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Quantitative analysis of fluorinated ethylchloroformate derivatives of non-protein amino acids using positive and negative chemical ionization gas chromatography–mass spectrometry

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Abstract

The GC–MS characterization of the ethylchloroformate derivatives of amino acids in an aqueous medium has been applied to non-protein amino acids. Derivatization of non-protein amino acids using ethylchloroformate, trifluoroethanol, and pyridine produced strong $[M+1]^+$ and $[M-1]^-$ ions in positive and negative chemical ionization (CI) modes, respectively. Twenty-one out of the twenty-three non-protein amino acids studied produced detectable ion chromatograms in both ionization modes when methane was used as the CI reagent gas. Mass spectra of these non-protein amino acid derivatives showed characteristic $[M-19]^+$, $[M+1]^+$, $[M+29]^+$, and $[M+41]^+$ peaks in the positive chemical ionization mode, and $[M-1]^-$, and $[M+35]^-$ peaks in the negative chemical ionization mode. The detection limits and the linear dynamic range of trifluorethanol ethylchloroformate derivatives of non-protein amino acids were studied using positive chemical ionization. The detection limits are mostly in the femtomole range.

1. Introduction

Recently, Husek introduced a derivatization procedure for gas chromatographic (GC) analysis of protein amino acids using ethylchloroformate (ECF) [1] which was based on his previous study of chloroformate-induced esterification of the carboxylic groups of fatty acids [2]. The main advantages of this technique include: (1) simultaneous N(O,S)-derivatization of amino acids in one step, (2) derivatization of amino acids in aqueous solution, (3) reaction time of only a few seconds, (4) derivatization with inexpensive reagents, and (5) GC [1–3] or GC–MS [4–6] identification of products.

It was shown by this lab [6] that ECF derivatization of protein amino acids in a solution of water, trifluoroethanol, and pyridine, followed by GC–MS analysis of the products using positive and/or negative chemical ionization (CI) mode increases the sensitivity of detection and simplifies the identification of these derivatized amino acids.

To date, most of the reported data shows the

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application of ECF derivatization to protein amino acids. Here we report the application of the ECF derivatization to non-protein amino acids using trifluoroethanol as the esterifying agent.

2. Experimental

The twenty-three non-protein amino acids were provided by Professor Mabry, Department of Botany, The University of Texas at Austin. A solution containing 250 ng/ml of each of the non-protein amino acids in 0.1 *M* HCl was used for the qualitative analysis. The ethylchloroformate was purchased from Sigma (St. Louis, MO, USA). The trifluoroethanol was purchased from Aldrich (Milwaukee, WI, USA). The ECF derivatives were prepared according to the procedure reported previously [1]. Analysis by GC–MS was carried out on a Finnigan MAT TSQ-70 MS (San Jose, CA, USA) coupled to a Varian 3400 GC (Palo Alto, CA, USA). The GC separation employed a 25-m DB-1701 fused-silica capillary column with 0.32 mm I.D. and 1 μm film coating (SGE, Austin, TX, USA). Mass spectrometric conditions were as follows: scan range of 120–500 u, scan rate of 1 scan/s (3 scans/s for quantitative analysis), methane gas at a pressure of ca. 2 Torr used as the CI reagent gas, and an interface temperature of 270°C. GC conditions were as follows: initial column temperature of 80°C, 2 min delay, final column temperature of 270°C, and a column ramp rate of 10°C/min. A 1-μl aliquot of the solution was injected in direct (splitless) injection mode for each analysis. For quantitative study, the stock solution (250 ng/ml, the upper limit of the dynamic range study) of non-protein amino acids was diluted by two to five orders of magnitude using 0.1 *M* HCl solution. These solutions were then derivatized and analyzed according to the above procedure.

3. Results and discussion

The reconstructed total ion chromatograms (RIC) of the twenty-one ECF + trifluoroethanol + pyridine derivatives of non-protein amino acids under positive and negative chemical ionization modes are shown in Figs. 1 and 2, respectively. Under the experimental conditions employed here, each derivatized non-protein

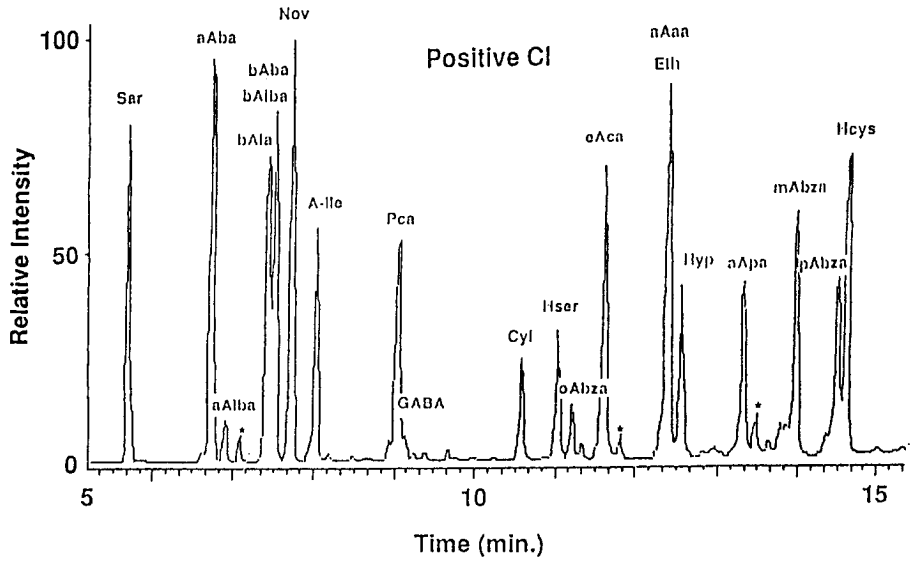

Fig. 1. Reconstructed TIC of positive chemical ionization GC–MS of the trifluoroethanol ECF derivatized non-protein amino acids. Peaks marked with an asterisk were not identified.

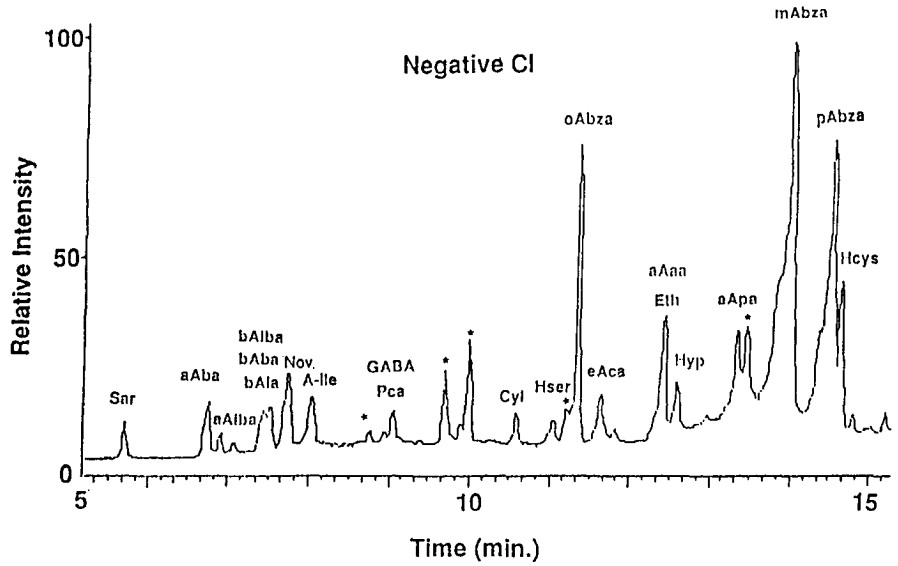

Fig. 2. Reconstructed TIC of negative chemical ionization GC–MS of the trifluoroethanol ECF derivatized non-protein amino acids. Peaks marked with an asterisk were not identified.

amino acid produced a separated peak except for three pairs whose peaks overlapped: β-aminoisobutyric acid and β-aminobutyric acid, DL-α-aminoadipic acid and DL-ethionine, and pipecolinic acid and γ-aminobutyric acid. However, the overlapping peaks were identified when their ion chromatograms were plotted. In cases where derivatized non-protein amino acids had the same molecular mass, their ion chromatograms and retention time were used for identification. Comparison between the GC–MS results in the positive and negative chemical ionization modes (Figs. 1 and 2, respectively) indicates that, in general, the positive CI chromatogram has lower background chemical noise and higher signal intensity than negative CI except for anthranilic acid, *p*-aminobenzoic acid, and *m*-aminobenzoic acid. Anthranilic acid in the positive CI mode did not produce a significant peak in the corresponding total ion chromatogram. In the negative CI mode, however, these three compounds showed strong peaks. The most likely explanation for this behavior is that these three non-protein amino acids all have an aromatic ring which increases the electron capture cross-section and/or stabilizes the negatively charged ions, therefore giving higher signal intensity with respect to non-aromatic non-protein amino acids. L-Citrulline and taurine did not produce detectable peaks in the total ion chromatograms of either ionization mode.

The results of the mass spectrometric study of the ECF derivatives of non-protein amino acids using trifluoroethanol in positive and negative CI modes are tabulated in Table 1. Similar to the analysis of the protein amino acids, $[M+1]^+$ is the base peak in all of the positive CI spectra. The positive CI spectra show a common fragmentation pattern for all of the non-protein amino acids, with major fragments originating from the loss of HF from $[M+1]^+$. In addition, with methane as a regent gas, all of the non-protein amino acid derivatives formed $[M+29]^+$ and $[M+41]^+$ ions, the $C_2H_5^+$ and $C_3H_5^+$ adducts of the molecules. Existence of the $[M-F]^+$, $[M+1]^+$, $[M+29]^+$ and $[M+41]^+$ peaks immediately distinguishes the fluorinated derivatives of non-protein amino acids from other side products or impurities which produce peaks in the chromatograms. As an example, Fig. 3 shows the mass spectrum of derivatized sarcosine (Sar) under the positive CI mode using methane as reagent gas.

The negative CI spectra show that $[M-1]^-$ is

Table 1
Characteristic ion peaks in positive CI spectra of trifluoroethanol ECF derivatives of non-protein amino acids

Non-protein amino acid	M_r	Derivative $[M+1]^+$	Base peak (m/z)	Other important ions (m/z)
Sarcosine (Sar)	89	244	244	224,272,284
β-Alanine (bAla)	89	244	244	224,272,284
α-Aminobutyric acid (aAba)	103	258	258	238,286,298
α-Aminoisobutyric acid (aAiba)	103	204	204	232,244
β-Aminobutyric acid (bAba)	103	258	258	238,286,298
β-Aminoisobutyric acid (bAiba)	103	258	258	238,286,298
L-Norvaline (Nov)	117	272	272	252,300,312
DL-Homoserine (Hser)	119	174	174	202,214
Pipecolinic acid (Pca)	129	284	284	264,312,324
Cycloleucine (Cyl)	129	230	230	258,270
ε-Amino-η-caproic acid (eAca)	131	286	286	266,314,326
L-*allo*-Isoleucine (A-Ile)	131	286	286	266,314,326
Hydroxy-L-proline (Hyp)	131	286	286	266,314,326
DL-Homocystein (Hcys)	135	362	362	342,390,402
m-Aminobenzoic acid (mAbza)	137	292	292	272
p-Aminobenzoic acid (pAbza)	137	292	292	272,320,332
Anthraniline (oAbza)	137	292	292	272,320,332
DL-α-Aminoadipic acid (aAaa)	161	398	398	378,426,438
DL-Ethionine (Eth)	163	318	318	346,358
γ-Aminobutyric acid GABA)	103	258	258	238,286,298
DL-α-Aminopinelic acid (aApa)	175	412	412	392,440,452

the base peak in all of the non-protein amino acids except DL-α-aminopinelic acid which gave the HCl adduct as the base peak. The negative CI spectra also show characteristic $[M+35]^-$ peaks arising from $[M+Cl]^-$ ions formed from the loss of a proton from the HCl adducts of the molecules under negative CI conditions. This is consistent with our previous results [6], that under the experimental conditions of this study protein and non-protein amino acids are present as HCl adducts.

In accordance with the previous studies of the

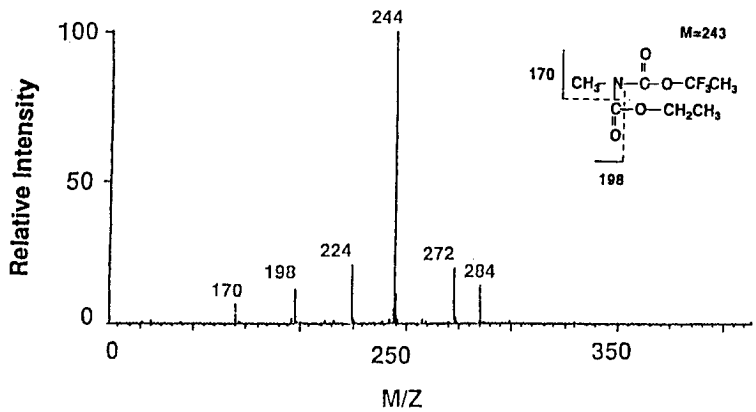

Fig. 3. Positive CI spectrum of derivatized sarcosine using methane as reagent gas. The m/z 224, 244, 272, and 284 are $[M-F]^+$, $[M+H]^+$, $[M+C_2H_5]^+$, and $[M+C_3H_5]^+$ peaks, respectively.

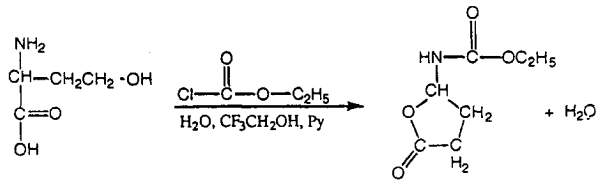

Fig. 4.

ECF derivatization of protein amino acids [5], the substituted alkyl group on the carboxylic side of the amino acids is provided by the alcohol, and the substituted alkyl group for the amine side is provided by the alkyl group of the chloroformate. A minor amount of derivatives are also found where the substituted alkyl group on both the carboxylic side and the amine side are provided by the alkyl group of the chloroformate. Derivatization of both the carboxylic side and the amine side by the alkyl group of ECF, however, gave the main products for α-aminoisobutyric acid and cycloleucine. In addition, we also observed that a lactone is the major derivatization product of DL-homoserine due to the stable structure of a five membered ring as shown in Fig. 4.

The detection limits and linear dynamic range of the ethylchloroformate derivatives of non-protein amino acids were studied using chemical ionization in the positive ion mode. The results are given in Table 2. As is shown, the detection limits are mostly in the femtomole range. Chemical ionization of the ECF trifluoroethyl ester derivatives of non-protein amino acids, therefore, is the technique of choice for detection of trace amounts of non-protein amino acids.

Acknowledgement

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References

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Table 2
Detection limits and linear dynamic ranges of trifluoroethyl ester ECF derivative non-protein amino acids

Non-protein amino acid	Detection limit (ng)	Linear dynamic range (orders of magnitude)	Correlation coefficient
α-Aminobutyric acid (aAba)	5.00E − 03	3.7	0.988
L-Norvaline (Nov)	5.00E − 03	3.7	0.9848
β-Alanine (bAla)	5.00E − 03	3.7	0.9854
DL-Homocystein (Hcys)	5.00E − 03	3.7	0.992
m-Aminobenzoic acid (mAbza)	5.00E − 03	3.7	0.9747
p-Aminobenzoic acid (pAbza)	5.00E − 03	3.7	0.9914
DL-Ethionine (Eth)	5.00E − 02	3	0.9917
DL-α-Aminopimelic acid (aApa)	5.00E − 02	3	0.9986
Sarcosine (Sar)	5.00E − 02	3	0.982
Hydroxy-L-proline (Hyp)	5.00E − 02	3	0.98
α-Aminoisobutyric acid (aAiba)	5.00E − 02	3	0.9933
DL-Homoserine (Hser)	5.00E − 02	3	0.9877
Pipecolinic acid (Pca)	5.00E − 02	3	0.9916
Cycloleucine (Cyl)	5.00E − 02	3	0.9924
L-*allo*-Isoleucine (Alle)	5.00E − 02	3	0.9975
ε-Amino-η-caproic acid (eAca)	0.25	2.7	0.9948
DL-α-Aminoadipic acid (aAaa)	0.25	2.7	0.9988

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