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Compositional analysis of the phenylthiocarbamyl amino acids by liquid chromatography–atmospheric pressure ionization mass spectrometry with particular attention to the cyst(e)ine derivatives

Karl Schmeer[a], Mohamed Khalifa[b], János Császár[c], Gyula Farkas[d], Ernst Bayer[a], Ibolya Molnár-Perl[b,*]

[a]*Institute of Organic Chemistry, Tübingen University, Auf der Morgenstelle 18, D-7400 Tübingen 1, Germany*
[b]*Institute of Inorganic and Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112, Hungary*
[c]*Institute of Organic Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112, Hungary*
[d]*Chinoin Pharmaceutical Works, P.O. Box 110, H-1325 Budapest, Hungary*

Abstract

An approach presented recently for the high-performance liquid chromatographic determination of DL- and L-cystines and cysteines as phenylthiocarbamyl (PTC) derivatives has been completed (i) with the determination of D-cystine/cysteine, (ii) by a study relating to the spectral characteristics of the PTC cystine/cysteine derivatives performed with diode-array detection and (iii) by the fragment analysis of the PTC-cyst(e)ine derivatives, applying the extremely soft conditions of the liquid chromatography–atmosphere pressure ionization mass spectrometry (LC–API-MS). In accordance with earlier experiences it has been repeatedly demonstrated that cystines and cysteines can be determined on the basis of two or four derivatives, with the same retention times and molar responses, without any special pretreatment, furnishing a simple possibility for the determination of the total cystine/cyteine in protein hydrolysates. The UV spectra of all four PTC derivatives provided by diode-array-detected purity parameters (PuP) showed that the corresponding PTC derivatives of DL-L- and D-cystine and -cysteine are identical and homogeneous, and do not show any characteristic differences. On the basis of the fragmentation patterns of seventeen PTC-amino acids and those of six PTC-cyst(e)ines, obtained under the very soft conditions of the LC-API-MS procedure, it can be stated that two characteristic PTC derivatives are formed: (i) the main product ("a" and "b" diastereoisomers, >80% of the total), shown to be the fragment of $m/z = 255$, a monomeric cysteine derivative, and (ii) the other characteristic constituent ("c" and "d" stereoisomers, <20% of the total), consisting of a fragments of $m/z = 255$ and its oxidised form of $m/z = 287$.

1. Introduction

The unusual behaviour of the D-, L- and DL-cysteines/cystines (monomers/dimers) in their interactions with phenyl isothiocyanate (PITC) has been reported recently [1–7]. It has been shown that independently of their original condition, i.e. from the monomeric cysteines and dimeric cystines equally, the same phenylthiocarbamyl (PTC) derivatives are formed. On the basis both of our experiences [1] and of literature data [2–4], we assumed that under the deri-

* Corresponding author.

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vatization conditions oxidative scission of the dimeric cystine disulphide bond takes place, resulting in two monomeric PTC-cysteine derivatives. The oxidative cleavage of the disulphide bond has been reported in the reaction of allyl isothiocyanate with cystine [2,3] and proved by NMR measurements [3]. Liquid chromatography–thermospray mass spectrometry (LC–TSP-MS) seemed to be to confirm this fact [4]; it has been demonstrated [4] that (i) reconstructed single-ion mass chromatograms corresponding to the protonated molecular ion for each PTC amino acid in the mixture, with a single exception, displayed a peak coinciding with the elution of the corresponding PTC amino acid and (ii) the exception was PTC-cystine, for which the largest ion observed within the scanned range was at m/z 255; this ion represents the monomer product formed from the PTC-derivatized cysteine dimer by fragmentation. Taking into consideration the forceful intervention of the LC–TSP-MS (thermospray nozzle and chamber at 320 and 340°C), the fragmentation can be presumed not only in the derivatization reaction but also in the thermospray evaporation process.

The aim of this study was to examine the composition and the behaviour of the different PTC-cyst(e)ine derivatives applying the well known mild conditions of API-MS.

2. Experimental

2.1. Materials

Triethylamine (TEA), PITC, amino acids and proteins were obtained from Sigma (St. Louis, MO, USA) and Serva (Heidelberg, Germany). HPLC-grade acetonitrile and methanol were purchased from Reanal (Budapest, Hungary). All other reagents of the highest purity available.

2.2. Apparatus

High-performance liquid chromatography (HPLC) with UV detection

The system used for the determination of the molar responses of the various racemates was a Liquochrom Model 2010 liquid chromatograph (Labor MIM, Budapest, Hungary), which consisted of a two-Liquopump 312/1 solvent-delivery system and a Type OE-308 UV detector with a wavelength range of 195–440 nm ("Liquochrom system"). Samples were injected in 20-μl volumes using an injector supplied by Labor MIM.

HPLC with diode-array detection

A Varian LC Star system was used, equipped with a Star 9020 workstation, a Star 9010 solvent-delivery system and a Polychrom 9065 diode-array detector (Varian, Walnut Creek Instrument Division, Walnut Creek, CA, USA). The same columns, eluents and elution programmes were applied as with the Liquochrom system.) The 150 mm × 4.0 mm I.D. columns (BST, Budapest, Hungary) contained Hypersil ODS bonded phase (5 μm; Shandon) ("Varian LC Star system").

Liquid chromatography–atmospheric pressure ionization mass spectrometry

The mass spectrometer used was an API III TAGA 6000 with an ionspray interface (Sciex, Thornhill, Toronto, Canada), with a Model 22 syringe infusion pump 22 (Harvard Apparatus, South Natick, MA, USA).

The HPLC column (100 × 2 mm I.D.) contained Nucleosil 120 C_{18} (5μm) (Grom, Herrenberg, Germany). The system consisted of a microbore HPLC pump, an ABI model 140A solvent-delivery system (Applied Biosystems, Foster City, CA, USA), Linear UV/VIS 204 Model 9550-0155 UV detector, (Linear Instruments, Reno, NV, USA), a C-R5A Chromatopac integrator, (Shimadzu, Analytical Instrument Division, Kyoto, Japan) and a Gilson Abimed Model 231 autosampler ("LC–API-MS system").

2.3. Hydrolysis system

A Pico Tag workstation (Millipore Waters, Milford, MA, USA) was used.

2.4. Hydrolysis of amino acids [simulated hydrolysis conditions ("hydrolysis")]

Authentic amino acids were dissolved in 0.1 *M* hydrochloric acid. Aliquots of 5 μl of stock solutions containing ca. 0.017 g of DL-, D- or L-cysteine · HCl or ca. 0.024 g of DL-, D- or L-cystine base per 10 cm^3 (weighed with 10^{-5} g accuracy) were pipetted into 50 × 6 mm I.D. tubes and up to twelve tubes were placed in a vacuum vial. The vial was then attached to the work station manifold and the solvent removed under vacuum. After drying, the vacuum was released and 200 or 900 μl of constant-boiling hydrochloric acid were pipetted into the bottom of the vacuum vial. The vacuum vial was then reattached to the manifold and, after treatment as recommended in the workstation manual, they were hydrolysed in the workstation at 145°C for 4 h. After this hydrolysis, the sample tubes were carefully removed from the vial and the hydrochloric acid wiped from the outside of each tube. The tubes were then transferred to a fresh reaction vial, attached to the workstation and evaporated to the constant minimal reading (about 65 mTorr). The hydrolysed samples were then ready for derivatization.

2.5. Derivatization of amino acids with PITC

Standards of individual cyst(e)ine samples (containing ca. 50.0 nmol of each amino acid), were placed in the 50 × 6 mm I.D. tubes and dried under vacuum. Free amino acids and hydrolysed proteins were redried after adding 10 μl of ethanol–water–TEA (2:2:1) to each tube. Thereafter, to each redried sample, 20 μl of derivatization reagent [ethanol–TEA–H_2O PITC (7:1:1:1)] was added and mixed by vortex mixing. The test-tubes containing the derivatized samples were then transferred in to the vial, installed again in the workstation, evaporated to a minimum reading of about 65 mTorr and held at this constant reading for 20 min. The derivatized standards were dissolved, in order of listing, in 50 μl of acetonitrile, 50 μl of water and 500 μl of 0.05 *M* sodium acetate solution with a pH of 7.2. Thus, the 20-μl aliquots of standards contained ca. 1700 pmol of each amino acid.

2.6. Separation of the PTC-amino acids by HPLC

The solvent system consisted of two eluents: (A) 0.05 *M* sodium acetate (pH 7.2) and (B) 0.1 *M* sodium acetate–acetonitrile–methanol (46:44:10) (mixed in volume proportions and titrated with glacial acetic acid or 50% sodium hydroxyde to pH 7.2). A gradient, which was optimized for the separation, from 0% to 100% B in 22 min was applied. After a 5-min washing step with 100% B, the eluent was programmed to 100% in 2 min. After an additional 3 min, elution with 100% A was performed. Thereafter the system was ready for the next injection.

3. Results and discussion

The new approach presented recently for the HPLC of DL- and L-cystine and -cysteine as their PTC derivatives [1] has been completed, in order to have a comprehensive overview, by a study of the D-racemates.

The reaction of all six racemates with PITC resulted in four derivatives both before and after hydrolysis (Figs. 1A and 2A, peaks a, b, c and d). The ratios of these four derivatives are characteristic of the initial compound.

Without any pretreatment, i.e., immediately after derivatization (Figs. 1A and 2A), the PTC derivatives of D- and L-cystine and -cysteine all elute as a main single peak (Figs. 1 and 2, peaks b) and as a minor accompanying peak (Figs. 1 and 2, peaks c). The trace amounts of compounds a and d, with D- and L-cystine and -cysteine are of negligible importance (Figs. 1A and 2A).

The DL-racemates reacted directly with PITC (Figs. 1A and 2A) furnished without exception all four derivatives with different distributions. The ratios of peaks a and b are characteristic of DL-cystine and DL-cysteine, respectively (Table 1, peak-area ratios, in order of listing: $a/b \approx 1.9$ and 1.17).

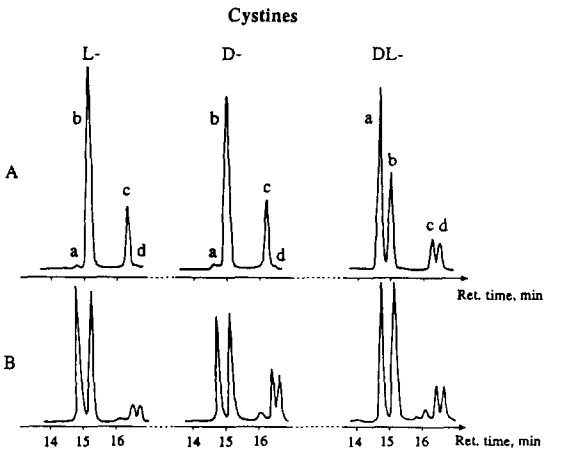

Fig. 1. HPLC of PTC-cystines derivatized (A) immediately and (B) after hydrolysis obtained with the Liquochrom system. Detailed data are given in Table 1.

After simulating the hydrolysis conditions (Figs. 1B and 2B), independently (a) of the parameters applied (varying the amount of HCl, reaction time and temperature) and (b) of the initial racemate form of cystines and cysteines reacted, the ratios of the two first-eluting PTC derivatives (Figs. 1B and 2B, peaks a and b, Table 1, peak-area ratios, column B) proved to be nearly the same ($a/b \approx 1$). The identity of the peak-area ratios after hydrolysis in all six cases ($a/b \approx 1$) can probably be attributed to the racemization of the D- and L-cystine and cysteine.

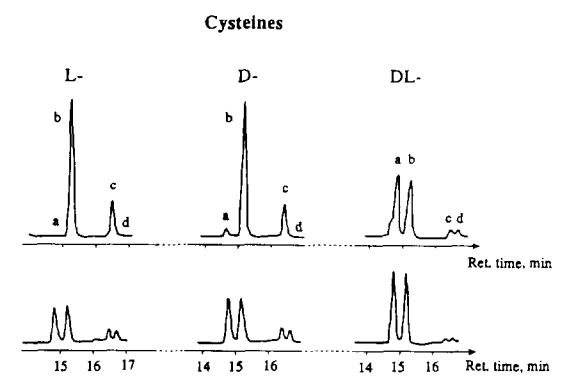

Fig. 2. HPLC of PTC-cysteines derivatized (A) immediately and (B) after hydrolysis, obtained with the Liquochrom system. Detailed data are given in Table 1.

Evaluating the detector responses of all six cyst(e)ines (Table 1, integration units/pmole amino acids), they are acceptable, with an experimental error of 7.2% or less (relative standard deviation). The same reproducibility can be calculated from the differences in the main values of the various racemates obtained before (Table 1, from the values in column A: 5.6%) or after hydrolysis (Table 1, from the values in column B 7.2%). It is worth mentioning that after hydrolysis the averages of the detector responses are smaller, probably owing to the destruction of cyst(e)ines. This effect can be avoided by the use of 3-(2-aminoethyl)indole as an additive in the HCl hydrolyses [5–7]. On the basis of the reproducibility data detailed above and published recently [1], the analytical applicability the proposed method has been demonstrated.

The characteristics of the spectra of PTC-cyst(e)ines in the UV region between 200 and 350 nm were analysed by diode-array detection (Table 2, Fig. 3). As can be seen, the maximum values of the absorbances of the corresponding PTC derivatives [Table 2, PuP (purity parameter) values for peaks a–d], independently of their origin, and within the experimental error of the measurements, are the same: the PuP of absorbances for peaks a and b varied from 254.63 to 254.84 nm, whereas for peaks c and d the corresponding values were higher, providing maximum absorbances between 255.60 and 256.19 nm. The small differences in the maximum values between the earlier (peaks a, b) and later eluting derivatives (peaks c and d) are probably due to the slightly different spectral characteristics of the two stereoisomer pairs, assuming that in addition to the NH_2 group, partly also the SH group can take part in any reaction.

Concerning the structure of the PTC-derivatives, it is obvious that we are measuring in all six cases either (i) the PTC-cystines (assuming that under hydrolysis/derivatization conditions the monomeric cysteines were oxidized to –S–S– bond-containing dimeric cystines) or (ii) the

Table 1
Distribution of the PTC derivatives of cystines and cysteines, measured by HPLC, derivatized (A) immediately and (B) after hydrolysis

Compound	Peak areas: integration units/ pmol amino acids (in total)[a]		Peak-area ratios: peak a/ peak b	
	A	B	A	B
DL-Cystine $\bar{X}$[b]	138	127	1.91/1	0.99/1
S.D.	4.6	1.0	0.11	0.068
R.S.D. (%)	3.3	0.8	3.9	6.8
DL-Cysteine $\bar{X}$[b]	125	110	1.17/1	1.01/1
S.D.	3.0	5.9	0.255	0.031
R.S.D. (%)	2.4	3.3	2122.8	3.1
D-Cystine $\bar{X}$[b]	137	123	0/1	0.96/1
S.D.	9.6	4.2		0.027
R.S.D. (%)	7.2	3.4		2.8
D-Cysteine $\bar{X}$[b]	121	114	0.1/1[c]	1.01/1
S.D.	7.0	2.8	0.576	0.079
R.S.D. (%)	5.8	2.5	58	7.8
L-Cystine $\bar{X}$[b]	133	129	0/1	0.95/1
S.D.	9.4	4.5		0.034
R.S.D. (%)	7.1	3.5		3.6
L-Cysteine $\bar{X}$[b]	124	110	0.1/1[c]	1.00/1
S.D.	4.1	6.2	0.098	0.056
R.S.D. (%)	3.3	5.3	98	5.6
Means of A and B $\bar{X}$	130	119		
S.D.	7.2	8.6		
R.S.D. (%)	5.6	7.2		

[a] In total: taking into account peaks a, b, c, d at (a) 936 s, (b) 966 s, (c) 1036 and (d) 1050 s (see also Fig. 1).
[b] $\bar{X}$: average of six separate tests.
[c] Averages obtained from the ratios of D- or L-cyteines, in order of listing, 0.072/1, 0.0065/1, 0.10/1, 0.23/1 (for D-cysteine) and 0.03/1, 0.003/1, 0.18/1, 0.19/1 (for L-cysteine), respectively.

Table 2
Evaluation of the spectra of various cyst(e)ines obtained by diode-array detection

Sample	Spectral analyses over of 200–350 nm for derivatives of peaks							
	Peak a		Peak b		Peak c		Peak d	
	PuP	S.D.	PuP	S.D.	PuP	S.D.	PuP	S.D.
DL-Cystine	254.84	0.047	254.78	0.050	255.87	0.507	255.60	0.195
L-Cystine	–	–	254.63	0.11	–	–	255.62	0.140
D-Cystine	–	–	254.68	0.073	–	–	255.62	0.086
D-Cysteine	254.79	0.006	254.65	0.122	255.72	0.045	256.19	0.138
D-Cysteine	253.96	0.028	254.68	0.112	–	–	–	–

Spectral Overlay Report

No.	Name PuP (nm)	tR (min)	Spectrum Type	Correction	Filename
1	DL-Cystine 311				
	254.831	17.092	Spectral Sum	--------	v0707003.run
2	DL-Cystine 311				
	254.700	17.534	Spectral Sum	--------	v0707003.run
3	D-Cystine 253				
	254.659	17.539	Spectral Sum	--------	v0707004.run
4	L-Cystine 338				
	254.610	17.506	Spectral Sum	--------	v0707005.run
5	DL-Cysteine 4				
	254.789	17.016	Spectral Sum	--------	v0707007.run
6	DL-Cysteine 4				
	254.639	17.432	Spectral Sum	--------	v0707007.run
7	D-Cysteine 113				
	254.681	17.262	Spectral Sum	--------	v0707009.run

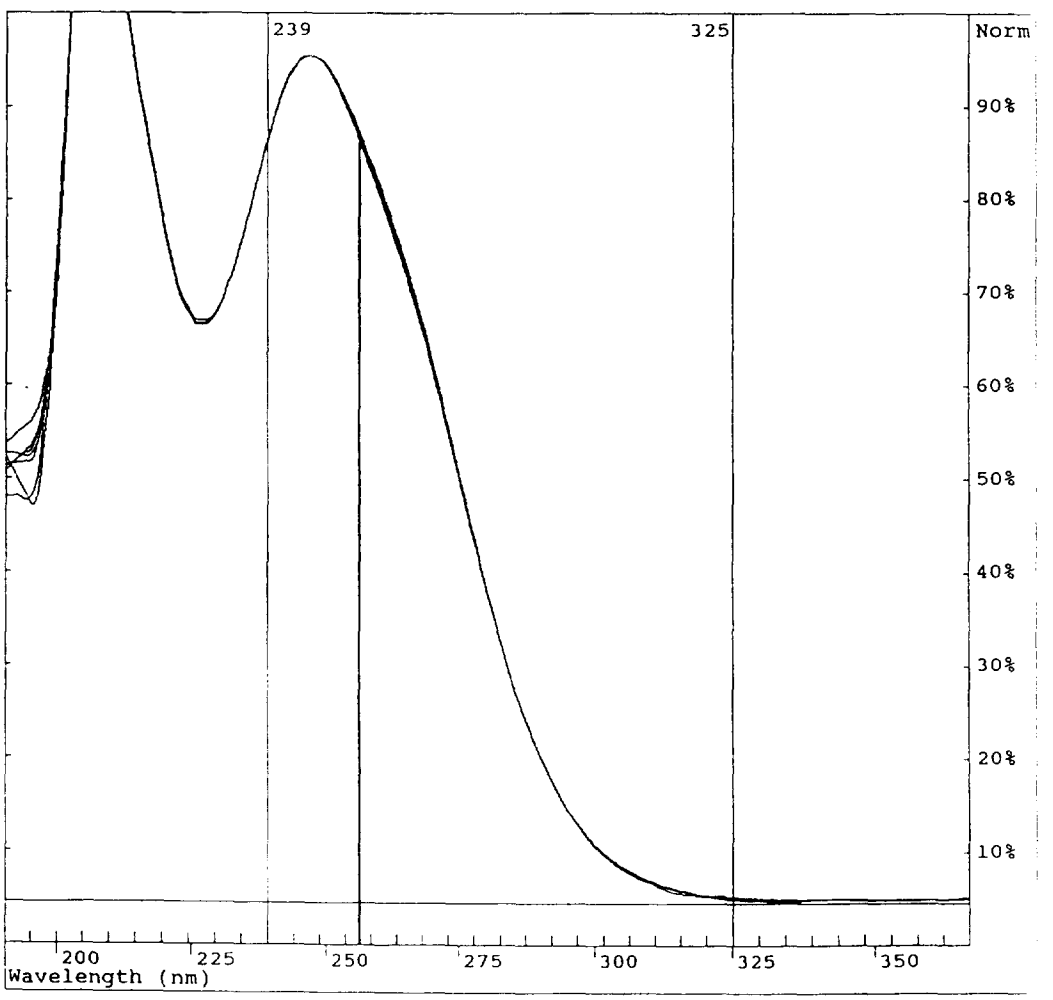

Fig. 3. Spectral overlay report on the PuP conditions of peaks a and b, obtained with the Varian LC Star system. Detailed data are given in Table 2.

PTC-cysteines. In accordance both with our spectrophotometric results [1] and with literature data [2–4], (a) we proved [1] that the characteristic shoulder peak of cysteines does not show any changes after hydrolysis conditions (oxidation to cystine under the hydrolysis conditions can be excluded); (b) Kawakishi and Namiki [2,3] verified by NMR spectrometry that the electrophilic attack of allyl isothiocyanate on cystine resulted in the scission of the disulphide bond yielding 2 mol of cysteine derivatives and, in addition to all of the above, (c) LC–MS studies [4], applying thermospray ionization, proved that the PTC derivative formed from cystine is a monomeric species of particularly high stability; Pramanik et al. [4] found "evidence for a pyrolytic cleavage process that yielded PTC and the free amino acid... in all cases except PTC-cys". All these results indicate that very likely with cysteine the composition of this PTC derivative must differ considerably from all others.

In order to determine the composition of the six PTC-cyst(e)ine derivatives, also in comparison with all other PTC-amino acids, a detailed LC-MS study was performed applying the very soft conditions of the API technique. Fragmentation patterns obtained both with seventeen PTC-amino acids (Table 3, Fig. 4) and with all six PTC-cyst(e)ines (Table 4, Fig. 5) derivatized immediately and after 'hydrolysis' conditions showed that concerning the components of the calibration standard (Table 3, Fig. 4), the main fragment proved to be, in all cases investigated, the protonated molecular ion. Chosen representatives, such as (A) methionine, (B) leucine, (C) phenylaline and (D) tryptophane, provided monomeric fragments of m/z 284.8, 266.8, 301.0 and 340.4, instead of the calculated $M + 1 = m/$

Table 3
Fragment patterns of PTC-amino acids obtained from full-scan LC–MS background-subtracted total ion current (TIC) chromatogram and positive-ion API mass spectra

Amino acid	Retention time[a] (min)	Molecular mass			
		M + 1		M + 1/D + 1	%D + 1[b]
		Calculated	Measured		
Aspartic acid	5.43	269.4	269.0	–[c]	
Glutamic acid	6.18	283.1	283.0	–	
Hydroxy-proline	6.75	267.1	267.0	–	
Serine	7.70	241.1	240.0	–	
Glycine	8.06	211.1	211.0	–	
Histidine	9.26	291.2	291.0	291/581	5
Threonine	9.49	255.1	255.0	255/509	3
Alanine	9.99	225.1	225.9	225/449	3
Proline	10.20	251.1	251.0	251/501	10
Arginine	10.76	310.2	310.0	310/619	2
Tyrosine	14.21	317.2	317.2	317/633	10
Valine	14.53	253.2	253.0	253/505	13
Methionine	15.21	285.2	284.8	285/569	16
Isoleucine-leucine	17.01	267.2	266.8	266/533	20
Phenylalanine	18.96	301.2	301.0	301/601	13
Tryptophan	19.69	340.2	340.4	340/679	12
Lysine	20.57	417.2	417.4	Out of mass range	

[a] Taken from the detailed mass spectra.
[b] Expressed as the total of [(M + 1) + (D + 1)].
[c] Dashes indicate that no dimer (D) was found.

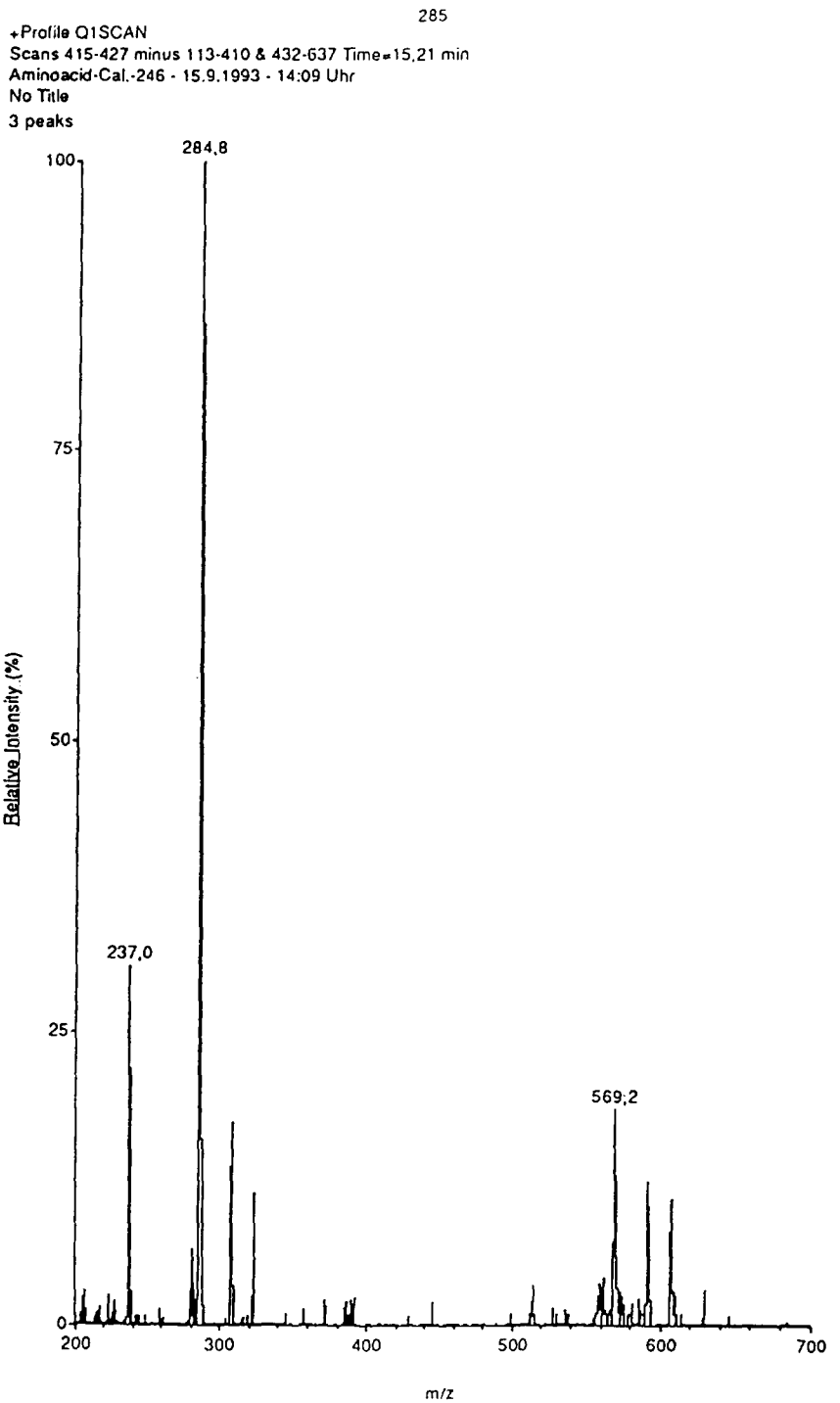

Fig. 4. Fragmentation patterns of PTC-leucine, obtained with the LC–API-MS system. Detailed data and also for all other amino acid derivatives are given in Table 3.

Table 4
Fragment patterns of the PTC-cyst(e)ines (DL-, D- and L-) derivatized before and after hydrolysis (H), obtained from peaks a, b, c and d by full-scan LC–MS background-subtracted total ion current (TIC) chromatograms and positive-ion API mass spectra

Initial compound	Mass fragments[a] expressed as % of the total obtained from peaks		
	Peak a (~15.6)[b] 255/511	Peak b (~16.0)[b] 255/511	Peaks c + d (~17.7–18.0)[b] 255/287/543
DL-Cysteine	85/15	85/15	44/38/18
DL-Cysteine (H)	85/15	85/15	45/44/11
DL-Cystine	83/17	92/8	0/78/22
DL-Cystine (H)	80/20	80/20	47/32/21
D-Cysteine		89/11	51/35/14
D-Cysteine (H)	87/13	85/15	46/41/13
D-Cystine		84/16	53/37/10
D-Cystine (H)	83/17	83/17	44/33/13
L-Cysteine		84/16	52/28/20
L-Cysteine (H)	87/13	87/13	41/41/18
L-Cystine		85/15	48/40/12
L-Cystine (H)	84/16	85/15	41/37/22

[a] Fragments: m/z 254.2–255, 286.6–287.2, 510.2–511.2 and 543.8–547.2, listed as 255, 511, 287 and 543.
[b] Retention times (min) are given in parentheses.

$z = 285.2$, 267.2, 301.2 and 340.2, respectively. The calculated and measured values (Table 3, columns 3 and 4) agree excellently, confirming that the API technique is a very low-energy process which does not induce fragmentations. In addition, the amounts of the protonated dimer ($2M + 1 = D + 1$), present in twelve cases out of seventeen, vary between 2 and 20%. The appearance of the dimers is probably attributable to the specificity of the PTC-amino acid derivatives and it is of primary importance from the point of view of the composition of the PTC-cyst(e)ine derivatives (Table 4, columns for peaks a, b and c + d).

As seen, for PTC-cystines and-cysteines, the ratios of the monomers to the dimers, in peaks a and b equally, vary between 8 and 20% (Table 4, columns 2 and 3, not exceeding the amount of dimer fragments found with other PTC-amino acids. In peaks c and d the amounts of dimers proved to be higher: as maximum values, for DL- and L-cystines 22% of the total were obtained (Table 4, last column). These results confirmed unambigously our earlier assumption [1], supported also by literature evidence [2–4], that in the interaction of PITC with cystines, as a result of the oxidative scission of the disulphide bond, moneric PTC-cysteine derivatives are formed.

A further question to be answered is to establish the exact composition of peaks a, b, c and d). Fragment patterns of the earlier eluting peak pair a and b, differ from that of the later pair c and d, providing the same fragments within the given pair. Thus, a and b and also c and d assumed to be diastereoisomer derivatives. Peaks a and b (instead of the calculated m/z 257 i.e., $M + 1 = 256 + 1$), furnished fragments of $m/z \approx 255$ (Table 4, columns 2 and 3), corresponding to the formula $C_{10}H_{10}N_2O_2S_2$. To describe the structure of this compound, containing two fewer protons than the regular PTC-cysteine, even with the knowledge of its outstandingly high stability [4], needs further study. (In contrast with all other PTC-amino acids, also under the extremely strong evaporation parameters of the thermospray ionization technique [4], the fragment of m/z 255, originating from cystine, proved to remain intact.)

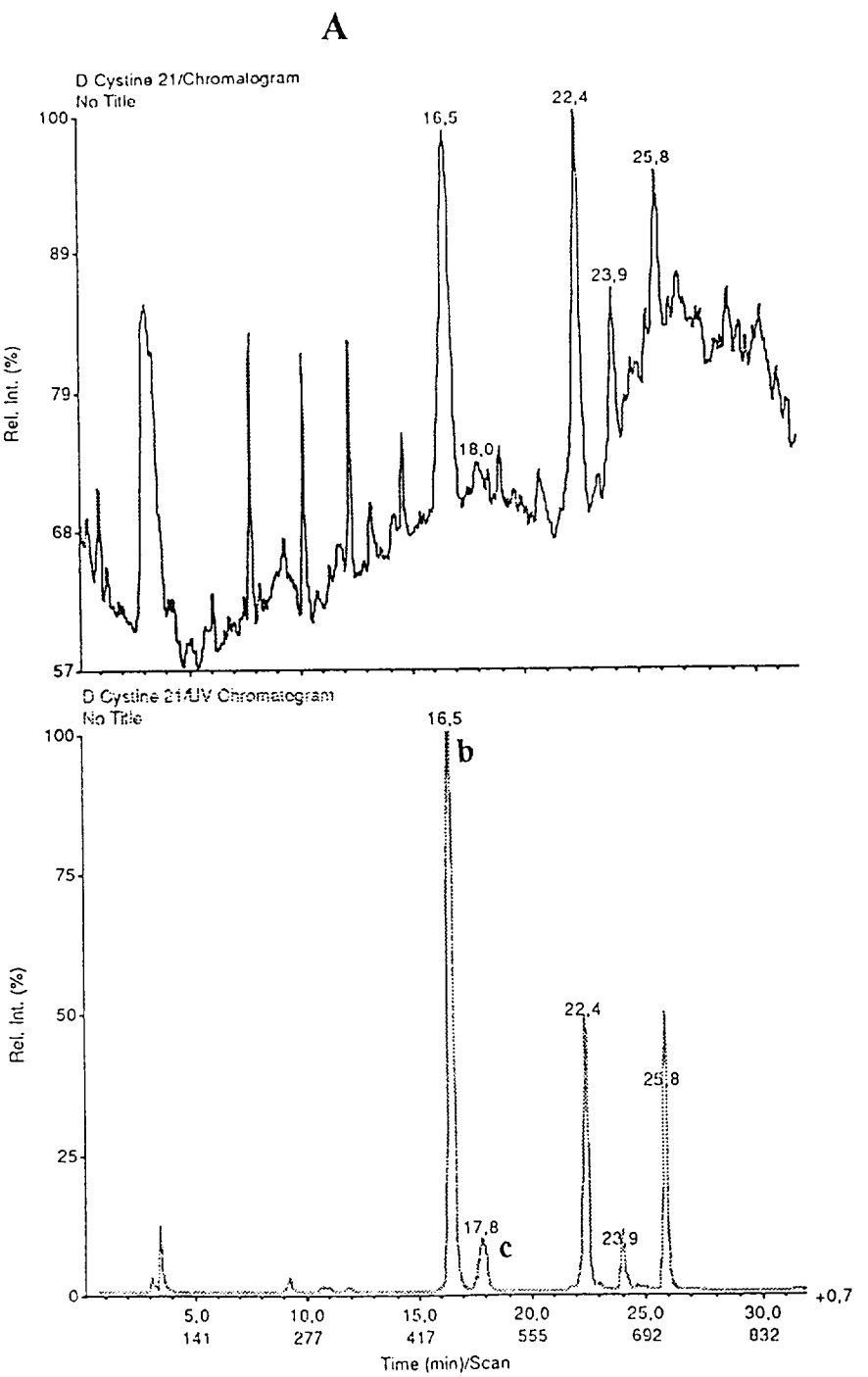

Fig. 5. (A,C) Ultraviolet absorption (254 nm) and total ion current (m/z 200–850) chromatograms and (B–D) fragmentation patterns of PTC-D-cystines. (A,B) Derivatized immediately. (A) Chromatograms of peaks b and c, retention times 16.5 and 17.8–18.0 min; (B) API-MS of peaks b and c. (C,D) Derivatized after simulating hydrolysis conditions. (C) chromatograms of peaks a, b, c and d, retention times 16.0, 16.4, 17.9 and 18.1–18.3 min; (D) API-MS of peaks a, b and c + d. Detailed data are given in Table 4. Chromatograms and mass spectra were obtained with the LC–API-MS system.

B

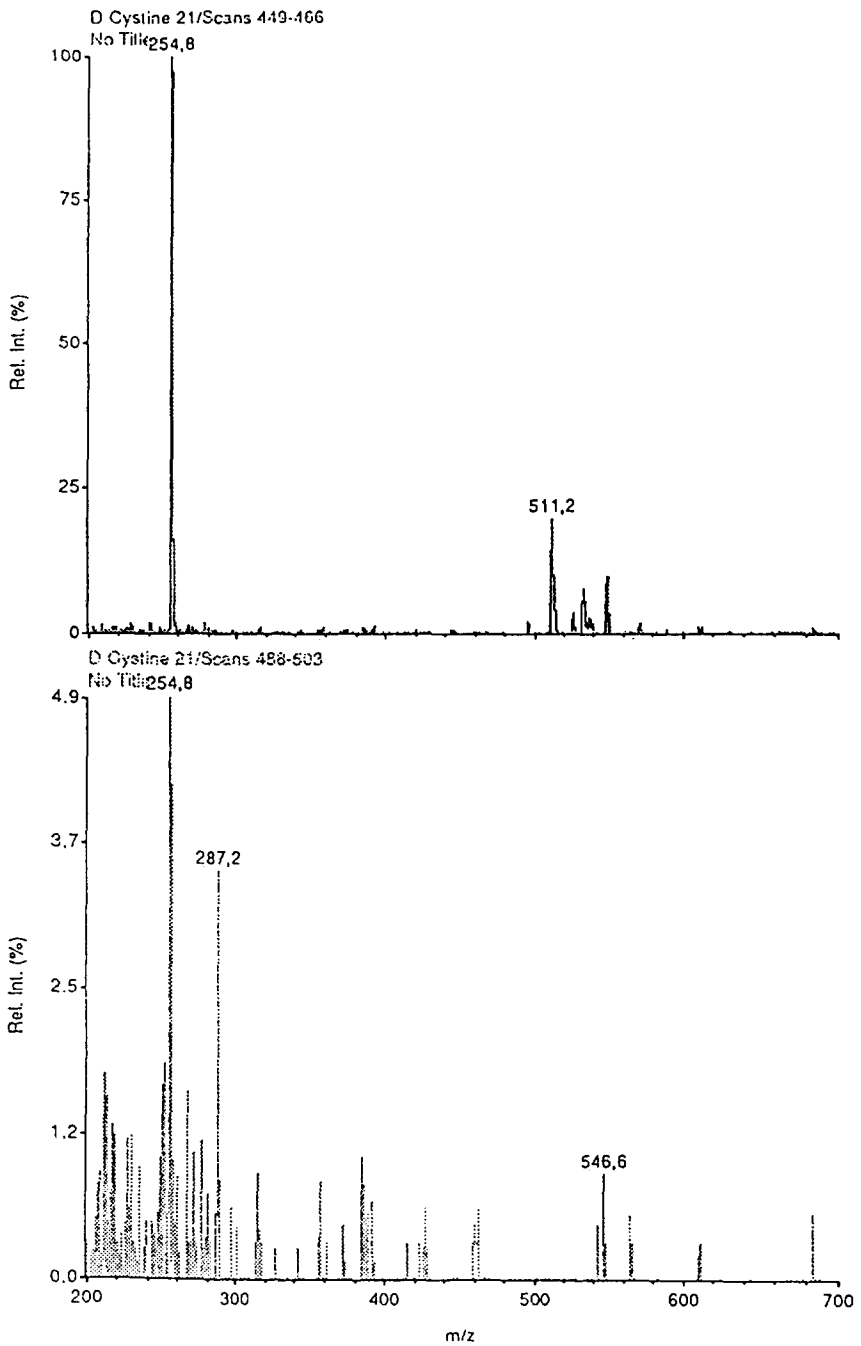

Fig. 5. (*Continued on p. 296*)

C

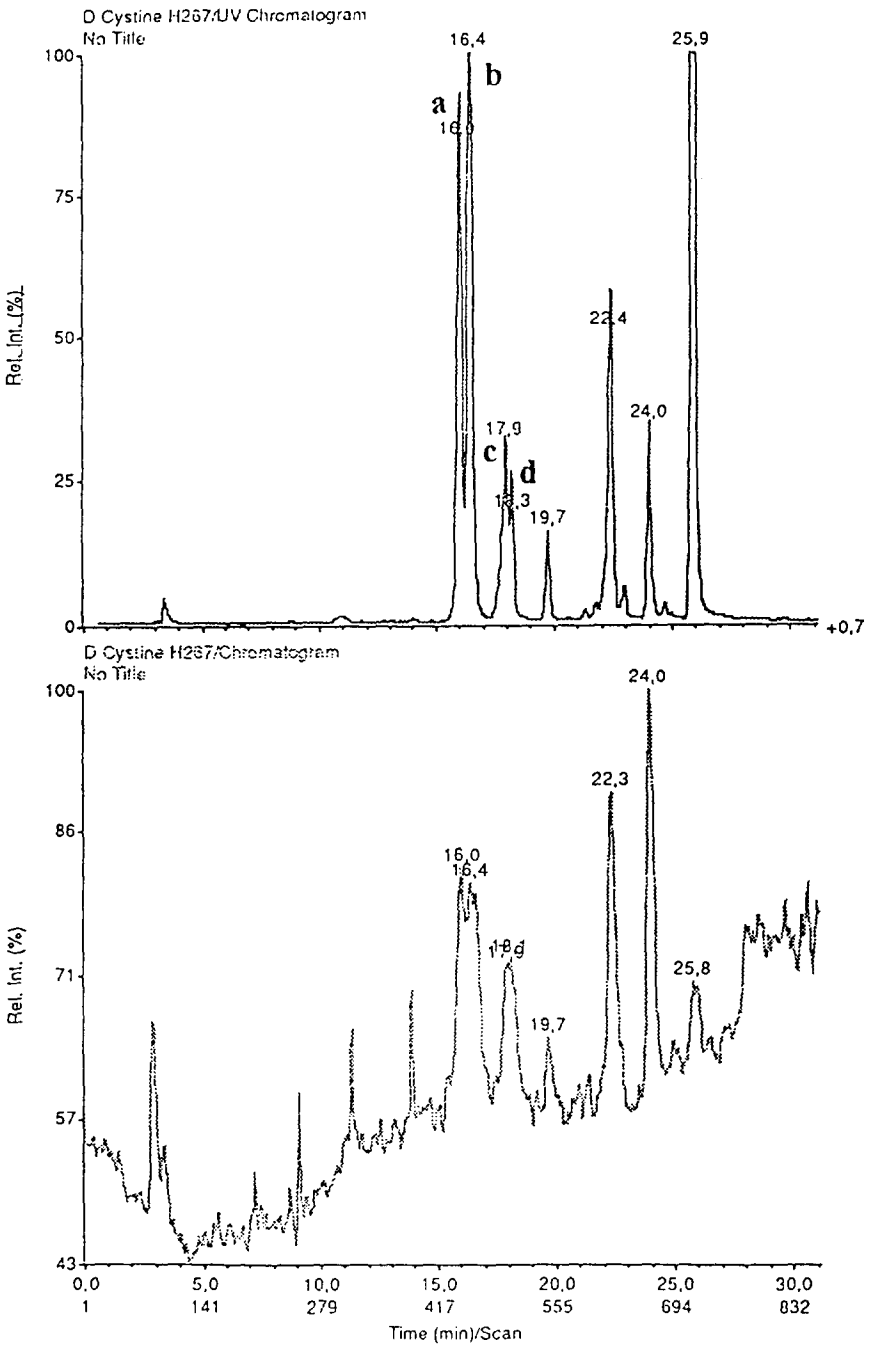

Fig. 5. (*Continued*)

D

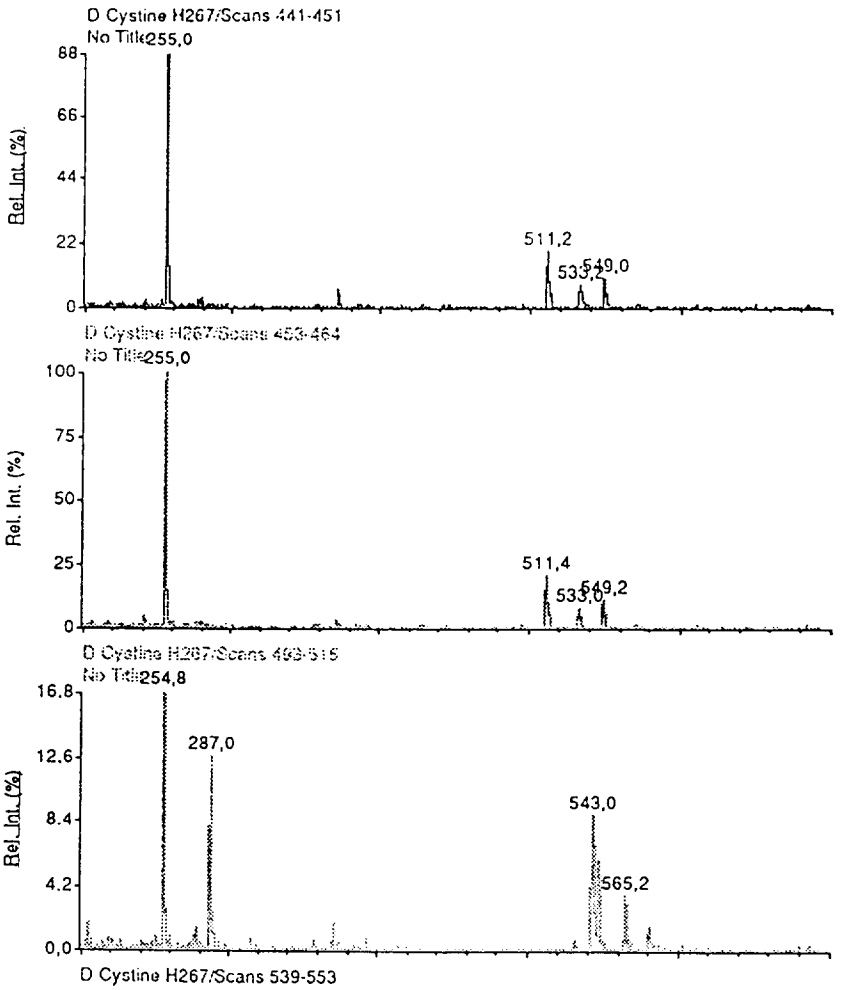

Fig. 5. (*Continued*)

Concerning the calculated masses of the monomeric (m/z 256 + 1) and dimeric (m/z 512 + 1) fragments of PTC-cyst(e)ines, shown by a detailed fragmentation profile of peak a obtained from derivatized L-cystine [Fig. 6A (monomers) and B (dimers)], they also exist, representing ca. 5–6% of the total, in case of both the monomeric (Fig. 6A, $M + 1 = m/z \approx$ 257) and the dimeric (Fig. 6B, $2M + 1 = m/z \approx$ 513) derivatives. Moreover, fragments with one proton less, indicating an intermediate position between the calculated (m/z 257) and abundant fragments (m/z 255), are also present (Fig. 6A, m/z 256; Fig. 6B, m/z 512), providing also 5–6% of the total of cyst(e)ine derivatives.

Regarding the composition of the c and d peak pair (Table 4, last column), they consist of a compound providing three fragments: such as

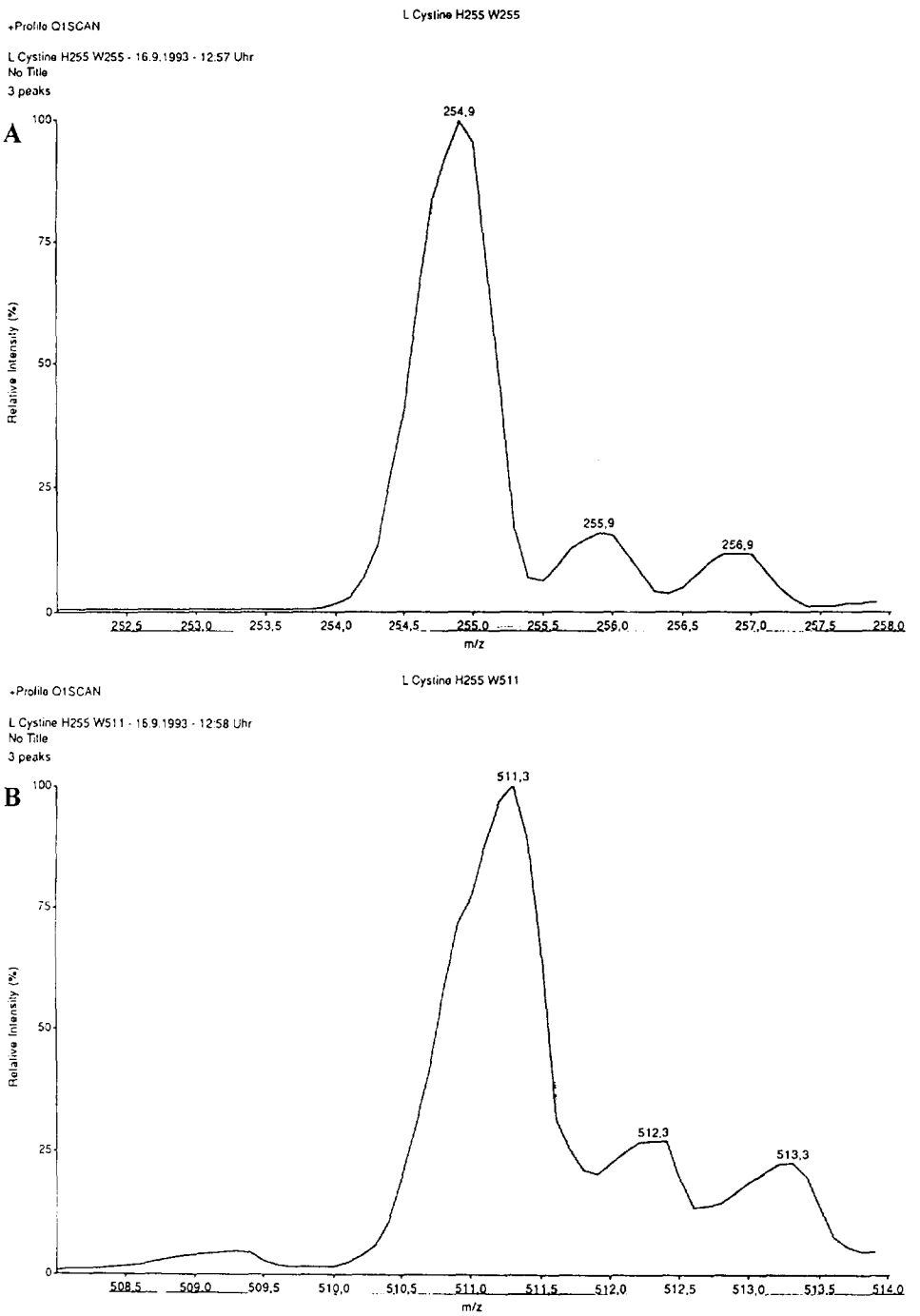

Fig. 6. Detailed fragmentation profile provided by (A) the monomeric and (B) the dimeric fragments obtained from peak a of PTC-L-cystine.

m/z 255, $m/z = 287$ (which corresponds to the protonated PTC cysteine sulphone), as well as their protonated dimer, $m/z = 543$.

4. Conclusions

On the basis of the fragmentation patterns of seventeen PTC-amino acids and six PTC-cyst(e)ines, obtained under the very soft conditions of the LC–API-MS procedure), we can state that two characteristic monomer PTC derivatives are formed. The PTC-cysteine monomers appear as two stereoisomer pairs: (i) the main product (a and b diastereoisomers, >80% of the total) proved to be a fragment of $m/z = 255$, a monomeric cysteine derivative; and (ii) the other characteristic constituent (c and d stereoisomers, <20% of the total) consisting of a fragment of $m/z = 255$ and its oxidized version of $m/z = 287$.

Taking into account all the literature data and our results detailed above, it can be concluded that the electrophilic attack of PITC on cystine results in oxidative scission of the disulphide bond, giving two PTC-cysteine derivatives.

In addition to the unambiguous evidences of the mechanism regarding the phenylthiocarbamylation of cyst(e)ines, the analytical utility of this work can be emphasized: the identical molar response values of the PTC derivatives of cystine and cysteine provide a simple approach for their determination in protein hydrolysates, without any special, previous pretreatment.

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