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QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF SULFUR CONTAINING AMINO ACIDS*

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SUMMARY

This research describes the analytical methods for the preparation, yield, structural confirmation, RMR a.a./phen. values***, and substrate interaction for the volatile trimethylsilyl (TMS) derivatives of twelve sulfur amino acids: S-methyl-L-cysteine, taurine, cysteic acid, homocystine, djenkolic acid, ethionine, methionine sulfoxide and sulfone, and L-2-thiolhistidine. For reference and comparison purposes the TMS derivatives for methionine, cysteine, and cystine were also prepared.

Bis(trimethylsilyl) acetamide (BSA) and bis(trimethylsilyl) trifluoracetamide (BSTFA) were employed as the silylating reagents. The TMS derivatives were also prepared on a macro scale, and the structural formulae were determined on the basis of elemental analysis and infrared spectra. The synthesized pure reference TMS derivatives were used in yield studies. The yields ranged from 95.5–99.2% with BSA as silylating reagent. Some difficulties due to oxidation of the sulfur atom were encountered during purification of the derivatives for the following sulfur amino acids: cysteic acid, L-2-thiolhistidine, cysteine, cystine, and homocystine. In this case working calibration curves were prepared. The TMS derivatives of the twelve sulfur amino acids can be quantitatively prepared by heating at 150° for five minutes in a closed tube. The silylation reagent–solvent ratio is $^1/_3$ v/v. BSTFA is recommended as the silylation reagent for all of the named sulfur amino acids except methionine, methionine sulfoxide, and S-methyl-L-cysteine.

The TMS derivatives were stable with respect to time in a closed tube. Separation of the derivatives was achieved on OV-1 and OV-17 columns. A reduced response was noted for methionine and L-2-thiolhistidine on an OV-22 column, and separation was not achieved for methionine sulfoxide and sulfone.

The chromatographic and instrumental conditions were: Column 0.50 w/w% OV-1 coated on 80–100 mesh acid-washed dimethylchlorosilane (DMCS) treated Chromosorb G packed into a 1 m × 3.5 mm I.D. U-shaped column, and temperature programmed from 75°–200° with rise of 4.6°/min.

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*** RMR a.a./phen. = relative molar response = $\frac{\text{area amino acid/mole}}{\text{area phenanthrene/mole}}$.

There are three natural protein sulfur amino acids (methionine, cysteine, and cystine); their gas–liquid chromatographic (GLC) and various derivative preparations have been well studied by different investigators[7–9,12]. In addition, there are various oxidized states of the natural protein sulfur amino acids, and many other sulfur amino acids that are found in plants, biological fluids, and synthetic peptides. The classification is vague and there is no set number of type of compounds in this group. A property in common is that all contain sulfur in various states of oxidation. At present, little research has been reported on the GLC and derivative preparation of these compounds. In the few literature references[2–5,10,11] the investigators have used ion-exchange chromatography, paper chromatography, and paper electrophoresis to study and to identify them.

Recently, CALDWELL AND TAPPEL[1] reported on the separation of silylated derivatives of some sulfo- and selenoamino acids and their oxidation products by gas–liquid chromatography. The trimethylsilyl (TMS) derivatives of cystine and methionine were prepared using bis(trimethylsilyl)acetamide (BSA) as silylating reagent, and heating at 90–100° for 1–15 min. Cystine and methionine were resolved from their selenium analogs on a column of 2% SE-30 coated on 90–100 mesh Anakrom SD. Also, retention times and partial resolution of the following compounds were reported: methionine sulfoxide and sulfone, cysteine, cysteinesulfinic acid, taurine, and cysteic acid.

In this investigation the sulfur amino acids were chosen on the basis of type of functional groups and the oxidation state of the sulfur and were: S-methyl-L-cysteine, taurine, cysteic acid, homocystine, djenkolic acid, ethionine, methionine sulfoxide and sulfone, and L-2-thiolhistidine. For reference and comparison purposes methionine, cysteine, and cystine were included.

The major aims of this research were to develop precise and quantitative derivatization methods for preparing the volatile TMS derivatives of the sulfur amino acids, to obtain structural information, and to investigate their chromatographic separation.

In preliminary investigations using the GEHRKE AND STALLING[8] procedure for the preparation of N-trifluoroacetyl *n*-butyl esters of the named sulfur amino acids no peaks were obtained for taurine and cysteic acid. When the acylation solvent was changed from methylene chloride to pyridine, peaks were obtained; also multiple peaks were obtained for methionine sulfone and sulfoxide, and the peaks for djenkolic acid and L-2-thiolhistidine were broad and had high retention temperatures.

In this research bis(trimethylsilyl) acetamide (BSA) and bis(trimethylsilyl) trifluoroacetamide (BSTFA)[7] were used as the silylating reagents. The pure TMS derivatives were prepared on a macro scale, and structural formulae were determined on the basis of elemental analysis and I.R. spectra. Various reaction times, temperatures, and chromatographic and instrumental conditions were investigated, and the pure TMS derivatives were used in yield studies.

EXPERIMENTAL METHOD

1. Apparatus and chromatographic conditions

(a) An F and M Model 300 linear temperature-programmed gas chromatograph with an F and M Model 1609 flame ionization attachment, and a column oven and

detector module of an F and M Model 400 biomedical gas chromatograph were used.

Conditions

Column temperature: initial 75°, final 200°
Program rate: 4.6°/min
Carrier flow N_2: 40 ml/min
Air (to detector): 450 ml/min
Hydrogen (to detector): 36 ml/min
Chart speed: $^1/_3$ in./min
Column: 1.00 m × 3.5 mm borosilicate glass column with 0.5% SE-30 coated on 60–80 mesh a.w. dimethylchlorosilane (DMCS) treated Chromosorb G.

(b) The I.R. spectra were made on a Beckman IR-10 instrument. The free acids were run by making a Nujol mull, and the TMS derivatives were run neat, or as a KBr pellet.

(c) A high vacuum all-glass molecular still was used to purify the reference standard derivatives.

(d) The elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

2. Reagents

(a) The following amino acids were purchased from the Mann Research Laboratories, New York, N.Y., and were chromatographically pure: L-djenkolic acid, L-cysteic acid monohydrate, taurine, DL-ethionine, L-methionine, DL-methionine sulfoxide, DL-methionine sulfone, L-2-thiolhistidine, S-methyl-L-cysteine, L-cysteine·HCl, L-cystine, and DL-homocystine.

(b) BSA was obtained from Aldrich Chemical Company, Inc. and it was purified by distillation before use. BSTFA was purchased from Regis Chemical Company, Inc., Chicago, Ill. Acetonitrile ("nanograde") was obtained from Mallinckrodt Co.

3. Sample preparation

(a) Preparative scale-synthesis of TMS derivatives. Two to three millimoles (0.500–1.000 g) of the amino acid were dissolved or suspended in a flask containing 5 ml of acetonitrile and 5 ml (*ca.* 25 mmoles) of BSA. The reaction mixture was refluxed under anhydrous conditions for 30 min. The excess reagents were removed by vacuum distillation, the oily residue was transferred with a capillary pipet to a micromolecular still and distilled. The purified reference derivative was stored in a sealed vial under N_2, and used for elemental analysis, I.R. studies, and recovery experiments.

(b) Analytical scale synthesis of TMS derivatives—BSA as silylating reagent. Ten (10.0) mg (*ca.* 0.04–0.08 mmole) of the sulfur amino acid and 10.0 mg of phenanthrene (internal standard) were placed in a 16 × 75 mm culture tube (screw cap with teflon liner), 0.50 ml (*ca.* 2.5 mmoles) of BSA and 1.50 ml of acetonitrile were added, the closed vial was placed in an oil bath immersed just to the top of the liquid level at 150° for 5 min, allowed to cool, and 5.0 μl were injected on the column.

(c) Analytical scale synthesis of TMS derivatives—BSTFA as silylating reagent. Ten (10.0) mg (*ca.* 0.04–0.08 mmole) of the sulfur amino acid and 10.0 mg of phenanthrene (I.S.) were placed in a 16 × 75 mm culture tube (screw cap with teflon liner), 0.50 ml (*ca.* 3.0 mmoles) of BSTFA and 1.50 ml of acetonitrile were added, the closed

vial was placed in an oil bath immersed just to the top of the liquid level at 150° for 5 min, allowed to cool, and 5.0 μl were injected on the column.

RESULTS AND DISCUSSIONS

To prepare volatile derivatives of the sulfur amino acids the N-trifluoroacetyl *n*-butyl ester derivatives were not satisfactory mainly for solubility reasons. Methionine sulfone and methionine sulfoxide gave multiple peaks, this was an indication of breakdown during the derivatization, and the derivatives of djenkolic acid and L-2-thiolhistidine were not very volatile. In 1966 KLEBE *et al.*[9] reported a new silylation reagent, bis(trimethylsilyl) acetamide. This reagent has been widely and successfully used for the preparation of volatile TMS derivatives of amino acids, sugars, steroids, and many other organic class molecules with an ionizable proton. Preliminary investigations yielded good derivatives with this reagent. The molar response relative to phenanthrene and retention temperatures of a number of different sulfur amino acids are presented in Table I.

TABLE I

RELATIVE MOLAR RESPONSE AND RETENTION TEMPERATURE OF SULFUR CONTAINING AMINO ACID TMS DERIVATIVES[a]

Amino acid	*RMR a.a./phen.*[b]				*Retention temperature (°C)*
	1	*2*	*3*	*Average*	
S-Methyl-L-cysteine	0.535	0.548	0.540	0.541	80
Cysteine	0.701	0.690	0.695	0.695	100
Taurine	0.305	0.325	0.312	0.314	108
Cysteic acid	0.587	0.613	0.592	0.597	118
Cystine	1.112	1.087	1.107	1.102	160
Homocystine	1.681	1.703	1.692	1.692	165
Djenkolic acid	1.076	1.066	1.070	1.070	175
Methionine	0.657	0.661	0.659	0.659	95
Ethionine	0.602	0.646	0.621	0.623	98
Methionine sulfoxide	0.566	0.557	0.562	0.561	115
Methionine sulfone	0.643	0.655	0.647	0.648	120
L-2-Thiolhistidine	0.477	0.478	0.479	0.478	155

[a] 0.50 ml BSA and 1.50 ml acetonitrile in a closed tube at 150° for 5 min.

[b] RMR a.a./phen. = relative molar response $= \frac{\text{area amino acid/mole}}{\text{area phenanthrene/mole}}$.

Average sigma of 0.009. RSD range of 0.46–3.53%.

To determine the most suitable reaction conditions for derivatization, experiments were conducted in which the samples were heated at various temperatures for different times. The RMR a.a./phen. was highest when the reaction vessel was heated at 150° for 5 min (Table II).

For recovery and structural studies the TMS derivatives were prepared on a macro scale, and purified by molecular distillation. The boiling point and mm Hg pressure at which these derivatives were obtained are listed in Table III. Five of the

TABLE II

RELATIVE MOLAR RESPONSE OF SULFUR CONTAINING AMINO ACID TMS DERIVATIVES PREPARED AT DIFFERENT TEMPERATURES[a]

Amino acid	*Silylation conditions and RMR a.a./phen.*		
	50° 5 min	*100° 5 min*	*150° 5 min*
S-Methyl-L-cysteine	0.491	0.541	0.541
Cysteine	0.570	0.632	0.695
Taurine	0.310	0.310	0.314
Cysteic acid	0.594	0.592	0.597
Cystine	0.871	0.920	1.102
Homocystine	0.720	0.817	1.692
Djenkolic acid	1.080	1.050	1.070
Methionine	..653	0.658	0.659
Ethionine	0.595	0.625	0.623
Methionine sulfoxide	0.549	0.563	0.561
Methionine sulfone	0.592	0.648	0.648
L-2-Thiolhistidine	0.466	0.496	0.478

[a] 0.50 ml BSA and 1.50 ml acetonitrile in a closed tube.

sulfur amino acid TMS derivatives were so prone to oxidation that they could not be purified under the conditions employed or even under a N_2 atmosphere (cysteic acid, L-2-thiolhistidine, cysteine, cystine, and homocystine). The pure TMS derivatives were analyzed for elemental composition and these data are given in Table IV. I.R. spectra of the TMS derivatives gave evidence that the NH_2, SO_3H, SH, and COOH groups were trimethylsilylated. The following absorption bands are an indication of silylation; the symmetrical $Si-CH_3$ deformation band at approx. 1250 cm^{-1}; the $Si-CH_3$ stretching band at approx. 755 and 840 cm^{-1}; and a shift in the carboxyl band from the free acid at approx. 1580–1720 cm^{-1} indicates that the carboxyl group has been silylated. Refer to Fig. 1 for a representative I.R. spectrum showing the indicated silylated functional groups.

In recovery experiments an *exact* amount of the synthesized reference sulfur

TABLE III

BOILING POINT OF SULFUR AMINO ACID REFERENCE TMS DERIVATIVES

Amino acid	*B.P. (°C) range*	*mm of Hg*
S-Methyl-L-cysteine	72– 75	0.020
Taurine	70– 72	0.010
Djenkolic acid	90– 93	0.020
Methionine	82– 84	0.005
Ethionine	88– 90	0.010
Methionine sulfoxide	91– 93	0.005
Methionine sulfone	110–112	0.005

The TMS derivatives were colorless, viscous, oily liquids when maintained under anhydrous conditions, on exposure to air they solidified to a yellowish wax.

TABLE IV

ELEMENTAL ANALYSIS OF SULFUR AMINO ACID TMS DERIVATIVES

Amino acid	*Empirical formula*	*Mol. weight*	*Analysis (%)*					
				C	*H*	*N*	*S*	*Si*
S-Methyl-L-cysteine	$C_{10}H_{25}NO_2Si_2S$	279.32	Calc.	42.99	8.95	5.01	11.47	20.10
			Found	42.50	8.62	5.12	11.24	19.97
Taurine	$C_{11}H_{31}NO_3Si_3S$[a]	341.41	Calc.	38.69	9.02	4.10	9.39	24.67
			Found	38.84	9.02	4.26	9.69	23.54
Djenkolic acid	$C_{19}H_{46}N_2O_4Si_4S_2$	542.51	Calc.	42.06	8.47	5.15	11.79	20.70
			Found	42.51	8.64	5.12	11.42	20.73
Methionine	$C_{11}H_{27}NO_2Si_2S$	293.33	Calc.	45.04	9.20	4.77	10.93	19.14
			Found	45.25	9.40	4.90	11.10	19.37
Ethionine	$C_{12}H_{29}NO_2Si_2S$	307.34	Calc.	46.89	9.44	4.55	10.43	18.27
			Found	46.90	9.49	4.53	10.69	18.61
Methionine sulfoxide	$C_{11}H_{27}NO_3Si_2S$	309.33	Calc.	42.70	8.73	4.53	10.36	18.15
			Found	42.61	8.68	4.50	10.20	18.30
Methionine sulfone	$C_{11}H_{27}NO_4Si_2S$	325.33	Calc.	40.60	8.30	4.30	9.85	17.26
			Found	40.14	8.12	4.11	10.02	17.36

[a] Note that both hydrogens of the NH_2 group were silylated.

amino acid TMS derivative was dissolved in acetonitrile in a 10 ml volumetric flask. To this flask 10.0 mg of phenanthrene were added as internal standard. The sample was mixed well and made to volume with acetonitrile. The area response of the reference TMS derivative was compared to the analytical sample prepared as follows:

Ten (10.0) mg of the sulfur amino acid and 10.0 mg of phenanthrene were derivatized using procedure 3b. After completion of the reaction the sample was made to 10.0 ml volume with acetonitrile and analyzed. Five (5) microliters were injected on the column.

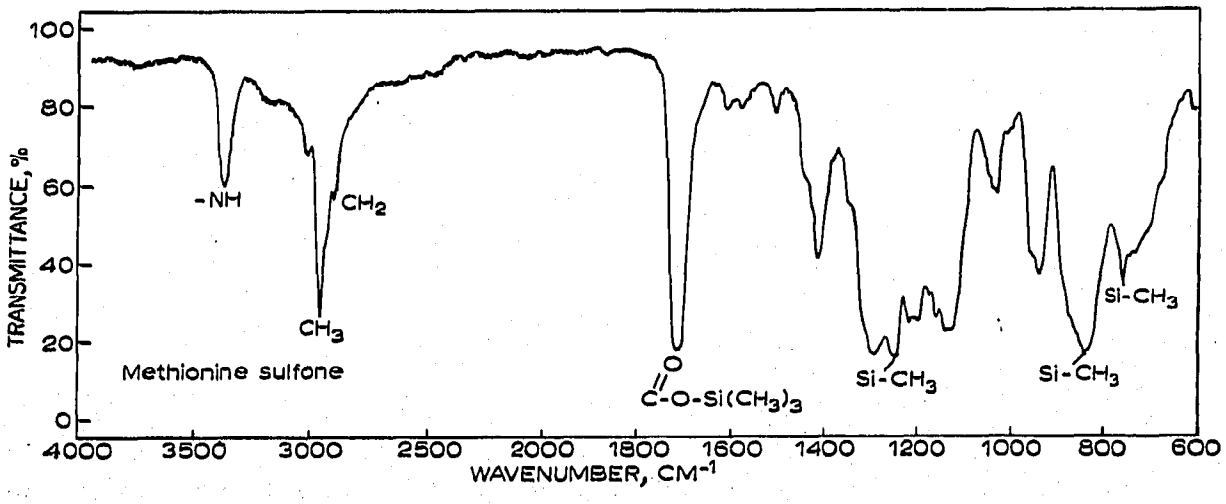


Fig. 1. I.R. spectrum of TMS derivative of methionine sulfone, neat film, Beckman IR-10.

The recovery experiments are based on the fact that *equal amounts* of internal standard (I.S.) were added to the sample and to the pure reference amino acid flasks.

The yields were calculated by comparing the detector response for the analytical sample taken through the method with that for the reference standard derivatives and are expressed in per cent.

Per cent conversion of amino acid to TMS derivative

$$\% \text{ Yield} = \frac{A_{\text{a.a.}}/A_{\text{I.S. with a.a.}}}{A_{\text{R}}/A_{\text{I.S. with R}}} \times \frac{W_{\text{R (used)}}}{W_{\text{R (calc.)}}} \times 10^2. \quad (1)$$

$W_{\text{R (calc.)}} \cong W_{\text{a.a. (used)}}$ is calculated from:

$$\frac{W_{\text{a.a.}}}{M_{\text{a.a.}}} = \frac{W_{\text{R (calc.)}}}{M_{\text{R}}}. \quad (2)$$

Therefore:

$W_{\text{R (calc.)}} = W_{\text{a.a.}} \cdot (M_{\text{R}}/M_{\text{a.a.}})$;

and substituted in (1)

$$\% \text{ Yield} = \frac{A_{\text{a.a.}} \times A_{\text{I.S.}_{\text{R}}} \times W_{\text{R}} \times M_{\text{a.a.}}}{A_{\text{I.S.}_{\text{a.a.}}} \times A_{\text{R}} \times W_{\text{a.a.}} \times M_{\text{R}}} \times 10^2. \quad (3)$$

$A_{\text{a.a.}}$ = Peak area for amino acid derivative in analytical method (*ca.* 10 mg),
$A_{\text{I.S.}_{\text{a.a.}}}$ = Peak area for internal standard in amino acid sample flask, 10 mg,
A_{R} = Peak area for weighed amount of pure reference amino acid,
$A_{\text{I.S.}_{\text{R}}}$ = Peak area for internal standard in pure reference amino acid flask, 10 mg,
$W_{\text{a.a.}}$ = Weight of amino acid used,
$M_{\text{a.a.}}$ = Molecular weight of amino acid,
W_{R} = Weight of pure reference amino acid used,
M_{R} = Molecular weight of the reference TMS derivative.

The percent conversion of seven of the sulfur amino acids to their respective TMS derivatives ranged from 95.5–99.2% (Table V). However, difficulties were encountered in synthesizing the reference TMS derivatives of cysteic acid, L-2-thiolhistidine, cysteine, cystine, and homocystine. During removal of solvent, excess BSA, and vacuum distillation, the products became dark colored and multiple peaks were obtained on chromatography. In this case calibration curves were prepared by making three independent analytical determinations at four different concentrations (1.0, 2.0, 5.0, and 10 mg of amino acid), and the experimental area for the amino acid divided by

TABLE V

PER CENT CONVERSION OF SULFUR AMINO ACID TO TMS DERIVATIVES

Amino acid	*Yield of derivative (%)*		*Average (%)*
	A	*B*	
S-Methyl-L-cysteine	96.9	97.1	97.0
Taurine	99.4	99.0	99.2
Djenkolic acid	98.6	98.7	98.6
Methionine	98.2	98.3	98.2
Ethionine	97.2	97.6	97.4
Methionine sulfoxide	95.2	95.8	95.5
Methionine sulfone	97.1	97.5	97.3

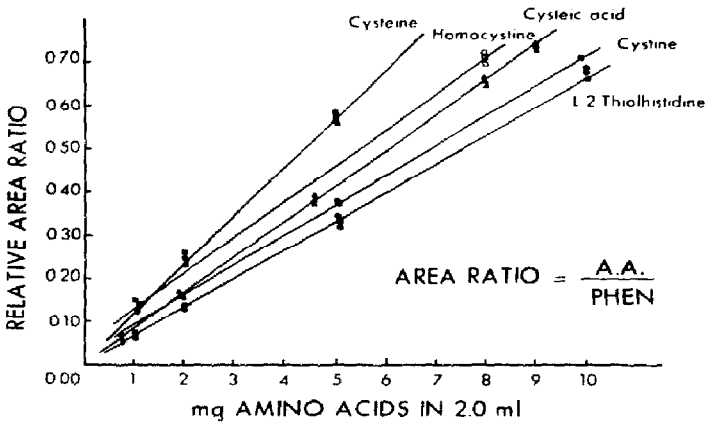


Fig. 2. Calibration curves for TMS sulfur amino acids.

the area for the phenanthrene (I.S.) was plotted as a function of concentration. Representative calibration curves are given in Fig. 2.

Experiments were made to investigate the stability of the sulfur amino acid TMS derivatives as a function of standing in an open vial under normal laboratory conditions. The derivatives were prepared by Method 3b, then a 5.0 μl aliquot was immediately injected on the column and the area determined.

The sample vial was left open and standing on the laboratory bench, 5.0 μl aliquots were injected on the column at the end of 1, 6, and 24 h. A very significant decrease in RMR a.a./phen. was observed after one hour for cysteic acid and taurine. Also, as expected, a gradual decrease was noted in RMR for the other sulfur amino acids, this is an indication of hydrolysis of the TMS derivative back to the free acid. The data are given in Table VI. After 24 h standing in an open vial at room temperature no peak was obtained for cysteic acid, L-2-thiolhistidine, cysteine, taurine, and cystine.

TABLE VI

STABILITY OF SULFUR AMINO ACID TMS DERIVATIVES AS A FUNCTION OF TIME[a,b]

Amino acid	*RMR a.a./phen. and time in hours*			
	0	*1*	*6*	*24*
S-Methyl-L-cysteine	0.541	0.401	0.345	[c]
Cysteine	0.695	0.413	0.347	[c]
Taurine	0.314	0.126	0.108	0.013
Cysteic acid	0.597	0.076	[c]	[c]
Cystine	1.102	0.920	0.766	[c]
Homocystine	1.692	1.530	1.400	0.981
Djenkolic acid	1.070	0.991	0.792	0.618
Methionine	0.659	0.488	0.442	0.335
Ethionine	0.623	0.598	0.522	0.442
Methionine sulfoxide	0.561	0.553	0.504	0.365
Methionine sulfone	0.648	0.628	0.607	0.534
L-2-Thiolhistidine	0.478	0.478	0.169	[c]

[a] Relative molar response with respect to phenanthrene as I.S.
[b] Sample allowed to stand in open vial.
[c] TMS derivative was hydrolyzed.

TABLE VII

RELATIVE MOLAR RESPONSE OF SULFUR AMINO ACIDS USING VARIOUS LIQUID PHASES[a]

Amino acid	*RMR a.a./phen. and substrates*		
	SE-30	*OV-17*	*OV-22*
S-Methyl-L-cysteine	0.541	0.548	0.550
Cysteine	0.695	0.695	0.699
Taurine	0.364	0.360	0.370
Cysteic acid	0.597	0.590	0.585
Cystine	1.102	1.070	1.080
Homocystine	1.692	1.711	1.720
Djenkolic acid	1.070	1.037	0.988
Methionine	0.659	0.652	0.490
Ethionine	0.623	0.635	0.669
Methionine sulfoxide	0.561	0.567	[b]
Methionine sulfone	0.648	0.650	[b]
L-2-Thiolhistidine[c]	1.019	1.012	0.793

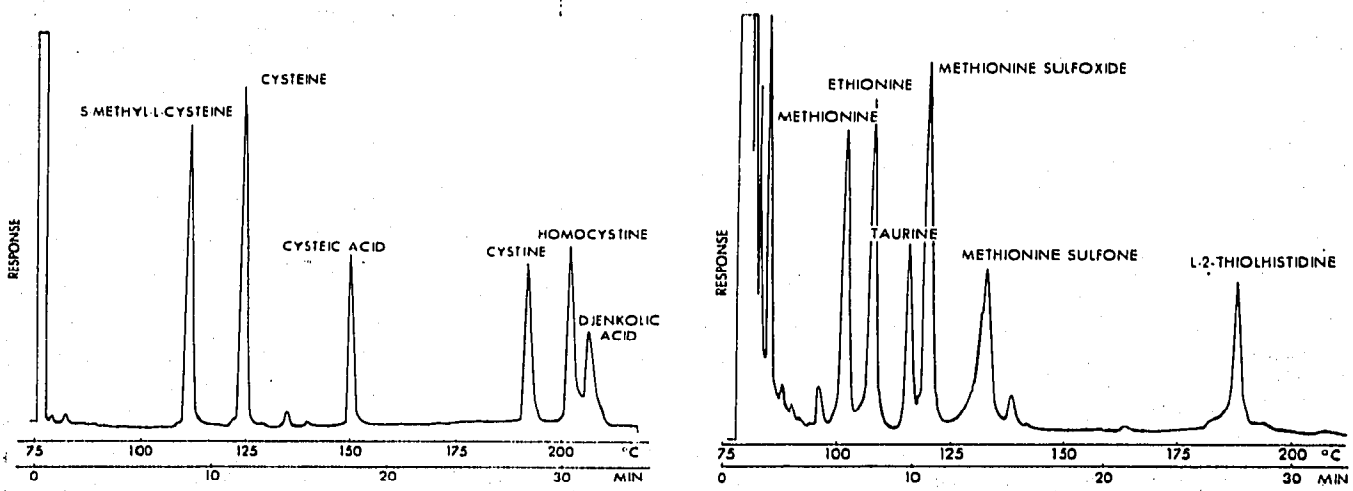
[a] 0.50 w/w % of liquid phase coated on 80–100 mesh acid washed DMCS treated Chromosorb G.

[b] Did not separate from phenanthrene.

[c] Derivative prepared using BSTFA as silylating reagent, all others with BSA.

A similar experiment was made on the stability of the derivatives as a function of time in a closed vial. No breakdown was observed on standing for 24 h.

The interaction of different substrates with the sulfur amino acid TMS derivative was investigated using siloxane polymer liquid phases of different polarity. The substrates were: SE-30 (OV-1), with 100% methyl groups; OV-17 (DC-550), a siloxane polymer with 50% methyl and 50% phenyl groups; and OV-22, a siloxane polymer with 26% methyl and 64% phenyl groups. The liquid phase was coated at a concentration of 0.5 w/w % on 60–80 mesh a.w. DMCS treated Chromosorb G and the results are expressed in terms of RMR a.a./phen (Table VII). It was concluded that the OV-1 or OV-17 liquid phases could be used interchangeably for the analysis of these



Figs. 3 and 4. Chromatography of TMS sulfur amino acids. Column: 0.50 w/w % SE-30 on 80–100 mesh a.w.; DMCS treated Chromosorb G; 1.0 m × 3.5 mm I.D. glass; initial temp. 75°; program. 4.6°/min; N_2 flow 40 ml/min. Injected mixture contained *ca.* 5 μg of each amino acid.

independent runs were made and the results were similar (Table IX). Methionine and S-methyl-L-cysteine had lower RMR values using BSTFA as the silylating reagent, and methionine sulfoxide was completely destroyed, five small peaks were observed on the chromatogram for this compound. The RMR values for L-2-thiolhistidine and taurine were larger with the BSTFA reagent.

Based on these studies BSTFA is recommended as the silylation reagent for all of the named sulfur amino acids except methioniné, methionine sulfoxide, and S-methyl-L-cysteine (Table VIII). Since it is very unlikely that all of these amino acids will be present in biological fluids at the same time the above recommendation is justified. The minimum detectable limit (MDL) at a 2/1 signal/noise with respect to the flame ionization detector was found to be *ca.* 5 ng injected on the column.

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