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Simultaneous determination of fatty, dicarboxylic and amino acids based on derivatization with isobutyl chloroformate followed by gas chromatography—positive ion chemical ionization mass spectrometry

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Abstract

Gas chromatography–mass spectrometry (GC–MS) with positive ion chemical ionization (PICI) using isobutane as reagent gas was applied for analysis of isobutoxycarbonyl/isobutyl derivatives of 13 fatty, 6 dicarboxylic and 13 amino acids in a single run. For all investigated compounds (except several amino acids) the quasimolecular ions $[MH]^+$ were registered. Asparagine underwent fragmentation via decarboxylation followed by elimination of OC_4H_9 ($[M-117]^+$), whereas serine and tyrosine produced the cluster ions $[M+C_4H_9OCO]^+$. Estimated detection limits were 6–250 pg in the total ion current (TIC) mode and 3–10 times lower using the selected-ion monitoring (SIM) mode.

Keywords: Derivatization, GC; Fatty acids; Dicarboxylic acids; Amino acids; Isobutyl chloroformate

1. Introduction

Identification and quantitation of fatty, dicarboxylic and amino acids in various matrices has become a typical analytical need. It is well-known that, with the exception of short-chain fatty acids (C_2–C_7) [1–3], a preliminary derivatization of these compounds is required to make them volatile and thereby eligible to GC. Nowadays, derivatization procedures for fatty acids include mostly methylation with methanol (in the presence of a catalyst) [4–9], sodium methylate or diazomethane [10]. Formerly, esterification with other alcohols (propanol [11] and butanol [11,12]) was used. Silylation is also the method of choice in some applications [13–16] due to its inherent versatility and wide coverage in the literature. In case of dicarboxylic acids, besides silylation [16] and esterification [11,12] the use of pentafluorobenzylbromide is reported [17] that enabled high-sensitive detection of the respective derivatives by GC—negative ion chemical ionization mass spectrometry.

Gas chromatographic detection of amino acid has included a number of techniques based on silylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA) [18] or *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [19–23], and esterification/acylation: in the two successive steps [24,25] or simultaneously via alkylchloroformate reaction [26–29]. Alkylchloroformate-induced esterification of several fatty, hydroxy, keto and dicarboxylic acids was also described in [30]. However, these results were obtained using flame ionization detector (FID) and should indispensably be proven by GC–MS. The use of alkylchloroformates is the most promising approach since derivatization occurs readily in aqueous media at room temperature, resulting in stable and easy-to-handle derivatives with excellent chromatographic properties. Unlike silylation, alkylchloroformates react only with NH_2 and carboxylic OH groups. It becomes of prime importance when working with biological samples that contain a large amount of sugars or other related compounds, since alkylchloroformates enable selective derivatization of organic and amino acids while keeping major components untouched, thereby simplifying a chromatogram to obtain.

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To date, most of the reported data concerning the analysis of fatty and dicarboxylic acids include gas chromatography with FID or mass spectrometers operating in the electron ionization (EI) mode. No data were found in the literature on simultaneous determination of these classes of compounds in a single run with mass spectrometric detection. Chemical ionization (CI) is rarely used [17], although the EI mass spectra of organic and amino acids do not contain the molecular ion peaks or their intensity is negligible. The same holds true for amino acids: a few publications deal with CI, both positive and negative ion [29,31]. However, this is not surprising because of the low inter-laboratory reproducibility of the CI mass spectra and the absence of the CI data in mass spectral libraries. Nevertheless, CI provides the unsurpassed selectivity and its application seems reasonable in target analysis when the molecular weight is known beforehand.

At the same time, the analysis of microbial samples, body fluids, foods and beverages often requires a simultaneous determination of amino, fatty and dicarboxylic acids since the presence and content of these compounds is a crucial factor that governs the quality or origin of the respective products.

Thus, the present paper is aimed at simultaneous determination of fatty, dicarboxylic and amino acids after their derivatization with isobutyl chloroformate and investigation of the respective mass spectra in the positive ion CI mode.

2. Experimental

GC–MS analysis was carried out on a *TRACE 2000* gas chromatograph (ThermoQuest, CE Instruments, Italy) connected to an *Automass Multi* quadrupole mass spectrometer (ThermoQuest, Finnigan, France).

The derivatives of the compounds in question were separated on a fused-silica capillary column (Restek RTX-5MS; 9.5 m × 0.25 mm × 0.1 μm) with helium (99.99%) carrier gas at a flow rate of 1.5 ml/min. Split/splitless injection was used (splitless time 30 s, then split 20:1) with an injector temperature of 280 °C. The column temperature was programmed as follows: isothermal 50 °C for 7 min, then to 230 °C at 10 °C/min.

Mass spectra were scanned over the range m/z = 150–500. The ion source temperature was 150 °C and the transfer line temperature was 280 °C. Isobutane (99.5%, Messer Griesheim GmbH, Germany) was used as reagent gas at the pressure in the ion source of ca. 6.5 Pa. Both the total ion current (TIC) and selected-ion monitoring (SIM) modes were used.

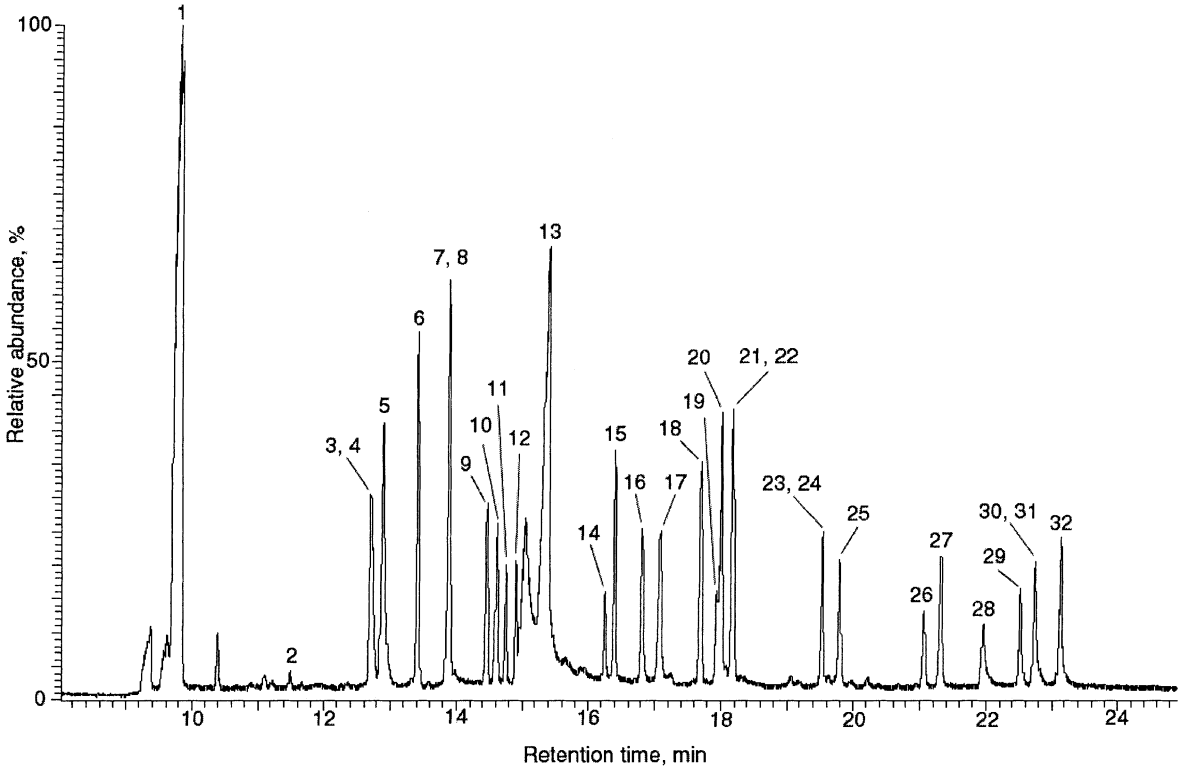

Fig. 1. Chromatogram for the model mixture of isobutoxycarbonyl isobutyl esters of amino acids, diisobutyl esters of dicarboxylic acids and isobutyl esters of fatty acids (1, malonic acid; 2, succinic acid; 3, alanine; 4, glutaric acid; 5, glycine; 6, undecanoic acid; 7, valine; 8, adipic acid; 9, dodecanoic acid; 10, leucine; 11, isoleucine; 12, proline; 13, asparagine; 14, *cis*-9-tetradecenoic acid; 15, myristic (tetradecanoic) acid; 16, azelaic acid; 17, methionine; 18, sebacic acid; 19, *cis*-9-hexadecenoic acid; 20, phenylalanine; 21, palmitic (hexadecanoic) acid; 22, serine; 23, linolenic acid; 24, oleic (*cis*-9-octadecenoic acid); 25, stearic (octadecanoic) acid; 26, *cis*-11-eicosenoic acid; 27, eicosanoic acid; 28, lysine; 29, *cis*-13-docosenoic acid; 30, docosanoic acid; 31, tryptophan; 32, tyrosine; all the compounds in the form of respective derivatives).

2.1. Chemicals

All the amino acids used in the study (alanine, glycine, valine, leucine, isoleucine, proline, asparagine, methionine, serine, phenylalanine, lysine, tryptophan and tyrosine) were supplied from Sigma (St. Louis, MO, USA). Isobutyl chloroformate (iBuCF), dicarboxylic acids (malonic acid, succinic acid, glutaric acid, adipic acid, azelaic acid and sebacic acid) and pyridine were purchased from Aldrich (Milwaukee, WI, USA). All the fatty acids (undecanoic acid, dodecanoic acid, *cis*-9-tetradecenoic acid, tetradecanoic acid, *cis*-9-hexadecenoic acid, hexadecanoic acid, *cis*-9-octadecenoic acid, linolenic acid, octadecanoic acid, *cis*-11-eisosenoic acid, eicosanoic acid, *cis*-13-docosenoic acid and docosanoic acid) were bought from Alltech Associates Inc. (Deerfield, IL, USA). 2-Methyl-1-propanol (iBuOH) and chloroform were purchased from Lecbiopharm (Moscow, Russia).

2.2. Solutions and derivatization procedure

A solution of amino acids was prepared in bidistilled water at a concentration of ca. 10^{-4} g/ml each. The solutions of fatty and dicarboxylic acids were prepared separately in pyridine at a concentration of ca. 10^{-5} g/ml each. A total of 100 μl of the amino acid solution was mixed with 60 μl of bidistilled water, 60 μl of iBuOH, 10 μl of fatty acid pyridine solution, 10 μl of dicarboxylic acid pyridine solution, 60 μl of iBuCF was added, and the mixture was sonicated for 30 s. The derivatives formed were then extracted with 500 μl of chloroform by vigorous shaking followed by centrifugation of the solution. One microliter of the chloroform layer was injected into the GC–MS.

3. Results and discussion

It is well-known that chemical ionization in mass spectrometry provides mild ionization conditions that often results in formation of the abundant molecular or quasi-molecular ions. Molecular masses of mixture components being rarely overlapped, CI enables carrying out the analyses of target compounds in the most selective way. The SIM in CI is a powerful tool for high-sensitive determination comparable in sensitivity with EI.

Using isobutyl chloroformate the volatile derivatives of 13 fatty acids, 6 dicarboxylic acids and 13 amino acids were prepared and separated on a short capillary column

Table 1
Retention times (RT) and *m*/*z* of characteristic ions in PICI mass spectra of isobutoxycarbonyl/isobutyl derivatives of fatty, dicarboxylic and amino acids

Compound	RT (min)	MW	*m*/*z* (relative abundance, %) and ion type
Malonic acid, diisobutyl ester	9.67	216	161 (9) $[M - 55]^+$, 217 (100) $[M + H]^+$
Succinic acid, diisobutyl ester	11.48	230	157 (8) $[M - 73]^+$, 231 (100) $[M + H]^+$
Alanine, *N*-isobutoxycarbonyl isobutyl ester	12.68	245	246 (100) $[M + H]^+$
Glutaric acid, diisobutyl ester	12.70	244	171 (16) $[M - 73]^+$, 245 (100) $[M + H]^+$
Glycine, *N*-isobutoxycarbonyl isobutyl ester	12.90	231	176 (3) $[M - 55]^+$, 232 (100) $[M + H]^+$
Undecanoic acid, isobutyl ester	13.42	242	187 (14) $[M - 55]^+$, 243 (100) $[M + H]^+$
Valine, *N*-isobutoxycarbonyl isobutyl ester	13.86	273	200 (3) $[M - 73]^+$, 274 (100) $[M + H]^+$
Adipic acid, diisobutyl ester	13.90	258	185 (14) $[M - 73]^+$, 259 (100) $[M + H]^+$
Dodecanoic acid, isobutyl ester	14.46	256	201 (12) $[M - 55]^+$, 257 (100) $[M + H]^+$
Leucine, *N*-isobutoxycarbonyl isobutyl ester	14.59	287	186 (11) $[M - 101]^+$, 288 (100) $[M + H]^+$
Isoleucine, *N*-isobutoxycarbonyl isobutyl ester	14.73	287	186 (6) $[M - 101]^+$, 288 (100) $[M + H]^+$
Proline, *N*-isobutoxycarbonyl isobutyl ester	14.89	271	170 (8) $[M - 101]^+$, 272 (100) $[M + H]^+$
Asparagine, *N*,*N*′-diisobutoxycarbonyl isobutyl ester	15.33	388	215 (10) $[M - 173]^+$, 271 (100) $[M - 117]^+$
cis-9-Tetradecenoic acid, isobutyl ester	16.25	282	227 (11) $[M - 55]^+$, 283 (100) $[M + H]^+$
Tetradecanoic acid, isobutyl ester	16.41	284	229 (19) $[M - 55]^+$, 285 (100) $[M + H]^+$
Azelaic acid, diisobutyl ester	16.82	300	227 (13) $[M - 73]^+$, 301 (100) $[M + H]^+$
Methionine, *N*-isobutoxycarbonyl isobutyl ester	17.05	305	204 (5) $[M - 101]^+$, 232 (20) $[M - 73]^+$, 306 (100) $[M + H]^+$
Sebacic acid, diisobutyl ester	17.70	314	185 (5) $[M - 129]^+$, 241 (13) $[M - 73]^+$, 315 (100) $[M + H]^+$
cis-9-Hexadecenoic acid, isobutyl ester	17.92	310	255 (7) $[M - 55]^+$, 311 (100) $[M + H]^+$
Phenylalanine, *N*-isobutoxycarbonyl isobutyl ester	17.98	321	220 (4) $[M - 101]^+$, 248 (7) $[M - 73]^+$, 322 (100) $[M + H]^+$
Hexadecanoic acid, isobutyl ester	18.15	312	257 (19) $[M - 55]^+$, 313 (100) $[M + H]^+$
Serine, *N*-isobutoxycarbonyl isobutyl ester	18.19	261	188 (55) $[M - 73]^+$, 244 (45) $[M - 17]^+$, 362 (100) $[M + 101]^+$
Linolenic acid, isobutyl ester	19.54	334	261 (13) $[M - 73]^+$, 279 (12) $[M - 55]^+$, 335 (100) $[M + H]^+$
cis-9-Octadecenoic acid, isobutyl ester	19.54	338	265 (6) $[M - 73]^+$, 283 (4) $[M - 55]^+$, 339 (100) $[M + H]^+$
Octadecanoic acid, isobutyl ester	19.80	340	267(4) $[M - 73]^+$, 285(48) $[M - 55]^+$, 341 (100) $[M + H]^+$
cis-11-Eicosenoic acid, isobutyl ester	21.07	366	293 (17) $[M - 73]^+$, 311 (7) $[M - 55]^+$, 367 (100) $[M + H]^+$
Eicosanoic acid, isobutyl ester	21.32	368	295 (7) $[M - 73]^+$, 313 (54) $[M - 55]^+$, 369 (100) $[M + H]^+$
Lysine, *N*,*N*′-diisobutoxycarbonyl isobutyl ester	21.96	402	329 (13) $[M - 73]^+$, 403 (100) $[M + H]^+$
cis-13-Docosenoic acid, isobutyl ester	22.53	394	321 (9) $[M - 73]^+$, 339 (6) $[M - 55]^+$, 395 (100) $[M + H]^+$
Tryptophan, *N*-isobutoxycarbonyl isobutyl ester	22.70	360	246 (13) $[M - 114]^+$, 287 (43) $[M - 73]^+$, 361 (100) $[M + H]^+$
Docosanoic acid, isobutyl ester	22.75	369	323 (8) $[M - 73]^+$, 341 (41) $[M - 55]^+$, 397 (100) $[M + H]^+$
Tyrosine, *N*-isobutoxycarbonyl isobutyl ester	23.14	337	320 (10) $[M - 17]^+$, 338 (3) $[M + H]^+$, 438 (100) $[M + 101]^+$

with low-polar stationary phase (contrary to commonly used semi-polar phases). The resultant chromatogram is presented in Fig. 1. As seen from Table 1, many derivatives had very close (or even the same) retention times. However, they were mass spectrometrically resolved owing to their characteristic ions. Table 1 shows that the mass spectra of most of the derivatives contained the intense quasimolecular ions, except for the derivatives of hydroxyl-containing amino acids (serine and tyrosine), and asparagine. The latter underwent fragmentation, probably via decarboxylation and elimination of C_4H_9O. Nevertheless, a high abundance of the fragment ion ($m/z = 271$) in mass spectrum of asparagine derivative favors the selective determination of this compound in a complex mixture. The derivatives of serine and tyrosine featured the formation of cluster ions corresponding to $[M + C_4H_9OCO]^+$. The presence of low-intensity quasimolecular ion in the PICI mass spectrum of tyrosine derivative and the ion $[M - H_2O + H]^+$ in case of serine indicates that isobutylchloroformate did not react with hydroxyl moieties of these amino acids, and these cluster ions were indeed formed in the very ion source.

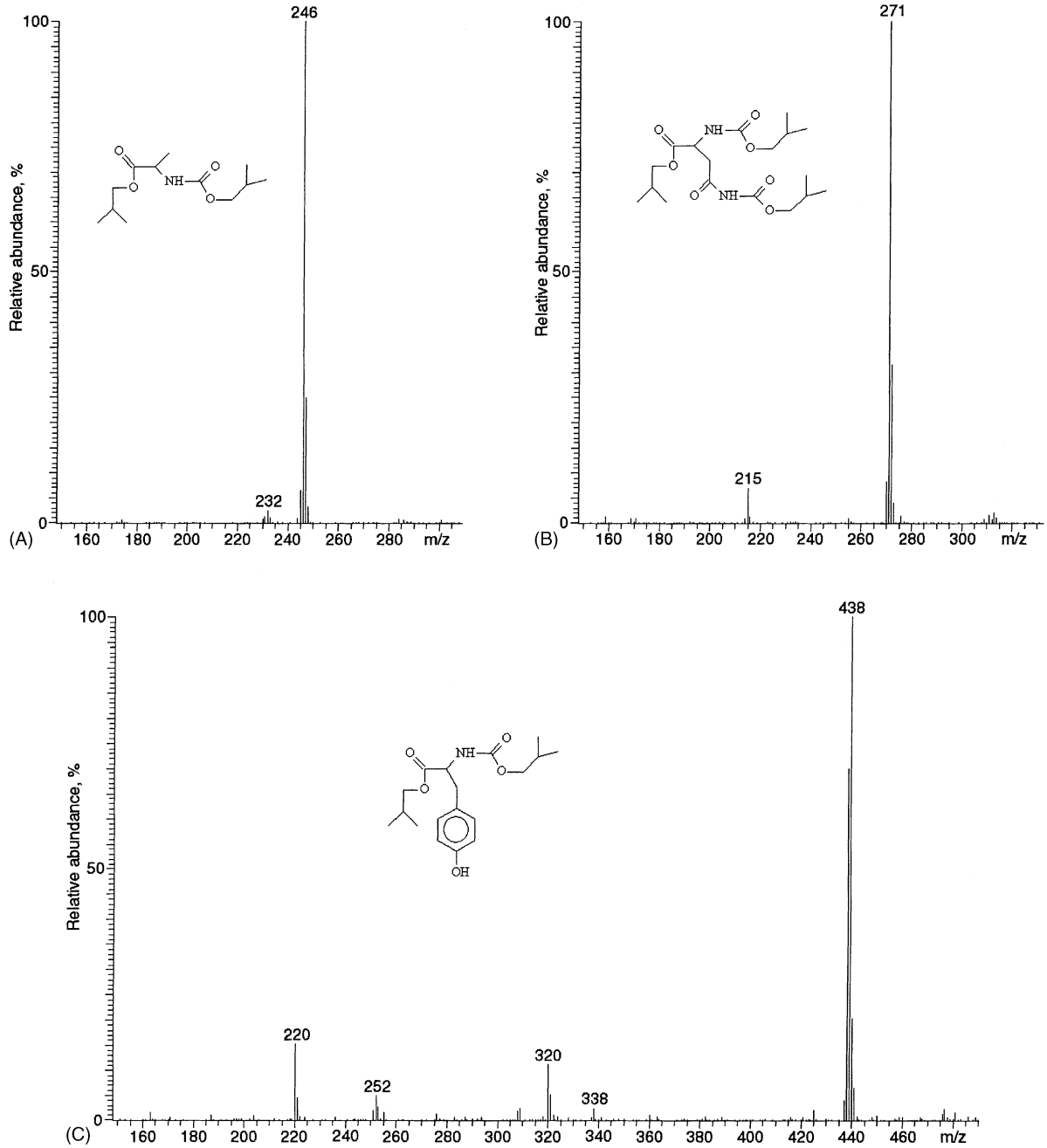

Fig. 2. The PICI mass spectra of (A) L-alanine *N*-isobutoxycarbonyl isobutyl ester; (B) L-asparagine *N*,*N*′-diisobutoxycarbonyl isobutyl ester; (C) L-tyrosine *N*-isobutoxycarbonyl isobutyl ester.

Besides the quasimolecular ion, also typical of fatty and dicarboxylic acid isobutyl/di-isobutyl esters were the ions with $m/z = 55$ and 73 that corresponded to ions $C_4H_7^+$ and $C_4H_9O^+$, respectively. Isobutoxycarbonyl isobutyl esters of leucine, isoleucine, proline, methionine, and phenylalanine eliminated C_4H_9OCO group resulting in the low-intensity ion $[M - 101]^+$.

The most indicative PICI mass spectra of isobutoxycarbonyl isobutyl esters of alanine, asparagine and tyrosine are given in Fig. 2. The PICI mass spectra of the first and the

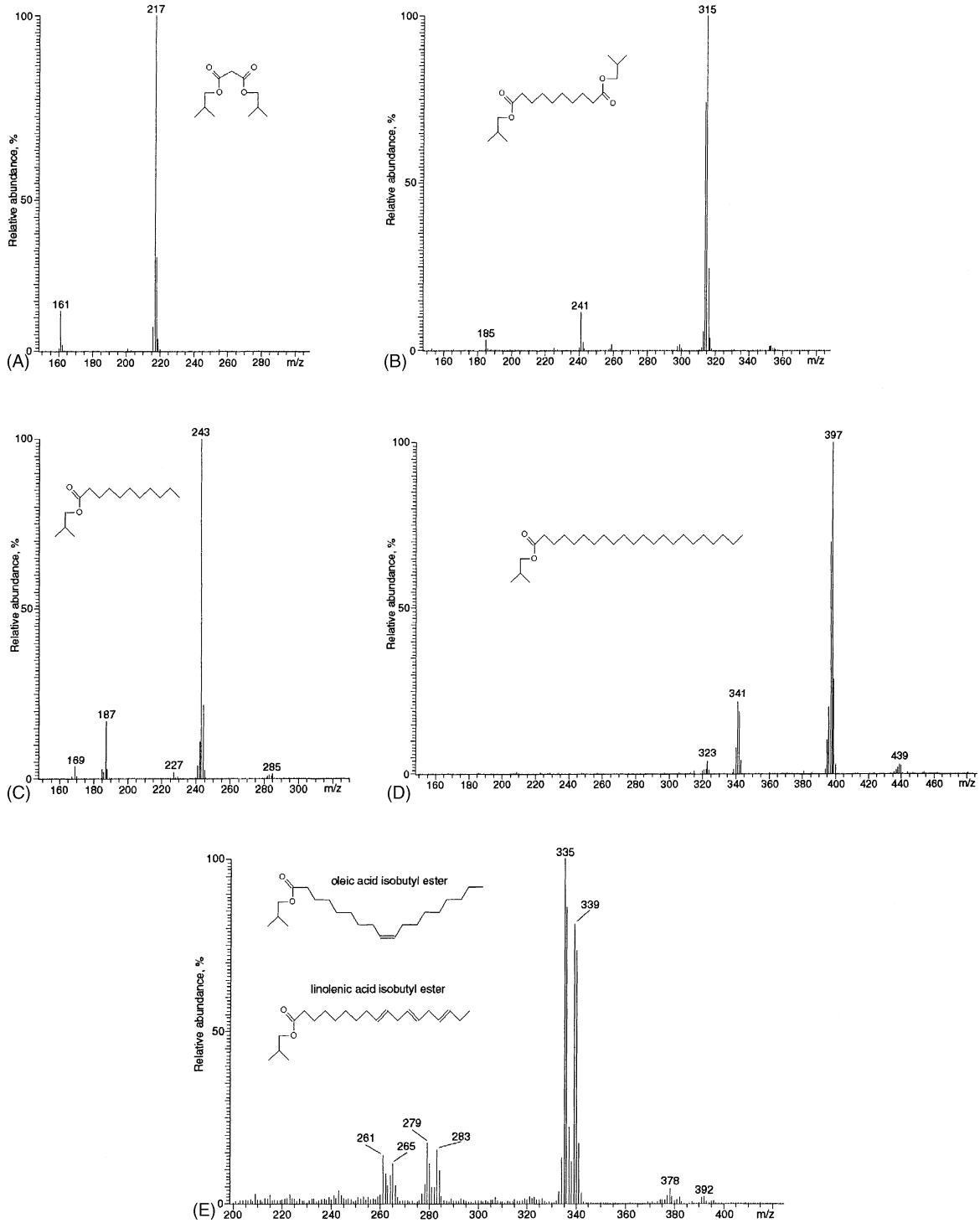

Fig. 3. The PICI mass spectra of (A) malonic acid diisobutyl ester; (B) sebacic acid diisobutyl ester; (C) undecanoic acid isobutyl ester; (D) docosanoic acid isobutyl ester; (E) oleic ($m/z = 339$) and linolenic ($m/z = 335$) acid isobutyl esters eluting simultaneously.

Table 2
Estimated detection limits obtained in TIC and SIM modes for isobutoxycarbonyl/isobutyl esters of the fatty, dicarboxylic and amino acids

Compound	Detection limit (pg)	
	TIC	SIM
Malonic acid, diisobutyl ester	50	5
Succinic acid, diisobutyl ester	250	22
Alanine, *N*-isobutoxycarbonyl isobutyl ester	26	3
Glutaric acid, diisobutyl ester	200	24
Glycine, *N*-isobutoxycarbonyl isobutyl ester	6	2
Undecanoic acid, isobutyl ester	30	5
Valine, *N*-isobutoxycarbonyl isobutyl ester	7	2
Adipic acid, diisobutyl ester	22	3
Dodecanoic acid, isobutyl ester	30	6
Leucine, *N*-isobutoxycarbonyl isobutyl ester	7	2
Isoleucine, *N*-isobutoxycarbonyl isobutyl ester	10	2
Proline, *N*-isobutoxycarbonyl isobutyl ester	34	4
Asparagine, *N*,*N*′-diisobutoxycarbonyl isobutyl ester	55	15
cis-9-Tetradecenoic acid, isobutyl ester	50	10
Tetradecanoic acid, isobutyl ester	60	20
Azelaic acid, diisobutyl ester	54	8
Methionine, *N*-isobutoxycarbonyl isobutyl ester	45	5
Sebacic acid, diisobutyl ester	70	11
cis-9-Hexadecenoic acid, isobutyl ester	75	15
Phenylalanine, *N*-isobutoxycarbonyl isobutyl ester	9	2
Hexadecanoic acid, isobutyl ester	40	15
Serine, *N*-isobutoxycarbonyl isobutyl ester	64	10
Linolenic acid, isobutyl ester	120	30
cis-9-Octadecenoic acid, isobutyl ester	120	20
Octadecanoic acid, isobutyl ester	75	20
cis-11-Eicosenoic acid, isobutyl ester	50	20
Eicosanoic acid, isobutyl ester	60	20
Lysine, *N*,*N*′-diisobutoxycarbonyl isobutyl ester	100	20
cis-13-Docosenoic acid, isobutyl ester	120	40
Tryptophan, *N*-isobutoxycarbonyl isobutyl ester	240	80
Docosanoic acid, isobutyl ester	120	40
Tyrosine, *N*-isobutoxycarbonyl isobutyl ester	30	6

last homologue in the series of dicarboxylic and fatty acid derivatives are shown in Fig. 3(A–D), respectively. Other investigated isobutyl/di-isobutyl esters of fatty and dicarboxylic acids produced analogous mass spectra consisting predominantly of the quasimolecular ion peaks.

Isobutyl esters of oleic and linolenic acids eluted with the same retention time. Fig. 3E shows that in spite of being unresolved chromatographically, these compounds could still be distinguished due to their characteristic ions.

The mass spectra of all the compounds were registered in the concentration range 10^{-8} to 10^{-10} g/μl, and the linearity of calibration curves in this concentration range has been confirmed. For detection limit estimation the most diluted standard solution was used. Detection limits were measured both in the total ion current (TIC) and SIM modes (Table 2).

As seen from Table 2, estimated detection limits measured in the TIC mode were 6 pg to 0.25 ng depending on compound, and 2–80 pg in case of SIM. Relative standard deviation was within 12% in the TIC mode and 20% in case of SIM calculated for four parallel runs.

4. Conclusion

Simultaneous GC-MS PICI determination of 13 fatty, 6 dicarboxylic and 13 amino acids was performed directly in aqueous media using isobutyl chloroformate derivatives in a single run. The sample preparation is selective and simple. It was shown that PICI with isobutane enabled registration of the mass spectra containing the intense quasimolecular ions for all the investigated fatty acid isobutyl esters, dicarboxylic acid di-isobutyl esters, and most of isobutoxycarbonyl isobutyl esters of the amino acids. Application of SIM in PICI mass spectrometry resulted in detection limits of 2–80 pg depending on a compound that is comparable with electron ionization, yet providing the ultimate sensitivity, which is rather important in analysis of complex real samples.

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