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Quantitative analysis of fluorinated ethylchloroformate derivatives of protein amino acids and hydrolysis products of small peptides using chemical ionization gas chromatography–mass spectrometry

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Abstract

The derivatization and extraction efficiencies of 16 protein amino acids were investigated using trifluoroethanol+ethylchloroformate+pyridine as the derivatization reagents and chloroform as the solvent. The derivatization efficiencies ranged from 90% to 99% for all the amino acids studied except aspartic acid (79%). The extraction efficiencies of the derivatized amino acids using chloroform were close to 100%. In addition, the detection limits and linear dynamic ranges of trifluoroethanol ethylchloroformate (TFE-ECF) derivatives of these amino acids were studied using positive-ion chemical ionization gas chromatography–mass spectrometry (GC–MS). The detection limits of the protein amino acids were mostly in the low femtomole range. The linear dynamic ranges of these amino acids were in the range of zero to three orders of magnitude. The GC–MS analysis of the derivatized amino acids was applied to the hydrolysis products of Equal, a sugar substitute containing aspartame and diprotin B, a tripeptide. The results demonstrate that the TFE-ECF derivatization of the hydrolysis products of small peptides followed by GC–MS analysis can be used for the identification of their amino acid composition (except arginine) and for their complete amino acid analysis.

Keywords: Derivatization, GC; Peptides; Amino acids; Ethylchloroformate

1. Introduction

The GC-based methods for amino acid analysis require derivatization of the amino acids to produce volatile adducts [1–7]. The most recent derivatization technique is that of Husek [8–10], in which a solution of ethylchloroformate (ECF)+ethanol+pyridine reacts instantaneously with the amino acids in aqueous solution. The reaction ethylates the amine and carboxyl functional groups in one step. The derivatized amino acids are then analyzed using a gas chromatograph and identified according to their retention time. This procedure is advantageous due to its uniquely rapid reaction time, simple sample handling (reaction in aqueous solution) and use of inexpensive reagents. All protein amino acids (except arginine) and 22 out of 24 non-protein amino acids have been derivatized and analyzed using this derivatization procedure [8–12].

Recently, it was also shown that fluorinated ECF derivatives of protein amino acids have better detection limits than their non-fluorinated counterparts in both positive- and negative-ion chemical ionization modes (PICI/NICI) [11,12]. While both ionization techniques showed similar detection limits for the fluorinated ethylchloroformate derivatives, the

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reconstructed ion chromatogram of the positive chemical ionization mode showed lower background chemical noise [11]. For this reason, PICI was used for the remainder of our study.

An interesting bioanalytical problem to which GC–MS can be applied is the characterization and quantitation of the amino acid compositions of small peptides, which play important biochemical roles [13]. Identification of the amino acid compositions of these small peptides is essential in determining their structures. Although determination of amino acids can be achieved using high-performance liquid chromatography [14–16] or capillary electrophoresis [17,18], GC–MS, with its faster speed and greater compound identification capability, is often a preferable analytical tool. Moreover, GC–MS provides molecular mass information which is important in characterizing hydrolysis products of peptides.

The amino acid products obtained from the hydrolysis of small peptides have been studied using GC–MS [19]. Mabbott successfully analyzed the hydrolysis products of small peptides using the Darbre and Islam derivatization procedure [20]. In this procedure, the carboxylic acid groups are first methylated and the free amino groups are converted to amides of trifluoroacetate. This technique, however, takes approximately one hour and involves a multistep derivatization procedure.

In this study, we applied the ECF derivatization technique to a qualitative and quantitative analysis of the hydrolysis products of two small peptides. To achieve this, we investigated: (1) the derivatization efficiencies of 16 protein amino acids, (2) extraction and ionization efficiencies of these amino acids, (3) the limits of detection, the limits of quantitation and linear dynamic ranges of trifluoroethanol (TFE)-ECF derivatives of the amino acids using GC–MS in PICI mode. The results of steps 1–3 were then used for the identification and quantitation of the amino acid compositions of a tripeptide, diprotin B (Val–Pro–Leu) and a real-world sample, Equal, a commonly-used sugar substitute containing aspartame (methyl ester of aspartyl-phenylalanine).

2. Experimental

The procedure for the derivatization of amino acids using ethanol was previously reported by Husek et al. [8–10]. In our experiment, however, trifluoroethanol was used as the esterification reagent for the carboxylic acids instead of ethanol [11,12]. The analyses were performed using a Varian 3400 gas chromatograph (Palo Alto, CA, USA) interfaced to a Finnigan-MAT TSQ 70 mass spectrometer (San Jose, CA, USA). The GC capillary (SGE, Austin, TX, USA) was a 7 m DB-5 fused-silica capillary column (cut from a 30-m-long column) with a 0.32 mm I.D. and a film thickness of 1 μm. Methane at a pressure of approximately 2 Torr (1 Torr=133.322 Pa) was used as the chemical ionization reagent gas. For each GC analysis, a 2-μl aliquot of the sample was injected into the GC injector in splitless injection mode. The GC column was held at an initial temperature of 80°C for 2 min then ramped to a final temperature of 280°C (300°C for the peptide analysis) at a rate of 30°C/min. The injector temperature was 280°C (290°C for the peptide analysis) throughout the experiment. The mass spectrometer was scanned in the mass range of 140–412 u (140–550 u for the peptide analysis) at a rate of 3 scans/s in PICI mode.

For the detection limits and quantitation study, a stock solution containing an equimolar amount of 17 protein amino acids, hereafter called AA-S-18 (2.5 mmol/ml, except L-cystine at 1.25 mmol/ml) (Sigma, St. Louis, MO, USA), was diluted by one to six orders of magnitude using a 0.1 *M* solution of HCl (Fisher Scientific, Fairlawn, NJ, USA). Each solution was derivatized according to the previously reported procedure [11], and the chloroform layer was removed. The remaining aqueous solution was dried under a stream of N_2 gas and redissolved in 20 μl of 0.1 *M* HCl, and was then rederivatized as before, using TFE+ECF+pyridine and chloroform. The chloroform layer from the second derivatization process was combined with the previously removed chloroform layer. This solution was dried under a stream of N_2 gas for 7–10 min and redissolved in 20 μl of chloroform which contained 1 μg of dichlorobenzene (Fisher Scientific) as an internal standard. A 2-μl aliquot of this solution was injected into the GC injector as described above. The amino acid standards used for calibration of the chemical ionization process were prepared as follows: 20 μL of the AA-S-18 solution was derivatized, diluted up to

three orders of magnitude, and analyzed using GC–MS in the PICI mode. For quantitative study, care must be taken when the derivatized amino acid solutions are dried under a stream of N_2 gas. Although no noticeable evaporation of small peptides was observed for a drying time of 7–10 min, evaporation of low-molecular-mass derivatized amino acids may occur if drying continues for longer periods of time.

To study the amino acid compositions of small peptides, 2.5 μmol of diprotin B (Sigma) was hydrolyzed using 1 ml of a 6 *M* HCl solution in a sealed glass tube under vacuum at 110°C for 24 h [21,22]. After hydrolysis, the acidic medium was evaporated with a rotary evaporator and redissolved in 1 ml of 0.1 *M* HCl solution. A 20-μl sample of this solution was rederivatized and analyzed. In addition, the same procedure was applied to 2.5 μmol of each of the three amino acids (valine, proline and leucine) making up the tripeptide. The hydrolysis and derivatization technique was also applied to Equal (NutraSweet, Deerfield, IL, USA). A solution of 100 mg Equal was hydrolyzed using 1 ml of 6 *M* HCl in a sealed glass tube under vacuum at 110°C for 24 h. The acidic medium was evaporated and the contents were redissolved in 1 ml of 0.1 *M* HCl solution, derivatized and then analyzed using GC–MS. For comparison, a 1-mg sample of the pure dipeptide, Asp-Phe (Sigma) in 1 ml of 6 *M* HCl and a sample of 0.446 mg of aspartic acid and 0.554 mg of phenylalanine (1:1 mole ratio) in 1 ml of 6 *M* HCl were identically treated. All experiments were repeated at least three times, and the averaged results were used in the linear dynamic range study.

3. Results and discussion

To accurately study the limits of detection, the limits of quantitation and the linear dynamic ranges of the derivatized amino acids using chemical ionization mode, several factors that can affect the quantitation study were examined. These factors included the derivatization efficiency of each amino acid, the extraction efficiency of each amino acid and the quantitative behavior of the chemical ionization procedure itself.

3.1. Derivatization and extraction efficiencies of amino acids

To study the derivatization efficiencies of the amino acids, the aqueous solution of amino acids was derivatized using the ethylchloroformate+trifluoroethanol+pyridine solution. The derivatized amino acids were extracted using 100 μl of chloroform. The chloroform solution was dried under a stream of nitrogen and the residue was redissolved in 20 μl chloroform and analyzed by GC–MS using PICI. The extraction of the leftover aqueous solution by chloroform and subsequent GC–MS analysis of this chloroform layer showed no significant signal for derivatized amino acids, indicating that the extraction efficiency was nearly 100%.

The aqueous solution remaining from the first derivatization process was rederivatized and analyzed using GC–MS. Examination of the remaining aqueous solution after the second derivatization process by direct insertion probe indicated that the amount of remaining underivatized amino acids was insignificant. That is, after two derivatization processes, almost 100% of the amino acids were derivatized. Table 1 shows the derivatization efficiency after one-step derivatization for each amino acid using a 0.1 μmol/ml solution of the amino acids mixture.

3.2. Relative intensities of the protonated molecular ions of derivatized amino acids under chemical ionization

The mass spectra and characteristic ion peaks of the protein amino acids have been previously reported [11]. It was shown that $[M-F]^+$, $[M+H]^+$, $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ ions immediately identify the fluorinated amino acid derivatives. To examine whether the $[M+H]^+$ ion peaks intensities could be used as a measure for a quantitative study of derivatized amino acids, the relative intensities of the four characteristic peaks of valine, proline and phenylalanine were examined under three different concentrations: 1, 10 and 100 ng/ml. At each concentration, the relative intensities of the four ions were constant (within the experimental error of approximately 10%) for each amino acid. The intensities of the protonated molecular ions of the deriva-

Table 1
Derivatization efficiencies of the 16 protein amino acids, and the relative intensities of the protonated molecular ions of the derivatized amino acids

Amino Acid	Derivatization efficiency (%) following the first derivatization	Relative intensity of $[M+H]^+$ of 0.2 nmol injected amino acid derivative[a]
Glycine (G)	97	58
Alanine (A)	93	44
Proline (P)	97	72
Valine (V)	99	47
Aspartic acid (D)	79	68
Threonine (T)	95	41
Leucine (L)	99	64
Isoleucine (I)	99	65
Serine (S)	93	10
Methionine (M)	99	60
Phenylalanine (F)	99	100
Cystine (C)	99	38[b]
Glutamic acid (E)	90	4
Lysine (K)	91	<0.1
Tyrosine (Y)	99	<0.01
Histidine (H)	–	<0.01

[a] Normalized with respect to the phenylalanine (F).
[b] This number is increased by a factor of 2 to compensate for the actual injection of 0.1 nmol of cystine.

tized amino acids can therefore be used for quantitative analysis, and their relative intensities can be used as a measure of their relative ionization (protonation) efficiencies. The relative intensities of the protonated molecular ions of each amino acid after the first and second derivatizations are tabulated in Table 1.

3.3. Quantitative study under chemical ionization mode

To date, the number of quantitative studies using chemical ionization are very limited. This is mainly because of (1) the limited amount of protonating reagent in the source of the mass spectrometer under chemical ionization conditions [23], and (2) instrumental parameters (temperature, pressure, design of the ion source, length of the ion path, etc.) that are difficult to reproduce from one instrument to another. Under chemical ionization, the ion source of the mass spectrometer is filled with approximately 2 Torr of a chemical reagent gas, in this case methane. The reagent gas is then bombarded with electrons to form a plasma. When methane is used as a reagent gas, the positive ions in the plasma are predominantly CH_5^+, $C_2H_5^+$ and $C_3H_5^+$. The ions will protonate and/or form positively charged adduct ions with the molecule of interest. The limited amount of reagent gas in the ion source of the mass spectrometer under chemical ionization conditions determines the analysis' linear dynamic range and the upper limit of its linear range.

To calibrate the linearity of the signal intensity versus concentration under chemical ionization conditions, several working curves were developed. These working curves were obtained by measuring the signal intensity of the $[M+H]^+$ from a series of amino acid standards. For example, Fig. 1 shows the signal intensity versus amount of injected derivatized leucine. As shown, the working curve is straight to approximately 100 ng of derivatized leucine, but above 100 ng the curve is no longer straight. The non-linearity of the line results from an inadequate amount of protonating agents in the ion source of the MS under CI conditions. The slope of the straight line was independent of the amino acid used. The working curve was then used to calibrate the linearity of the instrument under chemical ionization conditions.

Consequently, the standard calibration curve for

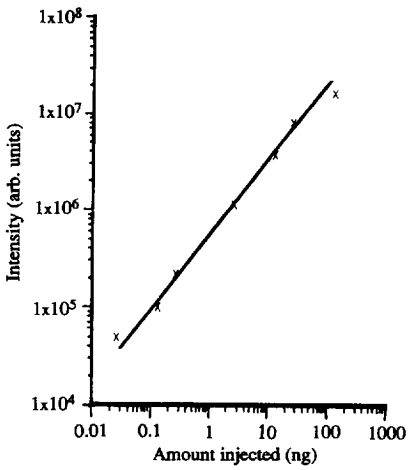

Fig. 1. Signal intensity versus amount of derivatized of leucine (Leu) injected, corresponding to the serial dilution of the derivative up to three orders of magnitude.

each amino acid was calculated by normalizing their sample calibration curves with respect to the chemical ionization working curve. For example, Fig. 2 shows the normalized linear dynamic range for ECF-derivatized glycine.

3.4. Quantitative analysis of amino acids

The standard calibration curve of glycine as a sample amino acid is shown in Fig. 2. The linear dynamic range was observed in a range from 100 pg to 100 ng for all amino acid derivatives with the exception of lysine, threonine and serine, which showed a linear range from 2 to 100 ng. The signal intensities of histidine and tyrosine were too weak for quantitative analysis within the concentration range studied. The detection limits ($S/N=3$), linear dynamic ranges, limits of quantitation (lowest point in linear dynamic range) and relative standard deviations of the N-ethoxycarbonyl trifluoroethyl ester derivatives of the protein amino acids are tabulated in Table 2. The results show that the detection limits of protein amino acids are mostly in the low picomole range. Considering the fact that these detection limits were obtained by scanning a wide mass range (140–412 u), significant improvement in detection limits are expected for the selected ion monitoring mode.

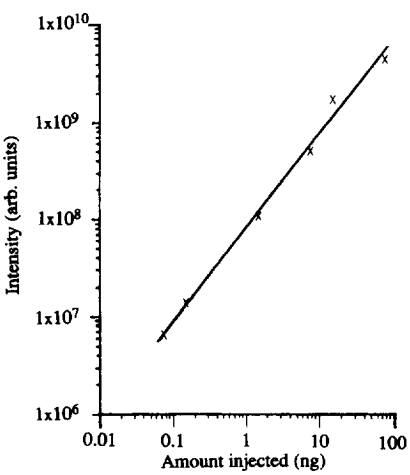

Fig. 2. Normalized linear dynamic range for ECF-derivatized glycine (Gly).

3.5. Application to hydrolysis products of small peptides

To investigate the application of the ethylchloroformate+trifluoroethanol+pyridine derivatization of amino acids to the hydrolysis products of small peptides, diprotin B, a tripeptide (Val–Pro–Leu), was analyzed. A solution of the three amino acids in diprotin B was identically treated for comparison. The ion chromatograms of the two solutions after 24 h hydrolysis with 6 *M* HCl at 110°C are shown in Fig. 3. The ion chromatograms of the derivatized amino acids derived from hydrolysis of the tripeptide and pure amino acids were very similar. The relative ratios of derivatization and ionization efficiencies of three amino acids from the peptide and amino acid mixture after hydrolysis were 71:100:91 and 66:100:83, respectively.

To study the utility of this derivatization technique in the analysis of dipeptide and amino acid composition of a real-world sample, Equal was analyzed. To quantify the amount of aspartame (methyl ester of aspartyl-phenylalanine) in the Equal sample, 20 μl of a 1 mg/ml Equal in 0.1 *M* HCl solution was derivatized with ethylchloroformate+trifluoroethanol+pyridine and analyzed using GC–MS. A standard containing 20 μl of a 1 mg/ml solution of pure Asp–Phe was also derivatized and analyzed using the same method for comparison. The results of the mass spectrometric study of the N-ethoxy-

Table 2
Detection limits, limits of quantitation, linear dynamic ranges and relative standard deviations of trifluoroethyl ester ethylchloroformate-derivatized amino acids

Amino Acid	Detection limit (ng)	Limit of quantitation (ng)	Linear dynamic range (orders of magnitude)	R.S.D. (%)
Glycine (G)	0.003	0.1	3.4	0.99
Alanine (A)	0.003	0.1	3.0	0.99
Proline (P)	0.004	0.1	3.4	0.99
Valine (V)	0.004	0.1	3.4	0.99
Aspartic acid (D)	0.001	0.1	3.4	0.99
Threonine (T)	0.1	1.0	2.4	0.99
Leucine (L)	0.001	0.1	3.0	0.99
Isoleucine (I)	0.001	0.1	3.0	0.99
Serine (S)	0.1	1.0	2.3	0.99
Methionine (M)	0.01	0.1	2.7	0.99
Phenylalanine (F)	0.001	1.0	3.4	0.99
Cystine (C)	0.01	0.1	3.0	0.99
Glutamic acid (E)	0.01	0.1	1.0	0.99
Lysine (K)	0.6	20.0	1.0	1.0
Tyrosine (Y)	2.5	–	–	–
Histidine(H)	25.0	–	–	–

[a] Consistent with previous studies [1,3,8], under experimental conditions employed here, arginine was not detected.

carbonyl trifluoroethyl ester derivatives of aspartame and Asp–Phe in the PICI mode are shown in Fig. 4a,b. The $[M-45]^+$, which arises from the loss of $-OCH_2CH_3$, is the base peak of the spectrum of both dipeptides.

However, the ratio of the intensity of the base peak of aspartame to that of the Asp–Phe was approximately 1:150, indicating that approximately only 1/150 of Equal is aspartame. This is consistent with the fact that the aspartame is approximately 160 times sweeter than sucrose in aqueous solution [24]. To analyze the amino acid contents of Equal, therefore, solutions containing 100 mg of Equal in 1 ml of

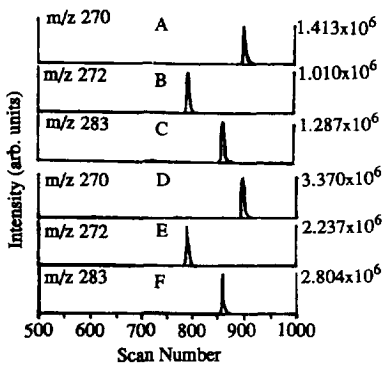

Fig. 3. Ion chromatograms for the two solutions of diprotin B after 24 h HCl hydrolysis at 110°C. A, B and C represent trifluoroethanol ethylchloroformate derivatives of valine, proline and leucine from the diprotin B, respectively. D, E and F represent trifluoroethanol ethylchloroformate derivatives of valine, proline and leucine from the standard, respectively.

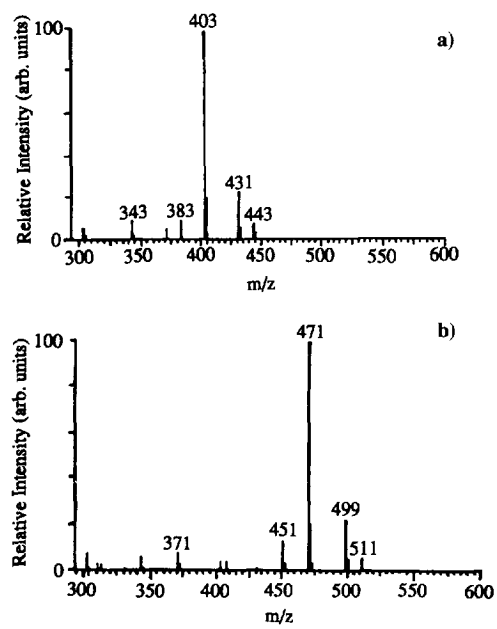

Fig. 4. MS (PICI mode) analyses of the N-ethoxycarbonyl trifluoroethyl ester derivatives of (a) aspartame and (b) Asp–Phe.

6 *M* HCl and 1 mg of pure dipeptide in 1 ml of 6 *M* HCl were hydrolyzed, derivatized and then analyzed using GC–MS. The ion chromatograms of the derivatized amino acids derived from hydrolysis of the Asp–Phe and Equal were very similar. The relative peak intensities of the amino acids (Phe:Asp) of the dipeptide Asp–Phe and Equal after hydrolysis were 1.35:1 and 1.31:1, respectively.

4. Conclusion

Derivatization with an ethylchloroformate+trifluoroethanol+pyridine solution is a useful technique for the identification and quantitation of amino acids and hydrolysis products of small peptides in aqueous solution. Limits of quantitation of the ECF-derivatized amino acids are mostly in the low femtomole range. Our preliminary results on analysis of the hydrolysis products of small peptides using trifluoroethanol+ethylchloroformate+pyridine derivatization indicate that this technique could be used to identify the amino acid contents (except arginine) of peptides. Further work, however, is required to quantify the amino acid contents of longer peptides by using this technique. For quantitative analysis, we suggest that the sample be derivatized and extracted and the remaining aqueous solution rederivatized to guarantee the complete derivatization of all amino acids. In addition, for quantitation under chemical ionization conditions, the chemical ionization calibration curve should be obtained using a serial dilution of a derivatized amino acid. The standard calibration curve can then be obtained by normalizing the calibration curve of the unknown with respect to the chemical ionization calibration curve.

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