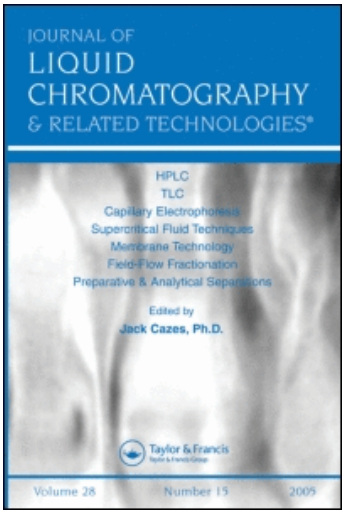

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Optimization of the Mobile Phase for HPLC Separation of S-Alk(en)yl-L-Cysteine Derivatives and Their Corresponding Sulfoxide Isomers

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OPTIMIZATION OF THE MOBILE PHASE FOR HPLC SEPARATION OF S-ALK(EN)YL-L-CYSTEINE DERIVATIVES AND THEIR CORRESPONDING SULFOXIDE ISOMERS

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ABSTRACT

Using the "PRISMA" optimization model, a total of 15 S-alk(en)yl-L-cysteine derivatives, including five thioethers and their corresponding sulfoxide isomers, could be separated following pre-column derivatization with o-phthaldialdehyde/*tert.*-butylthiol. Optimal isocratic separation of the sulfoxides was achieved with a limited number of experiments using a quaternary solvent system consisting of tetrahydrofuran, 1,4-dioxane, acetonitrile, and aqueous phosphate buffer of pH 7.15. Using a selective multisolvent gradient elution (SMGE) technique, which involves a simultaneous change in selectivity and solvent strength of the mobile phase, the full range of the examined sulfur amino acids (most of them are found in various *Allium* species) could be resolved in a single chromatographic run. Development and realization of the chromatographic optimization is shown and discussed. A simple computer program (written in Microsoft Basic) for the calculation of the actual mobile phase composition is added.

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INTRODUCTION

Separation development is still considered the most difficult, and sometimes most frustrating job in HPLC. In recent years much progress has been made in developing systematic procedures for chromatographic optimization, especially optimization of the mobile phase. Numerous papers, several reviews (1, 2) and books (3-5) have been published, presenting different approaches and strategies.

The "PRISMA" model, a geometrical design for solvent optimization, was recently developed. First used with HPLC (6), the method has been extended to other chromatographic methods such as thin-layer chromatography (TLC) (7), over-pressured layer chromatography (OPLC) (8), and centrifugal layer chromatography (CLC) (9). The procedure has been successfully applied to a variety of analytical and preparative separation problems in the chromatographic analysis of naturally occurring compounds (10, 11). Gradient possibilities in HPLC have also been demonstrated with the separation of 12 tobacco alkaloids which possessed closely related structures (12).

In this paper, we first describe optimization of the isocratic separation of five pairs of L-cysteine sulfoxide isomers achieved using the "PRISMA" model. In a second section, we demonstrate the efficiency of selective multisolvent gradient elution (SMGE) in separating the sulfoxides together with the more lipophilic thioether derivatives in a single chromatographic run. Most of these sulfur amino acids have been reported to occur in various *Allium* species (e.g., garlic or onion).

Application of the "PRISMA" Model

The theory of the "PRISMA" model and some gradient possibilities have been discussed elsewhere (6, 12). In this paper we would like to focus on some important features of the model which are necessary for the understanding of the

following. "PRISMA" is a solvent optimization model applicable to a given chromatographic system (defined column, temperature, pH, flow). It has a three-dimensional design which correlates solvent strength (S) with the selectivity of mobile phases. As many as five solvents may be included in the system. In the case of reversed-phase (RP) chromatography, there are generally three organic solvents (A, B, C); the fourth solvent is water and serves as solvent strength regulator (S = 0), and a fifth may be a modifier such as acetic or phosphoric acid. The total solvent strength (S_T) is represented by the height of the prism and may be calculated as follows:

$$S_T = \sum_{i=1} S_i Y_i$$

S_i : Solvent strength of the different components

Y_i : Volume fraction

At a constant S_T value in the regular prism - similar to the upper part of the model - the combination of solvents A, B, C may be characterized with selectivity points (P_S) and designated by a three-digit number or, if necessary, by three two-digit numbers. The concept of using selectivity points for the characterization of four-solvent systems as well as the procedure for calculating the actual mobile phase composition were described in detail in (13).

A simple computer program written in Microsoft Basic was developed to facilitate calculations. The self-explanatory program listing is given in Appendix I.

Using a four-solvent system, Kirkland and Glajch (14) described different types of gradients: I. *Isoselective multisolvent gradient elution* (IMGE), which means a change in solvent strength while holding the selectivity constant, and II. *selective multisolvent gradient elution* (SMGE) involving a change in both solvent strength and selectivity of the mobile

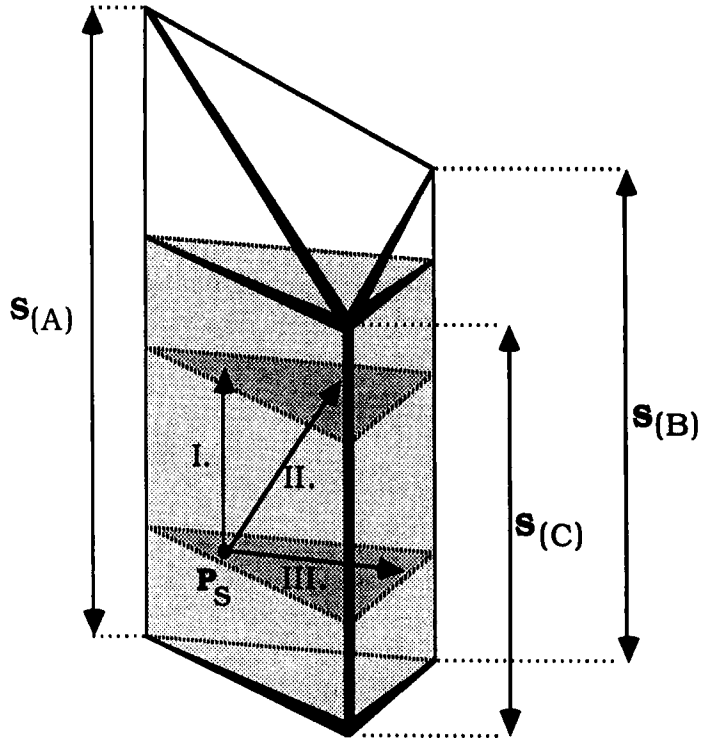


FIGURE 1. *The "PRISMA" optimization model showing three basic possibilities for multisolvent gradient elution.*
I: IMGE II: SMGE III: ICMGE

phase. IMGE (corresponding to a vertical shift in the prism) may be compared to gradient schemes of so-called linear solvent strength (LSS) as discussed by Snyder et al. (15). A third possibility (III) is called *isocratic multisolvent gradient elution* (ICMGE) (12). This term stands for a horizontal shift in the prism varying only the selectivity of the mobile phase at a constant S_T. All these three gradient elution techniques between selectivity points are possible using the "PRISMA" model as depicted in Figure 1.

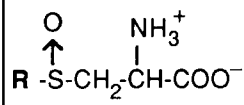
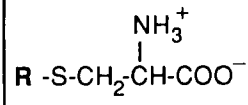
MATERIALS AND METHODS

Chemicals

Reference substances were prepared by basic alkylation of L-cysteine; the (±)-sulfoxide isomers were obtained by oxidation of the corresponding thioethers with hydrogen peroxide. Table I shows the chemical structures and abbreviations of all the synthesized compounds. The different substances were crystallized either from water/acetone or water/ethanol mixtures. (+)-ACSO ("alliin") could be separated from the (-)-form by fractional crystallization. Structural elucidation was done by elemental analysis and mass spectroscopy and partly by IR and NMR spectroscopy. Detailed procedures and structural data will be presented elsewhere.

TABLE 1

Chemical structures of the S-alk(en)yl-L-cysteines and their corresponding sulfoxides used in this study.

Cysteine derivatives	R				
	CH_3	C_2H_5	CH_2-CH=CH_2	C_3H_7	C_4H_9
	MCSO	ECSO	ACSO	PCSO	BCSO
Peak numbers (-/+ isomers)	P1/2	P3/4	P5/6	P7/8	P9/10
	MCS	ECS	ACS	PCS	BCS
Peak numbers	P11	P12	P13	P14	P15

Methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF), and 1,4-dioxane were of HPLC quality and were purchased from Romil Chemicals Ltd. (Shepshed, Leics, England). Pure water was obtained from a NANOpure Cartridge System (SKAN, Basel-Allschwil, Switzerland). Aqueous buffer solutions used for mobile phases were prepared with sodium dihydrogen phosphate dihydrate (0.045 M), adjusted to the desired pH value with sodium hydroxide, and finally membrane-filtered. Both chemicals, as well as o-phthaldialdehyde (OPA), *tert.*-butylthiol, and disodium hydrogen phosphate dihydrate were of analytical grade and obtained from Fluka (Buchs, Switzerland).

Apparatus

Two different chromatographic systems were employed in this work: (A) a programmable four-solvent delivery system (Model LC 41 including a LC 21 autosampler) from Bruker-Franzen Analytik (Bremen, F.R.G), and (B) a liquid chromatograph consisting of a Model 6000 A pump (Waters Assoc., Milford, MA, U.S.A), a low pressure gradient system Model 111 OPG/S (Autochrom Inc., Milford, MA, U.S.A), and a WISP 712 autosampler (Waters). Column temperatures were controlled using a PCRS Model 520 heater system (Kratos, Ramsey, NJ, U.S.A). Mobile phases were deaerated by purging with helium. The effluent was monitored either by a HP 1040 diode-array detector (Hewlett-Packard, Freiburg, F.R.G), pilot signal set at 337 nm, or by a HP 1046A fluorescence detector (excitation at 230 nm, emission recorded at 420 nm, cut-off filter 370 nm) (16).

Separations described in this work were carried out using Knauer (Berlin, F.R.G) column cartridges (100 x 4 mm i.d.) filled with Spherisorb ODS II (3 μm) RP material. Mobile phases were delivered at a flow rate of 1.2 ml/min. Column temperatures were usually held at 30 °C.

Methods

Preparation of the OPA/tert.-Butylthiol Buffered Reagent

The OPA/thiol reagent was made as follows: 140 mg of OPA was dissolved in 5 ml of methanol. This solution mixed with 100 µl of *tert.*-butylthiol was then diluted to 50 ml with pH 9.5 sodium phosphate buffer (0.05 M). This reagent was stored at room temperature and could be used for several weeks, although *tert.*-butylthiol had to be added from time to time to maintain its potency.

Standard Solutions

The sulfur amino acids were dissolved in 50% methanol/50% distilled water for a final concentration of about 0.5 mg/ml. Dilutions were made as necessary with methanol/water.

Derivatization Procedure

A volume of 180 µl of the reagent was added to 20 µl of sample solution in a autosampler micro vial and thoroughly mixed using a syringe. After a reaction time of at least 2 min, an aliquot (5-10 µl) was injected into the chromatographic system.

RESULTS AND DISCUSSION

Isocratic Separation of the Sulfoxide Isomers

The optimization procedure was started with the adjustment of the solvent strength, as described in (6), using a quaternary solvent system. Mobile phases consisted of THF (S_{THF} = 4.5), ACN (S_{ACN} = 3.2), and MeOH (S_{MeOH} = 2.6) representing the organic solvents which were most often employed for RP-HPLC, and aqueous phosphate buffer (S_{H_2O} = "0") (solvent strength weighing factors from Ref. 18). The pH of the buffer solution had

to be adjusted to a neutral pH in order to achieve good peak shapes. Mobile phases of acidic pH values gave tailing peaks which impaired resolution.

The four basic experiments using mobile phases of $S_T = 1.0$ and selectivity points 333, 811, 181, and 118 failed to resolve the full range of the sulfoxides (Figure 2). The major problem was the occurrence of fusing peaks of (+)-ACSO [P6] and (-)-PCSO [P7] which was more pronounced with higher proportions of ACN and MeOH (in addition, the extreme long elution time in selectivity point 118 is remarkable). On the other hand, large amounts of THF impaired the separation of (+)-MCSO [P2] and (-)-ECSO [P3]. Based on these findings, the organic modifier with the least selectivity - MeOH - was replaced. As expected, substitution with other aliphatic alcohols as ethanol or isopropanol resulted in little or no improvement; however, changing to other solvent classes is impeded by at least three factors:

1) HPLC requires mobile phases of high purity;
2) the solvents must not interfere with the detectors employed; and
3) RP chromatography needs organic solvents which are miscible with water.

The alternative was found when dioxane resulted in satisfactory separation of (+)-ACSO [P6] and (-)-PCSO [P7]. Subsequently, a new prism was "constructed" with THF, dioxane, and ACN as organic modifiers. The solvent strength of the dioxane was defined as 3.5 in accordance with (15). After adjusting the mobile phase of $P_S = 333$ to solvent strength 1.05, yielding a total elution time of about 14 min, the optimization procedure was continued with mobile phases represented by selectivity points $P_S = 811$, 181, and 118. Figure 3 depicts these four basic chromatograms. Best resolution of the sulfoxide mixture was obtained with the mobile phase described by $P_S = 181$. Using this mobile phase composition the more lipophilic

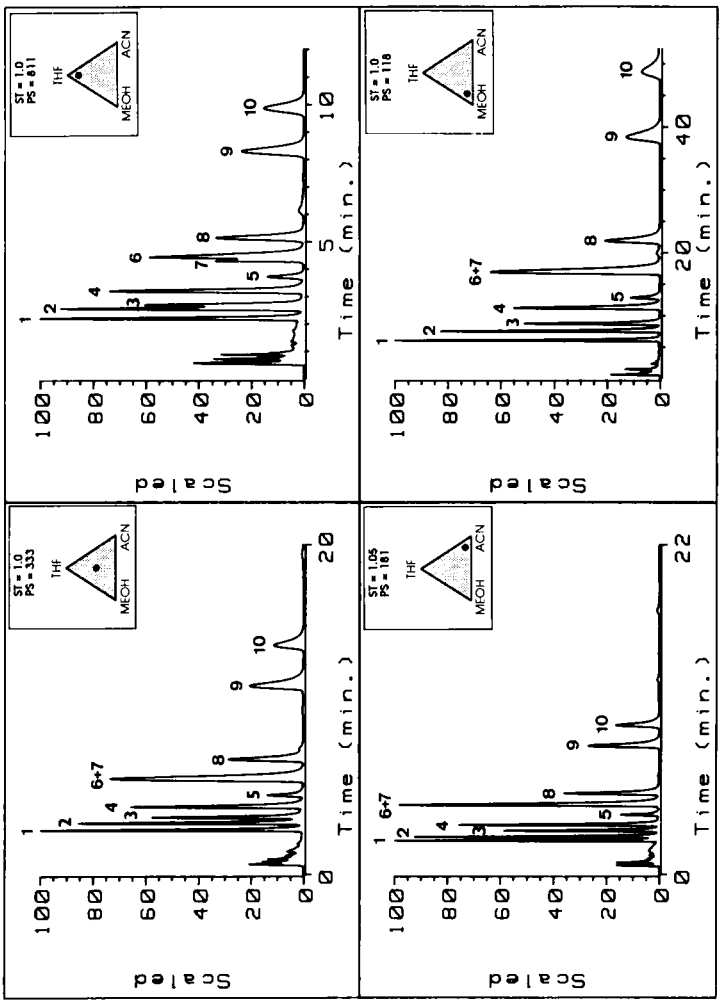


FIGURE 2. *The four basic experiments at the selected solvent strength for the isocratic separation of the (±)-sulfoxide isomers according to the "PRISMA" optimization strategy using mixtures of THF, MeOH, ACN, and aqueous phosphate buffer (pH 7.15). The effluent was monitored at 337 nm. Other chromatographic conditions as described in text.*

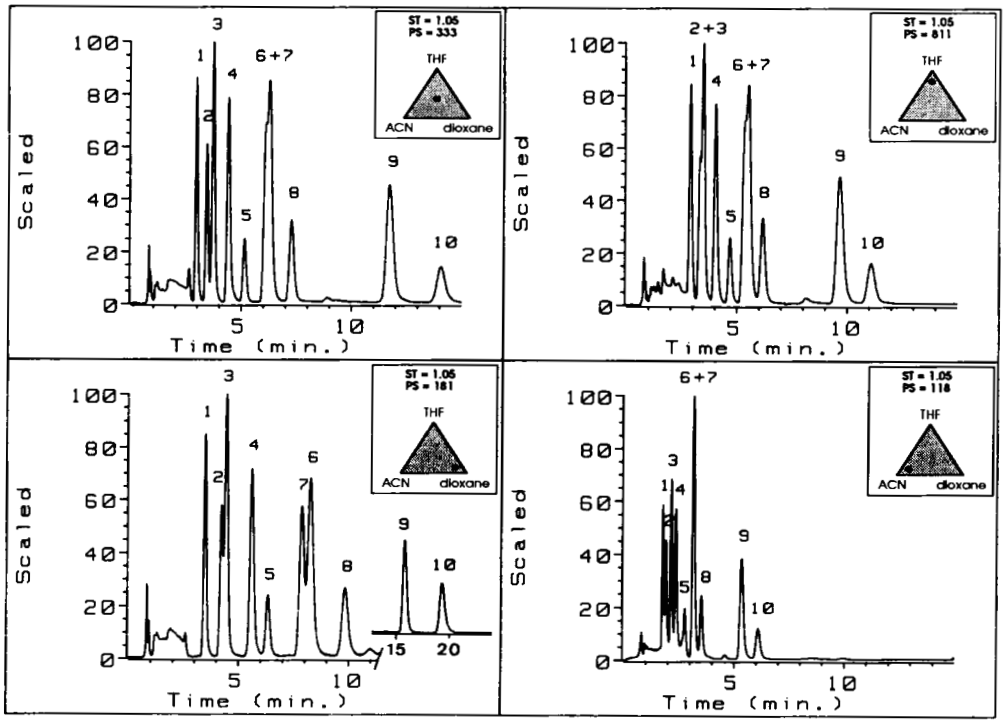


FIGURE 3. *The four basic experiments for the optimization of the isocratic separation of the (±)-sulfoxide isomers after replacing MeOH (see Figure 2) with 1,4-dioxane. New solvent system: THF, 1,4-dioxane, ACN, and aqueous phosphate buffer (pH 7.15). UV detection at 337 nm. Other chromatographic conditions as described in text.*

BCSO isomers [P9, P10] eluted after more than 20 min and were, therefore, favorably chromatographed using a gradient system as described later in this paper.

Further optimization was hindered by two factors which interfered with one another: separation of (+)-ACSO [P6] and (-)-PCSO [P7] required rather large proportions of 1,4-dioxane which coincidently impaired resolution of (+)-MCSO [P2] and (-)-ECSO [P3]. On the other hand, ACN exhibited good selectivity in separating the latter two sulfoxides, while (+)-ACSO [P6] and (-)-PCSO [P7] appeared as fusing peaks. These facts consequently determined the final optimization steps. A compromise was then found in selectivity point 06-83-11 with a reduced solvent strength of S_T = 0.95 (Figure 4). In fact, a further reduction slightly improved the resolution but resulted in impractically long elution times. The use of a ternary solvent system consisting only of 1,4-dioxane, ACN, and aqueous phosphate buffer, and leaving out THF, resulted in a slightly impaired separation.

The typical elution profile of a garlic sample is depicted in Figure 5 and will be discussed elsewhere (17). The main sulfur amino acid of interest (+)-ACSO (= alliin) was well separated and easily quantified.

Isocratic Separation of the Thioether Derivatives

Separation of the thioether homologues resulted in no problems. As compared with the sulfoxides, the more lipophilic character of the thioether derivatives required mobile phases of increased solvent strengths. In accordance with the theory, the elution order was correlated with the size and nature of the thioether side chain. Figure 6 shows a typical chromatogram obtained at S_T = 1.41 and P_S = 333 upon.

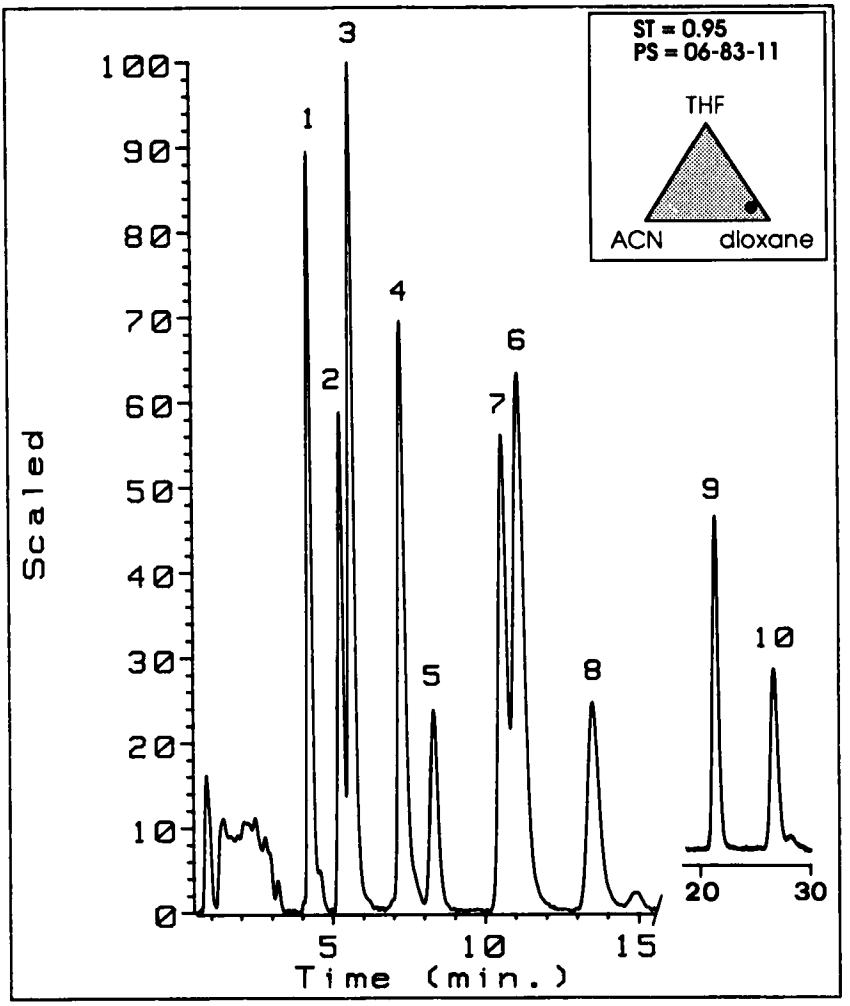


FIGURE 4. *Optimized isocratic separation of the (±)-sulfoxide isomers. The mobile phase employed was defined by* P_S = *06-83-11 and* S_T = *0.95. (1.3% THF, 22.5% 1,4-dioxane, 3.3% ACN, and 72.9% aqueous phosphate buffer (0.045 M, pH 7.15)).*

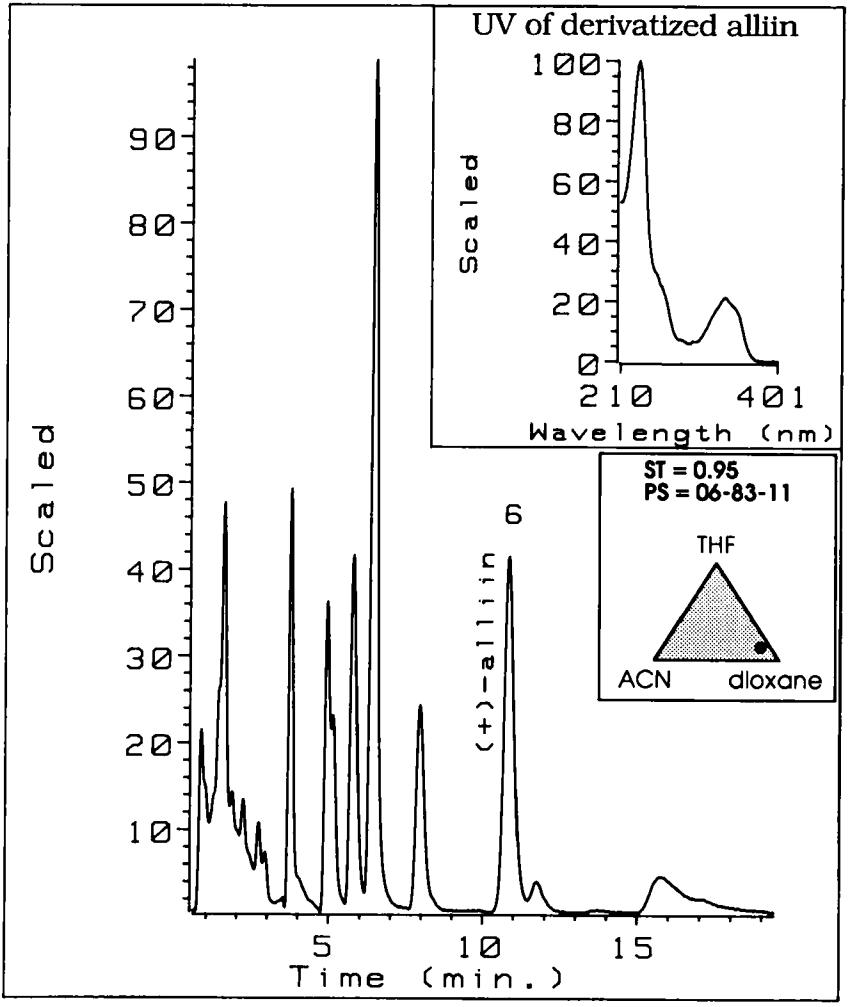


FIGURE 5. *Typical isocratic elution profile of a derivatized garlic sample. The UV spectrum of the isoindole derivative of (+)-alliin, depicted in the right upper edge, was taken on-line during the chromatographic run. Mobile phase as in Fig. 3. UV detection at 337 nm. Other chromatographic conditions as described in the experimental section.*

Selective Multisolvent Gradient Elution (SMGE) for the Separation of all the 15 Cysteine Derivatives

In a attempt to rapidly resolve both the sulfoxide and the thioether derivatives in a single chromatographic run, a suitable gradient system was developed. Two of three gradient techniques were possible: (1) Isoselective multisolvent gradient elution (IMGE) or (2) selective multisolvent gradient elution (SMGE). IMGE may be started with a mobile phase composition as was found for the isocratic separation of the sulfoxides. An increase in solvent strength will then rapidly elute the more lipophilic thioethers. Using a one step gradient, the separation may be made with a single HPLC pump. Considering the specific problems we experienced in separating the sulfoxides, a gradient system involving a change in selectivity seems more favorable. As described above, ACN showed good selectivity in resolving (+)-MCSO [P2] and (-)-ECSO [P3], while the separation of (-)-PCSO [P7] and (+)-ACSO [P6] required a large proportion of 1,4-dioxane in the mobile phase. Based on these observations, a strategy for the determination of selective gradient elution was conceived:

A) Start the gradient run using a mobile phase with sufficient ACN to resolve peaks 2 and 3.
B) Increase in the relative amount of dioxane while coincidently reducing the volume fraction of ACN to favor the separation of (-)-PCSO [P7] and (+)-ACSO [P6]
C) Increase the solvent strength in order to ensure rapid elution of the thioethers.

Preliminary experiments employing linear gradients started with mobile phases similar to the one which exhibited optimal selectivity for the isocratic separation of the sulfoxides (S_T = 0.95, P_S = 06-83-11). They were programmed to run within about 30 min to P_S = 00-100-00 with S_T = 1.75 (= 50% dioxane). The resulting chromatograms fit with the strategy

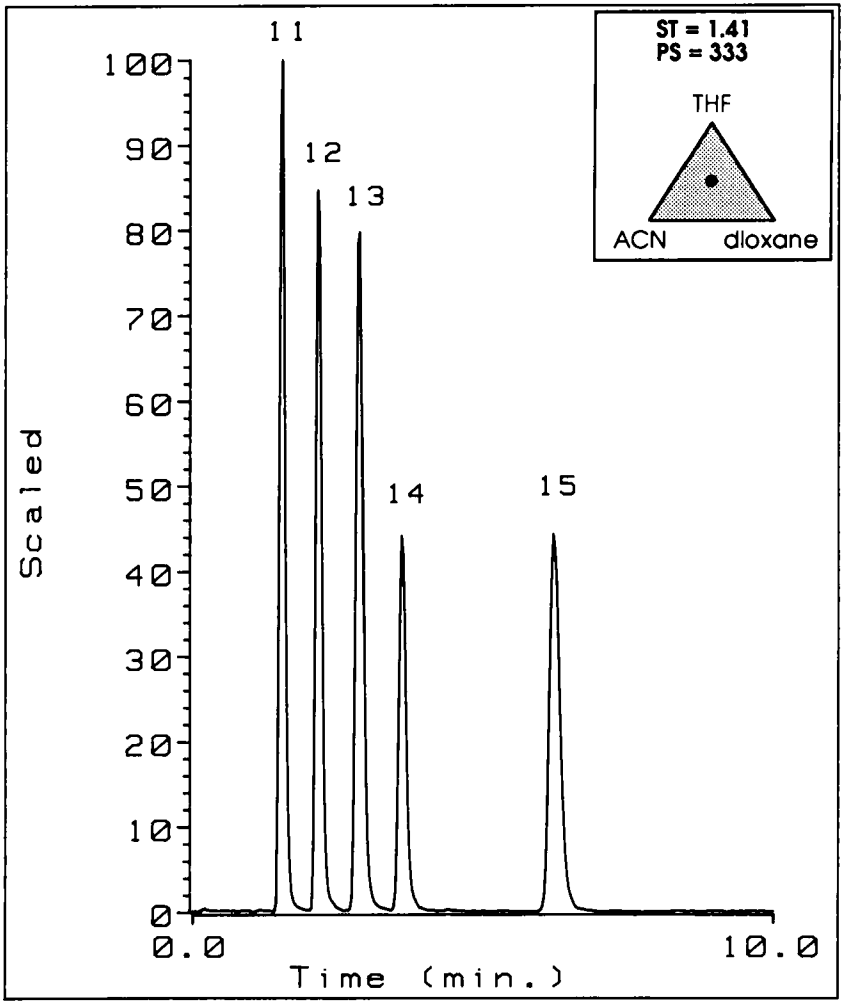


FIGURE 6. *Isocratic separation of L-cysteine thioether homologues. The mobile phase was defined by* $P_S = 333$ *and* $S_T = 1.41$ *(10.3% THF, 13.7% dioxane, 14.5% ACN, 61.4% aqueous phosphate buffer (pH 7.15)). Fluorescence detection (ex 230 nm, em 420 nm). Other parameters as described in text.*

outlined above and were easily optimized. The relative amounts of organic solvents in the mobile phase at the end of the gradient were limited to about 50% due to progressive precipitation of the buffer clogging the chromatographic system. The column temperature was increased to 30 ^{o}C in order to reduce high back pressures during gradient runs. It was found that the volume fraction of ACN could be lowered somewhat while coincidently reducing the solvent strength (S_T = 0.95 $\longrightarrow$ S_T = 0.75). This did not result in considerable loss of resolution between (-)-MCSO [P2] and (+)-ECSO [P3]. On the other side, the amount of dioxane could slightly be increased improving in combination with the programmed decrease in ACN (gradient end: 0% ACN) the separation of (+)-ACSO [P6] and (-)-PCSO [P7]. The last optimization step involved the resolution of fusing peaks of (+)-BCSO [P10] and ECS [P12]. Selective response was found when varying the relative amount of THF. Satisfactory separation was obtained by slightly increasing the proportion of THF in the mobile phase (gradient from 1.5% to 2.9% THF). In summary, the final conditions for the optimized separation of the 15 test substances, as shown in Figure 7, were as follows: linear gradient starting with a mobile phase of S_T = 0.75 and P_S = 09-87-04 and running within 32 min to S_T = 1.89 and P_S = 07-93-00.

CONCLUSIONS

The aim of this study was to rapidly develop a procedure for the HPLC separation of a total of 15 cysteine derivatives. At an early stage of the work, it became apparent that the evolution of an isocratic separation of the (±)-sulfoxide isomers was going to be difficult and could only be satisfactorily resolved by using a suitable optimization strategy. The "PRISMA" solvent optimization model, which takes advantage of the broad selectivity of a four-solvent system, proved to be an efficient tool enabling the

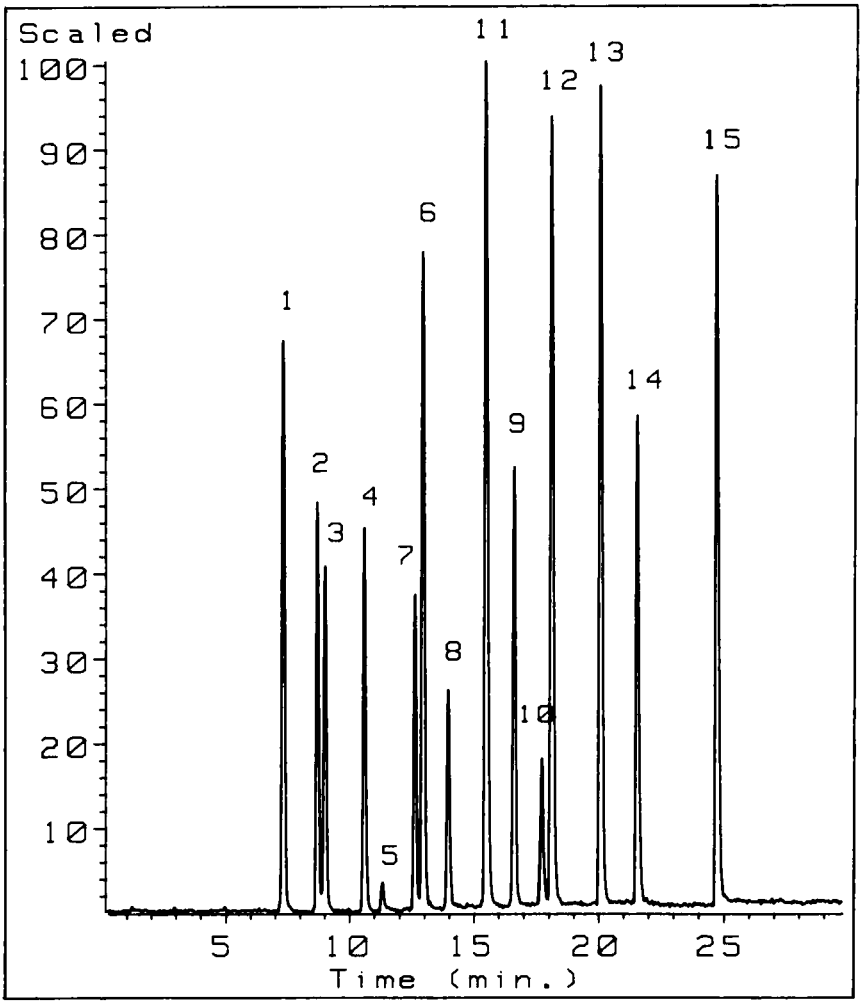


FIGURE 7. *Optimized separation of all 15 sulfur amino acids by selective multisolvent gradient elution (SMGE). Using a mixture of THF, 1,4-dioxane, ACN, and aqueous phosphate buffer of pH 7.15 the gradient was started with* $S_T = 0.75$ *at* P_S *= 09-87-04 and programmed to run linearly within 32 min to* P_S *= 07-93-00 and* $S_T = 1.89$. *Fluorescence detection (ex at 230 nm, em at 420 nm). Other chromatographic conditions as described in text.*

chromatographer to minimize the experimentation time required to search for an optimal mobile phase. It is easy to use and does not require highly sophisticated instrumentation or computers (although an automated four-solvent HPLC system is, of course, advantageous). Four-solvent systems used in RP-HPLC generally work with THF, ACN, and MeOH as organic solvents. According to the Snyder selectivity triangle (19), each represents a different group of solvents of similar properties. A change has to be considered when no satisfactory separation can be achieved with the initial optimization steps. This approach was used successfullly in this study by substituting MeOH for dioxane. It is noteworthy that both dioxane and ACN are classified in the same solvent group (VI) and were expected to provide similar chromatographic selectivity. In fact, the two solvents may be subdivided into subclasses (a, b) but there seemed to be no practical advantage to do so (19). However, the choice of a suitable substitute was empirical and was restricted to a limited number of solvents as described previously.

The efficiency of SMGE was first demonstrated by making a further improvement in the optimized isocratic separation of the (±)-sulfoxide isomers by designing a suitable change in selectivity and, secondly, in the rapid elution of the more lipophilic thioethers by increasing the solvent strength. This procedure offers the possibility to rapidly analyse the full range of the sulfur amino acids in a single chromatographic run. Further improvement of the visual approach for SMGE "optimization" by estimating more precisely optimum solvent compositions seems possible. This, however, requires adequate - and generally applicable - mathematical models and statistical algorithms for computer calculations, which to date are only partly available. On the other side, it should be emphasized that total automation of a four solvent system needs LC hardware with high composition accuracy and precision to convert theoretical optimization concepts or computer-simulated separations into practical results.

Both the isocratic and gradient procedures have successfully been applied for the analysis of garlic samples, including the quantitative determination of (+)-ACSO (= alliin). Details and characterization of the analytical procedure (extraction, sample pretreatment, calibration), as well as results of various analyses of plant extracts, will be presented in a subsequent paper (20).

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Appendix I

In the following a computer program listing is given for the calculation of the actual composition of the mobile phase defined by P_S and S_T in the regular part of the prism as described in (6). Mixtures of water (S_{H_2O} = 0) with up to 3 organic solvents may be calculated. The choice is made from a total of 7 organic solvents commonly used in RP-HPLC (solvent strength values are added in parentheses):

MeOH (2.6) / ACN (3.2) / THF (4.5) (values from Ref. 18)
ethanol (3.6) / acetone (3.4) /
1,4-dioxane (3.5) / isopropanol (4.2) (values from Ref. 15)

The program, written in Microsoft Basic 80 under CP/M 2.0B, is self-explanatory and can easily be adapted for use with other microcomputer systems. The printer subroutine (program statements 1010-1250) is optional and was written for use with a Panasonic KX-P1090 printer.

The data input for the volume fractions of the 3 selected organic solvents for a given selectivity point is shown for 2 examples:

Selectivity point (P_S)	Input of volume fractions ($\sum$=1.0)		
18-70-12	0.18	0.70	0.12
811	0.80	0.10	0.10

Program listing

```
10 REM PRISMA: CALCULATING THE COMPOSITION OF MOBILE PHASES
20 REM PROGRAM WRITTEN BY STEPHAN J. ZIEGLER IN MAY 87
30 REM   IN MICROSOFT BASIC 80, VERSION 5.0,
40 REM   UNDER CP/M, VERSION 2.08
50 REM   ON A ICM-2001 COMPUTER, ICM, TAIPEI,TAIWAN
60 REM
70 REM   PRINTER: PANASONIC KX-P1090
80 REM
90 REM
100 HOME
110 INVERSE
120 PRINT"PRISMA: SOLVENT OPTIMIZATION OF THE MOBILE PHASE"
130 PRINT
140 NORMAL
150 PRINT"Selection of 3 solvents"
160 PRINT
170 PRINT"(MEOH - ACN - ETOH - ACETONE - DIOXANE - ISOPROPANOL - THF)"
180 PRINT
190 INVERSE
200 PRINT"Solvent 1"
210 PRINT
220 NORMAL:INPUT "",SOLV$(1)
230 PRINT:INVERSE
240 PRINT"Solvent 2"
250 PRINT
260 NORMAL:INPUT "",SOLV$(2)
270 PRINT:INVERSE
280 PRINT"Solvent 3"
290 PRINT
300 NORMAL:INPUT "",SOLV$(3)
310 GOSUB 1260
320 FOR N = 1 TO 3
330 IF STRENGTH(N) <> 0 THEN GOTO 350
340 GOSUB 1440:HOME:INVERSE:PRINT "E R R O R   I N   INPUT":NORMAL:PRINT:PRINT:P
RINT "TRY AGAIN !":WAIT &HE000,128:RUN
350 NEXT
360 HOME
370 INVERSE
380 PRINT "PRISMA: SOLVENT OPTIMIZATION OF THE MOBILE PHASE"
390 PRINT
400 NORMAL
410 PRINT"Input of Volume Fractions"
420 PRINT
430 INVERSE
440 PRINT SOLV$(1),SOLV$(2),SOLV$(3)
450 NORMAL
460 PRINT
470 VTAB 7:INPUT "",VF(1)
480 IF VF(1) > 1 THEN GOSUB 1440:GOTO 470
490 VTAB 7:HTAB 15:INPUT "",VF(2)
500 IF VF(2) > (1-VF(1)) THEN GOSUB 1440:GOTO 490
510 VF(3)=1-VF(1)-VF(2)
520 IF VF(3) < .001 THEN VF(3) = 0
530 VTAB 7:HTAB 28:PRINT VF(3)
540 PRINT:PRINT:PRINT
550 INVERSE
560 PRINT"Solvent Strength of the Mobile Phase"
570 VTAB 13:NORMAL:INPUT "",ST
580 IF ST > STRENGTH(3) THEN GOSUB 1440:GOTO 570
590 PRINT:INVERSE
600 PRINT "Volume of the Mobile Phase (ml)"
610 PRINT
620 NORMAL:INPUT "",VOLTOT
630 PRINT
640 VOLFR(3) = VF(3)
650 VOLFR(2) = VF(2)*(1/STRENGTH(2)*STRENGTH(3))
660 VOLFR(1) = VF(1)*(1/STRENGTH(1)*STRENGTH(3))
670 VOL(3) = VOLFR(3)*ST/STRENGTH(3)*VOLTOT
680 VOL(2) = VOLFR(2)*ST/STRENGTH(3)*VOLTOT
690 VOL(1) = VOLFR(1)*ST/STRENGTH(3)*VOLTOT
700 HOH = VOLTOT-VOL(3)-VOL(2)-VOL(1)
710 HOME
720 INVERSE
730 PRINT"PRISMA: OPTIMIZATION OF THE MOBILE PHASE"
740 NORMAL:PRINT
750 PRINT"Calculation of the Solvent Composition"
760 PRINT:PRINT
770 PRINT "Solvent Strength of the Mobile Phase: ";ST
780 PRINT
790 PRINT "Volume: ";VOLTOT;"ml"
800 PRINT:PRINT "Volume Fractions:"
810 PRINT
820 PRINT VF(1),VF(2),VF(3)
830 PRINT
840 INVERSE
850 HOH$="WATER    "
860 PRINT SOLV$(1),SOLV$(2),SOLV$(3),HOH$
870 NORMAL
880 PRINT
```

Program listing (cont.)

```
890 PRINT USING "###.# ml       ";VOL(1),VOL(2),VOL(3),HOH
900 PRINT
910 PRINT USING "###.# %        ";100/VOLTOT*VOL(1),100/VOLTOT*VOL(2),100/VOLTOT*
VOL(3),100/VOLTOT*HOH
920 GOSUB 1440
930 INPUT "Copy on Printer: (Y / N)        ",COPY$
940 PRINT
950 IF COPY$ ="Y" THEN GOSUB 1010
960 INPUT "New Calculation: Other Solvents (S) / Other Composition (C) / END (E)
 ",NE$
970 IF NE$="E" THEN GOTO 1000
980 IF NE$="S" THEN GOTO 100
990 GOTO 360
1000 END
1010 REM SUBPROGRAM COPY TO PRINTER
1020 LPRINT CHR$(27);CHR$(87);CHR$(1)
1030 LPRINT "PRISMA: OPTIMIZATION OF THE MOBILE PHASE"
1040 LPRINT "**********************************"
1050 LPRINT CHR$(27);CHR$(87);CHR$(0)
1060 LPRINT:LPRINT
1070 LPRINT "Calculation of the Solvent Composition"
1080 LPRINT:LPRINT
1090 LPRINT "Solvent Strength of the Mobile Phase: ";ST
1100 LPRINT
1110 LPRINT "Volume: ";VOLTOT;"ml"
1120 LPRINT:LPRINT "Volume Fractions:"
1130 LPRINT:LPRINT VF(1),VF(2),VF(3)
1140 LPRINT:LPRINT
1150 LPRINT SOLV$(1),SOLV$(2),SOLV$(3),HOH$
1160 LPRINT "-------------------------------------------------"
1170 LPRINT
1180 LPRINT USING "###.# ml       ";VOL(1),VOL(2),VOL(3),HOH
1190 LPRINT
1200 LPRINT USING "###.# %        ";100/VOLTOT*VOL(1),100/VOLTOT*VOL(2),100/VOLTO
T*VOL(3),100/VOLTOT*HOH
1210 FOR K=1 TO 40
1220 LPRINT:NEXT K
1230 VTAB 21
1240 GOSUB 1440
1250 RETURN
1260 REM DATA INPUT OF SOLVENTS
1270 RESTORE
1280 FOR I=1 TO 7
1290 READ DA$,DA
1300 FOR J=1 TO 3
1310 IF DA$=SOLV$(J) THEN STRENGTH(J)=DA
1320 NEXT J
1330 NEXT I
1340 IF STRENGTH(1)>=STRENGTH(2) THEN GOTO 1370
1350 SWAP SOLV$(1),SOLV$(2)
1360 SWAP STRENGTH(1),STRENGTH(2)
1370 IF STRENGTH(1)>=STRENGTH(3) THEN GOTO 1400
1380 SWAP SOLV$(1),SOLV$(3)
1390 SWAP STRENGTH(1),STRENGTH(3)
1400 IF STRENGTH(2)>=STRENGTH(3) THEN GOTO 1460
1410 SWAP SOLV$(2),SOLV$(3)
1420 SWAP STRENGTH(2),STRENGTH(3)
1430 RETURN
1440 REM SUBPROGRAM BEEP
1450 FOR M= 1 TO 3:PRINT CHR$(7):NEXT M
1460 RETURN
1470 DATA "MEOH",2.6
1480 DATA "ACN",3.2
1490 DATA "ETOH",3.6
1500 DATA "ACETONE",3.4
1510 DATA "DIOXANE",3.5
1520 DATA "ISOPROPANOL",4.2
1530 DATA "THF",4.5
```