As it is known that the lifetime of silica-based stationary phases may be seriously reduced in the presence of aqueous buffer–mobile phase systems with a pH that is outside the range 2–7.5, the upper limit of pH is set at 7 as imposed by the stability of the stationary phase. The lower limit of pH is set at

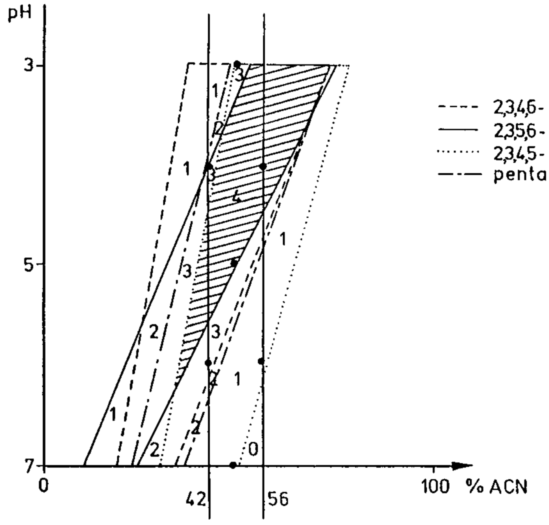

Fig. 3. Retention boundary map of the mixture of tetra- and pentachlorophenols. The numbers refer to the number of compounds for which $1 < k' < 10$. ACN = Acetonitrile.

3 because all compounds remain uncharged at lower pH. This pH region encompasses all pH values that range from pK_a − 1 to pK_a + 1 for the mixture of tetrachlorophenols and pentachlorophenol. The pK_a values of the fifteen chlorophenols range from 5.80 for 2,3,6-trichlorophenol to 9.92 for phenol [14] (see Table V). One can argue that in view of the lowest pK_a value of the sample set it is not reasonable to include a mobile phase pH as low as 3.0. However, it was decided to do so as several published RPLC separations of smaller mixtures of chlorophenols, obtained by trial-and-error, were achieved at pH values of 4.0 and lower [5,29,30]. In the low-pH region, there will be no pH effect. However, it is not necessary that this should be so: if a good separation is found that is not influenced by pH, this will be preferable as it leads to increased robustness.

The effective buffer range for a weak acid or base is approximately from pH = pK_a − 1 to pH = pK_a + 1 . This implies that a buffer system should be selected with successive pK_a values that differ by about 2 pH units to ensure adequate buffering ability at each pH within the selected region. When applying only one type of buffer, the buffer capacity will not be sufficient over one or more pH ranges in the selected pH region [31]. For phosphoric acid, for instance, the good buffering ranges between pH 3 and 7 are pH 3–3.2 and 6.2–7. For this reason, stoichiometric mixtures of citrate and phosphate buffers (1:1) were used.

EXPERIMENTAL CONDITIONS

Chromatographic equipment and parameters

All measurements were carried out with a Varian Model 5000 liquid chromatograph equipped with a Rheodyne injection valve (5-μl sample loop) and coupled with a Shimadzu SPD-2A UV detector. Detection was performed at 260 nm. The attenuation was set at 0.04 a.u.f.s. The chromatograms were recorded with a Spectra-Physics SP 4290 integrator. The column used was LiChrospher RP 18 (250 x 4 mm I.D.) from Merck with a particle size of 5 μm because octadecyl-modified stationary phases were found to be superior for the analysis of chlorinated phenols in reversed-phase chromatography [1,4]. The flow-rate was maintained at 1 ml/min. During chromatography, the column temperature was maintained at 30°C with a Prolabo Sup-Rs stabitherm column thermostat. pH measurements of buffer solutions were carried out with a Corning Model 240 pH meter.

Standards and reagents

All chlorophenols were of reference grade and were obtained from Aldrich. Standard solutions in the mobile phase with concentrations of ca. 300 mg/l were daily prepared by dilution of stock solutions in acetonitrile. Acetonitrile of HPLC gradient quality was purchased from Distrilab. The water used was purified in a Milli-Q system (Millipore). Sodium dihydrogenphosphate, phosphoric acid, citric acid, trisodium citrate and sodium hydroxide of analytical-reagent grade were obtained from Prolabo.

Citrate and phosphate buffers of pH 3, 4, 5, 6 and 7 and ionic strength 0.05 mol/l were prepared with purified water. At each pH value, the electrode was calibrated with standard solutions of pH 4 and 7. After controlling the pH, the buffers were filtered through a 0.45-μm Millipore filter under vacuum.

Procedures

Retention times were obtained from the peak maximum by the integrator. Peak widths at half-height were measured manually. The pH of the mobile phase was assumed to be the pH of the aqueous fraction. To avoid increasing the number of experiments too much, several standards with large enough differences in retention were chromatographed in one run.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE SEVEN EXPERIMENTS OF THE TWO DESIGNS

Experiment No.	pH	Acetonitrile (%)	
		15 phenols	4 phenols
1	7	38	49
2	5	38	49
3	3	38	49
4	6	45	56
5	4	45	56
6	6	31	42
7	4	31	42

The experimental conditions for the seven experiments of the two designs are given in Table I. The central experiment was carried out twice, on different days, to obtain an idea of the experimental error (see further). The seven experiments were performed in random order and, to avoid a memory effect from previous eluents, the column each time was equilibrated for 2 h with the next mobile phase at a flow-rate of 1 ml/ min.

Description of the software

A program for a Doehlert matrix design of experiments was developed by Hu and Massart [24]. As their program is limited to the use of at most ten compounds, a new version in Excell and Quickbasic was written. The program calculates the coefficients in eqn. 3 for y = retention time, t_R, and y = peak width at half-height, $w_{1/2}$, for each compound so that it can predict the values of both variables for a large set of experimental conditions (= different mobile phases). Subsequently the resolution of the worst separated peak pair, $R_{s_{\min}}$, is calculated as a chromatographic response. All values of $R_{s_{\min}}$, t_R and $w_{1/2}$ are saved in spreadsheets in Excell which allows the user to construct a three-dimensional graph of the optimization criterion or of each of the two variables as a function of the experimental parameters.

RESULTS AND DISCUSSION

Separation of a mixture of tetra- and pentachlorophenols

For the Doehlert design with tetra- and pentachlorophenols, the upper and lower limit are fixed at 56 and 42% of acetonitrile, respectively, using a retention boundary map (Fig. 3). The area where $1 < k' < 10$ for all compounds is large at pH 3 but becomes smaller with increasing pH and at pH 7 it is no longer possible to obtain $1 < k' < 10$ for 2,3,4,5-tetrachlorophenol and pentachlorophenol at the same time. Therefore, this area is combined with other areas each with as large a number of compounds as possible that fulfil the postulated requirements. As a result of combining areas where not all compounds have $1 < k' < 10$, the capacity factors of all solutes except 2,3,4,5-tetrachlorophenol will be smaller than 1 in the experiment at pH 7. In only two of the seven chromatograms of the experiments of the design (Table I) are four peaks observed. These chromatograms are obtained under experimental conditions 2 and 6.

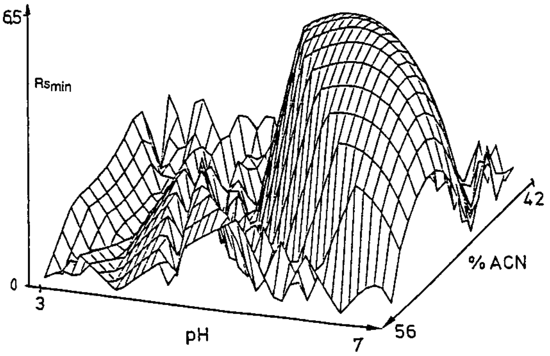

Fig. 4. Three-dimensional graph of $R_{s_{\min}}$ as a function of pH and concentration of acetonitrile for the mixture of tetra- and pentachlorophenols.

The resulting three-dimensional graph of $R_{s_{\min}}$ (Fig. 4) as a function of pH and concentration of acetonitrile and the graph in two dimensions representing areas of equal $R_{s_{\min}}$ (Fig. 5) show a wide optimum which allows the development of a rugged method. Both graphical presentations allow an evaluation of the ruggedness of the optimum. A maximum value of $R_{s_{\min}}$ of 6.63 is predicted at pH 6 and with a mobile phase that contains 49% of acetonitrile.

Two experiments were carried out with a mobile phase containing 49% of acetonitrile at pH 5.5 and 6.0. The predicted minimum resolution for the for-

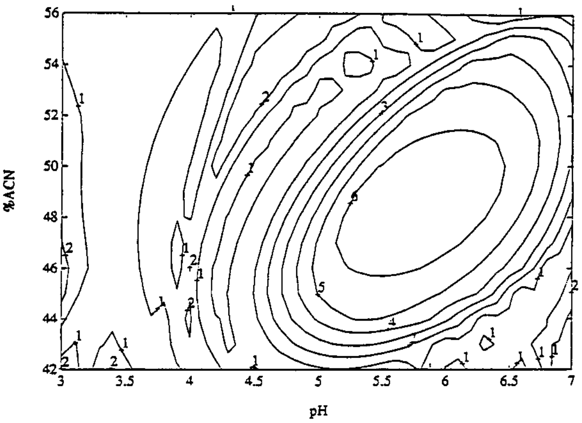

Fig. 5. Plot representing the interaction between pH and volume percentage of acetonitrile. The lines delimit areas of equal $R_{s_{\min}}$.

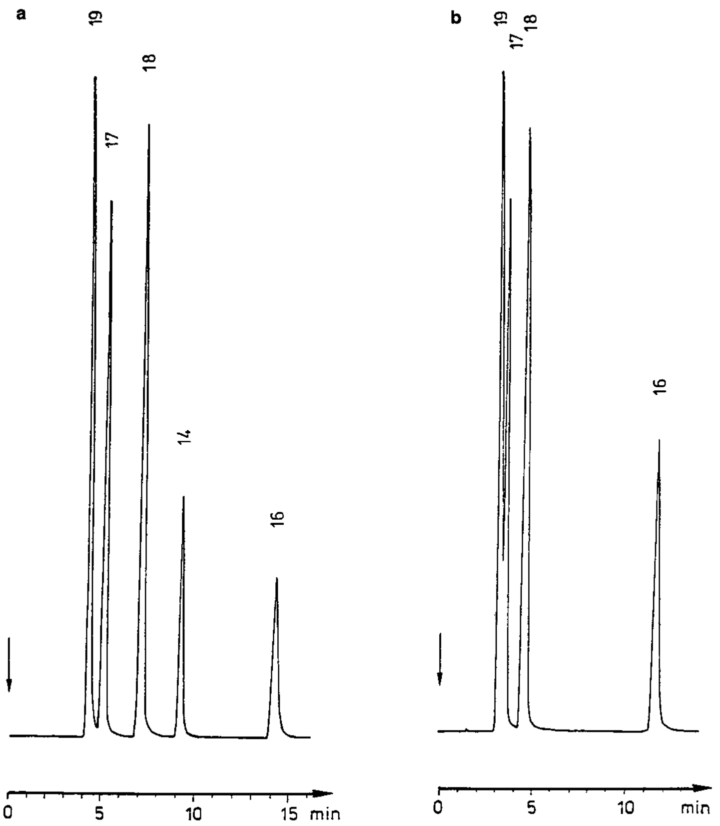

Fig. 6. Chromatogram of the mixture of tetra- and pentachlorophenols obtained with a volume fraction of acetonitrile of 49% at pH (a) 5.5 and (b) 6.0. Numbers of the solutes refer to Table II.

mer experimental conditions is 6.35. Baseline resolution was obtained by applying the first experimental conditions (Fig. 6a). Compared with reversed-phase isocratic separations of the same mixture that have been published [2,5], the resolution in Fig. 6a is superior, although the analysis time is about 4 min longer. The second experiment led to partial overlap of penta- and 2,3,5,6-tetrachlorophenol

TABLE II

COMPARISON BETWEEN PREDICTED RETENTION TIMES AND EXPERIMENTAL VALUES FOR THE MIXTURE OF TETRA- AND PENTACHLOROPHENOLS

Compound No.	Name[a]	pK_a	pH 5.5			pH 6		
			t_R (pred.)	t_R (exp.)	deviation (%)	t_R (pred.)	t_R (exp.)	deviation (%)
16	2,3,4,5-	5.64	13.7	14.3	4.3	11.1	11.6	3.78
18	2,3,4,6-	5.22	8.75	7.29	20	5.99	4.57	31.1
17	2,3,5,6-	5.02	6.62	5.29	25.1	4.17	3.48	19.8
19	Penta-	4.74	4.26	4.42	3.62	1.51	3.12	107

[a] Tetra- and pentachlorophenols.

(Fig. 6b). The pH of the mobile phase in the two experiments differed only by 0.5, which demonstrated that the separation of this, at first sight simple, mixture of four compounds requires the use of an optimization procedure in which pH is a very important parameter. At pH 6 also a complete separation of all compounds was predicted. The deviations of the predicted $R_{s_{\min}}$ must be due to deviations between the predicted values of retention and peak width and the results obtained by experiment. This might indicate a lack of fit of the experiments to the model. To investigate this the retention times of the compounds obtained experimentally are compared with the predicted values in Table II. The precision of predicted values of the peak width was investigated in the separation of the mixture of fifteen phenols. The deviations between predicted values of t_R and experimental results are larger in the experiment at pH 6, which indicates that the accuracy of the predictions is higher in experiments that are closer to the central experiment. For all solutes except 2,3,4,5-tetrachlorophenol the model does not provide accurate predictions. The pK_a values of the former compounds are nearly in the middle of the investigated pH range, so that an almost complete sigmoidal curve of retention *versus* pH is assumed in the selected parameter space. This was confirmed in later experiments performed with a larger set of pH-values for the computer-assisted determination of pK_a values of chlorophenols by means of HPLC [32]. For 2,3,4,5-tetrachlorophenol with a pK_a value of 5.64 there is only one "leg" of this curve that is better fitted by the second-order polynomial than a complete curve. This is illustrated in Fig. 7, which presents predicted and experimental values of t_R *versus* pH for 2,3,4,5-tetrachlorophenol and pentachlorophenol.

When a complete sigmoidal curve of retention *versus* pH is observed in the selected parameter space, other approaches are required. One such approach is to use non-linear regression using a model such as that by Horváth *et al.* [18]. The use of this method was described by us for pK_a determination in an optimization context [32]. The other approach is to transform the data to obtain a response surface that is more amenable to the usual experimental design approaches. We are currently investigating such transforms. Two of them are well known,

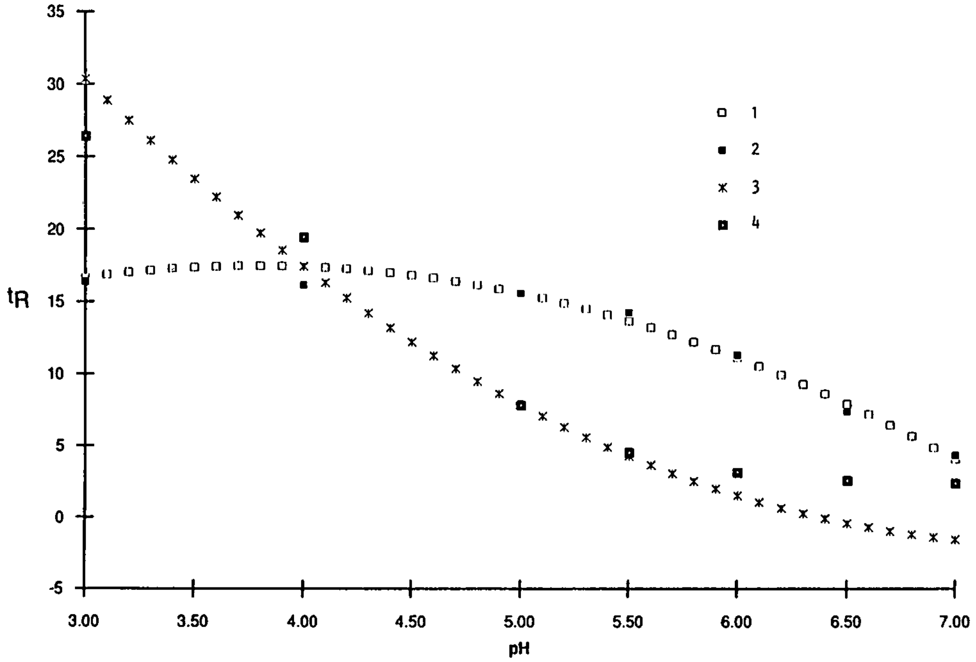

Fig. 7. Predicted and experimental retention times *versus* pH at 49% of acetonitrile. 2,3,4,5-Pentachlorophenol: 1 = predicted; 2 = experimental. Pentachlorophenol: 3 = predicted; 4 = experimental.

namely the probit transform and the logit transform. We chose to study the latter. In the present case it is carried out as follows:

$$z = \log [k'^*/(1 - k'^*)] \qquad (4)$$

where k'^* values are standardized k' values, obtained by transformation to a scale between 0 and 1 by applying the following equation:

$$k'^* = [Y_i^{(+)} - k']/[Y_i^{(+)} - Y_i^{(-)}] \qquad (5)$$

where $Y_i^{(+)}$ and $Y_i^{(-)}$ are the largest and smallest values of measured k' values which are respectively increased and decreased by 1% to avoid losing information of the experiments at the smallest and largest pH by k'^* equaling 0 or 1. A first- or second-order model should allow z to be accurately described as a function of the independent variable (here pH). We have verified this for a few examples, *e.g.*, on the data of Schoenmakers *et al.* [22] and for

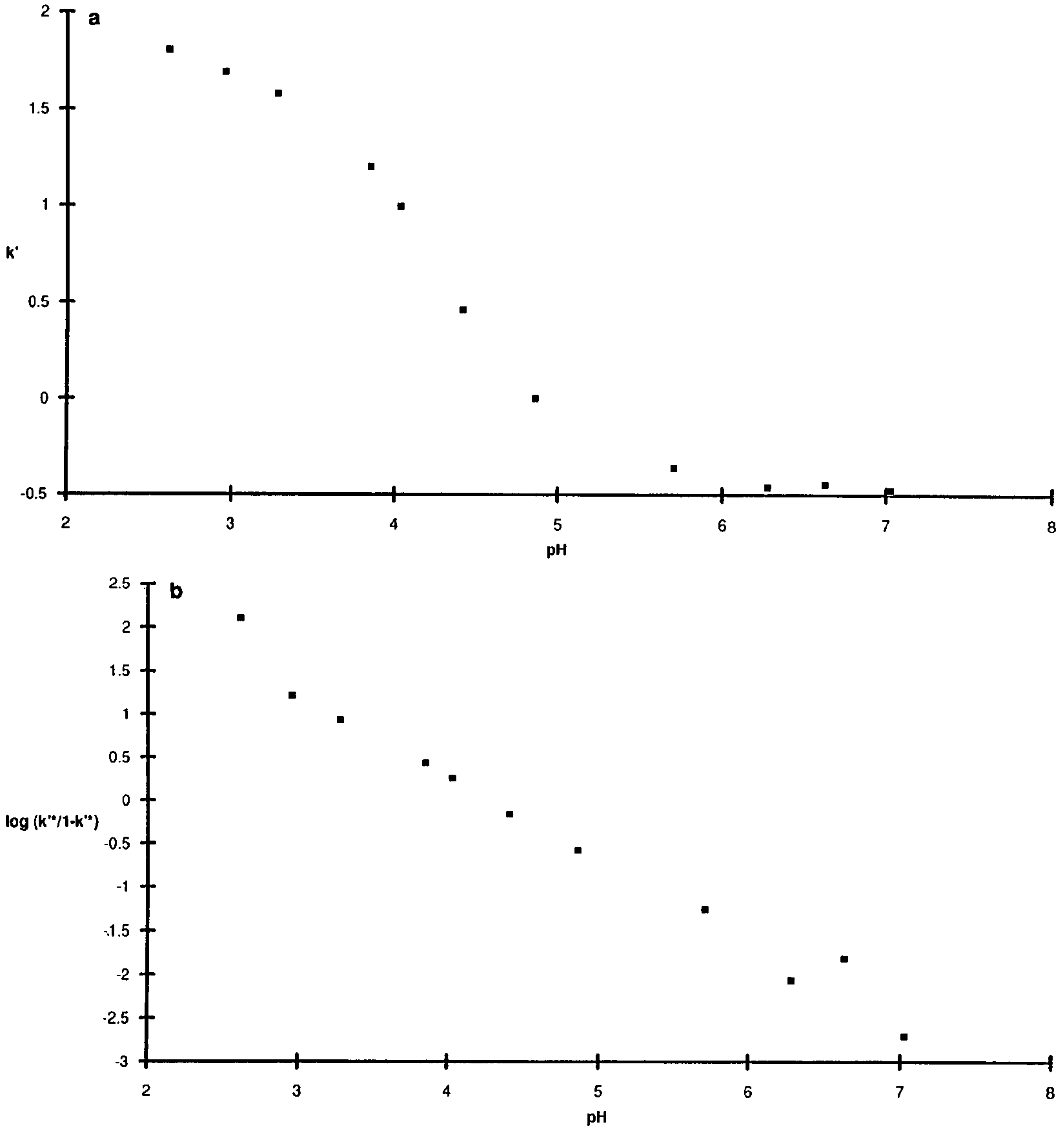

Fig. 8.

(Continued on p. 180)

2,3,4,5-tetrachlorophenol and pentachlorophenol. For these three solutes the curves of k' and z as a function of pH are represented in Fig. 8a–f. Both a first- and a second-order equation were applied to model z and the precision of the predictions was compared with that obtained by modelling k' *versus* pH with a quadratic equation. The precision was significantly better with the logit transform for benzoic acid and pentachlorophenol but not for 2,3,4,5-tetrachlorophenol (Table III, Fig. 9a and b). For the former solutes no difference in precision was obtained on applying a first- or second-order equation to model z *versus* pH (Table III). We are now verifying whether this transformation can be used in general and how to apply it to the bivariate optimization of pH and solvent strength.

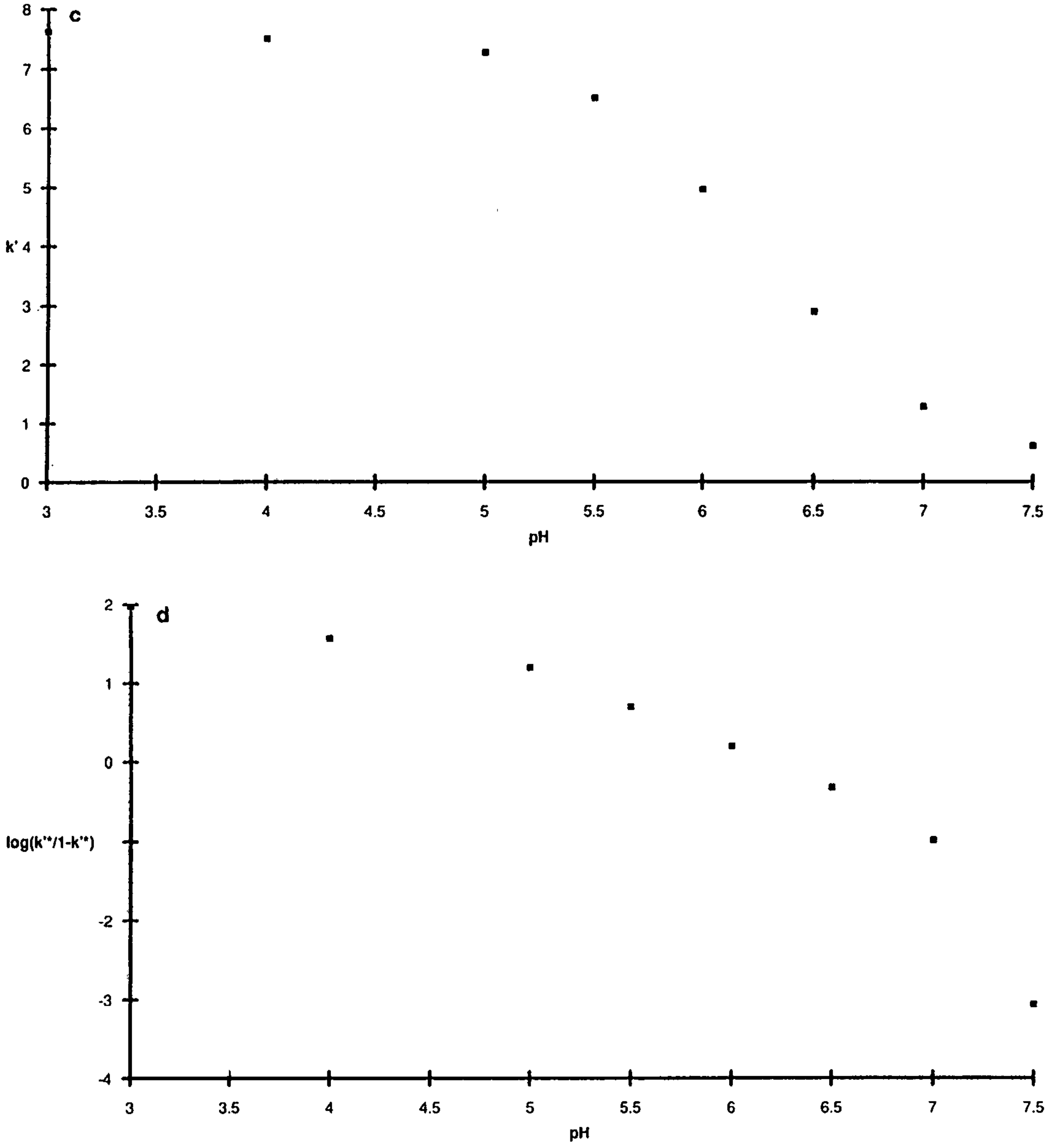

Fig. 8.

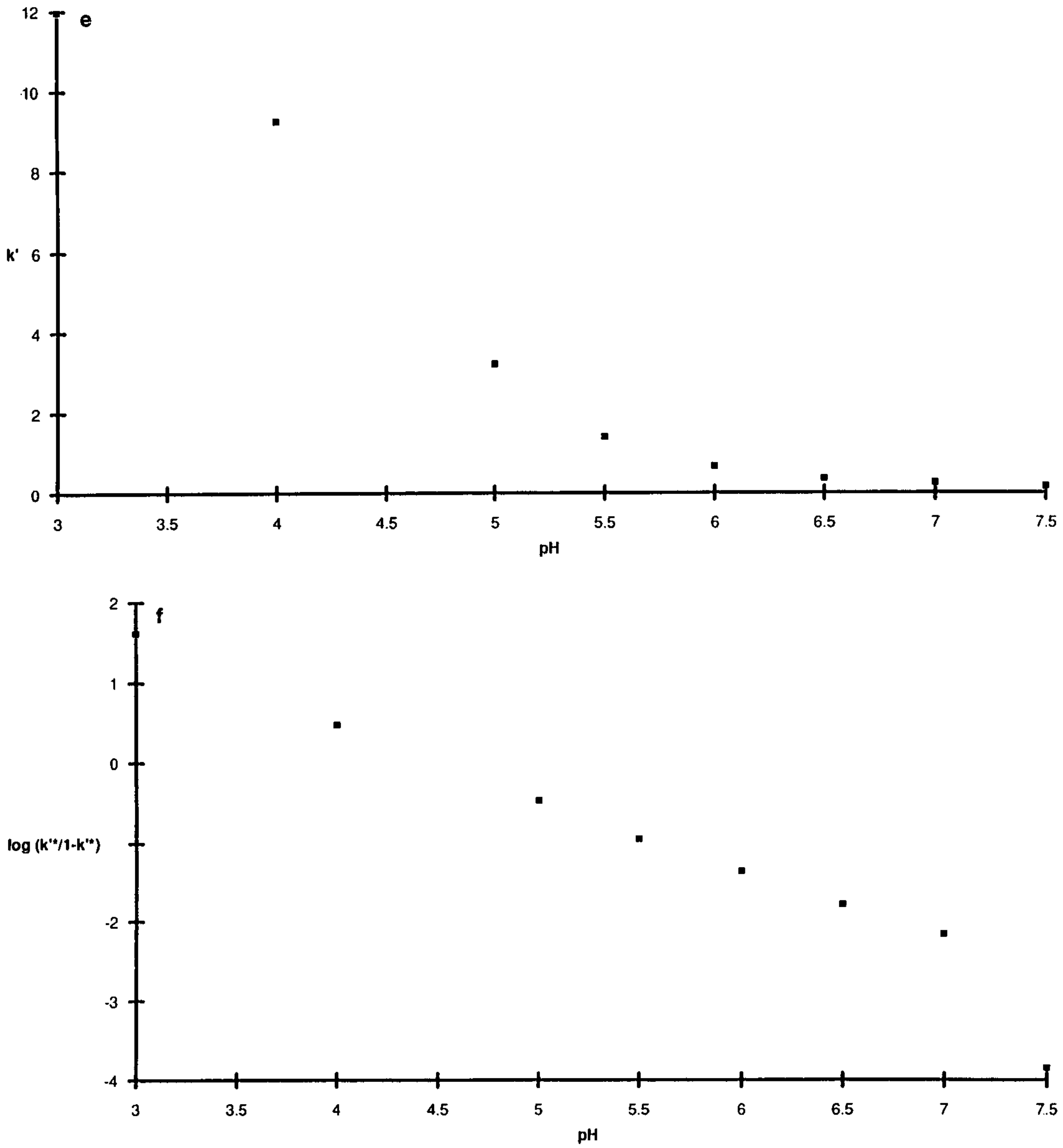

Fig. 8. Evolution of capacity factors and z values as a function of pH for (a, b) benzoic acid, (c,d) 2,3,4,5-tetrachlorophenol and (e,f) pentachlorophenol.

Separation of a mixture of phenol and fourteen chlorophenols

The requirement of $1 < k' < 10$ as imposed by applying eqn. 1 may be fulfilled if the optimization problem concerns a small set of compounds with similar polarities. However, this was not the case for the mixture of tetra- and pentachlorophenols. It is therefore not surprising that this requirement also was not fulfilled for the much more complex mixture of fifteen phenols. By performing two isocratic experiments at two different concentrations of acetonitrile at pH 3 and 7, an upper limit was set at 45% of acetonitrile so that phenol and 2,3,6-trichlorophenol, which have the shortest retention times at

TABLE III

COMPARISON BETWEEN EXPERIMENTAL VALUES OF CAPACITY FACTORS [k'(exp.)] AND VALUES PREDICTED BY FITTING A LINEAR MODEL [k'(a)] AND A SECOND-ORDER MODEL [k'(b)] TO z *VERSUS* pH, AND BY MODELLING THE EXPERIMENTAL DATA WITH A SECOND-ORDER EQUATION [k'(c)]

Compound	k'(exp.)	k'(a)	Deviation (%)	k'(b)	Deviation (%)	k'(c)	Deviation (%)
Pentachlorophenol	12.0	12.0	0.00	11.8	1.67	12.8	6.67
	9.25	9.89	6.86	10.1	9.19	7.44	19.6
	3.21	3.28	2.18	4.20	30.7	3.52	9.78
	1.39	1.27	8.35	1.70	22.6	2.11	51.4
	0.66	0.51	22.6	0.62	6.08	1.04	58.3
	0.36	0.27	25.0	0.28	21.8	0.34	5.14
	0.25	0.20	20.8	0.19	22.5	0.00	101
	0.17	0.18	4.06	0.17	2.06	0.01	94.5
2,3,4,5-Tetrachlorophenol	7.62	7.61	0.20	7.50	1.52	7.63	0.08
	7.51	7.48	0.36	7.52	0.10	7.80	3.80
	7.28	6.51	10.5	7.29	0.09	6.89	5.29
	6.52	5.06	22.4	6.70	2.74	6.04	7.31
	4.96	3.15	36.5	4.88	1.65	4.92	0.71
	2.89	1.71	41.0	2.07	28.5	3.54	22.4
	1.27	1.01	20.5	0.82	35.1	1.88	48.4
	0.61	0.75	22.1	0.63	3.11	−0.04	106
Benzoic acid[a]	1.81	1.78	1.16	1.79	0.78	2.06	14.3
	1.69	1.73	2.07	1.73	2.49	1.67	1.24
	1.58	1.63	3.42	1.63	3.23	1.39	11.8
	1.20	1.20	0.08	1.15	4.33	0.90	25.3
	1.00	1.09	9.45	1.03	3.02	0.82	17.6
	0.46	0.57	23.3	0.49	6.09	0.53	15.9
	−0.36	−0.39	9.10	−0.39	7.78	−0.18	48.7
	−0.46	−0.46	0.43	−0.45	1.98	−0.41	11.6
	−0.45	−0.47	5.39	−0.46	4.04	−0.49	10.6
	−0.48	−0.48	0.00	−0.47	0.63	−0.58	21.9

[a] Experimental data from ref. 22.

pH 3 and 7, respectively, should be eluted with a capacity factor of at least 0.5. The lower limit was relaxed and was set at 31% of acetonitrile, which should correspond to a capacity factor of about 40. This value, however, can only be used as a first approximation because extrapolations have been made outside the region $1 < k' < 10$. Once boundary values of the experimental parameters had been fixed, the seven experiments of the design were performed (Table I).

Only the experimental conditions of experiment 7 led to a chromatogram with 15 peaks but, as can be seen in Fig. 10, the peaks of 2,4- and 2,5-dichlorophenol show considerable overlap while the very large capacity factors cause peak broadening. Compared with the performance obtained in the chromatograms with tetra- and pentachlorophenols, the peaks show much less tailing. This is assumed to be due to reduced stereochemical effects and reduced interaction with residual silanol functions because of a smaller degree of chlorination.

Peak cross-overs occur as a function of pH (Fig. 11) but less when varying the volume fraction of acetonitrile at constant pH (Figs. 12 and 13). Most curves in Figs. 12 and 13 are convergent, as is expected when varying only the organic modifier content. The retention of compounds such as phenol and 2-chlorophenol as a function of pH remains almost constant as they are very weak acids, while the largest decreases in retention are observed for more acidic compounds, *e.g.*, trichlorophenols.

Three-dimensional graphs of $R_{s_{\min}}$ as a function

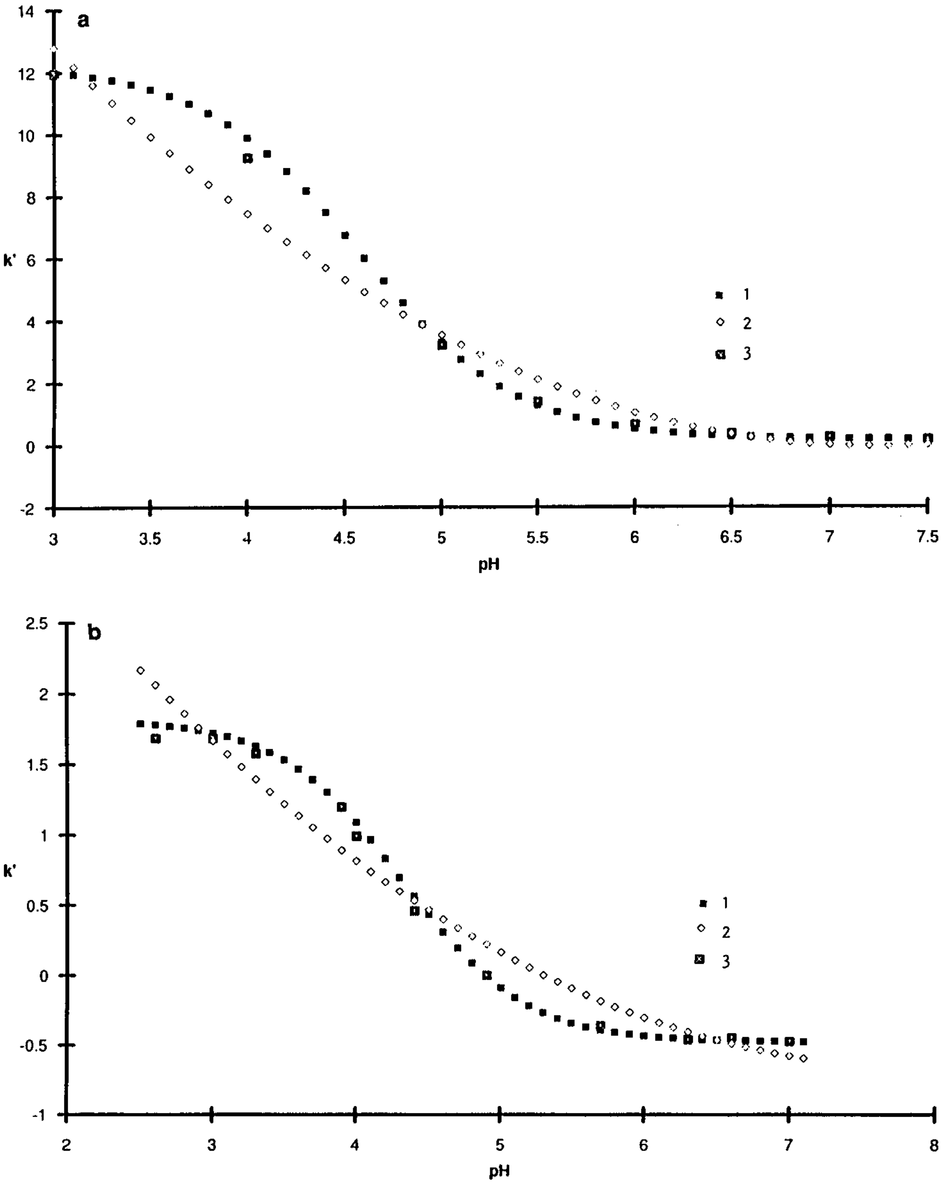

Fig. 9. Evolution of predicted capacity factors as a function of pH. 1 = capacity factors predicted by applying a linear model to z as a function of pH; 2 = capacity factors obtained by fitting a second-order model to the experimental data, represented by symbols 3. (a) Pentachlorophenol; (b) benzoic acid.

of pH and concentration of acetonitrile are represented in Fig. 14. The highest $R_{s_{\min}}$ value of 0.98 is predicted at pH 3.8 and 36% acetonitrile. Considering in more detail the spreadsheet of all $R_{s_{\min}}$ values (part of which is represented in Table IV) and Fig. 13, it can be seen that this optimum is not robust but should be very sensitive to small variations in mobile phase composition; when the volume fraction of acetonitrile is varied by only 1% the value of $R_{s_{\min}}$ is reduced to 0.4. An HPLC method under these conditions certainly will not be rugged.

An experiment was carried out at pH 3.9 and

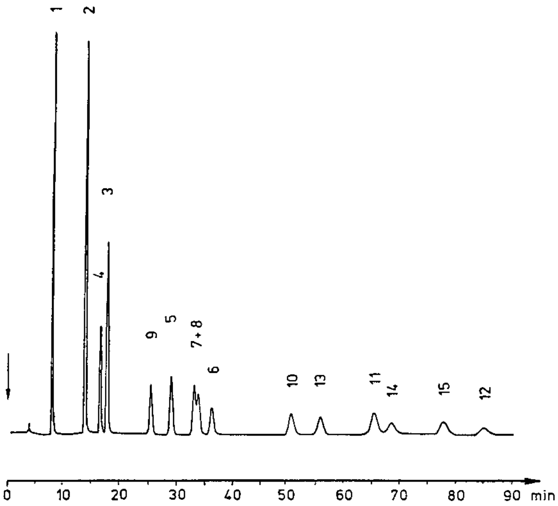

Fig. 10. Chromatogram of the mixture of fifteen chlorophenols , obtained in experiment 7 of the Doehlert design. For experimental conditions, see Table I.

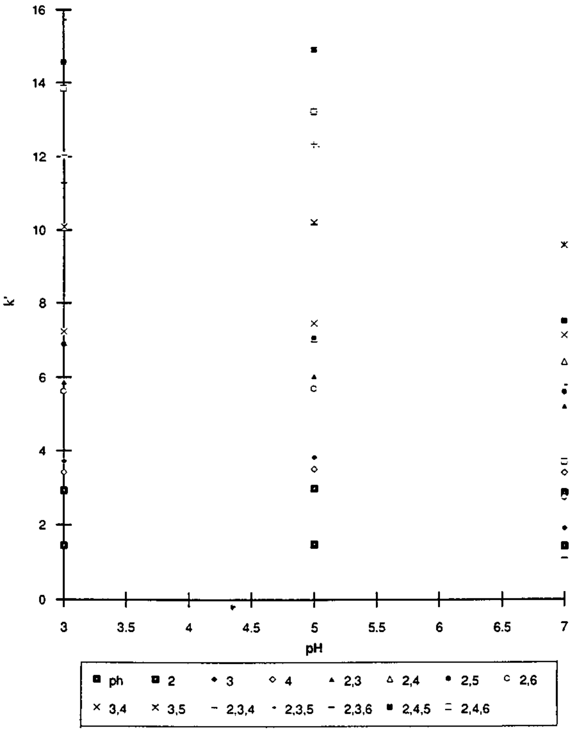

Fig. 11. Evolution of capacity factors as a function of pH for the mixture of fifteen chlorophenols.

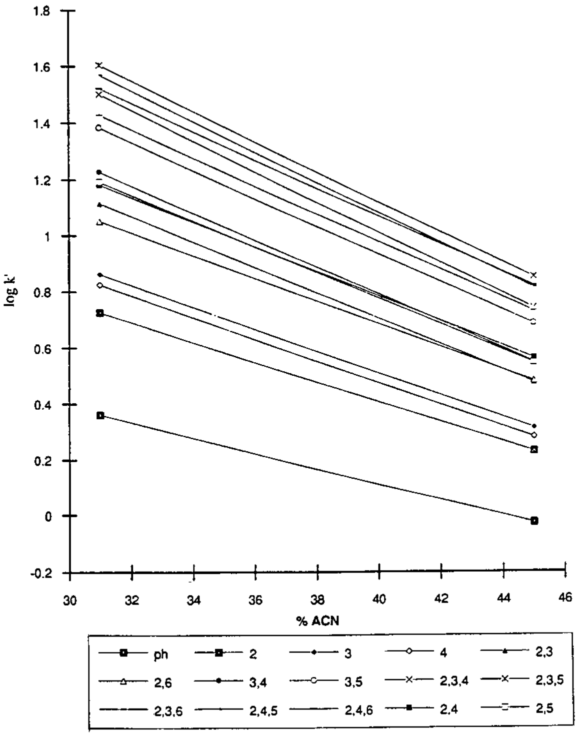

Fig. 12. Evolution of retention (log k') as a function of organic modifier concentration at pH 4.

with a mobile phase containing 36% of acetonitrile. These experimental conditions should lead to almost the same $R_{s_{\min}}$ as predicted at pH 3.8 but are slightly more robust (Table IV). In the resulting chromatogram (Fig. 15), fourteen compounds are well separated in an analysis time of 44 min. The peaks of 2,4- and 2,5-dichlorophenol completely overlap, which was not predicted. The retention times of the compounds obtained experimentally are compared with the predicted values in Table V. The predicted retention times agree with the actual retention times to within 5% for all but two compounds, *i.e.*, 2,4,6- and 2,3,6-trichlorophenol. For each compound the means of the percentage deviations between predicted values of t_R and $w_{1/2}$ and the experimental results for the seven experiments are given in Table V. Compared with the precision of predictions of retention times, a poorer precision is obtained when predicting peak widths. This may be explained by the larger inaccuracy of the measurements of the peak width, which is also included

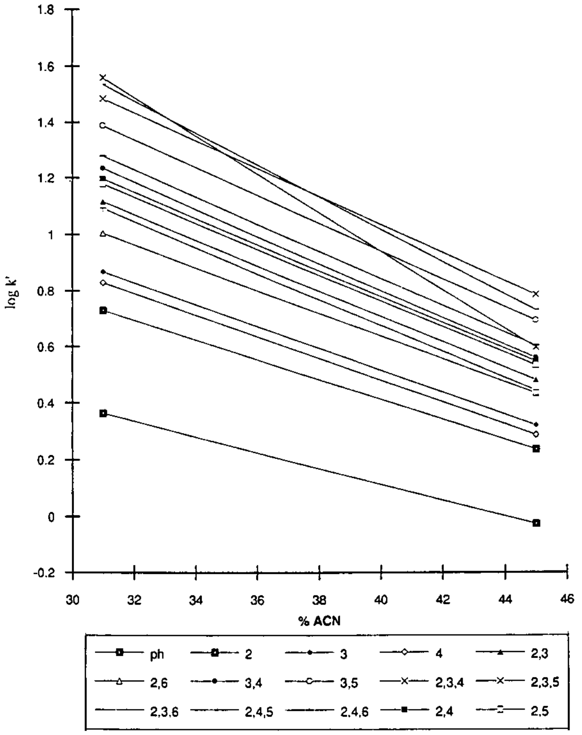

Fig. 13. Evolution of retention (log k') as a function of organic modifier concentration at pH 6.

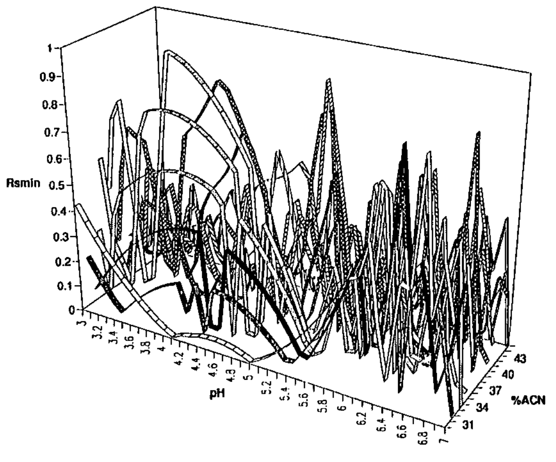

Fig. 14. Three-dimensional graphs of $R_{s_{\min}}$ as a function of pH and concentration of acetonitrile for the mixture of 15 chlorophenols.

in the mean percentage deviations in Table V, besides, of course, a lack of fit of the model. The quality of fit of the model depends mainly on the pK_a values of the compounds, but also on the order of elution (magnitude of retention times). In fact, both parameters are interdependent. The model is least accurate for 2,3,6- and 2,4,6-trichlorophenol. These compounds have the smallest pK_a values of the mixture and an almost complete sigmoidal curve of retention *versus* pH was observed in later experiments, as was the case for the tetra- and pentachlorophenols. Consequently, changes in retention times as a function of pH are pronounced for these trichlorophenols. This is illustrated in Fig. 16a and Fig. 16b, which represent the surface of retention times as a function of pH and concentration of acetonitrile for phenol (pK_a = 9.92) and 2,4,6-trichlorophenol (pK_a = 5.99), respectively. The pK_a value

TABLE IV

$R_{s_{\min}}$ VALUES FOR THE MIXTURE OF FIFTEEN CHLOROPHENOLS AS A FUNCTION OF pH AND VOLUME FRACTION OF ACETONITRILE

pH	Acetonitrile (%)									
	3.5	3.6	3.7	3.8	3.9	4	4.1	4.2	4.3	4.4
34	0.5072	0.5325	0.5526	0.5675	0.5773	0.5818	0.5811	0.5751	0.5638	0.5471
35	0.6683	0.7632	0.7748	0.7806	0.7805	0.7747	0.7629	0.7453	0.7217	0.6920
36	0.0840	0.3804	0.6826	0.9809	0.9681	0.9488	0.9229	0.8904	0.8512	0.8052
37	0.4260	0.1551	0.1213	0.4038	0.6708	0.7124	0.7637	0.8248	0.8958	0.8682
38	0.1547	0.1774	0.2108	0.0838	0.1556	0.3760	0.4529	0.4966	0.5451	0.6170
39	0.2581	0.2111	0.1115	0.0206	0.0026	0.0116	0.0878	0.0717	0.0304	0.0361
40	0.0245	0.1993	0.3410	0.3251	0.2081	0.0773	0.0256	0.1360	0.3059	0.4782

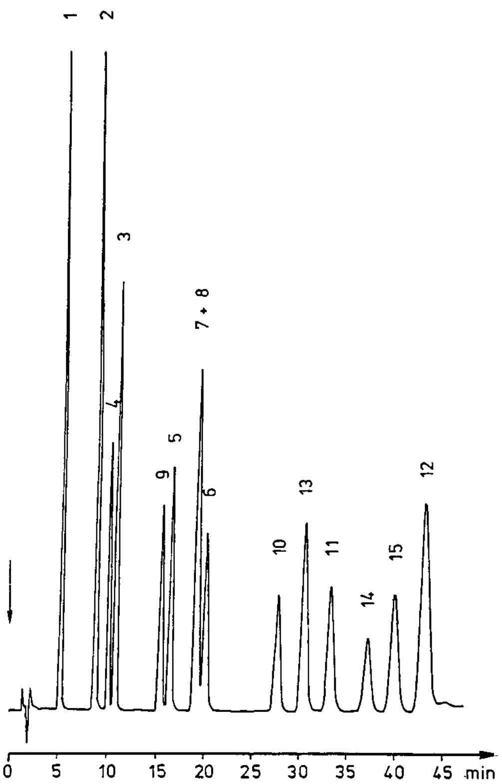

Fig. 15. Chromatogram of the mixture of fifteen chlorophenols obtained under the optimum experimental conditions: pH = 3.9; concentration of acetonitrile = 36%. For identification of the solutes, see Table V.

of phenol is more than two pH units greater than the upper limit of pH and small fluctuations of pH hardly change the retention times.

The experimental error was estimated by repeating the central experiment. Variations of retention times between different days were determined to be of the order of 0.7%, so that results seem to be fairly reproducible.

For the two overlapping compounds, 2,4- and 2,5-dichlorophenol, the deviations between predicted retention times and experimental results are less than 2.5%. Therefore, the difference between observed and predicted $R_{s_{\min}}$ values is probably due to the inaccuracy of the measurements of the peak width and also to small fluctuations in the experimental conditions, especially in the organic modifier content of the mobile phase (see Table V). The latter cause is illustrated in Figs. 12 and 13. At constant organic modifier concentration, differences in the retentions of 2,4- and 2,5-dichlorophenol are very small at pH 4 and 6. However, at pH 4 this difference depends on the concentration of acetonitrile.

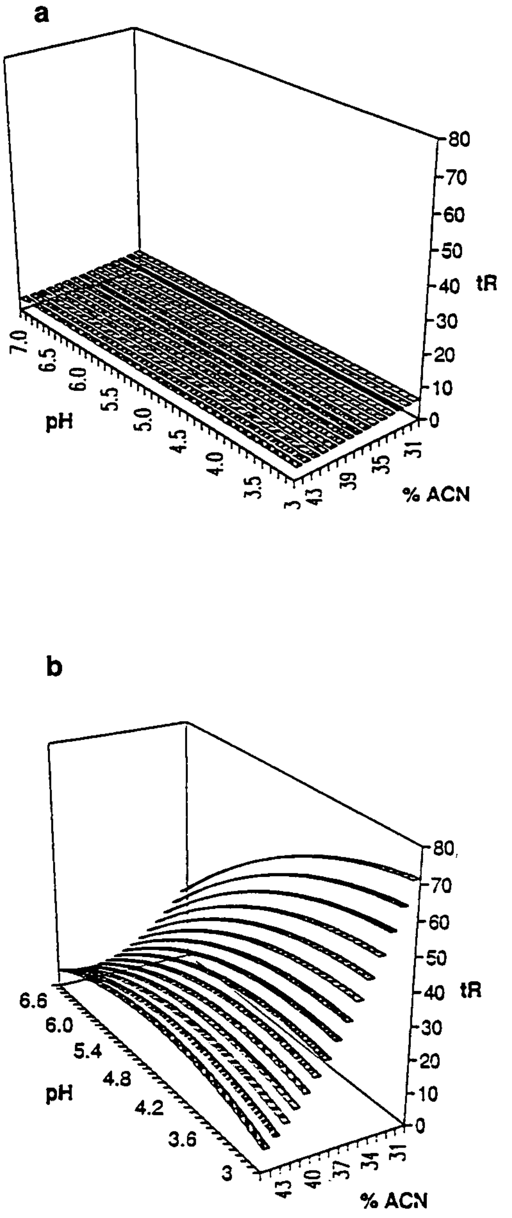

Fig. 16. Retention surfaces as a function of pH and concentration of acetonitrile. (a) Phenol; (b) 2,4,6-trichlorophenol.

After 4 weeks of continuous use, a decrease in

TABLE V

COMPARISON BETWEEN PREDICTED RETENTION TIMES AND EXPERIMENTAL VALUES FOR THE MIXTURE OF FIFTEEN CHLOROPHENOLS, OBTAINED WITH A VOLUME FRACTION OF ACETONITRILE OF 36% AT pH 3.9

In the last two columns the means of the percentage deviations between predicted and experimental t_R and $w_{1/2}$ values for the seven experiments of the design are given.

No.	Compound[a]	pK_a	t_R (pred.)	t_R (exp.)	Deviation (%)	Mean % dev. t_R[b]	Mean % dev. $w_{1/2}$[b]
1	Phenol	9.92	5.38	5.41	0.55	0.35	1.24
2	2-	8.52	9.06	9.12	0.66	0.45	10.9
4	4-	9.37	10.5	10.5	0.48	0.4	4.15
3	3-	8.97	11.4	11.3	0.88	6.07	0
9	2,6-	6.78	16.4	16.1	1.89	3.75	5.38
5	2,3-	7.71	17.3	17.1	0.93	1.36	3.64
7	2,4-	7.9	19.7	19.9	0.81	0.27	3.39
8	2,5-	7.51	20.4	19.9	2.41	2.22	2.77
6	3,4-	8.62	21.0	20.9	0.81	0.72	3
10	3,5-	8.25	28.6	28.6	0	1.23	0.97
13	2,3,6-	5.8	33.5	31.5	6.11	18.3	6.73
11	2,3,4-	6.97	35.5	34.3	3.29	4.7	9.15
14	2,4,6-	5.99	41.0	38.3	6.47	11.9	0.65
15	2,4,5-	6.72	42.6	41.1	3.54	4.1	1.77
12	2,3,5-	6.43	46.4	44.3	4.67	3.91	6.95

[a] Chloro substituent positions.

[b] The mean of the percentage deviations between predicted and experimental t_R and $w_{1/2}$ values for the seven experiments of the design.

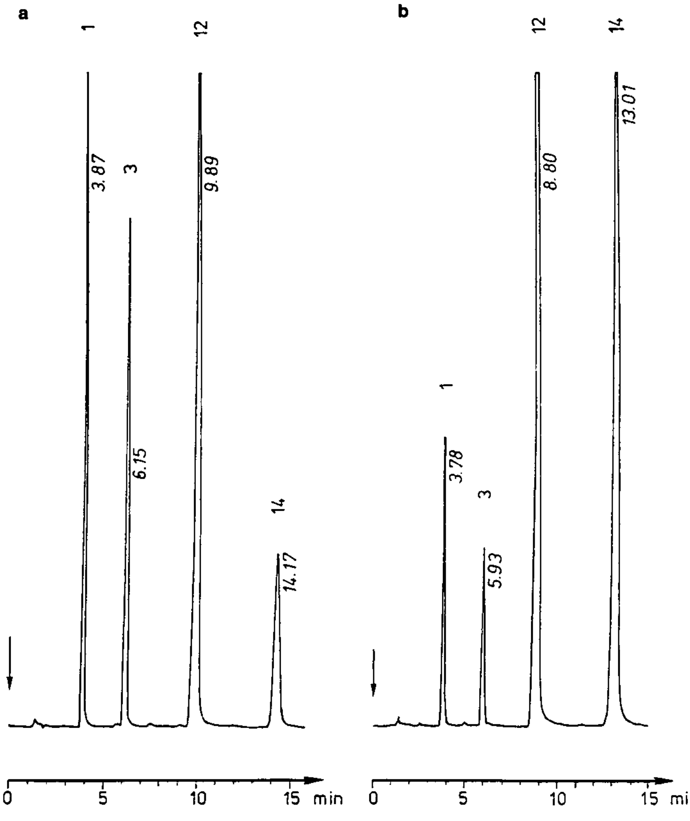

Fig. 17. Chromatograms illustrating the deterioration of the stationary phase. The experimental conditions are the same, *i.e.*, pH = 6 and acetonitrile = 46%, but chromatogram (b) was obtained 4 weeks later than (a). Numbers of the solutes refer to Table II.

retention times of about 10% was observed when repeating experiment 4 of the Doehlert design with fifteen compounds. The peak width remained almost the same and peak tailing also did not increase, as can be seen in Fig. 17. Washing the column with water and methanol, each for 5 h at a flow-rate of 1 ml/min, did not restore the previous retention times; the stationary phase had degraded. Such a decrease in retention is caused by hydrolysis of bonded ligands as solute retention in reversed-phase chromatography as a function of alkyl ligand density follows a linear relationship up to a certain limiting value [33]. Deterioration of the stationary phase was shown to be a cause of bad predictions in the experimental design, so the chromatographer should pay attention when applying conventional silica columns.

CONCLUSIONS

The large difference in retention times between tetra- and pentachlorophenols and the other chlorophenols makes the isocratic separation of all nineteen compounds on the same chromatogram impossible. By optimizing the pH of the mobile phase and the volume fraction of organic modifier using a Doehlert design, two mixtures of fourteen and four chlorophenols could be completely separated. In both separations the systematic approach allowed a short development time of about 2 weeks. One must determine the peak width and retention of each compound in each experiment. For the mixture of tetra- and pentachlorophenols, the deviations that were observed between the predicted values and results obtained by experiment are caused by a lack of fit of the quadratic model.

For the mixture of fifteen compounds, the quadratic model proved to be convenient for the simultaneous optimization of the two experimental parameters. The predictions were found to be accurate. The result obtained, a chromatogram with fourteen well separated peaks, does not correspond to what was predicted (fifteen peaks with an $R_{s_{\min}}$ of 0.96). However, the inaccuracy of the predicted $R_{s_{\min}}$ is not caused by the lack of fit.

In general, the results indicate that a quadratic model is adequate for describing only one leg of the sigmoidal curve of retention *versus* pH. If an (almost) complete curve is observed, a more complex model is required such as the model of Schoenmakers *et al.* [22]. Other approaches might prove worthwhile. One of these (the logit transformation) was described here. Another possibility is to use Drylab to determine an optimum region and to use the quadratic model with a D-optimum design in that region. The quadratic modelling is then an extension of the Drylab strategy. A very different approach based on an optimum mapping of the feasible region is also being investigated [34], but more detailed studies are needed to ascertain the value of such approaches.

The three-dimensional graph of $R_{s_{\min}}$ showed that the development of a rugged HPLC method to separate all the fifteen chlorophenols is not possible in the selected experimental domain and with a LiChrospher RP-18 stationary phase. To resolve this mixture in an isocratic experiment one will require a stationary phase with a higher efficiency and/or other types of organic modifier(s) or HPLC with photodiode-array detection in combination with a chemometric technique such as evolving factor analysis to be able to determine two substances, even when they overlap to a greater extent than usual [35]. It is our intention to explore these possibilities further.

ACKNOWLEDGEMENT

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CHROM. 24 636

Use of computer simulations in the development of gradient and isocratic high-performance liquid chromatography methods for analysis of drug compounds and synthetic intermediates

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ABSTRACT

DryLab G/plus and DryLab I/plus (LC Resources) are shown to be effective aids in the development and optimization of gradient and isocratic HPLC conditions for the assay of drug substances and related compounds. Data obtained after two experimental runs in the laboratory are entered into the appropriate program where HPLC conditions can be altered (*e.g.* flow-rate, column dimensions, mobile phase composition, gradient steepness and shape, etc.) to arrive at optimum separation conditions with less analyst time required. The computer simulations from DryLab G/plus are shown to be suitably accurate under "real life" conditions in the development of gradient purity methods for two drug substances (Zalospirone and WY-47 384) and two synthetic intermediates (cyclooctatetraene and 2-methylcarboxybenzaldehyde). Moreover, DryLab I/plus was shown to be accurate in predicting isocratic retention for the separation of impurities in cyclooctatetraene, both in scaling down to small columns for speed and scaling up to a semi-preparative separation for isolation of impurities.

INTRODUCTION

Typically, the development of rugged HPLC separations involves a significant investment of time and effort because the variety of stationary phases and mobile phase combinations provides for a broad array of possible separation conditions from which to choose. To assist chromatographers in this endeavor, a variety of HPLC method development schemes have been described [1–4]. The potential utility of computer simulation software packages as tools to help guide chromatographers to appropriate separation conditions is significant.

DryLab computer simulation programs (LC Resources, Lafayette, CA, USA) were designed to help chromatographers optimize separation conditions using fewer actual experimental runs. These software packages for isocratic and gradient HPLC methods development have been well documented by Snyder and co-workers [5,6]. In brief, data from two initial separations (isocratic or gradient) are entered into the appropriate program, then simulated experiments can be carried out at the computer to determine the effects on the separation of changing conditions such as flow-rate, percent organic modifier, gradient time and shape, column dimensions, etc. While taking advantage of this computer simulation software for HPLC method development, a chromatographer should be able to save considerable time and develop better HPLC separations.

EXPERIMENTAL

Instrumentation

The HPLC system used for these studies was

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composed of the following: a dual pump gradient (Model 590 and Model 6000A, Waters Assoc., Milford, MA, USA), a programmable variable-wavelength UV detector/gradient controller (Spectroflow 783G, Applied Biosystems, Ramsey, NJ, USA), an autoinjector (Model 710B WISP, Waters), and integration system (Model HP-3356, Hewlett-Packard, Avondale, PA, USA). DryLab I/plus and DryLab G/plus software was supplied by LC Resources, and was operated on an IBM PC-XT computer. The total dwell volume of the gradient system was about 3 ml.

Reagents and materials

All drug substances, intermediates and known impurities were obtained in-house (Wyeth-Ayerst Research). HPLC-grade methanol and acetonitrile (J. T. Baker, USA) were used as is. Distilled water was treated with a Milli-Q purification system (Millipore, USA) before use. Methanesulfonic acid (Aldrich, USA) was vacuum distilled before use. μBondapak C_{18} (Phenomenex, USA), Spherisorb C_8 and ODS-2 (Phase Separations, USA), Ultrasphere ODS (Beckman, USA), Supelco LC-18DB (Supelco, USA) and Pecosphere C_{18} (Perkin-Elmer, USA) columns were used.

Procedure

To demonstrate the practicality of computer simulations in the optimization of reversed-phase HPLC methods, DryLab G/plus and I/plus were used to aid in the development of four HPLC methods designed to assess the purity of drug compounds or synthetic intermediates. Overall, the separation goals were defined with an emphasis on the rugged separation of the maximum number of peaks. Of secondary importance was the speed of the separation.

Appropriate HPLC solvents were prepared to give good peak shapes and efficiencies for the compounds of interest. These mobile phase choices were based either on previous HPLC experience with the compounds of interest (examples 1 and 4) or on a "best guess" basis given the expected chromatographic behavior of the test compounds (examples 2 and 3). Two linear gradients were then run for each test compound over the same gradient range and with the same flow-rate. The gradient time of the first run was set at 15–20 min, while the gradient time for the second run was 3–4 times longer. Peaks were tracked during the optimization experiments based on their relative areas. Retention time data for observed peaks from the pairs of gradient test runs were entered into the DryLab G/plus program along with other data concerning the separation (flow-rate, temperature, organic solvent, column dimensions, etc.). Separation conditions including gradient shape, range, flow-rate, column dimensions, etc., could all be manipulated within the program, essentially to run separation experiments for projecting optimum conditions.

After the computer simulations were completed, the optimum predicted conditions were experimentally validated. The theoretical predictions from the computer simulations were then compared to the actual HPLC chromatograms. When possible, peaks were identified following optimization using authentic standards.

RESULTS

Gradient optimization

The first test involved a purity assay for the experimental drug substance zalospirone which, in its crude form, could have a number of impurities including synthetic intermediates and by-products. The initial linear gradient trials are shown in Fig. 1A and B. Changes in peak resolutions and even elution order between the two trials are evident. Using the information from these trials, 14 DryLab experiments were carried out before the optimum separation conditions depicted in Fig. 1C were arrived at with a total time of about 40 min at the computer. This is significantly less than the approximately 9 h of instrument time that would have been needed to perform the actual experiments.

Table I shows a comparison between the DryLab G/plus computer predicted retention times for the computer-optimized gradient separation and the experimentally observed retention times. Despite some minor differences, the final separation shown in Fig. 1C appears to be quite suitable with good resolution between all peaks.

A second use of DryLab G/plus involved the separation of the experimental drug substance WY-47 384 from possible synthetic impurities. Using basically the same mobile phases and column as for zalospirone, initial gradient trials were run as

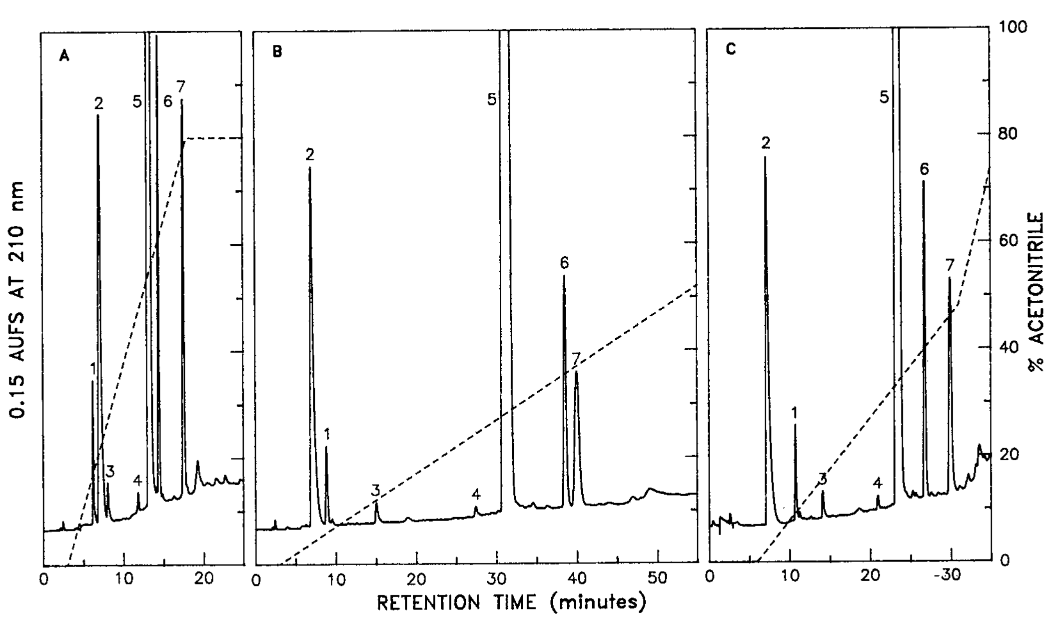

Fig. 1. (A, B) Results of initial gradient experiments run to acquire data for DryLab G/plus on crude zalospirone (peak 5). Mobile phase: 6 ml methanesulfonic acid/l water at pH 3.0 with KOH–acetonitrile. Column: 30 × 0.39 cm I.D. μBondapak C_{18}, 10 μm. Flow: 1.5 ml/min. Gradient: 0 to 80% acetonitrile in 15 min (A) and in 80 min (B). (C) Optimized gradient separation based upon DryLab G/plus predictions. Gradient: 0% acetonitrile for 3 min, then to 48% acetonitrile by 28 min, and to 80% acetonitrile by 33 min. Sample: 20 μl of crude zalospirone in methanol–dichloromethane (9:1). Peaks: 1 = synthetic precursor; 2 = dichloromethane; 3, 4, 7 = impurities; 5 = zalospirone; 6 = dimeric impurity.

shown in Fig. 2A and B. After 10 computer simulations were performed, an optimum gradient was arrived at as shown experimentally in Fig. 2C. The resolution between peak 2 and peak 3 (WY-47 384) is now very good with this optimized, segmented gradient. Table II compares the DryLab G/plus predicted retention times with found results.

Similarly, DryLab G/plus was utilized to develop a purity assay for 2-methylcarboxybenzaldehyde (2MCBA). Using acetonitrile–water, the initial gra-

TABLE I

COMPARISON OF ACTUAL RETENTION TIMES WITH DryLab G/plus PREDICTIONS FOR AN OPTIMIZED GRADIENT SEPARATION OF ZALOSPIRONE FROM IMPURITIES

Conditions: see Fig. 1.

Peak	Retention time (min)	
	DryLab	Actual
1	7.1	7.1
2	9.6	10.6
3	14.3	13.8
4	22.7	20.7
5	25.2	23.3
6	29.3	26.7
7	32.3	29.8

TABLE II

COMPARISON OF ACTUAL RETENTION TIMES WITH DryLab G/plus PREDICTIONS FOR AN OPTIMIZED GRADIENT SEPARATION OF WY-47 384 FROM IMPURITIES

Conditions: see Fig. 2.

Peak	Retention time (min)	
	DryLab	Actual
1	11.6	10.6
2	20.6	19.7
3	22.6	23.5
4	29.8	29.7
5	37.3	37.4

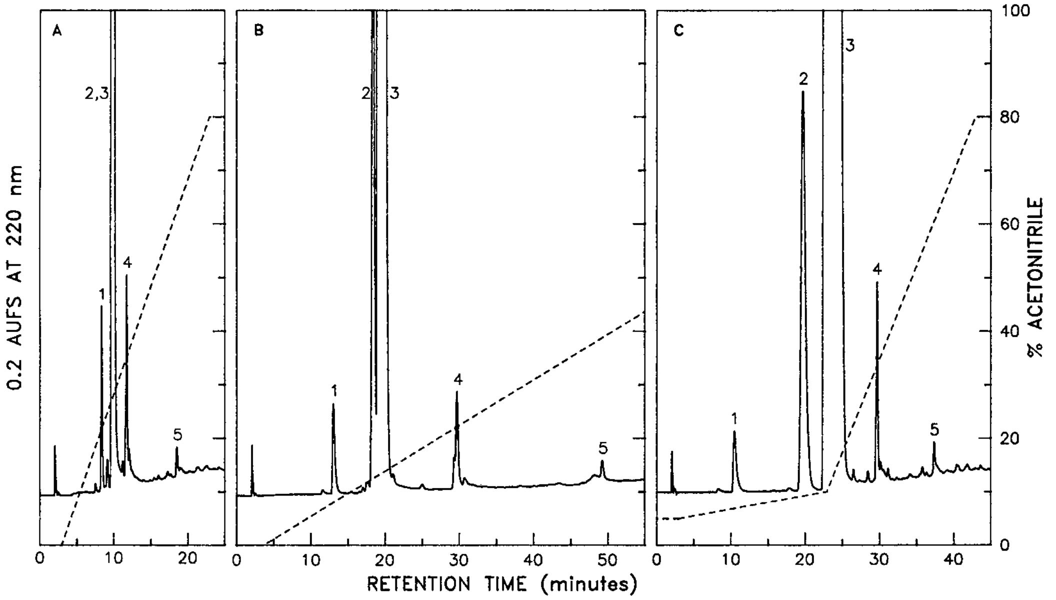

Fig. 2. (A, B) Results of initial gradient experiments run to acquire data for DryLab G/plus on crude WY-47 384 (peak 3). Mobile phase: 6 ml methanesulfonic acid/l water at pH 3.0 with KOH–acetonitrile. Column: 30 × 0.39 cm I.D. μBondapak C_{18}, 10 μm. Flow: 1.5 ml/min. Gradient: 0 to 80% acetonitrile in 20 min (A) and in 80 min (B). (C) Optimized gradient separation based upon DryLab G/plus predictions. Gradient: 5 to 10% acetonitrile in 20 min, then to 80% acetonitrile by 40 min. Sample: 20 μl of crude WY-47 384 in acetonitrile. Peaks: 1, 2 = synthetic precursors; 3 = WY-47 384; 4, 5 = synthetic by-products.

dient trials were performed as shown in Fig. 3A and B. Seven computer simulations gave the separation conditions used to generate the chromatogram shown in Fig. 3C which shows excellent resolution between impurity peaks 2 and 3 with the separation carried out in about 25 min. Comparisons between predicted and found retention times for this gradient separation are shown in Table III.

Finally, a method was developed to detect dimeric impurities in cyclooctatetraene. The initial methanol/water gradient trials (Fig. 4A and B) led to an optimized gradient shape shown in Fig. 4C after 8 computer simulations. Retention time comparisons are presented in Table IV.

TABLE III

COMPARISON OF ACTUAL RETENTION TIMES WITH DryLab G/plus PREDICTIONS FOR AN OPTIMIZED GRADIENT SEPARATION OF 2MCBA FROM IMPURITIES

Conditions: see Fig. 3C.

Peak	Retention time (min)	
	DryLab	Actual
1	8.6	10.9
2	11.7	13.2
3	12.6	14.2
4	15.1	15.8
5	17.2	17.7
6	18.6	18.9
7	23.0	23.2

TABLE IV

COMPARISON OF EXPERIMENTAL RETENTION TIMES WITH DryLab G/plus PREDICTIONS FOR THE OPTIMIZED GRADIENT SEPARATION OF COT FROM IMPURITIES

Conditions: see Fig. 4C.

Peak	Retention time (min)	
	DryLab	Actual
1	10.3	11.0
2	11.2	11.9
3	20.1	20.6
4	20.8	21.3

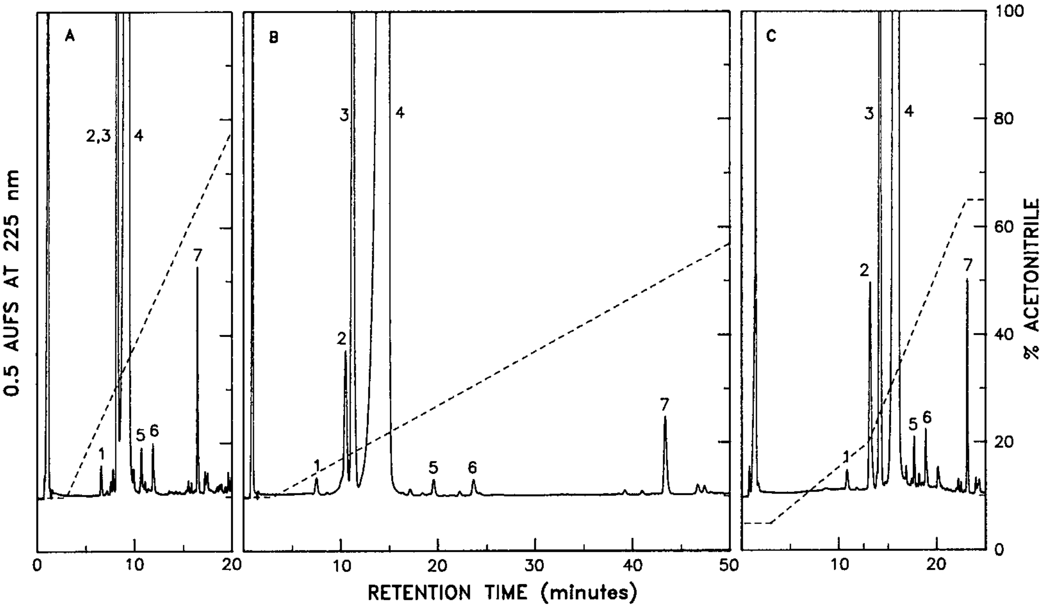

Fig. 3. (A, B) Results of initial gradient experiments run to acquire data for DryLab G/plus for 2-methylcarboxybenzaldehyde (peak 4). Mobile phase: water–acetonitrile. Column: 15 × 0.46 cm I.D. Spherisorb C_8, 5 μm. Flow: 1.5 ml/min. Gradient: 10 to 90% acetonitrile in water in 20 min (A) and in 80 min (B). (C) Optimized gradient separation based upon DryLab G/plus predictions. Gradient: 5 to 20% acetonitrile in 10 min then to 65% acetonitrile by 20 min. Sample: 10 μl of 2-methylcarboxybenzaldehyde at 10 mg/ml in acetonitrile. Peaks: 1–3 = impurities; 4 = 2MCBA; 5–7 = impurities.

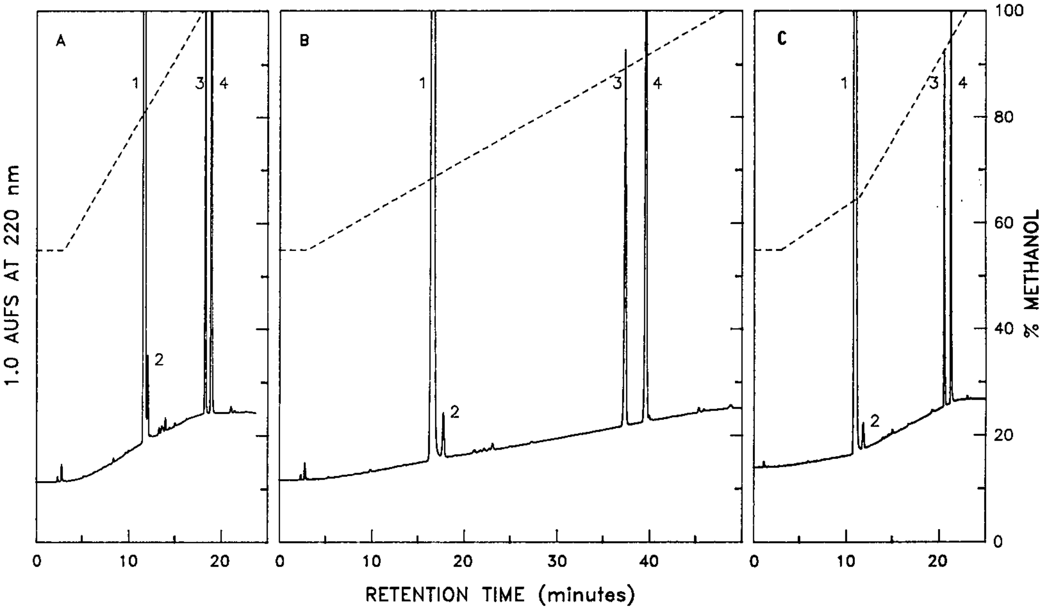

Fig. 4. (A, B) Results of initial gradient experiments run to acquire data for DryLab G/plus for cyclooctatetraene (COT, peak 1). Mobile phase: water–methanol. Column: 25 × 0.46 cm I.D. Spherisorb ODS-2, 5 μm cartridge. Flow: 1.25 ml/min. Gradient: 55 to 100% methanol in 15 min (A) and in 45 min (B). (C) Optimized gradient separation based upon DryLab G/plus predictions. Gradient: 55% to 65% methanol in 8.5 min then to 100% methanol from 8.5 to 20 min. Sample: 10 μl of COT at 5 mg/ml in methanol. Peaks: 1 = COT; 2 = unknown impurity; 3, 4 = dimeric impurities.

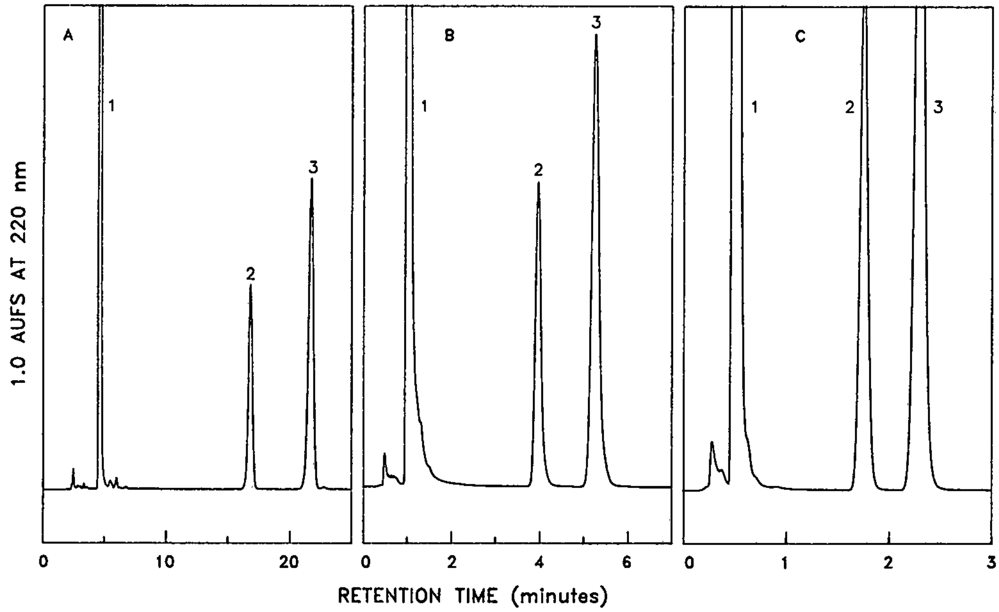

Fig. 5. Optimized analytical scale isocratic separation of dimers from COT based on DryLab I/plus predictions from the gradient separations shown in Fig. 4A and 4B. Mobile phase: methanol–water (83:17). Columns and flow-rates: (A) 25 × 4.6 cm I.D. Spherisorb ODS-2 (5 μm), 1.25 ml/min, (B) 7.5 × 0.46 cm I.D. Ultrasphere ODS (3 μm), 2 ml/min and (C) 3.3 × 0.46 cm I.D. Pecosphere C_{18} (3 μm), 2 ml/min. Sample: 10 μl of COT at 5 mg/ml in methanol. Peaks: 1 = COT; 2, 3 = dimers.

Isocratic optimization

Because a simple method to test only for dimers in COT was desired, computer simulations using the data generated from Fig. 4A and 4B (initial gradient trials) were performed with DryLab I/plus to arrive at suitable isocratic conditions for this assay. A reasonable separation could be performed on the 25 cm column used initially (Fig. 5A) with an optimized mobile phase composition of 83% methanol obtained directly from computer simulations; however, the column optimization functions of DryLab I/plus also suggested that satisfactory separation could be maintained on short, 3 μm particle columns with the same mobile phase composition to greatly reduce analysis time to under 3 min as shown in Figs. 5B and C. A comparison between DryLab I/plus predictions and confirming HPLC experiments is given in Table V. The resolutions be-

TABLE V

COMPARISON OF EXPERIMENTAL RETENTION TIMES WITH DryLab I/plus PREDICTIONS FOR THE OPTIMIZED ISOCRATIC SEPARATIONS OF COT FROM IMPURITIES ON VARIOUS COLUMNS

Conditions: see Fig. 5.

Peak	Retention time (min)					
	Spherisorb ODS-2		Ultrasphere ODS		Pecosphere C_{18}	
	DryLab	Actual	DryLab	Actual	DryLab	Actual
COT	4.2	4.6	0.9	1.0	0.4	0.5
Dimer 1	16.8	16.8	3.2	4.0	1.4	1.8
Dimer 2	21.5	21.7	4.1	5.3	1.8	2.3

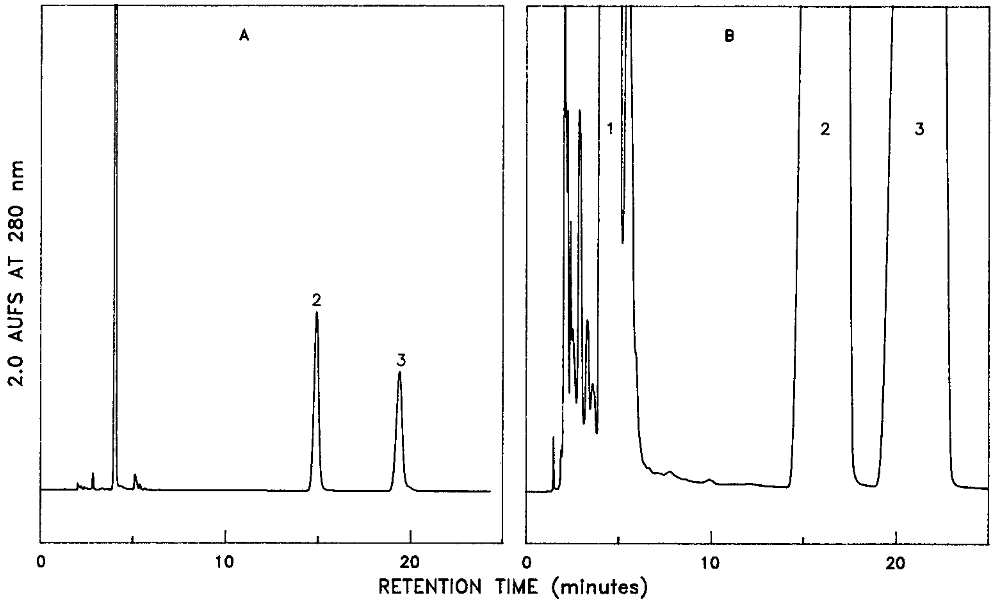

Fig. 6. Optimized semi-preparative isocratic separation of dimers from COT based on DryLab I/plus predictions from the gradient separations shown in Fig. 4A and 4B. Mobile phase: methanol–water (83:17). Column: 25 × 1 cm I.D. Supelco LC-18DB (5 μm) at a flow-rate of 6.0 ml/min. Sample: 2 μl (1.85 mg) of neat COT (A) and 200 μl (185 mg) (B). Peaks: 1 = COT; 2, 3 = dimers.

tween the peaks of interest are large enough to provide for a very rugged separation.

The utility of DryLab I/plus for scaling separations upward was also demonstrated for the separation of COT from dimers. In order to collect fractions of the COT dimer peaks for off-line identification by MS and NMR, the separation was moved to a semi-preparative system, again based directly on the predictions from the initial gradient trials of Fig. 4A and 4B. The results presented in Table VI and Fig. 6 show that the computer optimized separation was accurate and rugged enough to allow for the injection of 200 μl (about 185 mg) of neat COT on a 25 × 1 cm Supelco LC-18DB column at a flow-rate of 6.0 ml/min. It is apparent from Fig. 6B that more than 185 mg of COT could have been loaded onto the semi-preparative column while still maintaining good resolution between the dimer peaks; however the 200-μl injection size was convenient with the WISP autosampler used for the isolation procedure.

TABLE VI

COMPARISON OF EXPERIMENTAL RETENTION TIMES WITH DryLab I/plus PREDICTIONS FOR THE OPTIMIZED SEMI-PREPARATIVE ISOCRATIC SEPARATION OF COT FROM IMPURITIES

Conditions: see Fig. 6A (2-μl injection).

Peak	Retention time (min)	
	DryLab	Actual
1	10.3	11.0
2	11.2	11.9
3	20.1	20.6

DISCUSSION

Overall excellent predictions of retention behavior were observed using DryLab software as an aid in HPLC method development and optimization. Furthermore, many hours of analyst and instrument time and solvents were saved through the use of computer simulations. It is likely that better, more rugged separations were achieved using simulations since time restraints may not have allowed such a number of "real" experiments and a less optimized solution may have been chosen. To assess their impact on the desired separation, the simulations allow for a wide variety of gradient times, gradient profiles (linear *versus* segmented), column dimensions, particle sizes, flow-rates, etc. to be explored very rapidly and accurately.

Remarkably, separation predictions worked well for COT separations despite the fact that the column packing brands used for experimental HPLC separations were changed several times from that used to acquire initial data for the DryLab program. Indeed, many of the differences in observed *versus* predicted retention are probably due largely to the varying column packing chemistries used in the experiments, and not due to errors in the software algorithms.

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CHROM. 24 640

High-performance liquid chromatographic determination of polyamines in selected vegetables with postcolumn fluorimetric derivatization

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ABSTRACT

A rapid high-performance liquid chromatographic analysis for the simultaneous separation of five naturally occuring polyamines (agmatine, putrescine, cadaverine, spermidine and spermine) is described. Postcolumn derivatization with *o*-phthalaldehyde is used. The separation systems consisted of a strong cation-exchange column, an elution buffer consisting of 1 *M* sodium citrate (pH 5.4), a mixing coil for the chemical reaction and a spectrofluorimetric detector. The derivatized fluorescent compounds were detected at 340 nm (excitation) and 455 nm (emission). The recoveries of the polyamines were 94.5–107.0% with a standard deviation of 0.83–7.65%.

INTRODUCTION

Polyamines, such as agmatine, putrescine, cadaverine, spermidine and spermine, play an important role in cell fission, nucleotide biosynthesis and protein biosynthesis in both animals and plants. In fruits, many researchers have reported that free polyamine levels declined during fruit development and increased during fruit maturation and ripening [1–5]. These findings implied that free polyamine serves as an endogenous antisenescence agent in a number of plants [6]. On the other hand, ethylene is a senescence-promoting hormone and accelerates fruit and vegetable ripening. In contrast, free polyamines inhibited ethylene production in some tissues. It is suggested that polyamines and ethylene have opposite effects in relation to plant ripening and senescence [7–9].

Methods for the determination of polyamines in plants using thin-layer and high-performance liquid chromatography (HPLC) have been reported [10–14]. In these HPLC methods the polyamines were derivatized to either dansylates or benzoylates before the separation. Recently, *o*-phthalaldehyde reagent (OPA) has been successfully applied to polyamine assays as derivatization reagent in the precolumn [15] and postcolumn modes [16].

We have developed a rapid and sensitive HPLC method for the determination of the five natural polyamines agmatine (**I**), putrescine (**II**), cadaverine (**III**), spermidine (**IV**) and spermine (**V**) (Fig. 1) in vegetables with postcolumn fluorimetric derivatization. The objective was to establish the separation procedure and to determine polyamines of interest in vegetables in a single chromatographic run.

EXPERIMENTAL

Apparatus

The system consisted of two Model 880-51 degassers, two Model 880 PU LC pumps for the liquid chromatograph, a Model 850 AS autosampler, a

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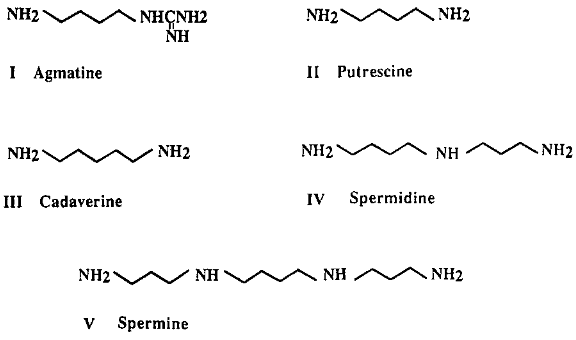

Fig. 1. Structures of polyamines **I–V**.

Polyaminepak (strong cation-exchange resin, 5-μm particle size) column (3.5 cm × 6 mm I.D.) (Japan Analytical Spectro, Tokyo, Japan), a Model 860 column over (70°C), a stainless-steel mixing coil (30 cm × 0.8 mm I.D.), an FP 210 spectrofluorimetric detector (excitation at 340 nm, emission at 455 nm) and a SIC Chromatorecordor 12 recorder. The system was controlled by a Model 801SC system controller. All instruments were purchased from Japan Analytical Spectro.

Reagents

Agmatine dihydrochloride and putrescine dihydrochloride were obtained from Wako (Osaka, Japan) and cadaverine dihydrochloride, spermidine trihydrochloride and spermine from Aldrich (Milwaukee, WI, USA). Other chemicals were of analytical-reagent grade (Wako).

Materials

Six vegetables, komatu-na (*Brassica rapa* L.), kaiware-daikon (Japanese radish; *Raphanus acanthiformis* Morel), broccoli (*Brassica oleracea* var. *italica, cv.* Charade), senpou-sai (*Brassica napus*), benri-na (*Brassica campestris* ssp.) and spinach (*Spinacia olèracea*), were grown and harvested at Chugoku National Agricultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries of Japan.

Sample preparation

Leaves of komatu-na, benri-na and spinach, edible cotyledon of kaiware-daikon and buds of broccoli were taken by cutting with a knife and collected at random, then used as samples. A 1.0-g amount of sample was placed in a 30-ml glass vial containing 7 ml of 5% perchloric acid ($HClO_4$), then homogenized for 1 min with a Polytron (Nichion Rika Equipment, Tokyo, Japan). The homogenate was placed for 1 h in an ice-cold water-bath (0°C) to accelerate protein precipitation [17], followed by centrifugation at 35 000 *g* for 20 min (2°C). The supernatant was stored at −20°C until analysis. A portion of the defrosted supernatant was filtered with a 0.45-μm filter (Millipore Japan, Tokyo, Japan). A 10-μl volume of filtrate was applied to the HPLC column. All treatments were performed in triplicate and average values are given.

HPLC conditions

Fig. 2 presents a schematic diagram of the HPLC system. A mixture of 100 ml of acetonitrile and 900 ml of 1.0 *M* sodium citrate (adjusted to pH 5.4 with 60% $HClO_4$) was pumped at a flow-rate of 0.65 ml

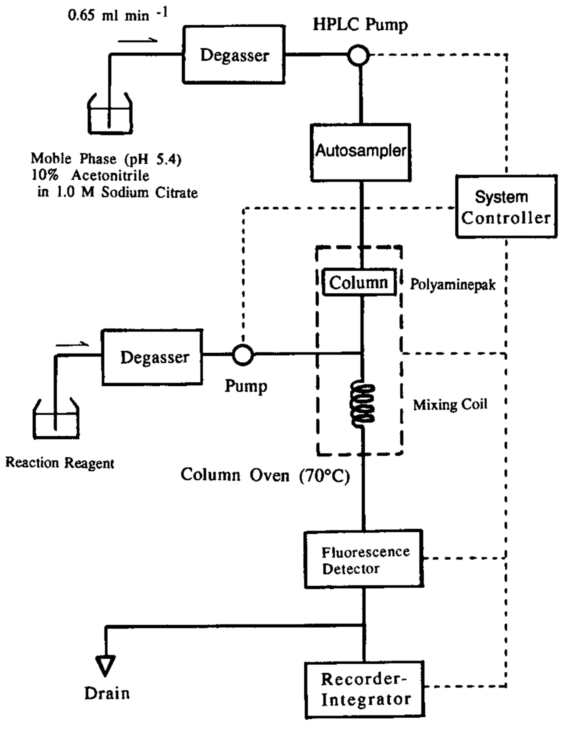

Fig. 2. Schematic diagram of the HPLC system. A postcolumn reaction solution was prepared by adding 2.0 ml of 2-mercaptoethanol, 2 ml of 30% Briji 35 and 0.8 g of OPA dissolved in 20 ml of ethanol to 980 ml of 0.4 *M* boric acid containing 0.4 *M* potassium hydroxide, and protected from light.

min^{-1} as the mobile phase. Duplicate 10-μl volumes of the sample filtrate described above were injected into the HPLC column. A postcolumn reaction solution was prepared by adding 2.0 ml of 2-mercaptoethanol, 2 ml of 30% Briji 35 and 0.8 g of OPA dissolved in 20 ml of ethanol to 980 ml of 0.4 *M* boric acid containing 0.4 *M* potassium hydroxide. The reaction solution was protected from light, pumped at a flow-rate of 0.7 ml min^{-1} and mixed with the column eluate to convert the polyamine into the fluorescent derivative. This compound was measured with a spectrofluorimeter. The column oven and the reaction coil were maintained at 70°C. The values of two injections were averaged.

Recovery studies

Recovery was estimated only using cotyledons of kaiware-daikon. Polyamines **I-IV** were dissolved in 5% $HClO_4$ to a final concentration of 0.2 m*M*. The samples were treated as described above, then 10 μl of the filtrate were injected into the HPLC column. The recoveries were determined by subtracting the values obtained for control vegetable tissue homogenization. The recovery experiment was performed with five replicates and mean values with the standard deviations are reported.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

Mobile phase. In order to separate five typical polyamines, we tried several elution systems, *i.e.*, acetonitrile–sodium dihyrogenphosphate and acetonitrile–sodium citrate, using a cation-exchange resin (PolyaminePak) and an isocratic elution system. The pH of the mobile phase was adjusted to 5.4 with 60% $HClO_4$. Both acetonitrile–1.0 *M* sodium dihyrogenphosphate (10:90, v/v) and acetonitrile–1.0 *M* sodium citrate (10:90, v/v) gave the best separation and resolution for all five compounds in 45 min. In this study, we selected 10% acetonitrile–1.0 *M* sodium citrate (pH 5.4) as the mobile phase. A typical chromatogram is shown in Fig. 3, the retention times for putrescine (**II**), cadaverine (**III**), spermidine (**IV**), agmatine (**I**) and spermine (**V**) being 4.8, 12.0, 16.5, 26.5 and 40.0 min, respectively.

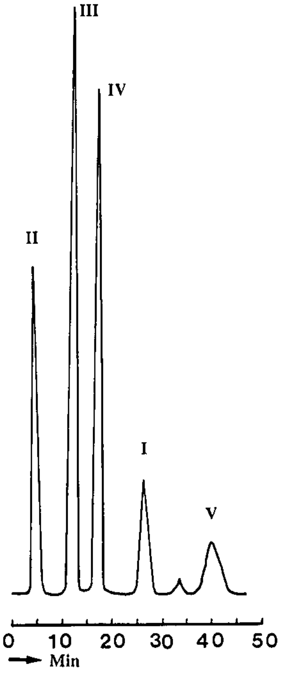

Fig. 3. Chromatogram of polyamines **I–V**. For operating conditions, see text.

Optimum concentration of reagent. We used OPA reagent as the reaction solution in order to convert polyamine into the fluorescent compounds. Fig. 4 shows the relationship between OPA concentration in the reaction solution and the peak area of four polyamines (**I–IV**) detected by spectrofluorimetry. In this study, 10 μl each of 125 m*M* polyamine stan-

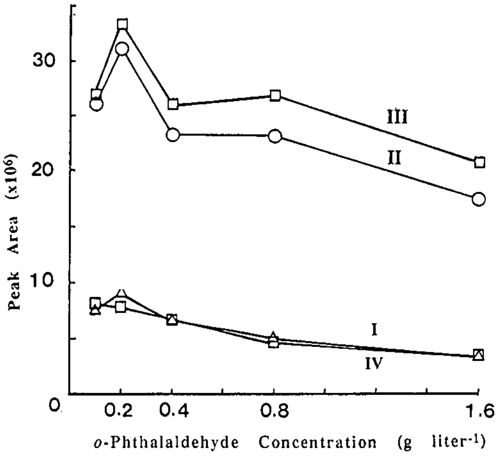

Fig. 4. Relationship between the peak area detected and the OPA reagent concentration. For operating conditions, see text.

dards were injected into the HPLC column. For the OPA concentration range studied (0.2–1.6 g l^{-1}), a 0.2 g l^{-1} concentration gave the maximum peak area except for **IV** in the concentration. However, the polyamine concentration studied was 1.25 nmol, and it would be necessary to increase the OPA reagent concentration in the presence of the higher concentrations of polyamines.

Linearity. In order to check the linearity of the relationship between the polyamine levels and the peak area using the above serparation system, 1 m*M* stock standard solutions of the five polyamines were prepared and suitably diluted with 5% $HClO_4$. Various amounts of the standard solution were injected into the HPLC column described above. Fig. 5 presents the calibration graphs for the five polyamines. All the graphs exhibited good linearity and obeyed Beer's law in the investigated concentration range of 0.06–5.00 nmol. The regression equation $y = ax + b$, where x is the concentration of polyamine (nmol) and y is the peak area, and the correlation coefficients (r) of the polyamines were as follows: for agmatine (**I**), $y = 4.573x + 0.0545$ ($r = 0.991$); for putrescine (**II**), $y = 10.882x + 2.2657$ ($r = 0.991$); for cadaverine (**III**), $y = 13.543x + 1.4795$ ($r = 0.996$); for spermidine (**IV**), $y = 4.838x + 0.5630$ ($r = 0.991$); and for spermine (**V**), $y = 0.481x + 0.0202$ ($r = 0.999$). These results suggest that the proposed HPLC method is sufficiently sensitive to detect the five polyamines. Spermine (**V**) had the lowest sensitivity.

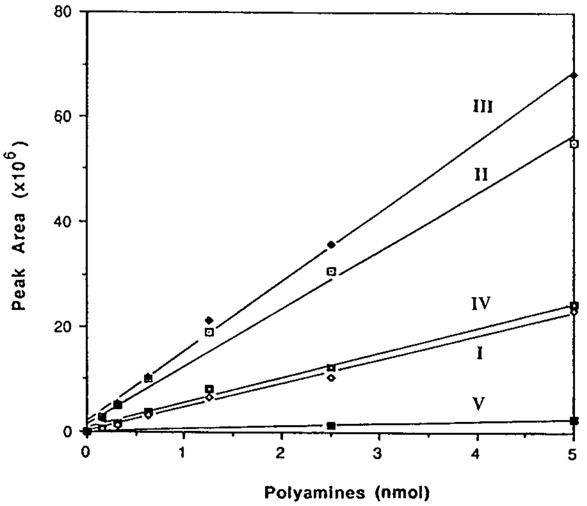

Fig. 5. Calibration graphs for polyamines I–V (peak area detected *versus* nanomoles of polyamines injected). **I** = Agmatine; **II** = putrescine; **III** = cadaverine; **IV** = spermidine; **V** = spermine. For conditions and regression equation, see text.

Recovery of polyamines from vegetable homogenates

Measured amounts of four polyamines (**I-IV**) were added to a sample homogenate of Japanese radish cotyledons (kaiware-diakon) with known levels of the compounds and were then determined by the proposed procedure. The recoveries of the polyamines were 94.5–107.0% with a standard deviation of 0.83–7.65% (Table I). Of the polyamines studied, it was appeared that the agmatine content varied more than the others.

Application

The contents of polyamines in the six selected vegetables were determined to demonstrate the validity of the method. The peaks were identified by adding a mixture of reference compounds to the sample solution before injection. Fig. 6 shows typical chromatograms of polyamines in the three vegetables. Table II gives the results expressed as nmol g^{-1} (fresh mass). Spermine (**V**) was not detected in any of the vegetables investigated. It has been reported that the contents of polyamines, particularly putrescine, spermidine and spermine, are abundant during seed germination of *Phaseolus mungo* [18] and that putrescine, cadaverine and agmatine are also abundant in bean seedlings [19]. In this study, the radish cotyledon (kaiware-daikon) contained more agmatine and putrescine, which agreed with the results reported by others [18,19]. Of the polyamines detected in the vegetables other than kaiware-daikon, spermidine was predominant, followed by cadaverine and/or putrescine in order of decreasing concentration.

CONCLUSIONS

A rapid useful HPLC analysis for five polyamines, agmatine, putrescine, cadaverine, spermidine and spermine, in vegetables was established. Polyamines were extracted from the vegetables by homogenization in 5% $HClO_4$, followed by centrifugation. The supernatant was filtered and injected into the HPLC column. The separated polyamines were converted into fluorescent derivatives reaction

TABLE I

RECOVERIES OF POLYAMINES I–IV FROM COTYLEDON OF KAIWARE-DAIKON (*RAPHANUS ACANTIFORMIS MOREL*)

For operating conditions, see text.

Polyamine	Added (nmol)	Recovered (nmol)	Recovery (%)	Mean recovery ± S.D. (%)
I	0.50	0.545	109.0	107.0 ± 7.65
	1.00	1.029	102.9	
	2.00	1.920	96.0	
	3.00	3.465	115.5	
	4.00	4.460	111.5	
II	0.50	0.480	96.0	96.2 ± 0.83
	1.00	0.975	97.5	
	2.00	1.922	96.1	
	3.00	2.856	95.2	
	4.00	3.852	96.3	
III	0.50	0.473	94.6	94.5 ± 0.93
	1.00	0.953	95.3	
	2.00	1.878	93.9	
	3.00	2.859	95.3	
	4.00	3.736	93.2	
IV	0.50	0.476	95.2	105.2 ± 6.41
	1.00	1.059	105.9	
	2.00	2.076	103.8	
	3.00	3.357	111.9	
	4.00	4.372	109.3	

TABLE II

CONTENTS OF POLYAMINES I-IV IN SIX VEGETABLES

For operating conditions, see text.

Vegetable	Polyamine[a] (nmol/g^{-1} fresh mass)				
	I	II	III	IV	V
Cotyledon of kaiware-daikon	4180	3887	–[b]	319.0	–
Broccoli	1.39	358.0	–	751.0	–
Komatsu-na	–	87.4	112.3	369.5	–
Senpou-sai	–	94.1	–	301.0	–
Benri-na	–	45.5	64.9	307.9	–
Spinach	–	27.5	–	98.4	–

[a] Average values of triplicate measurements.

[b] –, Not detected.

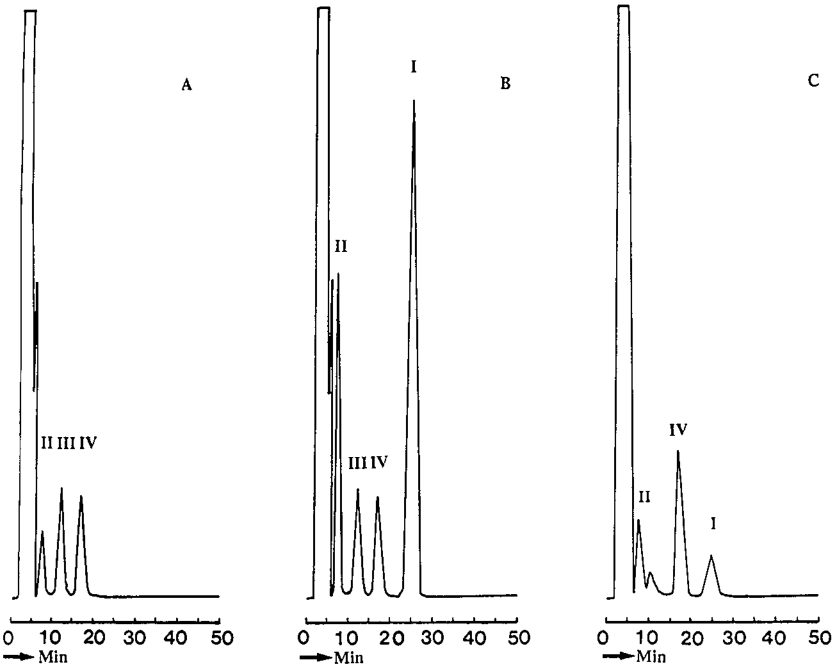

Fig. 6. Chromatograms of polyamines **I–IV** in (A) komatu-na, (B) kaiware-daikon and (C) broccoli. For the HPLC conditions, see text.

with OPA reagent, then detected by spectrofluorimetry.

ACKNOWLEDGEMENTS

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Partitioning of ecdysteroids using temperature-induced phase separation

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ABSTRACT

The partitioning behaviour of ecdysone and 20-hydroxyecdysone in aqueous two-phase systems was characterized. Primary systems were composed of an ethylene oxide–propylene oxide random copolymer, UCON 50-HB-5100, and dextran T500. The ecdysteroids were first partitioned in a two-phase system with a UCON-rich upper phase and a dextran-rich lower phase. After the phases had separated, the upper phase was removed and isolated in a separate container. This UCON solution was placed in a water bath and the temperature increased above the cloud point of the polymer. This resulted in the formation of a new two-phase system with an upper water–buffer phase and a lower UCON phase. Ecdysteroids partitioned mainly to the final upper water phase in this new two-phase system. Therefore, temperature-induced phase separation could be utilized to recover UCON polymer and obtain ecdysteroids in a water–buffer solution. The partitioning behaviour was manipulated by adding ethanol, sodium chloride or sodium sulphate to the primary two-phase systems. The recovery of ecdysteroids increased when ethanol was added to the system. In a two-phase system with an ethanol concentration of 20%, recovery was 73.6% for ecdysone and 85.6% for 20-hydroxyecdysone.

INTRODUCTION

Partitioning in aqueous two-phase systems is a standard technique for separation and purification of biomolecules [1–4]. Recently, temperature-induced phase separation has been introduced for enzyme purification [5,6]. This technique is possible because of the relatively low cloud point (50°C) of a random copolymer of 50% (w/w) ethylene oxide and 50% (w/w) propylene oxide (UCON 50-HB-5100). UCON is mixed with either dextran or hydroxypropyl starch to form an aqueous two-phase system with an upper UCON-rich phase and a lower phase enriched in either dextran or hydroxypropyl starch. When the target enzyme or other substance partitions to the upper UCON phase, this phase can be removed and the temperature increased above the cloud point of UCON. This results in the formation of a new aqueous two-phase system consisting of an upper water–buffer phase and a lower UCON-rich phase. Enzymes partition strongly to the upper phase of this new system, and the lower UCON phase can be recovered and recycled. The cloud point of UCON can be lowered by changing the ratio of ethylene oxide–propylene oxide groups or by addition of salts such as sodium sulphate or sodium chloride.

Ecdysteroids are a group of polyhydroxylated steroids derived from cholesterol through a variety of metabolic pathways found primarily in the Arthropoda [7,8]. These are phylogenetically old ste-

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roids [9] that are also found in plants and other invertebrate phyla [10,11]. Most ecdysteroids are precursors or metabolites of ecdysone (α-ecdysone: 2β,3β,14α,22*R*,25-pentahydroxy-5-β-cholest-7-en-6-one) and 20-hydroxyecdysone (β-ecdysone), the hormones that regulate the moulting cycle of anthropods [7]. 20-Hydroxyecdysone has also been implicated as a potential sex pheromone in some Crustacea [12–14]. Commercially, ecdysone and 20-hydroxyecdysone are important insecticides [15,16]. Recently these steroids have been detected in the urine of humans parasitized by helminths and nematods [17,18] or suffering from other medical disorders [19]. Consequently, ecdysteroids are medically important in the diagnosis of some pathological conditions in humans [19].

Karlson *et al.* [20] first obtained ecdysones from silkworm pupae, *Bombyx mori*, and described the original extraction and quantification procedures [21]. Two extraction procedures were described. One utilized methanol, butanol and light petroleum (b.p. 65–117°C) in several steps to obtain the desired compounds, while the other applied hot water (80–90°C) and butanol. In each, quantification of the ecdysones was by weight of the residue obtained in the final step after the solvent, methanol or butanol, was evaporated. Verification of the desired compound was obtained through a bioassay that tested its ability to initiate moulting in the pupae of a dipteran fly, *Calliphora erythrocephala* [21]. Since the work of Karlson and Shaaya [21], a few modifications have been made to the extraction procedures [8,11,22,23]. Methanol remains the primary solvent and the desired product is obtained after a number of steps. Detection and quantification of the ecdysones have become more sophisticated but increasingly complex and time-consuming [23–27]. The least complex and most sensitive method is the use of reversed-phase high-performance liquid chromatography (RP-HPLC) combined with UV absorption spectrophotometry [11,24,28–30].

One problem with using two-phase partitioning to purify steroids is that most steroids are insoluble in water. There have been two previous studies published on the partition of steroids [31,32]. Johansson and Joelsson [32] partitioned steroids in two-phase systems composed of polyethylene glycol (PEG), dextran and N,N-dimethylformamide. Shanbhag *et al.* [31] partitioned testosterone, cortisol and oestradiol, both free and bound to hormone receptor proteins, in a two-phase system composed of PEG and dextran. It is of interest to note that, while these steroids are relatively insoluble in water, they were soluble at low concentrations in an aqueous PEG–dextran two-phase system. Ecdysone and 20-hydroxyecdysone are unique among steroids because they are water-soluble. These molecules are identical in structure except for substitution of a hydroxyl group for a hydrogen at position 20 on 20-hydroxyecdysone. This substitution results in different hydrophobicities for these two molecules, with ecdysone being the most hydrophobic.

Aqueous two-phase systems offer an inexpensive and simple method for separation of many biomolecules [33]. A purification scheme for enzymes utilizing temperature-induced phase separation has also been developed [5,6]. By applying this system it is possible to recover the target molecules in a water phase free of polymers without additional ultrafiltration or dialysis steps. Therefore, application of an aqueous two-phase system and temperature-induced phase separation eliminates time-consuming and therefore expensive purification steps previously necessary to remove any organic solvents used in ecdysteroid extraction. In addition, separation using aqueous two-phase systems is a rapid process that does not require any specialized equipment, and the ecdysteroid is obtained in an inert aqueous solution. Therefore, it would be advantageous to develop a method utilizing this system for detection and purification of ecdysone and 20-hydroxyecdysone. However, before such a scheme can be developed, it is necessary to determine how these molecules are distributed in an aqueous two-phase system, and how to manipulate the system to achieve desirable partitioning. With this objective in mind, the partitioning behaviour of these molecules in both an initial UCON–dextran system and a new UCON–water system induced by increasing the temperature was studied. The systems were also modified by adding either salt or ethanol or a combination of these two and observing any effect on the molecules' partitioning.

EXPERIMENTAL

Chemicals

UCON 50-HB-5100 [monobutyl ether of ethylene oxide (EO)–propylene oxide (PO) random copolymer, EO/PO weight ratio 1:1, M_r 4000] was a kind gift from Union Carbide (New York, NY, USA). Before use in experiments, UCON polymer was ultrafiltered using a Filtron ultrafiltration unit (membrane cut-off 1000 molecular weight) (Filtron Technology Corporation, Clinton, MA, USA) to remove contaminants with light absorption at 280 nm. Dextran T500, M_r 500 000, was obtained from Pharmacia (Uppsala, Sweden). All other biochemicals were of analytical reagent grade.

Ecdysteroids

Both ecdysteroids were purchased from Sigma (St. Louis, MO, USA) in crystalline form (>95% purity). α-Ecdysone, $C_{27}H_{44}O_6$, molecular mass 464.6 (EC 3604-87-3) (**1**, Fig. 1), was dissolved in 20% methanol and water. β-Ecdysone, $C_{27}H_{44}O_7$, molecular mass 480.6 (EC 5289-74-7) (**2**, Fig. 2), was dissolved in water.

Ecdysteroid concentration was determined photometrically using a Shimadzu UV-240 double-beam spectrophotometer. The extinction coefficient for α-ecdysone was $\varepsilon_{280} = 5614\ M^{-1}\ \text{cm}^{-1}$ and for β-ecdysone $\varepsilon_{280} = 4478\ M^{-1}\ \text{cm}^{-1}$ [20].

Two-phase systems and temperature-induced phase separation

All polymer concentrations were calculated as weight percentages. The dextran T500 stock solution was 20% (w/w). The concentration of dextran was determined by polarimetry using an Optical Activity AA-10 automatic polarimeter (Optical Activity, UK) equipped with a sodium lamp set for 589 nm [2]. UCON stock solution was prepared as follows: after ultrafiltration, diluted UCON solution was placed in a water bath at 56°C for 30 min until a lower, concentrated UCON phase and an upper water phase were formed. The lower UCON phase was isolated and the UCON concentration determined using an immersion refractometer with an L1 prism (Carl Zeiss, Germany). This concentrated UCON was diluted to 40% (w/w) and used as stock solution for all experiments.

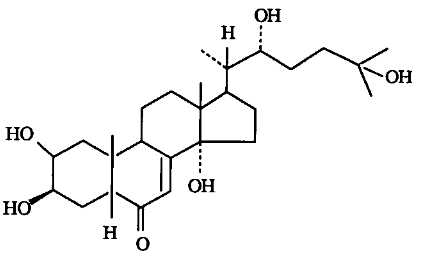

Fig. 1. Structure of α-ecdysone (compound **1**).

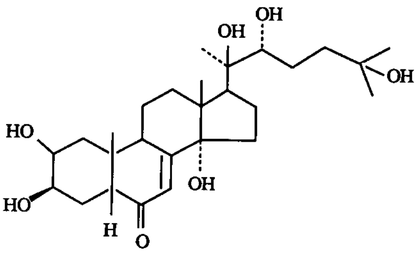

Fig. 2. Structure of 20-hydroxyecdysone (β-ecdysone; compound **2**).

Aqueous two-phase systems were prepared from the above stock solutions of polymers in water. Polymer solutions were weighed out and mixed with water, buffer, ecdysteroids and in some cases salt and/or ethanol. Phase systems were separated by centrifugation at 125 *g* for 10 min. The upper UCON-rich phase was removed and isolated in a separate container before being placed in a water bath at 56°C for 15 min. In one set of experiments (systems with 5% or 10% ethanol), sodium sulphate was added to this upper phase to a concentration of 0.1 *M* before the temperature increase. In a second set of experiments, sodium chloride was added to a concentration of either 0.05 *M* (systems with 5% or 10% ethanol) or 0.1 *M* (system with 20% ethanol) before temperature increase. This temperature increase resulted in the formation of a new phase system consisting of an upper water phase and an aqueous lower phase that was enriched in UCON [5,6]. Owing to its high viscosity, this lower UCON-rich phase was diluted by a factor of 10 before analysis, while the upper water-rich phase was analysed without dilution.

Methanol was added to a concentration of 45% to the lower, dextran-rich phase from the first partitioning step. The methanol caused dextran to precipitate out of solution, and the tubes were centrifuged for 15 min at 200 *g*. The methanol supernatant containing the ecdysteroids was removed for analysis, and the dextran pellet was discarded.

Two-phase partitioning of ecdysteroids

The partitioning of ecdysteroids between phases was determined by removing appropriate amounts from the methanol supernatant after dextran precipitation, the upper water phase at 56°C and the lower UCON-rich phase at 56°C, and subjecting them to RP-HPLC analysis (see below, *HPLC analysis*). The concentration in the upper UCON phase from the first partitioning step was calculated to be the sum of the amount of ecdysteroids obtained in the upper and lower phases formed by an increase in temperature. For all partitioning experiments, blank systems were made and samples were removed and injected to determine any interference. The partitioning of ecdysteroids between the two phases is defined by the partition coefficient, K:

$$K = C_t/C_b$$

where C_t and C_b are the concentrations of partitioned substance in the upper and lower phases, respectively, under equilibrium conditions [2]. The distribution ratio, G, is defined as:

$$G = K(V_t/V_b)$$

where V_t and V_b are the volumes of the upper and lower phases, respectively, and therefore G gives the ratio between total amount of ecdysteroid in each phase [2].

HPLC analysis

RP-HPLC was used to determine the concentration of ecdysteroids in all samples [11,24,28–30]. A Waters Delta Pak C_{18} 100-Å, 150 m × 3.9 mm I.D. column (Waters Chromatography Division, Tokyo, Japan) was coupled to a Merck-Hitachi L-6200 pump (Merck-Hitachi, Tokyo, Japan) attached to a Waters Model 441 absorbance detector with a fixed wavelength of 280 nm connected to a potentiometric recorder. Before use, the column was equilibrated with 60% methanol and water. The flow-rate was set at 0.7 ml min^{-1}, and the pressure ranged between 1680 and 1720 p.s.i. The injection volume was set at 0.1 ml.

The elution volume and absorption for ecdysteroids were determined by injecting α-ecdysone (1.0 mg ml^{-1}) and β-ecdysone (0.5 mg ml^{-1}), both separately and in a mixed sample. Elution volumes were 2.4 ml and 2.2 ml, respectively. Standard peak heights were 5.1 cm for 0.1 mg of α-ecdysone and 2.9 cm for 0.05 mg of β-ecdysone, with the detector sensitivity set at 0.2 absorbance units.

Blank injections containing no ecdysteroids were also made with UCON polymer, methanol supernatant from the dextran lower phase, the upper water phase from separation at 56°C and the lower UCON phase from separation at 56°C. None of these solutions eluted at the same volume as the ecdysteroids.

RESULTS AND DISCUSSION

Partitioning of ecdysteroids in a UCON–dextran system

Ecdysone (**1**) and 20-hydroxyecdysone (**2**) were partitioned in a two-phase system composed of 5.0% UCON 50-HB-5100, 4.0% dextran T500, and 0.012 *M* sodium phosphate buffer, pH 7.0 (Table I). The K value, which reflects the relative affinity of the compound for the two phases, is affected by many parameters, including hydrophobic interactions [2,34]. Since UCON is more hydrophobic than dextran, different partitioning behaviour can be predicted for the two ecdysteroids, depending on their hydrophobicity [35]. This trend was not reflected in their partitioning in the primary UCON–dextran system, with $K = 1.12$ for ecdysone and $K = 1.30$ for 20-hydroxyecdysone (Table IA). However, when the upper UCON phase was removed and the temperature increased to 56°C, the more hydrophobic ecdysone partitioned much more strongly to the lower UCON phase ($K = 0.59$) than 20-hydroxyecdysone ($K = 1.34$), which was enriched in the upper water phase.

Addition of salt to the two-phase systems increases hydrophobic interactions [36]. To observe this effect on the partitioning of ecdysone and 20-hydroxyecdysone, sodium chloride was added to the above system to a concentration of 0.04 *M*. As expected, this increased the affinity of ecdysone for the upper UCON-rich phase ($K = 1.21$), while the partition coefficient for more hydrophilic 20-hydroxyecdysone decreased to 1.14 (Table IB). Partitioning at 56°C between water and UCON phases for ecdysone was relatively unchanged ($K = 0.60$), while for 20-hydroxyecdysone the K value decreased to 1.04.

G values, which depend on the volume ratio between upper and lower phases, were also affected by addition of salt. This was partly the result of a slight

TABLE I

PARTITIONING OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 5.0% UCON 50-HB-5100, 4.0% dextran T500, 1.0 mg of α-ecdysone, 0.5 mg of β-ecdysone and 0.012 *M* sodium phosphate buffer, pH 7.0. *K* and *G* values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. (A) Primary system wihout sodium chloride added; (B) primary system with 0.04 *M* sodium chloride added. *Y* = percentage yield of ecdysones in the water phase. (A) Volume ratio (V_t/V_b) at 22°C = 1.8, at 56°C = 11.3; (B) volume ratio (V_t/V_b at 22°C = 1.9, at 56°C = 8.25.

Compound	*K* (22°C)	*G* (22°C)	*K* (56°C)	*G* (56°C)	*Y*
(A) Without sodium chloride					
α-Ecdysone	1.12	2.03	0.59	6.71	58.4
β-Ecdysone	1.30	2.37	1.34	15.16	65.9
(B) With sodium chloride					
α-Ecdysone	1.21	2.31	0.60	4.99	58.0
β-Ecdysone	1.14	2.17	1.04	8.56	61.3

increase in the volume of UCON phase from 0.3 ml to 0.4 ml when sodium chloride was added to 0.04 *M*. The *G* value for 20-hydroxyecdysone decreased from 15.16 to 8.56 and for ecdysone from 6.71 to 4.99.

Effect of ethanol on the partitioning of ecdysteroids

Ecdysone and 20-hydroxyecdysone were partitioned in a two-phase system composed of 6.0% UCON, 5.0% dextran T500 and 0.012 *M* sodium phosphate buffer, pH 7.0, and either 5.0%, 10.0% or 20.0% ethanol (Tables II and III). Since UCON is more hydrophobic than dextran, ethanol can be expected to partition preferentially to the upper UCON phase, thereby further increasing the hydrophobicity of this phase. After the UCON and dextran phases had separated, the upper UCON phase was isolated in a separate container and sodium chloride was added to this phase to a concentration of 0.05 *M* (Table II). For the 20.0% ethanol system sodium chloride was added to this upper phase to 0.1 *M* (Table III). The addition of salt was necessary in order to obtain phase separation when ethanol had been included in the original two-phase system. This UCON–water–salt solution was placed in a water bath at 56°C for 15 min to achieve formation of a new two-phase system.

In Fig. 3 the log *K* values at 22°C for ecdysone

TABLE II

PARTITIONING OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 6.0% UCON 50-HB-5100, 5.0% dextran T500, 1.0 mg of α-ecdysone, 0.5 mg of β-ecdysone and 0.012 *M* sodium phosphate buffer, pH 7.0; 0.05 *M* sodium chloride was added to the aqueous UCON phase prior to increasing the temperature. *K* and *G* values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. (A) Primary system with 5.0% ethanol added; (B) primary system with 10.0% ethanol added. *Y* = percentage yield of ecdysones in the water phase. (A) Volume ratio (V_t/V_b) at 22°C = 3.25, at 56°C = 5.5; (B) Volume ratio (V_t/V_b) at 22°C = 2.60, at 56°C = 4.8.

Compound	*K* (22°C)	*G* (22°C)	*K* (56°C)	*G* (56°C)	*Y*
(A) With 5% ethanol					
α-Ecdysone	1.28	4.15	0.17	0.95	39.3
β-Ecdysone	1.04	3.38	0.39	2.17	52.8
(B) With 10% ethanol					
α-Ecdysone	1.79	4.72	0.43	2.07	55.6
β-Ecdysone	1.34	3.54	0.68	3.30	59.9

TABLE III

PARTITIONING OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 6.0% UCON 50-HB-5100, 5.0% dextran T500, 1.0 mg α-ecdysone, 0.5 mg of β-ecdysone, 20.0% ethanol and 0.012 *M* sodium phosphate buffer, pH 7.0; 0.1 *M* sodium chloride was added to the aqueous UCON phase prior to increasing the temperature. *K* and *G* values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. *Y* = percentage yield of ecdysones in the water phase. Volume ratio (V_t/V_b) at 22°C = 4.0, at 56°C = 6.3.

Compound	*K* (22°C)	*G* (22°C)	*K* (56°C)	*G* (56°C)	*Y*
α-Ecdysone	3.01	12.02	0.62	3.94	73.6
β-Ecdysone	3.18	12.72	1.91	12.09	85.6

and 20-hydroxyecdysone obtained from Tables IA, IIA, IIB and III have been plotted as a function of ethanol concentration. For ecdysone log *K* increased from 0.05 with no ethanol to 0.48 with 20% ethanol. Log *K* for 20-hydroxyecdysone exhibited a different tendency, first decreasing with 5% ethanol (log *K* = 0.02), then increasing to 0.50 when 20% ethanol was included in the system. The different partitioning behaviour of ecdysteroids with 5% ethanol most likely reflects their different hydrophobicities. While ecdysone was drawn more strongly into the hydrophobic UCON phase, 5% ethanol had much less effect on 20-hydroxyecdysone, even pushing it slightly more into the hydrophilic dextran phase. However, as the concentration of ethanol was raised this effect diminished, with both

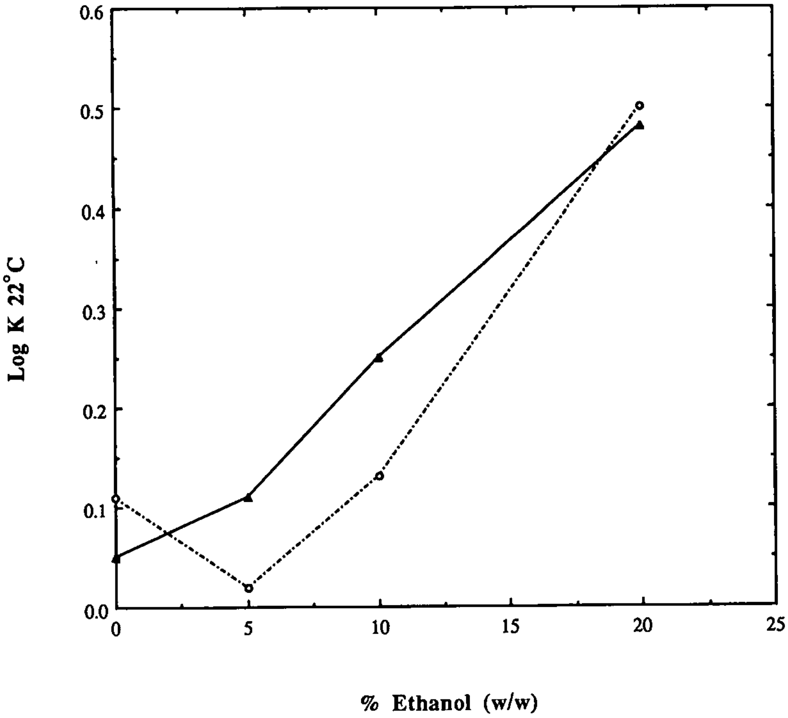

Fig. 3. Effect of ethanol concentration on the partioning at 22°C of α-ecdysone (▲) and β-ecdysone (○). Two-phase systems containing ethanol were composed of 6.0% UCON 50-HB-5100, 5% dextran T500 and 0.01 *M* sodium phosphate buffer, pH 7.0. The system without ethanol was 5.0% UCON 50-HB-5100, 4.0% dextran T500 and 0.01 *M* sodium phosphate buffer, pH 7.0. Data were obtained from Tables IA, IIA, IIB and III.

ecdysteroids being drawn increasingly into the upper UCON-rich phase.

In Fig. 4 log K values from Tables IB, IIA, IIB and III for the partition of ecdysone and 20-hydroxyecdysone at 56°C have been plotted as a function of ethanol concentration. At 56°C it is harder to determine the effect of ethanol on the partitioning of ecdysteroids owing to addition of salt to this upper phase. Partitioning of both ecdysteroids at 56°C to the UCON-rich phase increased dramatically with the addition of 5% ethanol (log $K = -0.77$ for ecdysone and -0.41 for 20-hydroxyecdysone), while further addition of ethanol increasingly pulled them into the upper water–ethanol–buffer phase. At 20% ethanol the partition coefficient at 56°C for 20-hydroxyecdysone was higher (log K = 0.28) than when no ethanol had been added (log K = 0.02). For ecdysone the two values were roughly equal, with log $K = -0.22$ when no ethanol was present and log $K = -0.21$ when 20% ethanol was included in the system. This result was consistent throughout the experiments, with the more hydrophobic ecdysone displaying a greater affinity for the UCON phase than 20-hydroxyecdysone.

The yield of ecdysteroids was calculated from the amount of ecdysteroid recovered in the upper water phase after separation at 56°C. For the phase system without ethanol or salt added, the yield of ecdysone was 58.4% and of 20-hydroxyecdysone 65.9% (Table IA). Addition of sodium chloride to the system lowered the yield of ecdysone to 58.0% and of 20-hydroxyecdysone to 61.3% (Table IB). When 5% ethanol was included in the system the yields of ecdysone and 20-hydroxyecdysone were 39.3% and 52.8%, respectively (Table IIA). Increasing ethanol to 10% increased the yield for ecdysone to 55.6% and for 20-hydroxyecdysone to 59.9% (Table IIB). The best yield of both ecdysones

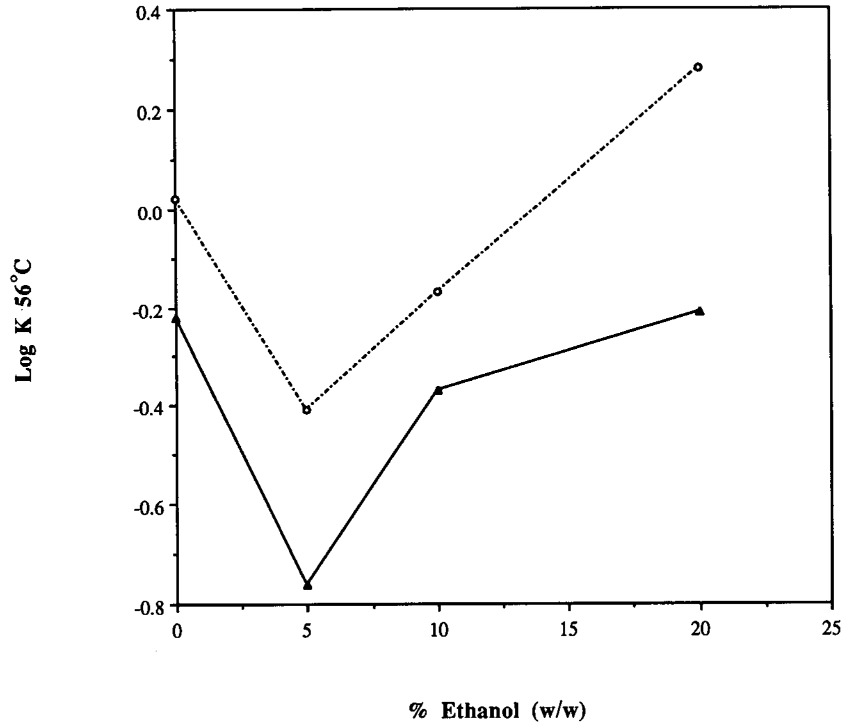

Fig. 4. Effect of ethanol concentration on the partitioning at 56°C of α-ecdysone (▲) and β-ecdysone (○). Primary systems were the same as described in Fig. 3. Sodium chloride was added to the removed upper phase from primary separation before increasing the temperature. The salt concentrations were as follows: 0.04 M, upper phase from primary system with no ethanol; 0.05 M, upper phase from primary systems with 5% and 10% ethanol; 0.1 M, upper phase from primary system with 20% ethanol. Data were obtained from Tables IB, IIA, IIB and III.

was obtained in a system which included 20% ethanol where recovery for ecdysone was 73.6% and for 20-hydroxyecdysone 85.6% (Table III).

Addition of sodium sulphate to two-phase systems containing ethanol

Adding a salt containing a divalent anion, sodium sulphate (0.1 *M*), to the upper UCON-rich phase before increasing the temperature had an effect on the partitioning of ecdysteroids. Partitioning experiments were carried out in systems containing either 5% or 10% ethanol (Table IV). In the system with 5% ethanol, K at 56°C was 0.25 for ecdysone and 0.46 for 20-hydroxyecdysone. K values at 56°C for the system with 10% ethanol were lower (K = 0.26 for ecdysone and 0.52 for 20-hydroxyecdysone) than in a system with 10% ethanol and sodium chloride (K = 0.43 for ecdysone and 0.68 for 20-hydroxyecdysone, Table IIB). These results reflect an increased hydrophilicity of upper water–salt phases at 56°C when sodium chloride was replaced with sodium sulphate. The yield of ecdysteroids was also fairly low in systems containing sodium sulphate. With 5% ethanol the yield was 47.7% for ecdysone and 56.2 for 20-hydroxyecdysone. Increasing the ethanol concentration to 10% had little effect on total recovery of ecdysteroids, with yields of 46.4% for ecdysone and 58.6% for 20-hydroxyecdysone.

TABLE IV

PARTITIONING AT 56°C OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 6.0% UCON 50-HB-5100, 5.0% dextran T500, 1.0 mg of α-ecdysone, 0.5 mg of β-ecdysone and 0.012 *M* sodium phosphate buffer, pH 7.0; 0.1 *M* sodium sulphate was added to the aqueous UCON phase prior to increasing the temperature. K and G values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. (A) Primary system with 5.0% ethanol added; (B) primary system with 10.0% ethanol added. Y = percentage yield of ecdysones in the water phase. (A) Volume ratio (V_t/V_b) at 56°C = 5.3; (B) volume ratio (V_t/V_b) at 56°C = 4.4.

Compound	K (56°C)	G (56°C)	Y
(A) With 5% ethanol			
α-Ecdysone	0.25	1.35	47.7
β-Ecdysone	0.46	2.46	56.2
(B) With 10% ethanol			
α-Ecdysone	0.26	1.16	46.4
β-Ecdysone	0.52	2.32	58.6

Purification scheme for ecdysone and 20-hydroxyecdysone

The results obtained in partitioning these ecdysteroids show that it would be possible to purify them using temperature-induced phase separation. A purification scheme for enzymes using this technique has already been developed [5,6]. In these studies it has been shown that in an initial UCON–dextran or UCON–hydroxypropyl starch phase system most proteins, cell particles and other cell debris partition strongly to the lower phase, leaving an upper UCON phase with little contamination. Based on these findings, it can be expected that when a cell homogenate containing ecdysteroids is added to a UCON–dextran or UCON–hydroxypropyl starch two-phase system the ecdysteroids will partition to the upper UCON-rich phase, while most proteins and other macromolecules will partition to the lower dextran-rich or hydroxypropyl starch-rich phase. Also, addition of ethanol to the phase system should increase this effect, as increased hydrophobicity of upper UCON-rich phase would push hydrophilic biomolecules even more into the lower phase at 22°C and draw the hydrophobic ecdysteroids more into the UCON upper phase. This will result in even greater purification of ecdysteroids. The lower dextran or hydroxypropyl starch phase goes to waste. The upper UCON phase is removed and placed in a water bath at 56°C for 15 min. This temperature increase forms a new two-phase system with an upper water–buffer phase and a lower, highly viscous UCON phase. Ecdysteroids which are recovered in this water phase can be used without further purification. The lower UCON phase is recovered and can be used for future extractions. A system composed of 6.0% UCON 50-HB-5100, 5.0% dextran T500, 20% ethanol and 0.012 *M* sodium phosphate buffer, pH 7.0, shows greatest promise as a possible purification technique for these ecdysteroids (Table III). K values were the highest observed at both 22°C and 56°C. The percentage recovery (yield) in the final water–buffer phase was also very high, 73.6% for ecdysone and 85.6% for 20-hydroxyecdysone. The present purification techniques use high concentrations of organic solvents and repeated extractions, with normal yields of 50–80% [25,28]. However, this study shows that a high yield and purity of ecdysteroids can be achieved by using an aqueous two-phase system combined with temperature-induced phase separation.

CONCLUSIONS

It is possible to partition water-soluble ecdysteroids in an aqueous two-phase system composed of UCON 50-HB-5100 and dextran T500. The use of temperature-induced phase separation allows these molecules to be recovered in an aqueous phase composed of water and buffer that is free of polymers or other contaminants. Since the ecdysteroids are recovered in a water–buffer phase virtually free of contaminants or organic solvents, it should be possible to use them without further purification. The high level of ecdysteroids recovered, 73.6% and 85.6% for α-ecdysone and β-ecdysone, respectively, would allow their detection even when present in small quantities. For these reasons, the use of aqueous two-phase partition combined with temperature-induced phase separation offers great potential as either an analytical or preparative technique for ecdysteroids.

The partitioning behaviour of ecdysteroids in both the primary phase system and in the new temperature-induced system can be manipulated by addition of salt or ethanol. Because of their different hydrophobicities, addition of a small amount of salt or ethanol had a different effect on the partitioning behaviour of ecdysone and 20-hydroxyecdysone. The distribution of the molecules seemed to be influenced by hydrophobic interactions with the upper UCON-rich phase in the primary system and with the lower UCON-rich phase at 56°C. In almost all instances, the more hydrophobic ecdysone partitioned more strongly to the UCON-rich phases than did 20-hydroxyecdysone.

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Liquid chromatography–electrospray ionization-mass spectrometry of cyanobacterial toxins

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ABSTRACT

Cyanobacterial neurotoxins such as anatoxin-a and saxitoxin, peptide hepatotoxins including microcystin variants and nodularin were studied by electrospray ionization-mass spectrometry following direct infusion of samples into the mass spectrometer. This technique was further exemplified when analysing the cyclic hepatotoxic peptides by means of on-line liquid chromatography–mass spectrometry. Peptide toxins were identified in extracts from toxic bloom material and cell cultures by comparison of spectral and chromatographic data to that of authentic materials. When authentic materials were not available, several peptides were tentatively identified by their molecular-weight information provided by this technique.

INTRODUCTION

Many freshwater cyanobacteria (blue-green algae) produce toxins (Fig. 1) which include: (a) the cyclic heptopeptides of microcystin and its analogues; (b) the cyclic pentapeptide nodularia toxin and (c) the neurotoxic alkaloids anatoxins and saxitoxin. Microcystins have the common structure cyclo(-D-Ala–L-X–D-MeAsp–L-Y–Adda–D-Glu–Mdha) where X and Y are variable amino acids (labelled X and Y, Fig. 1) [1], Nodularin contains four of the five amino acids found in microcystin and dehydrobutyrine [2]. Blue-green algae are found in eutrophic lakes and drinking water reservoirs and have been responsible for the deaths of domestic, farm and wild animals in many countries over several decades [3–5]. Consumption of drinking water contaminated by the toxins can seriously affect human health, and there is an interest in the tumor-promoting potential of the microcystins [6]. Complications arise when more than one species of cyanobacteria co-exist in the algal bloom, and in addition each species can produce more than one kind of toxin. Hence there is a need to detect, quantify and confirm the identity of these compounds for safety evaluation.

The most commonly used analytical method available for detecting the peptide hepatotoxins is high-performance liquid chromatography (HPLC) with ultraviolet detection. Those peaks collected which were shown to be toxic to mice were further characterised by mass spectrometry (MS), using fast atom bombardment ionization (FAB) [7]. Gas chromatography–mass spectrometry (GC–MS) seems to be a suitable technique for analysing anatoxins, but the toxins must be derivatized prior to

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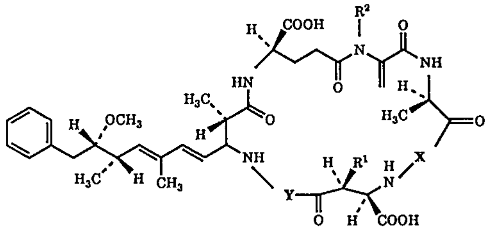
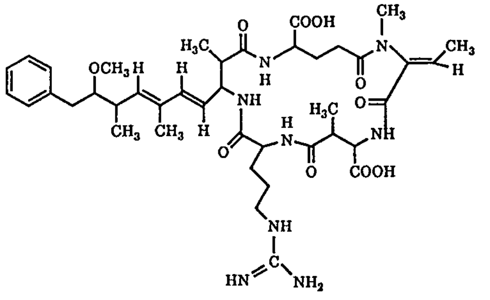
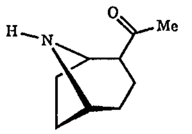


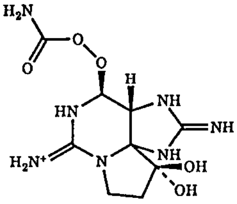
					M_r
MCYST-LR:	X = Leu;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	994
MCYST-FR:	X = Phe;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1028
MCYST-RR:	X = Arg;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1037
MCYST-RR: ([D-Asp^3])	X = Arg;	$R^1 = H$;	Y = Arg;	R^2 = CH3	1023
MCYST-RR: ([D-Asp^3,Dha^7])	X = Arg;	$R^1 = H$;	Y = Arg;	$R^2 = H$	1009
MCYST-YR:	X = Tyr;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1044
MCYSY-M(O)R:	X = Met(O);	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1028
MCYST-WR:	X = Trp;	$R^1 = CH_3$;	Y = Arg;	$R^2 = CH_3$	1067



Nodularin



(+)-Anatoxin-a



Saxitoxin

Fig. 1. Structures of cyanobacterial toxins. MCYST = microcystin.

analysis [8–10]. Saxitoxin and its analogues are routinely analysed by fluorescence detection of their oxidised products [11].

It is feasible to study the peptide class of toxins by on-line LC–MS using FAB ionization [generally known as continuous flow (CF)-FAB] [12,13]. Since FAB requires matrices to assist ionization it is necessary either to develop a HPLC method with the matrix being incorporated into the mobile phase, or to introduce the matrix coaxially into the mass spectrometer ion source [14]. Care has to be taken to ensure a steady film of liquid being delivered to the FAB target. The amount of sample required by FAB to acquire a spectrum of the purified toxin is in the nanogram range.

Recently Fenn and co-workers [15,16] have successfully interfaced electrospray ionization with mass spectrometry (ESI–MS), a development which has proved invaluable in the analysis of thermally labile polar molecules, especially for peptides and proteins. This report demonstrates that on-line ESI-MS is a simple, specific, reliable and rapid technique to monitor precisely and simultaneously various algal toxins in extracts from laboratory cultures or algal blooms present in eutrophic waters.

EXPERIMENTAL

Chemicals

Anatoxin-a was obtained from Biometric Systems (Eden Prairie, MN, USA). Saxitoxin was purchased from Calbiochem Novabiochem (Nottingham, UK). Microcystin-LR and microcystin-desmethyl-3,7-RR were purified from lyophilized cells of *Microcystis aeruginosa* PCC 7813 and Nodularin was purified from a laboratory culture of *Nodularia spumigena* as described by Lawton [17]. Microcystin-RR was a gift from Professor H.W. Siegalman (Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA).

HPLC grade solvents and glacial acetic acid were purchased from Romil Chemicals (Shepshed, UK) and Fisons PLC (Loughborough, UK), respectively.

Algal material

Microcystis aeruginosa PCC 7813 was grown in batch culture on BG 11 media as described [18]. Cells were harvested by continuous centrifugation and freeze dried. Toxic bloom material containing *Microcystis aeruginosa* was collected from Hartbeespoort Dam in South Africa in August 1987 and Water Lane fish farm, Wessex, UK in November 1990. Toxic bloom material containing *Nodularia spumigena* was collected from the River Humber in 1990.

Preparation of samples from algal cells

Lyophilized cells (0.5 g/50 ml) were extracted twice in 5% (w/v) acetic acid for 30 min with continuous agitation. The extracts were centrifuged at 3000 *g* for 10 min. Both supernatants were pooled and filtered through a GF/C filter to remove particulate material. The filtrate was applied to a preconditioned environmental C_{18} Sep-Pak (Millipore) at a rate of 2 ml/min. The cartridge was washed with 20 ml 25% methanol and the peptides were eluted with 100% methanol. Solvent was removed *in vacuo* at 40°C and residues were retained for analysis. For the PCC 7813 0.5 g freeze dried material, 1/2000 μl was used for analysis, whereas for the *Nodularia*, South African and fish farm studies, each injection contained approximately 100, 135 and 400 ng equivalent amount of nodularin or microcystin-LR toxins, respectively (assessed by their extinction coefficients from the UV spectra).

Detection of cyanobacterial toxins by ESI-MS

Authentic toxin standards were dissolved in a mixture of methanol–water (50:50, v/v) containing 1% acetic acid. Each of these solutions was infused at 1 μl/min with a Harvard Apparatus infusion pump (model No. 22, Harvard Apparatus, Cambridge, MA, USA) into a Finnigan TSQ 700 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) fitted with an electrospray ion source (Analytica, Branford, CT, USA). A potential difference of 3500–3600 V was applied between the grounded needle and the metalized ends of the glass capillary tube that passes ions into the analyser. Nitrogen heated to 60°C was used as the drying gas. Data analysis was controlled by DEC 2100 data system. The deconvolution program is based on the Finnigan ICIS software version 5 [19]. Full scan spectra were acquired in the positive ion peak centroid or profile modes over the mass range of *m/z* 400–1200 at 6 s/scan.

Detection of cyanobacterial peptides by LC-ESI-MS

The HPLC system used was an ABI model 140A dual syringe pump (Applied Biosystems, San Jose, CA, USA) fitted with a Rheodyne 7125 injection valve equipped with a 5-μl sample loop. Separation was achieved using a 150 x 1 mm I.D. microbore column packed with Spherisorb 5-μm ODS material (Phase Separations Ltd., Clwyd, UK). The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% acetic acid. A gradient elution was employed: 0–10 min 40% B; 10–20 min, 40–55% B; 20–35 min, 55–65% B and then isocratic for 5 min at 65% B. The flow-rate was maintained at 45 μl/min. The effluent was directed to a Valco tee splitter. One outlet was connected directly with a fused silica capillary (1 m × 100 μm I.D., J & W Scientific) to the UV detector (Applied Biosystems, Model 785A) fitted with a micro flow cell (2.4 μl volume, 6 mm pathlength). The eluent was monitored by UV detection at 240 nm. The other outlet was linked to the stainless steel electrospray needle via a fused silica capillary 1.5 m × 50 μm I.D. The split ratio was maintained at 11:1 (UV:MS), so that approximately 3.5 μl/min of the effluent entered the ESI ion source. Full scan mass spectra were obtained in the peak centroid mode over the mass range of *m/z* 400–1200 at 6 s/scan. The individual samples were dissolved in an appropriate volume of methanol and an aliquots of 1 μl were analysed by LC–MS.

RESULTS

Detection of cyanobacterial toxins by ESI-MS

The mass spectrum obtained by direct infusion of anatoxin-a in methanol containing 1% acetic acid gave a strong protonated molecule at *m/z* 166 $[M + H]^+$ and a natriated adduct at *m/z* 188 $[M + Na]^+$ (Fig. 2a). A weak signal at *m/z* 181 suggested the presence of a methylene analogue of anatoxin. This component has been characterised by Skulberg *et al.* [20], but further studies are required to confirm its presence.

The electrospray mass spectrum of saxitoxin is presented in Fig. 2b, approximately 2 picomol sample was consumed to give this full scan spectrum. The protonated molecule at *m/z* 300 and the methylated adduct at *m/z* 314 dominate the spectrum. The fragment ion at *m/z* 282 could be attrib-

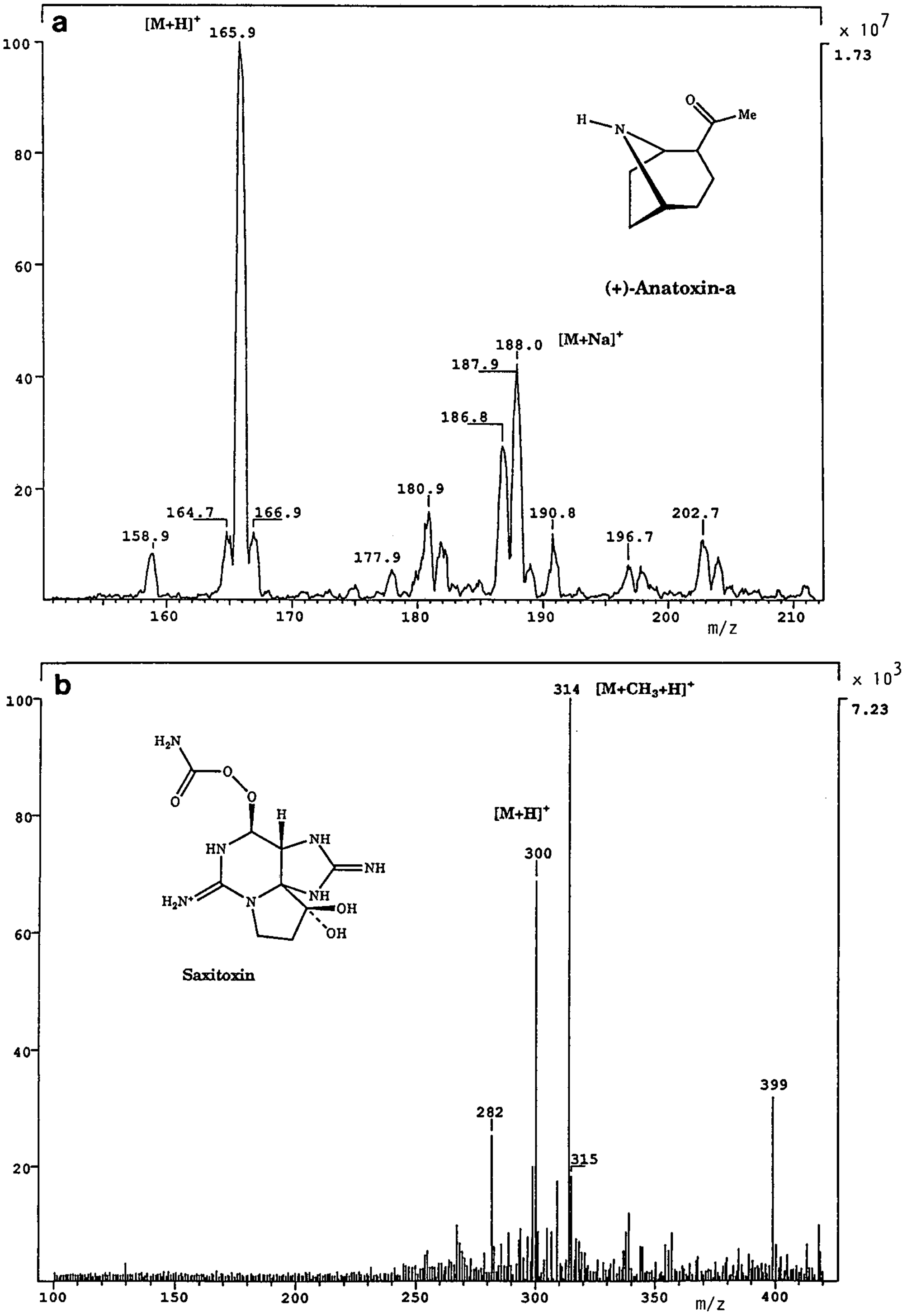

a
[M+H]⁺ 165.9
x 10^7
1.73
H
N
O
Me
(+)-Anatoxin-a
188.0 [M+Na]⁺
187.9
186.8
180.9
164.7
166.9
158.9
177.9
190.8
196.7
202.7
100
80
60
40
20
160
170
180
190
200
210
m/z
b
314 [M+CH3+H]⁺
x 10^3
7.23
H2N
O
O
O
H
HN
NH
NH
H2N+
N
NH
OH
OH
Saxitoxin
[M+H]⁺
300
282
315
399
100
80
60
40
20
100
150
200
250
300
350
400
m/z

Fig. 2.

uted to either loss of water from m/z 300 or the loss of methanol from m/z 314. A similar observation has been reported by Quilliam *et al.* [21] during LC–ionspray-MS analysis.

When the peptide toxins are analysed by ESI-MS, compounds with molecular weights less than 1000 are more likely to give strong singly charged protonated molecules $[M + H]^+$. This can be illustrated by nodularin which has a molecular weight of m/z 824 (figure 2c), whereas for toxins with molecular weights over 1000, their corresponding doubly charged ions $[M + 2H]^{2+}$ predominate. In the case of microcystin-LR, the singly charged ($[M + H]^+$ at m/z 995), doubly charged ($[M + 2H]^{2+}$ at m/z 498) ions as well as the natriated ($[M + H + Na]^{2+}$ at m/z 509) and kaliated ($[M + H + K]^{2+}$ at m/z 517) adducts of the doubly charged ions were all observed in the spectrum (Fig. 2d). A deconvoluted spectrum around the doubly charged cluster ions at m/z 500 gave m/z 995 ($[M + H]^+$), m/z 1017 ($[M + Na]^+$) and m/z 1033 ($[M + K]^+$) (Fig. 2e). Approximately 100 femtomoles of the sample were consumed to obtain a full scan mass spectrum, with signal-to-noise level in the order of 20:1 or better. The amount consumed to produce a full scan spectrum could easily be reduced.

Detection of cyanobacterial peptides by LC-ESI-MS

For the LC–MS system, a split ratio of 11:1 was applied and 3.5 μl/min of the LC eluent was directed into the mass spectrometer. The rest of the flow was diverted to the UV detector fitted with a 2.4-μl flow cell. Since a capillary flow cell (90 nl) suitable to monitor a low LC flow-rate was not available, the peaks on the chromatograms tended to be rather broad. There was a 20-s delay in receiving the signal at the mass spectrometer compared to the UV detector. The five standards available for this study, mcyst-LR, mcyst-YR, mcyst-desmethyl-3,7-RR, mcyst-RR and nodularin, were separated by reversed phase LC with mass spectrometric and UV detection (Fig. 3a and b). Although the compounds did not have baseline separation on the UV chromatogram, the reconstructed ion chromatogram

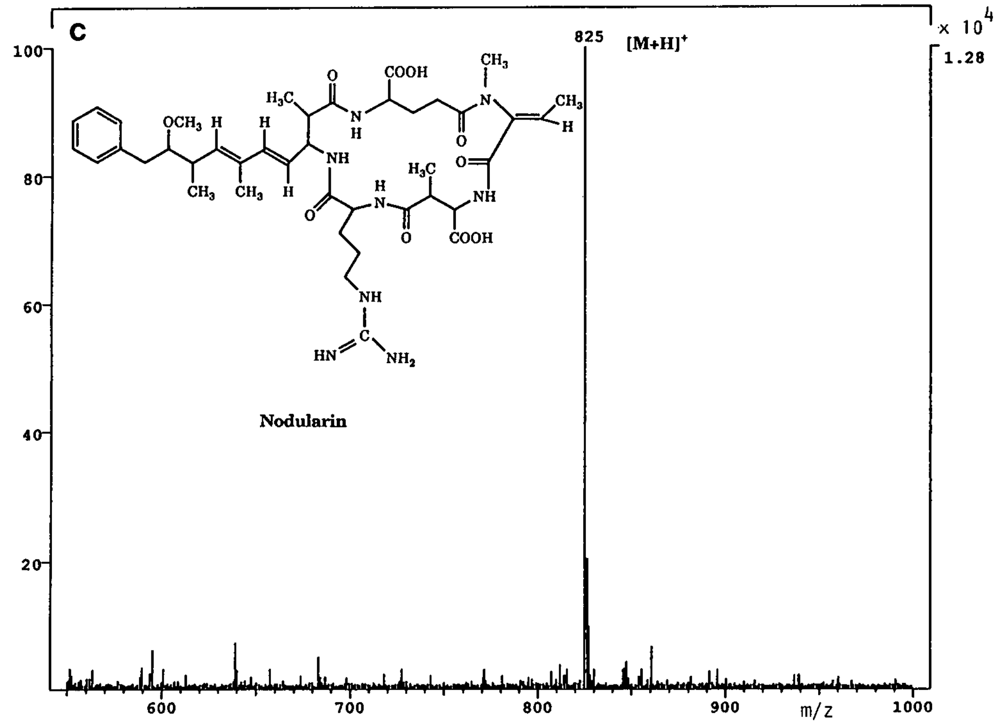


Fig. 2.

(Continued on p. 220)

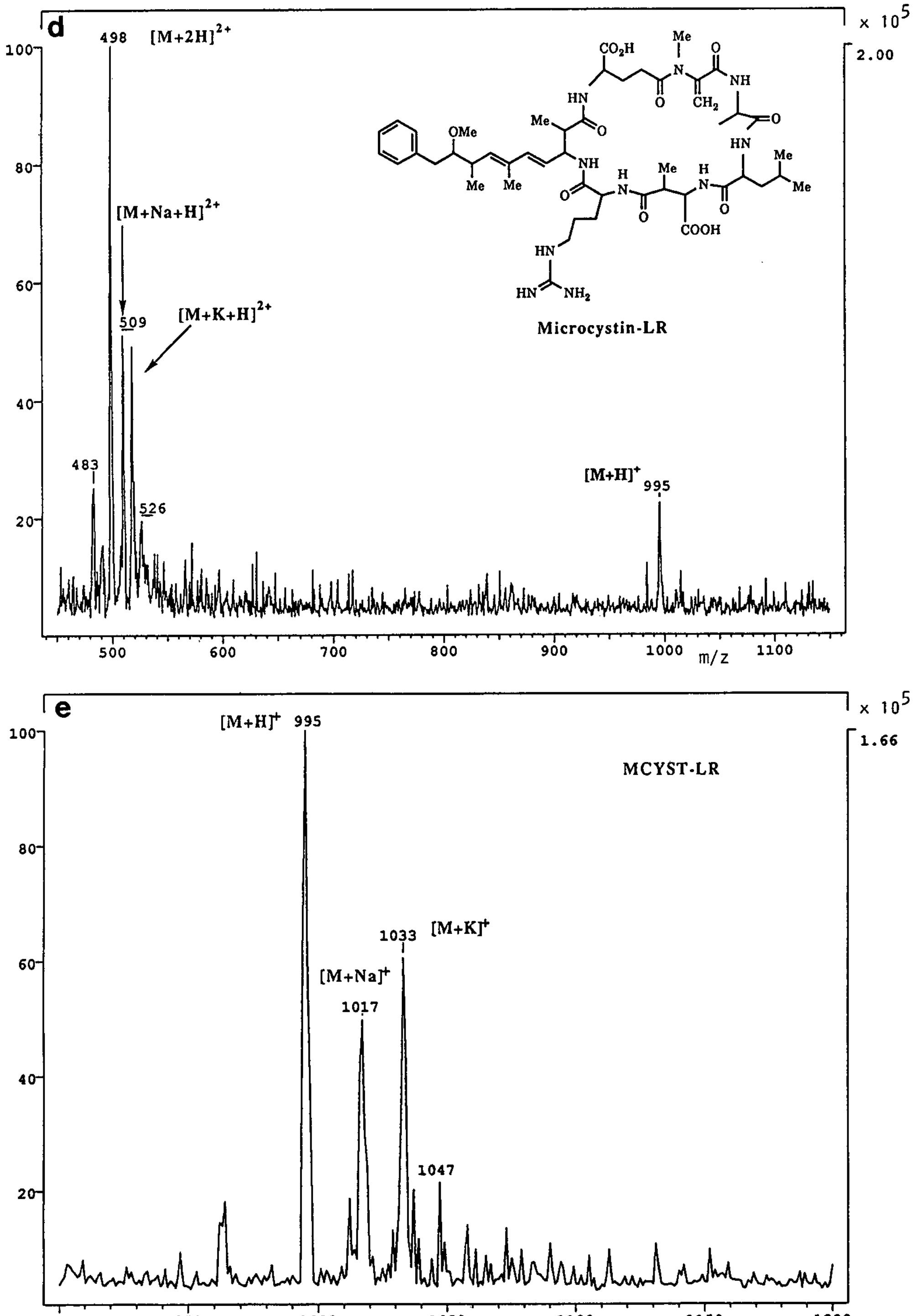


Fig. 2. Electrospray mass spectra obtained from freshwater cyanobacterial toxins, (a) (+)-anatoxin-a, (b) saxitoxin, (c) Nodularin and (d) microcystin-LR. (e) Deconvolution of the spectrum around the doubly charged cluster ions at m/z 500 gave m/z 995 ($[M + H]^+$), m/z 1017 ($[M + Na]^+$) and m/z 1033 ($[M + K]^+$).

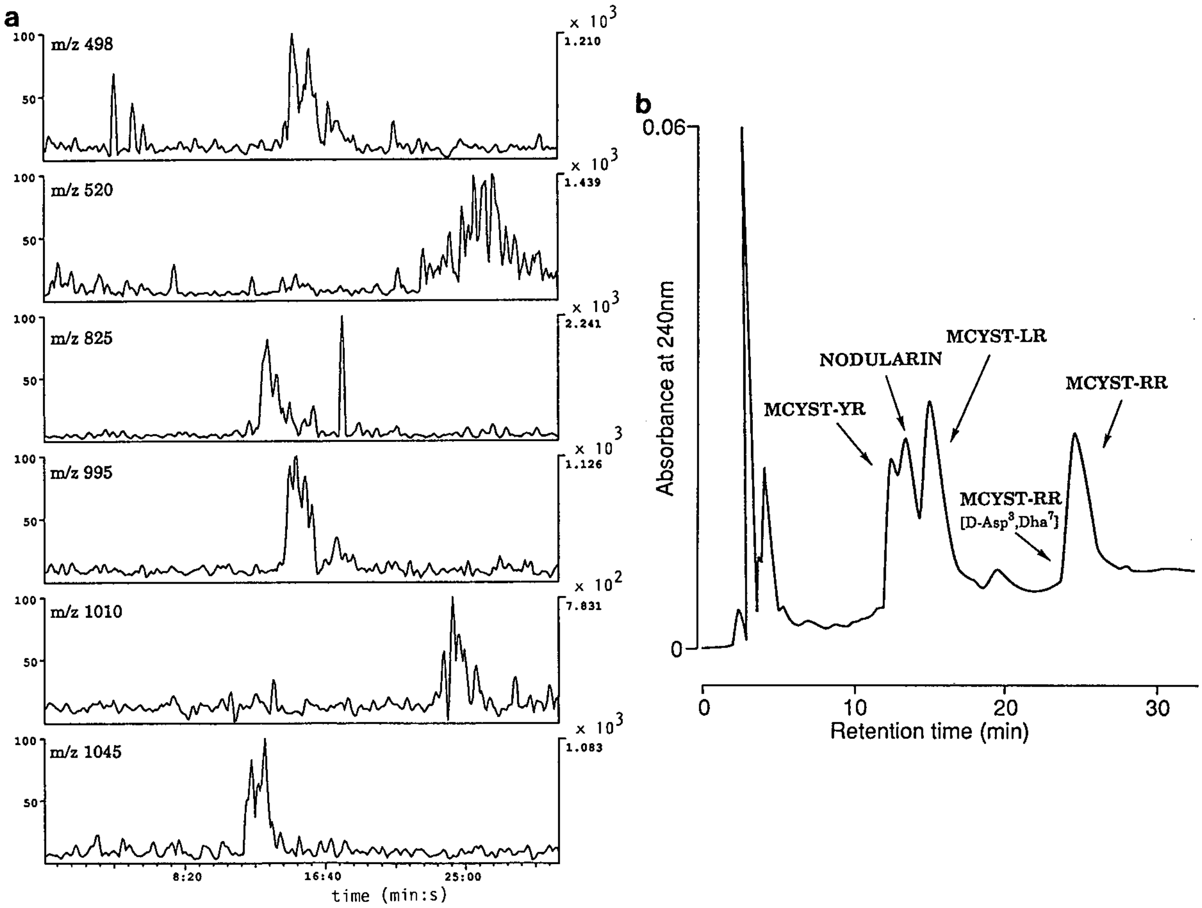


Fig. 3. Separation of toxic peptide standards by reversed-phase LC and displayed as (a) reconstructed ion chromatograms; (b) LC chromatogram with UV detection.

shows the compounds to be adequately resolved. They were easily recognised either as their singly or doubly charged protonated ions. Nodularin had a mass spectrum with a predominant peak at *m/z* 825 ($[M + H]^+$) (data not shown). The mass spectra of mcyst-LR, -YR and desmethyl-3,7-RR consisted of doubly and singly charged ions. Mcyst-RR only formed a doubly charged ion at *m/z* 520 $[M + 2H]^{2+}$ but deconvolution of this ion gave the protonated ion at *m/z* 1038.

Once the system was shown to be satisfactory with the known standards, extracts from cyanobacterial cultures and blooms were analysed.

The extract from the bloom material collected from Hartbeespoort Dam contained several microcystin variants as shown by the reconstructed ion chromatograms and UV chromatogram (Fig. 4a and b). At t_R 13.30 min mcyst-YR was observed which gave a protonated ion at *m/z* 1045.7 and a doubly charged ion at *m/z* 523. Mcyst-LR was detected at t_R 16.00 min with *m/z* 995, followed by mcyst-FR at t_R 20 min. The mass spectrum of mcyst-FR contained mainly a singly charged protonated ion at *m/z* 1029 and a weak signal (20% intensity) of its doubly charged ion at *m/z* 514. Mcyst-WR was detected at t_R 21.30 min (*m/z* 1069). Finally a broad peak of mcyst-RR was present as its doubly charged ion at *m/z* 520. All toxins mentioned above provide very good mass spectra as shown in Fig. 5.

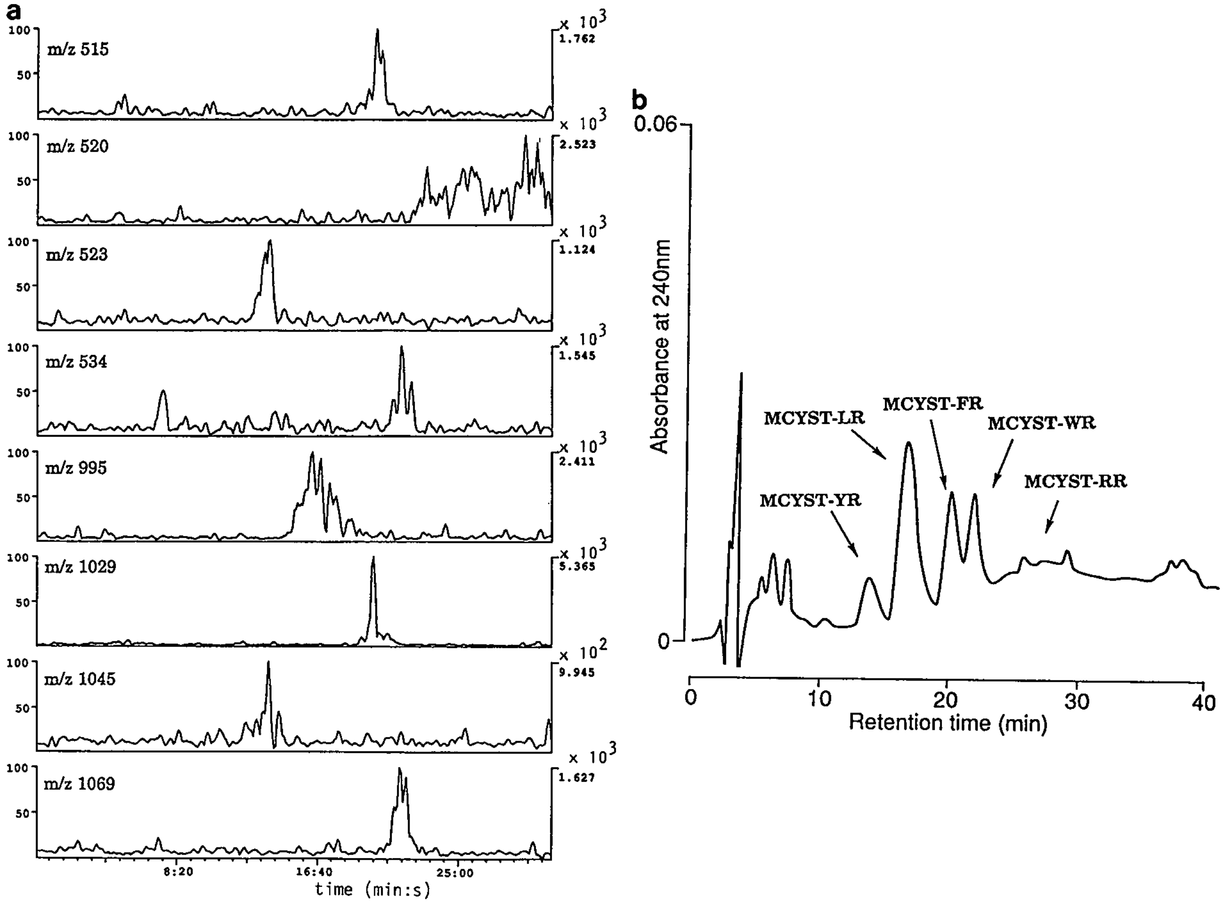


Fig. 4. Separation of microcystins in an extract from bloom material from Hartbeespoort Dam, South Africa. (a) Reconstructed ion chromatograms; (b) LC chromatogram with UV detection.

Analysis of the extract of the bloom containing predominantly *Nodularia spumigena* showed the presence of the pentapeptide, nodularin at t_R 12.70 min (Fig. 6a and b). Examination of the reconstructed ion chromatograms (Fig. 6a) demonstrated the presence of trace amounts of mcyst-LR (*m/z* 995 at t_R 16.00 min) and possibly mcyst-RR [D-Asp^3] at t_R 28 min which had a mass spectrum with a prominent ion at *m/z* 512 and a deconvoluted spectrum with the protonated ion at *m/z* 1024 (Fig. 6d).

Several microcystin analogues were detected in the extract from the bloom material collected from Water Lane Fish Farm (Fig. 7a and 7b) including mcyst-LR and -YR at t_R 11.30 min and t_R 14.80 min, respectively. Several other peaks were detected which may be other microcystin variants. A weak spectrum with *m/z* 1029 was tentatively identified as mcyst-M(O)R (Fig. 7c). At low concentration, no *m/z* 515 doubly charged ion was obtained. However at a high concentration, the *m/z* 515 ion was present, but it was masked by the two abundant doubly charged ions at *m/z* 537 and 519 (data not shown). This component has the same molecular weight as mcyst-FR but differs in retention time. The relatively short retention time suggests the presence of a sulphoxide functional group in the molecule. Another component was detected at t_R 12.30 min which had a strong doubly charged ion at *m/z* 519 and a deconvoluted ion at *m/z* 1037. This compound could be mcyst-L-Harg (Fig. 7d and e) as

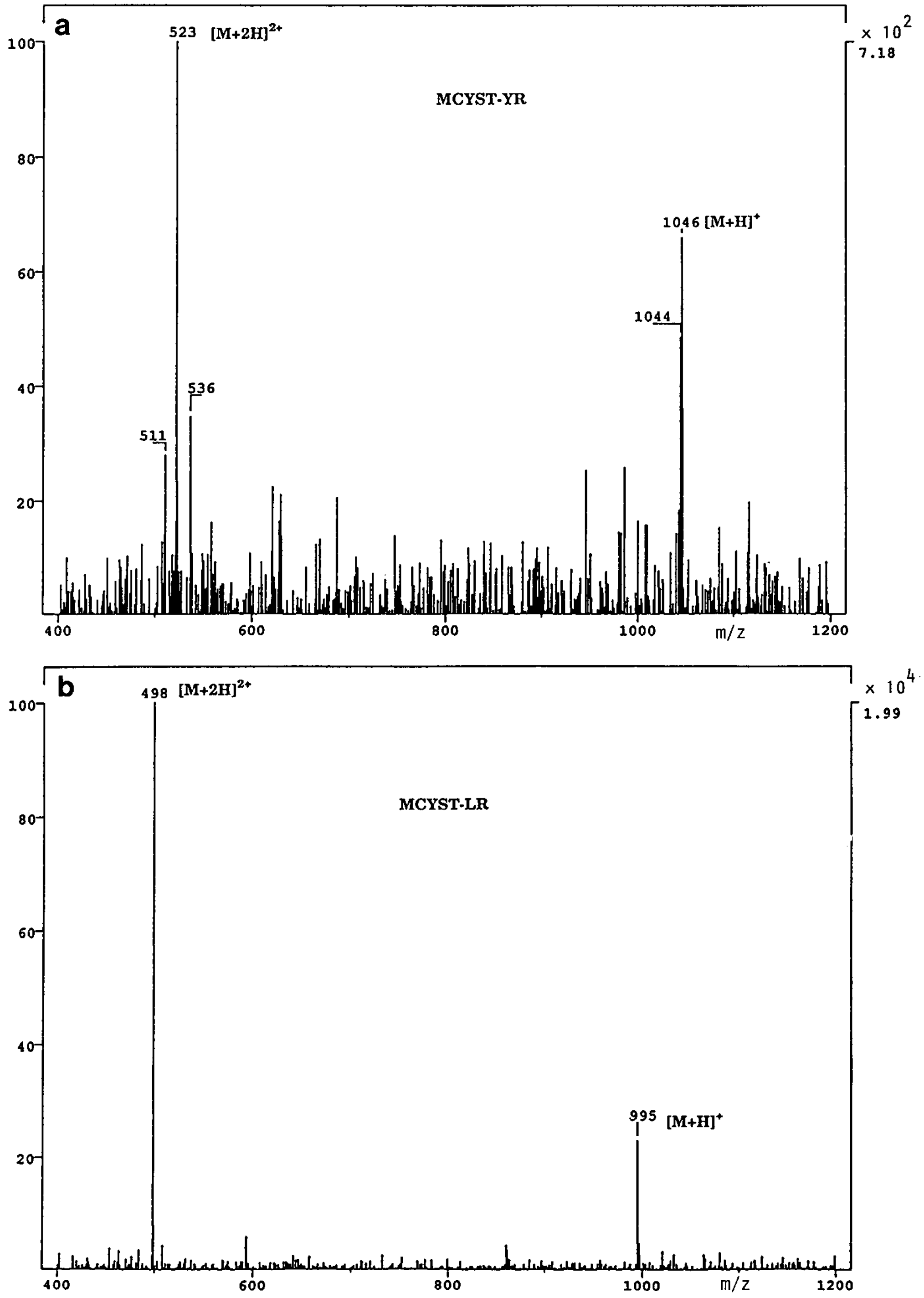


Fig. 5.

(*Continued on p. 224*)

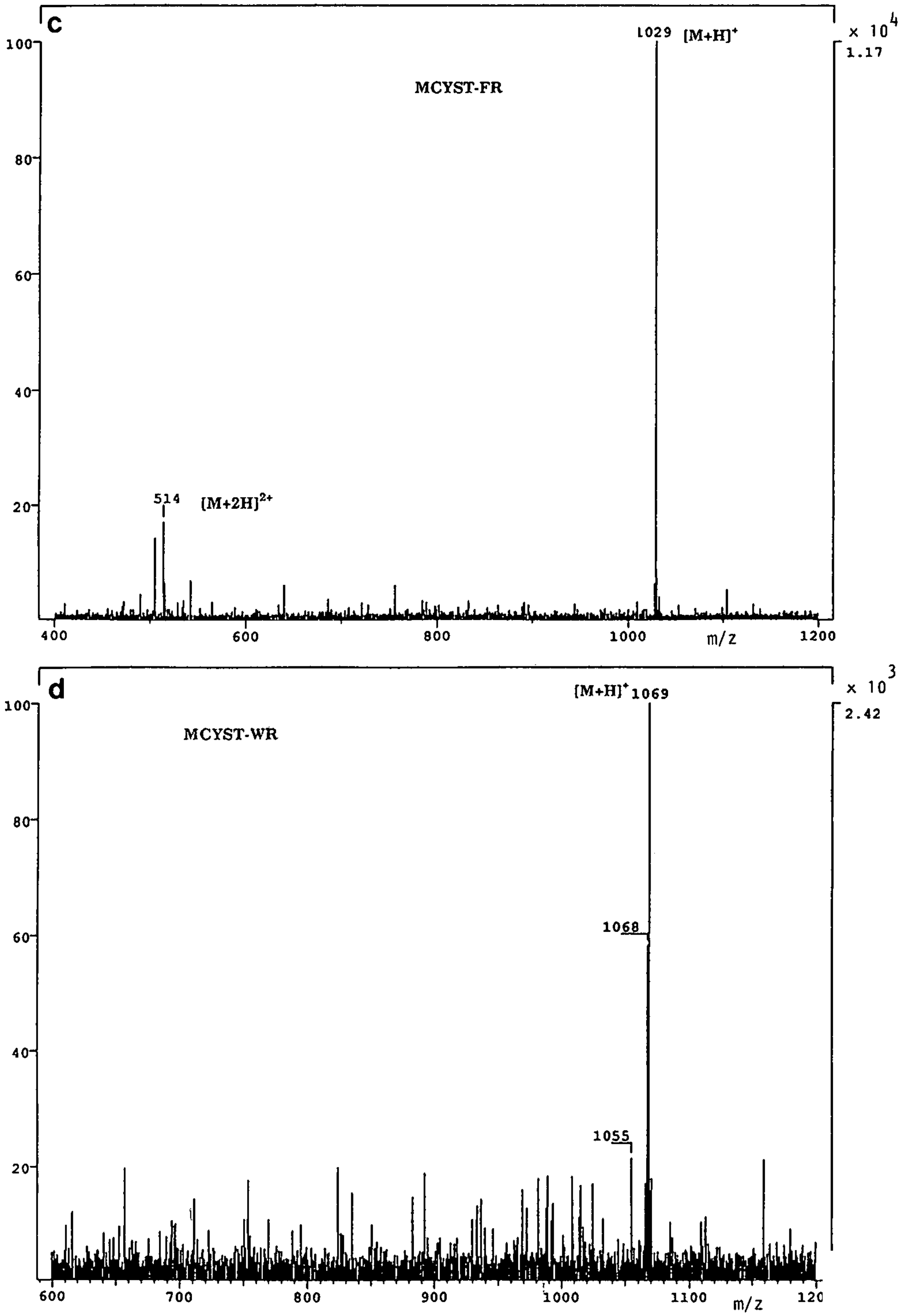

c
MCYST-FR
1029 [M+H]+
514 [M+2H]2+
x 10^4
1.17
100
80
60
40
20
400
600
800
1000
m/z
1200
d
MCYST-WR
[M+H]+ 1069
1068
1055
x 10^3
2.42
100
80
60
40
20
600
700
800
900
1000
1100
m/z
120

Fig. 5.

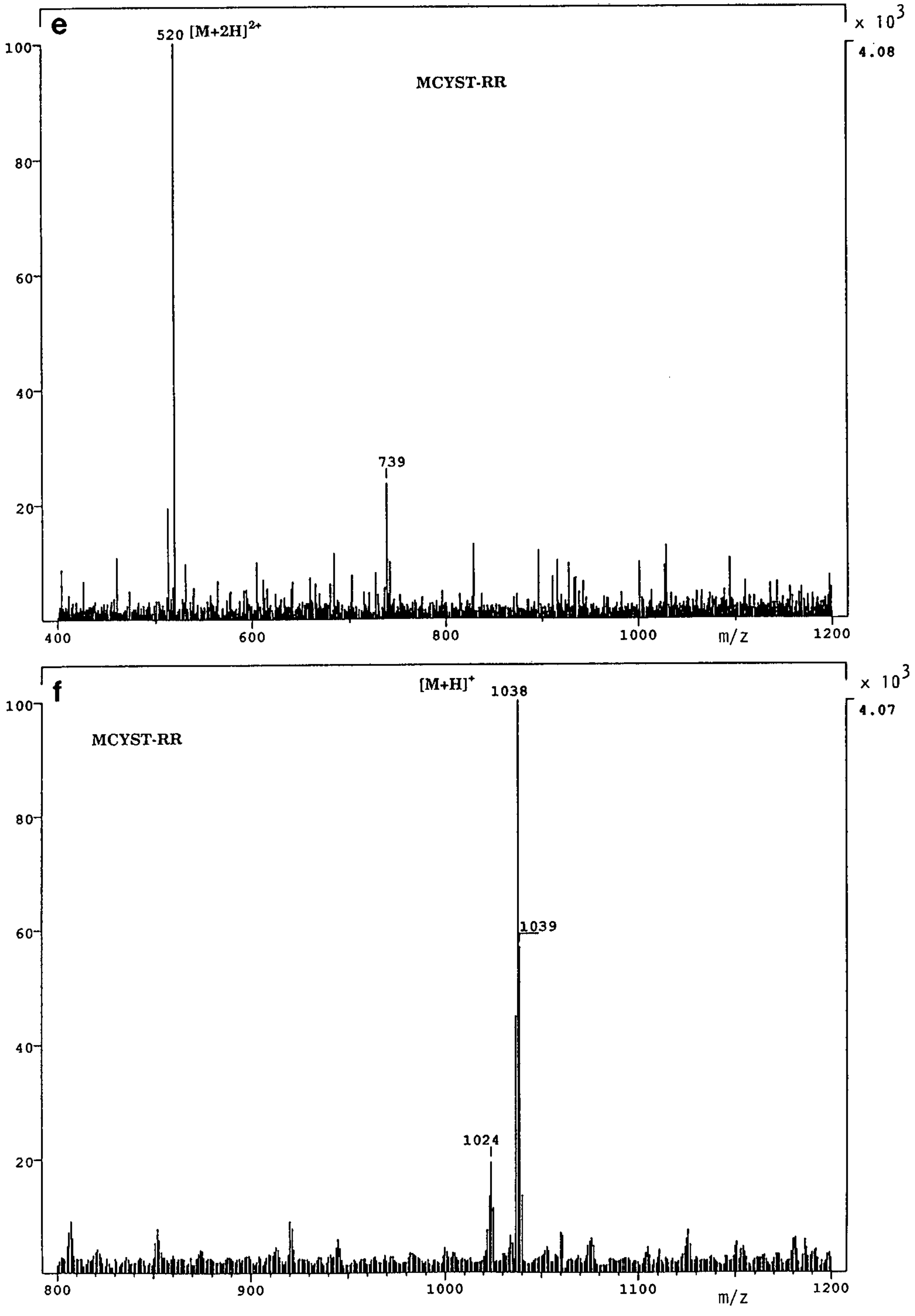


Fig. 5. Mass spectra of toxic peptides identified in bloom material from Hartbeespoort Dam including (a) mcyst-YR, (b) mcyst-LR, (c) mcyst-FR, (d) mcyst-WR and (e and f) mcyst-RR.

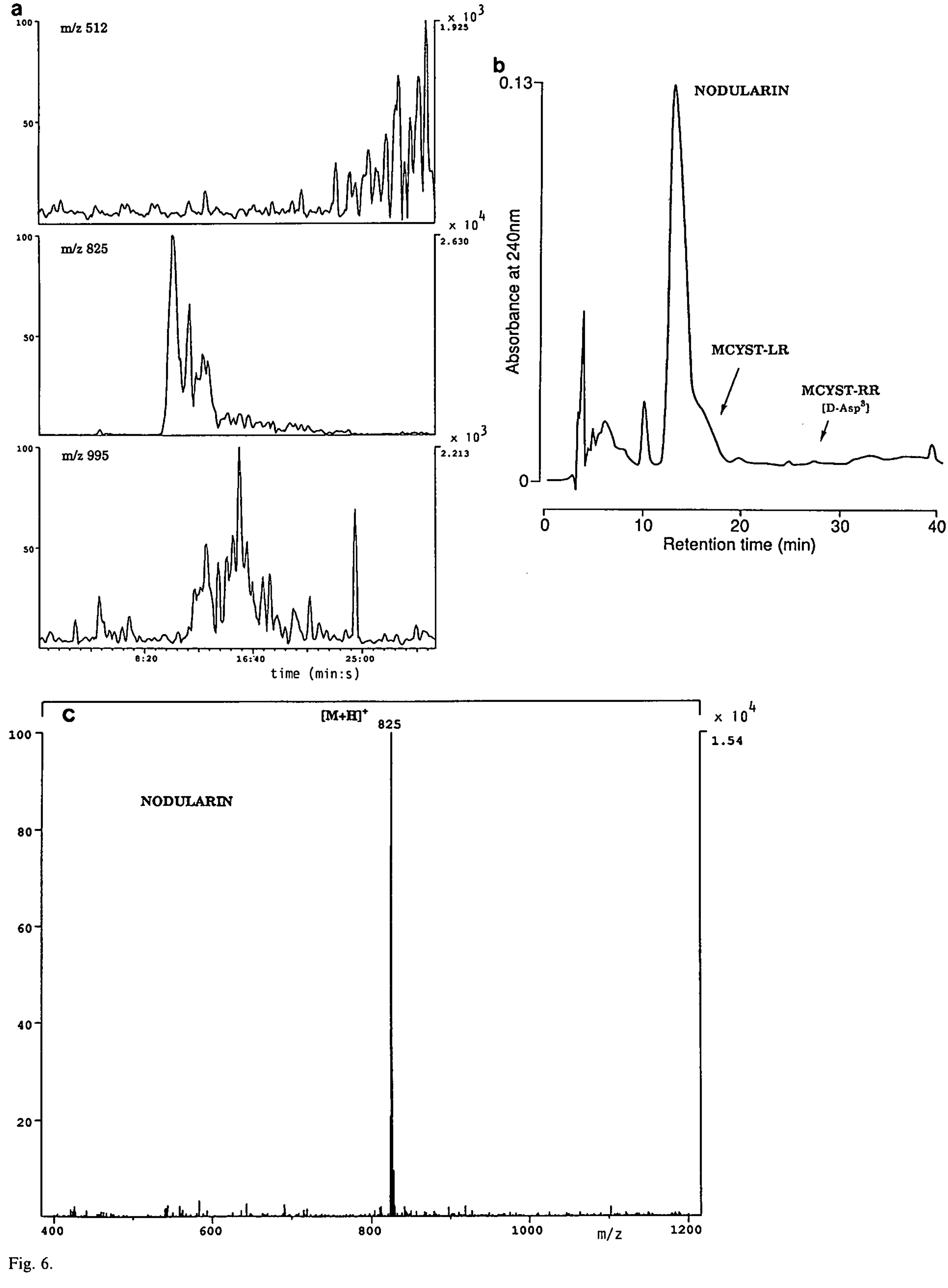

a
m/z 512
x 10^3
1.925
m/z 825
x 10^4
2.630
m/z 995
x 10^3
2.213
100
50
8:20
16:40
25:00
time (min:s)
b
0.13
0
Absorbance at 240nm
NODULARIN
MCYST-LR
MCYST-RR
[D-Asp³]
0
10
20
30
40
Retention time (min)
c
[M+H]⁺
825
x 10^4
1.54
NODULARIN
100
80
60
40
20
400
600
800
1000
1200
m/z

Fig. 6.

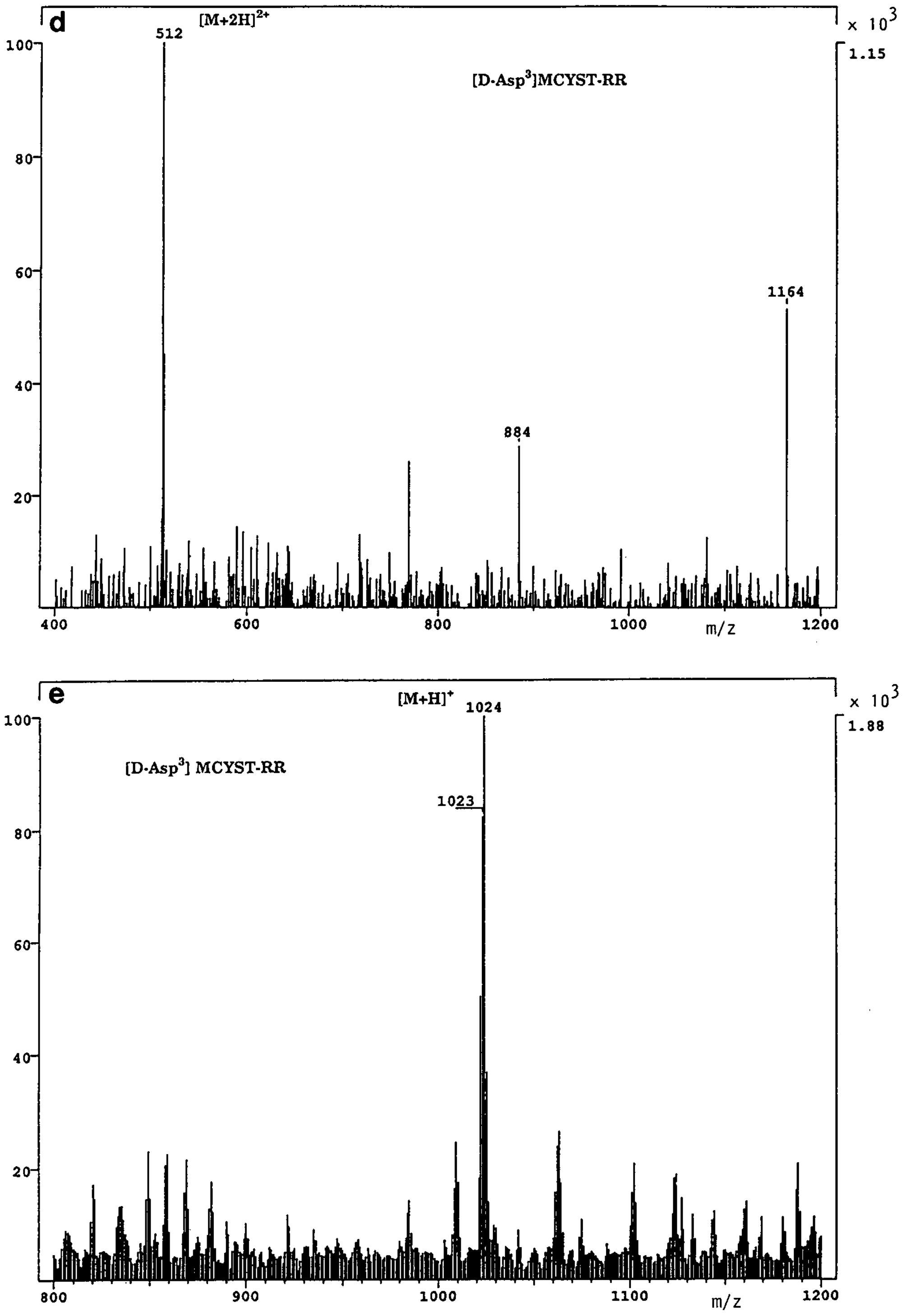


Fig. 6. (a) Reconstructed ion chromatograms; (b) reversed-phase LC chromatogram of extract from bloom containing *Nodularia spumigena*. Toxic peptides detected were (c) nodularin, (d and e) mcyst-LR and [D-Asp^3] mcyst-RR.

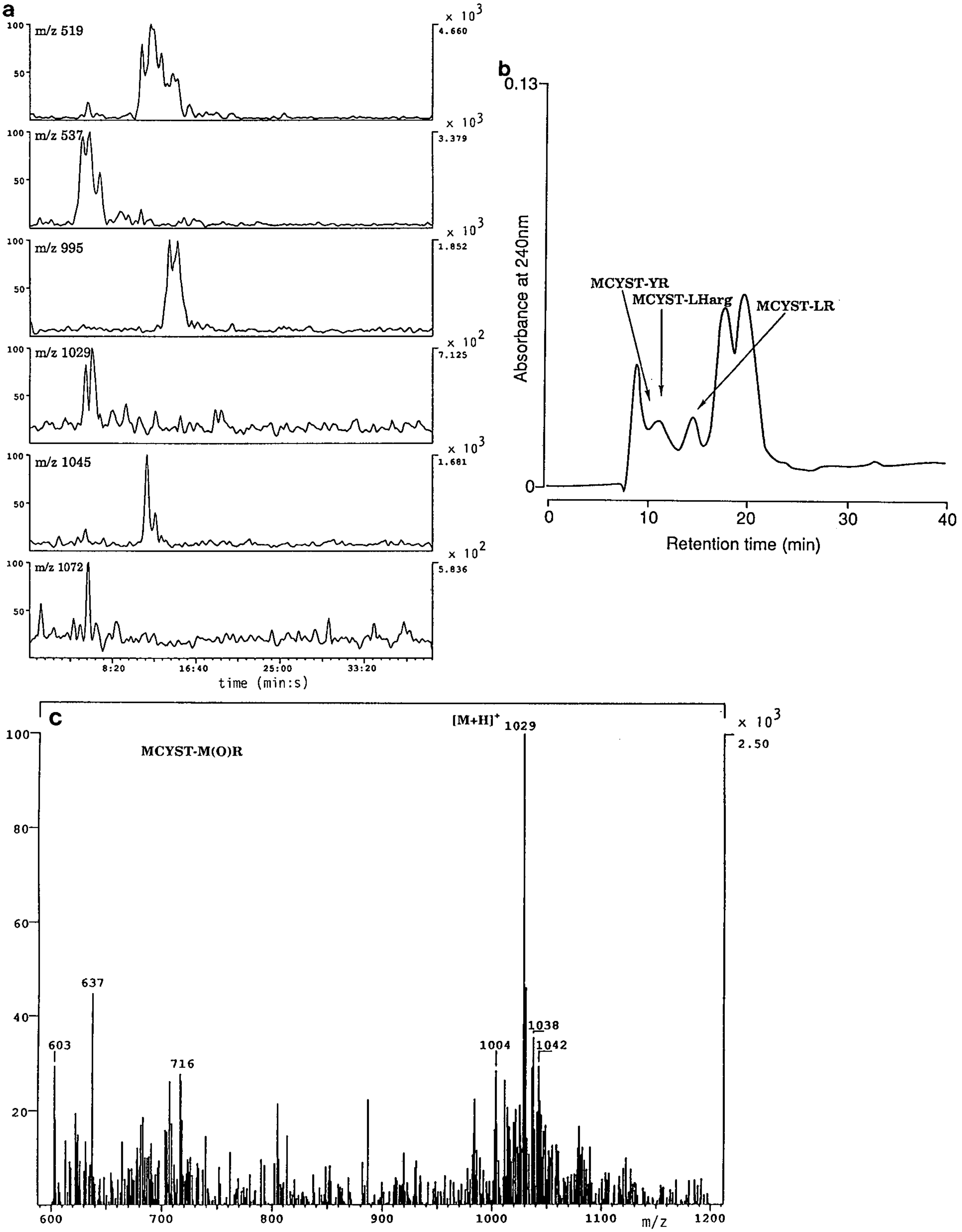

a
m/z 519
x 10^3
4.660
m/z 537
x 10^3
3.379
m/z 995
x 10^3
1.852
m/z 1029
x 10^2
7.125
m/z 1045
x 10^3
1.681
m/z 1072
x 10^2
5.836
100
50
8:20
16:40
25:00
33:20
time (min:s)
b
0.13
0
Absorbance at 240nm
MCYST-YR
MCYST-LHarg
MCYST-LR
0
10
20
30
40
Retention time (min)
c
MCYST-M(O)R
[M+H]+ 1029
x 10^3
2.50
100
80
60
40
20
637
603
716
1004
1038
1042
600
700
800
900
1000
1100
1200
m/z

Fig. 7.

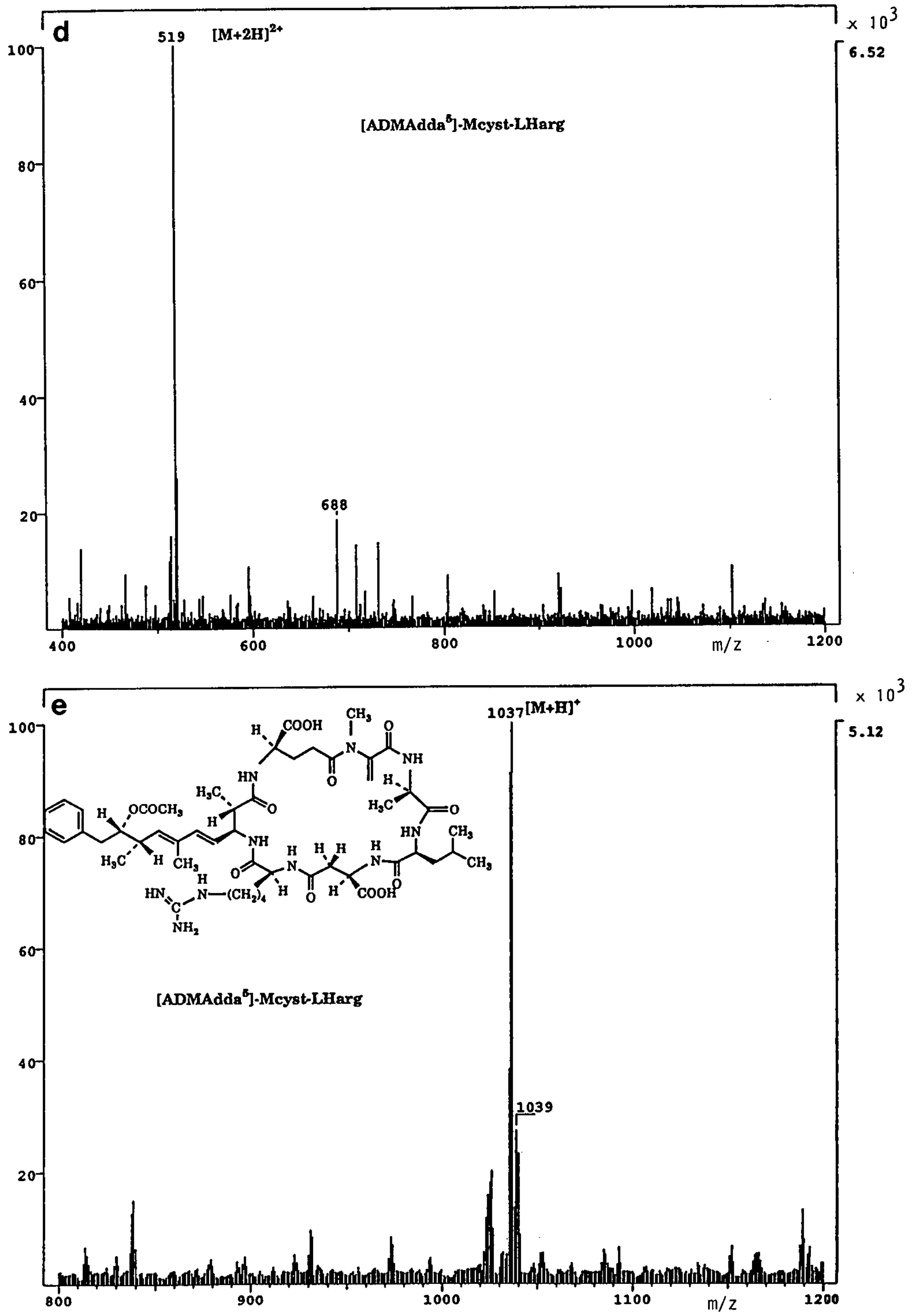


Fig. 7.

(*Continued on p. 230*)

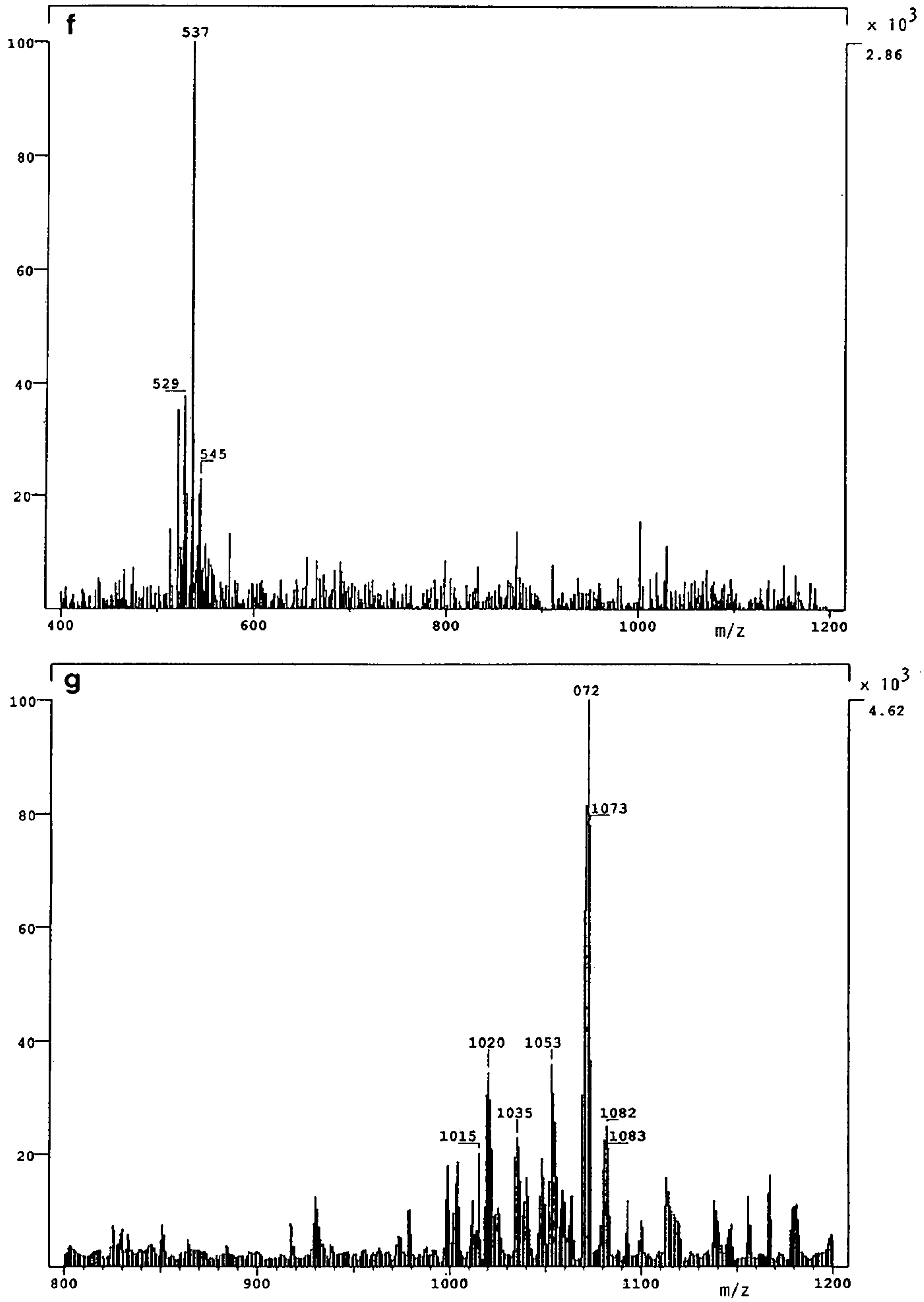


Fig. 7. (a) Reconstructed ion chromatograms; (b) reversed-phase LC chromatogram indicating toxins present in the extract from Water Lane Fish Farm. As well as mcyst-LR and -YR, two peptides were tentatively identified on the basis of their mass spectra as (c) mcyst-M(O)R and (d and e) mcyst-L-Harg. Another compound with $[M + 2H]^{2+}$ at *m/z* 537 and $[M + H]^+$ *m/z* 1072 (f and g, respectively) may be another peptide variant.

described by Namikoshi *et al.* [22]. A strong signal at *m/z* 537 with t_R 5.50 min could be the doubly charged ion of a toxin which has previously been observed but not characterised. The deconvoluted spectrum gave a protonated molecule at *m/z* 1072 (Fig. 7f and g). It is highly likely that this corresponds to another variant since it has previously been detected in extracts from laboratory cultures of *Microcystis aeruginosa* by FAB mass spectrometry.

In the extract from the laboratory culture of *M. aeruginosa* mcyst-LR was the major component (Fig. 8a and b) but at t_R 19.10 min a second component, possibly mcyst-RR [D-Asp^3, D-Ha^7], was detected, giving an $[M + H]^+$ at *m/z* 1010 and an $[M + 2H]^{2+}$ ion at *m/z* 505 (Fig. 8c).

DISCUSSION

Traditionally different analytical methods such as UV detection (for toxins with UV chromophore), fluorescence detection for saxitoxin [11], GC–MS for anatoxin [8-10], static FAB-MS [7] and on-line CF-FAB-MS detection for microcystins [12,13], are required for studying different classes of cyanobacterial toxin. Mass spectrometry, unlike UV spectroscopy, has the advantage that it can handle compounds which lack the UV chromophore. Furthermore GC–MS is applicable to study anatoxin, although samples have to be derivatized before analysis. FAB-MS has been a method of choice to analyse microcystins. It requires a suitable matrix for sample ionization and subsequently, LC–CF-FAB-MS has to incorporate the matrix in the LC

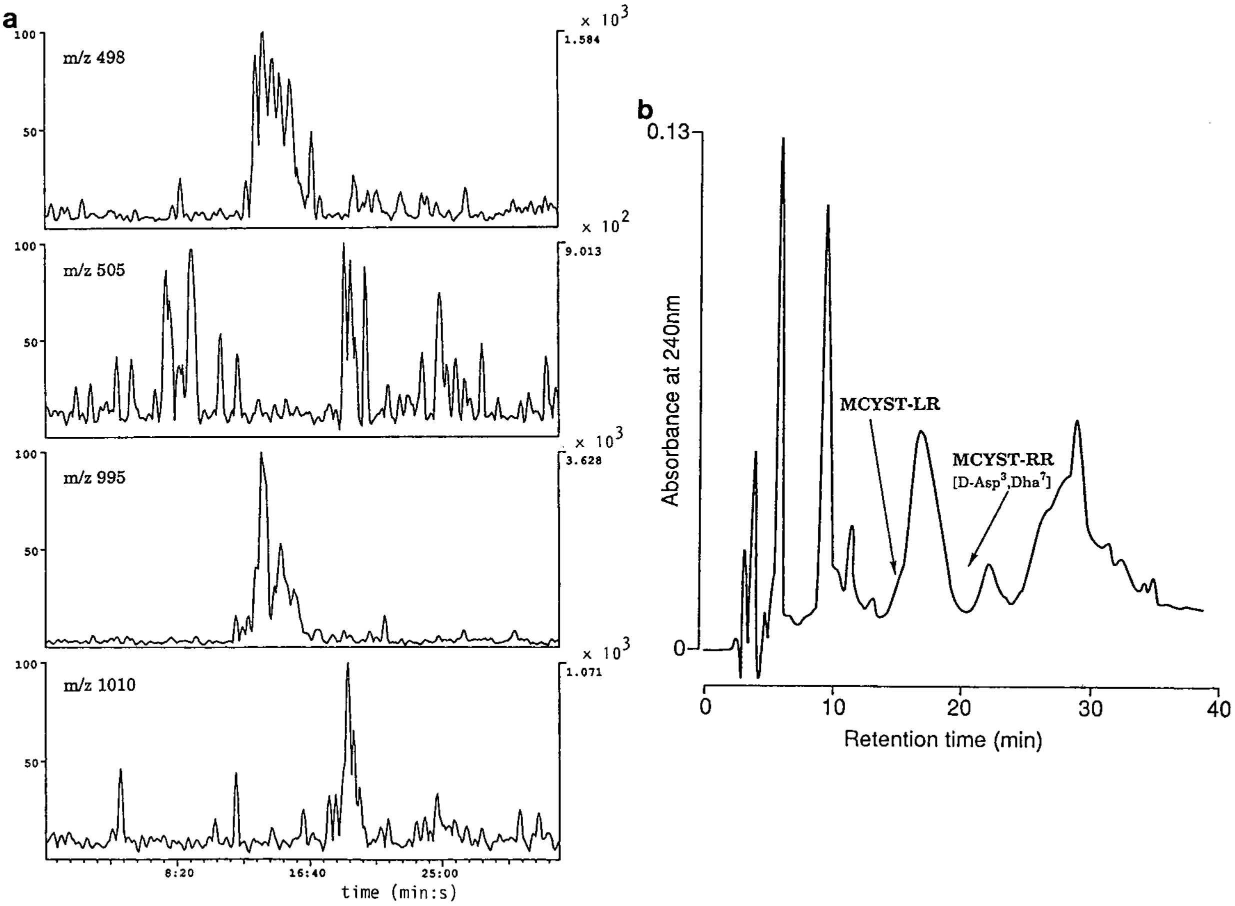


Fig. 8.

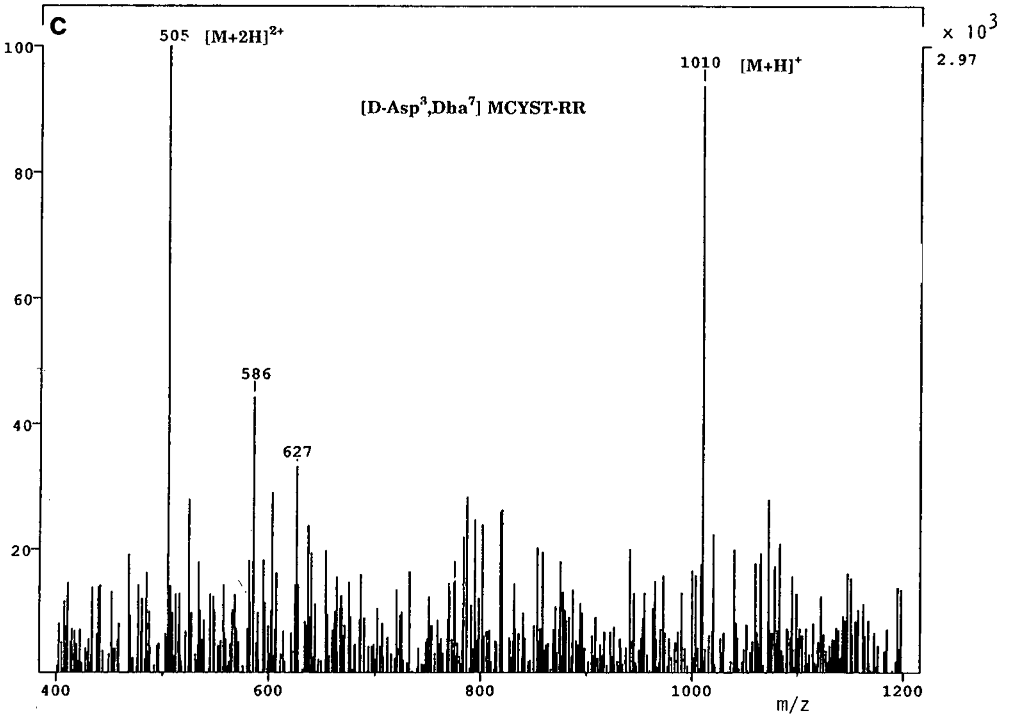


Fig. 8. (a) Reconstructed ion chromatograms; (b) reversed-phase LC chromatogram indicating the presence of toxins in an extract from a culture of *Microcystis aeruginosa* PCC 7813 as detected by (a) mass spectrometry and (b) UV absorption at 240 nm. As well as mcyst-LR another peptide, mcyst-RR [D-Asp^3, D-Ha^7] was identified on the basis of its mass spectrum (c) and comparison the retention time to that of the standard.

separation. Due to changes in polarity and viscosity of the mobile phase, the matrix will affect the chromatographic separation. In addition, not all matrices are amenable to LC–MS because they are not capable of forming a stable and consistent film on the FAB target. High matrix backgrounds often hinder the detection of compounds of interest and severely decrease the limit of detection. While various techniques are normally required to achieve efficient ionization for different classes of toxins, this study demonstrates that ESI-MS is practicable for all the compounds, especially when different classes of toxins are known to coexist in a bloom sample. ESI-MS experiences less ion suppression effect in comparison to FAB, hence it is a more attractive technique for analysing mixtures. Furthermore, on-line LC–MS analysis is less time consuming than off-line LC–MS and with a microbore LC system, less sample is required for the analysis in comparison to the conventional LC set up [13]. ESI-MS was developed primarily for molecular weight determination of proteins and other large biomolecules. This study shows the technique is also applicable for analysis of low-molecular-weight compounds with good sensitivity. Further studies are in progress to determine the suitability of this method for analysis of other cyanobacterial toxins.

These results show that developments and innovations in mass spectrometry require sophisticated (and sometimes expensive) equipment. However, the combination of LC–MS for the identification of peptide toxins exploits the separation capability of liquid chromatography with the molecular weight information provided by the mass spectrometer, and offer a much wider scope in the analysis of algal toxin. However in a system where authentic reference compounds are not available, tandem mass spectrometry (MS–MS) [23] can be considered and thereby further information on the identity of these compounds could be obtained.

Although LC–MS of other cyanobacterial toxins was not examined in this study their ability to be ionized by electrospray and to be separated on reversed-phase systems would suggest this is feasible.

ACKNOWLEDGEMENTS

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Determination of paraquat and diquat by liquid chromatography–thermospray mass spectrometry

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ABSTRACT

Thermospray liquid chromatography–mass spectrometry can be used for identifying and determining both paraquat ($C_{12}H_{14}N_2^{2+}$) and diquat ($C_{12}H_{12}N_2^{2+}$). Reversed-phase liquid chromatography was performed using a 15-cm Shim-pack CLC-ODS column, with methanol–water (80:20) + 0.1 *M* ammonium acetate (adjusted to pH 5 with trifluoroacetic acid) as buffers, at a flow-rate of 1.0 ml/min. The mass spectral sensitivity was best when the temperatures of the vaporizer, block and tip heater of the ion source block were set at 160, 310 and 320°C, respectively. When thermospray ionization was used, ions of *m/z* 186 and 183 were obtained as base peaks for paraquat and diquat, respectively. Detection limits by selected ion monitoring were of the order of 20 ng (*S/N* = 3.5). The mass spectra are influenced by temperature and therefore, precise temperature control is essential.

INTRODUCTION

Paraquat and diquat can be determined by methods such as spectrophotometry [1–5], gas chromatography [6–10], high-performance liquid chromatography [11–19], gas chromatograph–mass spectrometry (GC–MS) [20,21] and fast atom bombardment (FAB) MS [22]. However, these methods, with the exception of MS [5,6,22] and GC–MS, do not give a sufficiently complete identification of unknown compounds.

Recently, thermospray liquid chromatography–mass spectrometry (LC–TSP-MS) has been used in analysis for drugs, lipids, nucleotides, steroids and carbohydrates. In this work, the identification and determination of paraquat and diquat using this method was attempted. These herbicides could be detected without the need for derivatization.

EXPERIMENTAL

A liquid chromatograph–tandem quadrupole mass spectrometer (Shimadzu LC-MS QP1000EX) equipped with a Vestec thermospray interface was used for recording mass spectra and selected ion monitoring. The column used was a 15 cm × 4.6 mm I.D. stainless-steel tube packed with a totally porous matrix, prepared by chemically binding octadecyl groups to the surface of spherical silica particles [Shim-pack CLC-ODS (Shimadzu), particle diameter 5 μm, pore diameter 100 Å]. The mobile phase was methanol–water (80:20, v/v) containing 0.1 *M* ammonium acetate and adjusted to pH 5 with trifluoroacetic acid (TFA). This was injected by a Shimadzu LC-9A pump at a flow-rate of 1.0 ml/min. Samples were injected using a Rheodyne Model 7125 injector fitted with a 100-μl loop or with a Shimadzu SIL-7A autoinjector.

The exit temperature of the vaporizer was 140–180°C . The block and tip heater temperature of the ion source block was 270–310°C. Positive-ion thermospray mass spectra were obtained using the thermospray ionization mode or the thermospray on

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filament ionization mode (filament-on ionization mode). Typical conditions for thermospray MS were a scan range of *m/z* 135–600 in 1 s and electron multiplier voltage 1400 V.

RESULTS AND DISCUSSION

Temperature of the ion source block and vaporizer

The areas of the fragment ions and the molecular ions change depending on the temperatures of the vaporizer and the ion source block. The temperature of the latter consists of the block and the tip heater temperatures, and these were examined by the use of selected ion monitoring. For this examination, paraquat was used owing to its lower detection sensitivity than diquat. In this context, the optimum operating conditions for paraquat were investigated.

Both the thermospray mode and filament-on mode methods of ionization were similar with respect to the vaporizer temperature change (Fig. 1). The areas of the fragment ions and the molecular ions were maximum when the vaporizer temperature was 161°C. The area of the ions measured using the thermospray ionization mode was greater than that with the filament-on ionization mode. Further, a high detection sensitivity was obtained when the vaporizer temperature was 161°C in the thermospray ionization mode.

The relationship between the temperature of the ion source block and the paraquat ion area is shown in Fig. 2. The ion area tended to increase with increase in temperature. The thermospray ionization mode was the best ionization method. However, the temperature there must be kept constant so that the ionization is controlled by the ion source block temperature, which means that for every temperature change the detection sensitivity was changed. Therefore, the temperature of the tip heater was set higher than that of the block in order to compensate for any temperature change during flow-rate changes of the mobile phase.

Based on the above, the temperatures giving maximum ion intensities were 161°C for the vaporizer, 310°C for the block and 320°C for the tip heater.

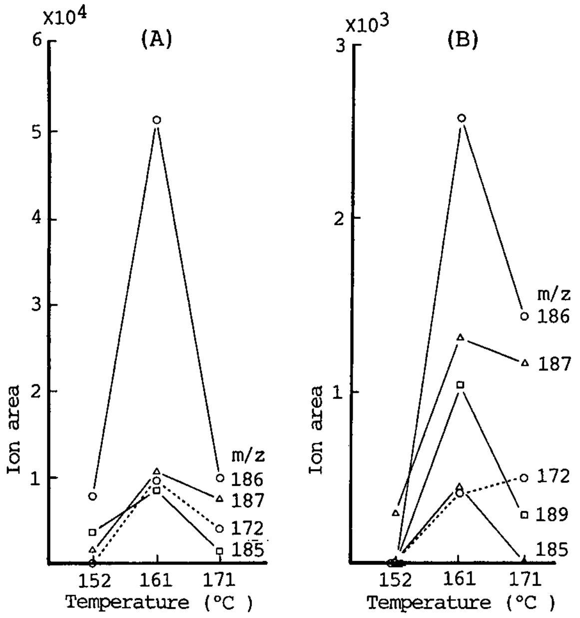

Fig. 1. Relationship between temperature of the vaporizer and ion area. A 200-ng paraquat sample was injected; single determination by selected ion monitoring. The temperatures of the block and tip heater were both 310°C. (A) Thermospray ionization mode; (B) filament-on ionization mode. Ion area represents peak area.

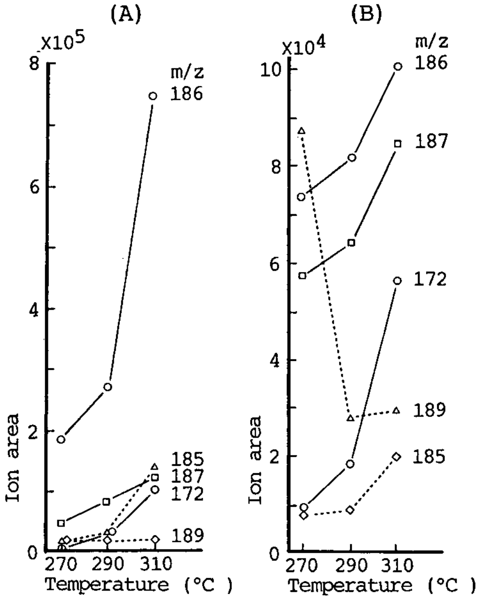

Fig. 2. Relationship between temperature of the ion source block and ion area. Temperature of the ion source block consists of the block and tip heater temperatures. A 200-ng paraquat sample was injected; single determination by selected ion monitoring. The temperature of the vaporizer was 161°C. (A) Thermospray ionization mode; (B) filament-on ionization mode. Ion area represents peak area.

Mass spectrum and mass chromatogram

Mass spectra in the thermospray ionization mode of paraquat and diquat are shown in Fig. 3. Ions of *m/z* 186 and 183 were obtained as base peaks for paraquat and diquat, respectively, under thermospray ionization conditions. The ions obtained in the mass spectrum of paraquat using the thermospray ionization mode were similar to those obtained by FAB-MS [22], whereas the ions for diquat were different from those obtained by FAB-MS. Mass chromatograms of paraquat and diquat obtained in the analysis of a standard mixture are illustrated in Fig. 4. Peaks representing paraquat were seen at *m/z* 186 and 171 at a retention time of 5.5 min and for diquat at *m/z* 183, 184 and 157 at a retention time of 4.8 min. The amounts injected to obtain the data in Figs. 3 and 4 were 200 ng each of paraquat and diquat. Hence, LC–TSP-MS analysis enabled paraquat and diquat to be identified.

However, the relative intensities of the fragment ions of paraquat in the mass spectra differed according to the scan number. This may be due to the peak of *m/z* 171 from the mass chromatogram, which was flat compared with that of *m/z* 186. Hence the mass spectrum of paraquat was obtained where the scan number indicated the peak top of the *m/z* 186 ion in the mass chromatogram.

In the filament-on ionization mode, the mass spectrum of paraquat was very similar to that obtained using the thermospray ionization mode. However, the intensity of the *m/z* 184 molecular ion of diquat was increased, so that the mass spectrum of diquat changed slightly. As filament-on ionization is similar to a chemical ionization process, the base peak of diquat in this mode was obtained at *m/z* 184, but the fragment ions were similar to those in the thermospray ionization mode.

We therefore decided to use both ionization modes because of their accuracy. Paraquat and diquat did not interfere with each other so that for quantification the fragment ions and the molecular ions of both were used.

Calibration

Typical calibration graphs for paraquat and diquat with selected ion monitoring, using the thermospray ionization mode, are shown in Fig. 5. The graphs are linear over the range 30–500 ng. When

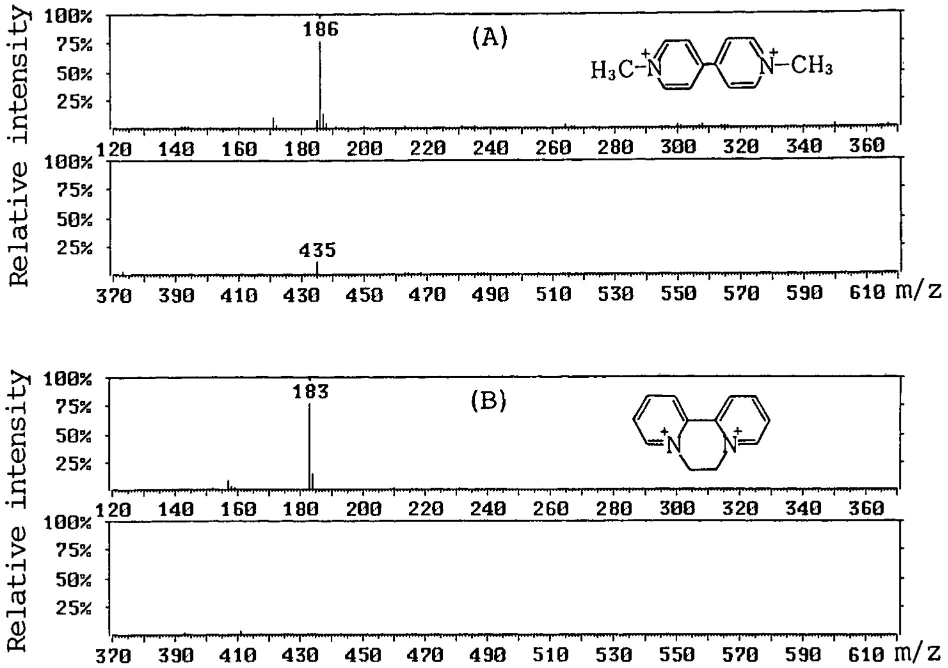

Fig. 3. Mass spectra of (A) paraquat and (B) diquat. The temperatures of the vaporizer, block and tip heater were 161, 310 and 320°C, respectively. Thermospray ionization mode; 200 ng of each sample injected.

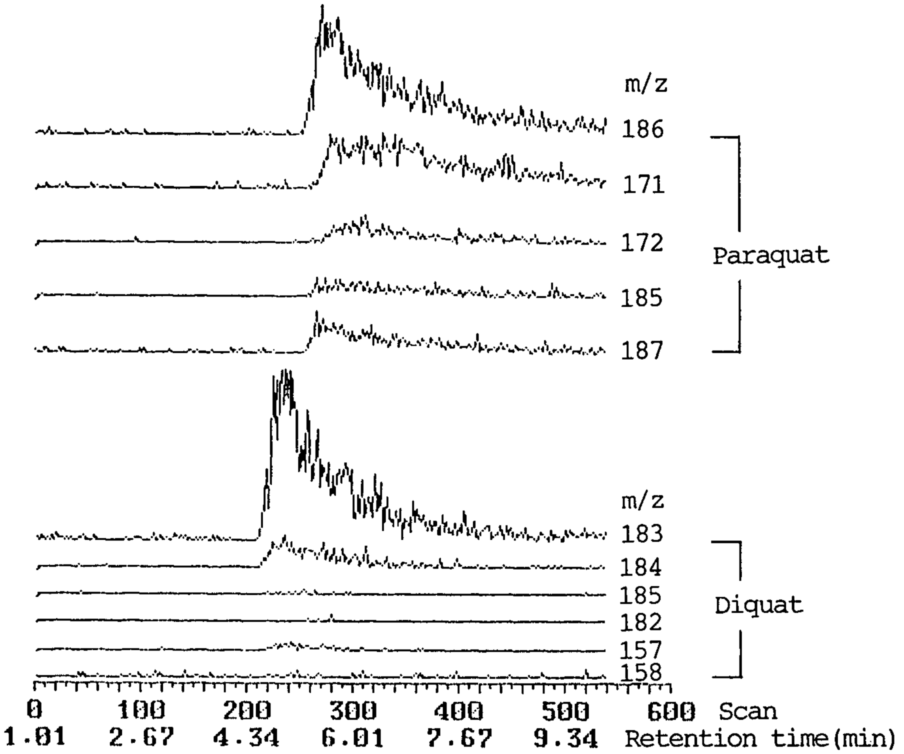

Fig. 4. Mass chromatograms of paraquat and diquat. The temperatures of the vaporizer, block and tip heater were 161, 310 and 320°C, respectively. Thermospray ionization mode; 200 ng of each sample injected.

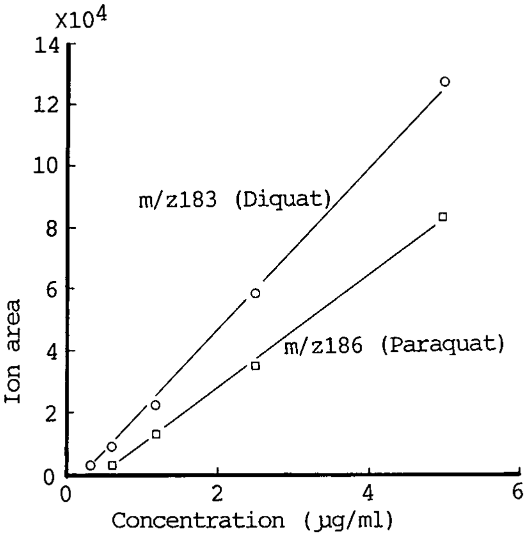

Fig. 5. Calibration graphs for paraquat and diquat. A 100-μl volume was injected; single determination by selected ion monitoring. The temperatures of the vaporizer, block and tip heater were 161, 310 and 320°C, respectively. Thermospray ionization mode. Ion area represents peak area.

the volume of sample injected was 100 μl, a linear response was observed over a concentration range 0.3–5 μg/ml; the detection limit was 20 ng (S/N = 3.5). The detection sensitivity changed, however, with variations in the temperature of the ion source block and vaporizer. The measurement error was about 10%. Quantitative analysis by this method was possible whit appropriate temperature control and when measurement of a known concentration of the compound was interspersed at frequent intervals.

The filament-on ionization mode was not appropriate for quantitative analysis because the sensitivity was lower than that of the thermospray ionization mode.

Sample preparation

Extraction methods have been reported in detail previsously [2,3,14,15,23–25], hence these were not included in this study. The extraction cartridge used was either a Sep-Pak C_{18} or a Bond Elut C_{18}, which permitted direct injection into the LC–TSP-MS system. Extraction cartridges are suitable for practical use.

CONCLUSIONS

LC with UV detection has mostly been used for the determination of paraquat and diquat, whereas LC with diode-array detection has been used for the identification of these compounds. However, LC–TSP-MS enabled quantitative and qualitative analyses of paraquat and diquat to be achieved without the need for individual compound isolation and derivatization. The maximum ion intensities for mass spectrometry were obtained with the vaporizer at 160°C, the block at 310°C, and the tip heater at 320°C. Quantitative analysis was made possible by sensitive control of the temperature of the ion source block and vaporizer. A standard sample is measured regularly in order to obtain accurate readings.

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Temperature dependence of the anion-exchange chromatographic behaviour of cyclic phosphate oligomers and their anion-exchange separation

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ABSTRACT

The temperature dependence of the chromatographic behaviour of a series of cyclic condensed phosphates with degree of polymerization from 3 to 8 on an anion-exchange column was investigated. The efficiency of Jandera's equation in predicting the elution behaviour was confirmed and the elution conditions for separating cyclic phosphates were established with the aid of computer simulations. An increase in column temperature resulted in a significant increase in the number of theoretical plates. Conditions for separating the six phosphates were not found at 30°C; however, the computer simulations suggested that suitable conditions for their separation existed at 70°C and this was confirmed experimentally. The enthalpy changes of the ion-exchange reaction of all phosphates were determined from the temperature dependences of their retention times and varied in proportion to their anionic charges. The ion-exchange reaction of these cyclic phosphates was governed by the dehydration of the anions and partial dissociation of ion pairs between cyclic phosphate anions and K^+ ions in the eluent.

INTRODUCTION

Cyclic phosphates, belonging to inorganic condensed phosphates and represented as $M^{I}_{n}P_{n}O_{3n}$ (M = metal), from the viewpoint of electrolyte solution chemistry are very interesting materials, having high negative charges on their relatively compact molecules [1]. They also can be used for other purposes, such as complexing agents with various cations or food additives, in place of higher membered linear polyphosphates. Of these cyclic phosphates, cyclotri- and cyclotetraphosphate have long been known and can be easily prepared. The existence of cyclic phosphates above the tetramer was confirmed in 1956 [2]. Since that time, the chemistry of cyclic phosphates has developed considerably. We have studied various chemical properties of these oligomers with degree of polymerization less than eight [3–5].

Recently, HPLC has been successfully applied to studies of the hydrolysis of cyclic oligophosphates [6–8]. Further, we have reported on the rapid optimization of the separation conditions for four inorganic cyclic phosphates under gradient elution conditions in anion-exchange chromatography [9].

In this work, the separation of six cyclic phosphate anions from each other and the chromatographic behaviour of these anions were investigated. Some of the condensed phosphates, including cyclotri- and cyclotetraphosphate have been used to

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derive the equations describing their elution behaviour and to test the plate theory [10–12]. Since these condensed phosphate oligomers are useful polyprotic acids in which each of the PO_4 tetrahedra units is linked to each of its neighbours by an oxygen atom, their degree of protonation can be changed with the pH of the eluents except for cyclic phosphates. Despite extensive studies of their chromatographic behaviour, few theoretical approaches to their selectivities in ion exchangers have been reported.

It has been said that the enthalpy changes in ion-exchange reactions are usually small and temperature does not have a great effect on the ion-exchange equilibria [13]. However, the importance of column temperature was pointed out in the ion-exchange chromatographic separation of linear polyphosphate anions having highly negative charges [14,15]. In this work, the effect of temperature on the chromatographic behaviour of cyclic phosphate anions was studied in detail. The retention time of each of the cyclic phosphate increases with increase in temperature. The theoretical plate number also increases with increase in temperature. We found that the four cyclic phosphate mixtures mentioned above can be separated at 70°C faster and with a better resolution owing to the increase in the number of theoretical plates than at 30°C.

Further, the separation of six cyclic oligomers from the trimer to octamer has been achieved, where the pentamer and heptamer were isolated by the column chromatography of the fractionated mixture of Graham's salt [3].

From the temperature dependence of the retention times of six cyclic phosphate anions, the enthalpy changes of the anion-exchange reaction were determined. The enthalpy changes were positive, and hence the anion-exchange reactions were endothermic. Large differences in the enthalpy changes per unit anionic charge on the cyclic phosphate anions were not observed. The enthalpy changes can be regarded as corresponding to desolvation energies and the dissociation energies of K^+ which form ion pairs with cyclic phosphate anions when the anions enter into the exchanger phase. These steps are important in the anion-exchange reaction of these cyclic phosphate anions. The effect of temperature should be considered for the ion-exchange reactions of highly charged ions such as condensed phosphate ions.

EXPERIMENTAL

Chemicals

Sodium salts of cyclotri- (P_{3m}), $Na_3P_3O_9{\cdot}6H_2O$, and cyclotetra phosphate (P_{4m}), $Na_4P_4O_{12}{\cdot}4H_2O$, were prepared by the usual methods [16]. Sodium cyclohexa- (P_{6m}) and cyclooctaphosphate (P_{8m}) were prepared by the methods of Griffith and Buxton [17] and Schülke [18], respectively. Cyclopenta- (P_{5m}) and cycloheptaphosphate (P_{7m}) were isolated from the fractionated solution of Graham's salt by the use of a QAE-Sephadex A-25 column [3]. All other chemicals were of analytical-reagent grade.

Chromatographic system

The anion exchanger TSK Gel SAX, 10 μm, was packed into a 500 mm × 4 mm I.D. column. A Hitachi L-6200 liquid chromatograph was used and a Hitachi 655A-13 reaction pump was connected to mix the Mo(V)–Mo(VI) reagent with the column effluents for the spectrophotometric determination of the phosphates with a Hitachi L-4200 UV–Vis detector. The flow system was similar to that described previously [14]. The separation column was surrounded by a jacket, the temperature of which was kept constant within ± 0.1°C by circulating water.

Elution procedure

The sample solution (0.5 ml) was injected into a separation column and chromatographed at a flow-rate of 1.0 ml/min. The concentrations of the samples were $(1.5\text{–}4.5)\cdot10^{-5}$ *M*. The eluent consisted of appropriate concentrations of potassium chloride and 0.1% (w/v) Na_2EDTA buffered at pH 10.2. The column interstitial volume and the extra-column volume were 2.88 and 2.04 ml, respectively.

Computer simulations

The computer simulation procedure and the notations used in the equations have been described previously [9,19].

RESULTS AND DISCUSSION

In a previous paper [9], we predicted the elution behaviour of four cyclic phosphates, cyclotri-, -tetra-, -hexa- and -octaphosphate, which can be relatively easily prepared, at 30°C. The four were separated from each other as predicted by the computer simulations. However, the retention times for the phosphates did not differ much and the separation of the six cyclic phosphate anions known so far (the above four plus cyclopenta- and -heptaphosphate) seemed to be difficult at that temperature. Therefore, the elution behaviour of these cyclic phosphates at higher temperatures was investigated. It was found that the retention volume of the cyclic phosphate anions increased with increase in the column temperature. The temperature dependence of the retention volume of cyclooctaphosphate is shown in Fig. 1. The number of plates in the column used (N) was calculated from the analysis of the elution curves with the usual plate theory. The retention volume increased with increase in elution temperature and the number of plates also increased considerably. The separation of four cyclic phosphates at 70°C was examined for comparison with the data at 30°C. To find the optimum conditions for separating the four cyclic phosphates by computer simulation, the dependence of the capacity factor on the eluent concentration at 70°C was determined. As described previously [9], a plot of the logarithm of the capacity factor (k') against the logarithm of the concentration of eluting ion (c') was found to be linear:

$$k' = ac'^{-b} \qquad (1)$$

The plot of ln k' against ln c' at 70°C is shown in Fig. 2. The values of a and b obtained are given in Table I. The slope, b, is referred to as the ionic charge of cyclic phosphate anion in the exchanger phase [3]. Substituting the above a and b values in Jandera's equation [9,19,20] and the computer simulations yielded the best conditions for the rapid and complete separation of the four cyclic phos-

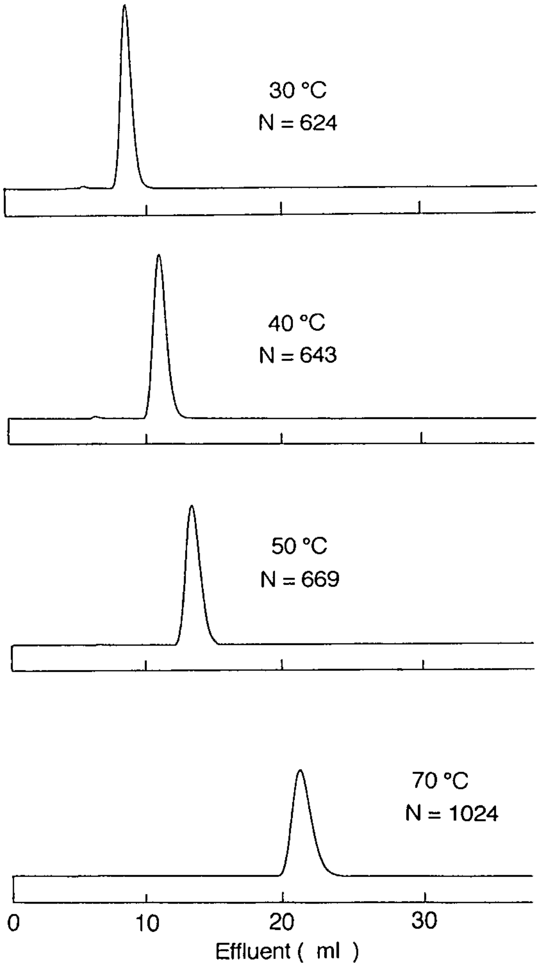

Fig. 1. Chromatograms of cyclooctaphosphate with 0.4 M KCl as eluent at various temperatures.

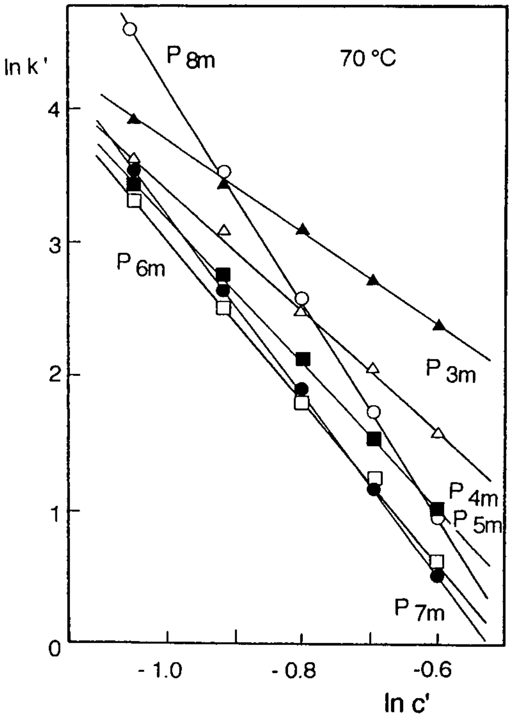

Fig. 2. Plots of ln k' $vs.$ ln c' at a column temperature of 70°C.

TABLE I

CONSTANTS *a* AND *b* IN EQN. 1 AT A COLUMN TEMPERATURE OF 70°C

Solute	*a*	*b*
$P_3O_9^{3-}$	1.56	−3.29
$P_4O_{12}^{4-}$	0.351	−4.48
$P_5O_{15}^{5-}$	0.115	−5.36
$P_6O_{18}^{6-}$	0.0631	−5.78
$P_7O_{21}^{7-}$	0.0342	−6.58
$P_8O_{24}^{8-}$	0.0241	−7.86

TABLE II

OBSERVED AND PREDICTED RETENTION VOLUMES FOR FOUR CYCLIC PHOSPHATES UNDER GRADIENT ELUTION CONDITIONS AS GIVEN IN FIG. 3 AT 70°C

Phosphate	Retention volume (ml)		Difference (%)
	Observed	Predicted	
P_{3m}	36.7	39.8	+8.3
P_{4m}	22.3	23.6	+5.8
P_{6m}	13.6	13.5	−0.7
P_{8m}	16.7	17.0	+1.6

phates with gradient conditions as C_i = 0.5, C_f = 0.6, t_f = 40 min, x = 5 [9].

Fig. 3 shows the observed and predicted chromatograms under the optimum gradient conditions. The four cyclic phosphates are completely separated within 40 min, which is half of the separation time at 30°C. The retention times observed are in fair agreement with the predicted values, as shown in Table II. The increase in the column temperature from 30 to 70°C resulted in an increase in the plate number and hence the column separation efficiency.

Next, the separation of the six cyclic phosphates from cyclotri- to cyclooctaphosphate was examined. As the preparation of cyclopenta- and cycloheptaphosphate is difficult by the usual method, they were isolated by column chromatography as described previously [3]. Computer simulations at lower column temperatures did not give satisfactory results. However, a good separation at 70°C was predicted with gradient conditions of C_i = 0.4, C_f = 0.5, t_f = 350 min, x = 1 [9]. The chromatogram predicted by the computer simulation is shown in Fig. 4. The chromatogram observed is also shown in Fig. 4, and the separation of the six cyclic phosphates was satisfactory as predicted. The predicted and observed retention volumes of each phosphate are given in Table III, showing good agreement.

It can be concluded that by increasing the column temperature to 70°C and by considering the gradient profile of the eluent, the six cyclic phosphates can be separated from each other. As shown in the chromatograms finally obtained (Fig. 4), the cyclic

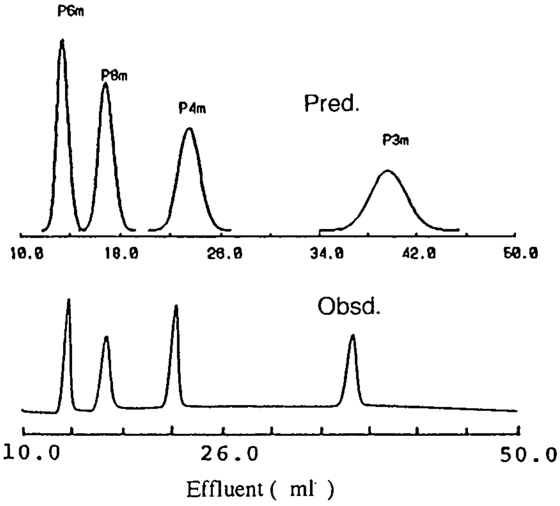

Fig. 3. Predicted and observed chromatograms for a mixture of four cyclic phosphates with gradient elution condtions of C_i = 0.5, C_f = 0.6, t_f = 40 min, x = 5.

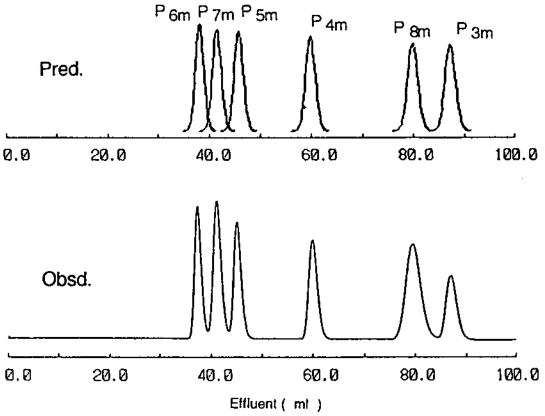

Fig. 4. Predicted and observed chromatograms for a mixture of six cyclic phosphates with gradient elution conditions of C_i = 0.4, C_f = 0.5, t_f = 350 min, x = 1.

TABLE III

OBSERVED AND PREDICTED RETENTION VOLUMES FOR SIX CYCLIC PHOSPHATES UNDER THE GRADIENT ELUTIONS CONDITION GIVEN IN FIG. 5 AT 70°C

Phosphate	Retention volume (ml)		Difference (%)
	Observed	Predicted	
P_{3m}	88.1	88.6	+0.5
P_{4m}	61.1	60.7	−0.8
P_{5m}	46.6	46.4	−0.4
P_{6m}	38.9	38.7	−0.6
P_{7m}	42.7	42.2	−1.2
P_{8m}	80.6	82.4	+2.2

phosphates were not eluted in order of increasing charge. The cyclic phosphates from the trimer to hexamer are eluted in order of decreasing charge, contrary to the elution order of linear phosphate oligomers [19]. However, the heptamer and octamer are eluted in order of increasing charge. The elution order depends completely on the relationship between the capacity factors and the eluent concentration. From the usual law of mass action for ion exchange, a linear relationship can be derived for the plots of ln k' *vs.* ln $[Cl^-]$ and the values of the slopes of the straight lines correspond to the charge of each cyclic phosphate anion (eqn. 1). Finally, the ln k' *vs.* ln c' relationships in Fig. 2 are governed by the free energy changes of ion-exchange reactions of the cyclic phosphate anions, but at the present stage it is not possible to determine or predict true free energy changes of such high-valent anions.

The mechanism of the ion-exchange reaction of these cyclic phosphate anions was considered in some detail from the temperature dependence of the capacity factors. When the concentration of the eluting reagent, KCl, was kept constant at 0.5 M, the results for the variation of ln k' for each phosphate with the reciprocal of absolute temperature of the column are as shown in Fig. 5. Good linearity was obtained. As mentioned previously, the capacity factor can be related to column temperature by eqn. 2, as the ionic strength of the system was kept constant:

$$\ln k' = -\Delta H/RT + \Delta S/R + \text{constant} \qquad (2)$$

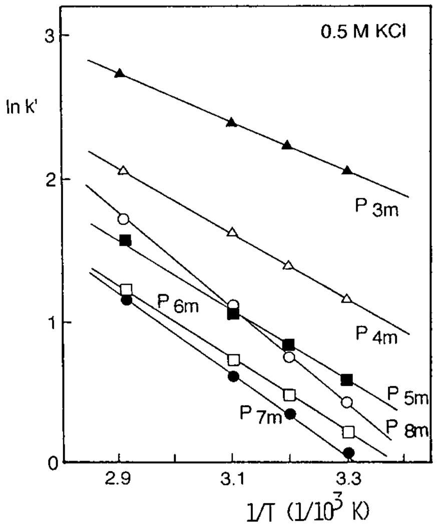

Fig. 5. Temperature dependence of the logarithm of the capacity factor with 0.5 M KCl as an eluent.

In this study, only the capacity factors were determined as a function of the concentration of KCl and temperature. Although selectivity coefficients were not obtained, the enthalpy changes for the anion-exchange reactions were calculated.

The enthalpy changes for the ion-exchange reaction were calculated from the slopes of the linear ln k' *versus* $1/T$ plots. We measured the temperature dependence of the capacity factors of the cyclic

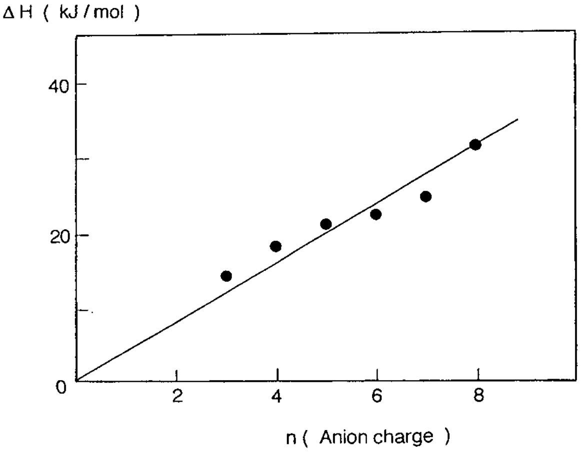

Fig. 6. ΔH *vs.* charge of cyclic phosphate anions in 0.5 M KCl.

phosphates at constant KCl concentrations from 0.3 to 0.5 *M*. The enthalpy changes thus obtained for cyclotetraphosphate were 21.2, 20.5, 21.9 and 19.8 kJ/mol at KCl concentrations of 0.3, 0.4, 0.45 and 0.5 *M*, respectively. For the remaining solutes, the ΔH values did not change so much with KCl concentration, but for the six cyclic phosphates they strongly depended on the charge of the cyclic phosphate anions. The positive values of ΔH indicated that the ion-exchange reaction is endothermic and as an example ΔH values in 0.5 *M* KCl solution are shown in Fig. 6 as a function of the anionic charges. The experimental data yielded an approximately linear plot passing through the origin. Thus, the ΔH values per unit anionic charge were almost same for each cyclic phosphate anion.

No theory has been proposed to predict the selectivity order for a series of cyclic phosphate oligomers on anion exchangers because of the difficulty involved in the determination of thermodynamic selectivity coefficients. The anionic selectivity has been discussed for some oxygen-containing anions by Chu *et al.* [21]. They attributed the stronger absorption of the anions to their weaker hydration. In our case, the enthalpy changes seemed to involve energy necessary for the partial dissociation of the ion pair between the phosphate anion and K^+ ion in the solution phase and also the dehydration energy of the phosphate anion entering into the ion-exchanger phase, and the dehydration step of the phosphate anion on adsorption might be one of the important factors that control the ion-exchange reaction of these cyclic phosphate anions.

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Quantitative structure–retention relationship approach to prediction of linear solvation energy relationship coefficients

I. H-Bond acceptor capability of gas chromatography stationary phases in McReynolds data set

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ABSTRACT

Linear solvation energy relationships (LSER) have been used successfully in the characterization of GC stationary phases. The various terms in the LSER are related to the ability of the stationary phase to engage in different types of intermolecular interactions. Using the 77 phase McReynolds set, structural features of the stationary phases have been correlated with the a value in an LSER, which reflects the H-bond acceptor capabilities of the stationary phase. Structural descriptors consisted of the fraction of each phase that was represented by a given functional group. For the coatings in this data set, only five descriptors were identified as contributing to the observed acceptor behavior: siloxane, ester, ether, hydroxyl and methylene (CH_2). These terms were included in the equation $a = c_1(\text{siloxane}) + c_2(\text{ester}) + c_3(\text{ether}) + c_4(\text{OH}) + c_5(CH_2)$, where the coefficients (c_i) were determined by multiple linear regression analysis. The R^2 for the above equation was 0.990. The values of the first four coefficients are a direct indication of the ability of these functional groups to act as H-bond acceptors, while the fifth term is believed to contribute via inductive effects.

INTRODUCTION

The importance of solubility, solvation processes, and solvent-mediated interactions in nearly all fields of chemistry has fueled research directed toward the development of predictive methods to enhance our understanding of such phenomena. Because the retention of solutes in chromatographic separations provides a quantitative measure of solute-solvent interactions, chromatographic methods have been used extensively for the investigation of solubility processes. Consequently, the characterization of stationary phases for chromatography is an active research area. The ability to predict the solubility/retention behavior of these materials facilitates the process of selecting a stationary phase for a given application.

The various approaches for predicting chromatographic behavior can be categorized as either theoretical or empirical. The theoretical approaches attempt to explain the interactions that give rise to retention by way of fundamental chemical theory, especially the thermodynamics of solubility behavior [1–4]. In addition to their predictive ability, such models also assist in our understanding of the chromatographic process; the better the model is at accounting for the relevant factors in the system, the

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greater its predictive accuracy. Empirical approaches rely on the statistical correlation of known or measurable characteristics of the compounds under study with their observed retention behavior. Such correlations usually take the form of multiple linear regression relationships [5,6]. In the majority of cases, the properties used for correlation are molecular structural descriptors, and these studies have been labelled as quantitative structure–retention relationships (QSRRs) [7–12]. While these methods do not enjoy the same fundamental theoretical basis as the former approach, their predictive ability has been well demonstrated. Unfortunately, the QSRR results are only valid for compound classes that have been included in the original correlation. Use of the derived regression equation to predict the behavior of *other* compound classes produces questionable results.

A compromise between the two approaches discussed above is the development of solubility factors or parameters to approximate or describe the solvation process [13–17]. Like the empirical approaches, these methods rely on the development of a regression relationship with terms and coefficients that are characteristic of the individual components in the system . However, they have a more fundamental, theoretical basis in that the terms in the regression equation represent the relative ability of the components to engage in solubility interactions. By far, the most successful and widely applied of these methods is the linear solvation energy relationships (LSER) of Kamlet, Abraham and Taft and co-workers which utilize solvatochromic parameters [18–22]. The LSER usually take a form similar to the following,

$$\log SP = c + r_1 R_2 + s_1 \pi_2^* + a_1\alpha_2 + b_1\beta_2 + l_1 \log L^{16} \qquad (1)$$

where SP refers to the solvation property under study, which in chromatographic investigations is usually the specific retention volume (V_g) or the partition coefficient (K_c). The coefficients with the subscript 1 are descriptors for the solvent (or stationary phase), and the terms with subscript 2 are descriptors for the solutes. For example, the α and β terms refer to the ability of a given solute to act as a hydrogen bond donor (acid) and hydrogen bond acceptor (base), respectively. The L^{16} term is the Ostwald solubility (partition) coefficient of the solute in hexadecane, and provides a measure of the dispersion interaction. The π^* measures the dipolarity/polarizability interactions, and the R term represents non-bonded electron interactions. The coefficients r, s, a, b and l represent the corresponding ability of the solvent phase to engage in these interactions, while c is a regression constant. Hence, the overall equation represents a model of the solvation process. The number and identity of parameters included in the LSER varies with the system under study.

The advantage of the LSER approach is that, once the relevant terms and coefficients have been determined for the solutes and solvents/phases in question, the solubility behavior of any solute/solvent pair can be calculated, *i.e.* the results are not necessarily limited to a closed system as in other empirical methods. The development of the terms (α, β, π^*, L^{16}, R) and coefficients (a, b, r, s, l) requires extensive data collection and reduction, and every compound/stationary phase must be studied in order to be characterized.

In this paper we demonstrate an alternative approach to the calculation of the coefficient in the LSER equation above for chromatographic stationary phases. Since the ability of a compound to engage in solubility interactions is determined largely by its molecular structure and functionality, we propose to use a simplified QSRR approach to the calculation/prediction of these values. The advantages of such an approach are that it permits rapid prediction of solubility behavior based on structural features, and it may assist in the rational design of materials for applications where solubility behavior is relevant.

METHODOLOGY

The approach demonstrated herein consists of correlating, via multiple linear regression, the coefficients determined for an LSER with structural features of the stationary phase materials. For the work reported here, we have focused on prediction of the coefficient a, which is a measure of H-bond acceptor basicity of a stationary phase interacting with an H-bond donor solute (characterized by α). In order for a material to act as an H-bond acceptor it must contain atoms that have lone electrons (*e.g.* oxygen, nitrogen) or functional groups that have sufficient electron density (*i.e.* multiple bonds, aromatic rings).

The first requirement of this approach is a fully characterized set of stationary phases, *i.e.*, a set for which the LSER coefficient values have already been determined. A variety of GC stationary phases have recently been characterized using solvatochromic LSERs [23,24], including the McReynolds 77-phase set [25], which is probably the most extensively documented set of GC stationary phases. The second requirement is documentation of the structural features of these phases so that these materials can be adequately represented by a set of descriptors. A thorough description of the materials, by general chemical class, included in the McReynolds set is given below. In cases where a material could be listed under more than one class, it is included under the heading which represents the more significant contribution to the observed solubility behavior.

Hydrocarbons

Included in this class are squalane and the Apiezon greases. Squalane, a saturated hydrocarbon, contains no functional groups capable of engaging in H-bond acceptor interactions and thus has an *a* value of 0.0. The Apiezon phases contain undetermined amounts of alkene and aromatic groups which convey some weak acceptor behavior to these phases (*a* = 0.10–0.13). The actual structure(s) of these materials is not well known, however, which prevents their inclusion in the study set.

Polysiloxanes

These materials are dimethyl siloxane-based polymers, usually terminated with trimethyl silyl groups [$-Si(CH_3)_3$]. The methyl group substituents can be replaced with other functional groups to alter the solubility properties of the material. Included in this group are SE-30 (100% methyl substituted), SE-30-NPGA (same as SE-30, with neopentyl glycol adipate terminal groups), SE-31 (1% vinyl), SE-52 (5% phenyl), DCF-550 (25% phenyl), XF-1150 (50% cyanoethyl), DCFS-1265 (50% trifluoropropyl), and Versilube F-50 (chlorophenyl groups, unknown%). These phases are mostly weak acceptors, having *a* values in the range of 0.17 to 0.33; the only moderately strong acceptor is XF-1150, with an *a* value of 1.38.

Phosphates

Included in this group are tricresyl phosphate and Kroniflex-THPP, which have *a* values of 1.23 and 2.28, respectively. The relatively large *a* values are consistent with the multiple oxygens in the phosphate which are capable of acceptor behavior.

Nitrogen (amides/amines)

The only stationary phase which contains amine groups is Quadrol (N,N,N′,N′-tetrakis(2-hydroxypropyl)ethylene diamine), which has an *a* value of 2.38. The amides in the data set include Flexol-8N8 (2,2'-(2-ethyl hexamido)diethyl di(2-ethyl)hexoate, and the Hallcomids M18/180L (N,N-dimethyl stearamide and N,N-dimethyl oleylamide). The *a* values for these phases are 1.55 and 1.59, respectively.

Oxygen (esters/ethers/hydroxyls)

This is by far the best represented set of functional groups in the data set. Within this subheading are several well-defined classes of materials, which are worth describing further.

Esters/polyesters. Most of the esters are manufactured by the reaction of aliphatic alcohols with dicarboxylic acids (adipic, sebacic and phthalic). Included in this set are dioctyl sebacate (DOSb), di-2-ethylhexyl sebacate (DEHb), di-2-ethylhexyl adipate (DEHA), isooctyl decyl adipate (IODA), diisodecylphthalate (DIDP), dioctylphthalate (DOP), bis(2-ethoxyethyl)phthalate, and dibutyl-tetrachlorophthalate. These are moderate acceptors (*a* = 0.60–1.25). Three of the phases are produced by the esterification of sucrose [sucrose diacetate hexaisobutanoate (SAIB), sucrose octaacetate, and octakis-2(hydroxypropyl) sucrose (Hyprose SP80)], which tend to be stronger acceptors (*a* = 1.29–2.40). Additional ester phases are tripelargonate, a triester produced by the reaction of glycerol with pelargonic acid (*a* = 0.82), and Citroflex A-4 (acetyl tributyl citrate) (*a* = 0.94).

Nine coatings in the set are polyesters, produced by the polymerization of various glycols with adipic (A), succinic (S) and sebacic (Sb) acids. Glycols used to produce these materials included ethylene glycol (EG), diethylene glycol (DEG), triethylene glycol (TEG), and neopentyl glycol (NPG). Abbreviated names for these phases are given as NPGA (terminated and unterminated), EGSb, DEGSb, NPGS, EGA, DEGA, TEGS, and DEGS. Most of the polymer chains terminate in –OH groups, but the terminal group for the NPGA term. phase was undetermined. While these phases were very popular until the 1970s, there is no information available as to the molecular weight range of the materials used. Attempts to obtain such information from the manufacturer(s) revealed that even they did not know for sure, and there was variation from one lot to the next.

Polyglycol ethers. Included under this heading are the Pluronic phases and the Carbowaxes (CW). The Carbowaxes are linear polyethylene glycols (PEGs), terminated by hydroxyl groups, with the phase number providing information about the approximate relative molecular mass (M_r) range. Carbowax 1000, for example, has an M_r of 950–1050, whereas Carbowax 20M has an M_r between 15 000 and 20 000.

The Pluronic phases consist of block copolymers of PEG and polypropylene glycol (PPG). The letter in the phase suffix refers to the physical appearance of the phase as liquid (L), paste (P) or flake (F). The first number in the suffix, when multiplied by 300, yields the approximate M_r of the phase represented by the PPG block, while the second number, when multiplied by 10, yields the % of the total M_r represented by the PEG. Pluronic L63, therefore, is a liquid phase having a PPG weight of approximately 1800, which represents 70% of the total M_r with 30% of the polymer being PEG. The total M_r of this phase is about 2600. There is some disagreement in the literature regarding the terminal groups on the polymer chains [15,26], but the more commonly accepted structure contains hydroxyl terminal groups.

Other PPG- and PEG-based polymer phases in this class include UCON LB-1715 and UCON HB-2000. The latter is a butoxy-terminated PPG with the average number of repeating PPG units equal to 28 or 29. In general, the PPG-based polymers tend to be water insoluble. The former is also butoxy-terminated, but is approximately 50% (w/w) in PEG and PPG units with a total M_r of about 2000; the high percentage of PEG groups renders these polymers water soluble.

Phenyl ethers. Eight of the remaining phases are characterized by ether linkages. Two of these are polyphenyl ethers (5 ring and 6 ring, PPE-5 and PPE-6). Once again, there is some variation in structures reported in the literature, with one reference reporting hydroxyl terminated chains [17], and another reported no hydroxyl terminals [15]. The most likely structure is the former. The remaining six ether phases are often used as detergents/surfactants; these include Polytergent-J300, Oronite NIW, Ethofat 60/25, Tergitol NPX, Igepal CO-880 and Triton X-305. These phases are di-substituted phenyls; one substituent consists of a saturated hydrocarbon (HC), and the other substituent is an hydroxyl terminated PEG chain. The number of carbons in the saturated HC substituent varies from 8 (Triton X-305) to 17 (Ethofat 60/25), and the number of ethylene glycol units in the PEG substituent varies from 10 (Tergitol NPX) to 30 (Triton X-305). The exact structure of the Polytergent-J300 could not be verified.

Alcohols. Included in this group are docosanol ($C_{22}H_{45}OH$), diglycerol [$(HOCH_2CH(OH)CH_2)_2–O$], and sorbitol. The structure of sorbitol can be written either as a straight chain or as a 5-membered ring, which is formed upon the elimination of a water molecule. These phases are unique in the 77-phase set because they also exhibit significant H-bond donor acidity and, thus, have relatively large b values (0.34–0.52).

Miscellaneous phases

The remaining two phases can not be classified in any of the above groups. Castor wax is hydrogenated castor oil [$CH_3(CH_2)_{16}COOH$], with the carboxylic acid group providing significant H-bond acceptor ability ($a = 1.09$). The final remaining phase is Zonyl E-7 which can be described as a polyfluorinated ester of aromatic acid (1,2,4,5-tetracarboxylic acid), although the exact structure could not be verified.

DATA SET DEVELOPMENT

The a values used in this work were taken from Abraham *et al.* [25] for the 77-phase McReynolds data set at 120°C. Structures for as many coatings in this set as possible were obtained from a variety of literature sources and supplier documentation [15,17,26–30]. Unfortunately, structures for several of the phases in the data set could not be verified, and these coatings were not included in the final data set. Non-verified phases included Zonyl E-7, Polytergent-J300, Versilube F50, and the four Apiezon phases. In addition, the terminal group for NPGA-(term.) could not be verified, so this phase was also eliminated. This reduced the number of phases in the set to 69. A set of structural descriptors were calculated for the remaining coatings based on the relative abundance of individual functional groups, expressed as a percentage of the molecular mass. Potential H-bond acceptor groups include alkenes (C=C), phenyls, siloxanes (Si–O), phosphates (PO_4), nitriles (C≡N), amides ($CONR_2$), amines (NR_3), esters (COOR), ethers (C–O–C), and hydroxyls (R–OH).

Functional groups for the remaining phases were considered as descriptors to be included in the regression equation. In order for the regression results to be statistically valid each functional groups should be adequately represented within the data set to prevent skewing of the result. Once again, several of the remaining phases were eliminated because of inadequate representation. Among the functional groups that were under-represented were the phosphates (2 phases), the amides/amines (3 phases), the alkene/vinyl (3 phases), and the cyano/nitriles (1 phase). Phases eliminated at this stage were tricresyl phosphate, Kroniflex THPP, Flexol 8N8, the Hallcomids (18,180L), Quadrol, SE-31 and XF-1150. This reduced the data set to 61. Two of the remaining phases contained halogens (dibutyltetrachlorophthalate, DCFS-1265); because the acceptor behavior of chlorine is likely to be negligible, and the fluorine will likely affect acceptor behavior via inductive effects, these coatings were retained for the preliminary regression. Chlorine and fluorine were not, however, included among the functional groups considered for the regression equation.

The remaining 61 coatings in the data set are listed in Table I. Simplified descriptors for the functional groups in these coatings were determined as follows. Fractional representations for each functional groups were calculated by multiplying the number of functional groups in the molecule by the amu for that functional group, and dividing by the total M_r. Hydroxyls and ethers, for example, contain one oxygen at 16 amu, whereas each ester contains two oxygens for a total of 32 amu. Siloxanes (Si–O) contained one oxygen at 16 amu; since Si does not participate as an acceptor, its mass was not included. Phenyls were represented as (C_6H_x) with the total number of hydrogens being determined by the degree of substitution. Monosubstituted phenyls, for example, were C_6H_5 for 77 amu. Also included in the table are fractions of CH_3, CH_2 and CH groups. Although these CH_x functionalities cannot engage in acceptor behavior, they provide information regarding the extent of branching within the molecule, which can affect the molar volume and/or potential steric or inductive effects. The resulting fractions and the M_r used for each phase are included in Table I. It should be noted that the actual mass for the polyester phases was not known. The data for these phases are for $M_r \approx 2000$ ($n \approx 10$; n = number of monomer repeat units). The phases in the data set are predominantly represented by oxygenated functional groups (ester, ether, hydroxyl), with siloxanes and phenyl groups being marginally represented. The number of coatings containing these functional groups are given in Table II.

RESULTS

Statistical evaluation of the data set was performed using two different software packages. A complete statistical system (CSS), obtained from Statsoft (Tulsa, OK, USA), was used for correlation analysis of the regression variables, for calculation of standard and deleted residuals, and for calculation of F values. The R^2 values obtained using the CSS package used (N) rather than ($N-1$), and were thus not reported in this work. Additional statistical analyses were performed using the regression subroutines available in Quattro Pro 3.0 (Borland International). R^2 values reported herein were obtained using the QPro software. Values for residuals, as well as for the regression coefficients and associated standard errors, were identical for both software packages. In all cases, results are for the linear regression with the intercept forced to zero.

The data in Table I were used to develop a regression equation of the following form

$$a_i = c_1\,(\text{descriptor}_1)_i + c_2\,(\text{descriptor}_2)_i + c_3\,(\text{descriptor}_3) + \ldots + c_n\,(\text{descriptor}_n)_i \quad (2)$$

where a is the coefficient from the LSER from ref. 25, $c_{1,2\ldots}$ are the regression coefficients, (descriptor) is the fraction of the total M_r represented by a given functionality (phenyl, siloxane, etc.) as given in Ta-

TABLE I

PHASE DATA SET WITH DESCRIPTORS (POLYESTER M_r SET TO $n = 10$)

Coating	M_r	Coef. *a*	Phenyl	Siloxane	Ester	Ether	OH	CH_2	CH	CH_3
DCFS-1265	24 600	0.2900	0.0000	0.1020	0.0000	0.0000	0.0000	0.1784	0.0000	0.0992
SE-52	100 000	0.2200	0.0959	0.1992	0.0000	0.0000	0.0000	0.0000	0.0000	0.3554
DCF-550	5186	0.2900	0.3563	0.1481	0.0000	0.0000	0.0000	0.0000	0.0000	0.2260
SE-30	1 998 100	0.2900	0.0000	0.2162	0.0000	0.0000	0.0000	0.0000	0.0000	0.4054
SE-30(NPGA)	10 032	0.3300	0.0000	0.2089	0.0128	0.0000	0.0000	0.0001	0.0000	0.4053
PPE-5	446	0.5400	0.8565	0.0000	0.0000	0.1435	0.0000	0.0000	0.0000	0.0000
PPE-6	538	0.5600	0.8513	0.0000	0.0000	0.1487	0.0000	0.0000	0.0000	0.0000
DBTCP	416	0.6000	0.1731	0.0000	0.1538	0.0000	0.0000	0.2019	0.0000	0.0721
DIDP	446	0.7300	0.1704	0.0000	0.1435	0.0000	0.0000	0.3139	0.1166	0.2018
DOP	390	0.7700	0.1949	0.0000	0.1641	0.0000	0.0000	0.3590	0.0667	0.1538
bis-(2EOE)Ph	310	1.2500	0.2452	0.0000	0.2065	0.1032	0.0000	0.2710	0.0000	0.0968
Oronite-NIW	906	1.4600	0.0839	0.0000	0.0000	0.2472	0.0177	0.5872	0.0143	0.0497
Tergitol NPX	660	1.4700	0.1152	0.0000	0.0000	0.2424	0.0242	0.5939	0.0000	0.0227
IgepalCO-880	880	1.6100	0.0864	0.0000	0.0000	0.2727	0.0182	0.6045	0.0000	0.0170
Triton-X305	1526	1.6500	0.0498	0.0000	0.0000	0.3145	0.0105	0.6147	0.0000	0.0098
Ethofat 60/25	988	1.5400	0.0000	0.0000	0.0324	0.2591	0.0162	0.6802	0.0000	0.0152
DOSb	426	0.7900	0.0000	0.0000	0.1502	0.0000	0.0000	0.7230	0.0000	0.0704
IODA	398	0.8100	0.0000	0.0000	0.1608	0.0000	0.0000	0.5276	0.0760	0.1884
DEHSb	426	0.8300	0.0000	0.0000	0.1502	0.0000	0.0000	0.5915	0.0610	0.1408
DEHA	370	0.8700	0.0000	0.0000	0.1730	0.0000	0.0000	0.5297	0.0703	0.1622
EGSb	2343	1.4400	0.0000	0.0000	0.2733	0.0000	0.0137	0.6097	0.0000	0.0000
DEGSb	2826	1.4500	0.0000	0.0000	0.2265	0.0623	0.0113	0.6143	0.0000	0.0000
NPGA	2244	1.4700	0.0000	0.0000	0.2852	0.0000	0.0143	0.3868	0.0000	0.1471
NPGS	1964	1.4900	0.0000	0.0000	0.3259	0.0000	0.0163	0.2994	0.0000	0.1527
DEGS	1986	1.6800	0.0000	0.0000	0.3222	0.0886	0.0161	0.4512	0.0000	0.0000
EGA	1782	1.7200	0.0000	0.0000	0.3591	0.0000	0.0180	0.4871	0.0000	0.0000
DEGA	2266	1.7300	0.0000	0.0000	0.2824	0.0777	0.0141	0.5190	0.0000	0.0000
TEGS	2470	1.8700	0.0000	0.0000	0.2591	0.1425	0.0130	0.4874	0.0000	0.0000
UCON-LB1715	1698	1.2200	0.0000	0.0000	0.0000	0.2638	0.0094	0.2556	0.2144	0.2562
PL-L81	2660	1.2900	0.0000	0.0000	0.0000	0.2767	0.0120	0.2789	0.2004	0.2312
PL-L72	2634	1.4100	0.0000	0.0000	0.0000	0.2855	0.0121	0.3189	0.1777	0.2050
PL-L61	2080	1.4200	0.0000	0.0000	0.0000	0.2769	0.0154	0.2894	0.1938	0.2236
PL-P84	3980	1.4400	0.0000	0.0000	0.0000	0.3055	0.0080	0.3975	0.1339	0.1545
PL-L42	1588	1.4500	0.0000	0.0000	0.0000	0.2821	0.0202	0.3262	0.1719	0.1984
PL-P85	4860	1.4600	0.0000	0.0000	0.0000	0.3160	0.0066	0.4407	0.1097	0.1265
PL-L63	2608	1.4600	0.0000	0.0000	0.0000	0.2945	0.0123	0.3597	0.1545	0.1783
UCON-HB2000	2028	1.4800	0.0000	0.0000	0.0000	0.3077	0.0079	0.4418	0.1090	0.1331
PL-P65	3664	1.5000	0.0000	0.0000	0.0000	0.3144	0.0087	0.4394	0.1100	0.1269
PL-L44	2027	1.5200	0.0000	0.0000	0.0000	0.3000	0.0158	0.3937	0.1347	0.1554
PL-P46	3084	1.5600	0.0000	0.0000	0.0000	0.3217	0.0104	0.4767	0.0885	0.1021
PL-F88	12 076	1.6000	0.0000	0.0000	0.0000	0.3445	0.0026	0.5576	0.0441	0.0509
PL-F68	8388	1.6100	0.0000	0.0000	0.0000	0.3433	0.0038	0.5558	0.0449	0.0519
PL-F77	6946	1.6100	0.0000	0.0000	0.0000	0.3340	0.0046	0.5160	0.0674	0.0777
CW-20M	17 508	1.7800	0.0000	0.0000	0.0000	0.3623	0.0018	0.6357	0.0000	0.0000
CW-6000	6772	1.8000	0.0000	0.0000	0.0000	0.3603	0.0047	0.6347	0.0000	0.0000
CW-4000	3362	1.8500	0.0000	0.0000	0.0000	0.3569	0.0095	0.6330	0.0000	0.0000
CW-1540	1470	1.8700	0.0000	0.0000	0.0000	0.3483	0.0218	0.6286	0.0000	0.0000
CW-1000	1008	1.8900	0.0000	0.0000	0.0000	0.3413	0.0317	0.6250	0.0000	0.0000
CW-600	612	2.1200	0.0000	0.0000	0.0000	0.3268	0.0523	0.6176	0.0000	0.0000
CW-400	414	2.1800	0.0000	0.0000	0.0000	0.3092	0.0773	0.6087	0.0000	0.0000
CW-300	304	2.2800	0.0000	0.0000	0.0000	0.2895	0.1053	0.5987	0.0000	0.1020
Hyprose-SP80	806	2.4000	0.0000	0.0000	0.0000	0.2184	0.1588	0.1911	0.2581	0.1489
SAIB	846	1.2900	0.0000	0.0000	0.3026	0.0567	0.0000	0.0496	0.1998	0.2482

TABLE I *(continued)*

Coating	M_r	Coef. *a*	Phenyl	Siloxane	Ester	Ether	OH	CH_2	CH	CH_3
Sucrose(Ac)$_8$	678	1.5900	0.0000	0.0000	0.3776	0.0708	0.0000	0.0619	0.1534	0.177
Citroflex A4	388	0.9400	0.0000	0.0000	0.3299	0.0000	0.0000	0.3608	0.0000	0.1546
Docosanol	327	0.7500	0.0000	0.0000	0.0000	0.0000	0.0490	0.9002	0.0000	0.0459
Diglycerol	166	2.7700	0.0000	0.0000	0.0000	0.0964	0.3855	0.3373	0.1566	0.0000
Sorbitol	164	1.7700	0.0000	0.0000	0.0000	0.0974	0.9898	0.1705	0.3167	0.0000
Squalane	422	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5308	0.1848	0.2844
Tripelargonate	512	0.8200	0.0000	0.0000	0.1875	0.0000	0.0000	0.6289	0.0254	0.0879
Castorwax	284	1.0900	0.0000	0.0000	0.0563	0.0000	0.0563	0.7887	0.0000	0.0528

ble I. The 61-phase data set was used to develop a preliminary regression equation. Of the 8 functionality factors in the table, only 5 were determined to be significant. These 5 were siloxane, ester, ether, hydroxyl, and methylene (CH_2). The regression coefficients and their respective standard errors are given in Table III. Examination of residuals using this equation revealed that the *a* values for two coatings (sorbitol and Citroflex A4) were severely over-predicted, while one coating (Hyprose SP80) was severely under-predicted. The over-predictions could be partly due to steric effects; some of the oxygens in these compounds may have limited accessibility. In addition, the sorbitol has a high *b* value due to its ability to act as a H-bond donor. The possibility of intramolecular interactions, *i.e.* self-association, may reduce the availability of the hydroxyls for H-bond acceptor behavior with solutes. An iterative process of regression, followed by eliminating of outliers due to steric hinderance and/or self-association, reduced the data set to 55 coatings. Eliminated at this round were the three alcohol phases (docosanol, diglycerol, sorbitol), Citroflex A4, and tripelargonate. The elimination of the first three coatings can be justified due to self-association; each has a significant *b* value. The latter two coatings were eliminated on the basis of unusual steric hinderance. It is worth noting that these 5 coatings tend to be of low M_r (164 – 512) compared with the majority of coatings in the data set. Any steric or self-association phenomena are likely to have a larger effect on smaller molecules than on larger ones. One additional coating, squalane, was eliminated due to the fact that it contained no H-bonding capability ($a = 0.00$), yet had a CH_2 fraction of 0.52. Inclusion of this coating would skew the coefficient for CH_2.

TABLE II

REPRESENTATION OF FUNCTIONAL GROUPS IN 61-PHASE DATA SET

No. of coatings	Functional groups				
	Phenyl	Siloxane	Ester	Ether	OH
	12	5	23	40	41

Upon elimination of these coatings the regression coefficients were redetermined and are given in Ta-

TABLE III

COMPARISON OF REGRESSION RESULTS FOR 61-PHASE AND 55-PHASE DATA SETS

Data set	R^2 (standard error)	Siloxane	Ester	Ether	OH	CH_2	F
61 phases	0.922	1.409	3.423	4.090	5.009	0.549	902
	(0.167)	(0.415)	(0.189)	(0.170)	(0.295)	(0.088)	
55 phases	0.990	1.412	3.517	3.872	8.969	0.514	7893
	(0.054)	(0.134)	(0.068)	(0.064)	(0.275)	(0.036)	

ble III for comparison. The R^2 for the regression improved from 0.922 (standard error = 0.167) to 0.990 (standard error = 0.054). The regression coefficients for siloxane and ester increased slightly, whereas the coefficients for ether and CH_2 decreased slightly. The largest effect was observed for the coefficient for hydroxyl, which increased dramatically from 5.009 to 8.969. As a result of this large increase in the hydroxyl coefficient some of the coatings that were under-predicted using the original regression equation, most notably Hyprose SP80, were now more accurately predicted. Using these coefficients yields the following regression equation

$$a = 1.412\,(\text{siloxane}) + 3.517(\text{ester}) + 3.872(\text{ether}) + 8.969(\text{OH}) + 0.514(\text{CH}_2) \quad (3)$$

The F value of 7893 for eqn. 3 strongly indicates that this equation is statistically significant and a useful predictor for a. A plot of predicted a values using the regression equation *versus* observed a values from the LSER study [25] is given in Fig. 1.

The factors used in developing the regression equation were evaluated for correlation between factors; the correlation data for the 55-phase set are presented in Table IV. The only sizable positive correlation is between siloxane and CH_3; such a correlation is not unusual since most of the siloxanes have a large number of methyl substituents. Among the five factors included in the final regression equation several negative correlations are indicated, but the only one worthy of note is between ester and ether and is a consequence of the coating structures within the data set. Whereas there are a large number of coatings that have either ester *or* ether groups (23 and 40, respectively) only 8 coatings have *both* an ester and ether groups. Thus, intercorrelation of the regression factors was not considered to be above an acceptable level.

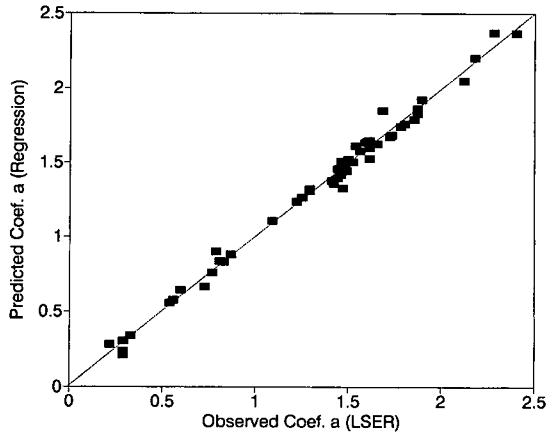

Fig. 1. Plot of the a values calculated using eqn. 3 (predicted) *versus* a values determined for the LSER from ref. 25 (observed). R^2 for the regression is 0.990, with a standard error of 0.054.

The residuals from the regression were examined to determine if they were truly random, or whether there was some systematic bias in the data. The average and standard deviation for the calculated residuals (using eqn.3) were 0.0007 and 0.051, respectively. Of the 55 coatings in the data set, 29 coatings were over-predicted while 26 were under-predicted. A plot of the residuals *versus* a in Fig. 2 indicates that there is no systematic trend as a function of a. Plots of residuals *versus* the various regression factors and *versus* M_r resulted in similarly random plots. Finally, the coatings were arranged in groups

TABLE IV

REGRESSION FACTOR CORRELATION TABLE

Variable	Phenyl	Siloxane	Ester	Ether	OH	CH_2	CH	CH_3
Phenyl	1.000							
Siloxane	0.0407	1.000						
Ester	−0.1157	−0.1970	1.000					
Ether	−0.1633	−0.3811	−0.6466	1.000				
OH	−0.1789	−0.1777	−0.1759	0.2269	1.000			
CH_2	−0.4608	−0.5556	−0.0357	0.3721	0.1966	1.000		
CH	−0.2076	−0.2240	−0.1212	0.2324	0.1467	−0.3023	1.000	
CH_3	−0.1358	0.6375	−0.0918	−0.3181	−0.1604	−0.6625	0.4826	1.000

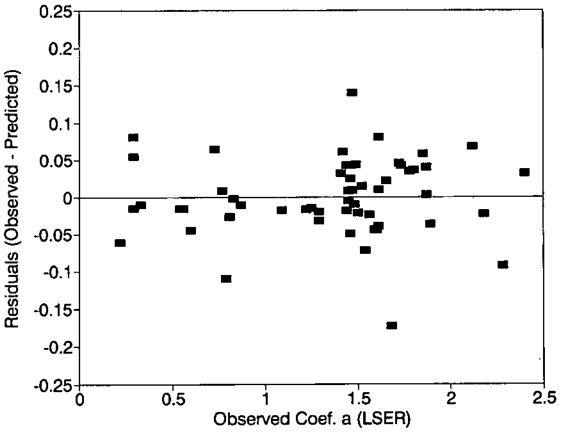

Fig. 2. Plot of residual values for *a* (observed − predicted) *versus* observed value, indicating random ditribution of errors.

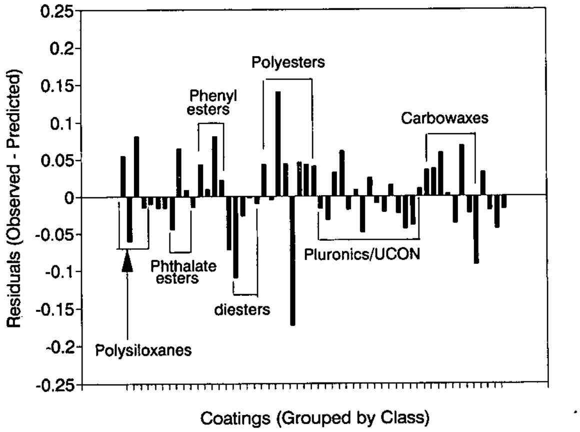

Fig. 3. Residuals for 55-phase data set grouped according to coating compound classes.

based on chemical class to determine if there were trends in residuals as a function of coating class. The results are presented in Fig. 3. While most of the coating classes exhibit fairly random distribution of residuals, some of the ester classes exhibit systematic behavior. The four diesters, for example, are over-predicted, whereas the phenyl esters tend to be under-predicted. The polyesters are, for the most part, under-predicted even though the largest *over*-predicted coating (DEGS) is a member of this class. For the most part, the number of coatings in each class is rather limited and the magnitude of the residuals is small; any conclusions regarding systematic trends in the residuals as a function of chemical class would be tenuous. The influence of any individual coating class on the regression results was investigated further.

The first coating class investigated was the polyesters. As stated in the introduction, the actual M_r for the 8 polyester coatings was not known; the regression results given above were calculated using estimated M_r of 2000 ($n = 10$) . Additional calculations were performed to determine what effect the M_r of the polyester coatings would have on the regression results. The functionality fraction descriptors in Table I were recalculated for the 8 polyester coatings using estimated M_r of approximately 5000 and 10 000. Most of the descriptors change only slightly, on the order of 1–3%. The hydroxyl fractional descriptor, however, decreases dramatically

TABLE V

COMPARISON OF REGRESSION RESULTS (POLYESTER M_r VARIABLE)

M_r	R^2	Standard error	Siloxane	Ester	Ether	OH	CH_2
2000	0.990	0.0539	1.412 (0.134)	3.517 (0.068)	3.872 (0.064)	8.969 (0.275)	0.513 (0.036)
5000	0.989	0.0579	1.409 (0.144)	3.619 (0.073)	3.868 (0.069)	8.948 (0.297)	0.517 (0.038)
10000	0.988	0.0601	1.409 (0.149)	3.654 (0.075)	3.866 (0.072)	8.919 (0.309)	0.0520 (0.040)
–[a]	0.994	0.0438	1.414 (0.109)	3.423 (0.075)	3.899 (0.054)	8.906 (0.225)	0.502 (0.030)

[a] Regression results for 48 coating set after elimination of polyester coatings.

TABLE VI

RESIDUAL (STANDARD AND DELETED) FOR 55-PHASE DATA SET

Coating	*a*		Standard residual	Deleted residual	% change residual
	Observed	Predicted			
DCFS-1265	0.2900	0.23558	0.05442	0.05893	8.3
SE-52	0.2200	0.28117	−0.06117	−0.08097	−32.4
DCF-550	0.2900	0.20904	0.08096	0.09361	156
SE-30	0.2900	0.30517	−0.01517	−0.02130	−40.4
SE-30(NPGA)	0.3300	0.33993	−0.00993	−0.01360	−36.9
PPE-5	0.5400	0.55566	−0.01566	−0.01613	−3.0
PPE-6	0.5600	0.57580	−0.01580	−0.01631	3.2
DBTCP	0.6000	0.64461	−0.04461	−0.04558	−2.2
DIDP	0.7300	0.66590	0.06410	0.06580	2.6
DOP	0.7700	0.76151	0.00849	0.00879	3.5
Bis-(2EOE)Ph	1.2500	1.26506	−0.01506	−0.01588	−5.4
Oronite-NIW	1.4600	1.41748	0.04252	0.04426	4.1
Tergitol-NPX	1.4700	1.46063	0.00937	0.00977	4.2
IgepalCO-880	1.6100	1.52959	0.08041	0.08381	42
Triton-X305	1.6500	1.62762	0.02238	0.02348	49
Ethofat 60/25	1.5400	1.61182	−0.07182	−0.07547	−5.1
DOSb	0.7900	0.89953	−0.10953	−0.12778	−16.7
IODA	0.8100	0.83648	−0.02648	−0.02842	−7.3
DEHSb	0.8300	0.83201	−0.00201	−0.00221	−10.0
DEHA	0.8700	0.88047	−0.01047	−0.01123	−7.3
EGSb	1.4400	1.39719	0.04281	0.04717	10.2
DEGSb	1.4500	1.45466	−0.00466	−0.00499	−7.1
NPGA	1.4700	1.32997	01.4003	0.15109	7.9
NPGS	1.4900	1.44617	0.04383	0.04892	11.6
DEGS	1.6800	1.85239	−0.17239	−0.19088	−10.7
EGA	1.7200	1.67457	0.04543	0.05139	13.1
DEGA	1.7300	1.68708	0.04292	0.04614	7.5
TEGS	1.8700	1.82996	0.04004	0.04282	6.9
UCON-LB1715	1.2200	1.23704	−0.01704	−0.01768	−3.7
PL-L81	1.2900	1.32228	−0.03228	−0.06654	−106.2
PL-L72	1.4100	1.37779	0.03221	0.03344	3.8
PL-L61	1.4200	1.35894	0.06106	0.06336	38
PL-P84	1.4400	1.45882	−0.01882	−0.01960	−4.1
PL-L42	1.4500	1.44102	0.00898	0.00930	3.5
PLP85	1.4600	1.50911	−0.04911	−0.05128	−4.4
PL-L63	1.4600	1.43538	0.02462	0.02554	3.7
UCON-HB2000	1.4800	1.48919	−0.00919	−0.00956	−4.0
PL-P65	1.5000	1.52108	−0.02108	−0.02197	−4.2
PL-L44	1.5200	1.50553	0.01447	0.01500	3.7
PL-P46	1.5600	1.58375	−0.02375	−0.02476	−4.3
PL-F88	1.6000	1.64362	−0.04362	−0.04617	−5.8
PL-F68	1.6100	1.64881	−0.03881	−0.04101	−5.7
PL-F77	1.6100	1.59954	0.01046	0.01100	5.1
CW-20M	1.7800	1.74547	0.03453	0.03696	7.0
CW-6000	1.8000	1.76322	0.03678	0.03919	6.6
CW-4000	1.8500	1.79223	0.05777	0.06118	5.9
CW-1540	1.8700	1.86699	0.00301	0.00316	4.9
CW-1000	1.8900	1.92682	−0.03682	−0.03860	−4.8
CW-600	2.1200	2.05163	0.06837	0.07285	6.6
CW-400	2.1800	2.20312	−0.02312	−0.02606	−12.7
CW-300	2.2800	2.37283	−0.09283	−0.11770	−26.8
Hyprose-SP80	2.4000	2.36803	0.03197	0.07790	143.7
SAIB	1.2900	1.30932	−0.01932	−0.02298	−19.0
Sucrose(Ac)8	1.5900	1.63402	−0.04402	−0.05857	−33.1
Castorwax	1.0900	1.10794	−0.01794	−0.02406	−3.41

by a factor of between 4 and 6 as the M_r increases from *ca.* 2000 to 10 000. Since the coefficient for the hydroxyl is the largest factor in the regression equation, such variations in this factor may significantly affect the regression results.

Regression equations were developed for the 55 coating data set using the three different M_r ranges for the polyesters, independently. Regression results for the three data sets are compared in Table V. The effect on siloxane, ether and CH_2 coefficients is relatively small, but the effect on ester and hydroxyl coefficients is larger. As the M_r of the polyester increases the value for the hydroxyl coefficient decreases and the value for the ester coefficient increases. Such an effect is reasonable in light of the large decrease in the hydroxyl fractional descriptor as M_r increases. Increasing the M_r of the polyester decreases the hydroxyl fraction of the molecule, and the relative weight given to this factor (the coefficient) decreases accordingly, while the ester coefficient increases to make up the difference. The standard error for all coefficients increases as M_r increases. The R^2 for the regression equation decreases only slightly, from 0.990 to 0.988.

Elimination of the polyester coatings from the data set improved the regression equation dramatically. The R^2 improved to 0.994 (standard error = 0.0438), and the standard error for 4 out of 5 of the regression coefficients decreased. The standard error for ester increased slightly from 0.068 to 0.075. These results are also included in Table V.

Other classes of coatings were removed from the data set and regression results were compared with eqn. 3. In most cases, removal of a given class resulted in a slight improvement in the R^2, but with an increase in the standard error of the regression coefficients. The only exception occurred upon removal of the siloxanes. Removal of this class necessitated reducing the number of factors in the regression equation by 1. The R^2 for the resulting 4-term regression was 0.986 (standard error = 0.0534), although the values for the 4 remaining coefficients differ by 2% or less from the values in eqn. 3.

A complete list of the residuals for the 55-phase data set is given in Table VI. Included in this list are deleted residuals, *i.e.* the residual that would be calculated for that particular coating if that coating were eliminated from the training set. The deleted residuals provide an indication of the relative importance of that particular coating in the regression. For most of the coatings in the set the deleted residual differs from the standard residual by 10% or less. Only 13 of the 55 coatings had deleted residuals that differed by greater than 10%. Of these, 4 were siloxanes which are marginally represented in the data set. All three of the sucrose esters also exhibited changes in the deleted residual greater than 10%, with the deleted residual for Hyprose SP80 changing by 144%. The only other unusually large variation was observed for Pluronic-L81 which changed by 106%. It should be noted that, even though the% change in the residual appears to be large, the absolute change in the predicted *a* value was rather small in most cases.

To test the validity of the regression model the coatings in the data set were divided into two subsets. This was accomplished by placing the coatings in order of increasing *a* value, and then assigning

TABLE VII

SUMMARY OF SUBSET REGRESSION PREDICTIVE UTILITY

Training set		+/−[a]	R^2 for Regression[b] (standard error)	Residuals average ± S.D.
Subset I	I	15 / 13	0.991 (0.056)	0.0029 ± 0.051
	II	16 / 11	0.989 (0.054)	0.0206 ± 053
Subset II	I	9 / 19	0.987 (0.064)	−0.0233 ± 0.062
	II	13 / 14	0.993 (0.044)	−0.0027 ± 0.044

[a] + is the number of coatings under-predicted (residual is positive); − is the number of coatings over-predicted (residual is negative).

[b] R^2 and standard error values for the subset used as the training set are taken from the regression results. Values for the predicted subset are calculated as regression results of observed (LSER) values *versus* predicted (regression) *a* values.

alternating coatings to subset I or subset II. Subset I had 28 coatings (a = 0.22–2.40), while subset II had 27 coatings (a = 0.29–2.28). Subset I was then used to generate regression coefficients, which were used to calculate a values for the coatings in subset II. This process was then reversed, and subset II was used to generate regression coefficients, which were used to calculate a values for the coatings in subset I. The results are summarized in Table VII. Even though the R^2 values for the predicted set are lower than for the training set, the results are still well within acceptable limits.

DISCUSSION

Although the statistical results indicate that the regression equation provides an accurate prediction for a values of the GC stationary phases, it would be more intellectually satisfying if these results (*i.e.*, regression coefficients) made sense on a molecular level. On a strictly qualitative basis, we can compare the values for the regression coefficients in eqn. 3 in light of the relative abilities of these functional groups to act as H-bond acceptors.

The coefficients for the oxygen-containing functional groups increase in the order

siloxane < ester < ether < OH

We can rationalize this trend, keeping in mind that the ability of a functional group to act as a H-bond acceptor will depend on the number and availability of lone pairs of electron. The siloxane is structurally similar to the ether, in that both are systems exhibit a tetrahedral bond arrangement around the Si or C atom. The decreased ability of the Si–O–Si functionality to act as a H-bond acceptor can be explained in terms of the extent of association of the non-bonding electron pairs with the O atom. In the siloxane system there is some back-bonding of these lone-pair electrons with the *d*-orbitals of the Si. This is evident in the Si–O bond strength (370 kJ/mol) compared to the C–O bond (336 kJ/mol) in which there is no back-bonding. This back-bonding decreases the availability of the lone-pair electrons and, hence, significantly decreases their tendency for H-bond acceptance.

The value for ester (3.517) is slightly less than that for ether (3.872). These coefficient values are normalized against the number of O atoms in each functional group, so that the ability of an ester group to accept H-bonds is actually a factor of two greater than indicated by the coefficient. The fact that the H-bond acceptor ability of an *individual* O atom in an ester group is less than that of an O in ether is likely due to the bond orders. In ethers, the average bond order for a C–O bond is 1; in esters, the average C–O bond order is 1.5. This increase in bond order decreases the availability of electrons for H-bonding. Finally, the tremendous ability of hydroxyls to act as H-bond acceptors is consistent with the large coefficient (8.969) in eqn. 3.

It should be noted that the values of the regression coefficients are somewhat dependent on the structural descriptors used to represent these functional groups. In this work we chose to only consider those atoms which actively engaged in H-bond acceptor behavior, *i.e.*, oxygens. Alternatively, these descriptors could have included the secondary atoms (*i.e.* Si and C), which may exert inductive effects on H-bond behavior. The esters, for example, could have been represented as $-CO_2-$, having a mass of 44 amu. While use of alternative descriptors in developing the regression equation would produce different coefficient values, the relative rankings of functional groups in terms of H-bond acceptor behavior would not change. In addition, the predictive value of the resulting relationship, implied by the R^2 values, is not affected.

The fact that CH_2 was determined to be a statistically significant factor in the regression equation was, initially, disconcerting. Obviously, there are no lone electron pairs or any other structural feature that would permit CH_2 to engage in H-bond interactions directly. Removing the CH_2 term from the regression, however, decreases the R_2 for the resulting equation to 0.951 (standard error = 0.121). Clearly, this term contains information relevant to the H-bonding ability of the coatings, although the nature of this information is not intuitively obvious. A possible explanation for this observation would be the inductive effect of CH_2 on the acceptor behavior of other functional groups in the coating structure. As the length of hydrocarbon chains increases (*i.e.* as the fraction of CH_2) the polarizability of the oxygen-containing functional groups increases and, as a consequence, their acceptor behavior increases. The positive sign and relatively small magnitude of the CH_2 coefficient in eqn. 3 are consistent with such inductive effects.

CONCLUSIONS

Clearly, the regression equation discussed above permits the accurate prediction of the ability of GC stationary phases to act as H-bond acceptors, as indicated by their *a* values in the LSER of Abraham *et al.* [25]. The general applicability of this approach is currently limited by the fact that several functional groups (nitrile, amide, amine, phosphate, etc.) were not adequately represented, which precludes determination of statistically significant regression coefficients. Furthermore, this approach is constrained to those coating materials that have been characterized by LSERs. Before universal applicability can be demonstrated, both of these limitations must be addressed; subsequent refinements of the regression equation(s) may be necessary. The success of this approach for the oxygen-containing coatings is remarkable in light of the relatively simple model and the fact that the structures for many of the coatings were approximated.

The ability of a given functional group to engage in H-bonding is likely to be sensitive to changes in the structure of the molecule. More sophisticated computer modeling methods are available which permit the calculation of electron density maps, which allow for refinement of the molecular descriptors used in the development of our regression equation. Such refinements would possibly obviate the need for the CH_2 term by correcting for inductive effects. The major advantage of the approach presented here is that results can be obtained relatively quickly (and easily) without requiring access to sophisticated modelling software.

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CHROM. 24 558

Preconcentration of volatile components of foods: optimization of the steam distillation–solvent extraction at normal pressure

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ABSTRACT

Optimization of the experimental conditions affecting the simultaneous steam distillation–solvent extraction of volatile components of foods was carried out by using the modified sequential simplex method. For this purpose, a new micro steam distillation–extraction device was constructed. The apparatus includes an enlarged surface condenser which contributes to preventing eventual losses of high-volatility components. Determination in the μg/l range of compounds having different polarities and volatilities are accomplished with high recoveries. The construction of the apparatus is such that the use of extraction solvents having densities either higher or lower than that of the solvent sample is feasible with only one configuration.

INTRODUCTION

Numerous techniques for isolating and concentrating volatile chemicals prior to GC analysis have recently been published and several methods have been applied to obtain a representative concentrate of the sample to be analysed, *i.e.*, liquid–liquid extraction, headspace analysis and simultaneous steam distillation–solvent extraction (SDE). The last method, introduced by Likens and Nickerson [1] in 1964, has received great attention. Some modifications have been made to the original apparatus, including the incorporation of a vacuum jacket in the arm which conducts the solvent vapour to the extractor body [2] and the use of more efficient cooling surfaces [3,4].

Godefroot *et al.* [5] reported a micro SDE device which allows operation with a small volume of solvent without requiring a subsequent concentration of the extract by solvent evaporation. This configuration has been used in different investigations, mainly related to food flavour research [6–8]. Several studies of the effects of different process factors on recovery efficiencies [9] and minimum concentrations that can be efficiently extracted [10] by using the SDE procedure have already been reported, but as far as we know the optimization of the variables that influence the process has not been thoroughly investigated.

The optimization of analytical techniques usually leads to an enormous experimental effort as the number of variables involved increases. In that situation it is of the utmost importance to apply a suitable optimization procedure for simultaneous handling of experimental variables. The sequential simplex method [11] has been broadly recognized as a very efficient empirical optimization procedure [12,13] since it allows the optimized conditions affecting a process to be obtained with a moderate number of experimental runs. This method has been successfully used in chromatographic research, as reported in a previous paper [14].

The objective of this work was to optimize the

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simultaneous distillation–extraction procedure at normal pressure, in order to attain a minimum error in determinations of the extracted solutes. To this end, the modified sequential simplex method [15] was applied. A micro SDE apparatus was specially constructed for this study. The new design allows effective mixing of the solvent vapour with sample vapour and includes an enlarged surface water condenser.

EXPERIMENTAL

Steam distillation–solvent extraction

The test mixture included fourteen compounds, previously reported as food volatile components, which were selected by considering differences in polarity, volatility and water solubility (see Table III). The stock solution consisted of a pure mixture of about 7.14% per component and was stored at −30°C in the dark. Synthetic mixtures were prepared directly in the extraction flask by adding the appropriate volume of the stock solution to water purified in a Milli-Q system (Millipore) in order to obtain a concentration of 1 mg/l or 10 μg/l of each compound. The reagents, all of GC grade, were purchased from Merck (Darmstadt, Germany) (isoamyl acetate, ethyl hexanoate, ethyl octanoate, benzaldehyde, diethyl succinate, ethyl dodecanoate, 2-phenylethanol), Aldrich Chemie (Steinheim, Germany) (1-hexanol, linalool, α-terpineol, γ-decalactone) and Sigma (St. Louis, MO, USA) (β-ionone, ethyl tetradecanoate).

According to our previous experience [8], doubly distilled dichloromethane (Merck) was initially selected as the extraction solvent for the optimization study. Depending on the concentration range to be investigated, a 1- or 2-ml volume of the solvent was used. In all instances cleaned boiling chips were added to the sample and solvent flasks. The sample heating bath temperature, solvent heating bath temperature, coolant temperature and extraction time were varied according to the optimization procedure. Further concentration was not needed and a 2-μl volume of the extract was subsequently analysed by capillary GC.

Between successive runs the SDE apparatus was cleaned by rinsing with acetone (Merck) and Milli-Q-purified water.

Capillary gas chromatography

The GC analyses were performed on a Perkin-Elmer Model 8500 gas chromatograph equipped with a PTV Perkin-Elmer injector and a flame ionization detector. The system was coupled to a Model 2600 chromatography software system (Nelson Analytical). A 50 m × 0.22 mm I.D. fused-silica capillary column (SGE, Ringwood, Australia) coated with a 0.25-μm layer of cross-linked BP-21 (FFAP) was used. Helium at 276 kPa over atmospheric pressure served as the carrier gas. Injections were carried out in the cold split (1/10) mode by introducing the sample at 30°C and subsequently heating the body injector (at 14°C/s) to 250°C. The detector was operated at 250°C. The oven temperature was initially 70°C for 5 min, then programmed at 5°C/min to 180°C and maintained there for 15 min.

The SDE apparatus used (Fig. 1) was specially constructed for this study by modifying the device previously designed by Godefroot *et al.* [5]. In order to minimize losses of the most volatile compounds, the condenser surface has been enlarged by the introduction of a jacket water, concentric with the cold finger, which acts as a second cooling system. A chamber on the top of the device facilitates effective mixing between the sample and the extraction solvent vapour. The distillation solvent and distillation sample arms enter the mixing chamber at the same height, thereby allowing the use of both high- and low-density extraction solvents.

Simplex optimization

The effect of modifying the experimental conditions on the overall performance of the analytical method was evaluated in terms of the differences in concentrations of the compounds present in the extract, calculated by the internal standard method, and their corresponding expected values. In order to improve the discrimination between different experimental runs, errors affecting determinations were squared, thus magnifying small differences between their summations. Moreover, two different internal standards, methyl octanoate and methyl decanoate (Fluka, Buchs, Switzerland) were used and the concentrations obtained with each one of them were averaged. The response function (Y) can be expressed as:

$$Y = \sum (C_i - C_i^0)^2 \qquad (1)$$

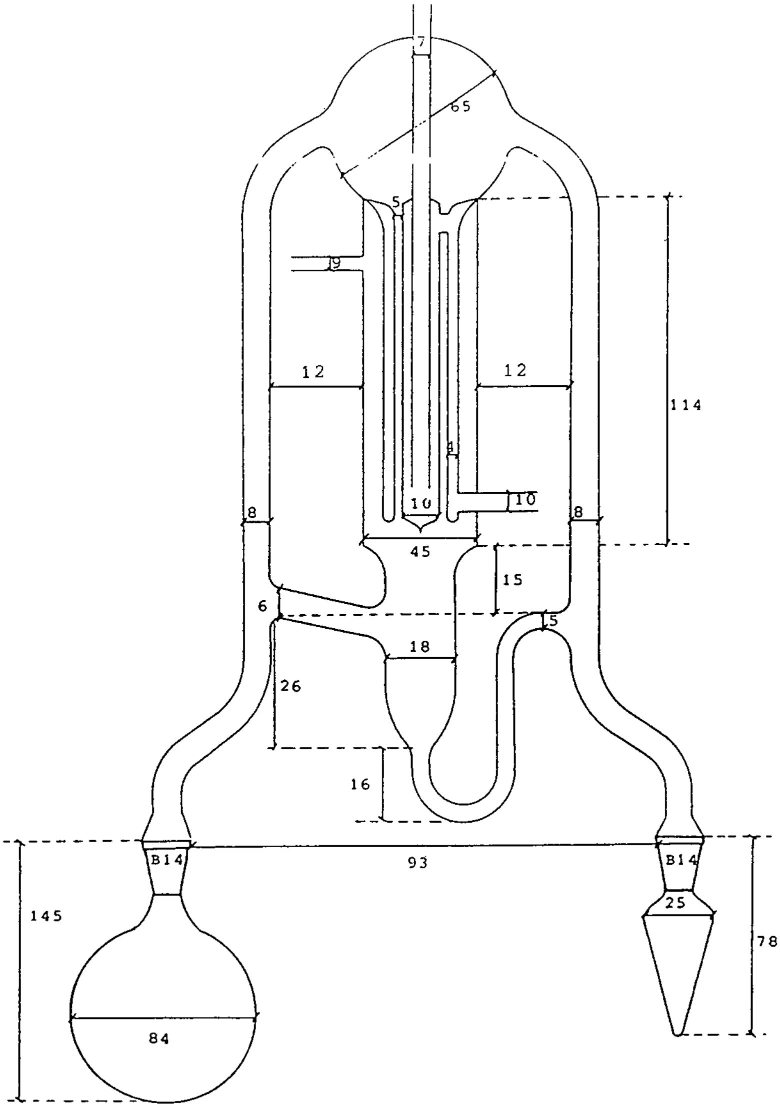

Fig. 1. Design of proposed simultaneous steam distillation–solvent extraction (SDE) device. Dimensions in mm.

where C_i is the concentration of the compound corresponding to the ith chromatographic peak and C_i^0 is the expected concentration for the same compound. Obviously, our objective was to minimize the value of the response function.

The initial experimental design was established according to Spendley *et al.* [16]. Physical values of factors were calculated from their mathematical coordinates using the equation

$$x_{\text{phys}} = x_0 + x_{\text{math}} \left(\frac{x_2 - x_1}{s} \right) \quad (2)$$

where x_{phys} is the physical value of the variable x, x_{math} the corresponding mathematical coordinate, x_0 its base level (starting physical value), x_1 and x_2 the lower and upper limits, respectively, of the range studied and s is the number of mathematical units into which the range has been divided.

The initial and successive simplexes were moved in the directions given by the rules of movement of the modified simplex method [15] and the corresponding responses were subsequently evaluated. Accordingly, different sets of variables were tested until no further improvement was achievable. In all instances, at least two replicates of each analysis were carried out. The coordinates of every new vertex were calculated according to the expression

$$V_i^* = C + \alpha (C - V_i) \tag{3}$$

where V_i^* is the new vertex, C the centroid of the retained vertices in the movement, V_i the rejected vertex and α a factor with different values depending on whether a reflection ($\alpha = 1$), an expansion ($\alpha > 1$) or a contraction ($\alpha < 1$) was performed.

It should be pointed out that the self-directing nature of the optimization method makes possible a boundary violation (*i.e.*, a movement outside the experimental range initially considered). Then, the corresponding vertex must be rejected without experimentation and so the simplex is subsequently forced to move back inside the boundaries by applying a factor $\alpha = -0.5$ [17].

As far as this study is concerned, no variable except the sample heating bath temperature was considered to be object of upper boundary, as values higher than 150°C might eventually lead to artefact formation.

RESULTS AND DISCUSSION

The experimental variables to be optimized, their studied ranges and their starting values, for the 1 mg/l mixture, are given in Table I.

Table II summarizes the sets of experimental values tested throughout the optimization procedure. The base level of each variable (*i.e.*, the starting point) is also included (vertex no. 1). The *s* value (eqn. 2) was set to 3. Values obtained for the response function (eqn. 1) are included in the response column in Table II.

The optimization study was initiated by performing the first five experiments defined in Table II, which constitute the initial simplex. The assessment of the values obtained for the response function in each analysis allows the worst vertex (no. 5) to be rejected. Then, a new simplex was formed with the retained vertices and a new one resulting from the mirror image of the rejected vertex ($\alpha = 1$). The procedure must be repeated to move from one simplex into another by rejecting the worst observation and by selecting an adequate α value.

Vertex 23 proceeds from a boundary violation of the sample heating bath temperature occurring in vertex 22, which was rejected, as previously mentioned, without experimentation.

Searching was stopped after vertex 23 for a variety of reasons: (a) the differences between responses of vertices 15, 18, 19, 21 and 23 are small, thus indicating that a region had been already reached in which the influence of the variables in the response value was not important (see Fig. 2 and Table II), (b) no significant improvement was achieved in the last movements (Fig. 2) and (c) vertex 15 was maintained throughout the performance of seven successive simplices, which according to Spendley *et al.* [16] is indicative of the attainment of a true optimum, provided that four variables are involved in the optimization procedure. Bearing in mind the small differences occurring between experimental variables defining four vertices of the last simplex, we finally selected the experimental conditions corresponding to vertex 18, even though the best response value actually results from conditions corresponding to vertex 15, because the analysis is less time consuming. It should be emphasized that the optimum zone has been achieved by carrying out only 22 experimental runs.

TABLE I

VARIABLES INCLUDED IN THE 1 mg/l MIXTURE OPTIMIZATION STUDY

Variable	Minimum value	Maximum value	Base level
Sample heating bath temperature, T (°C)	100	150	125
Solvent heating bath temperature, T_s (°C)	40	70	55
Coolant temperature, T_c (°C)	−5	17	6
Extraction time, t (min)	15	180	97.5

TABLE II

EXPERIMENTAL RUNS AND RESULTS FOR THE SIMPLEX OPTIMIZATION OF THE SDE PROCEDURE WITH THE 1 mg/l MIXTURE

Vertex No.	Simplex No.	Retained vertices	Experimental variable levels				Response Y
			T (°C)	T_s (°C)	T_c (°C)	t (min)	
1	1	–	125.0	55.0	6.0	97.5	2572
2	1	–	140.4	57.2	7.6	109.5	2656
3	1	–	128.6	64.3	7.6	109.5	1652, 1887[a]
4	1	–	128.6	57.2	12.8	109.5	2896
5	1	–	128.6	57.2	7.6	148.4	4804
6	2	1, 2, 3, 4	132.7	59.6	9.4	64.6	2353, 2298[a]
7	3	1, 2, 3, 6	134.7	60.8	2.5	81.1	1291, 1461[a]
8[b]	3	1, 2, 3, 6	137.8	62.7	−2.6	66.8	5782
9	4	1, 3, 6, 7	120.1	62.7	5.1	66.8	2590
10[c]	4	1, 3, 6, 7	125.2	61.3	5.8	77.5	2089
11	5	3, 6, 7, 10	135.6	68.0	6.6	68.8	1018, 1103[a]
12[b]	5	3, 6, 7, 10	141.0	74.5	6.9	54.5	1707
13	6	3, 7, 10, 11	129.4	67.6	1.9	103.8	2330
14[d]	6	3, 7, 10, 11	131.9	61.6	7.5	74.4	2072
15	7	3, 7, 11, 14	140.3	66.1	6.4	89.4	947, 828[a]
16[e]	7	3, 7, 11, 14	144.0	67.2	6.5	92.4	1570
17	8	3, 7, 11, 15	137.8	68.0	4.0	100.0	1389
18	9	7, 11, 15, 17	148.7	66.8	3.4	56.3	956, 927[a]
19	10	11, 15, 17, 18	146.4	73.6	7.7	76.2	1047
20	11	11, 15, 18, 19	147.8	69.3	8.0	45.4	1246
21[c]	11	11, 15, 18, 19	145.3	69.0	7.0	59.1	1042
22	12	15, 18, 19, 21	154.7	69.7	5.6	71.7	–
23[d]	12	15, 18, 19, 21	140.4	68.4	6.4	69.6	893

[a] The second response value was obtained by performing a new run to check a vertex maintained in $k + 1$ movements.

[b] Obtained from expansion $\alpha = 2$.

[c] Obtained from contraction $\alpha = 0.5$.

[d] Obtained from contraction $\alpha = -0.5$.

[e] Obtained from expansion $\alpha = 1.5$.

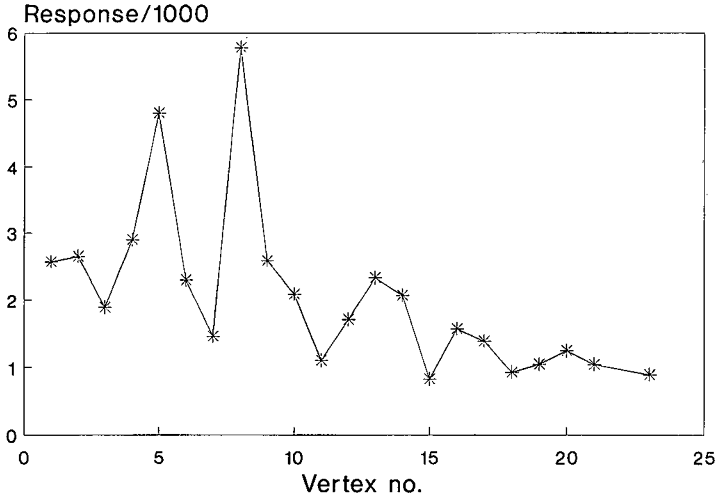

Fig. 2. Evolution of the response values obtained for the simplex optimization of the SDE procedure.

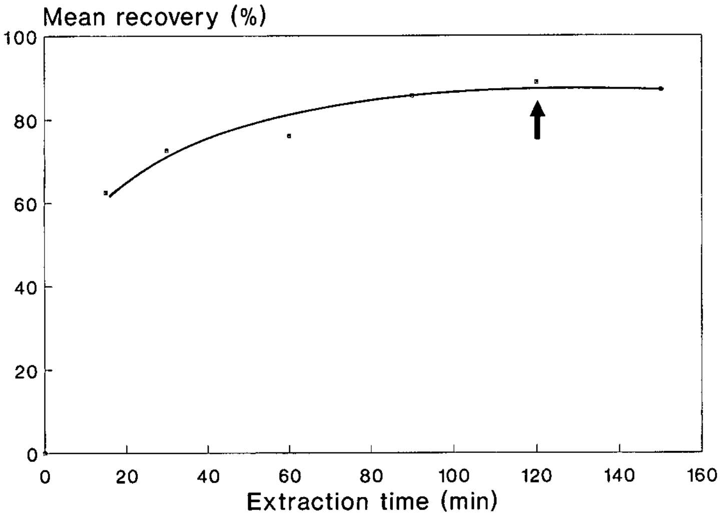

Fig. 3. Recovery of components by SDE at a concentration of 10 μg/l for each test compound as a function of the extraction time.

Fig. 3 depicts the effect of the extraction time, using dichloromethane as solvent, on the recoveries of the investigated compounds occurring at a concentration of 10 μg/l. From these data, a 2-h extraction time was considered to be the best option to determine components in the μg/l range. Other experimental conditions were fixed at the values previously optimized by applying the simplex procedure.

Tables III and IV show for concentrations of 1 mg/l or 10 μg/l for each test compound, respectively, the recoveries obtained by SDE, using dichloromethane as solvent, under the experimental conditions corresponding to the base level and under the optimized conditions. As can be seen, near 100% values were obtained after optimization, except for 2-phenylethanol, probably owing to its high solubility in water. However, it is noteworthy that the optimization method proposed also improves the recovery obtained for this compound. In order to check the possibility of using the modified SDE apparatus with solvents lighter than water, Table III also includes recoveries obtained by using *n*-pentane as solvent. In all instances, relative standard deviations, calculated from a minimum of six replicates, of less than 10% were obtained.

Fig. 4 shows the chromatogram obtained from the aroma extract resulting from a Muscat grape juice by using the SDE procedure under the optimized conditions for determining compounds in the μg/l range. Tentative peak identification of several compounds characteristic of juice was carried out by matching retention times with those of reference solutes.

TABLE III

RECOVERIES OBTAINED FOR THE COMPOUNDS SELECTED IN THE mg/l RANGE, UNDER THE EXPERIMENTAL CONDITIONS CORRESPONDING TO THE BASE LEVEL OF THE SIMPLEX OPTIMIZATION AND THE OPTIMUM CONDITIONS SELECTED FOR DICHLOROMETHANE OR *n*-PENTANE

Recovery as percentage of initial amount. Mean value of a minimum of six replicates.

Compound	Base level CH_2Cl_2	Optimum CH_2Cl_2	Optimum *n*-pentane
Isoamyl acetate	94.24	100.86	89.50
Ethyl hexanoate	93.76	102.66	90.68
Terpinolene	76.96	98.84	85.36
1-Hexanol	102.34	101.32	73.54
Ethyl octanoate	83.96	100.46	87.56
Benzaldehyde	105.96	102.46	87.18
Linalool	105.40	101.74	90.12
Diethyl succinate	80.56	97.00	75.04
α-Terpineol	105.04	102.84	91.42
Ethyl dodecanoate	68.18	99.10	94.58
2-Phenylethanol	18.36	55.28	11.26
β-Ionone	98.82	99.20	89.62
Ethyl tetradecanoate	74.82	101.48	75.20
γ-Decalactone	73.94	98.62	85.42

TABLE IV

RECOVERIES OBTAINED BY SDE FOR THE COMPOUNDS SELECTED AT A CONCENTRATION OF 10 μg/l

Recovery as a percentage of initial amount. Mean value of a minimum of six replicates.

Compound	Base level CH_2Cl_2	Optimum CH_2Cl_2
Isoamyl acetate	67.33	98.00
Ethyl hexanoate	86.50	97.50
Terpinolene	95.50	97.25
1-Hexanol	55.83	94.50
Ethyl octanoate	95.00	94.50
Benzaldehyde	48.33	98.50
Linalool	78.83	97.00
Diethyl succinate	17.33	79.00
α-Terpineol	55.00	97.75
Ethyl dodecanoate	96.33	85.00
2-Phenylethanol	16.50	26.00
β-Ionone	60.83	82.00
Ethyl tetradecanoate	83.50	100.90
γ-Decalactone	17.66	87.50

CONCLUSIONS

The use of the sequential simplex method allows the performance of the SDE procedure to be significantly improved. The proposed modified apparatus operated under the optimized conditions renders, in general, excellent recovery efficiencies for the investigated compounds in the mg/l or μg/l range. A notable advantage is the fact that the use of solvents denser or lighter than the solvent sample is possible with only one configuration. Moreover, the proposed micro SDE apparatus may work efficiently in different approaches including operation at normal pressure, at reduced pressure and concentration of the dynamic headspace from the sample. These aspects will be the topics of forthcoming papers.

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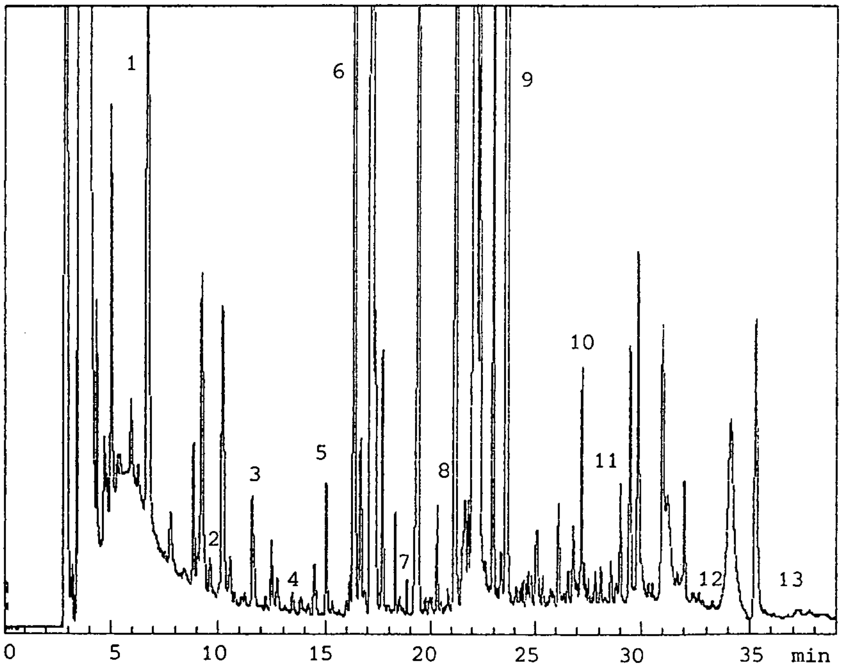

Fig. 4. Chromatogram of the aroma concentrate obtained from simultaneous steam distillation–solvent extraction (SDE) of a Muscat grape juice by using dichloromethane. Experimental conditions as optimized for determining components occurring in the μg/l range. Peaks: 1 = isoamyl acetate; 2 = ethyl hexanoate; 3 = terpinolene; 4 = 1-hexanol; 5 = methyl octanoate; 6 = ethyl octanoate; 7 = benzaldehyde; 8 = methyl decanoate; 9 = α-terpineol; 10 = ethyl dodecanoate; 11 = 2-phenylethanol; 12 = ethyl tetradecanoate; 13 = γ-decalactone. See text for further details.

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Separation and identification of free amino acid enantiomers in peat by capillary gas chromatography

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ABSTRACT

Racemization of free amino acids in a native peat bog at seven depths from 0 to 5.3 m was studied using glass capillary gas chromatography with a chiral liquid phase, Chirasil-L-Val. Fifteen L-enantiomers were identified: α-Ala, Val, Thr, Ile, Pro, Leu, Ser, Asp, Phe, Glu, Tyr, Orn, Met, Asn and Lys. Five D-enantiomers were also found: α-Ala, Ser, Asp, Glu and Phe. The D/L ratios at different depths were determined and are discussed in terms of racemization, plant and microbial degradation, and the physical and chemical conditions in the peat bog. In addition, three optically inactive amino acids were identified: β-Ala, Gly and γ-aminobutyric acid.

INTRODUCTION

The amino acids in active living tissues exist mostly as L-enantiomers, which is the only form used by animal enzyme systems. However, the cell walls of bacteria also contain some D-enantiomers, especially D-α-Ala, D-Glu and D-Asp [1]. After the tissue ceases to be metabolically active, both free and bonded amino acids eventually start to racemize in the soil. This process continues until the ratio of enantiomers is 1:1. The rate of the process has been used to date some fossil proteins in ocean sediments, archaeological bones and fossil shells [1–5].

Aspartic acid has been found to be a suitable indicator up to 80 000 years and the D-Allo/L-Ile ratio has been used for even older samples [2,3,6].

The rate of racemization depends on the structure of the amino acid, especially on the side chain at the α-carbon, and on the chemical and physical conditions of the environment, for example temperature, trace elements and acidity [1,5]. In aqueous solutions the rate of racemization decreases in the order Ser > Thr > Asp > Phe > α-Ala > Glu > Leu > Ile > Val [7].

This study is a sequel to our earlier work on the free amino acids in a typical Finnish peat bog [8]. The aim of the present study was to separate and identify the D- and L-enantiomers of the optically active free amino acids and to study the effect of the age of the peat on their D/L ratios. As far as we know, there have been no previous studies on the enantiomers of free amino acids in native peat bogs.

EXPERIMENTAL

Reagents and solutions

DL- and L-amino acids were obtained from Sigma (St. Louis, MO, USA) and pentafluoropropionic anhydride (PFP) from Fluka (Buchs, Schwitzerland). The other reagents were analytical grade and were obtained from Merck (Darmstadt, Germany). Aqueous solutions were prepared using deionized and distilled water.

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Samples and isolation of amino acids

The peat samples were collected from various depths, 0–5.3 m, in the Kaitalamminsuo bog, at Eno, Finland (E30°07′, N63°01′). Characterization of the samples and the method used for separation of amino acids are presented elsewhere [8].

Gas chromatography

The gas chromatographic measurements were made using a Perkin-Elmer F22 capillary gas chromatograph with two flame ionization detectors. The structures of the amino acids were confirmed with a Jeol JMS-D300/JMA-3500 mass spectrometer.

Amino acids were separated on a silica glass capillary column (25 m × 0.22 mm I.D.) using a 0.12-μm Chirasil-L-Val liquid phase.

The carrier gas was nitrogen, which had a flowrate of 1.8 ml/min and a split ratio of 1:32. Splitless injection was used, and the split was opened 40 s after injection of the sample. The temperatures of the injector and the detector were 220 and 230°C, respectively. The column oven was kept at 90°C for 4 min and then heated to 190°C at a rate of 4°C/min.

Derivatization of amino acids

The esters of amino acids were synthesized by mixing 2.0–3.5 mg of sample with 29 μl of acetyl chloride and 71 μl of isopropanol. The mixture was heated in a closed bottle at 110°C for 30 min, after which it was cooled to 50°C and the volatile reagents were evaporated carefully with nitrogen gas. The dry residue was dissolved in 50 μl of dichloromethane, and 50 μl of pentafluoropropionic anhydride were added. The mixture was heated for 10 min at 150°C in a closed bottle. After it was cooled to room temperature, volatile reagents were removed under a stream of nitrogen. To prevent the evaporation of α-Ala, Val and Thr, excessive drying was avoided. The residue was then dissolved in an appropriate volume of dichloromethane and the sample was chromatographed immediately [9,10].

RESULTS AND DISCUSSION

Kaitalamminsuo bog is an *Eriophorum–Sphagnum* mire. The samples for this study were taken from seven depths up to a depth of 5.3 m. The peat was classified and characterized by determining its water, element and ash contents as well as the pH and degree of humification on Von Post's scale (see ref. 8).

Because the chiral centres of the liquid phase, Chirasil-L-Val, had an L-configuration, the D-enantiomers eluted from the capillary column first. The net retention times, resolution factors (L/D) and mass spectrometric data for D,L-amino acid standards are presented in Table I. The resolution factors for all the amino acids were high enough to identify the enantiomers by their retention times.

Two typical gas chromatograms for amino acid extracts from peat are presented in Fig. 1A and B. Fig. 1A represents the surface layer, 20–40 cm, where the conditions were aerobic; Fig. 1B is from a depth of 3.0–3.3 m, at which the conditions were anaerobic. Eighteen different amino acids were separated and identified with the column used.

L-Enantiomers were found for all the optically active amino acids. For five of them, the D-enantiomer also occurred in detectable amounts. Of the epimers of isoleucine, only the *threo*-L-isomer was found. The method of derivatization used here was not suitable for arginine and histidine.

The D/L ratios for the enantiomers at various depths are presented in Table II as the means of five measurements. In the surface layer of the peat, where microbial degradation of plant and microbial material is most effective, the L-enantiomers were the most abundant enantiomers for all five amino acids. The living organisms and enzyme systems contain and use L-enantiomers of the amino acids almost exclusively. However, the microbial cell walls contain small amounts of D-α-Ala and some other D-enantiomers [1,11]; this together with racemization explains the low positive values of D/L ratios, which were also found in the surface layer of the bog.

At a depth of 40–100 cm there is a stepwise change in the properties of peat and in the total amount and qualitative composition of free amino acids [8]. Between these depths the D/L ratios for all five amino acids increase markedly, from 1.5 times for serine to 7.3 times for glutamic acid. An important reason for this may be changes in conditions in the bog from aerobic to anaerobic, where the microbial activity as well as the supplementary production of free amino acids is low.

Below 100 cm the proportions of D-enantiomers increase at different rates, but they seem to reach

TABLE I

NET RETENTION TIMES (t'_R), RESOLUTION FACTORS ($\alpha = t'_L/t'_D$), MOLECULAR MASSES (M_r) AND MASS SPECTROMETRIC DATA OF THE ISOPROPYL ESTERS OF N-(O,S)-PENTAFLUOROPROPIONYL-D,L-AMINO ACIDS

Amino acid	t'_R (min)	α	M_r	Main peaks in the mass spectra
D-α-Ala	1.28	1.390	277	190(100), 43(47), 191(43), 41(13), 119(11)
L-α-Ala	1.79			
D-Val	2.62	1.237	305	218(100), 55(87), 43(54), 219(34), 203(25), 41(14)
L-Val	3.24			
D-Thr	3.39	1.150	453	43(100), 203(70), 202(45), 57(26), 41(23), 119(16)
L-Thr	3.92			
Gly	3.97	–	263	43(100), 177(63), 176(62), 41(24)
β-Ala	4.10	–	277	55(100), 218(67), 43(48), 189(24)
D-*threo*-Ile	3.98	1.128	319	69(100), 232(59), 42(31), 203(30), 40(28), 233(21), 221(18), 57(11)
L-*threo*-Ile	4.49			
D-Pro	5.72	1.031	303	216(100), 217(12), 43(10), 41(10), 119(8), 71(7)
L-Pro	5.90			
D-Leu	6.04	1.273	319	43(100), 69(85), 190(48), 232(41), 189(27), 41(26)
L-Leu	7.69			
D-Ser	6.69	1.085	439	43(100), 189(68), 188(45), 41(20), 119(19), 230(16)
L-Ser	7.26			
γ-Aminobutyric acid	10.39	–	291	232(100), 43(59), 41(44), 204(39)
D-Asp	11.99	1.019	363	43(100), 234(64), 262(46), 235(35), 189(33)
L-Asp	12.22			
L-Asn	13.85		466	43(100), 215(51), 189(40), 216(35)
D-Met	13.67	1.081	337	61(100), 75(70), 221(34), 43(32), 203(31), 263(20)
L-Met	14.78			
D-Phe	15.67	1.054	353	91(100), 190(75), 148(65),103(30), 43(26), 266(25)
L-Phe	16.52			
D-Glu	16.14	1.051	377	248(100), 202(78), 230(72), 43(69), 85(55), 276(54)
L-Glu	16.97			
D-Tyr	21.17	1.033	515	253(100), 310(91), 352(88), 43(48), 248(30)
L-Tyr	21.86			
D-Orn	22.28	1.043	466	216(100), 43(27), 67(22), 231(11)
L-Orn	23.25			
D-Lys	25.45	1.024	480	230(100), 43(27), 67(22), 231(11), 110(11), 176(10)
L-Lys	26.07			

TABLE II

D/L RATIOS OF FREE AMINO ACIDS AT VARIOUS DEPTHS IN THE KAITALAMMINSUO PEAT BOG

Peat types: 1 = *Eriophorum-Sphagnum*; 2 = *Bryales–Carex*; 3 = *Sphagnum–Carex*; 4 = *Carex*; 5 = *Carex–Bryales*; 6 = *Carex–Phragmites–Equisetum*

Sample	K1	K2	K3	K4	K5	K6	K7	K8
Peat type	1	2	1	3	3	4	5	6
Depth	0–20 cm	0–20 cm	20–40 cm	1.0–1.3 m	2.0–2.3 m	3.0–3.3 m	4.0–4.3 m	5.0–5.3 m
Degree of humification [8]	H 0–1	H 0–1	H 2–3	H 5–6	H 5	H 5	H 5	H 5
pH [8]	5.3	5.4	5.8	3.8	3.9	3.7	5.9	2.8
Amino acid	D/L ratio							
α-Ala	0.414	0.144	0.272	1.270	1.190	1.130	1.690	1.230
Ser	0.090	0.106	0.043	0.064	0.089	0.235	0.161	0.123
Asp	0.013	0.008	0.016	0.093	0.115	0.157	0.172	0.164
Phe	0.033	0.024	0.043	0.128	0.340	0.376	0.378	0.320
Glu	0.012	0.011	0.014	0.102	0.084	0.099	0.194	0.157

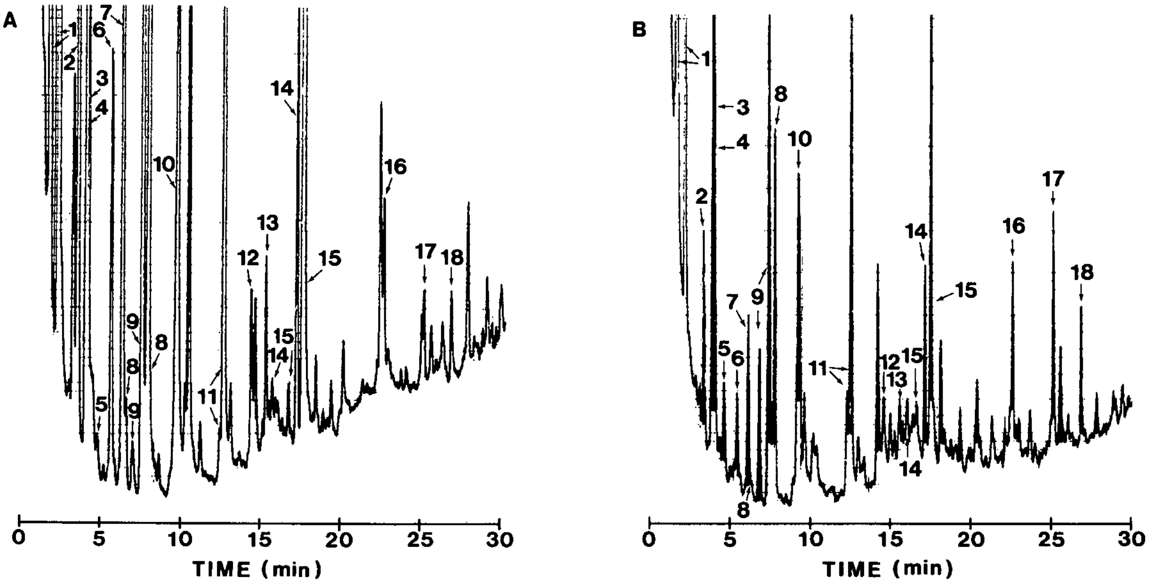

Fig. 1. Two typical gas chromatograms of isopropyl esters of N-(O,S)-pentafluoropropionyl-D,L-amino acids extracted from native peat. Depths of the samples were: A = 20–40 cm; B = 3.0–3.3 m. Peaks: 1 = α-Ala; 2 = Val; 3 = Gly; 4 = Thr; 5 = β-Ala; 6 = *threo*-Ile; 7 = Pro; 8 = Leu; 9 = Ser; 10 = γ-aminobutyric acid; 11 = Asp; 12 = Asn; 13 = Met; 14 = Phe; 15 = Glu; 16 = Tyr; 17 = Orn; 18 = Lys.

equilibrium values typical for each amino acid. Racemization probably continues at such a low rate that the process is impossible to follow owing to the large variation in results and the relatively young age of the peat, 9000 years [12].

The most rapid rate of racemization is that of α-alanine, for which equilibrium is reached already at a depth of 1 m and the D-enantiomer is predominant. Because the D/L ratio of α-alanine is greater than 1, the degradation of plants and microbes must produce not only the L-enantiomer but also the D-enantiomer.

The greatest changes in D/L ratios are for Asp, Phe and Glu. For these amino acids, at a depth of 5 m the proportion of D-enantiomer is 10–13 times higher than in the surface layer. However, the L-enantiomer is still clearly more abundant. On the other hand, the D/L ratio of serine does not change as much as that of the other four amino acids. It is impossible to say to what extent D-Asp, D-Phe and D-Glu are degradation products of plants and microbes or whether the racemization of these amino acids takes place in peat at an exceptionally high rate. The levelling off of the change at greater depths indicates that in the upper peat layer the degradation might provide an additional source of these D-enantiomers.

The type of peat has an obvious effect on the D/L ratios. The differences between Bryales (K2, K7) and *Sphagnum* types (K1, K3, K4, K5, K6, K8) are especially great. The same distribution was also found in the quantitative amounts of amino acids [8].

On the other hand, there are reports on the D/L ratios of bound amino acids in ocean sediments [1,4]. The order of the results for samples from different sources is for the most part the same, but there are some obvious differences. For example, in peat samples the D/L ratio of α-alanine is high compared with that in the ocean sediments, because D-α-Ala in peat is at least partly a degradation product of microbial material. In samples from the Antarctic Ocean sediments, ornithine razemices at a very high rate [1]. In peat, however, D-Orn was not found at all. The reason for this must be related to environmental factors. For example, the pH of the ocean sediments was 7–8, but of the peat bog was 2.8–5.9. The differences in acidity might change the racemization rates by several orders of magnitude [5]. In addition, the peat bogs contain trace metals such as copper, zinc, manganese, magnesium, etc., and when chelated with amino acids they have the same effect on the racemization rates [5,13].

With the exception of threonine, the six amino acids that racemized at the highest rate in aqueous solutions also had the highest D/L ratios in peat [7]. D-Threonine was not found at all in these peat samples.

In light of these results, it seems unrealistic to estimate the age of peat from the D/L ratios of free amino acids. Obviously, too many environmental factors influence the D/L ratios in natural conditions, and the age of the peat in our samples was too low, at a depth of 5 m only about 9000 years, to allow us to obtain reliable results on pure racemization without an additional source of D-enantiomers from the destruction of plant and microbial material.

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Packed-column supercritical fluid chromatography of omeprazole and related compounds

Selection of column support with triethylamine- and methanol-modified carbon dioxide as the mobile phase[☆]

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ABSTRACT

Using standardized chromatographic conditions with respect to carbon dioxide and methanol containing 1% of triethylamine as modifier, the chromatographic behaviour of omeprazole and four of its analogues was investigated on six types of chromatographic support at 40°C. Superior selectivity was obtained for omeprazole *versus* its analogues with NH_2-modified silica and also good peak symmetry (LiChrosorb and Kromasil). Using the present chromatographic system it is possible to detect and separate the analogues added to omeprazole in the 0.5% (w/w) range within 10 min.

INTRODUCTION

Omeprazole (Fig. 1) is a gastric proton-pump inhibitor (Losec). The methods used for the control of the stability of the substance and its content in the pharmaceutical formulations are based on normal-phase liquid chromatography with UV detection using a silica column and dichloromethane containing methanol and ammonia as the mobile phase [1,2]. For omeprazole sodium a reversed-phase system with acetonitrile–water as the mobile phase and Microspher C_{18} as the support is preferred [3]. We were interested in exploring whether it was possible to use modified carbon dioxide as the mobile phase for the chromatography of omeprazole and related compounds. As capillary columns have limited sample capacity and the compounds of interest here are polar and thermolabile we preferred packed columns.

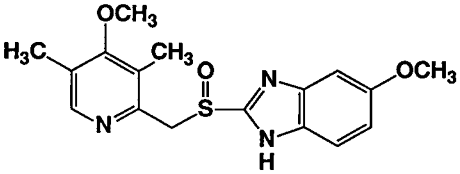

Fig. 1. Structure of omeprazole. The methoxy group of the pyridine can be absent (as in H 180/29) and the nitrogens of the imidazole can be methylated (isomers, H 193/61).

Supercritical fluid chromatography (SFC) has attracted considerable interest for the separation and determination of a wide range of different compounds [4]. The greatest number of publications have been based on studies using capillary columns

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and the analytes are often fairly lipophilic in nature. Another reason is that most commercial instruments have been constructed with capillary columns or narrow-bore packed columns.

For improved sample capacity and speed of analysis, packed columns are attractive. Howevere, pure carbon dioxide and ordinary columns packed with silica particles are not useful for most polar and protolytic compounds. Therefore, polar modifiers are mixed with the mobile phase. They work both by deactivating active sites on the support, mainly acidic silanol groups, and by increasing the solvating power of the mobile phase [5–8]. The most frequently used modifier is methanol. As our compounds of interest have basic nitrogens we included a tertiary amine in the main modifier, as is common practice in liquid chromatography [9].

Most of the supports used for packed-column SFC were originally intended for liquid chromatography. Often a certain type of column is chosen without any experimental evidence for its superiority, although some exceptions where different supports have been investigated in detail have been published [10–17]. For pharmaceutical substances the most commonly used supports seems to be the cyano [16,18–22] and amino [10,22–27] types of modified silicas. The silica can also be modified for SFC use but from the reported chromatograms it seems clear that residual activity from the support still persists [28,29]. Papers have also been published on the use of polymeric supports with SFC [11,20,30,31] but their retaining properties are high and they suffer from being sensitive to repeated pressure changes [31].

The aim of this work was to evaluate and select a suitable chromatographic support for the separation and determination of omeprazole and some of its possible contaminants and degradation products.

EXPERIMENTAL

Instrumentation

The chromatographic system was constructed from the following separate units. The pump for CO_2 was an LKB (Bromma, Sweden) Model 2150 with pump heads cooled by a circulating water–glycol mixture (2°C) (MGW Lauda RM6, Lauda-Königshofen, Germany). A jacket in the form of an E was shaped from a block of aluminium to fit snugly on the pump heads and made hollow by drilling several holes at different angles and plugging all except two (inlet and outlet) with screws. A second pump, ISCO (Lincoln, NE, USA) Model μLC-500 was used for delivery of the modifier. Carbon dioxide and the modifier were merged in a tee using tubing that almost faced each other. Mixing was accomplished using a magnetic stirrer in a small vessel (*ca.* 1.5 ml), previously used as slurry reserve.

A Rheodyne (Cotati, CA, USA) Model 7010 injector with a 20-μl loop was used for sample introduction. The columns were kept in a water-bath at 40°C (Thermomix 1441; Braun, Melsungen, Germany). The detector was a Jasco (Tokyo, Japan) UV 875 with a high-pressure cell (4 μl). The wavelength monitored was 300 nm and the absorbance range 0.04. A third pump was connected to the system downstream of the detector as a pressure monitor (ISCO μLC-500). A piece of fused-silica tubing (15–20 cm × 50 μm I.D.) was used as restrictor. To prevent clogging by solid carbon dioxide, most of it was kept in the water-bath. A Hewlett-Packard Model 3390A or a Spectra-Physics Model 4400 integrator was used to display the signal from the UV detector.

Columns

Standard commercial liquid chromatographic columns were used. Initially they were tested with pure carbon dioxide and polyaromatic hydrocarbons (naphthalene, anthracene and pyrene) as test substances dissolved in hexane prior to chromatography with modifiers in order to check the efficiency (wavelength 254 nm, 0.04 absorbance). The following dimensions and sources were used: 125 × 4.0 mm I.D. (NH_2, DIOL, CN, RP-18, Si-60; Merck, Darmstadt, Germany); 125 × 4.6 mm I.D. (NH_2; Eka Nobel, Bohus, Sweden); 200 × 4.6 mm I.D. [NH_2 and $N(CH_3)_2$; Macherey–Nagel, Düren, Germany]; and 150 × 4.6 mm I.D. (CN; Supelco, Bellefonte, PA, USA). The size of the particles was 5 μm throughout.

Chemicals and reagents

The carbon dioxide used was 3.5 grade with dipper tube from AGA (Lidingö, Sweden), methanol and dichloromethane of analytical-reagent grade were obtained from Merck and triethylamine zur Synthese from Merck was glass distilled.

Omeprazole, omeprazole sodium and the corresponding sulphide (H 168/22), sulphone (H 168/66), N-methylated (H 193/61) and desmethoxylated (H 180/29) (the pyridine) compounds were from the Department of Organic Chemistry, Astra Hässle (Mölndal, Sweden). Their structures can be understood from that of omeprazole in Fig. 1.

Methods

The substituted benzimidazoles were dissolved in dichloromethane at *ca.* 500 μg/ml. Samples were chromatographed using a pump flow setting of CO_2 of 1.20 ml/min and modifier of 60 μl/min (1% of triethylamine in methanol). The system was allowed to equilibrate for about 1 h before any measurements were made. For each column the separation factors with respect to omeprazole were calculated from the capacity factors, $k' = (t_r - t_0)/t_0$. When possible this was done from the injection of a complete mixture of the compounds of interest. The hold-up time was measured from the point of injection to the apex of the negative peak caused by the injection. The symmetry was calculated up one tenth of the peak height from the baseline. Normally a minimum of two new columns of each type were investigated in order to confirm the results obtained.

RESULTS AND DISCUSSION

The present chromatographic system

This system was preceded by a slightly different instrumental set-up in which 250 × 1 mm I.D. glass-lined columns were used and carbon dioxide was delivered by an ISCO pump working under constant pressure [32]. The columns used were packed in-house as some purchased columns were unacceptable regarding efficiency and peak symmetry. However, as the selectivity with the preferred packing material was poor [32] and we encountered problems in making reproducible columns, the system was changed to one in which standard-size packed columns could be used. Also, an advantage is the less critical demand on the tubing and the connections compared with using small I.D. (50 μm) fused-silica tubing and microbore columns.

The weak point of this system is the regulation of the back-pressure. It was not possible to keep the back-pressure at the same value for all columns but as the percentage of modifier was of greater importance for the capacity factors than was the actual outlet pressure, and hence the density in the system, this was thought to be a minor problem at this stage of development. The back-pressure will be under control in the future [33,34]. Minor changes in modifier concentration affect the capacity factor more than do minor changes of pressure [23], although this does not exclude the possibility that the selectivity might change with varying density.

The mobile phase used throughout was carbon dioxide with methanol as the main modifier and triethylamine added as base. The presence of an amine or base in the modifier is commonly used in packed-column SFC [10,14,18,22,23,25,35,36] when the analytes contain aliphatic nitrogens and is in analogy with practice in liquid chromatography [9].

Selection of column support with suitable selectivity

The separation factors (α) relative to omeprazole for the investigated columns and substances are illustrated in Fig. 2. The actual capacity factors for omeprazole, and the inlet pressures and linear velocity of the mobile phase, are given in Table I. The columns were tested in duplicate except for the Si-60, dimethylamino and the diol columns. The last-mentioned type has been extensively investigated previously [32].

From Fig. 2 it is evident that the selectivity is not adequate with LiChrosorb RP-18 or Superspher Si-60 (bare silica). With the former support there is insufficient selectivity between omeprazole and its desmethoxylated analogue, and with bare silica the last-mentioned compound and methylated omeprazole co-elute (Fig. 2). With the diol support most compounds are close to omeprazole (Fig. 2). This is in agreement with previous results [32].

It is interesting to observe the reasonably good agreement between the aminopropylsilica columns from the three different suppliers, Macherey–Nagel, Merck and Eka Nobel. For this kind of support the order of elution is according to increasing polarity of the analytes. The elution order is illustrated with the chromatogram in Fig. 3. With dimethylamino-modified silica an increase in selectivity is found between omeprazole and the sulphide and the sulphone. Selectivity factors from columns run in duplicate agree reasonably well considering the absence of control of the back-pressure.

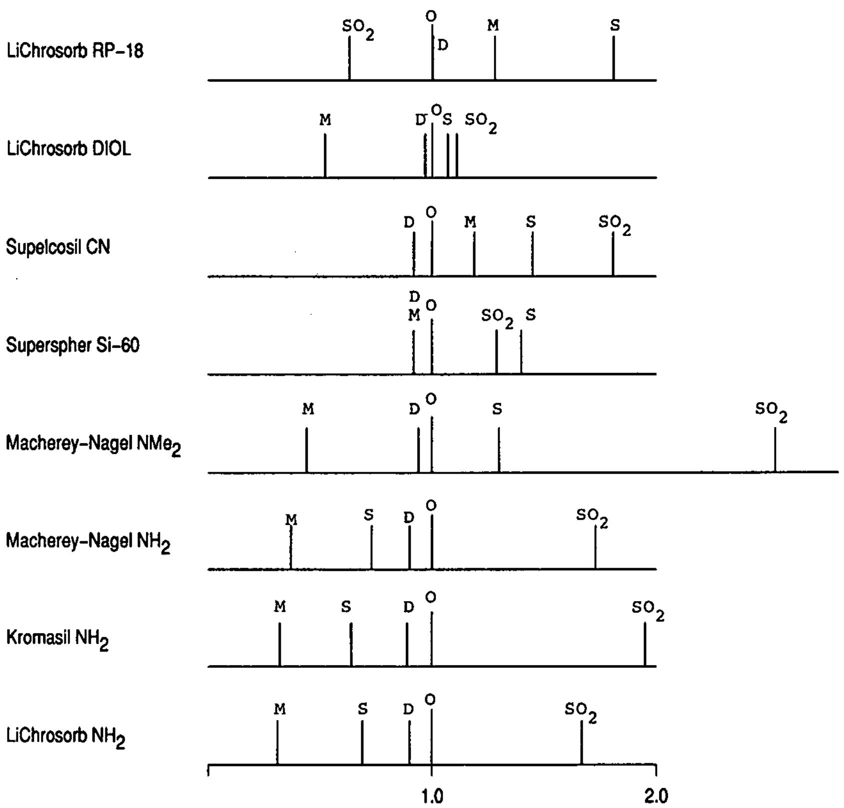

Fig. 2. Illustration of the selectivity factors relative to omeprazole with different column supports. Compounds: O = omeprazole; M = methylated omeprazole isomers (H 193/61); D = desmethoxyomeprazole (H 180/29); S = corresponding sulphide (H 168/22); SO_2 = corresponding sulphone (H 168/66) (*cf.*, Fig. 1). For conditions, see Experimental section and Table I.

The capacity factors for omeprazole show high values using bare silica and aminopropylsilica from Macherey–Nagel (Table I). In the former instance a stable equilibrium was first reached after about 6 h of continuous work. The capacity factors decreased by about 30% over this period. It is possible that a significant layer of adsorbed methanol acts as a stationary phase that retains the analytes to a larger extent than on, *e.g.*, RP-18 (Table I). With the amine column mentioned the high capacity factor may partly be due to a fairly low outlet pressure (Table I).

Selection of column support with the least residual activity

The asymmetry factors for omeprazole and its analogues were measured in order to obtain an indication of remaining active sites on the support. Most columns gave good symmetry for methylated omeprazole (Table I), which lacks the slightly acidic nitrogen in the imidazole ring (Fig. 1). However, with the diol column poor symmetry (*ca.* 2) was observed also for this compound (Table I). Although the peak shape is reasonable using RP-18 or Superspher silica, they do not have sufficient selectivity (see above). Of the columns tested, the aminopropylsilica columns from Merck and Eka Nobel showed the best overall symmetry for the compounds of interest (Table I). As the latter contains *ca.* 30% more packing material and the capacity factors are a slightly high, the former was preferred for testing with actual omeprazole samples. The chromatogram in Fig. 3 shows the separation and good peak symmetry obtained with this column. The greater inertness of the aminopropyl type of silica has been reported elsewhere [26,37] and is evident from other reports [23,25,27].

TABLE I

SELECTION OF SUITABLE COLUMN SUPPORTS: ASYMMETRY FACTORS AND CHROMATOGRAPHIC DETAILS

Pump flows, 1.20 ml/min CO_2 and 60 μl/min of modifier (1% TEA in methanol). For other details and conditions, see Experimental section. Structures in Fig. 1.

Column (support)	Asymmetry factors				k' (omeprazole)	Linear flow-rate (cm/s)	Inlet/outlet pressure (bar)
	Methylated omeprazole[a]	Omeprazole	Sulphide (H 168/22)	Sulphone (H 168/66)			
LiChrosorb RP-18	1.4	1.5	1.6	1.15	2.57	0.15	122/120
LiChrosorb Diol	2.1	2.1	2.8	2.2	5.56	0.13	273/269
Supelco CN	0.9	1.8	>4	1.2	5.06	0.16	95/96
Superspher Si-60	1.2	1.45	nm[b]	1.33	28.6	0.17	142/130
Macherey–Nagel $N(CH_3)_2$	1.22	1.68	nm	nm	7.23	0.14	126/126
Macherey–Nagel NH_2	1.0	nm	>2	>2	31.3	0.15	118/83
Kromasil NH_2	1.0	0.95	0.94	nm	14.6	0.13	119/105
LiChrosorb NH_2	0.9	1.22	1.37	1.44	9.13	0.15	175/nm

[a] Given for the second isomer of the pair.
[b] nm = Not measured.

Chromatographic analysis of omeprazole substance

Using the selected aminopropylsilica column, the possibility of determining omeprazole of different origins was tested. In order to reduce the time of analysis, the flow-rate and the percentage of modifier were increased. The last aspect is crucial, as the separation of omeprazole and its desmethoxylated analogue is easily destroyed with too much modifier. In Fig. 4 a successful separation within 10 min where minute amounts of possible contaminants had been added to omeprazole is shown. Regulatory requirements require that at least 0.1% of impu-

Fig. 3. Chromatogram of omeprazole and related compounds on LiChrosorb NH_2 using 1.2 ml/min of CO_2 and 60 μl/min of 1% TEA in methanol. Peaks as in Fig. 2. Inlet pressure, 175 bar.

TABLE II

COMPARISON OF LC AND SFC IN THE STUDY OF DEGRADATES FORMED FROM OMEPRAZOLE SODIUM UNDER ACCELERATED CONDITIONS

The degradates are given as a percentage of the omeprazole peaks, as the chromatographic pattern of the other degradates was not identical.

Compound	Sample A[a]		Sample B[a]	
	LC	SFC	LC	SFC
Omeprazole	100	100	100	100
Methylated omeprazole	3.66[b]	1.23, 2.39, 3.62	0.23[b]	0.07, 0.14, 0.21
Sulphide	nm[c]	0.35	nm[c]	nd[d]
Sulphone	2.02	2.33	3.00	2.62

[a] A is omeprazole sodium from storage at 50°C and B is from a rejected batch.
[b] The isomers of methylated omeprazole were not separated by LC [3].
[c] The sulphide was not measured by LC; retention time about 25 min [3].
[d] nd = Not detected.

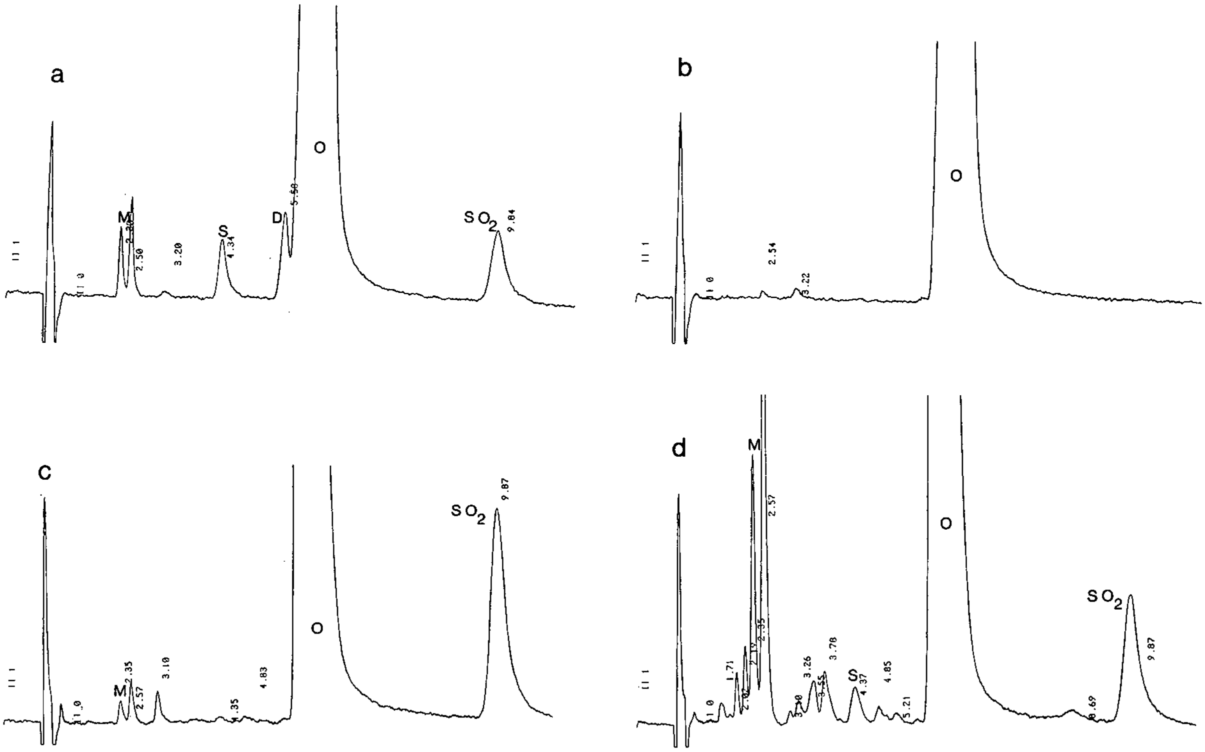

Fig. 4. Chromatogram of different batches of omeprazole substance, *ca.* 4 mg/ml in dichloromethane. Conditions: CO_2 2.0 ml/min and 1% TEA in methanol 120 μl/min; column, LiChrosorb NH_2 at 40°C; inlet pressure, 183 bar; outlet pressure, 175 bars; linear velocity, 0.27 cm/s. Peaks as in Fig. 2. (a) With possible impurities added (0.4–0.6%, w/w); (b) pure omeprazole substance; (c) omeprazole sodium from a rejected batch; (d) omeprazole sodium that had been stored at 50°C.

rities can be determined. It should also be noted that the isomers of methylated omeprazole have different ε values. A corresponding chromatogram with pure omeprazole is given in Fig. 4b and here the sum of extraneous peaks constitutes less than 0.1 area-% at 300 nm. Fig. 4c and d show chromatograms from omeprazole sodium of different origin. In Fig. 4c the substance had been freeze-dried but rejected after inspection after storage for 3 months at ambient temperature. The chromatogram in Fig. 4d is for omeprazole sodium that had been stored at 50°C for a certain period of time. Here more of the methylated omeprazole isomers are formed than shown in Fig. 4c. Analysis by liquid chromatography gave the same patterns, as can be seen from Table II. However, the liquid chromatographic method cannot discriminate between the isomers of methylated omeprazole although they elute after about 12 min [3]. As the sulphide requires 25 min for elution, the SFC system is about 2.5 times faster and has a higher degree of selectivity.

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Simulated quantitative and qualitative isotachophoretic indices of 73 amino acids and peptides in the pH range 6.4–10

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ABSTRACT

Qualitative and quantitative isotachophoretic indices of 73 amino acids, dipeptides and tripeptides were simulated under 24 leading electrolyte conditions covering the pH range 6.4–10. The R_E values and time-based zone lengths are tabulated together with the absolute mobility (m_0) and pK_a values used. The leading electrolyte used was 10 m*M* HCl and the pH buffers were imidazole, tris(hydroxymethylamino)methane, 2-amino-2-methyl-1,3-propanediol and ethanolamine. The simulated indices will be useful in the assessment of the separability and determination of the listed and related compounds.

INTRODUCTION

In 1983 we published qualitative and quantitative isotachophoretic (ITP) indices for 287 ions in the pH range 3–10 in a tabular form [1]. The validity of the simulation has been confirmed for many samples. For example, Oefner *et al.* [2] measured qualitative and quantitative indices of 52 anions and compared them with the simulated values and correlation factors of 0.993 and 0.92 were obtained for qualitative and quantitative indices, respectively. In addition to this extensive comparative work, many researchers have utilized the table for separability assessment.

In this work, the qualitative and quantitative ITP indices of 73 amino acids and peptides as anions were simulated on the basis of the absolute mobilities and thermodynamic dissociation constants. Most of them have been evaluated by the ITP technique [3–5] and several of them were refined using new R_E data. The methods used for the evaluation of physico-chemical constants have been reviewed by Pospichal *et al.* [6].

SIMULATION

Method

The simulation techniques were the same as in the previous work [1]; iterative calculation using the RFQ method [7] was carried out to fulfil ITP conditions. The effect of ionic strength on the mobilities and dissociation constants was considered.

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TABLE I

ELECTROLYTE SYSTEMS USED IN SIMULATION FOR AN ANIONIC ANALYSIS

Leading ion = 10 m*M* Cl^-. Gly-Gly = glycylglycine; β-Ala = β-alanine; ε-AMC = ε-aminocaproic acid; CR = creatinine; HIS = histidine; IM = imidazole; TRIS = tris(hydroxymethylamino)methane (Tris); AMEDIOL = 2-amino-2-methyl-1,3-propanediol; EA anolamine.

Buffers	Number and the pH of leading solutions						
Gly-Gly	(1)2.6	(2)2.8	(3)3.0	(4)3.2	(5)3.4	(6)3.6	
β-Ala	(7)3.0	(8)3.2	(9)3.4	(10)3.6	(11)3.8	(12)4.0	
ε-AMC	(13)3.8	(14)4.0	(15)4.2	(16)4.4	(17)4.6	(18)4.8	
CR	(19)4.2	(20)4.4	(21)4.6	(22)4.8	(23)5.0	(24)5.2	(25)5.4
HIS	(26)5.4	(27)5.6	(28)5.8	(29)6.0	(30)6.2	(31)6.4	
IM	(32)6.4	(33)6.6	(34)6.8	(35)7.0	(36)7.2	(37)7.4	
TRIS	(38)7.4	(39)7.6	(40)7.8	(41)8.0	(42)8.2	(43)8.4	
AMEDIOL	(44)8.2	(45)8.4	(46)8.6	(47)8.8	(48)9.0	(49)9.2	
EA	(50)9.0	(51)9.2	(52)9.4	(53)9.6	(54)9.8	(55)10.	

Electrolyte system

The leading electrolyte was 10 m*M* HCl containing an appropriate pH buffer. The pH range of the leading electrolyte (pH_L) was varied in the range of 6.4–10 (0.2 pH steps, electrolytes 32–55) using four different pH buffers as shown in Table I. For a few acidic amino acids, the pH range was 3–10 and eight kinds of buffers were used (electrolytes 1–55).

TABLE II

SIMULATED EFFECTIVE MOBILITIES AND CONCENTRATIONS OF LEADING ZONE CONSTITUENTS

Leading ion = 10 m*M* Cl^-. pH_L = pH of leading electrolyte; m_L = effective mobility of leading ion (10^{-5} cm ^{2} V^{-1} s^{-1}); $C^t_{B,L}$ = total concentration (m*M*) of buffer ion; $m_{B,L}$ = effective mobility of buffer ion (10^{-5} cm^2 V^{-1} s^{-1}); R_L = specific resistance of leading electrolyte (Ω m); E_L = potential gradient of leading zone (V cm^{-1}); v = isotachophoretic velocity (10^{-3} cm s^{-1}). Capillary I.D. = 0.5 mm.

Buffer	pH_L	m_L	$C^t_{B,L}$	$m_{B,L}$	R_L	E_L	v
IM	(32) 6.4	−74.70	11.60	41.51	8.435	42.96	32.09
	(33) 6.6	−74.70	12.54	38.41	8.435	42.96	32.09
	(34) 6.8	−74.70	14.03	34.34	8.435	42.96	32.09
	(35) 7.0	−74.70	16.38	29.40	8.435	42.96	32.09
	(36) 7.2	−74.70	20.12	23.94	8.435	42.96	32.09
	(37) 7.4	−74.70	26.03	18.50	8.435	42.96	32.09
TRIS	(38) 7.4	−74.70	11.90	21.95	10.28	52.35	39.11
	(39) 7.6	−74.70	13.01	20.07	10.28	52.35	39.10
	(40) 7.8	−74.70	14.78	17.68	10.28	52.35	39.10
	(41) 8.0	−74.70	17.57	14.87	10.28	52.34	39.10
	(42) 8.2	−74.70	22.00	11.88	10.28	52.33	39.09
	(43) 8.4	−74.70	29.02	9.00	10.27	52.33	39.09
AMEDIOL	(44) 8.2	−74.70	12.37	23.10	10.03	51.10	38.17
	(45) 8.4	−74.70	13.76	20.77	10.03	51.09	38.16
	(46) 8.6	−74.70	15.96	17.91	10.03	51.07	38.15
	(47) 8.8	−74.70	19.45	14.70	10.02	51.04	38.13
	(48) 9.0	−74.70	24.99	11.45	10.01	51.00	38.10
	(49) 9.2	−74.70	33.77	8.47	10.00	50.94	38.05
EA	(50) 9.0	−74.69	12.88	31.58	8.969	45.68	34.12
	(51) 9.2	−74.69	14.56	27.94	8.959	45.63	34.08
	(52) 9.4	−74.69	17.24	23.62	8.942	45.54	34.01
	(53) 9.6	−74.69	21.49	18.98	8.915	45.41	33.91
	(54) 9.8	−74.68	28.24	14.47	8.874	45.19	33.75
	(55) 10.0	−74.69	39.01	10.51	8.808	44.86	33.50

TABLE III

PHYSICO-CHEMICAL CONSTANTS OF BUFFERS USED IN SIMULATIONS (25°C)

m_0 = Absolute mobility (cm^2 V^{-1} s^{-1}); pK_a = thermodynamic dissociation constant.

Buffer	m_0	pK_a
Gly-Gly	31.5	3.140
β-ala	36.7	3.552
ε-AMC	28.8	4.373
CR	37.2	4.828
HIS	29.6	6.040
IM	52.0	7.150
TRIS	29.5	8.076
AMEDIOL	29.5	8.780
EA	44.3	9.498
Cl$^-$	79.1	–

Table II summarizes the simulated effective mobilities and concentrations of the leading zone constituents. Table III gives the absolute mobilities and thermodynamic dissociation constants of the buffers used. Most of them were obtained by ITP but some were taken from the literature [8–11]. The names and abbreviations of substances treated are given in Table IV.

Migration conditions

The inner diameter of the separation tube was 0.5 mm, the migration current was 100 μA and the temperature of the separation tube was 25°C.

CONTENTS OF SIMULATED DATA

The contents of the simulated data given in Table V, pH$_L$, pH$_S$, m, R_E, t, R_S and IS, are explained briefly below.

pH$_L$

This is the pH of the leading electrolyte used in the simulation. The leading electrolytes used are summarized in Table I. Eight different buffers were used to adjust the pH of 10 mM HCl. To maintain a good buffering capacity of the leading electrolyte, the pH$_L$ should satisfy the relationship pK_Q − 0.5 < pH$_L$ < pK_Q + 0.5, where pK_Q is the pK_a of the buffers used. The maximum buffering capacity is obtained at pH$_L$ = pK_Q.

pH$_S$

This is the pH of the sample zone at the steady state evaluated by simulation. When pH$_S$ differs significantly from pH$_L$ (approximately over 1–1.5), the

TABLE IV

NAMES OF SAMPLES AND ABBREVIATIONS USED

No.[a]	Name	Abbreviation
400	Alanylalanine	Ala-Ala
401	L-Alanyl-L-alanyl-L-alanine	Ala-Ala-Ala
402	Alanyl-α-amino-*n*-butyric acid	Ala-Amin
403	Alanylasparagine	Ala-Asn
404	Alanylglycine	Ala-Gly
405	DL-Alanylglycylglycine	Ala-Gly-Gly
406	β-Alanylhistidine	β-Ala-His
407	Alanylleucine	Ala-Leu
408	DL-Alanyl-DL-leucylglycine	Ala-Leu-Gly
409	Alanylmethionine	Ala-Met
410	β-Alanine	β-Ala
411	Alanine	Ala
412	Alanylphenylalanine	Ala-Phe
413	Alanylserine	Ala-Ser
414	Alanylvaline	Ala-Val
415	α-Amino-*n*-nutyric acid	Amin
417	Asparagine	Asn
418	Aspartic acid	Asp
419	Cysteine	Cys
420	Cystine	CysH

(Continued on p. 286)

TABLE IV *(continued)*

No.[a]	Name	Abbreviation
421	Glutamic acid	Glu
422	Glutamine	Gln
423	Glycylalanine	Gly-Ala
424	Glycyl-α-amino-*n*-butyric acid	Gly-Amin
425	Glycylasparagine	Gly-Asn
426	Glycine	Gly
427	Diglycine	Gly-Gly
428	Triglycine	Gly-Gly-Gly
429	Tetraglycine	Gly-Gly-Gly-Gly
430	Pentaglycine	Gly-Gly-Gly-Gly-Gly
431	Hexaglycine	Gly-Gly-Gly-Gly-Gly-Gly
432	Glycylglycyl-L-isoleucine	Gly-Gly-Ile
433	Glycylglycyl-D-leucine	Gly-Gly-Leu
434	Glycylglycyl-L-phenylalanine	Gly-Gly-Phe
435	Glycylglycyl-L-valine	Gly-Gly-Val
436	Glycyl-L-histidylglycine	Gly-His-Gly
437	Glycylisoleucine	Gly-Ile
438	Glycyl-L-leucine	Gly-Leu
439	Glycyl-DL-leucyl-DL-alanine	Gly-Leu-Ala
440	Glycyl-L-leucyl-L-tyrosine	Gly-Leu-Tyr
441	Glycylphenylalanine	Gly-Phe
442	Glycyl-L-phenylalanyl-L-phenylalanine	Gly-Phe-Phe
443	Glycyl-L-proline	Gly-Pro
444	Glycyl-L-prolyl-L-alanine	Gly-Pro-Ala
445	Glycylserine	Gly-Ser
446	Glycyl-D-threonine	Gly-Thr
447	Glycyltryptophane	Gly-Trp
448	Glycyltyrosine	Gly-Tyr
449	Glycylvaline	Gly-Val
450	Histidine	His
451	Hydroxyproline	Hyp
452	Isoleucine	Ile
453	Leucine	Leu
454	Leucylglycine	Leu-Gly
455	L-Leucylglycylglycine	Leu-Gly-Gly
456	DL-Leucylglycyl-DL-phenylalananine	Leu-Gly-Phe
457	Leucylleucine	Leu-Leu
458	L-Leucyl-L-leucyl-L-leucine	Leu-Leu-Leu
459	Leucylphenylalanine	Leu-Phe
460	L-Leucyl-L-tyrosine	Leu-Tyr
461	Leucylvaline	Leu-Val
463	Methionine	Methionine
464	Ornithine	Ornithine
465	Phenylalanine	Phenylalanine
466	Proline	Proline
467	Serine	Serine
468	L-Seryl-L-seryl-L-serine	Ser-Ser-Ser
469	Taurine	Taurine
470	Threonine	Threonine
471	Tryptophan	Tryptophan
472	Tyrosine	Tyrosine
473	Valine	Valine
474	3,5-Diiodotyrosine	I2-Tyr

[a] In our database, 416 and 462 correspond to arginine and lysine, respectively. As they are cations in the pH range 6.4–10, they are not included here.

buffering ability of the counter ion might not be so good and also the experimental reproducibility of R_E and R_s may not be good.

m_S

This is the effective mobility of the sample at the steady state 10^{-5} cm^2 V^{-1} s^{-1}. When the effective mobility of the sample is very small, the pH shift is very large and therefore the isotachophoretic migration may be unstable.

R_E

The ratios of potential gradients can be qualitative indices of the separated zones. R_E is one of the qualitative indices, defined as

$$R_E = E_S/E_L = m_L/m_S = R_S/R_L \; (= h_S/h_L)$$

where E, m, R and h denote the potential gradient, the effective mobility, the specific resistance and the step height in the recorder trace, respectively, and the subscripts L and S denote the leading and sample zones, respectively. In order to convert the step heights into R_E values, the step heights of the leading zones, h_L, should be estimated by the equation

$$h_L = h_{std}/(R_{E,std} - 1)$$

where h_{std} is the observed step height of a standard sample for the conversion and $R_{E,std}$ is the R_E value of the standard. It should be noted that a linear relationship is assumed between step heights and potential gradients. The identification power of isotachophoresis using qualitative R_E indices has been confirmed theoretically by Kenndler [12].

t

This is the time-based zone length of a 10-nmol sample in seconds. As the migration current was 100 μA, it corresponds to the electric charge in 10^{-4}C; for t = 28.65 s, it means that the zone length of a 10-nmol sample will be 28.65 s when a migration current of 100 μA is applied and the electric charge will be 2.865 mC. This might be useful for estimating the time-based zone length when the migration current is different from 100 μA.

R_S

This is the ionic specific resistance of the separated zones in Ω m. This parameter is newly added based on the wide use of conductivity detectors.

IS

This is the strength of the separated zones in m*M*. When the migration order of the zones is simulated, the effective mobility of the ith ion in the jth zone ($m_{i,j}$) and that of the jth ion in the ith zone ($m_{j,i}$) should be evaluated and compared [13,14]. The ionic strength is necessary in the correction of absolute mobility.

TABLE V

ISOTACHOPHORETIC INDICES OF 73 AMINO ACIDS AND PEPTIDES

The first line for each sample shows the following information:

No.	Chemical formula	Abbreviation	m_0	pK_a	Relative molecular mass
400	$C_6H_{12}N_2O_3$	Ala-Ala	−27.0	8.490	160.2

pH$_L$ = pH of the leading electrolyte used in simulation; pH$_S$ = pH of the sample zone at the steady state; m_S = effective mobility of the sample at the steady state (10^{-5} cm^2 V^{-1} s^{-1}; R_E = ratio of potential gradients; t = time-based zone length of a 10-nmol sample (s); R_S = specific resistance of the separated zones (Ω m); IS = ionic strength of the separated zones (m*M*).

400	C6H12N2O3			Ala-Ala				-27.0		8.490	160.2	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.89	7.92	7.97	8.03	8.10	8.19	8.38	8.42	8.49	8.57	8.67	8.79
m_S	5.32	5.64	6.11	6.76	7.62	8.74	11.31	11.95	12.82	13.96	15.33	16.86
R_E	14.04	13.24	12.23	11.06	9.80	8.55	6.60	6.25	5.82	5.35	4.87	4.43
t	28.65	28.66	28.68	28.70	28.73	28.76	20.32	20.32	20.33	20.34	20.36	20.39
R_S	118.5	111.7	103.2	93.28	82.65	72.11	67.88	64.26	59.87	54.99	50.06	45.51
I_S	1.14	1.21	1.32	1.46	1.65	1.90	2.89	3.06	3.29	3.59	3.96	4.37
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.81	8.89	9.00	9.13	9.28	9.45	9.39	9.53	9.70	9.89	10.08	10.29
m_S	17.13	18.03	19.10	20.23	21.30	22.20	21.97	22.62	23.18	23.61	23.93	24.16
R_E	4.36	4.14	3.91	3.69	3.51	3.36	3.40	3.30	3.22	3.16	3.12	3.09
t	21.36	21.38	21.41	21.47	21.56	21.70	26.45	26.63	26.91	27.39	28.22	29.69
R_S	43.74	41.55	39.22	37.00	35.12	33.65	30.50	29.58	28.81	28.20	27.70	27.23
I_S	4.34	4.57	4.85	5.14	5.42	5.63	5.07	5.21	5.31	5.36	5.35	5.24

401	C9H17N3O4			Ala-Ala-Ala				-22.2		8.245	231.3	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.78	7.82	7.87	7.94	8.02	8.12	8.28	8.33	8.41	8.51	8.63	8.77
m_S	5.50	5.86	6.38	7.07	7.98	9.09	10.86	11.49	12.31	13.32	14.45	15.60
R_E	13.57	12.74	11.71	10.56	9.36	8.21	6.88	6.50	6.07	5.61	5.17	4.79
t	32.97	32.99	33.02	33.06	33.12	33.18	22.79	22.80	22.82	22.84	22.88	22.92
R_S	114.5	107.4	98.81	89.08	78.98	69.28	70.69	66.85	62.38	57.65	53.13	49.21
I_S	1.26	1.35	1.47	1.63	1.85	2.11	3.06	3.24	3.48	3.78	4.11	4.45
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.75	8.85	8.98	9.13	9.30	9.48	9.40	9.56	9.75	9.95	10.16	10.38
m_S	15.46	16.19	16.97	17.71	18.32	18.79	18.69	19.06	19.34	19.55	19.71	19.84
R_E	4.83	4.61	4.40	4.22	4.08	3.97	4.00	3.92	3.86	3.82	3.79	3.76
t	24.10	24.14	24.20	24.29	24.44	24.66	30.48	30.78	31.27	32.12	33.63	36.60
R_S	48.48	46.29	44.14	42.28	40.82	39.75	35.84	35.11	34.53	34.06	33.63	33.15
I_S	4.28	4.49	4.71	4.92	5.08	5.18	4.62	4.69	4.71	4.68	4.58	4.37

402	C7H14N2O3			Ala-Amin				-25.8		8.495	174.2	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.89	7.93	7.97	8.03	8.11	8.20	8.38	8.43	8.49	8.57	8.68	8.80
m_S	5.07	5.38	5.84	6.46	7.30	8.38	10.78	11.39	12.24	13.33	14.65	16.11
R_E	14.74	13.88	12.80	11.55	10.23	8.91	6.93	6.56	6.10	5.60	5.10	4.64
t	29.57	29.58	29.60	29.62	29.66	29.70	20.85	20.86	20.87	20.88	20.90	20.93
R_S	124.3	117.0	108.0	97.47	86.26	75.19	71.24	67.39	62.73	57.58	52.40	47.63
I_S	1.11	1.18	1.28	1.42	1.60	1.85	2.81	2.98	3.21	3.51	3.87	4.27
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.82	8.90	9.01	9.14	9.29	9.46	9.40	9.55	9.72	9.90	10.10	10.31
m_S	16.34	17.21	18.23	19.32	20.33	21.18	20.97	21.59	22.12	22.53	22.83	23.05
R_E	4.57	4.34	4.10	3.87	3.67	3.53	3.56	3.46	3.38	3.32	3.27	3.24
t	21.95	21.97	22.01	22.08	22.18	22.33	27.32	27.52	27.84	28.39	29.33	31.04
R_S	45.86	43.54	41.08	38.76	36.79	35.27	31.95	30.99	30.19	29.56	29.03	28.54
I_S	4.22	4.45	4.73	5.01	5.27	5.48	4.92	5.05	5.15	5.19	5.16	5.03

403	C7H13N3O4			Ala-Asn				-25.5		8.470	203.2	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.88	7.92	7.96	8.02	8.10	8.19	8.37	8.42	8.48	8.57	8.67	8.80
m_S	5.13	5.45	5.91	6.55	7.40	8.48	10.83	11.45	12.30	13.39	14.70	16.14
R_E	14.56	13.71	12.64	11.41	10.10	8.81	6.90	6.52	6.07	5.58	5.08	4.63
t	29.81	29.82	29.84	29.87	29.90	29.95	20.99	21.00	21.01	21.02	21.04	21.07
R_S	122.8	115.6	106.6	96.25	85.19	74.30	70.88	67.04	62.42	57.33	52.23	47.56
I_S	1.12	1.20	1.30	1.44	1.63	1.88	2.85	3.01	3.25	3.54	3.90	4.30
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.81	8.89	9.00	9.14	9.29	9.47	9.40	9.55	9.72	9.91	10.14	10.32
m_S	16.32	17.18	18.19	19.24	20.21	21.02	20.82	21.41	21.91	22.30	22.58	22.79
R_E	4.58	4.35	4.11	3.88	3.70	3.55	3.59	3.49	3.41	3.35	3.31	3.28
t	22.10	22.13	22.17	22.23	22.34	22.50	27.54	27.75	28.08	28.65	29.62	31.41
R_S	45.92	43.61	41.19	38.92	37.01	35.54	32.18	31.25	30.48	29.86	29.35	28.86
I_S	4.24	4.47	4.74	5.02	5.27	5.47	4.91	5.03	5.12	5.15	5.11	4.98

404	$C_5H_{10}N_2O_3$			Ala-Gly				-28.8		8.390	146.1	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.91	7.97	8.05	8.14	8.33	8.38	8.44	8.52	8.63	8.75
m_S	6.20	6.57	7.09	7.82	8.80	10.05	12.88	13.58	14.53	15.76	17.23	18.84
R_E	12.04	11.37	10.53	9.55	8.49	7.44	5.80	5.50	5.14	4.74	4.34	3.97
t	27.42	27.43	27.45	27.47	27.49	27.52	19.60	19.60	19.61	19.62	19.63	19.65
R_S	101.6	95.94	88.85	80.56	71.61	62.72	59.63	56.55	52.83	48.70	44.55	40.74
I_S	1.31	1.38	1.50	1.65	1.87	2.14	3.19	3.37	3.62	3.94	4.32	4.74
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.77	8.85	8.96	9.10	9.25	9.43	9.35	9.50	9.67	9.86	10.06	10.27
m_S	19.03	19.97	21.07	22.21	23.25	24.10	23.85	24.48	25.00	25.40	25.69	25.90
R_E	3.93	3.74	3.55	3.36	3.21	3.10	3.13	3.05	2.99	2.94	2.91	2.88
t	20.55	20.57	20.60	20.65	20.72	20.84	25.28	25.42	25.67	26.07	26.75	27.95
R_S	39.39	37.52	35.55	33.71	32.18	30.99	28.09	27.34	26.71	26.21	25.80	25.40
I_S	4.68	4.92	5.20	5.49	5.75	5.95	5.39	5.52	5.61	5.66	5.65	5.56

405	$C_7H_{13}N_3O_4$			Ala-Gly-Gly				-25.0		8.254	203.2	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.78	7.81	7.86	7.93	8.01	8.10	8.28	8.33	8.40	8.49	8.61	8.75
m_S	6.11	6.50	7.04	7.78	8.76	9.97	12.16	12.83	13.73	14.85	16.12	17.44
R_E	12.22	11.50	10.61	9.60	8.53	7.49	6.14	5.82	5.44	5.03	4.63	4.28
t	30.25	30.27	30.29	30.32	30.36	30.40	21.22	21.22	21.23	21.25	21.27	21.30
R_S	103.0	97.00	89.54	80.98	71.96	63.18	63.14	59.83	55.91	51.70	47.61	44.00
I_S	1.35	1.44	1.56	1.73	1.95	2.23	3.24	3.43	3.68	3.99	4.35	4.71
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.73	8.83	8.95	9.10	9.27	9.45	9.36	9.53	9.71	9.90	10.11	10.32
m_S	17.36	18.17	19.06	19.93	20.66	21.23	21.07	21.51	21.85	22.11	22.29	22.43
R_E	4.30	4.11	3.92	3.75	3.62	3.52	3.54	3.47	3.42	3.38	3.35	3.33
t	22.35	22.37	22.42	22.48	22.59	22.76	27.89	28.11	28.46	29.06	30.09	31.99
R_S	43.18	41.24	39.29	37.57	36.20	35.19	31.79	31.11	30.56	30.12	29.73	29.32
I_S	4.57	4.79	5.04	5.26	5.45	5.59	5.01	5.10	5.15	5.14	5.08	4.92

406	$C_9H_{14}N_4O_3$			β-Ala-His				-24.4		9.664	226.2	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.78	7.81	7.86	7.93	8.01	8.74	8.95	8.99	9.03	9.09	9.16	9.25
m_S	0.00	0.00	0.00	0.00	0.00	2.57	3.91	4.16	4.51	5.01	5.69	6.56
R_E	0.00	0.00	0.00	0.00	0.00	29.10	19.10	17.96	16.55	14.90	13.14	11.39
t	0.00	0.00	0.00	0.00	0.00	31.59	22.27	22.29	22.31	22.34	22.38	22.45
R_S	0.00	0.00	0.00	0.00	0.00	245.5	196.3	184.7	170.1	153.1	135.0	117.0
I_S	0.00	0.00	0.00	0.00	0.00	0.55	0.99	1.06	1.15	1.28	1.45	1.68
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.34	9.39	9.45	9.53	9.63	9.74	9.80	9.88	9.98	10.11	10.26	10.43
m_S	7.65	8.19	8.94	9.91	11.14	12.57	13.38	14.29	15.41	16.68	17.97	19.19
R_E	9.76	9.12	8.36	7.53	6.71	5.94	5.58	5.23	4.85	4.48	4.16	3.89
t	23.62	23.67	23.75	23.86	24.03	24.29	29.97	30.27	30.75	31.54	32.92	35.51
R_S	97.91	91.46	83.83	75.51	67.18	59.45	50.09	46.83	43.34	39.93	36.87	34.27
I_S	1.91	2.05	2.24	2.48	2.79	3.14	3.02	3.22	3.45	3.68	3.88	3.97

407	$C_9H_{18}N_2O_3$			Ala-Leu				-23.9		8.505	202.3	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.90	7.94	7.98	8.04	8.12	8.21	8.39	8.44	8.50	8.59	8.69	8.82
m_S	4.67	4.97	5.40	6.00	6.79	7.80	9.93	10.51	11.31	12.33	13.56	14.92
R_E	16.00	15.03	13.83	12.45	11.00	9.57	7.53	7.11	6.61	6.06	5.51	5.01
t	31.21	31.23	31.25	31.28	31.32	31.37	21.81	21.81	21.83	21.85	21.87	21.91
R_S	134.9	126.8	116.7	105.1	92.78	80.76	77.36	73.06	67.91	62.26	56.61	51.45
I_S	1.04	1.11	1.21	1.35	1.53	1.76	2.68	2.85	3.07	3.36	3.71	4.09
No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.83	8.91	9.02	9.16	9.31	9.48	9.42	9.57	9.74	9.93	10.13	10.35
m_S	15.08	15.90	16.86	17.86	18.79	19.57	19.39	19.96	20.44	20.81	21.09	21.30
R_E	4.95	4.70	4.43	4.18	3.98	3.82	3.85	3.74	3.65	3.59	3.54	3.51
t	23.02	23.05	23.10	23.18	23.30	23.49	28.89	29.14	29.54	30.22	31.41	33.66
R_S	49.69	47.12	44.44	41.92	39.81	38.18	34.55	33.52	32.67	31.99	31.43	30.88
I_S	4.03	4.25	4.52	4.79	5.03	5.23	4.67	4.79	4.87	4.89	4.84	4.68

408 $C_{11}H_{21}N_3O_4$ Ala-Leu-Gly -21.3 8.272 259.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.79	7.83	7.89	7.95	8.04	8.14	8.29	8.35	8.43	8.52	8.64	8.78
m_S	5.17	5.51	6.00	6.67	7.54	8.60	10.26	10.86	11.65	12.63	13.72	14.83
R_E	14.46	13.55	12.44	11.20	9.91	8.68	7.28	6.88	6.41	5.92	5.45	5.04
t	33.99	34.02	34.05	34.10	34.16	34.23	23.39	23.41	23.43	23.45	23.49	23.54
R_S	121.9	114.3	104.9	94.44	83.60	73.24	74.83	70.69	65.88	60.81	55.96	51.75
I_S	1.20	1.28	1.40	1.56	1.76	2.02	2.94	3.12	3.35	3.64	3.97	4.30

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.86	8.99	9.14	9.31	9.50	9.41	9.58	9.77	9.97	10.18	10.40
m_S	14.70	15.41	16.17	16.89	17.49	17.96	17.87	18.22	18.50	18.71	18.86	19.01
R_E	5.08	4.85	4.62	4.42	4.27	4.16	4.18	4.10	4.04	3.99	3.96	3.93
t	24.78	24.82	24.89	24.99	25.15	25.40	31.48	31.82	32.38	33.34	35.08	38.61
R_S	50.99	48.64	46.33	44.33	42.77	41.61	37.50	36.72	36.10	35.59	35.13	34.60
I_S	4.14	4.34	4.56	4.76	4.92	5.03	4.47	4.53	4.55	4.51	4.40	4.16

409 $C_8H_{16}N_2O_3S$ Ala-Met -24.2 8.463 220.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.88	7.91	7.96	8.02	8.10	8.19	8.37	8.42	8.48	8.57	8.68	8.80
m_S	4.91	5.23	5.68	6.30	7.12	8.17	10.33	10.93	11.75	12.80	14.05	15.41
R_E	15.21	14.29	13.16	11.86	10.49	9.14	7.23	6.83	6.36	5.84	5.32	4.85
t	30.94	30.95	30.98	31.01	31.05	31.09	21.64	21.65	21.66	21.68	21.71	21.74
R_S	128.3	120.6	111.0	100.1	88.46	77.11	74.31	70.22	65.33	59.98	54.65	49.80
I_S	1.10	1.17	1.27	1.41	1.60	1.84	2.78	2.95	3.18	3.47	3.82	4.21

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.81	8.90	9.01	9.15	9.30	9.48	9.41	9.56	9.73	9.92	10.13	10.34
m_S	15.54	16.36	17.32	18.30	19.21	19.96	19.78	20.33	20.79	21.14	21.40	21.60
R_E	4.81	4.57	4.31	4.08	3.89	3.74	3.78	3.67	3.59	3.53	3.49	3.46
t	22.83	22.86	22.91	22.98	23.10	23.28	28.62	28.85	29.24	29.89	31.04	33.18
R_S	48.23	45.79	43.26	40.90	38.94	37.44	33.87	32.92	32.13	31.50	30.97	30.46
I_S	4.13	4.36	4.62	4.89	5.12	5.31	4.74	4.86	4.93	4.95	4.90	4.74

410 $C_3H_7NO_2$ β-Alanine 36.7 3.552 89.1
-30.8 10.241

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.88	7.91	7.96	8.02	8.10	8.19	9.25	9.28	9.31	9.36	9.42	9.50
m_S	0.00	0.00	0.00	0.00	0.00	0.00	2.84	3.00	3.24	3.58	4.05	4.68
R_E	0.00	0.00	0.00	0.00	0.00	0.00	26.35	24.92	23.09	20.89	18.45	15.95
t	0.00	0.00	0.00	0.00	0.00	0.00	21.15	21.16	21.19	21.22	21.26	21.33
R_S	0.00	0.00	0.00	0.00	0.00	0.00	270.8	256.2	237.3	214.7	189.6	163.9
I_S	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.64	0.69	0.76	0.86	1.00

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.63	9.66	9.71	9.78	9.85	9.94	10.06	10.11	10.18	10.27	10.38	10.51
m_S	5.99	6.38	6.95	7.73	8.77	10.11	11.84	12.68	13.81	15.28	17.05	19.07
R_E	12.48	11.71	10.76	9.67	8.52	7.39	6.31	5.89	5.41	4.89	4.38	3.92
t	22.37	22.42	22.50	22.61	22.78	23.04	27.95	28.21	28.63	29.28	30.34	32.18
R_S	125.2	117.4	107.9	96.87	85.28	73.92	56.59	52.79	48.35	43.59	38.86	34.49
I_S	1.25	1.34	1.45	1.62	1.83	2.10	2.29	2.44	2.65	2.90	3.19	3.46

411 $C_3H_7NO_2$ Alanine -32.7 9.862 89.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.88	7.91	7.96	8.02	8.10	8.80	9.04	9.07	9.11	9.16	9.23	9.30
m_S	0.00	0.00	0.00	0.00	0.00	2.61	4.27	4.51	4.87	5.38	6.07	7.00
R_E	0.00	0.00	0.00	0.00	0.00	28.59	17.51	16.56	15.34	13.89	12.30	10.67
t	0.00	0.00	0.00	0.00	0.00	26.09	19.17	19.18	19.19	19.20	19.23	19.26
R_S	0.00	0.00	0.00	0.00	0.00	241.2	180.0	170.2	157.7	142.8	126.4	109.6
I_S	0.00	0.00	0.00	0.00	0.00	0.51	0.93	0.98	1.06	1.17	1.33	1.54

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.42	9.46	9.51	9.58	9.67	9.77	9.85	9.91	9.99	10.10	10.22	10.37
m_S	8.58	9.14	9.94	11.02	12.41	14.13	15.59	16.65	18.03	19.70	21.57	23.49
R_E	8.70	8.17	7.52	6.78	6.02	5.28	4.79	4.49	4.14	3.79	3.46	3.18
t	20.12	20.15	20.19	20.25	20.35	20.49	24.66	24.81	25.05	25.43	26.04	27.08
R_S	87.30	81.93	75.37	67.97	60.27	52.86	42.96	40.19	37.05	33.80	30.72	28.00
I_S	1.86	1.98	2.15	2.39	2.69	3.07	3.15	3.36	3.63	3.95	4.28	4.59

412 $C_{12}H_{16}N_2O_3$ Ala-Phe -23.9 8.502 236.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.90	7.93	7.98	8.04	8.12	8.21	8.39	8.44	8.50	8.59	8.69	8.81
m_S	4.68	4.98	5.42	6.01	6.81	7.82	9.95	10.53	11.33	12.35	13.58	14.94
R_E	15.95	14.99	13.79	12.42	10.97	9.55	7.51	7.09	6.59	6.05	5.50	5.00
t	31.21	31.23	31.25	31.28	31.32	31.37	21.81	21.81	21.83	21.85	21.87	21.91
R_S	134.6	126.4	116.3	104.8	92.54	80.56	77.21	72.92	67.78	62.14	56.51	51.38
I_S	1.05	1.12	1.21	1.35	1.53	1.77	2.69	2.85	3.08	3.37	3.71	4.10

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.83	8.91	9.02	9.16	9.31	9.48	9.42	9.57	9.74	9.93	10.13	10.35
m_S	15.10	15.92	16.87	17.87	18.80	19.58	19.40	19.97	20.45	20.82	21.09	21.30
R_E	4.95	4.69	4.43	4.18	3.97	3.82	3.85	3.74	3.65	3.59	3.54	3.51
t	23.02	23.05	23.10	23.18	23.30	23.49	28.89	29.14	29.54	30.22	31.41	33.66
R_S	49.62	47.07	44.39	41.89	39.79	38.16	34.53	33.51	32.66	31.99	31.43	30.88
I_S	4.04	4.26	4.52	4.79	5.04	5.23	4.67	4.79	4.87	4.89	4.84	4.68

413 $C_6H_{12}O_4N_2$ Ala-Ser -26.2 8.297 176.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.80	7.83	7.88	7.94	8.02	8.11	8.29	8.34	8.41	8.50	8.61	8.75
m_S	6.16	6.53	7.07	7.81	8.79	10.02	12.42	13.10	14.02	15.18	16.52	17.93
R_E	12.13	11.43	10.56	9.56	8.50	7.45	6.02	5.70	5.33	4.92	4.52	4.17
t	29.27	29.28	29.30	29.32	29.36	29.40	20.65	20.66	20.67	20.68	20.70	20.72
R_S	102.3	96.43	89.11	80.65	71.67	62.87	61.83	58.60	54.76	50.58	46.47	42.80
I_S	1.34	1.43	1.55	1.71	1.93	2.21	3.24	3.42	3.67	3.98	4.35	4.74

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.74	8.84	8.96	9.10	9.26	9.44	9.36	9.52	9.70	9.89	10.09	10.30
m_S	17.92	18.78	19.74	20.69	21.51	22.17	21.98	22.47	22.87	23.16	23.37	23.53
R_E	4.17	3.98	3.78	3.61	3.47	3.37	3.40	3.32	3.27	3.22	3.20	3.17
t	21.72	21.74	21.78	21.84	21.93	22.08	26.98	27.16	27.48	28.00	28.89	30.52
R_S	41.82	39.90	37.95	36.19	34.77	33.70	30.49	29.78	29.21	28.75	28.36	27.95
I_S	4.62	4.85	5.10	5.35	5.56	5.71	5.14	5.24	5.31	5.32	5.27	5.14

414 $C_8H_{16}N_2O_3$ Ala-Val -25.2 8.500 188.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.90	7.93	7.98	8.04	8.11	8.20	8.39	8.43	8.50	8.58	8.68	8.81
m_S	4.93	5.24	5.69	6.31	7.13	8.18	10.49	11.10	11.93	13.00	14.29	15.72
R_E	15.14	14.25	13.13	11.85	10.48	9.13	7.12	6.73	6.26	5.75	5.23	4.75
t	30.06	30.07	30.09	30.12	30.15	30.20	21.14	21.14	21.15	21.17	21.19	21.22
R_S	127.7	120.2	110.8	99.92	88.37	77.00	73.17	69.17	64.36	59.05	53.72	48.82
I_S	1.09	1.15	1.25	1.39	1.58	1.82	2.77	2.93	3.16	3.46	3.81	4.21

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.82	8.90	9.01	9.14	9.30	9.47	9.41	9.55	9.72	9.91	10.11	10.32
m_S	15.93	16.78	17.79	18.84	19.83	20.66	20.47	21.07	21.58	21.98	22.27	22.49
R_E	4.69	4.45	4.20	3.96	3.77	3.61	3.65	3.54	3.46	3.40	3.35	3.32
t	22.27	22.30	22.34	22.41	22.51	22.68	27.79	28.00	28.35	28.93	29.94	31.80
R_S	47.04	44.65	42.11	39.73	37.71	36.16	32.74	31.76	30.94	30.29	29.75	29.24
I_S	4.16	4.39	4.66	4.94	5.20	5.40	4.84	4.97	5.06	5.09	5.06	4.92

415 $C_5H_{11}O_2N$ α-Amino-n-butyric acid -30.5 9.827 117.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.90	7.93	7.98	8.04	8.11	8.79	9.03	9.06	9.10	9.15	9.21	9.29
m_S	0.00	0.00	0.00	0.00	0.00	2.57	4.13	4.38	4.73	5.23	5.91	6.82
R_E	0.00	0.00	0.00	0.00	0.00	29.09	18.07	17.07	15.80	14.29	12.63	10.95
t	0.00	0.00	0.00	0.00	0.00	27.27	19.84	19.85	19.86	19.88	19.91	19.95
R_S	0.00	0.00	0.00	0.00	0.00	245.4	185.8	175.5	162.4	146.8	129.8	112.5
I_S	0.00	0.00	0.00	0.00	0.00	0.51	0.93	0.99	1.07	1.19	1.34	1.55

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.41	9.45	9.50	9.57	9.66	9.76	9.84	9.91	9.99	10.10	10.23	10.38
m_S	8.28	8.83	9.61	10.65	12.00	13.65	14.95	15.97	17.29	18.86	20.60	22.36
R_E	9.02	8.46	7.78	7.01	6.22	5.47	5.00	4.68	4.32	3.96	3.62	3.34
t	20.88	20.91	20.96	21.03	21.14	21.30	25.80	25.98	26.26	26.71	27.45	28.72
R_S	90.51	84.85	77.98	70.27	62.32	54.73	44.81	41.90	38.64	35.30	32.16	29.42
I_S	1.85	1.98	2.16	2.39	2.70	3.07	3.10	3.31	3.57	3.88	4.19	4.45

416 $C_6H_{14}N_4O_2$ Arginine

417 $C_4H_8N_2O_3$ Asparagine -32.1 9.051 132.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.16	8.19	8.22	8.27	8.34	8.41	8.64	8.67	8.72	8.78	8.86	8.95
m_S	3.62	3.83	4.13	4.56	5.16	5.95	8.77	9.27	9.97	10.95	12.23	13.83
R_E	20.61	19.50	18.08	16.37	14.49	12.55	8.52	8.06	7.49	6.82	6.11	5.40
t	25.59	25.60	25.61	25.62	25.63	25.65	18.64	18.64	18.64	18.65	18.66	18.68
R_S	173.8	164.5	152.5	138.1	122.2	105.9	87.55	82.87	76.98	70.12	62.75	55.49
I_S	0.72	0.77	0.83	0.91	1.04	1.20	2.02	2.13	2.30	2.53	2.84	3.22

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.04	9.09	9.16	9.26	9.37	9.51	9.51	9.61	9.74	9.89	10.06	10.25
m_S	15.20	16.11	17.32	18.83	20.56	22.35	22.47	23.60	24.83	26.01	27.02	27.81
R_E	4.92	4.64	4.31	3.97	3.63	3.34	3.32	3.17	3.01	2.87	2.76	2.69
t	19.50	19.51	19.54	19.58	19.64	19.74	23.75	23.86	24.05	24.36	24.87	25.77
R_S	49.31	46.51	43.24	39.76	36.39	33.43	29.82	28.36	26.90	25.60	24.53	23.65
I_S	3.48	3.70	3.98	4.34	4.74	5.16	4.80	5.04	5.29	5.52	5.68	5.76

418 $C_4H_7NO_4$ Aspartic Acid -30.1 3.900 133.1
-56.8 10.017

No.	1	2	3	4	5	6	7	8	9	10	11	12
pH_L	2.60	2.80	3.00	3.20	3.40	3.60	3.00	3.20	3.40	3.60	3.80	4.00
pH_S	3.56	3.65	3.74	3.83	3.93	4.04	3.74	3.83	3.92	4.03	4.16	4.30
m_S	8.48	9.99	11.48	13.01	14.66	16.45	11.44	12.95	14.57	16.34	18.20	20.06
R_E	8.81	7.47	6.50	5.74	5.10	4.54	6.53	5.77	5.13	4.57	4.10	3.72
t	35.45	30.27	26.89	24.42	22.55	21.16	25.77	23.32	21.45	20.05	19.06	18.39
R_S	50.44	51.42	51.30	49.87	47.30	44.07	52.66	51.41	48.94	45.70	42.28	39.12
I_S	2.09	2.36	2.63	2.97	3.38	3.87	2.68	3.02	3.44	3.94	4.49	5.05

No.	13	14	15	16	17	18	19	20	21	22	23	24
pH_L	3.80	4.00	4.20	4.40	4.60	4.80	4.20	4.40	4.60	4.80	5.00	5.20
pH_S	4.32	4.42	4.53	4.67	4.82	5.00	4.62	4.73	4.87	5.03	5.21	5.40
m_S	20.41	21.48	22.59	23.68	24.66	25.46	23.38	24.20	24.98	25.67	26.20	26.58
R_E	3.66	3.48	3.31	3.15	3.03	2.93	3.19	3.09	2.99	2.91	2.85	2.81
t	19.96	19.70	19.47	19.28	19.13	19.03	22.25	22.12	22.01	21.93	21.87	21.83
R_S	36.01	34.86	33.54	32.23	31.09	30.21	29.99	29.18	28.39	27.71	27.19	26.83
I_S	5.05	5.29	5.57	5.87	6.14	6.36	5.45	5.64	5.83	6.01	6.14	6.24

No.	25	26	27	28	29	30	31
pH_L	5.40	5.40	5.60	5.80	6.00	6.20	6.40
pH_S	5.59	5.59	5.78	5.97	6.16	6.36	6.56
m_S	26.84	26.77	26.93	27.05	27.12	27.17	27.20
R_E	2.78	2.79	2.77	2.76	2.75	2.75	2.75
t	21.81	19.17	19.16	19.15	19.14	19.14	19.14
R_S	26.58	28.61	28.46	28.35	28.28	28.23	28.20
I_S	6.31	6.69	6.73	6.76	6.78	6.80	6.81

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	6.62	6.82	7.02	7.22	7.42	7.62	7.57	7.77	7.97	8.17	8.37	8.57
m_S	27.39	27.41	27.43	27.46	27.49	27.53	27.34	27.40	27.49	27.63	27.85	28.20
R_E	2.73	2.72	2.72	2.72	2.72	2.71	2.73	2.73	2.72	2.70	2.68	2.65
t	26.89	26.90	26.91	26.92	26.95	26.99	19.18	19.23	19.32	19.45	19.65	19.95
R_S	23.00	22.99	22.97	22.95	22.92	22.89	28.09	28.02	27.93	27.78	27.56	27.22
I_S	5.90	5.90	5.91	5.92	5.93	5.94	6.85	6.87	6.90	6.94	7.01	7.11

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.40	8.60	8.79	8.99	9.18	9.37	9.28	9.45	9.62	9.79	9.97	10.15
m_S	27.93	28.29	28.84	29.63	30.75	32.25	31.58	33.08	34.87	36.91	39.06	41.18
R_E	2.67	2.64	2.59	2.52	2.43	2.32	2.37	2.26	2.14	2.02	1.91	1.81
t	20.56	20.89	21.36	22.02	22.91	24.02	27.87	29.03	30.30	31.67	33.07	34.59
R_S	26.84	26.49	25.98	25.26	24.32	23.17	21.21	20.23	19.15	18.04	16.96	15.97
I_S	6.89	7.00	7.16	7.40	7.74	8.18	7.46	7.90	8.43	9.03	9.66	10.25

419 $C_3H_7NO_2S$ Cysteine -31.7 8.600 121.2
-62.7 10.280

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.94	7.97	8.01	8.06	8.13	8.21	8.42	8.46	8.52	8.59	8.69	8.80
m_S	5.64	5.96	6.43	7.09	7.99	9.18	12.61	13.32	14.31	15.65	17.36	19.39
R_E	13.24	12.53	11.61	10.53	9.34	8.14	5.92	5.61	5.22	4.77	4.30	3.85
t	25.84	25.86	25.88	25.90	25.94	26.00	18.97	19.00	19.05	19.13	19.24	19.42
R_S	111.7	105.7	97.97	88.84	78.82	68.66	60.88	57.65	53.64	49.05	44.23	39.58
I_S	1.14	1.21	1.31	1.45	1.63	1.88	2.96	3.14	3.38	3.71	4.12	4.63

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.85	8.92	9.01	9.13	9.27	9.43	9.40	9.52	9.67	9.82	9.99	10.16
m_S	20.30	21.52	23.10	25.00	27.17	29.53	29.26	31.08	33.25	35.75	38.48	41.31
R_E	3.68	3.47	3.23	2.99	2.75	2.53	2.55	2.40	2.25	2.09	1.94	1.81
t	20.33	20.48	20.71	21.06	21.59	22.33	26.31	27.00	27.92	29.11	30.46	31.94
R_S	36.91	34.82	32.43	29.94	27.53	25.30	22.90	21.53	20.08	18.63	17.22	15.92
I_S	4.77	5.07	5.45	5.93	6.47	7.06	6.56	7.02	7.57	8.20	8.90	9.63

420 $C_6H_{12}N_2O_4S_2$ Cystine -27.0 8.405 240.3
-53.9 9.845

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.85	7.88	7.93	7.98	8.06	8.15	8.34	8.39	8.45	8.53	8.64	8.76
m_S	5.86	6.22	6.74	7.46	8.43	9.69	12.63	13.38	14.43	15.81	17.53	19.55
R_E	12.75	12.01	11.09	10.01	8.86	7.71	5.91	5.58	5.18	4.73	4.26	3.82
t	28.87	28.90	28.95	29.01	29.09	29.21	20.99	21.07	21.19	21.38	21.65	22.04
R_S	107.5	101.3	93.51	84.44	74.71	65.05	60.79	57.37	53.21	48.56	43.79	39.25
I_S	1.27	1.35	1.46	1.62	1.84	2.12	3.25	3.45	3.73	4.11	4.58	5.14

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.79	8.87	8.98	9.11	9.26	9.42	9.38	9.51	9.65	9.81	9.97	10.15
m_S	20.04	21.34	23.02	25.02	27.31	29.81	29.38	31.32	33.51	35.80	38.05	40.06
R_E	3.73	3.50	3.25	2.99	2.74	2.51	2.54	2.39	2.23	2.09	1.96	1.86
t	23.12	23.45	23.92	24.60	25.47	26.53	31.22	32.09	33.11	34.18	35.37	36.67
R_S	37.40	35.10	32.54	29.92	27.39	25.06	22.80	21.37	19.93	18.60	17.42	16.42
I_S	5.17	5.53	5.99	6.56	7.22	7.93	7.30	7.86	8.49	9.16	9.80	10.35

421 $C_5H_9NO_4$ Glutamic Acid -26.8 4.324 147.1
-55.7 9.966

No.	1	2	3	4	5	6	7	8	9	10	11	12
pH_L	2.60	2.80	3.00	3.20	3.40	3.60	3.00	3.20	3.40	3.60	3.80	4.00
pH_S	3.77	3.86	3.94	4.02	4.11	4.20	3.94	4.02	4.10	4.20	4.31	4.43
m_S	5.19	6.20	7.23	8.31	9.53	10.93	7.20	8.26	9.46	10.84	12.40	14.12
R_E	14.40	12.04	10.33	8.99	7.84	6.83	10.38	9.04	7.90	6.89	6.02	5.29
t	38.32	32.67	29.00	26.31	24.25	22.72	27.74	25.07	23.02	21.47	20.36	19.61
R_S	82.44	82.84	81.51	78.07	72.77	66.32	83.73	80.58	75.40	68.92	62.05	55.59
I_S	1.36	1.55	1.74	1.99	2.30	2.69	1.78	2.02	2.34	2.74	3.22	3.74

No.	13	14	15	16	17	18	19	20	21	22	23	24
pH_L	3.80	4.00	4.20	4.40	4.60	4.80	4.20	4.40	4.60	4.80	5.00	5.20
pH_S	4.49	4.57	4.66	4.78	4.91	5.07	4.77	4.86	4.97	5.11	5.27	5.45
m_S	15.02	16.02	17.14	18.37	19.63	20.78	18.32	19.21	20.18	21.14	22.00	22.68
R_E	4.97	4.66	4.36	4.07	3.81	3.59	4.08	3.89	3.70	3.53	3.40	3.29
t	21.19	20.95	20.73	20.54	20.40	20.29	23.84	23.73	23.63	23.55	23.49	23.45
R_S	48.94	46.74	44.20	41.55	39.07	37.01	38.28	36.75	35.14	33.63	32.38	31.45
I_S	3.93	4.17	4.46	4.80	5.16	5.49	4.48	4.70	4.94	5.19	5.41	5.59

No.	25	26	27	28	29	30	31
pH_L	5.40	5.40	5.60	5.80	6.00	6.20	6.40
pH_S	5.64	5.66	5.82	6.00	6.20	6.39	6.59
m_S	23.17	23.15	23.44	23.66	23.81	23.91	23.98
R_E	3.22	3.23	3.19	3.16	3.14	3.12	3.11
t	23.43	20.45	20.44	20.42	20.42	20.42	20.42
R_S	30.79	33.09	32.70	32.41	32.21	32.07	31.99
I_S	5.72	6.12	6.21	6.27	6.31	6.34	6.36

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	6.66	6.86	7.06	7.26	7.45	7.65	7.60	7.79	7.99	8.19	8.39	8.59
m_S	24.19	24.23	24.27	24.31	24.35	24.41	24.20	24.28	24.40	24.59	24.88	25.32
R_E	3.09	3.08	3.08	3.07	3.07	3.06	3.09	3.08	3.06	3.04	3.00	2.95
t	29.18	29.18	29.19	29.21	29.24	29.29	20.47	20.54	20.63	20.79	21.02	21.37
R_S	26.05	26.00	25.96	25.92	25.88	25.81	31.73	31.62	31.46	31.22	30.85	30.31
I_S	5.42	5.43	5.44	5.45	5.47	5.48	6.43	6.45	6.48	6.54	6.61	6.74

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.44	8.63	8.83	9.02	9.21	9.39	9.32	9.48	9.64	9.81	9.98	10.16
m_S	24.99	25.46	26.16	27.16	28.53	30.31	29.65	31.36	33.36	35.59	37.89	40.12
R_E	2.99	2.93	2.86	2.75	2.62	2.46	2.52	2.38	2.24	2.10	1.97	1.86
t	22.07	22.44	22.96	23.68	24.58	25.67	30.05	31.08	32.18	33.34	34.52	35.83
R_S	29.99	29.43	28.63	27.56	26.22	24.65	22.59	21.34	20.02	18.71	17.49	16.40
IS	6.49	6.63	6.82	7.10	7.48	7.97	7.26	7.73	8.29	8.92	9.56	10.16

422 $C_5H_{10}N_2O_3$ Glutamine -30.1 9.308 146.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.29	8.32	8.35	8.40	8.47	8.54	8.76	8.80	8.84	8.90	8.98	9.07
m_S	2.62	2.77	3.00	3.32	3.76	4.36	6.59	6.98	7.54	8.31	9.34	10.66
R_E	28.54	26.96	24.94	22.52	19.86	17.14	11.33	10.70	9.91	8.99	8.00	7.01
t	26.79	26.80	26.81	26.82	26.84	26.87	19.39	19.39	19.40	19.41	19.43	19.45
R_S	240.7	227.4	210.3	190.0	167.5	144.6	116.4	110.0	101.9	92.40	82.18	72.02
IS	0.53	0.56	0.61	0.68	0.77	0.89	1.55	1.64	1.78	1.96	2.21	2.53

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.16	9.21	9.27	9.36	9.46	9.59	9.62	9.71	9.82	9.96	10.12	10.30
m_S	12.07	12.84	13.89	15.24	16.85	18.64	19.12	20.23	21.52	22.87	24.12	25.18
R_E	6.19	5.82	5.38	4.90	4.43	4.01	3.91	3.69	3.47	3.27	3.10	2.97
t	20.34	20.37	20.40	20.45	20.53	20.66	25.00	25.15	25.38	25.76	26.40	27.51
R_S	62.08	58.34	53.92	49.13	44.39	40.07	35.04	33.08	31.03	29.12	27.47	26.13
IS	2.82	3.01	3.26	3.58	3.97	4.39	4.14	4.38	4.65	4.91	5.13	5.25

423 $C_5H_{10}N_2O_3$ Gly-Ala -28.8 8.435 146.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.86	7.89	7.94	7.99	8.07	8.16	8.35	8.40	8.46	8.54	8.64	8.77
m_S	5.95	6.30	6.81	7.51	8.46	9.67	12.51	13.19	14.13	15.35	16.81	18.43
R_E	12.55	11.85	10.97	9.94	8.83	7.73	5.97	5.66	5.29	4.87	4.44	4.05
t	27.42	27.43	27.45	27.46	27.49	27.52	19.61	19.61	19.61	19.62	19.63	19.65
R_S	105.8	99.96	92.55	83.87	74.51	65.17	61.39	58.20	54.33	50.02	45.66	41.64
IS	1.25	1.33	1.44	1.59	1.79	2.05	3.10	3.28	3.52	3.83	4.21	4.63

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.79	8.87	8.97	9.11	9.26	9.43	9.36	9.51	9.68	9.86	10.06	10.27
m_S	18.69	19.63	20.75	21.93	23.02	23.93	23.67	24.33	24.90	25.33	25.64	25.87
R_E	4.00	3.80	3.60	3.41	3.24	3.12	3.16	3.07	3.00	2.95	2.91	2.89
t	20.56	20.58	20.61	20.65	20.73	20.85	25.29	25.43	25.67	26.08	26.76	27.96
R_S	40.10	38.16	36.10	34.15	32.50	31.22	28.31	27.50	26.83	26.29	25.85	25.43
IS	4.59	4.84	5.12	5.41	5.69	5.90	5.34	5.48	5.59	5.64	5.63	5.55

424 $C_6H_{12}N_2O_3$ Gly-Amin -27.2 8.421 160.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.85	7.89	7.93	7.99	8.07	8.16	8.35	8.39	8.46	8.54	8.65	8.77
m_S	5.71	6.05	6.55	7.24	8.16	9.33	11.93	12.59	13.49	14.66	16.05	17.57
R_E	13.09	12.34	11.41	10.32	9.16	8.01	6.26	5.93	5.54	5.10	4.65	4.25
t	28.51	28.52	28.54	28.56	28.59	28.62	20.23	20.23	20.24	20.25	20.27	20.29
R_S	110.4	104.1	96.23	87.07	77.26	67.54	64.39	60.99	56.90	52.37	47.83	43.67
IS	1.23	1.30	1.41	1.56	1.76	2.02	3.04	3.22	3.46	3.77	4.14	4.54

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.79	8.87	8.98	9.11	9.27	9.44	9.37	9.52	9.69	9.88	10.08	10.29
m_S	17.75	18.65	19.71	20.80	21.80	22.63	22.40	23.01	23.52	23.90	24.19	24.39
R_E	4.21	4.00	3.79	3.59	3.43	3.30	3.33	3.25	3.18	3.12	3.09	3.06
t	21.25	21.28	21.31	21.36	21.45	21.59	26.30	26.47	26.75	27.22	28.03	29.46
R_S	42.22	40.16	38.01	35.99	34.31	33.01	29.91	29.08	28.40	27.85	27.40	26.97
IS	4.49	4.72	5.00	5.28	5.53	5.73	5.17	5.29	5.38	5.42	5.40	5.29

425 $C_6H_{11}N_3O_4$ Gly-Asn -27.5 8.388 189.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.98	8.05	8.14	8.33	8.38	8.44	8.53	8.63	8.76
m_S	5.94	6.30	6.81	7.52	8.47	9.68	12.31	12.99	13.92	15.10	16.51	18.04
R_E	12.56	11.85	10.96	9.93	8.82	7.72	6.07	5.75	5.37	4.95	4.52	4.14
t	28.30	28.31	28.32	28.34	28.37	28.41	20.10	20.11	20.11	20.12	20.14	20.16
R_S	106.0	100.0	92.48	83.73	74.36	65.08	62.35	59.09	55.17	50.83	46.50	42.55
IS	1.27	1.35	1.46	1.62	1.83	2.09	3.13	3.31	3.55	3.86	4.24	4.64

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.77	8.86	8.97	9.11	9.26	9.44	9.36	9.51	9.69	9.88	10.08	10.28
m_S	18.18	19.09	20.14	21.22	22.20	23.00	22.77	23.36	23.86	24.23	24.49	24.69
R_E	4.11	3.91	3.71	3.52	3.36	3.25	3.28	3.20	3.13	3.08	3.05	3.02
t	21.11	21.13	21.17	21.22	21.30	21.44	26.09	26.26	26.53	26.99	27.77	29.15
R_S	41.22	39.25	37.19	35.28	33.69	32.48	29.42	28.64	28.00	27.48	27.06	26.64
I_S	4.57	4.81	5.08	5.36	5.61	5.80	5.23	5.36	5.44	5.48	5.45	5.35

426 $C_2H_5NO_2$ Glycine -37.4 9.780 75.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.98	8.68	8.75	8.99	9.02	9.06	9.11	9.17	9.25
m_S	0.00	0.00	0.00	0.00	2.74	3.17	5.24	5.53	5.95	6.55	7.38	8.48
R_E	0.00	0.00	0.00	0.00	27.24	23.58	14.26	13.52	12.56	11.40	10.12	8.81
t	0.00	0.00	0.00	0.00	23.77	23.79	17.77	17.78	17.78	17.79	17.81	17.83
R_S	0.00	0.00	0.00	0.00	229.8	198.9	146.6	138.9	129.1	117.2	104.0	90.52
I_S	0.00	0.00	0.00	0.00	0.51	0.59	1.07	1.13	1.22	1.34	1.52	1.75

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.37	9.41	9.46	9.53	9.61	9.72	9.80	9.86	9.94	10.04	10.17	10.31
m_S	10.41	11.06	11.98	13.24	14.87	16.87	18.48	19.67	21.23	23.12	25.21	27.34
R_E	7.18	6.76	6.23	5.64	5.02	4.43	4.04	3.80	3.52	3.23	2.96	2.73
t	18.55	18.57	18.60	18.64	18.70	18.80	22.35	22.45	22.61	22.86	23.26	23.93
R_S	72.02	67.76	62.52	56.55	50.32	44.30	36.26	34.02	31.46	28.80	26.28	24.06
I_S	2.12	2.26	2.45	2.71	3.05	3.46	3.57	3.80	4.10	4.46	4.84	5.20

427 $C_4H_8N_2O_3$ Gly-Gly -31.5 8.400 132.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.91	7.97	8.04	8.13	8.33	8.37	8.44	8.52	8.62	8.74
m_S	6.70	7.08	7.62	8.39	9.42	10.74	13.99	14.73	15.75	17.07	18.66	20.42
R_E	11.15	10.55	9.80	8.90	7.93	6.95	5.34	5.07	4.74	4.38	4.00	3.66
t	25.85	25.86	25.87	25.88	25.90	25.92	18.70	18.70	18.70	18.71	18.71	18.73
R_S	94.01	89.00	82.63	75.10	66.90	58.66	54.88	52.12	48.75	44.98	41.14	37.58
I_S	1.37	1.44	1.56	1.72	1.93	2.21	3.31	3.49	3.74	4.07	4.46	4.90

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.85	8.95	9.08	9.24	9.41	9.33	9.48	9.65	9.83	10.03	10.23
m_S	20.73	21.74	22.94	24.21	25.39	26.36	26.05	26.77	27.37	27.84	28.17	28.41
R_E	3.60	3.44	3.26	3.09	2.94	2.83	2.87	2.79	2.73	2.68	2.65	2.63
t	19.55	19.57	19.59	19.62	19.68	19.78	23.82	23.93	24.13	24.44	24.98	25.90
R_S	36.16	34.46	32.65	30.93	29.47	28.34	25.71	25.00	24.40	23.92	23.53	23.16
I_S	4.88	5.13	5.42	5.73	6.01	6.23	5.68	5.83	5.94	6.01	6.02	5.96

428 $C_6H_{11}N_3O_4$ Gly-Gly-Gly -26.1 8.102 189.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.70	7.74	7.79	7.86	7.94	8.04	8.21	8.26	8.34	8.44	8.56	8.71
m_S	7.26	7.70	8.32	9.17	10.27	11.62	13.83	14.56	15.52	16.68	17.96	19.23
R_E	10.29	9.70	8.98	8.15	7.27	6.43	5.40	5.13	4.81	4.48	4.16	3.88
t	29.37	29.38	29.40	29.43	29.46	29.50	20.69	20.69	20.70	20.71	20.73	20.76
R_S	86.77	81.84	75.73	68.72	61.35	54.21	55.51	52.73	49.47	46.02	42.74	39.91
I_S	1.59	1.69	1.83	2.02	2.27	2.57	3.63	3.83	4.09	4.41	4.76	5.11

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.68	8.78	8.92	9.07	9.25	9.43	9.33	9.50	9.69	9.88	10.09	10.30
m_S	19.02	19.82	20.67	21.44	22.07	22.53	22.40	22.77	23.05	23.25	23.39	23.51
R_E	3.93	3.77	3.61	3.48	3.39	3.31	3.34	3.28	3.24	3.21	3.19	3.18
t	21.75	21.78	21.82	21.87	21.97	22.12	27.02	27.21	27.53	28.05	28.96	30.60
R_S	39.39	37.79	36.25	34.92	33.90	33.15	29.91	29.39	28.98	28.64	28.33	27.98
I_S	4.93	5.14	5.37	5.57	5.73	5.83	5.26	5.33	5.36	5.35	5.28	5.14

429 $C_8H_{14}N_4O_5$ Gly-Gly-Gly-Gly -23.3 8.142 246.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.73	7.77	7.82	7.89	7.97	8.07	8.23	8.29	8.37	8.47	8.59	8.74
m_S	6.30	6.70	7.26	8.03	9.03	10.25	12.08	12.75	13.62	14.67	15.83	16.97
R_E	11.86	11.15	10.28	9.30	8.27	7.29	6.18	5.86	5.48	5.09	4.72	4.40
t	31.84	31.86	31.89	31.92	31.97	32.03	22.12	22.13	22.14	22.16	22.19	22.23
R_S	100.1	94.08	86.73	78.42	69.78	61.50	63.55	60.22	56.37	52.32	48.50	45.22
I_S	1.43	1.52	1.65	1.83	2.06	2.35	3.34	3.53	3.78	4.08	4.42	4.75

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.71	8.81	8.95	9.10	9.28	9.47	9.37	9.54	9.73	9.93	10.13	10.35
m_S	16.77	17.50	18.26	18.96	19.53	19.96	19.86	20.19	20.45	20.63	20.77	20.89
R_E	4.46	4.27	4.09	3.94	3.82	3.74	3.76	3.70	3.65	3.62	3.60	3.58
t	23.35	23.39	23.44	23.52	23.64	23.84	29.36	29.62	30.05	30.78	32.07	34.52
R_S	44.70	42.82	41.01	39.48	38.30	37.43	33.73	33.14	32.66	32.27	31.91	31.49
IS	4.56	4.77	4.98	5.17	5.32	5.41	4.84	4.90	4.92	4.89	4.80	4.60

430 $C_{10}H_{17}N_5O_6$ Gly-Gly-Gly-Gly-Gly -21.2 8.167 303.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.74	7.78	7.84	7.91	8.00	8.10	8.25	8.31	8.39	8.49	8.62	8.76
m_S	5.63	6.01	6.54	7.25	8.17	9.29	10.85	11.47	12.27	13.24	14.30	15.34
R_E	13.26	12.43	11.42	10.30	9.14	8.04	6.89	6.51	6.09	5.64	5.22	4.87
t	34.13	34.16	34.20	34.25	34.31	34.38	23.46	23.47	23.49	23.52	23.56	23.61
R_S	111.9	104.9	96.36	86.87	77.11	67.84	70.78	66.96	62.56	57.98	53.69	50.04
IS	1.31	1.40	1.53	1.70	1.92	2.19	3.12	3.31	3.55	3.84	4.16	4.47

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.73	8.84	8.97	9.13	9.31	9.49	9.40	9.57	9.77	9.97	10.18	10.40
m_S	15.13	15.81	16.51	17.15	17.66	18.05	17.98	18.28	18.51	18.68	18.82	18.94
R_E	4.94	4.72	4.52	4.36	4.23	4.14	4.15	4.08	4.03	4.00	3.97	3.94
t	24.85	24.89	24.96	25.06	25.22	25.47	31.57	31.91	32.48	33.46	35.22	38.82
R_S	49.53	47.39	45.36	43.66	42.35	41.39	37.26	36.59	36.07	35.64	35.22	34.73
IS	4.28	4.48	4.68	4.85	4.99	5.07	4.51	4.56	4.56	4.52	4.40	4.15

431 $C_{12}H_{20}N_6O_7$ Gly-Gly-Gly-Gly-Gly-Gly -19.3 8.107 360.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.72	7.76	7.82	7.90	7.99	8.10	8.23	8.29	8.38	8.49	8.62	8.77
m_S	5.42	5.80	6.32	7.02	7.91	8.97	10.21	10.80	11.55	12.43	13.37	14.26
R_E	13.78	12.89	11.82	10.64	9.44	8.33	7.32	6.92	6.47	6.01	5.59	5.24
t	36.68	36.72	36.77	36.83	36.91	37.00	24.94	24.96	24.99	25.03	25.07	25.14
R_S	116.2	108.7	99.67	89.73	79.67	70.25	75.21	71.10	66.47	61.74	57.43	53.84
IS	1.30	1.39	1.52	1.69	1.91	2.17	3.06	3.24	3.47	3.75	4.04	4.32

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.72	8.84	8.98	9.15	9.33	9.52	9.43	9.61	9.80	10.01	10.22	10.46
m_S	14.01	14.61	15.21	15.73	16.14	16.44	16.42	16.66	16.83	16.97	17.08	17.21
R_E	5.33	5.11	4.91	4.75	4.63	4.54	4.55	4.48	4.44	4.40	4.37	4.34
t	26.50	26.56	26.64	26.77	26.97	27.29	34.03	34.48	35.23	36.55	39.05	44.68
R_S	53.47	51.27	49.24	47.59	46.35	45.45	40.80	40.18	39.68	39.24	38.79	38.21
IS	4.11	4.29	4.47	4.61	4.72	4.77	4.23	4.25	4.23	4.16	4.00	3.68

432 $C_{10}H_{19}N_3O_4$ Gly-Gly-Ile -21.9 8.096 245.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.71	7.75	7.80	7.88	7.96	8.07	8.22	8.28	8.36	8.46	8.59	8.74
m_S	6.17	6.57	7.14	7.90	8.88	10.06	11.64	12.29	13.13	14.12	15.18	16.21
R_E	12.11	11.37	10.46	9.45	8.41	7.43	6.41	6.08	5.69	5.29	4.92	4.61
t	33.33	33.36	33.39	33.44	33.49	33.56	22.98	23.00	23.01	23.04	23.07	23.11
R_S	102.2	95.89	88.27	79.74	70.97	62.65	65.94	62.47	58.50	54.39	50.57	47.35
IS	1.43	1.52	1.66	1.84	2.07	2.35	3.31	3.50	3.75	4.04	4.36	4.66

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.70	8.81	8.95	9.11	9.29	9.48	9.38	9.56	9.75	9.95	10.16	10.39
m_S	15.97	16.64	17.33	17.94	18.42	18.78	18.72	19.00	19.21	19.37	19.49	19.60
R_E	4.68	4.49	4.31	4.16	4.05	3.98	3.99	3.93	3.89	3.86	3.83	3.81
t	24.31	24.35	24.41	24.50	24.65	24.88	30.77	31.08	31.60	32.48	34.06	37.19
R_S	46.93	45.02	43.23	41.73	40.60	39.78	35.79	35.22	34.77	34.38	34.01	33.56
IS	4.46	4.66	4.85	5.02	5.14	5.21	4.65	4.70	4.70	4.65	4.54	4.31

433 $C_{10}H_{19}N_3O_4$ Gly-Gly-Leu -21.9 8.116 245.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.72	7.76	7.81	7.88	7.97	8.07	8.22	8.28	8.37	8.47	8.60	8.74
m_S	6.07	6.46	7.02	7.78	8.74	9.91	11.52	12.16	13.00	13.99	15.06	16.10
R_E	12.32	11.56	10.64	9.61	8.55	7.54	6.48	6.14	5.75	5.34	4.96	4.64
t	33.33	33.35	33.39	33.43	33.49	33.55	22.98	23.00	23.01	23.04	23.07	23.11
R_S	103.9	97.49	89.73	81.03	72.08	63.57	66.65	63.12	59.08	54.88	50.97	47.65
IS	1.40	1.50	1.63	1.81	2.04	2.32	3.27	3.46	3.71	4.00	4.32	4.63

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.71	8.82	8.95	9.12	9.29	9.48	9.38	9.56	9.75	9.95	10.16	10.39
m_S	15.88	16.56	17.26	17.88	18.38	18.75	18.68	18.98	19.19	19.36	19.48	19.60
R_E	4.71	4.51	4.33	4.18	4.06	3.98	4.00	3.94	3.89	3.86	3.83	3.81
t	24.31	24.35	24.41	24.51	24.65	24.88	30.78	31.09	31.60	32.48	34.06	37.19
R_S	47.21	45.25	43.41	41.87	40.70	39.84	35.86	35.26	34.79	34.40	34.02	33.56
I_S	4.44	4.63	4.83	5.00	5.13	5.20	4.64	4.69	4.69	4.65	4.54	4.31

434 $C_{13}H_{19}N_3O_4$ Gly-Gly-Phe -21.9 8.041 281.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.68	7.72	7.78	7.85	7.94	8.05	8.19	8.26	8.34	8.45	8.58	8.73
m_S	6.45	6.87	7.46	8.25	9.26	10.46	11.98	12.64	13.48	14.46	15.50	16.48
R_E	11.57	10.87	10.01	9.05	8.07	7.14	6.23	5.91	5.54	5.17	4.82	4.53
t	33.34	33.37	33.40	33.45	33.51	33.57	22.98	23.00	23.01	23.04	23.07	23.11
R_S	97.62	91.65	84.42	76.35	68.07	60.24	64.07	60.75	56.97	53.10	49.53	46.57
I_S	1.49	1.59	1.73	1.92	2.16	2.45	3.41	3.60	3.85	4.15	4.45	4.75

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.68	8.80	8.94	9.11	9.29	9.48	9.38	9.56	9.75	9.95	10.16	10.39
m_S	16.21	16.87	17.52	18.09	18.53	18.85	18.80	19.06	19.25	19.39	19.50	19.61
R_E	4.61	4.43	4.26	4.13	4.03	3.96	3.97	3.92	3.88	3.85	3.83	3.81
t	24.31	24.35	24.41	24.50	24.64	24.88	30.76	31.07	31.59	32.47	34.05	37.17
R_S	46.22	44.42	42.75	41.39	40.36	39.63	35.63	35.10	34.69	34.33	33.98	33.54
I_S	4.54	4.72	4.91	5.06	5.17	5.23	4.68	4.71	4.71	4.66	4.55	4.32

435 $C_9H_{17}N_3O_4$ Gly-Gly-Val -22.6 8.125 231.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.72	7.76	7.81	7.88	7.97	8.07	8.23	8.28	8.36	8.47	8.59	8.74
m_S	6.20	6.60	7.17	7.93	8.92	10.11	11.83	12.49	13.34	14.36	15.47	16.56
R_E	12.04	11.31	10.42	9.42	8.38	7.39	6.31	5.98	5.60	5.20	4.83	4.51
t	32.56	32.58	32.61	32.65	32.70	32.76	22.54	22.55	22.56	22.59	22.62	22.66
R_S	101.6	95.41	87.89	79.43	70.67	62.31	64.90	61.49	57.56	53.46	49.61	46.34
I_S	1.42	1.51	1.65	1.83	2.06	2.34	3.31	3.50	3.75	4.05	4.38	4.70

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.70	8.81	8.95	9.11	9.29	9.47	9.38	9.55	9.74	9.94	10.15	10.37
m_S	16.34	17.05	17.78	18.44	18.97	19.36	19.28	19.59	19.83	20.00	20.13	20.24
R_E	4.57	4.38	4.20	4.05	3.94	3.86	3.87	3.81	3.77	3.74	3.71	3.69
t	23.82	23.85	23.91	23.99	24.13	24.34	30.04	30.33	30.80	31.60	33.02	35.78
R_S	45.86	43.95	42.13	40.61	39.44	38.59	34.75	34.16	33.69	33.30	32.93	32.49
I_S	4.51	4.71	4.91	5.09	5.23	5.31	4.75	4.80	4.81	4.77	4.67	4.46

436 $C_{10}H_{15}N_5O_4$ Gly-His-Gly -22.5 8.279 269.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.79	7.83	7.88	7.95	8.03	8.13	8.29	8.35	8.42	8.52	8.63	8.77
m_S	5.41	5.76	6.27	6.95	7.85	8.95	10.79	11.41	12.23	13.25	14.41	15.59
R_E	13.81	12.96	11.92	10.74	9.52	8.34	6.92	6.55	6.11	5.64	5.18	4.79
t	32.64	32.66	32.69	32.73	32.78	32.84	22.60	22.61	22.63	22.65	22.69	22.73
R_S	116.5	109.3	100.5	90.63	80.31	70.37	71.16	67.29	62.75	57.93	53.28	49.21
I_S	1.24	1.32	1.44	1.60	1.81	2.07	3.02	3.20	3.44	3.73	4.07	4.42

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.85	8.98	9.13	9.30	9.48	9.40	9.56	9.75	9.94	10.15	10.37
m_S	15.49	16.23	17.04	17.82	18.48	18.99	18.87	19.26	19.57	19.80	19.97	20.11
R_E	4.82	4.60	4.38	4.19	4.04	3.93	3.96	3.88	3.82	3.77	3.74	3.71
t	23.90	23.93	23.99	24.08	24.22	24.44	30.18	30.46	30.94	31.75	33.20	36.01
R_S	48.39	46.16	43.95	42.01	40.48	39.35	35.50	34.73	34.13	33.64	33.19	32.71
I_S	4.27	4.48	4.71	4.92	5.09	5.21	4.64	4.72	4.75	4.73	4.63	4.43

437 $C_8H_{16}N_2O_3$ Gly-Ile -25.2 8.412 188.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.85	7.89	7.94	8.00	8.07	8.17	8.35	8.39	8.46	8.55	8.65	8.78
m_S	5.35	5.68	6.16	6.83	7.71	8.82	11.12	11.75	12.61	13.71	15.00	16.41
R_E	13.96	13.14	12.12	10.94	9.69	8.47	6.72	6.36	5.92	5.45	4.98	4.55
t	30.06	30.08	30.10	30.13	30.16	30.21	21.13	21.13	21.15	21.16	21.18	21.21
R_S	117.8	110.8	102.2	92.30	81.76	71.42	69.05	65.34	60.88	56.00	51.16	46.76
I_S	1.18	1.25	1.36	1.51	1.71	1.96	2.94	3.12	3.35	3.65	4.01	4.40

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.79	8.88	8.99	9.13	9.29	9.46	9.39	9.54	9.72	9.91	10.11	10.32
m_S	16.51	17.36	18.33	19.33	20.23	20.96	20.77	21.32	21.77	22.11	22.36	22.55
R_E	4.52	4.30	4.08	3.86	3.69	3.56	3.60	3.50	3.43	3.38	3.34	3.31
t	22.25	22.28	22.33	22.39	22.50	22.66	27.77	27.97	28.32	28.90	29.91	31.77
R_S	45.39	43.17	40.86	38.73	36.98	35.64	32.25	31.39	30.68	30.12	29.64	29.17
I_S	4.32	4.55	4.81	5.08	5.31	5.49	4.92	5.03	5.11	5.13	5.08	4.94

438 $C_8H_{16}N_2O_3$ Gly-Leu -25.1 8.432 188.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.86	7.90	7.94	8.01	8.08	8.17	8.36	8.40	8.47	8.55	8.66	8.79
m_S	5.23	5.56	6.03	6.68	7.55	8.65	10.93	11.56	12.41	13.50	14.79	16.19
R_E	14.27	13.43	12.38	11.18	9.90	8.64	6.83	6.46	6.02	5.53	5.05	4.61
t	30.15	30.16	30.18	30.21	30.25	30.29	21.18	21.19	21.20	21.21	21.23	21.27
R_S	120.4	113.3	104.5	94.29	83.48	72.88	70.23	66.43	61.87	56.88	51.91	47.39
I_S	1.15	1.23	1.33	1.48	1.68	1.92	2.90	3.07	3.30	3.60	3.96	4.35

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.80	8.88	8.99	9.13	9.29	9.47	9.39	9.55	9.72	9.91	10.11	10.32
m_S	16.32	17.16	18.14	19.15	20.06	20.82	20.62	21.18	21.64	21.99	22.25	22.44
R_E	4.58	4.35	4.12	3.90	3.72	3.59	3.62	3.53	3.45	3.40	3.36	3.33
t	22.31	22.34	22.38	22.45	22.56	22.72	27.85	28.06	28.41	29.00	30.02	31.91
R_S	45.93	43.66	41.29	39.10	37.28	35.89	32.48	31.59	30.86	30.28	29.79	29.31
I_S	4.27	4.50	4.77	5.03	5.27	5.46	4.89	5.01	5.08	5.10	5.06	4.92

439 $C_{11}H_{21}N_3O_4$ Gly-Leu-Ala -21.1 8.259 259.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.79	7.83	7.88	7.95	8.03	8.13	8.29	8.35	8.42	8.52	8.64	8.78
m_S	5.18	5.53	6.02	6.69	7.56	8.62	10.24	10.84	11.63	12.60	13.67	14.77
R_E	14.42	13.51	12.40	11.16	9.88	8.66	7.29	6.89	6.42	5.93	5.46	5.06
t	34.23	34.26	34.30	34.34	34.40	34.48	23.53	23.55	23.57	23.60	23.64	23.69
R_S	121.6	114.0	104.6	94.17	83.37	73.07	74.95	70.81	66.00	60.95	56.13	51.97
I_S	1.21	1.29	1.41	1.57	1.78	2.03	2.95	3.12	3.36	3.65	3.97	4.30

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.86	8.99	9.14	9.31	9.50	9.41	9.58	9.77	9.97	10.18	10.41
m_S	14.62	15.32	16.07	16.77	17.36	17.80	17.72	18.07	18.33	18.53	18.69	18.83
R_E	5.11	4.88	4.65	4.45	4.30	4.19	4.22	4.13	4.07	4.03	4.00	3.97
t	24.93	24.98	25.05	25.15	25.31	25.57	31.71	32.06	32.63	33.62	35.42	39.10
R_S	51.25	48.90	46.61	44.64	43.10	41.96	37.81	37.04	36.43	35.93	35.46	34.94
I_S	4.14	4.34	4.55	4.75	4.90	5.00	4.44	4.50	4.52	4.48	4.36	4.12

440 $C_{17}H_{25}N_3O_5$ Gly-Leu-Tyr* -21.0 8.405 351.4

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.86	7.90	7.95	8.01	8.10	8.19	8.35	8.41	8.48	8.57	8.68	8.81
m_S	4.53	4.84	5.28	5.87	6.66	7.64	9.33	9.89	10.64	11.59	12.68	13.84
R_E	16.50	15.44	14.16	12.72	11.22	9.78	8.01	7.55	7.02	6.45	5.89	5.40
t	34.33	34.35	34.39	34.44	34.50	34.57	23.61	23.62	23.65	23.68	23.72	23.77
R_S	139.1	130.3	119.4	107.3	94.62	82.48	82.33	77.64	72.14	66.25	60.53	55.45
I_S	1.05	1.13	1.23	1.37	1.56	1.80	2.68	2.84	3.07	3.35	3.68	4.03

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.81	8.90	9.02	9.17	9.33	9.51	9.44	9.60	9.78	9.98	10.19	10.41
m_S	13.81	14.54	15.35	16.15	16.85	17.41	17.31	17.73	18.07	18.33	18.52	18.69
R_E	5.41	5.14	4.87	4.63	4.43	4.29	4.32	4.21	4.13	4.08	4.03	4.00
t	25.03	25.08	25.15	25.26	25.42	25.69	31.87	32.22	32.81	33.82	35.65	39.43
R_S	54.26	51.55	48.81	46.36	44.39	42.91	38.71	37.74	36.96	36.34	35.78	35.19
I_S	3.90	4.11	4.34	4.57	4.76	4.89	4.34	4.42	4.45	4.43	4.32	4.08

441 $C_{11}H_{14}N_2O_3$ Gly-Phe -24.7 8.310 222.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.81	7.84	7.89	7.95	8.03	8.13	8.30	8.35	8.42	8.51	8.63	8.76
m_S	5.75	6.11	6.63	7.34	8.27	9.44	11.62	12.27	13.15	14.25	15.52	16.85
R_E	12.98	12.22	11.27	10.18	9.03	7.91	6.43	6.09	5.68	5.24	4.81	4.43
t	30.50	30.52	30.54	30.57	30.61	30.66	21.37	21.38	21.39	21.40	21.43	21.46
R_S	109.5	103.0	95.04	85.86	76.18	66.75	66.09	62.57	58.39	53.89	49.48	45.55
I_S	1.28	1.36	1.48	1.64	1.85	2.12	3.11	3.29	3.54	3.84	4.20	4.57

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* The mobility was refined as a monoanion (a nonovalent anion).

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.85	8.97	9.11	9.28	9.46	9.38	9.54	9.72	9.91	10.11	10.33
m_S	16.82	17.63	18.54	19.44	20.22	20.83	20.66	21.13	21.50	21.78	21.98	22.13
R_E	4.44	4.24	4.03	3.84	3.69	3.59	3.61	3.53	3.47	3.43	3.40	3.37
t	22.52	22.55	22.59	22.66	22.77	22.95	28.15	28.37	28.74	29.35	30.43	32.41
R_S	44.57	42.49	40.39	38.51	37.00	35.87	32.42	31.67	31.06	30.58	30.16	29.72
I_S	4.45	4.67	4.92	5.16	5.36	5.50	4.93	5.03	5.08	5.08	5.02	4.86

442 $C_{20}H_{23}N_3O_4$ Gly-Phe-Phe -19.7 8.216 369.4

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.77	7.81	7.87	7.94	8.03	8.13	8.27	8.33	8.41	8.52	8.64	8.79
m_S	5.04	5.39	5.88	6.54	7.39	8.42	9.81	10.39	11.15	12.05	13.05	14.03
R_E	14.82	13.86	12.70	11.42	10.11	8.87	7.61	7.19	6.70	6.20	5.72	5.32
t	36.07	36.11	36.15	36.21	36.28	36.37	24.60	24.62	24.65	24.68	24.73	24.79
R_S	125.0	116.9	107.2	96.34	85.28	74.83	78.26	73.89	68.88	63.69	58.83	54.69
I_S	1.20	1.28	1.40	1.56	1.77	2.02	2.91	3.08	3.32	3.60	3.90	4.21

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.75	8.86	9.00	9.15	9.33	9.52	9.43	9.61	9.80	10.00	10.21	10.45
m_S	13.84	14.49	15.16	15.77	16.27	16.65	16.60	16.89	17.11	17.28	17.42	17.56
R_E	5.40	5.15	4.93	4.74	4.59	4.49	4.50	4.42	4.36	4.32	4.29	4.25
t	26.13	26.18	26.27	26.39	26.58	26.89	33.49	33.91	34.62	35.86	38.17	43.25
R_S	54.13	51.71	49.40	47.46	45.97	44.88	40.36	39.61	39.02	38.53	38.05	37.46
I_S	4.02	4.22	4.41	4.59	4.72	4.79	4.24	4.29	4.28	4.23	4.08	3.78

443 $C_7H_{12}N_2O_3$ Gly-Pro -27.8 8.746 172.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.01	8.04	8.09	8.14	8.21	8.29	8.50	8.54	8.59	8.66	8.75	8.86
m_S	4.28	4.54	4.92	5.45	6.17	7.10	9.70	10.26	11.05	12.10	13.42	14.98
R_E	17.43	16.44	15.19	13.71	12.11	10.51	7.70	7.28	6.76	6.17	5.57	4.99
t	28.09	28.10	28.11	28.13	28.16	28.19	20.03	20.03	20.04	20.05	20.07	20.09
R_S	147.0	138.7	128.1	115.7	102.2	88.69	79.12	74.81	69.49	63.46	57.20	51.24
I_S	0.91	0.96	1.04	1.16	1.31	1.52	2.43	2.57	2.77	3.04	3.39	3.79

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.91	8.98	9.07	9.19	9.32	9.48	9.44	9.57	9.72	9.90	10.09	10.29
m_S	15.66	16.56	17.69	19.00	20.34	21.58	21.40	22.24	23.05	23.73	24.25	24.63
R_E	4.77	4.51	4.22	3.93	3.67	3.46	3.49	3.36	3.24	3.15	3.08	3.03
t	21.04	21.06	21.10	21.15	21.23	21.37	26.00	26.16	26.43	26.88	27.64	28.99
R_S	47.86	45.24	42.33	39.41	36.78	34.62	31.31	30.08	28.97	28.06	27.33	26.70
I_S	3.88	4.11	4.40	4.74	5.07	5.38	4.87	5.05	5.21	5.32	5.36	5.30

444 $C_{10}H_{17}N_3O_4$ Gly-Pro-Ala -22.5 8.492 243.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.90	7.93	7.98	8.05	8.12	8.22	8.39	8.44	8.50	8.59	8.70	8.82
m_S	4.46	4.76	5.18	5.76	6.53	7.51	9.43	10.00	10.77	11.75	12.91	14.18
R_E	16.74	15.70	14.42	12.96	11.43	9.95	7.92	7.47	6.94	6.36	5.79	5.27
t	32.61	32.63	32.66	32.69	32.74	32.80	22.62	22.63	22.65	22.67	22.70	22.75
R_S	141.2	132.4	121.6	109.3	96.43	83.90	81.40	76.80	71.32	65.36	59.45	54.11
I_S	1.02	1.09	1.18	1.32	1.50	1.73	2.62	2.78	3.00	3.29	3.63	4.00

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.83	8.92	9.03	9.17	9.32	9.50	9.43	9.59	9.76	9.95	10.16	10.38
m_S	14.29	15.06	15.96	16.89	17.74	18.44	18.30	18.81	19.25	19.58	19.83	20.02
R_E	5.23	4.96	4.68	4.42	4.21	4.05	4.08	3.97	3.88	3.81	3.77	3.73
t	23.92	23.96	24.02	24.11	24.25	24.48	30.24	30.52	31.00	31.82	33.27	36.10
R_S	52.46	49.75	46.93	44.33	42.17	40.51	36.62	35.56	34.70	34.01	33.43	32.85
I_S	3.92	4.13	4.39	4.64	4.87	5.04	4.49	4.59	4.66	4.66	4.59	4.40

445 $C_5H_{10}N_2O_4$ Gly-Ser -28.1 8.350 162.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.82	7.85	7.90	7.96	8.03	8.12	8.31	8.36	8.43	8.51	8.62	8.75
m_S	6.28	6.65	7.19	7.93	8.92	10.18	12.89	13.59	14.54	15.76	17.19	18.74
R_E	11.89	11.23	10.39	9.42	8.37	7.34	5.80	5.50	5.14	4.74	4.34	3.99
t	27.89	27.90	27.91	27.93	27.96	27.99	19.86	19.87	19.87	19.88	19.89	19.91
R_S	100.3	94.70	87.65	79.45	70.64	61.92	59.58	56.51	52.80	48.72	44.65	40.95
I_S	1.34	1.42	1.53	1.69	1.91	2.18	3.24	3.42	3.67	3.99	4.37	4.78

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.84	8.96	9.09	9.25	9.43	9.35	9.50	9.68	9.87	10.07	10.27
m_S	18.85	19.77	20.83	21.90	22.87	23.65	23.41	23.99	24.47	24.83	25.08	25.27
R_E	3.96	3.78	3.59	3.41	3.27	3.16	3.19	3.11	3.05	3.01	2.98	2.95
t	20.84	20.86	20.89	20.94	21.02	21.15	25.70	25.85	26.11	26.54	27.27	28.57
R_S	39.76	37.90	35.97	34.18	32.71	31.59	28.62	27.89	27.29	26.82	26.42	26.03
I_S	4.70	4.93	5.21	5.48	5.72	5.91	5.34	5.46	5.54	5.58	5.55	5.45

446 $C_6H_{12}N_2O_4$ Gly-Thr -26.3 8.334 176.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.81	7.85	7.90	7.96	8.03	8.13	8.31	8.36	8.43	8.51	8.62	8.76
m_S	5.98	6.34	6.87	7.59	8.55	9.75	12.19	12.86	13.77	14.93	16.27	17.71
R_E	12.49	11.77	10.88	9.84	8.74	7.66	6.13	5.81	5.42	5.00	4.59	4.22
t	29.18	29.20	29.22	29.24	29.27	29.31	20.61	20.61	20.62	20.64	20.65	20.68
R_S	105.4	99.30	91.75	83.01	73.72	64.59	63.01	59.70	55.75	51.43	47.17	43.33
I_S	1.30	1.38	1.50	1.66	1.87	2.14	3.17	3.35	3.59	3.91	4.27	4.67

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.85	8.96	9.10	9.27	9.45	9.36	9.52	9.70	9.89	10.09	10.30
m_S	17.75	18.62	19.60	20.58	21.45	22.15	21.95	22.47	22.89	23.21	23.44	23.61
R_E	4.21	4.01	3.81	3.63	3.48	3.37	3.40	3.32	3.26	3.22	3.19	3.16
t	21.67	21.70	21.74	21.79	21.89	22.04	26.91	27.10	27.41	27.92	28.81	30.41
R_S	42.22	40.25	38.22	36.38	34.87	33.73	30.53	29.78	29.17	28.69	28.28	27.86
I_S	4.56	4.79	5.05	5.31	5.53	5.70	5.13	5.23	5.30	5.32	5.28	5.15

447 $C_{13}H_{15}N_3O_3$ Gly-Trp -23.6 8.359 261.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.83	7.87	7.92	7.98	8.06	8.15	8.33	8.38	8.45	8.54	8.65	8.78
m_S	5.27	5.61	6.09	6.76	7.63	8.73	10.77	11.39	12.23	13.28	14.50	15.79
R_E	14.17	13.31	12.26	11.05	9.78	8.55	6.93	6.56	6.11	5.63	5.15	4.73
t	31.51	31.53	31.55	31.59	31.63	31.68	21.96	21.97	21.98	22.00	22.03	22.07
R_S	119.5	112.3	103.4	93.21	82.53	72.15	71.26	67.38	62.78	57.81	52.95	48.60
I_S	1.19	1.26	1.38	1.53	1.73	1.99	2.94	3.12	3.35	3.65	4.00	4.37

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.78	8.87	8.99	9.13	9.30	9.48	9.40	9.56	9.74	9.93	10.13	10.35
m_S	15.78	16.58	17.47	18.35	19.13	19.75	19.60	20.07	20.44	20.72	20.93	21.10
R_E	4.73	4.51	4.28	4.07	3.90	3.78	3.81	3.72	3.65	3.60	3.57	3.54
t	23.18	23.21	23.27	23.34	23.47	23.66	29.13	29.38	29.79	30.50	31.73	34.08
R_S	47.48	45.20	42.88	40.79	39.10	37.82	34.18	33.35	32.67	32.13	31.66	31.18
I_S	4.25	4.47	4.72	4.96	5.17	5.31	4.75	4.84	4.89	4.89	4.82	4.64

448 $C_{12}H_{14}N_2O_5$ Gly-Tyr -19.7 8.211 266.3
-39.4 9.981

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.77	7.81	7.87	7.94	8.02	8.13	8.27	8.33	8.41	8.51	8.64	8.78
m_S	5.12	5.47	5.98	6.66	7.54	8.62	10.17	10.80	11.64	12.67	13.87	15.16
R_E	14.60	13.64	12.49	11.22	9.91	8.67	7.35	6.92	6.42	5.90	5.39	4.93
t	36.21	36.26	36.33	36.42	36.54	36.69	25.05	25.13	25.26	25.45	25.73	26.13
R_S	123.1	115.1	105.4	94.61	83.58	73.11	75.52	71.10	65.99	60.59	55.36	50.64
I_S	1.22	1.31	1.43	1.60	1.82	2.09	3.03	3.23	3.49	3.83	4.21	4.64

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.76	8.87	9.00	9.15	9.31	9.48	9.44	9.58	9.73	9.90	10.07	10.25
m_S	15.03	15.93	17.02	18.24	19.62	21.14	20.84	22.16	23.64	25.22	26.81	28.29
R_E	4.97	4.69	4.39	4.09	3.81	3.53	3.58	3.37	3.16	2.96	2.79	2.64
t	27.43	27.80	28.34	29.09	30.09	31.35	37.89	39.05	40.42	41.91	43.72	45.94
R_S	49.88	47.02	44.02	41.03	38.13	35.34	32.15	30.20	28.25	26.41	24.72	23.25
I_S	4.46	4.76	5.13	5.55	6.03	6.56	5.84	6.29	6.79	7.32	7.80	8.19

449 $C_7H_{14}N_2O_3$ Gly-Val -26.0 8.385 174.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.98	8.06	8.15	8.33	8.38	8.45	8.53	8.64	8.77
m_S	5.65	6.00	6.49	7.18	8.10	9.26	11.67	12.32	13.21	14.34	15.68	17.12
R_E	13.22	12.46	11.50	10.40	9.22	8.06	6.40	6.06	5.65	5.21	4.76	4.36
t	29.41	29.43	29.45	29.47	29.50	29.54	20.75	20.75	20.76	20.78	20.80	20.82
R_S	111.5	105.1	97.01	87.70	77.78	68.03	65.80	62.30	58.11	53.52	48.96	44.82
I_S	1.23	1.31	1.42	1.57	1.78	2.04	3.04	3.22	3.46	3.77	4.13	4.53

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.78	8.86	8.98	9.12	9.28	9.45	9.38	9.53	9.70	9.89	10.10	10.31
m_S	17.21	18.08	19.07	20.08	20.99	21.73	21.53	22.08	22.53	22.87	23.12	23.30
R_E	4.34	4.13	3.92	3.72	3.56	3.44	3.47	3.38	3.32	3.27	3.23	3.20
t	21.83	21.86	21.89	21.96	22.05	22.20	27.14	27.33	27.65	28.19	29.10	30.77
R_S	43.54	41.45	39.27	37.28	35.63	34.38	31.12	30.31	29.64	29.11	28.67	28.23
I_S	4.44	4.67	4.94	5.20	5.44	5.62	5.05	5.16	5.24	5.26	5.22	5.09

450 $C_6H_9N_3O_2$ Histidine 29.6 6.040 155.2
-28.8 9.342

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.98	8.06	8.15	8.79	8.82	8.86	8.92	9.00	9.09
m_S	0.00	0.00	0.00	0.00	0.00	0.00	6.14	6.50	7.02	7.75	8.72	9.96
R_E	0.00	0.00	0.00	0.00	0.00	0.00	12.17	11.49	10.64	9.64	8.57	7.50
t	0.00	0.00	0.00	0.00	0.00	0.00	20.01	20.00	19.99	19.98	19.98	19.99
R_S	0.00	0.00	0.00	0.00	0.00	0.00	125.1	118.1	109.3	99.08	88.03	77.04
I_S	0.00	0.00	0.00	0.00	0.00	0.00	1.47	1.56	1.69	1.87	2.11	2.42

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.18	9.23	9.29	9.38	9.48	9.61	9.64	9.73	9.84	9.98	10.14	10.32
m_S	11.30	12.03	13.03	14.32	15.86	17.58	18.07	19.15	20.40	21.71	22.93	23.98
R_E	6.61	6.21	5.73	5.22	4.71	4.25	4.13	3.90	3.66	3.44	3.26	3.11
t	20.92	20.94	20.97	21.03	21.12	21.25	25.82	25.99	26.25	26.68	27.41	28.69
R_S	66.31	62.26	57.48	52.29	47.17	42.50	37.07	34.95	32.74	30.67	28.90	27.43
I_S	2.69	2.87	3.11	3.43	3.80	4.21	3.97	4.20	4.47	4.72	4.93	5.03

451 $C_5H_9NO_3$ Hydroxyproline -30.1 9.816 131.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.98	8.06	8.79	9.02	9.05	9.09	9.14	9.21	9.29
m_S	0.00	0.00	0.00	0.00	0.00	2.57	4.13	4.37	4.72	5.22	5.91	6.81
R_E	0.00	0.00	0.00	0.00	0.00	29.05	18.11	17.10	15.82	14.30	12.64	10.96
t	0.00	0.00	0.00	0.00	0.00	27.50	19.97	19.98	19.99	20.01	20.04	20.08
R_S	0.00	0.00	0.00	0.00	0.00	245.0	186.1	175.8	162.6	147.0	129.9	112.7
I_S	0.00	0.00	0.00	0.00	0.00	0.51	0.94	1.00	1.08	1.19	1.35	1.56

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.40	9.45	9.50	9.57	9.66	9.76	9.84	9.90	9.99	10.10	10.23	10.38
m_S	8.25	8.80	9.58	10.62	11.97	13.60	14.87	15.88	17.19	18.75	20.46	22.18
R_E	9.05	8.49	7.80	7.03	6.24	5.49	5.02	4.70	4.35	3.98	3.65	3.37
t	21.02	21.05	21.10	21.18	21.29	21.45	26.01	26.19	26.48	26.95	27.71	29.03
R_S	90.82	85.12	78.21	70.48	62.52	54.92	45.06	42.13	38.86	35.52	32.39	29.66
I_S	1.86	1.99	2.16	2.40	2.71	3.08	3.10	3.31	3.57	3.87	4.18	4.43

452 $C_6H_{13}NO_2$ Isoleucine -26.7 9.765 131.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.98	8.06	8.79	9.00	9.03	9.07	9.13	9.20	9.28
m_S	0.00	0.00	0.00	0.00	0.00	0.00	3.87	4.11	4.45	4.94	5.60	6.46
R_E	0.00	0.00	0.00	0.00	0.00	0.00	19.29	18.18	16.77	15.13	13.35	11.57
t	0.00	0.00	0.00	0.00	0.00	0.00	21.29	21.30	21.32	21.34	21.38	21.43
R_S	0.00	0.00	0.00	0.00	0.00	0.00	198.3	186.9	172.4	155.5	137.2	118.8
I_S	0.00	0.00	0.00	0.00	0.00	0.00	0.94	0.99	1.08	1.20	1.36	1.57

No.	1.23	1.31	1.42	1.57	1.78	0.51	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.39	9.43	9.49	9.56	9.65	9.76	9.83	9.90	10.00	10.11	10.25	10.42
m_S	7.70	8.22	8.96	9.95	11.21	12.71	13.75	14.70	15.90	17.30	18.79	20.26
R_E	9.71	9.08	8.33	7.51	6.66	5.88	5.43	5.08	4.70	4.32	3.97	3.69
t	22.51	22.55	22.62	22.72	22.86	23.08	28.27	28.52	28.91	29.55	30.63	32.58
R_S	97.39	91.10	83.56	75.22	66.74	58.76	48.72	45.52	42.01	38.49	35.26	32.47
I_S	1.84	1.96	2.14	2.38	2.68	3.03	3.00	3.20	3.44	3.71	3.95	4.12

453 $C_6H_{13}NO_2$ Leucine -26.4 9.728 131.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.98	8.06	8.76	8.98	9.01	9.06	9.11	9.18	9.27
m_S	0.00	0.00	0.00	0.00	0.00	2.55	3.97	4.21	4.56	5.06	5.73	6.61
R_E	0.00	0.00	0.00	0.00	0.00	29.30	18.83	17.74	16.37	14.76	13.03	11.29
t	0.00	0.00	0.00	0.00	0.00	29.96	21.36	21.37	21.39	21.41	21.45	21.50
R_S	0.00	0.00	0.00	0.00	0.00	247.1	193.6	182.4	168.3	151.7	133.9	116.0
I_S	0.00	0.00	0.00	0.00	0.00	0.53	0.97	1.03	1.11	1.24	1.40	1.62

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.37	9.41	9.47	9.55	9.64	9.75	9.82	9.89	9.98	10.10	10.25	10.41
m_S	7.84	8.38	9.13	10.13	11.40	12.90	13.88	14.83	16.02	17.40	18.86	20.27
R_E	9.53	8.92	8.18	7.37	6.55	5.79	5.38	5.04	4.66	4.29	3.96	3.68
t	22.58	22.63	22.69	22.79	22.94	23.16	28.39	28.64	29.03	29.68	30.77	32.76
R_S	95.63	89.45	82.05	73.90	65.64	57.89	48.27	45.12	41.68	38.26	35.14	32.46
I_S	1.89	2.02	2.20	2.44	2.75	3.11	3.05	3.25	3.49	3.75	4.00	4.15

454 $C_8H_{16}N_2O_4$ Leu-Gly -25.0 8.269 204.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.78	7.82	7.87	7.93	8.01	8.11	8.28	8.33	8.41	8.50	8.61	8.75
m_S	6.03	6.41	6.95	7.68	8.65	9.85	12.05	12.72	13.62	14.73	16.01	17.34
R_E	12.38	11.65	10.75	9.72	8.64	7.58	6.20	5.87	5.49	5.07	4.67	4.31
t	30.25	30.27	30.29	30.32	30.35	30.40	21.22	21.22	21.23	21.25	21.27	21.30
R_S	104.4	98.28	90.71	82.02	72.86	63.94	63.71	60.35	56.39	52.11	47.95	44.27
I_S	1.34	1.42	1.54	1.71	1.93	2.20	3.21	3.40	3.65	3.96	4.31	4.68

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.74	8.83	8.96	9.10	9.27	9.45	9.37	9.53	9.71	9.90	10.11	10.32
m_S	17.27	18.08	18.99	19.86	20.61	21.19	21.03	21.48	21.83	22.09	22.28	22.43
R_E	4.33	4.13	3.93	3.76	3.62	3.52	3.55	3.48	3.42	3.38	3.35	3.33
t	22.35	22.38	22.42	22.49	22.59	22.76	27.90	28.11	28.46	29.06	30.09	31.99
R_S	43.40	41.43	39.45	37.70	36.29	35.25	31.85	31.15	30.59	30.14	29.75	29.33
I_S	4.55	4.77	5.01	5.25	5.44	5.58	5.00	5.09	5.14	5.14	5.08	4.92

455 $C_{10}H_{19}N_3O_4$ Leu-Gly-Gly -21.5 7.992 245.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.66	7.70	7.76	7.83	7.93	8.03	8.17	8.24	8.33	8.44	8.57	8.73
m_S	6.60	7.03	7.63	8.44	9.45	10.65	12.06	12.71	13.54	14.49	15.48	16.40
R_E	11.32	10.62	9.79	8.86	7.91	7.01	6.19	5.88	5.52	5.15	4.82	4.55
t	33.82	33.85	33.88	33.93	33.99	34.06	23.25	23.27	23.28	23.31	23.34	23.39
R_S	95.48	89.62	82.55	74.70	66.68	59.15	63.67	60.40	56.71	52.97	49.57	46.78
I_S	1.54	1.64	1.78	1.98	2.22	2.51	3.46	3.66	3.91	4.19	4.49	4.77

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.67	8.79	8.94	9.11	9.29	9.48	9.38	9.56	9.75	9.96	10.17	10.40
m_S	16.12	16.74	17.35	17.87	18.27	18.55	18.52	18.75	18.92	19.04	19.15	19.25
R_E	4.63	4.46	4.31	4.18	4.09	4.03	4.03	3.98	3.95	3.92	3.90	3.88
t	24.60	24.65	24.71	24.81	24.96	25.20	31.20	31.53	32.07	33.00	34.68	38.06
R_S	46.49	44.75	43.17	41.90	40.95	40.27	36.18	35.69	35.30	34.96	34.61	34.17
I_S	4.55	4.73	4.90	5.04	5.14	5.19	4.63	4.66	4.65	4.60	4.47	4.23

456 $C_{17}H_{25}N_3O_4$ Leu-Gly-Phe -19.3 7.938 335.4

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.64	7.69	7.75	7.83	7.92	8.04	8.16	8.23	8.32	8.44	8.59	8.75
m_S	6.22	6.64	7.23	8.00	8.95	10.06	11.11	11.72	12.47	13.32	14.16	14.92
R_E	12.02	11.25	10.34	9.34	8.34	7.42	6.72	6.37	5.99	5.61	5.27	5.01
t	36.74	36.78	36.83	36.89	36.97	37.06	24.95	24.97	24.99	25.03	25.08	25.13
R_S	101.4	94.89	87.19	78.79	70.37	62.61	69.09	65.50	61.55	57.64	54.19	51.43
I_S	1.49	1.60	1.74	1.93	2.17	2.45	3.34	3.53	3.77	4.03	4.30	4.53

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.68	8.80	8.96	9.13	9.32	9.51	9.41	9.60	9.80	10.00	10.22	10.46
m_S	14.64	15.18	15.67	16.08	16.39	16.61	16.62	16.80	16.93	17.03	17.12	17.24
R_E	5.10	4.92	4.77	4.64	4.56	4.50	4.49	4.45	4.41	4.39	4.36	4.33
t	26.49	26.55	26.63	26.76	26.96	27.28	34.00	34.45	35.20	36.52	39.01	44.63
R_S	51.19	49.37	47.79	46.54	45.64	44.98	40.31	39.84	39.46	39.11	38.71	38.16
I_S	4.31	4.47	4.61	4.73	4.80	4.82	4.28	4.29	4.26	4.18	4.02	3.69

457 $C_{12}H_{24}N_2O_3$ Leu-Leu -21.6 8.397 244.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.85	7.89	7.94	8.01	8.09	8.18	8.35	8.40	8.47	8.56	8.67	8.81
m_S	4.68	5.00	5.45	6.06	6.86	7.87	9.64	10.21	10.98	11.95	13.08	14.27
R_E	15.95	14.94	13.71	12.33	10.88	9.49	7.75	7.31	6.80	6.25	5.71	5.24
t	33.62	33.64	33.67	33.72	33.77	33.84	23.19	23.20	23.22	23.25	23.29	23.34
R_S	134.5	126.0	115.7	104.0	91.81	80.07	79.67	75.18	69.89	64.22	58.70	53.79
I_S	1.08	1.16	1.26	1.41	1.60	1.84	2.73	2.90	3.13	3.42	3.75	4.10

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.80	8.89	9.01	9.16	9.32	9.50	9.43	9.59	9.77	9.97	10.17	10.40
m_S	14.25	14.99	15.82	16.64	17.37	17.94	17.83	18.26	18.61	18.87	19.07	19.24
R_E	5.24	4.98	4.72	4.49	4.30	4.16	4.19	4.09	4.01	3.96	3.92	3.88
t	24.56	24.60	24.67	24.77	24.92	25.17	31.17	31.50	32.03	32.96	34.62	37.96
R_S	52.60	49.99	47.35	44.99	43.07	41.64	37.58	36.64	35.89	35.28	34.75	34.18
I_S	3.98	4.19	4.43	4.66	4.85	4.99	4.43	4.52	4.56	4.54	4.44	4.22

458 $C_{18}H_{35}N_3O_4$ Leu-Leu-Leu -17.6 7.730 357.5

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.55	7.60	7.67	7.76	7.87	8.00	8.09	8.17	8.28	8.42	8.57	8.75
m_S	6.66	7.12	7.75	8.54	9.48	10.51	11.10	11.67	12.33	13.01	13.64	14.15
R_E	11.21	10.49	9.64	8.75	7.88	7.11	6.73	6.40	6.06	5.74	5.48	5.28
t	39.60	39.65	39.72	39.80	39.90	40.00	26.59	26.61	26.65	26.69	26.75	26.82
R_S	94.58	88.46	81.35	73.80	66.48	59.94	69.19	65.82	62.29	59.00	56.28	54.22
I_S	1.65	1.76	1.92	2.12	2.36	2.62	3.47	3.66	3.87	4.10	4.30	4.47

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.65	8.79	8.96	9.15	9.34	9.54	9.44	9.63	9.84	10.05	10.27	10.54
m_S	13.90	14.29	14.63	14.88	15.06	15.19	15.24	15.35	15.43	15.50	15.58	15.72
R_E	5.37	5.23	5.11	5.02	4.96	4.92	4.90	4.87	4.84	4.82	4.79	4.75
t	28.30	28.37	28.47	28.63	28.88	29.29	36.70	37.29	38.30	40.12	43.77	53.65
R_S	53.90	52.42	51.21	50.32	49.67	49.20	43.95	43.60	43.29	42.96	42.54	41.85
I_S	4.24	4.36	4.46	4.52	4.55	4.54	4.02	4.00	3.95	3.84	3.63	3.18

459 $C_{15}H_{22}N_2O_3$ Leu-Phe -21.8 8.413 278.4

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.86	7.90	7.95	8.01	8.09	8.19	8.35	8.41	8.48	8.57	8.68	8.81
m_S	4.66	4.97	5.41	6.02	6.82	7.82	9.63	10.20	10.97	11.95	13.08	14.29
R_E	16.04	15.03	13.80	12.40	10.95	9.55	7.76	7.32	6.81	6.25	5.71	5.23
t	33.38	33.41	33.44	33.48	33.54	33.60	23.06	23.07	23.09	23.12	23.15	23.20
R_S	135.3	126.8	116.4	104.6	92.37	80.53	79.76	75.26	69.96	64.25	58.68	53.71
I_S	1.07	1.15	1.25	1.39	1.58	1.82	2.72	2.89	3.11	3.40	3.73	4.09

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.81	8.90	9.02	9.16	9.32	9.50	9.43	9.59	9.77	9.96	10.17	10.39
m_S	14.29	15.04	15.88	16.73	17.47	18.07	17.95	18.40	18.76	19.04	19.25	19.42
R_E	5.23	4.97	4.70	4.47	4.27	4.13	4.16	4.06	3.98	3.92	3.88	3.85
t	24.41	24.46	24.52	24.62	24.77	25.01	30.96	31.27	31.79	32.69	34.30	37.52
R_S	52.45	49.82	47.16	44.76	42.81	41.34	37.32	36.37	35.59	34.97	34.43	33.87
I_S	3.98	4.19	4.43	4.66	4.86	5.01	4.45	4.54	4.58	4.57	4.48	4.26

460 $C_{15}H_{22}N_2O_4$ Leu-Tyr -18.2 7.828 294.3
-36.4 10.065

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.60	7.64	7.71	7.79	7.90	8.01	8.12	8.20	8.30	8.43	8.58	8.74
m_S	6.44	6.88	7.50	8.29	9.26	10.36	11.22	11.84	12.61	13.46	14.34	15.20
R_E	11.61	10.85	9.96	9.01	8.07	7.21	6.66	6.31	5.93	5.55	5.21	4.92
t	38.59	38.65	38.72	38.82	38.95	39.10	26.24	26.31	26.43	26.61	26.87	27.25
R_S	97.90	91.53	84.05	76.00	68.05	60.81	68.44	64.83	60.91	57.04	53.54	50.50
I_S	1.58	1.69	1.84	2.05	2.29	2.58	3.48	3.68	3.93	4.22	4.52	4.82

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.67	8.81	8.97	9.14	9.32	9.50	9.44	9.60	9.76	9.93	10.10	10.29
m_S	14.87	15.54	16.30	17.15	18.14	19.31	19.03	20.15	21.42	22.82	24.26	25.66
R_E	5.02	4.81	4.58	4.35	4.12	3.87	3.92	3.71	3.49	3.27	3.08	2.91
t	28.55	28.92	29.47	30.25	31.29	32.63	39.61	41.00	42.56	44.36	46.43	49.17
R_S	50.41	48.20	45.95	43.65	41.24	38.69	35.20	33.22	31.18	29.18	27.32	25.63
I_S	4.55	4.79	5.06	5.37	5.74	6.17	5.45	5.83	6.28	6.74	7.19	7.53

461 $C_{11}H_{22}N_2O_3$ Leu-Val -22.3 8.364 230.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	7.87	7.92	7.99	8.07	8.17	8.33	8.38	8.45	8.55	8.66	8.79
m_S	4.97	5.30	5.77	6.41	7.25	8.30	10.15	10.75	11.55	12.55	13.71	14.92
R_E	15.02	14.09	12.95	11.65	10.30	9.00	7.36	6.95	6.47	5.95	5.45	5.01
t	32.84	32.86	32.89	32.93	32.98	33.04	22.73	22.74	22.76	22.79	22.82	22.86
R_S	126.7	118.8	109.2	98.30	86.91	75.91	75.61	71.42	66.48	61.17	56.01	51.43
I_S	1.14	1.22	1.32	1.47	1.67	1.92	2.84	3.02	3.25	3.54	3.88	4.24

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-------------------------------------------------------------------------------
 No.   44    45    46    47    48    49    50    51    52    53    54    55
 pHL  8.20  8.40  8.60  8.80  9.00  9.20  9.00  9.20  9.40  9.60  9.80 10.00
 pHS  8.79  8.88  9.00  9.15  9.31  9.49  9.41  9.57  9.76  9.95 10.16 10.38
 mS  14.89 15.65 16.49 17.32 18.05 18.62 18.50 18.93 19.28 19.54 19.73 19.90
 RE   5.02  4.77  4.53  4.31  4.14  4.01  4.04  3.95  3.87  3.82  3.78  3.75
 t   24.05 24.08 24.14 24.24 24.38 24.60 30.40 30.70 31.19 32.02 33.51 36.44
 RS  50.33 47.89 45.43 43.22 41.45 40.12 36.22 35.35 34.64 34.08 33.58 33.06
 IS   4.11  4.32  4.56  4.79  4.98  5.12  4.56  4.64  4.68  4.67  4.58  4.37
_______________________________________________________________________________
 462   C6H14N2O2     Lysine
_______________________________________________________________________________
 463   C5H11NO2S     Methionine                   -30.4    9.376   149.2
 No.   32    33    34    35    36    37    38    39    40    41    42    43
 pHL  6.40  6.60  6.80  7.00  7.20  7.40  7.40  7.60  7.80  8.00  8.20  8.40
 pHS  7.84  8.35  8.39  8.44  8.50  8.57  8.80  8.83  8.87  8.93  9.00  9.09
 mS   0.00  2.61  2.82  3.12  3.53  4.10  6.27  6.63  7.16  7.90  8.89 10.16
 RE   0.00 28.67 26.53 23.97 21.13 18.23 11.92 11.27 10.43  9.46  8.41  7.35
 t    0.00 26.67 26.68 26.69 26.71 26.73 19.33 19.34 19.34 19.35 19.37 19.40
 RS  126.7 241.9 223.8 202.2 178.3 153.8 122.6 115.8 107.2 97.22 86.38 75.57
 IS   1.14  0.53  0.57  0.63  0.72  0.83  1.46  1.55  1.68  1.85  2.09  2.39
-------------------------------------------------------------------------------
 No.   44    45    46    47    48    49    50    51    52    53    54    55
 pHL  8.20  8.40  8.60  8.80  9.00  9.20  9.00  9.20  9.40  9.60  9.80 10.00
 pHS  9.19  9.24  9.30  9.38  9.48  9.61  9.65  9.73  9.84  9.97 10.13 10.30
 mS  11.62 12.37 13.39 14.72 16.32 18.14 18.74 19.86 21.18 22.60 23.95 25.11
 RE   6.43  6.04  5.58  5.08  4.58  4.12  3.99  3.76  3.53  3.31  3.12  2.97
 t   20.28 20.30 20.34 20.39 20.47 20.60 24.92 25.06 25.29 25.66 26.29 27.38
 RS  64.48 60.58 55.94 50.88 45.83 41.19 35.76 33.70 31.53 29.47 27.67 26.19
 IS   2.70  2.87  3.11  3.43  3.81  4.24  4.03  4.27  4.54  4.82  5.06  5.20
_______________________________________________________________________________
 464   C5H12N2O2     Ornithine                     28.4    8.712   132.2
                                                  -28.4   10.755
 No.   32    33    34    35    36    37    38    39    40    41    42    43
 pHL  6.40  6.60  6.80  7.00  7.20  7.40  7.40  7.60  7.80  8.00  8.20  8.40
 pHS  7.84  8.35  8.39  8.44  8.50  8.57  8.80  8.83  8.87  8.93  9.00  9.09
 mS   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
 RE   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
 t    0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
 RS   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
 IS   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
-------------------------------------------------------------------------------
 No.   44    45    46    47    48    49    50    51    52    53    54    55
 pHL  8.20  8.40  8.60  8.80  9.00  9.20  9.00  9.20  9.40  9.60  9.80 10.00
 pHS  9.19  9.24  9.30  9.38  9.48 10.32  9.65  9.73 10.56 10.64 10.74 10.90
 mS   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00 10.50 11.69 13.36 15.90
 RE   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  7.12  6.39  5.59  4.70
 t    0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00 56.00 60.38 70.55 112.2
 RS   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00 63.62 56.95 49.60 41.37
 IS   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  1.43  1.53  1.62  1.60
_______________________________________________________________________________
 465   C9H11NO2      Phenylalanine                -26.9    9.262   165.2
 No.   32    33    34    35    36    37    38    39    40    41    42    43
 pHL  6.40  6.60  6.80  7.00  7.20  7.40  7.40  7.60  7.80  8.00  8.20  8.40
 pHS  7.84  8.30  8.34  8.39  8.46  8.53  8.75  8.78  8.83  8.89  8.97  9.06
 mS   0.00  2.62  2.85  3.16  3.60  4.18  6.16  6.54  7.07  7.81  8.79 10.02
 RE   0.00 28.47 26.25 23.62 20.76 17.88 12.12 11.43 10.56  9.56  8.50  7.45
 t    0.00 28.89 28.90 28.92 28.95 28.98 20.59 20.60 20.61 20.63 20.65 20.69
 RS  126.7 240.2 221.4 199.3 175.1 150.8 124.6 117.5 108.6 98.30 87.34 76.57
 IS   1.14  0.56  0.60  0.67  0.76  0.89  1.53  1.63  1.77  1.96  2.21  2.52
-------------------------------------------------------------------------------
 No.   44    45    46    47    48    49    50    51    52    53    54    55
 pHL  8.20  8.40  8.60  8.80  9.00  9.20  9.00  9.20  9.40  9.60  9.80 10.00
 pHS  9.14  9.20  9.27  9.36  9.47  9.60  9.62  9.71  9.83  9.98 10.15 10.33
 mS  11.18 11.91 12.90 14.14 15.61 17.20 17.52 18.53 19.67 20.82 21.86 22.72
 RE   6.68  6.27  5.79  5.28  4.79  4.34  4.26  4.03  3.80  3.59  3.42  3.29
 t   21.69 21.72 21.77 21.84 21.94 22.10 27.00 27.19 27.50 28.02 28.90 30.48
 RS  67.04 62.90 58.08 52.94 47.93 43.45 38.23 36.11 33.95 31.98 30.32 28.95
 IS   2.76  2.94  3.19  3.51  3.87  4.27  3.96  4.18  4.42  4.64  4.80  4.84
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466 $C_5H_9O_2N$ Proline -29.0 10.640 115.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.84	8.30	8.34	8.39	8.46	8.53	8.75	8.78	8.83	8.89	9.67	9.74
m_S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.78	3.21
R_E	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	26.92	23.28
t	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	27.32	27.50
R_S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	276.6	239.2
I_S	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.52	0.60

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.89	9.92	9.96	10.02	10.09	10.18	10.33	10.37	10.43	10.51	10.62	10.75
m_S	4.33	4.60	4.99	5.55	6.31	7.31	9.30	9.93	10.83	12.06	13.67	15.74
R_E	17.26	16.25	14.97	13.47	11.84	10.22	8.03	7.52	6.89	6.19	5.46	4.74
t	29.12	29.26	29.48	29.81	30.30	31.04	38.51	39.30	40.59	42.75	46.67	55.15
R_S	173.2	163.0	150.1	135.0	118.6	102.2	72.05	67.35	61.64	55.21	48.48	41.78
I_S	0.79	0.83	0.90	1.00	1.13	1.29	1.52	1.61	1.72	1.87	2.03	2.15

467 $C_3H_7NO_3$ Serine -34.3 9.329 105.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.29	8.32	8.36	8.40	8.46	8.54	8.77	8.80	8.84	8.90	8.97	9.06
m_S	2.90	3.06	3.29	3.63	4.10	4.74	7.35	7.76	8.35	9.19	10.31	11.76
R_E	25.78	24.44	22.69	20.58	18.21	15.76	10.17	9.63	8.94	8.13	7.24	6.35
t	24.64	24.65	24.65	24.66	24.67	24.69	18.14	18.15	18.15	18.16	18.17	18.18
R_S	217.5	206.1	191.4	173.6	153.6	133.0	104.5	98.96	91.90	83.54	74.44	65.29
I_S	0.56	0.59	0.64	0.70	0.80	0.92	1.61	1.71	1.84	2.03	2.28	2.61

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.16	9.21	9.27	9.35	9.45	9.57	9.61	9.69	9.80	9.93	10.08	10.26
m_S	13.50	14.33	15.47	16.96	18.77	20.80	21.45	22.68	24.15	25.72	27.21	28.50
R_E	5.53	5.21	4.83	4.40	3.98	3.59	3.48	3.29	3.09	2.90	2.74	2.62
t	18.96	18.97	18.99	19.03	19.09	19.18	22.96	23.06	23.23	23.50	23.94	24.70
R_S	55.52	52.28	48.40	44.14	39.86	35.91	31.23	29.50	27.65	25.89	24.35	23.08
I_S	2.96	3.14	3.40	3.73	4.14	4.60	4.41	4.67	4.96	5.27	5.54	5.73

468 $C_9H_{17}N_3O_7$ Ser-Ser-Ser -22.0 7.385 279.3

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	7.38	7.44	7.51	7.60	7.72	7.86	7.94	8.04	8.16	8.31	8.48	8.66
m_S	10.39	11.01	11.83	12.84	13.99	15.19	15.69	16.33	17.02	17.68	18.23	18.65
R_E	7.19	6.79	6.32	5.82	5.34	4.92	4.76	4.57	4.39	4.23	4.10	4.01
t	33.41	33.44	33.48	33.52	33.58	33.63	22.93	22.94	22.96	22.98	23.00	23.04
R_S	60.62	57.23	53.28	49.07	45.03	41.49	48.93	47.03	45.11	43.43	42.10	41.15
I_S	2.43	2.58	2.78	3.02	3.30	3.59	4.51	4.70	4.91	5.11	5.28	5.40

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	8.54	8.70	8.88	9.07	9.26	9.46	9.34	9.53	9.73	9.94	10.15	10.38
m_S	18.40	18.74	19.00	19.19	19.32	19.41	19.47	19.55	19.60	19.65	19.70	19.77
R_E	4.06	3.99	3.93	3.89	3.87	3.85	3.84	3.82	3.81	3.80	3.79	3.78
t	24.22	24.26	24.31	24.40	24.54	24.77	30.61	30.91	31.42	32.29	33.83	36.89
R_S	40.72	39.98	39.42	39.01	38.72	38.49	34.41	34.23	34.07	33.89	33.64	33.28
I_S	5.18	5.27	5.34	5.38	5.40	5.39	4.85	4.84	4.80	4.73	4.60	4.36

469 $C_2H_7NO_3S$ Taurine -37.9 9.182 125.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.22	8.24	8.28	8.32	8.38	8.46	8.69	8.73	8.77	8.83	8.90	8.99
m_S	3.71	3.91	4.20	4.61	5.19	5.98	9.23	9.73	10.45	11.45	12.80	14.51
R_E	20.12	19.11	17.80	16.19	14.38	12.49	8.09	7.68	7.15	6.52	5.84	5.15
t	23.12	23.13	23.13	23.14	23.14	23.16	17.24	17.24	17.24	17.24	17.25	17.26
R_S	169.7	161.2	150.1	136.6	121.3	105.4	83.20	78.94	73.50	67.04	59.98	52.88
I_S	0.69	0.73	0.78	0.86	0.97	1.12	1.93	2.04	2.19	2.41	2.70	3.07

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.09	9.13	9.20	9.28	9.39	9.52	9.54	9.63	9.74	9.88	10.04	10.21
m_S	16.45	17.41	18.73	20.42	22.44	24.66	25.12	26.45	28.00	29.59	31.05	32.26
R_E	4.54	4.29	3.99	3.66	3.33	3.03	2.97	2.82	2.67	2.52	2.41	2.32
t	17.94	17.95	17.97	18.00	18.04	18.10	21.48	21.56	21.68	21.89	22.22	22.79
R_S	45.56	43.02	39.99	36.66	33.33	30.30	26.67	25.30	23.86	22.50	21.34	20.39
I_S	3.44	3.64	3.93	4.29	4.73	5.20	4.98	5.24	5.55	5.86	6.12	6.31

470 $C_4H_9NO_3$ Threonine -31.6 9.229 119.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.25	8.27	8.31	8.36	8.42	8.50	8.72	8.76	8.80	8.86	8.94	9.03
m_S	2.97	3.14	3.39	3.75	4.25	4.91	7.41	7.84	8.45	9.30	10.43	11.87
R_E	25.12	23.76	22.01	19.91	17.59	15.21	10.08	9.53	8.84	8.03	7.16	6.29
t	25.92	25.92	25.93	25.94	25.96	25.98	18.86	18.86	18.87	18.88	18.89	18.91
R_S	211.8	200.4	185.6	168.0	148.4	128.3	103.6	97.96	90.86	82.55	73.59	64.67
I_S	0.60	0.63	0.68	0.75	0.85	0.99	1.71	1.81	1.95	2.15	2.42	2.76

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.12	9.17	9.23	9.32	9.43	9.56	9.58	9.67	9.79	9.93	10.09	10.27
m_S	13.35	14.18	15.31	16.74	18.44	20.30	20.69	21.84	23.16	24.51	25.74	26.75
R_E	5.59	5.27	4.88	4.46	4.05	3.68	3.61	3.42	3.22	3.05	2.90	2.79
t	19.75	19.77	19.80	19.84	19.91	20.01	24.12	24.25	24.45	24.77	25.32	26.27
R_S	56.13	52.83	48.93	44.72	40.56	36.80	32.38	30.64	28.84	27.17	25.75	24.59
I_S	3.06	3.26	3.52	3.86	4.26	4.69	4.42	4.66	4.93	5.20	5.41	5.53

471 $C_{11}H_{12}N_2O_2$ Tryptophan -26.8 9.635 204.2

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.25	8.27	8.31	8.36	8.42	8.71	8.93	8.97	9.01	9.07	9.14	9.22
m_S	0.00	0.00	0.00	0.00	0.00	2.84	4.38	4.65	5.04	5.58	6.32	7.28
R_E	0.00	0.00	0.00	0.00	0.00	26.30	17.04	16.06	14.82	13.38	11.82	10.26
t	0.00	0.00	0.00	0.00	0.00	29.46	21.01	21.02	21.04	21.06	21.09	21.14
R_S	0.00	0.00	0.00	0.00	0.00	221.9	175.2	165.1	152.3	137.5	121.4	105.5
I_S	0.00	0.00	0.00	0.00	0.00	0.60	1.07	1.14	1.23	1.37	1.55	1.79

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.32	9.37	9.43	9.51	9.60	9.71	9.77	9.85	9.95	10.07	10.22	10.39
m_S	8.53	9.11	9.92	10.99	12.32	13.89	14.75	15.73	16.95	18.32	19.73	21.05
R_E	8.76	8.20	7.53	6.80	6.06	5.38	5.06	4.75	4.41	4.08	3.78	3.55
t	22.18	22.22	22.28	22.37	22.50	22.70	27.78	28.00	28.36	28.95	29.94	31.73
R_S	87.86	82.23	75.51	68.15	60.72	53.80	45.42	42.53	39.40	36.34	33.58	31.24
I_S	2.06	2.20	2.40	2.66	2.98	3.36	3.25	3.46	3.71	3.98	4.22	4.36

472 $C_9H_{11}NO_3$ Tyrosine -20.7 9.165 181.2
-42.0 10.207

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.25	8.27	8.31	8.37	8.44	8.52	8.71	8.75	8.80	8.87	8.95	9.05
m_S	0.00	0.00	2.56	2.88	3.30	3.87	5.51	5.90	6.44	7.20	8.20	9.48
R_E	0.00	0.00	29.16	25.97	22.60	19.29	13.55	12.67	11.59	10.38	9.11	7.88
t	0.00	0.00	35.12	35.20	35.30	35.44	24.69	24.77	24.88	25.04	25.27	25.59
R_S	0.00	0.00	246.0	219.1	190.7	162.7	139.2	130.2	119.1	106.6	93.61	81.00
I_S	0.00	0.00	0.59	0.67	0.77	0.90	1.56	1.67	1.83	2.06	2.35	2.74

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.12	9.17	9.25	9.34	9.46	9.59	9.61	9.70	9.82	9.95	10.10	10.27
m_S	10.47	11.30	12.43	13.89	15.68	17.76	18.20	19.69	21.52	23.58	25.74	27.86
R_E	7.13	6.61	6.01	5.38	4.76	4.20	4.10	3.79	3.47	3.17	2.90	2.68
t	27.14	27.38	27.74	28.25	28.94	29.85	36.48	37.28	38.40	39.76	41.40	43.55
R_S	71.57	66.30	60.26	53.90	47.70	42.06	36.82	33.98	31.03	28.24	25.75	23.61
I_S	2.96	3.21	3.55	4.00	4.55	5.20	4.87	5.31	5.85	6.47	7.11	7.69

473 $C_5H_{11}NO_2$ Valine -28.4 9.710 117.1

No.	32	33	34	35	36	37	38	39	40	41	42	43
pH_L	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pH_S	8.25	8.27	8.31	8.37	8.44	8.74	8.97	9.00	9.04	9.10	9.17	9.25
m_S	0.00	0.00	0.00	0.00	0.00	2.75	4.32	4.58	4.95	5.48	6.20	7.15
R_E	0.00	0.00	0.00	0.00	0.00	27.20	17.31	16.33	15.08	13.63	12.04	10.45
t	0.00	0.00	0.00	0.00	0.00	28.41	20.44	20.45	20.46	20.48	20.51	20.55
R_S	0.00	0.00	0.00	0.00	0.00	229.4	177.9	167.8	155.0	140.0	123.8	107.4
I_S	0.00	0.00	0.00	0.00	0.00	0.56	1.02	1.08	1.17	1.30	1.47	1.70

No.	44	45	46	47	48	49	50	51	52	53	54	55
pH_L	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pH_S	9.36	9.40	9.46	9.53	9.62	9.73	9.80	9.87	9.96	10.08	10.22	10.38
m_S	8.50	9.07	9.87	10.94	12.29	13.91	14.95	15.96	17.23	18.70	20.26	21.76
R_E	8.79	8.23	7.57	6.83	6.08	5.37	5.00	4.68	4.34	3.99	3.69	3.43
t	21.54	21.58	21.63	21.71	21.83	22.01	26.81	27.01	27.33	27.84	28.70	30.22
R_S	88.18	82.59	75.87	68.43	60.85	53.71	44.81	41.93	38.77	35.61	32.72	30.23
I_S	1.99	2.12	2.31	2.56	2.88	3.26	3.22	3.43	3.68	3.97	4.24	4.44

474	C9H9I2NO3		I2-Tyr					-21.0 -42.0		6.500 9.690	433.0	
No.	32	33	34	35	36	37	38	39	40	41	42	43
pHL	6.40	6.60	6.80	7.00	7.20	7.40	7.40	7.60	7.80	8.00	8.20	8.40
pHS	7.04	7.13	7.26	7.41	7.58	7.76	7.74	7.90	8.08	8.27	8.46	8.65
mS	14.97	15.62	16.33	17.02	17.62	18.13	17.91	18.27	18.64	19.05	19.54	20.19
RE	4.99	4.78	4.57	4.39	4.24	4.12	4.17	4.09	4.01	3.92	3.82	3.70
t	34.91	34.95	35.01	35.08	35.17	35.29	23.92	24.05	24.25	24.56	25.01	25.64
RS	42.08	40.34	38.59	37.02	35.75	34.76	42.88	42.02	41.18	40.30	39.27	38.00
IS	3.60	3.76	3.93	4.11	4.27	4.40	5.32	5.44	5.57	5.71	5.89	6.13
No.	44	45	46	47	48	49	50	51	52	53	54	55
pHL	8.20	8.40	8.60	8.80	9.00	9.20	9.00	9.20	9.40	9.60	9.80	10.00
pHS	8.53	8.71	8.89	9.07	9.25	9.43	9.36	9.51	9.67	9.84	10.02	10.21
mS	19.77	20.44	21.33	22.46	23.85	25.43	24.95	26.35	27.86	29.37	30.77	31.98
RE	3.78	3.65	3.50	3.33	3.13	2.94	2.99	2.83	2.68	2.54	2.43	2.33
t	26.47	27.13	27.98	29.02	30.22	31.46	37.44	38.56	39.69	40.94	42.32	44.06
RS	37.91	36.66	35.12	33.33	31.36	29.37	26.86	25.39	23.97	22.67	21.53	20.57
IS	5.81	6.05	6.38	6.79	7.29	7.85	7.03	7.51	8.04	8.54	8.96	9.25

EXAMPLE OF APPLICATION

Fig. 1 shows the pH dependence of the effective mobility of polyglycines, butyric acid and carbonic acid at infinite dilution (ionic strength = 0) The curves were plotted using the values shown in Table V. The curves are useful for the assessment of separability to a first approximation.

Fig. 2. shows the simulated R_E *vs.* pH_L curves. The values were taken from Table V. It was expected from Fig. 2 that the separation of these samples might be possible in the pH range 8.3–9 (pH buffer = Tris or amediol).

Fig. 3 shows the simulated and the observed isotachopherograms at pH 8.4 (pH buffer = Tris). Carbonic acid was contained in the terminating electrolyte. Good agreement was obtained, suggesting the utility of the simulation.

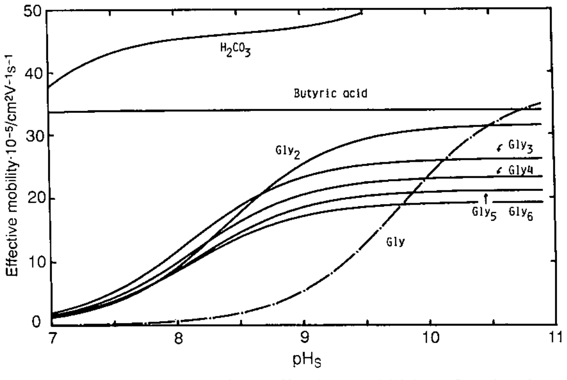

Fig. 1. pH dependence of the effective mobilities of carbonic acid (H_2CO_3), butyric acid, diglycine (Gly_2), triglycine (Gly_3), tetraglycine (Gly_4), pentaglycine (Gly_5), hexaglycine (Gly_6) and glycine (Gly). The curves are not for the isotachophoretic steady state. pH_S = pH of samples.

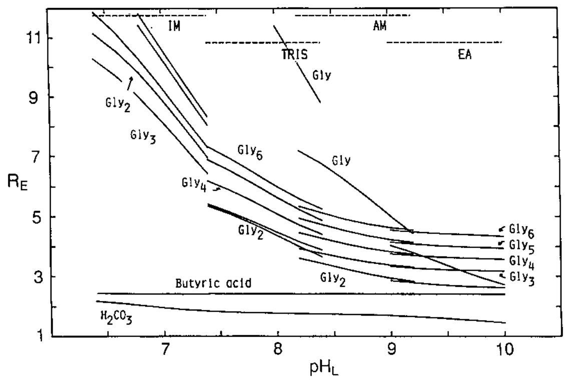

Fig. 2. pH dependence of the R_E values simulated for the samples shown in Fig. 1. pH buffers: IM = imidazole; Tris = tris(hydroxymethyl)aminomethane (Tris); AM = amediol; EA = ethanolamine.

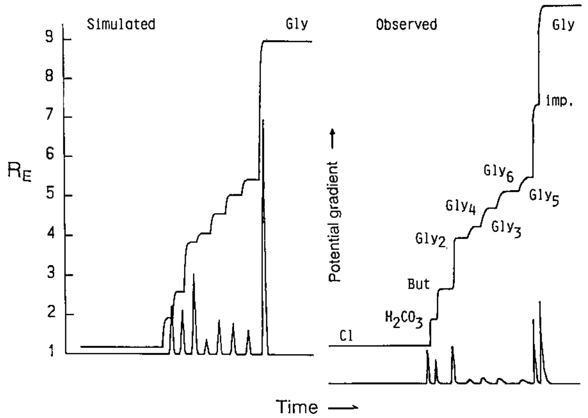

Fig. 3. Simulated and observed isotachopherograms for the samples in Figs. 1 and 2 at pH_L = 8.4 (Tris buffer). The terminator was glycine. The leading electrolyte contained 0.1% of hydroxypropylcellulose.

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Separation of polymyxins by micellar electrokinetic capillary chromatography

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ABSTRACT

High-efficiency separations of basic polypeptides from the polymyxin group with equal or nearly equal mass to charge ratios were achieved by micellar electrokinetic chromatography in dynamically modified capillaries using a zwitterionic surfactant as an additive to the running buffer. The peptides are probably separated on the basis of differences in their partition coefficients to the surface of the pseudo-phase ion exchanger and to the lipophilic core of the micelles. Samples of polymyxins B, D and E (colistin) from different sources were examined.

INTRODUCTION

Polymyxins (Fig. 1) are a group of closely related decapeptide antibiotics produced by strains of *Bacillus polymyxa*. They have a general structure composed of a cyclic heptapeptide moiety and a side-chain consisting of a tripeptide acylated at the N-terminus. The polymyxins differ from each other in amino acid composition and each polymyxin consists of a mixture of polypeptides with an identical amino acid composition but with a different terminating fatty acid. Thus, each polymyxin may be subdivided corresponding to at least three fatty acid components: 6-methyloctanoic acid, 6-methylheptanoic acid and *n*-octanoic acid. The characteristic feature of polymyxins, in addition to the hydrophobic fatty acid moiety, is their strong basicity due to four or five unmasked amino groups from 2,4-diaminobutyric acid. The peptides in each group of the polymyxins (A, B, D and E) have equal or nearly equal mass to charge ratios and therefore exhibit very similar electrophoretic mobilities.

The complex composition of the polymyxins has earlier been demonstrated by HPLC [1,2] and TLC [3], and a difference in the potency of polymyxin B_1 and B_2 fractions has been demonstrated by microbiological assays [4]. The fatty acids present in polymyxins have been measured by GC after hydrolysis [5]. Samples of polymyxins from different sources have been examined for potency and composition by use of microbiological and chemical assays [6]. Complete separation of the peptides has not been achieved with any of the above-mentioned methods. However, in order to identify these antibiotics, to examine their purity and to determine the relative contents of their components, a complete separation of the peptide will be very useful.

About 10 years ago, capillary electrophoresis (CE) was shown to be very suitable for separating complex peptide mixtures [7], especially when problems with adsorption to the capillary wall were taken into consideration [8]. The affinity of peptides toward the capillary wall may be reduced by appropriate pH adjustment [9,10], by the action of surface modifiers [11] and by the addition of surfactants to

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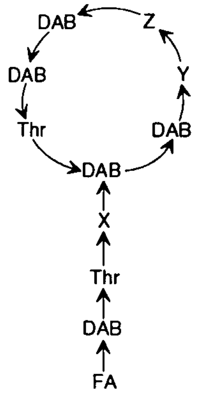

Polymyxins	X	Y	Z	FA	m/z
A_1	DAB	Leu	Thr	6-MOA	231
A_2	DAB	Leu	Thr	6-MHA	
B_1	DAB	Phe	Leu	6-MOA	241
B_2	DAB	Phe	Leu	6-MHA	
B_3	DAB	Phe	Leu	n-OA	
D_1	Ser	Leu	Thr	6-MOA	286
D_2	Ser	Leu	Thr	6-MHA	
E_1	DAB	Leu	Leu	6-MOA	234
E_2	DAB	Leu	Leu	6-MHA	
E_3	DAB	Leu	Leu	n-OA	

DAB = 2,4-diaminobutyric acid

6-MOA = 6-methyloctanoyl; 6-MHA = 6-methylheptanoyl; n-OA = n-octanoyl

Fig. 1. Structure of the polymyxins.

the running buffer [12]. Further, additions of surfactants above their critical micelle concentration (CMC) also generate the possibility of an additional separation mechanism, micellar electrokinetic capillary chromatography, which can be beneficial in separating substances with similar electrophoretic mobilities. However, high concentrations of these surfactants are often necessary and, owing to the high ionic strength of the buffer, only lower voltages can then be applied. This problem has been overcome by the use of non-ionic or zwitterionic surfactants [13], which have no net charge and, therefore, do not contribute to the conductivity of the buffer.

Recently, it was shown that 3-(N,N-dimethylhexadecylammonium)propanesulphonate (PAPS) dynamically modifies the silica surface of the capillary as well as it forms micelles [14]. High-efficiency separations of polymyxin B were achieved using PAPS as an additive to the running buffer.

In this study, micellar electrokinetic capillary chromatography was used to separate polymyxins A, B, D and E and to examine the composition of samples of polymyxins from different sources.

EXPERIMENTAL

Chemicals

3-(N,N-Dimethylhexadecylammonium)propanesulphonate (PAPS), octanoic acid and Dowex 50W-X8 (100–200 mesh) were obtained from Fluka (Buchs, Switzerland). Sudan III was purchased from Merck (Darmstadt, Germany) and methanesulphonic acid from Sigma (St. Louis, MO, USA). Diazomethane solution was prepared by mixing 35 ml of diethylene glycol monoethyl ether (Fluka), 20 ml of diethyl ether and a solution of potassium hydroxide (6 g) in water (10 ml). During the subsequent distillation of the mixture, a solution of 21.5 g of N-methyl-N-nitroso-*p*-toluenesulphonamide (Aldrich, Steinheim, Germany) in about 200 ml of diethyl ether was added.

Polymyxins A, B, D and E sulphate, 6-methylheptanoic acid and 6-methyloctanoic acid were kindly provided by Dumex (Copenhagen, Denmark). Polymyxin B sulphate was also obtained from Pfizer (Karlsruhe, Germany) and polymyxin E from Smit (Turin, Italy), Sumitomo Shoji Kaisha (Tokyo, Japan) and Warner-Lambert Research Institute (Morris Plains, NJ, USA).

CE apparatus

A Waters Quanta 4000 capillary electrophoresis system (Millipore, Milford, MA, USA) was used and on-column detection was performed by UV absorption at 214 nm. Electropherograms were recorded on a DP 700 data processor (Carlo Erba, Valencia, CA, USA).

A fused-silica capillary of 75 μm I.D. and 360 μm O.D. was obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillaries used was 60 cm and 52.4 cm to the detector.

Procedure

The fused-silica column was treated with 0.1 *M* sodium hydroxide solution for 30 min and subsequently with distilled water for 10 min before introducing the electrophoresis buffer for 10 min. When changing buffer composition, the capillary was rinsed for 10 min with 0.1 *M* sodium hydroxide and subsequently with distilled water for 5 min before introducing the new buffer.

For the preparation of the running buffer, 0.2 *M* sodium phosphate stock solution (pH 2.5) was used. The buffer system was composed of appropriate amounts of PAPS, buffer stock solution and distilled water without further readjustment of pH.

Sudan III in methanol (0.01%) was used as an indicator for the migration of the micelles.

Polymyxins A, B, D and E sulphate were dissolved in distilled water to a concentration of 0.5, 1.0 or 5.0 m*M* and kept refrigerated when not in use.

Sample injection was accomplished by hydrostatic injection (9.8 cm) for 5 or 10 s. All analyses were performed using an applied potential of 15 kV (90 μA).

Preparative HPLC

The chromatographic system consisted of a Merck Hitachi Model 655-A12 liquid chromatograph equipped with a Model 655 variable-wavelength UV monitor operated at 220 nm and a Rheodyne (Berkeley, CA, USA) Model 7120 injector with a 1.5-ml loop. The column (250 × 8 mm I.D.) was packed with Spherisorb ODS 2 (5 μm).

The experiments were carried out at ambient temperature using methanol–methanesulphonic acid–water (49.5:1.0:49.5, v/v/v) as the eluent [2] at a flow-rate of 3 ml/min. Polymyxin B or E sulphate was dissolved in the eluent to a concentration of 100 mg/ml and kept at about 4°C when not in use. A total amount of 4 mg of polymyxin B or E was injected each time and six major peaks were collected.

A sample from each isolated compound was injected into the capillary electrophoresis system in order to identify the peaks obtained in the electropherograms of polymyxin B and E.

The pooled fractions of each compound were, adjusted both before and after evaporation to half the volume, to neutral pH with sodium hydroxide solution and subsequently transferred on to a 5 mm I.D. cation-exchange column packed with 2 g of Dowex 50W-X8. The column was then washed with 3 ml of distilled water and the peptide was eluted from the column using 7 ml of 2 *M* ammonia solution followed by 20 ml of distilled water. The ion-exchange column was regenerated with 3 ml of 4 *M* hydrochloric acid followed by 7 ml of distilled water.

The collected fraction was evaporated on a rotary evaporator for 15 min at ambient temperature to remove ammonia and then freeze-dried.

GC–MS

A few milligrams of the freeze-dried residue were hydrolysed with 1 ml of 6 *M* hydrochloric acid at 110°C for 20 h. The hydrolysate was extracted with three 2-ml portions of diethyl ether and the ethereal extract was dried over anhydrous sodium sulphate. With a slow stream of nitrogen at 25°C, the dried extract was evaporated to a final concentration of 10–20 ng/μl.

Fatty acid samples and standard solutions were derivatized with diazomethane solution. The methyl esters produced were analysed by injection on to a 30 m × 0.26 mm I.D. fused-silica DB-5 column (J&W, Folsom, CA, USA) with a film thickness of 0.25 μm and coupled to a Finnigan MAT (Bremen, Germany) Model 4515 B mass spectrometer operated in the electron impact ionization mode at 70 eV. The GC oven temperature was held at 35°C for 2 min, then increased linearly at 12°C/min to 260°C, where it was maintained for 30 min.

RESULTS AND DISCUSSION

Separation of polymyxins B_1, B_2 and B_3

The polymyxins B are a group of peptides having equal or nearly equal mass-to-charge ratios and therefore they exhibit very similar electrophoretic mobilities. Recently, the separation mechanisms involved in CE with PAPS was studied [14] and the ability of PAPS to separate these almost identical peptides was shown.

Fig. 2. shows the electropherograms of polymyxin B with no and with 30 m*M* of PAPS added to the running buffer. No separation of the peptides was obtained in plain buffer whereas the addition of 30 m*M* PAPS provided a significant increase in the separation of the peptides, resulting in at least ten peaks.

Based on a comparison of the electrophoretic mobilities of the peptides with those of the isolated fractions and taking the identified free fatty acids into account, the following can be concluded: the peak labelled 3 in Fig. 2b corresponds to polymyxin B_1 with the 6-methyloctanoyl residue, peak 1 was identified as polymyxin B_2 with the 6-methylheptanoyl residue and peak 2 appears to be due to polymyxin B_3 with an *n*-octanoyl residue.

The electrophoretic mobilities of polymyxins B_1 and B_2 were calculated based on peaks 3 and 1, respectively, and plotted against the concentration of PAPS (Fig. 3). With increasing concentration of PAPS, the electrophoretic mobility of the two peptides decreases, probably owing to increased interaction with the micellar pseudo-phase, the concentration of which increases proportionally to that of PAPS. Simultaneously, the relative mobility of the two peptides increases.

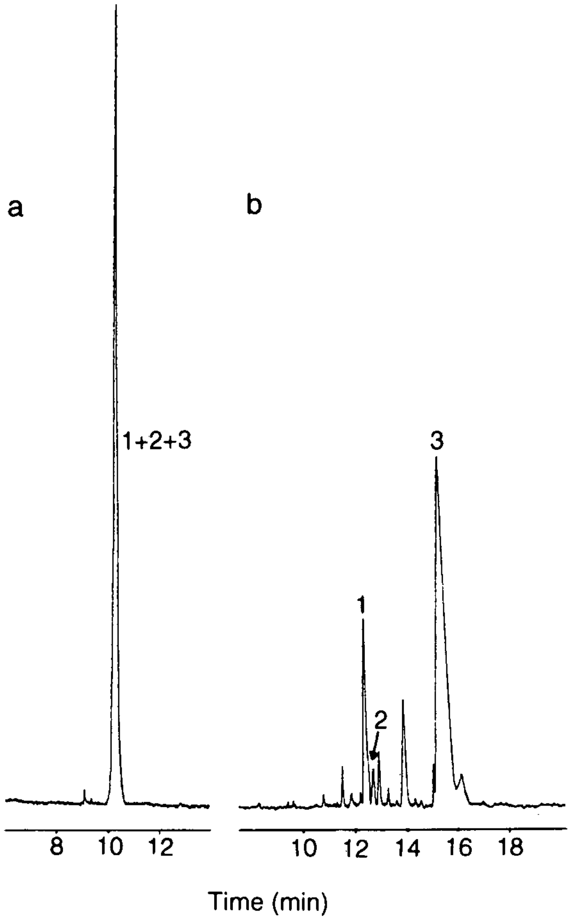

Fig. 2. Electropherograms of polymyxin B (0.5 m*M* in water) obtained (a) with no PAPS and (b) with 30 m*M* PAPS added to the running buffer. Capillary, 75 μm I.D.; buffer, 0.2 *M* sodium phosphate (pH 2.5)–water (1:1, v/v); voltage, 15 kV; hydrostatic injection, 10 s. Peaks: 1 = polymyxin B_2; 2 = polymyxin B_3; 3 = polymyxin B_1.

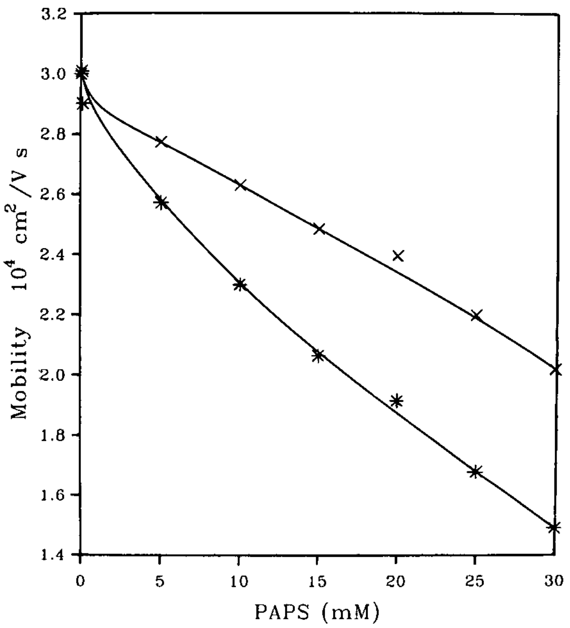

Fig. 3. Electrophoretic mobility of (∗) polymyxin B_1 and (×) polymyxin B_2 *versus* the concentration of PAPS in the running buffer. Conditions as in Fig. 2.

Separation of polymyxins A, B, D and E (colistin)

Polymyxin A differs from polymyxin B in the substitution of Phe and Leu with Leu and Thr, respectively. Polymyxin D differs from the other polymyxins investigated because a DAB group is replaced by Ser. In consequence, the polymyxins D have a higher mass to charge ratio than the other polymyxins included in this study. Fig. 4a and b show the electropherograms of polymyxins A and D, each being separated into two main peaks. A tentative assignment of the peaks has been made.

Fig. 4c shows the electropherogram of the polymyxins E (colistin), which differ from polymyxin B only in the substitution of a single amino acid. The assignments of the main peaks, E_1 (colistin A) with 6-methyloctanoyl, E_2 (colistin B) with 6-methylheptanoyl and E_3 with the *n*-octanoyl residue, were based on a comparison of the electrophoretic mo-

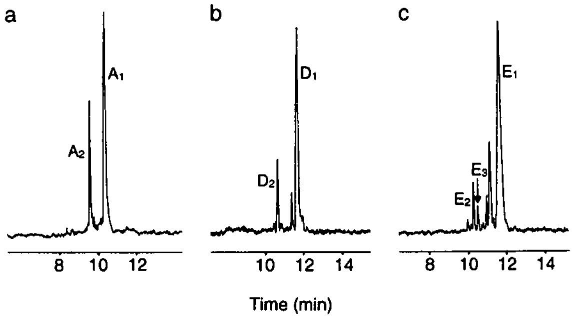

Fig. 4. Electropherograms of (a) polymyxin A, (b) polymyxin D and (c) polymyxin E. Sample concentrations, 1.0 m*M*; capillary, 75 μm I.D.; buffer, 0.2 *M* sodium phosphate (pH 2.5)–water (1:1, v/v) + 30 m*M* PAPS; voltage, 15 kV; hydrostatic injection, 5 s.

bilities of the peptides with those of the isolated fractions, and the identification of the free fatty acids.

Proposed separation mechanism

As described previously [14], above the CMC of PAPS, separation depends on the partitioning of solute molecules between the bulk solvent and the micellar pseudo-phase in the buffer solution. The separation also depends on the difference in electrophoretic mobility of the bulk solvent *versus* the micelle. Micelles formed from zwitterionic surfactants have no net charge and are therefore expected to migrate with the electroosmotic flow. However, surfaces in contact with aqueous media are more often negatively than positively charged, because the smaller, less hydrated and more polarizing anions have a greater tendency to be specifically adsorbed than the cations [15]. Adsorption of anions on the micelles gives rise to mobility in an applied field and the micelles migrate towards the anode. This is illustrated with Sudan III, which elutes about 20 min after the electroosmotic flow marker.

In free solution, the peptides migrate with a speed given by the sum of their electrophoretic mobility and the electroosmotic flow. When zwitterionic surfactants are added to the buffer in high concentrations, the zwitterionic associations with the peptide molecules and with the silica capillary surface may both prevent peptide adsorption on the fused silica and help to break up peptide–peptide interactions. In the presence of PAPS at concentrations above its CMC, the peptides show ion-exchange partitioning to the surface of the micelles (Fig. 5). While associated with the micelles, the peptides migrate with the speed of the micelles.

The hydrophobic moiety of the peptides may

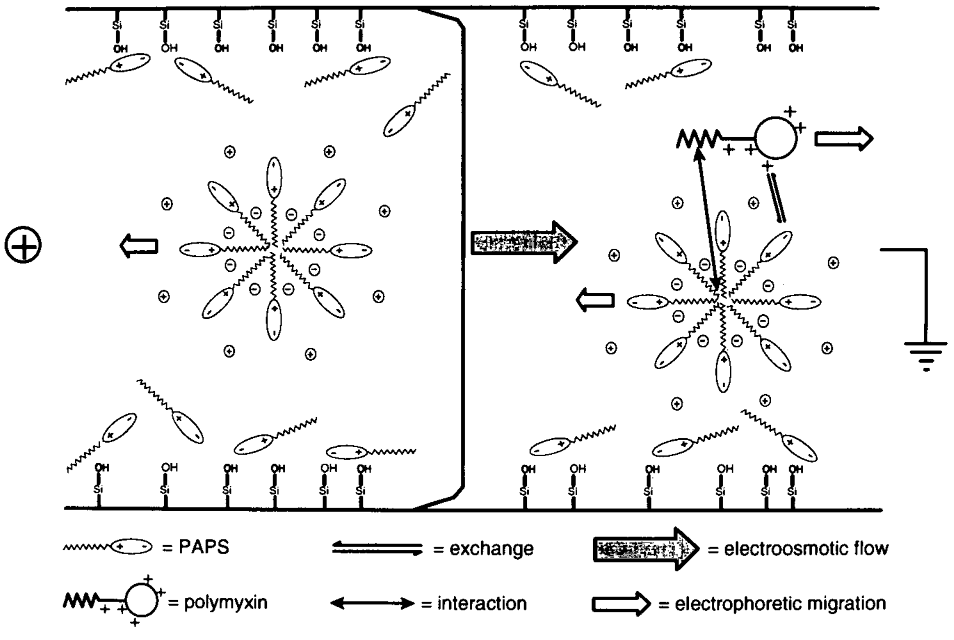

Fig. 5. Schematic representation of the proposed separation mechanism of polymyxins.

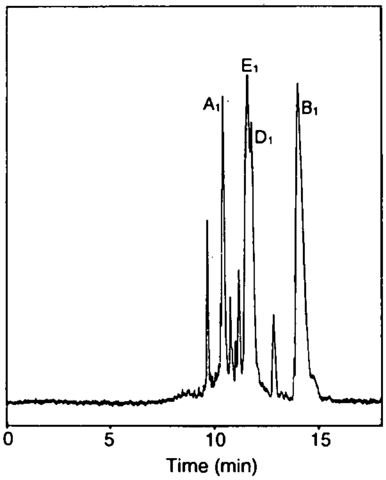

Fig. 6. Electropherogram of polymyxins A, B, D and E mixed in equal amounts. Conditions as in Fig. 4.

show affinity for the inner core of the micelles (Fig. 5), while the remaining polar parts of the peptides stay outside. Consequently, polymyxins with an identical peptide moiety and with equal mass to charge ratios are eluted in order of increasing degree of hydrophobicity, 6-methylheptanoyl < *n*-octanoyl. The polymyxins having a 6-methyloctanoyl residue show a higher affinity for the micelles owing to the higher mass to charge ratio and to an increased degree of hydrophobicity of the fatty acid moiety. The peptides are therefore separated on the basis of differences in their partition coefficients to the surface of the pseudo-phase ion exchanger and to the lipophilic core of the micelles.

In plain buffer, the polymyxins migrate as single peaks in the order A < E < B < D (not shown), in accordance with the increasing mass to charge ratios. When PAPS is added to the buffer, the migra-

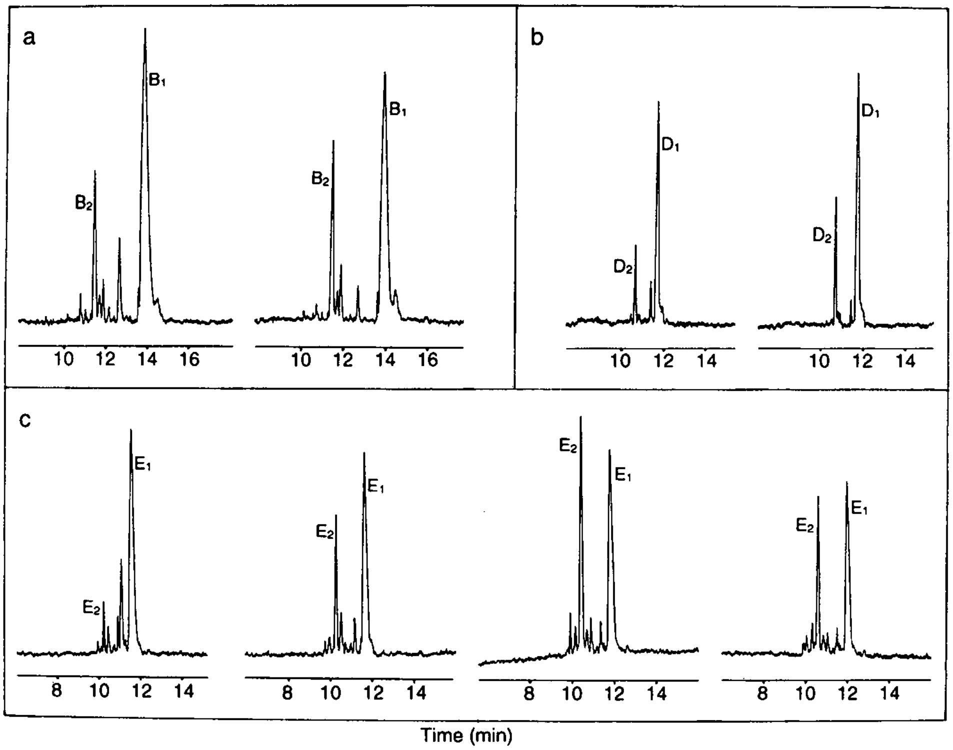

Fig. 7. Electropherograms of (a) two samples of polymyxin B, (b) two samples of polymyxin D and (c) four samples of polymyxin E from different sources. Conditions as in Fig. 4.

tion order of polymyxins B_1 and D_1 is reversed. The contribution of an amino acid residue to the order of elution can be evaluated based on a comparison of the migration times of polymyxins A_1, B_1, D_1 and E_1, which have an identical fatty acid group. As the polymyxins migrate in the order $A_1 < E_1 < D_1 < B_1$ (Fig. 6), the migration times increase in the order Thr < Leu and Leu < Phe. Polymyxin D_1 has a slower migration rate than polymyxin A_1, as expected because of a higher mass to charge ratio, but for the same reason polymyxin D_1 was also expected to have a slower rate of migration than polymyxin B_1 as observed in plain buffer. The probably explanation is that the replacement of DAB by Ser, Phe by Leu and Leu by Thr makes polymyxin D more polar than polymyxin B and therefore it shows less affinity for the micelles. Consequently, in the presence of PAPS at concentrations above its CMC, the order of migration of the polymyxins is determined by the mass to charge ratios and by the polarity of the peptides.

Polymyxins from different sources

Two samples each of polymyxins B and D and four samples of polymyxin E from different sources were separated by CE (Fig. 7). The elution profile of each sample was characteristic and sufficiently distinct to identify the samples by manufacturer. The variations between the relative peak areas of the main peaks in the electropherograms of the four samples of polymyxin E from different sources are very high, but correspond to results found by GC and HPLC analysis [6].

CONCLUSIONS

The method developed is useful for both qualitative and quantitative measurements on polymyxins. As some of the major components of each polymyxin may possibly be present as impurities in samples of other polymyxins, the method is also valuable for purity testing of the polymyxins.

ACKNOWLEDGEMENTS

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CHROM. 24 668

Discussion

High-performance liquid chromatographic determination of urinary 2,5-hexanedione

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(Received August 31st, 1992)

In a recent paper in this journal, entitled "Determination of total 2,5-hexanedione by reversed-phase high-performance liquid chromatography", Marchiseppe *et al.* [1] proposed a method for the HPLC of an *n*-hexane metabolite by using UV detection at 233 nm. Our more recent paper [2] demonstrated that such a wavelength was specific for 3-methylcyclopent-2-enone, an impurity often present in commercial 2,5-hexanedione. This contaminant was confirmed by comparing the gas and high-performance liquid chromatographic, mass spectrometric, infrared and ultraviolet data of the unknown compound with those of a synthesized reference sample.

Subsequently, in a paper entitled "Rapid and sensitive determination of urinary 2,5-hexanedione by reversed-phase high-performance liquid chromatography", Colombini *et al.* [3] proposed some valid modifications to the preparation of the sample, but, unfortunately used a wavelength similar to that of Marchiseppe *et al.* [1], which is unsuitable for identifying 2,5-hexanedione.

In Figs. 1 and 2 the UV and GC–MS analyses of Aldrich and Eastman Kodak 2,5-hexanediones are reported. The UV spectra of these two commercial 2,5-hexanediones (0.47 mg/ml), obtained by means

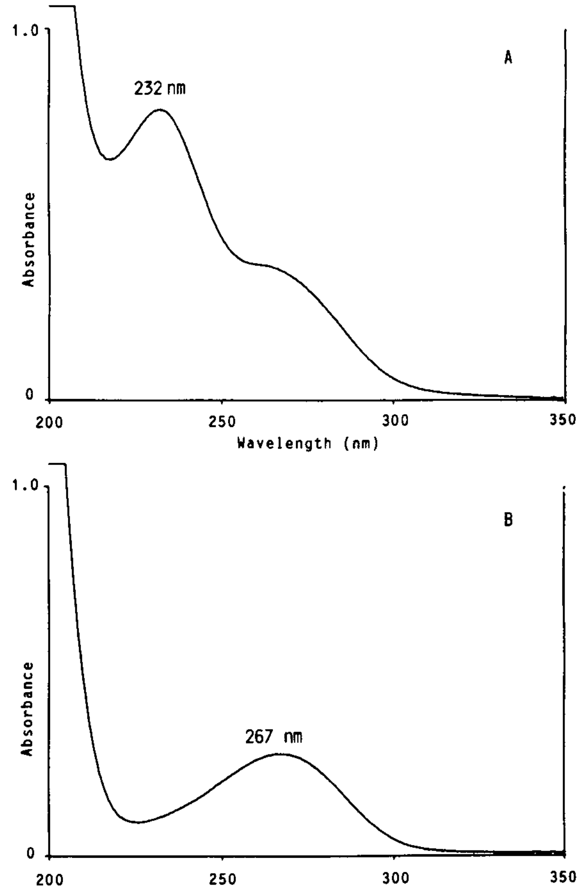

Fig. 1. UV spectra of 2,5-hexanedione from (A) Aldrich and (B) Eastman Kodak in water–acetonitrile (85:15), recorded from 200 to 350 nm with a Hitachi U-3200 spectrophotometer.

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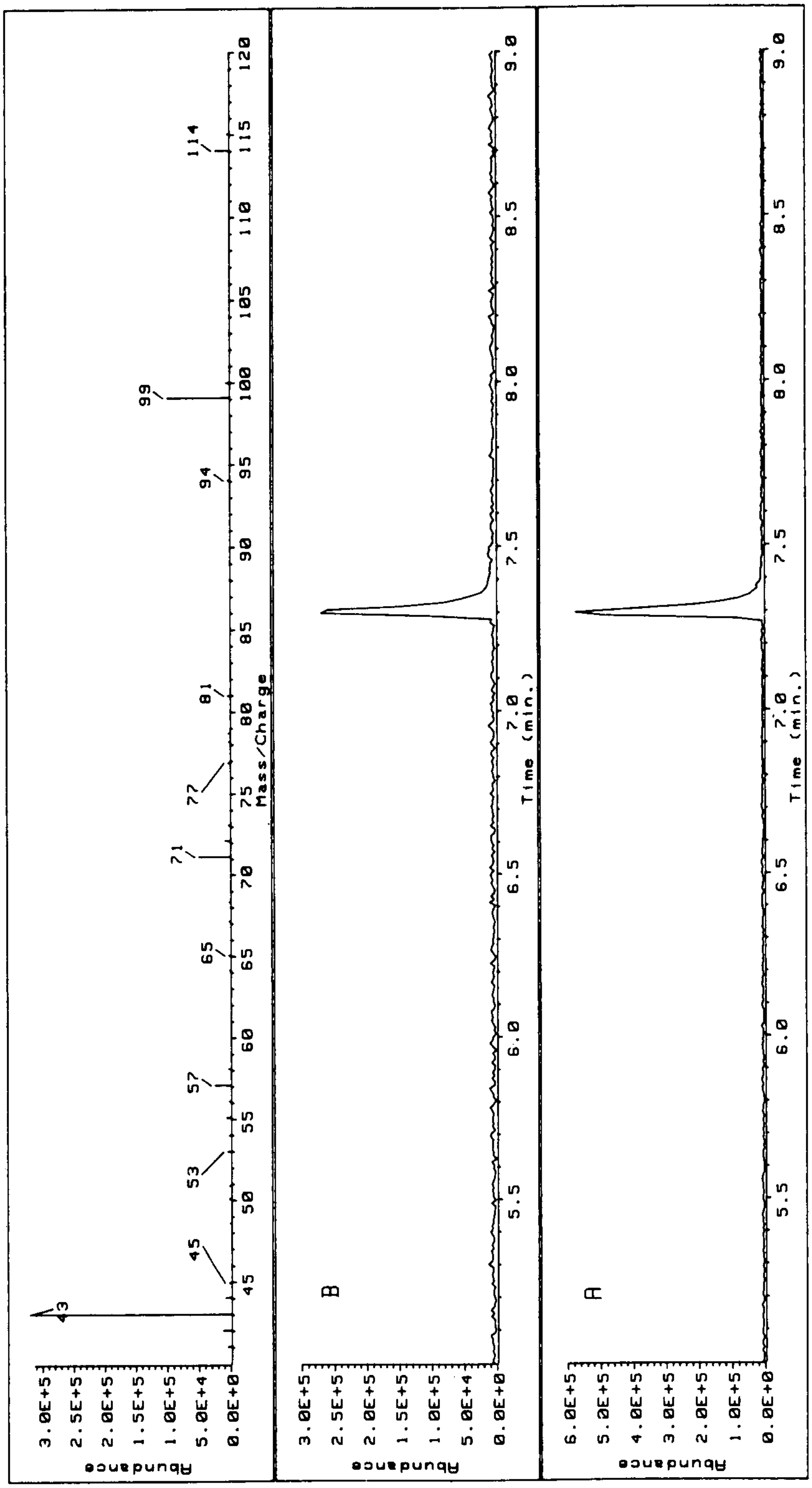

Fig. 2. GC–MS chromatograms and mass spectrum corresponding to the detected peaks in the analysis of 2,5-hexanedione samples from (A) Aldrich and (B) Eastman Kodak.

of a Hitachi U-3200 spectrophotometer in the same solvent as used by Colombini *et al.* [3], confirmed the definite difference due to the presence of the impurity, whereas under GC–MS conditions the same commercial samples (1.4 mg/ml) were identical. On the basis of the results, the maximum absorption wavelength of 2,5-hexanedione is 267 nm only [2,4].

In our opinion, in spiked urines 3-methylcyclopent-2-enone was introduced involuntarily as misleading interferent with 2,5-hexanedione. The impurity was thought to be the metabolite owing to the similar HPLC behaviour and high molar absorptivity [5]. In fact, this parameter greatly influences the response factor of the analyte in HPLC–UV determination and therefore a strong signal is associated with a small concentration of 3-methylcyclopent-2-enone owing to its high molar absorptivity, which is about 150 times higher than that of 2,5-hexanedione.

The presence of 3-methylcyclopent-2-enone in the urine of exposed workers may be attributed to the alkaline environment produced by sodium hydroxide and carbonate, which favours the cyclization of the present 2,5-hexanedione, according to the literature synthesis [5].

The GC–MS measurements were performed using a Hewlett-Packard Model 5890 gas chromatograph equipped with an Ultra-1 fused-silica capillary column (25 m × 0.2 mm I.D., 0.33 μm). The chromatographic system was coupled on-line with a Hewlett-Packard Model 5971 A quadrupole which recorded the electron ionization mass spectra by cyclically scanning from 40 to 250 mass units every 0.49 s. For GC separations the column temperature was programmed from 50°C (isothermal for 3 min) at 10°C/min to 150°C, maintained for 5 min. The injection volume was 1 μl under split conditions.

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CHROM. 24 669

Book Review

Ion chromatography —principles and applications (*Journal of Chromatography Library*, Vol. 46), by P. R. Haddad and P. E. Jackson, Elsevier, Amsterdam, 1990, XXI + 776 pp., price Dfl. 335.00, ISBN 0-444-88232-4.

The intensity of interest in separation science is evident from the number of publications on the subject that have appeared in many scientific journals. When any aspect of the field reaches a point of maturity, books on the subject soon appear.

In 1975, ion chromatography was introduced as a new and powerful separation method for ion determinations. This was a special version of the well established ion-exchange chromatography among other high-performance liquid chromatographic methods. This technique opened up a new direction in analytical chemistry, namely the simultaneous determination of ionic species in complex matrices. The applications and publications in the area of ion chromatography have expanded very rapidly since this analytical process was commercialized. The authors of this book indicate that there have been well over 1800 publications since the inception of the method.

The present book on ion chromatography is Volume 46 in the *Journal of Chromatography Library*. It is well edited and presented to give a comprehensive overview of this separation method. The concepts are divided into five logical parts and 22 chapters, which follow a helpful introduction. The parts are as follows: Part I, Ion-exchange separation methods (stationary phases, eluents, retention models); Part II, Ion-interaction, ion-exclusion and miscellaneous separation methods; Part III, Detection methods; Part IV, Practical aspects (sample handling, matrix elimination, optimization); and Part V, Applications of ion chromatography.

The text with over 700 pages is wide in scope and relatively large and presents in collected systematized form a large amount of information. Part I begins with a short introduction to the basic principles of ion exchange and the characteristics of ion exchangers (capacity, selectivity). A minor criticism is that it is surprisingly short, merely 9 pages. Chapters 3 and 4 in Part I contain a detailed discussion of ion-exchange stationary phases (silica-based, resin-based, agglomerated ion exchangers) and different eluent characteristics. This part takes a fresh look at the subject of retention models. The problem of optimizing the eluent composition and eluent selection can be solved on the basis of valid models of retention mechanisms. The various separation principles, *e.g.*, ion-interaction, ion-exclusion and miscellaneous separation methods, are discussed separately and illustrated with a number of examples in Part II. These alternative approaches can be used for the separation of ionizable species (carboxylic acids, organometallics, coordination compounds). Part III gives an excellent overview of detection methods, on the basis of which the reader should easily be able to decide which method is best suited to the analytes in which he or she is interested. Conductivity, electrochemical, potentiometric, spectroscopic and postcolumn detection modes are presented in terms of principles of operation, cell designs and performance characteristics of detectors. Part IV provides valuable information on sample handling (collection, extraction, clean-up, ultra-trace analysis, matrix elimination) and methods development (selection of chromatographic parameters, optimization, column switching, coupled methods, automation). The overall analytical problem must be carefully defined before the analysis. A series of questions can provide an evaluation of the sample and the capabilities and limitations of given separation method. These questions and answers are detailed in Part V.

Analytical chemists and users of ion-chromatographic instrumentation will profit from the extensive information in the applications part. Part V consists of seven chapters covering environmental, industrial, foods and plants, clinical and pharmaceutical, metals and metallurgical solutions, treated waters and miscellaneous applications, with very informative tables, listing experimental conditions, sample type, approximate retention time for each solute, sample preparation procedures, the column (and its dimensions), the eluent composition (and the flow-rate used) and the detection methods employed. These descriptions of routine and advanced methods in tabulated form were derived from over 1200 publications. The first four parts contain 480 pages of the text and Part V on applications fills another 250 pages.

The book ends with an Appendix concerning aspects of statistical information on publications on ion chromatography. It can be seen how the scope of the method has changed dramatically in the past and that the subject is currently expanding rapidly.

This is an extremely well organized book written by two well known experts in the field of ion chromatography. There are many helpful and very illustrative figures, chromatograms and schematic overviews. The book is highly recommended to analytical, environmental, industrial and clinical chemists, university students and all those interested in modern separation science.

Lausanne (Switzerland) Peter Hajos

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CHROM. 24 655

Book Review

Laboratory experiments in liquid chromatography, by W. V. Willis, CRC Press, Boca Raton, FL, 1991, II + 260 pp., price £ 42.50, ISBN 0-8493-0177-7.

Everyone who is involved in teaching chromatography is probably familiar with the problem of how to design a practical course for newcomers in this field. How should one organize experiments that cover all the different aspects of chromatography without carrying out time-consuming laboratory work to evaluate whether a certain experiment "works"? In my opinion, this book of William Willis will be a great help to all those who are faced with this problem, especially those who are involved in both research and teaching.

The book is divided into six chapters, which cover the theory of chromatography, different separation techniques (reversed- and normal-phase, ion-exchange/ion-pairing and size-exclusion) and preparative chromatography. Each chapter includes a large number of experiments which deal with virtually all facets of liquid chromatography. The experiments vary in degree of difficulty and range from the relatively simple determination of caffeine in beverages to the determination of the molecular mass of proteins by size-exclusion chromatography. The clear organization of the book is an excellent feature. Each experiment is headed by an introduction, which gives a clear and compact overview of the theoretical background and the goals of the experiment. The experimental part includes a list of materials required, the methodology of the experiment, notes on how to evaluate the results and some additional suggestions and practical tips. Even if one intends to alter the experiments by using different analytes, for example, it is still possible to use the framework presented by this book.

Despite some (mainly editorial) errors, *e.g.*, Fig. 5.1 represents the sulphonic acid groups on a cation exchanger as SO_3^+, I can recommend this book to all those who are either involved in the teaching of chromatography or who want to learn more about chromatography without following expensive courses.

Leiden (Netherlands) Hubertus Irth

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CHROM. 24 706

Book Review

Advances in applied lipid research, Vol. 1, edited by F. B. Padley, JAI Press, London, UK, 1992, IX + 271 pp., price £ 54.00, ISBN 1-55938-317-8.

This is the first volume of a new annual series aimed at the applied area of lipid research. Rather than focusing on a single theme it covers a varied spectrum of topics including chemical and biochemical transformations of lipids of industrial interest, and separation technology.

The first chapter deals with enzymatic modifications of lipids, and in particular the nature and function of lipases in degradative and synthetic modifications to lipids, both from a fundamental standpoint and in real commercial applications. A second chapter takes a similar approach to discussion of the biosynthesis of lipids in yeasts, and the use of these organisms in industrial processes. Similarly, the sources of long-chain polyunsaturated fatty acids, together with their biochemistry and nutritional properties are thoroughly reviewed. The interaction of oxidised lipids with proteins in foods on storage or cooking is a topic of great current interest, not least because of implications for the health of the consumer. The discussion here deals largely with the chemistry involved. All of these chapters are by recognised experts in their fields and are authoritative accounts.

Readers of the *Journal of Chromatography* will probably find the final chapter by Bartle and Clifford to be of greatest interest, since it deals with supercritical fluid extraction and chromatography of lipids and related compounds. A large number of papers are now appearing on applications of these techniques, and many laboratories must be considering purchase of instruments. The theory of the use of supercritical fluids in chromatography especially is discussed at considerable length and in some depth here, and indeed I cannot imagine a better introduction to the topic. However, only eight pages are devoted to applications to lipids with a few more on comparisons with other techniques. To my mind, the authors make the best of a not very good case for the technique; I have yet to find a published application to lipids that could not be done better by gas chromatography or high-performance liquid chromatography in my view. Supercritical fluid extraction is another matter and there do seem to be some real advantages, especially when extraction and chromatography can be performed in a concerted manner, although it is evident from this report that problems remain. However, I strongly recommend that this review be read by everyone with even a passing interest in the area.

In short, this volume is a promising start to the series and I look forward to seeing future volumes.

Ayr (UK) William W. Christie

Author Index

Akasaka, K., Ohrui, H. and Meguro, H.
Normal-phase high-performance liquid chromatography with a fluorimetric postcolumn detection system for lipid hydroperoxides 628(1993)31
Alred, P. A., Tjerneld, F. and Modlin, R. F.
Partitioning of ecdysteroids using temperature-induced phase separation 628(1993)205
André, P., see Morin, P. 628(1993)161
Ayyangar, N. R., see Tambe, A. S. 628(1993)143
Baba, Y., see Kura, G. 628(1993)241
Ballantine, Jr., D. S.
Quantitative structure–retention relationship approach to prediction of linear solvation energy relationship coefficients. I. H-Bond acceptor capability of gas chromatography stationary phases in McReynolds data set 628(1993)247
Bank, S., see Zhu, M. 628(1993)37
Baumeister, K., see Weinmann, W. 628(1993)111
Beattie, K. A., see Poon, G. K. 628(1993)215
Berezkin, V. G.
Analysis of substances in the gaseous phase (Comprehensive Analytical Chemistry, VOl. XXVIII) (by E. Smolková-Keulemansová and L. Feltl) 628(1993)170
Betts, T.J.
Two-solute method for indicating polarity changes of conventional and novel gas chromatographic stationary phases with temperature increase 628(1993)138
Biswas, S., see Tambe, A. S. 628(1993)143
Blanch, G. P., Tabera, J., Herraiz, M. and Reglero, G.
Preconcentration of volatile components of foods: optimization of the steam distillation–solvent extraction at normal pressure 628(1993)261
Bourguignon, B., Marcenac, F., Keller, H. R., De Aguiar, P. F. and Massart, D. L.
Simultaneous optimization of pH and organic modifier content of the mobile phase for the separation of chlorophenols using a Doehlert design 628(1993)171
Ćelap, M. B., see Tešić, Ž. L. 628(1993)148
Chen, X., see Zhu, M. 628(1993)37
Christie, W. W.
Advances in applied lipid research, volume 1 (edited by F. B. Padley) (Book Review) 628(1993)322
Codd, G.A., see Poon, G.K. 628(1993)215
Daniel, T., see Tambe, A. S. 628(1993)143
De Aguiar, P. F., see Bourguignon, B. 628(1993)171
DeCaprio, A. P., see Zhu, M. 628(1993)37
Doretti, L., see Sturaro, A. 628(1993)316
Dorsey, T. F., see Eganhouse, R. P. 628(1993)81
Drandarov, K. and Hais, I. M.
Thin-layer chromatography of the positional isomers of some 1,2,3,4-tetrahydro-2-naphthol and 3-amino-1,2,3,4-tetrahydro-2-naphthol derivatives 628(1993)103
Dreux, M., see Morin, P. 628(1993)153
Dreux, M., see Morin, P. 628(1993)161
Edwards, C., see Poon, G. K. 628(1993)215
Eganhouse, R. P., Dorsey, T. F., Phinney, C. S. and Westcott, A. M.
Determination of C_6–C_{10} aromatic hydrocarbons in water by purge-and-trap capillary gas chromatography 628(1993)81
Elmroth, I., Fox, A. and Larsson, L.
Determination of bacterial muramic acid by gas chromatography–mass spectrometry with negative-ion detection 628(1993)93
Fox, A., see Elmroth, I. 628(1993)93
Gao, R.-Y., see Wang, Q.-S. 628(1993)127
Garrigues, P., see Pyell, U. 628(1993)3
Gaš, B., see Hirokawa, T. 628(1993)283
Gilpin, R. K., see Heuchel, M. 628(1993)59
Griggs, L. J., see Poon, G. K. 628(1993)215
Gügel, A. and Müllen, K.
Separation of C_{60} and C_{70} on polystyrene gel with toluene as mobile phase 628(1993)23
Gyllenhaal, O. and Vessman, J.
Packed-column supercritical fluid chromatography of omeprazole and related compounds. Selection of column support with triethylamine- and methanol-modified carbon dioxide as the mobile phase 628(1993)275
Hais, I. M., see Drandarov, K. 628(1993)103
Hajos, P.
Ion chromatography. Principles and applications (by P. R. Haddad and P. E. Jackson) (Book Review) 628(1993)319
Hansen, S. H., see Kristensen, H. K. 628(1993)309
Hargitai, T., Kaida, Y. and Okamoto, Y.
Preparation and chromatographic evaluation of 3,5-dimethylphenyl carbamoylated β-cyclodextrin stationary phases for normal-phase high-performance liquid chromatographic separation of enantiomers 628(1993)11
Hendrix, C., Wijsen, C., Yun, L. M., Roets, E. and Hoogmartens, J.
Column liquid chromatography of cefadroxil on poly(styrene–divinylbenzene) 628(1993)49
Herraiz, M., see Blanch, G. P. 628(1993)261
Heuchel, M., Jaroniec, M. and Gilpin, R. K.
Application of a new numerical method for characterizing heterogeneous solids by using gas–solid chromatographic data 628(1993)59
Hirokawa, T., Kiso, Y., Gaš, B., Zusková, I. and Vacík, J.
Simulated quantitative and qualitative isotachophoretic indices of 73 amino acids and peptides in the pH range 6.4–10 628(1993)283
Hoogmartens, J., see Hendrix, C. 628(1993)49
Irth, H.
Laboratory experiments in liquid chromatography (by W. V. Willis) (Book Review) 628(1993)321
Ishida, N., see Yoshida, M. 628(1993)235
Janjić, T. J., see Tešić, Ž. L. 628(1993)148
Jaroniec, M., see Heuchel, M. 628(1993)59
Jauhiainen, T.-P., see Kunnas, A. V. 628(1993)269
Kaida, Y., see Hargitai, T. 628(1993)11

(Continued overleaf)

Kaufmann, I., see Weinmann, W. 628(1993)111
Keller, H. R., see Bourguignon, B. 628(1993)171
Kiso, Y., see Hirokawa, T. 628(1993)283
Kitamura, E., see Kura, G. 628(1993)241
Kristensen, H. K. and Hansen, S. H
Separation of polymyxins by micellar electrokinetic capillary chromatography 628(1993)309
Kunnas, A. V. and Jauhiainen, T.-P.
Separation and identification of free amino acid enantiomers in peat by capillary gas chromatography 628(1993)269
Kura, G., Kitamura, E. and Baba, Y.
Temperature dependence of the anion-exchange chromatographic behaviour of cyclic phosphate oligomers and their anion-exchange separation 628(1993)241
Larsson, L., see Elmroth, I. 628(1993)93
Marcenac, F., see Bourguignon, B. 628(1993)171
Massart, D. L., see Bourguignon, B. 628(1993)171
Meguro, H., see Akasaka, K. 628(1993)31
Mizuno, K., see Takagi, K. 628(1993)122
Modlin, R. F., see Alred, P. A. 628(1993)205
Morin, P., Villard, F. and Dreux, M.
Borate complexation of flavonoid-O-glycosides in capillary electrophoresis. I. Separation of flavonoid-7-O-glycosides differing in their flavonoid aglycone 628(1993)153
Morin, P., Villard, F., Dreux, M. and André, P.
Borate complexation of flavonoid-O-glycosides in capillary electrophoresis. II. Separation of flavonoid-3-O-glycosides differing in their sugar moiety 628(1993)161
Müllen, K., see Gügel, A. 628(1993)23
Nogata, Y., see Ohta, H. 628(1993)199
Ohrui, H., see Akasaka, K. 628(1993)31
Ohta, H., Takeda, Y., Yoza, K.-I. and Nogata, Y.
High-performance liquid chromatographic determination of polyamines in selected vegetables with postcolumn fluorimetric derivatization 628(1993)199
Okamoto, Y., see Hargitai, T. 628(1993)11
Parvoli, G., see Sturaro, A. 628(1993)316
Phinney, C. S., see Eganhouse, R. P. 628(1993)81
Poon, G. K., Griggs, L. J., Edwards, C., Beattie, K. A. and Codd, G. A.
Liquid chromatography–electrospray ionization-mass spectrometry of cyanobacterial toxins 628(1993)215
Przybylski, M., see Weinmann, W. 628(1993)111
Pyell, U., Garrigues, P. and Rayez, M. T.
Influence of shape parameters on the separation of tetrachlorodibenzo-*p*-dioxin isomers on reversed-phase columns 628(1993)3
Rayez, M. T., see Pyell, U. 628(1993)3
Reglero, G., see Blanch, G. P. 628(1993)261
Roets, E., see Hendrix, C. 628(1993)49
Sabljić, A., see Sekušak, S. 628(1993)69
Saito, Y., see Takagi, K. 628(1993)122
Satoh, S., see Takagi, K. 628(1993)122
Sekušak, S. and Sabljić, A.
Calculation of retention indices by molecular topology. III. Chlorinated dibenzodioxins 628(1993)69
Shimizu, M., see Takagi, K. 628(1993)122
Spink, D. C., see Zhu, M. 628(1993)37
Sturaro, A., Parvoli, G. and Doretti, L.
High-performance liquid chromatographic determination of urinary 2,5-hexanedione 628(1993)316
Tabera, J., see Blanch, G. P. 628(1993)261
Takagi, K., Toyoda, M., Saito, Y., Mizuno, K., Shimizu, M. and Satoh, S.
Determination of kinetin in callus of *Panax ginseng* by liquid chromatography 628(1993)122
Takeda, Y., see Ohta, H. 628(1993)199
Tambe, A. S., Daniel, T., Biswas, S. and Ayyangar, N. R.
Gas chromatographic method for the determination of chlorobenzophenone isomers 628(1993)143
Tešić, Ž. L., Janjić, T. J. and Ćelap, M. B.
Thin-layer chromatography on silica gel of a homologous series of bis(alkylxanthato)nickel(II) complexes 628(1993)148
Tjerneld, F., see Alred, P. A. 628(1993)205
Tokiyasu, T., see Yoshida, M. 628(1993)235
Toyoda, M., see Takagi, K. 628(1993)122
Vacík, J., see Hirokawa, T. 628(1993)283
Vessman, J., see Gyllenhaal, O. 628(1993)275
Villard, F., see Morin, P. 628(1993)153
Villard, F., see Morin, P. 628(1993)161
Wang, Q.-S., Gao, R.-Y. and Yan, B.-W.
Optimization of the separation of technical aldicarb by semi-preparative reversed-phase high-performance liquid chromatography 628(1993)127
Wang, Y.-C. and Whang, C.-W.
High-performance liquid chromatography of inorganic mercury and organomercury with 2-mercaptobenzothiazole 628(1993)133
Watabiki, T., see Yoshida, M. 628(1993)235
Weinmann, W., Baumeister, K., Kaufmann, I. and Przybylski, M.
Structural characterization of polypeptides and proteins by combination of capillary electrophoresis and ^{252}Cf plasma desorption mass spectrometry 628(1993)111
Westcott, A. M., see Eganhouse, R. P. 628(1993)81
Whang, C.-W., see Wang, Y.-C. 628(1993)133
Wijsen, C., see Hendrix, C. 628(1993)49
Wrisley, L.
Use of computer simulations in the development of gradient and isocratic high-performance liquid chromatography methods for analysis of drug compounds and synthetic intermediates 628(1993)191
Yan, B.-W., see Wang, Q.-S. 628(1993)127
Yoshida, M., Watabiki, T., Tokiyasu, T. and Ishida, N.
Determination of paraquat and diquat by liquid chromatography–thermospray mass spectrometry 628(1993)235
Yoza, K.-I., see Ohta, H. 628(1993)199
Yun, L. M., see Hendrix, C. 628(1993)49
Zhu, M., Spink, D. C., Bank, S., Chen, X. and DeCaprio, A. P.
Analysis of alkylpyrrole autoxidation products by high-performance liquid chromatography with thermospray mass spectrometry and UV photodiode-array detection 628(1993)37
Zusková, I., see Hirokawa, T. 628(1993)283

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Journal of Chromatography and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1992	N 1992	D 1992	J 1993	F 1993	
Journal of Chromatography	623/1 623/2 624/1+2	625/1 625/2	626/1 626/2 627/1+2	628/1 628/2 629/1 629/2	630/1+2 631/1+2 632/1+2 633/1+2	The publication schedule for further issues will be published later.
Cumulative Indexes, Vols. 601–650						
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