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Gas chromatography–chemical ionization mass spectrometry in amino acid analysis of pyoverdins

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Abstract

The application and advantages of positive chemical ionization with methane [CI(CH_4)] for gas chromatography–mass spectrometry (GC–MS) of perfluoroacyl amino acid alkyl esters are discussed. All investigated amino acids show $[M+H]^+$, $[M+C_2H_5]^+$, and $[(M+H)-C_3H_6]^+$. By a comparison of the electron ionization (EI) and CI(CH_4) mass spectra the analysis of amino acid mixtures by GC–MS will give reliable results also in the case of isomeric compounds or of (partial) decomposition during analysis. © 1997 Elsevier Science B.V.

Keywords: Detection, GC; Derivatization, GC; Mass spectrometry; Pyoverdins; Amino acids; Histidine; Hydroxyhistidine

1. Introduction

Pyoverdins are the typical siderophores (ion chelating compounds) which are produced by the fluorescent group of the bacterial genus *Pseudomonas* when grown in an ion-deficient surrounding [1]. They comprise a peptide chain of six to 12 partially non-proteinogenic and/or modified amino acids. Their identification by gas chromatography–mass spectrometry (GC–MS) after total hydrolysis is an important step in structure elucidation. It will be shown that the combination of EI and CI(CH_4) mass spectrometry gives the best results, but selection of the most adequate derivatization technique may be essential especially for polyfunctional amino acids.

GC–MS is the method of choice for the analysis of amino acids after the total hydrolysis of proteins and other substances containing peptide chains [2,3]. Amino acids usually are analyzed by GC–MS after suitable derivatization. The most frequently used method is the two-step preparation of (perfluoro)acyl alkyl esters, such as *N*(*O*,*S*)-trifluoroacetyl amino acid *n*-butyl esters [4], *N*-acetyl amino acid *n*-propyl esters [3], *N*-pentafluoropropionyl-amino acid *n*-propyl esters [5], *N*-pivaloyl-isopropyl esters [3]. Silylation allows one-step derivatization, especially the preparation of *tert.*-butyldimethylsilyl derivatives [6]. Another approach involves simultaneous *N*(*O*,*S*)-derivatization with ethyl chloroformate in water–ethanol–pyridine [7].

The complete electron impact (EI) mass spectra for 48 *N*-trifluoroacetyl amino acid *n*-butyl esters have been reported [4]. In several cases similar mass spectra of non-identical amino acid derivatives are obtained and GC retention times or indices are required as additional information. The molecular mass of amino acid derivatives can be confirmed by chemical ionization (CI) mass spectrometry with methane as reagent gas [7]. The application of GC–negative-ion chemical ionization–mass spectrometry

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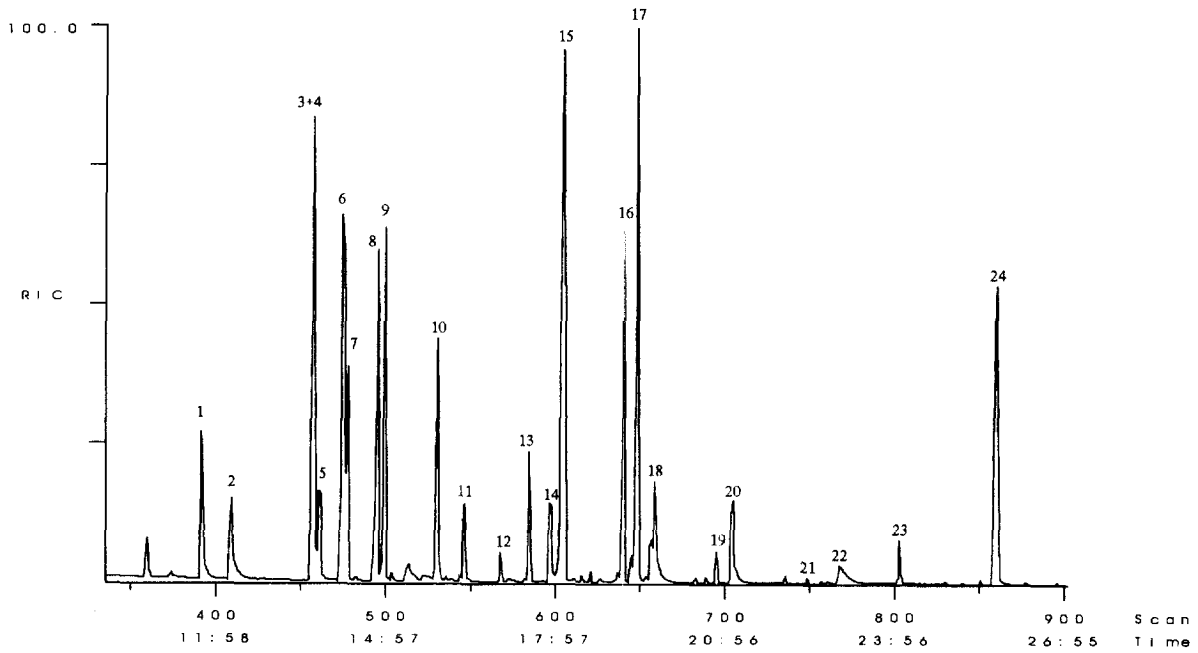


Fig. 1. Gas chromatogram of 24 amino acids as trifluoroacetyl-isopropyl esters.

(NICI–MS) was tested for some amino acid pentafluoropropionyl-hexafluoroisopropyl (PFP-HFIP) derivatives [8], which gave a limit of detection below the ng level for phenylalanine, tyrosine, and dihydroxyphenylalanine.

We report here about our experience in amino acid analysis using GC–MS with chemical ionization with methane, CI(CH_4). The amino acids were investigated mainly as *N*(*O*,*S*)-trifluoroacetyl isopropyl esters and especially for histidine and β-hydroxyhistidine pentafluoropropionyl-isopropyl (PFPP) derivatives were prepared. The specimens investigated were standard amino acid mixtures and hydrolysis products of pyoverdins [1].

2. Experimental

2.1. Instrumentation

The measurements were performed with a Finnigan Incos 500 mass spectrometer (Finnigan MAT, San Jose, CA, USA) directly coupled with a Varian 3400 gas chromatograph (Varian Analytical Instruments, Sunnyvale, CA, USA) with split/splitless injector, and a SE-54 capillary column (25 m×0.25 mm, 0.25 μm film thickness) (CS-Chromatography Service, Langerwehe, Germany). The carrier gas (He) flow was 1 ml/min (head pressure 55 kPa). Injection was performed manually using combined splitless/split (split-ratio 10:1) mode. The sampling time was 0.6 min, the injection volume 1 μl, injection temperature 280°C. The following oven temperature program was used: initial 30°C, held for 6 min; increased from 30 to 250°C at 10°C/min, held at 250°C for 2 min. The transfer line temperature was 250°C. Incos 500 was equipped with two different ion sources for EI and CI. MS parameters were: ion source set temperature 180°C for EI and 0°C for CI. The real measured temperature during CI measurements was between 60 and 90°C. The MS was scanned from 50 to 600 u in 1.7 s. The electron multiplier voltage was 1200 V (EI) and 1400 V (CI). GC–MS data were acquired using Data General DG-20.

2.2. Samples and standard mixtures

Amino acid *N*(*O*,*S*)-trifluoroacetyl isopropyl esters (TAP derivatives) were prepared as described elsewhere [3,8] using amino acid standard mixture in water containing 24 amino acids (about 1 μmol per

amino acid). To determine the retention indices (I) of the amino acid TAP derivatives a standard mixture of saturated hydrocarbons C_9 to C_{23} in hexane was prepared. The I values were calculated from the measured retention times of saturated hydrocarbons and an interpolation curve using Microsoft Excel 5.0 software. The chemicals used were purchased mainly from Sigma–Aldrich (Deisenhofen, Germany) and Fluka (Buchs, Switzerland) and were all of analytical grade.

3. Results and discussion

In amino acid analyses of pyoverdins proteinogenic and uncommon amino acids as well as artifacts or decomposition products have to be identified. For the systematic analysis a mixture with 24 amino acids was prepared. The chromatogram of this standard mixture (about 4 nmol of TAP ester from each amino acid) is presented in Fig. 1. The retention times (RT) and calculated I as well as the most abundant and characteristic fragments of their EI and CI(CH_4) spectra are assembled in Table 1. From the CI(CH_4), molecular masses can be readily obtained. Especially in spectra of arginine and histidine, whose EI spectra are rather uncharacteristic, the base peak in the CI spectra (see Figs. 2 and 3) represents the protonated molecule. CI(CH_4) spectra allow also to determine the number of carboxylic groups in amino

Table 1
Characteristic fragments in EI and CI(CH_4) mass spectra, retention times and retention indices of trifluoroacetyl amino acid isopropyl esters (the numbers correspond to those in Fig. 1)

No.	Name	M_r	EI mass spectrum[a]	CI(CH_4) mass spectrum[b]	RT (min)	RT/RT_{Ala}	I
1	Alanine	227	**140**, 141,168	228, *256*, **186**, 140	11.75	1.00	1054
2	Glycine	213	**126**, 127, 154	214, *242*, **172**, 126	12.28	1.05	1082
3	β-Alanine	227	**168**, 126, 139, 186	228, *256*, **168**, 186	13.67	1.16	1160
4	Valine	255	**168**, 153, 194, 186	256, *284*, **214**, 168	13.67	1.16	1160
5	Serine	339	**139**, 138, 110, 184, 252, 280	340, *368*, **298**, 139	13.92	1.18	1175
6	Norvaline	255	**168**, 126, 153, 114, 140	256, *284*, **214**, 168	14.17	1.20	1190
7	*allo*-Threonine	353	**153**, 152, 170, 198	354, *382*, **312**, 198	14.37	1.22	1203
8	Leucine	269	**182**, 140, 153	270, *298*, **228**, 182	14.90	1.27	1236
9	Isoleucine	269	**182**, 153, 171	270, *298*, 228, **182**	15.02	1.28	1244
10	Cysteine	355	**140**, 268, 103, 170, 154	356, *384*, **314**, 268	15.88	1.35	1302
11	Proline	253	**166**, 167, *96*	254, *282*, **212**, 166	16.30	1.39	1331
12	Hydroxyproline	365	**164**, 278, 210, 209	366, *394*, **324**, 164	17.00	1.45	1381
13	Aspartic acid	313	**184**, 139, 212, 140, 166, 226	314, *342*, 272, **230**[c]	17.50	1.49	1419
14	2,4-Diaminobutanoic acid	352	**152**, 140, 126, 127, 265, 292	353, *381*, **311**, 291	17.95	1.53	1453
15	Methionine	287	*61*, *75*, 153, 171, 213, 131	288, *316*, **246**, 228	18.10	1.54	1465
16	Glutamic acid	327	**152**, 180, 198, 226, *85*	328, *356*, 286, **244**[c]	19.22	1.64	1555
17	Phenylalanine	303	*91*, 148, 190, 216, 103	304, *332*, **262**, 216	19.42	1.65	1571
18	Ornithine	366	**166**, 167, 126, 211, 306	367, *395*, **325**, 166	19.80	1.69	1603
19	Tyrosine	415	**203**, 260, 302, 328, 175	416, *444*, **374**, 302	20.83	1.77	1693
20	Lysine	380	**180**, 126, 181, 140, 168	381, *409*, **339**, 180	21.12	1.80	1719
21	Arginine	504	**166**, 304, 292, 139, 140, 209	**505**, *533*, 463, 435	22.35	1.90	1832
22	Histidine (*monoacyl*)	293	*81*, 206, *82*, 109	**294**, *322*, 252, 206	23.32	1.98	1926
23	Tryptophan	438	**226**, 283, 325, 351, 438, 129	439, *467*, **397**, 226	24.00	2.04	1994
24	Cystine	516	**184**, 226, 138, 104, 170, 516	517, *545*, 475, **433**[c]	25.75	2.19	2176
	Threonine[e]	353	**153**, 266, 294, 198, 180	354, *382*, **312**, 198	13.52	1.15	1151
	Succinic acid[d,e]	202	**101**, 119, 143, 161	203, 161, 119[c], **101**	15.18	1.29	1255
	Hydroxyornitine artifact[e]	251	**164**, *94*, 209, 251	252, *280*, **210**, 164	15.48	1.32	1275
	Hydroxyaspartic acid[e]	425	**138**, 183, 166, 324	426, *454*, 384, **342**[c]	16.60	1.41	1352

[a] **The most abundant ion with *m/z* over 100**, *characteristic fragments with m/z below 100.*
[b] $[M+H]^+$, $[M+C_2H_5]^+$, $[(M+H)-C_3H_6]^-$, **the most abundant ion.**
[c] $[(M+H)-2C_3H_6]^+$.
[d] Included as a typical hydrolylis product of pyoverdins.
[e] Amino acid derivatives not contained in Fig. 1.

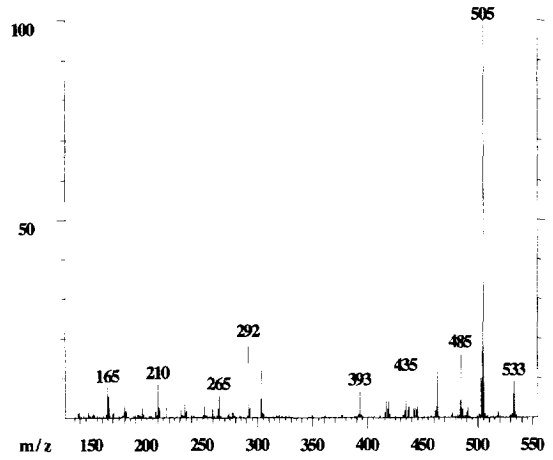


Fig. 2. CI(CH_4) mass spectrum of the TAP derivative of arginine ($[M+H]^+$=505).

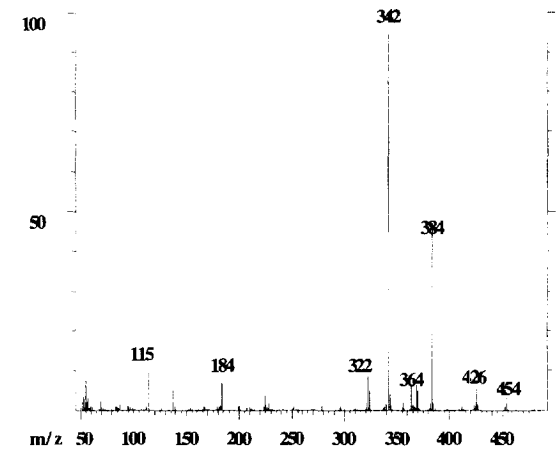


Fig. 4. CI(CH_4) mass spectrum of the TAP derivative of hydroxyaspartic acid ($[M+H]^+$=426).

acids due to characteristic successive eliminations of C_3H_6 from the $[M+H]^+$ ion giving the abundant fragments $[M+H-mC_3H_6]^+$, where $m=1,\ldots,N_{COOH}$. For examples see CI(CH_4) spectra of β-hydroxyaspartic acid and cystine (Figs. 4 and 5). For GC–MS investigation with EI the following problems may occur: (1) similar mass spectra and/or absence of molecular ions (e.g. ornithine and proline; hydroxyproline and an artifact from N^5-hydroxyornithine); (2) incompletely separated gas chromatographic peaks (e.g. valine and serine; norvaline and *allo*-threonine); (3) similar mass spectra and unseparated chromatographic peaks (e.g. valine and β-alanine); (4) similar mass spectra of isomers (e.g. leucine and isoleucine; threonine and *allo*-threonine; valine and norvaline); (5) decomposition (rearrangement) products (see β-hydroxyhistidine); (6) unidentified products from hydrolysis and/or derivatization.

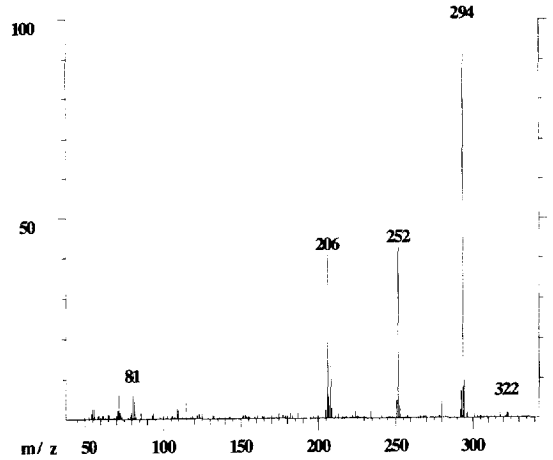


Fig. 3. CI(CH_4) mass spectrum of the TAP derivative of histidine ($[M+H]^+$=294).

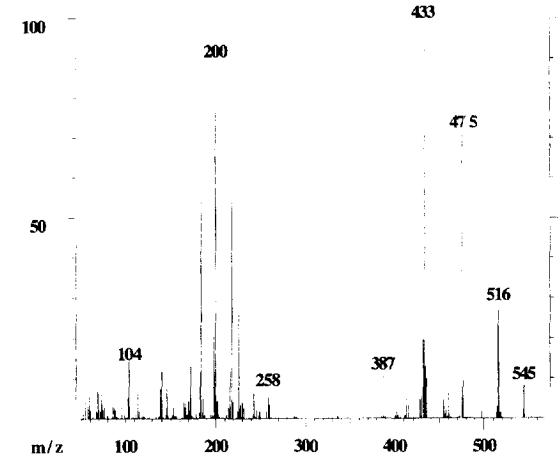


Fig. 5. CI(CH_4) mass spectrum of the TAP derivative of cystine ($[M+H]^+$=517).

3.1. Amino acids with similar EI mass spectra

3.1.1. Proline and ornithine

The EI mass spectra are presented in Fig. 6. The base peak (*m/z* 166), in both cases is formed by the loss of $\dot{C}OOC_3H_7$ from proline and after cyclization (loss of NH_3) from ornithine. In contrast, the CI(CH_4) spectra (Fig. 7) can be distinguished readily by their $[M+H]^+$ ions *m/z* 254 (Pro) and 367 (Orn) and the $[M+H-C_3H_6]^+$ ions *m/z* 212 and 325. The ion responsible for the base peak (*m/z* 166) in both EI spectra has only an intensity of about 15–30% in the CI(CH_4) spectra.

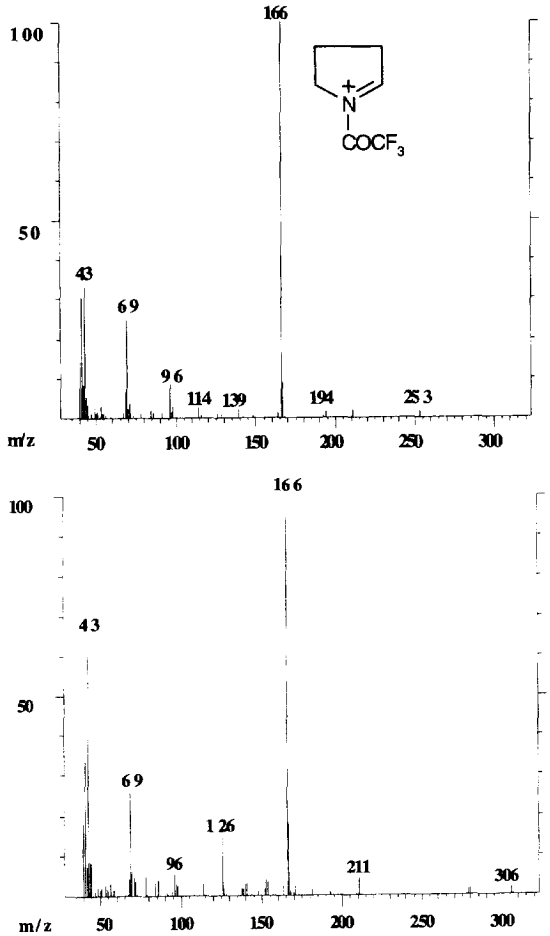


Fig. 6. EI mass spectra of the TAP derivatives of proline (top, M_r 253) and ornithine (bottom, M_r 366).

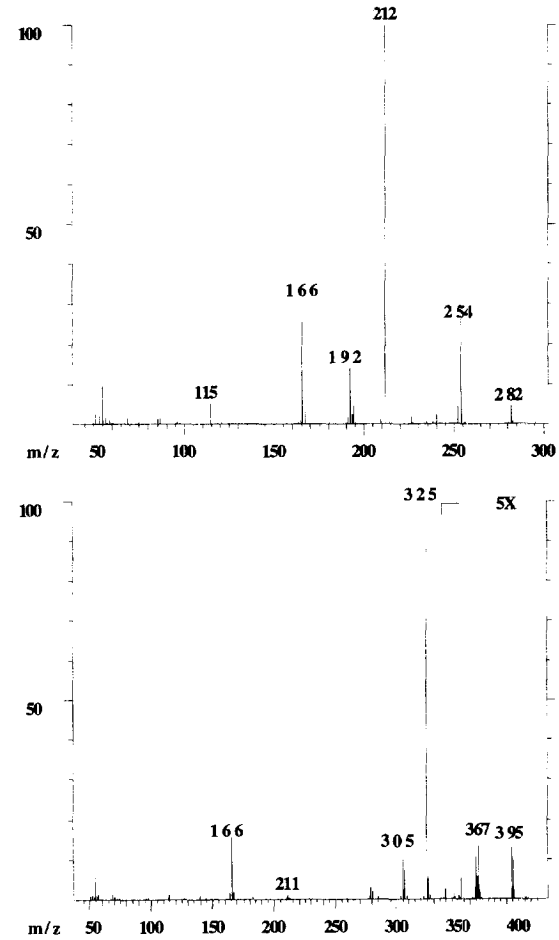


Fig. 7. CI(CH_4) mass spectra of the TAP derivatives of proline (top, $[M+H]^+=254$) and ornithine (bottom, $[M+H]^+=367$).

3.1.2. 4-Hydroxyproline and an artifact from N^5-hydroxyornithine

The EI spectra of both substances are presented in Fig. 8 showing a base peak at *m/z* 164 due to the elimination of CF_3COOH and loss of $\dot{C}OOC_3H_7$ from the TAP derivative of 4-hydroxyproline. Due to preceding cyclization (loss of NH_3 and H_2O) the same fragment is formed from the second substance. The CI(CH_4) spectra of both substances are presented in Fig. 9. The molecular mass of the TAP derivative of the artifact is found to be 251 u. Its structure is, therefore, *N*-trifluoroacetyl-2,3- (or 3,4)-dehydroproline-isopropyl ester.

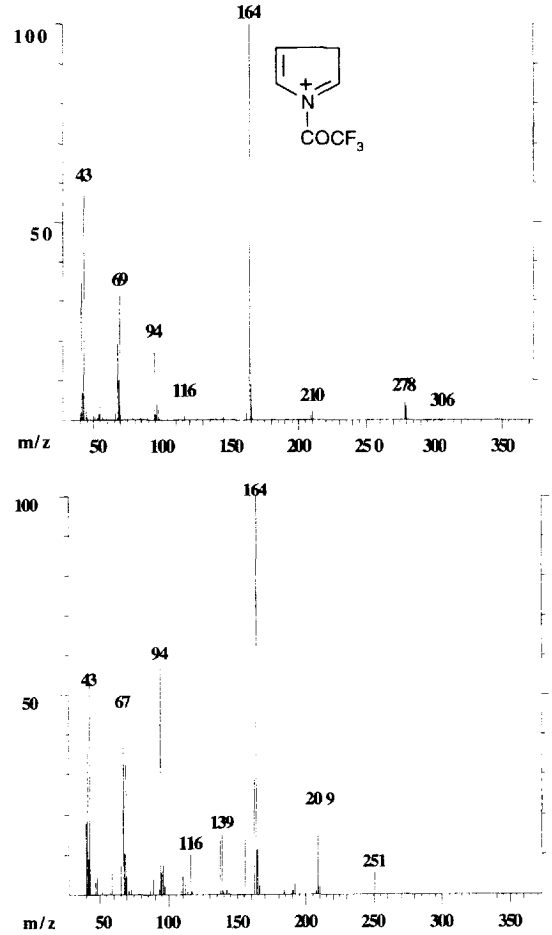


Fig. 8. EI mass spectra of the TAP derivatives of 4-hydroxyproline (top, M_r 365) and N^5-hydroxyornithine artifact (bottom, M_r 251).

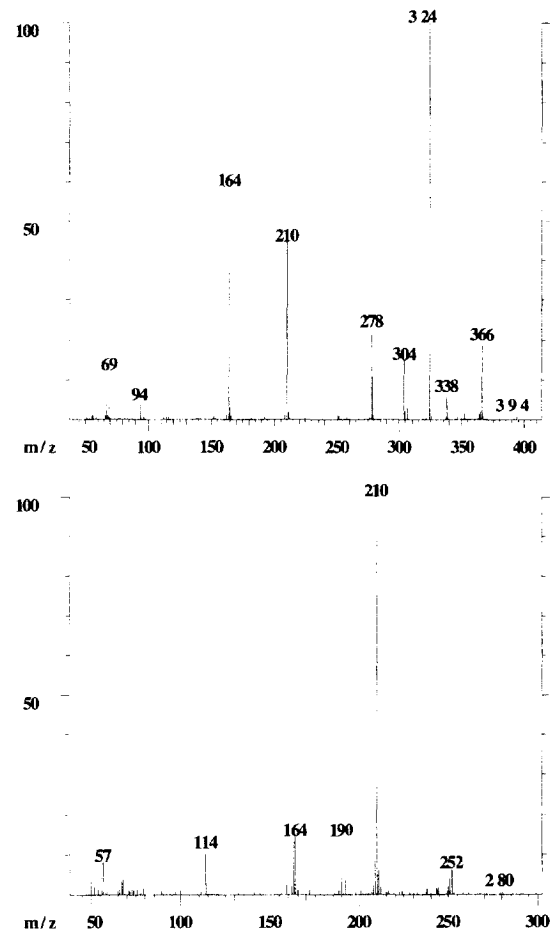


Fig. 9. CI(CH_4) mass spectra of the TAP derivatives of 4-hydroxyproline (top, $[M+H]^+=366$) and N^5-hydroxyornithine (bottom, $[M+H]^+=252$).

3.2. Incompletely and non-separated GC peaks

In the case of incompletely (base line) separated GC peaks (valine and serine; norvaline and *allo*-threonine) the $[M+H]^+$ ions and the characteristic fragments will be sufficient for identification, if necessary after spectra substraction. A more difficult case is the pair β-alanine and valine whose EI spectra are rather similar. Their CI(CH_4) spectra are sufficiently different for an identification if only one compound is present or if both compounds occur in comparable amounts, but minor components may be overlooked.

3.3. Isomeric amino acids

As can be seen from Figs. 10 and 11 (leucine/isoleucine) and Figs. 12 and 13 (threonine/*allo*-threonine) both the EI and CI(CH_4) spectra differ only in the relative intensities of several peaks. For the interpretation of the EI spectra see Ref. [3]. In the CI(CH_4) spectra of leucine/isoleucine, mainly

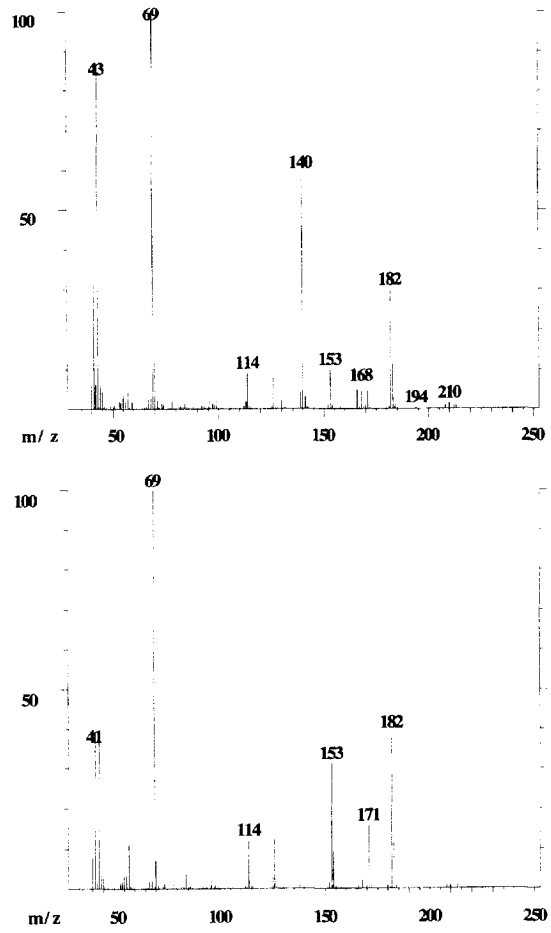


Fig. 10. EI mass spectra of the TAP derivatives of leucine (top) and isoleucine (bottom), M_r 269.

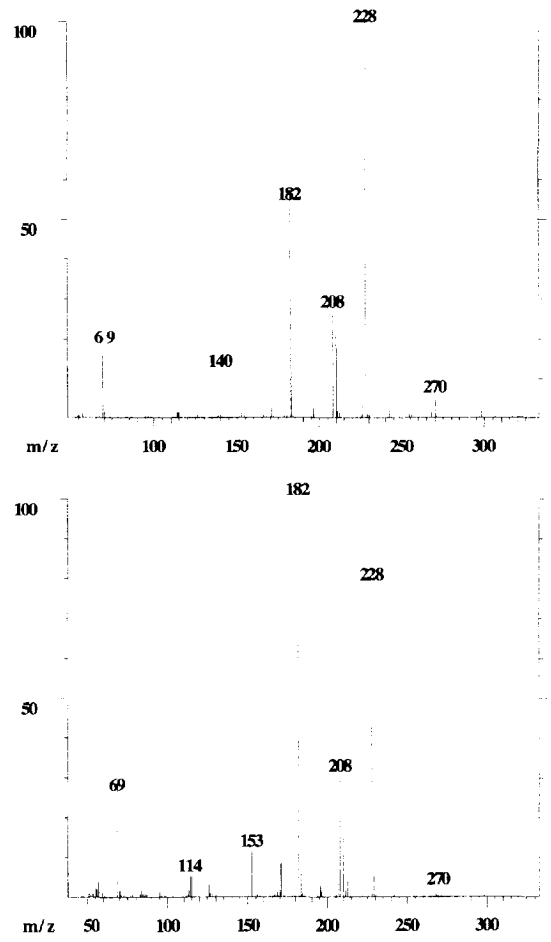


Fig. 11. CI(CH_4) mass spectra of the TAP derivatives of leucine (top) and isoleucine (bottom), $[M+H]^+=270$.

$[M+H]^+$ (m/z 270), $[M+H-C_3H_6]^+$ (m/z 228), and $[M-COOC_3H_7]^+$ (m/z 182), in those of threonine/*allo*-threonine $[M+H]^+$ (m/z 354), $[M+H-C_3H_6]^+$ (m/z 312), and [m/z 312$-CF_3COOH]^-$ (m/z 198) are observed.

3.4. Histidine and β-hydroxyhistidine

From histidine only the GC peak of its *mono*-trifluoroacetyl derivative will be observed due to an equilibrium between the *mono*- and *di*-acyl derivatives [9,10]. The pentafluoropropionyl and heptabutanoyl derivatives which are obtained more readily show the same behavior. Therefore, for the derivatization of β-hydroxyhistidine [1,11], pentafluoropropionic anhydride was used. The gas chromatogram of this compound in dichloromethane shows two major peaks. The molecular masses of these two components were found to be 341 and 445 u. The mass spectra of these two components are presented in Fig. 14. Instead of the expected derivatization product A (see Table 2) only decomposition products were formed. The first one (B) with a molecular mass of 341 is formed by the loss of pentafluoropropionic acid (C_2F_5COOH) from A, and

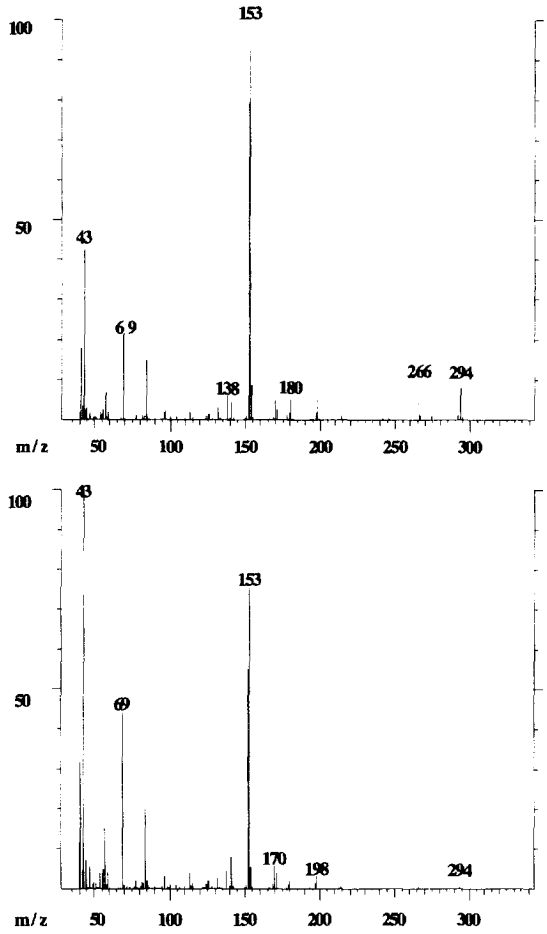


Fig. 12. EI mass spectra of the TAP derivatives of threonine (top) and *allo*-threonine (bottom), M_r 353.

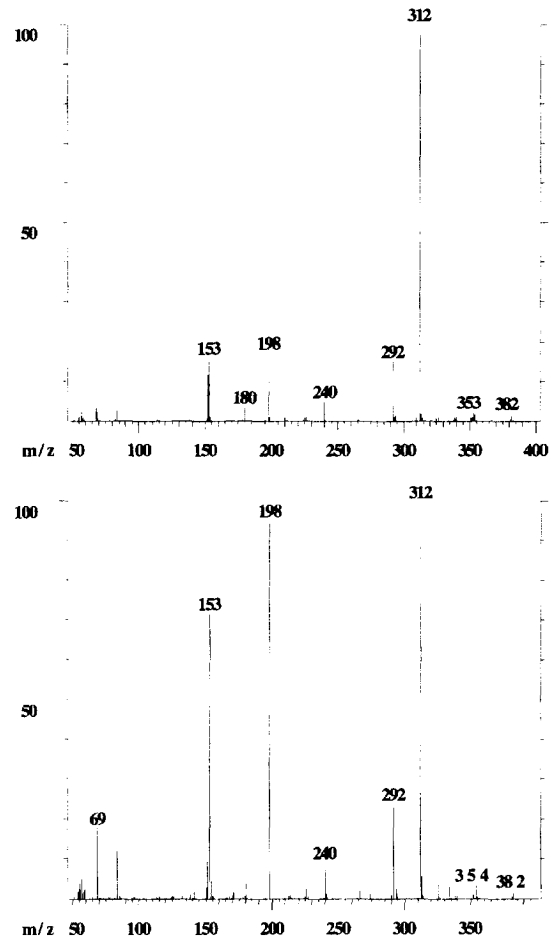


Fig. 13. CI(CH_4) mass spectra of the TAP derivatives of threonine (top) and *allo*-threonine (bottom), $[M+H]^+=354$.

the second one with a molecular mass of 445 by the loss of isopropanol under cyclization.

It should be briefly mentioned here that the silylation of histidine and β-hydroxyhistidine using *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) proceeded under different reaction temperatures (from 80 to 130°C) and reaction times (from 0.5 to 2 h) and gave for each compound two main derivatization products, viz. *O*-mono- and *O,N,N*-tri-trimethylsilyl derivatives for histidine and *O,O*-di- and *O,O,N,N*-tetra-trimethylsilyl-derivatives for β-hydroxyhistidine.

4. Conclusion

CI(CH_4) was shown to be very suitable to identify standard α-amino acids as well as artifacts and rearrangement products after the isopropylation followed by *N*(*O,S*)-trifluoroacetylation or pentafluoropropionylation. All measured amino acids show protonated molecules $[M+H]^+$, cluster ions $[M+C_2H_5]^+$, and characteristic fragmentation ions: $[M+H-mC_3H_6]^+$, *m* changes from 1 to the number of carboxylic groups; $[M+H-C_3H_9O]^+$. CI(CH_4) mass spectra of amino acid TAP derivatives show

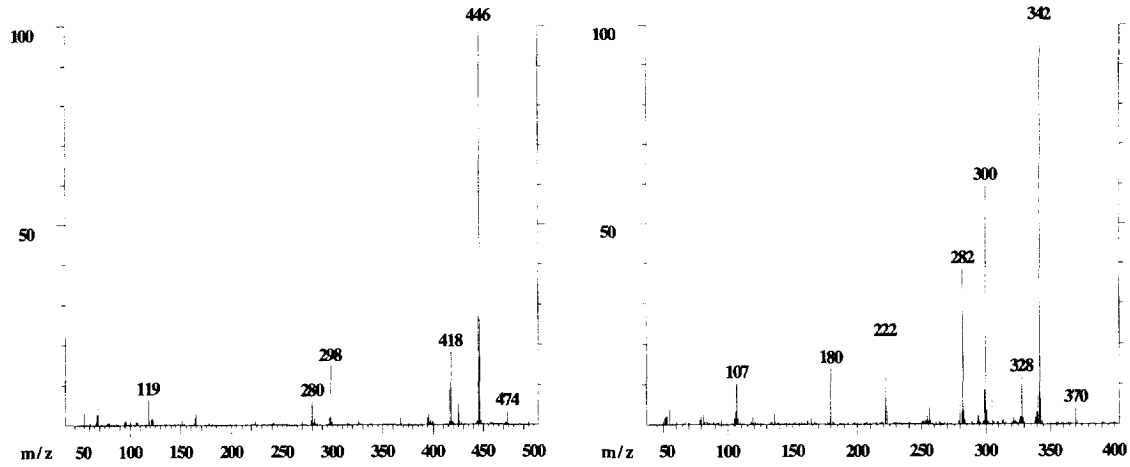
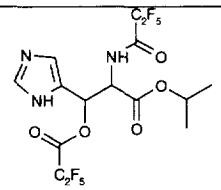
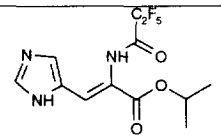
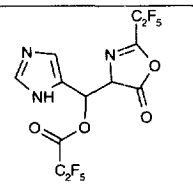


Fig. 14. CI(CH_4) mass spectra of two derivatization products of β-hydroxyhistidine after the derivatization with isopropanol followed by pentafluoropropionylation with $[M+H]^+$ 446 (left) and 342 (right).

molecular ions and characteristic fragmentation paths. The GC–CI(CH_4)–MS was shown to be selective and sensitive in detection of amino acids as perfluoroacyl isopropyl derivatives after hydrolysis of pyoverdins.

Table 2
The structure of *N,O*-di-pentafluoropropionyl-β-hydroxyhistidine isopropyl ester (A) and two proposed stuctures of derivatization products (B, C)

Compound	Molecular mass, u	Structure
A	505	
B	341	
C	445	

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