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Gas Chromatography–Mass Spectrometry Method for the Determination of Free Amino Acids as Their Dimethyl-*tert*-butylsilyl (TBDMS) Derivatives in Animal Source Food

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Supporting Information

ABSTRACT: The suitability of a one-step derivatization procedure using *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide for the simultaneous assay of 22 free amino acids and its application for their analysis in six animal source foods (pork, dry cured ham, chicken stock, fresh cheese, ripened cheese, and dry salted sardine) by GC-MS were studied. All 22 free amino acid derivatives were correctly detected and resolved. Reproducibility (%RSD) of the method was in the range of 1.9–12.2%. Detection and quantitation limits of the analytical procedure ranged from 0.01 to 0.46 mg/100 g dry weight and from 0.02 to 1.55 mg/100 g dry weight, respectively. The calibration curves were linear within the range 0.1–15.0 mg/100 g with correlation coefficient values (R^2) from 0.9891 to 0.9983. All analyzed food products showed free amino acid contents similar to those found in the scientific literature. The proposed GC-MS method for the determination of free amino acids in animal source food can be used in routine for both analytical and research purposes.

KEYWORDS: *free amino acids, GC-MS, MTBSTFA, animal source food*

INTRODUCTION

Amino acids are organic compounds present in animal tissues not only forming peptides, proteins, and nonpeptide polymers but also as free molecules. There are large amounts of free amino acids that play important roles in animal source food. From the nutritional point of view, the determination of the free amino acid content of food is an interesting tool to complete food label information. In addition, it is known that some free amino acids have a direct role in food flavor,[1] whereas others have an enhancement effect on palatability.[2] Moreover, free amino acids participate in the formation of amines and volatile compounds as a consequence of decarboxylation and Maillard reactions, respectively.[3] Free amino acid content is also of interest in the study of dynamic changes throughout food processing, being a useful index of proteolytic and enzymatic hydrolysis reactions in some products such as cheese, dry-cured ham, or fish.[4−12] Because of the influence of free amino acids on sensory and technological characteristics, and due to the important weight of these characteristics in acceptance and consumption of food products, the determination of free amino acids in food science is of great concern.

Current chromatographic procedures to separate and quantitate amino acids in food and derivates include ion exchange chromatography, gas chromatography (GC), and high-performance liquid chromatography (HPLC).[13] Reverse phase HPLC with a precolumn derivatization process is perhaps the most popular of the current techniques to quantitate amino acids in food. However, gas chromatography–mass spectrometry (GC-MS) has been proven to be a useful and alternative technique, and it is also a suitable method to analyze amino acids in food. Besides, GC-MS is a simple, versatile, rapid, and widespread methodology.

When GC was first used for the analysis of amino acids, there was the need for two-stage derivatization steps (esterification and acylation), which decreased the acceptance of the GC technique. Later, a one-step derivatization method with the silylation of both the amino and carboxyl groups using bis(trimethylsilyl)trifluoroacetamide (BSTFA) as derivative reagent was developed.[14] Silylation is a derivatization procedure suitable for application to a high variety of polar molecules, based on the substitution of the active hydrogen atoms of OH, NH, and SH groups by a silyl group. The consequent reduction of the dipole–dipole interaction of the target molecules results in their transformation into molecules of low polarity, increased volatility, and high thermal stability, making them suitable for being analyzed by GC-MS with enhancement of the resolution.[15]

N-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) has also been successfully used to derivatize amino acids of protein and peptide hydrolysates.[16] MTBSTFA produces dimethyl-*tert*-butylsilyl (TBDMS) derivatives, which are characterized by a higher molecular weight compared with the trimethylsilyl (TMS) derivatives produced by BSTFA. As a consequence, the elution of these TBDMS derivatives requires longer running times. However, TBDMS derivatives are considerably more stable not only to moisture but also to hydrolysis, hydrogenolysis, mild reduction, and oxidation

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reactions than the corresponding TMS derivatives,[15] being more appropriate for the GC-MS analysis. In addition, MTBSTFA allows the use of milder derivatization conditions. The reaction takes place rapidly even at room temperature, except for the more basic amino acids (lysine, arginine, histidine, glutamine, and asparagine) when using MTBSTFA:/acetonitrile (1:1).[16] Moreover, TBDMS derivatives can be identified with high selectivity and specificity using selected ion monitoring analysis (SIM) due to their characteristic EI mass spectra. MTBSTFA is the second most widely used derivative agent in the performance of GC-MS,[15] and some authors recommend its use when there is the necessity of a silylation procedure of amino acids.[17]

Most studies focused on MTBSTFA for derivatizing amino acids have been developed in model systems.[18,19] Only a few publications in the scientific literature show the use of MTBSTFA for derivatizing amino acids from biological samples. MTBSTFA has been used to analyze amino acid content in neurochemicals[20] and soil.[21] However, the only paper found devoted to its use in foodstuffs is that by Starke et al.,[22] on potato juice samples, and no information for its use on animal source foods has been found in the scientific literature. Moreover, we have not found any work carrying out the validation of this analytical procedure. Thus, the aim of this work was to study the suitability of the MTBSTFA derivatization procedure for the analysis of complex free amino acid mixtures and for its use in diverse animal source food complex matrices.

MATERIALS AND METHODS

Chemicals. Hydrochloric acid (HCl), 37% extra pure (Scharlau, Barcelona, Spain), and glass wool, washed (Scharlau), were used in the free amino acid extraction. Acetonitrile of HPLC-gradient grade (Panreac, Barcelona, Spain) and dry dichloromethane, maximum 0.01% water, stabilized with amylene (Panreac, Barcelona, Spain), were required for the deproteinization and derivatization. Standard amino acids (Sigma-Aldrich, Madrid, Spain) purchased for preparing the standard solutions were alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, phenylalanine, aspartic acid, hydroxyproline, cysteine, glutamic acid, arginine, asparagine, lysine, glutamine, histidine, tyrosine, tryptophan, and cystine. DL-Norleucine (Sigma-Aldrich) was used as internal standard. MTBSTFA (Sigma-Aldrich) was used as derivatization agent.

Standard Solutions and Calibration. Stock solution of norleucine at 5 μg/mL was prepared by dissolving 0.1 g of norleucine in 200 mL of 0.1 N HCl. An initial standard solution at 200 μg/mL of each individual amino acid was prepared. For that, 0.05 g of each amino acid was individually dissolved in 0.01 N HCl with an ultrasonic apparatus. Subsequently, the 22 dissolved amino acids were mixed and more 0.1 N HCl was added to obtain a total volume of 250 mL. Then, seven increasing dilutions of the initial standard solution were made (150, 100, 50, 25, 10, 5, and 1 μg/mL). These standard calibration solutions were freshly made the day of the analysis. Three replications of each concentration level were analyzed to construct the calibration curves. Calibration curve equations (Table 1) ($y = ax + b$) and their corresponding correlation coefficients were obtained for each individual free amino acid using a spreadsheet application, with the ratio of the free amino acid peak area/norleucine peak area (y) and the concentration levels (x). The final results, expressed in milligrams per 100 g of dry weight (dw), take into account the moisture content and the exact weight of the sample.

Samples. Six different source animal food products were analyzed in this work to demonstrate the suitability of the developed method. The selected products were lean pork, Iberian dry-cured ham, chicken stock, fresh cheese, ripened cheese, and dry salted sardine. They were chosen to cover a wide range of free amino acid concentration, with fresh products (fresh cheese, lean pork), ripened products (Iberian dry-cured ham, ripened cheese, and dry salted sardine), and a product that is expected to have a high amount of free amino acids (chicken stock). These food products also differed in their animal source (dairy, fish, and meat products) and moisture content. All products were purchased from a local supermarket. With the exception of chicken stock, products (300 g) were previously ground using a commercial grinder. Subsequently, the moisture content of the six products was determined according to a method of the Association of Official Analytical Chemists (AOAC, 2000) (moisture reference 935.29).[23] The rest of the ground samples and chicken stock sample were stored at −80 °C until free amino acid analysis.

Table 1. Retention Times (Rt), Calibration Equations, and Corresponding Correlation Coefficients (R^2) of the Analyzed Free Amino Acids

amino acid	Rt	calibration eq	R^2
alanine	13.12	$y = 0.021x + 0.0327$	0.9954
glycine	13.64	$y = 0.0212x + 0.0412$	0.9941
valine	16.95	$y = 0.0214x - 0.0359$	0.9983
leucine	18.24	$y = 0.0207x - 0.0008$	0.9982
isoleucine	19.31	$y = 0.0195x - 0.0387$	0.9982
norleucine (IS)	19.79		
proline	20.72	$y = 0.0207x - 0.076$	0.9972
methionine	26.52	$y = 0.0207x - 0.0399$	0.9973
serine	27.08	$y = 0.032x - 0.004$	0.9961
threonine	28.01	$y = 0.0215x - 0,0274$	0.9941
phenylalanine	29.88	$y = 0.0209x - 0.0098$	0.9937
aspartic acid	31.29	$y = 0.0291x + 0.012$	0.9937
hydroxyproline	32.08	$y = 0.005x - 0.022$	0.9965
cysteine	32.61	$y = 0.0025x - 0.0049$	0.9916
glutamic acid	34.13	$y = 0.0045x - 0.0112$	0.9938
arginine	34.16	$y = 0.0003x - 0.0016$	0.9918
asparagine	34.83	$y = 0.0221x + 0.006$	0.9948
lysine	36.42	$y = 0.0161x - 0.0958$	0.9934
glutamine	37.48	$y = 0.0086x + 0.0414$	0.9929
histidine	40.68	$y = 0.0257x - 0.0057$	0.9915
tyrosine	41.28	$y = 0.0016x + 0.0003$	0.9891
tryptophan	42.08	$y = 0.0215x - 0.0764$	0.9931
cystine	50.56	$y = 0.0111x - 0.0409$	0.9912

Free Amino Acid Extraction. Five grams each of ground samples and of chicken stock were prepared following a modification of the method described by Aristoy and Toldra.[24] First, samples were homogeneized for 4 min in a Stomacher 400 (Lab-Blender, Barcelona, Spain) with 25 mL of 0.1 N HCl. The content of the Stomacher bag was transferred to a plastic tube and centrifuged at 10000 rpm for 50 min at 4 °C. The supernatants were filtered through glass wool and stored at −80 °C until analysis.

Free Amino Acid Deproteinization and Derivatization. Standard solutions and food samples were analyzed following exactly the same process. One hundred microliters of the filtered homogenates of each food sample and of each standard solution was placed into conical tubes. Next, 250 μL of acetonitrile was added to deproteinize the samples. Tubes were subsequently centrifuged at 10000 rpm for 3 min at 4 °C. Then, 100 μL of the supernatant was transferred to heat-resistant microcentrifuge tubes with screw lids, and 100 μL of a DL-norleucine solution (5 ng/mL) was added as internal standard. Tubes were dried in a speed vacuum model SVC200 (Savant, Barcelona, Spain) coupled to a refrigerated condensation trap model RT4104 (Savant) for 120 min. The residual water was removed by adding 50 μL of dichloromethane to the dried samples and using the speed vacuum again for 30 min. Finally, 50 μL of the derivatization agent (MTBSTFA) and 50 μL of acetonitrile were added to the dried tubes, which were manually shaken and subsequently incubated at 100 °C for 60 min to induce the derivatization reaction to occur.[19] Then,

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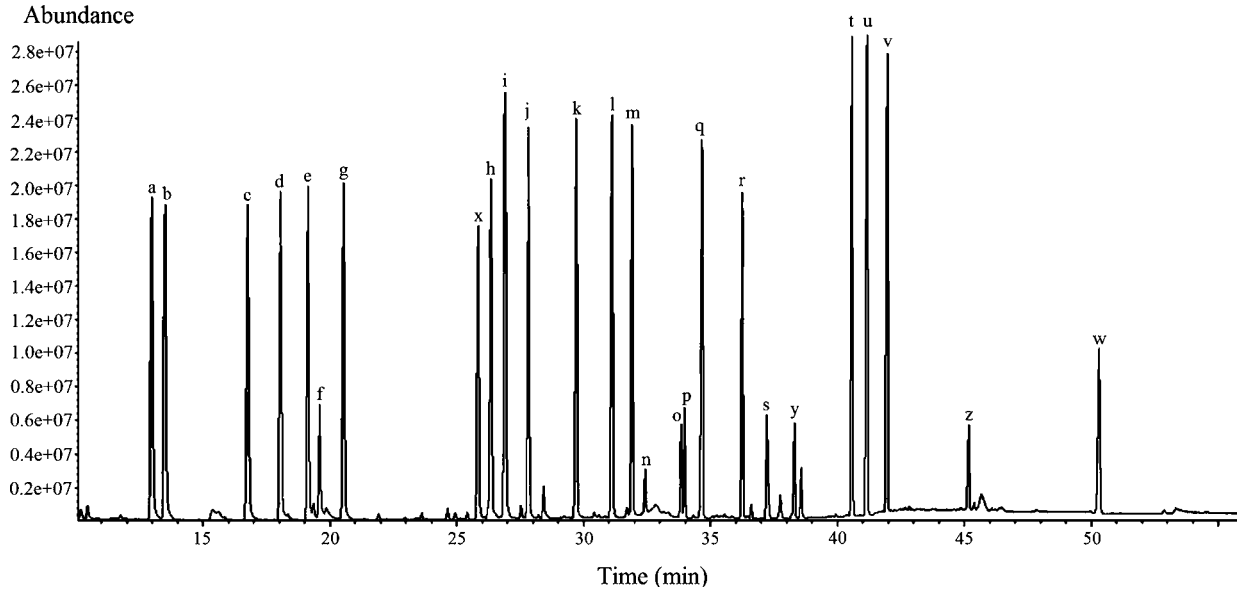

Figure 1. GC-MS separation of a standard mixture (15.00 mg/100 mL) of alanine (a), glycine (b), valine (c), leucine (d), isoleucine (e), norleucine internal standard (f), proline (g), methionine (h), serine (i), threonine (j), phenylalanine (k), aspartic acid (l), hydroxyproline (m), cysteine (n), glutamic acid (o), arginine (p), asparagine (q), lysine (r), glutamine (s), histidine (t), tyrosine (u), tryptophan (v), and cystine (w). Extra derivatives were detected for glutamine (x), arginine (y), and tryptophan (z).

tubes where stored at −18 °C and analyzed by GC-MS within the next 24 h.

Free Amino Acid Analysis. The chromatographic analysis was carried out in GC equipment 5890 series II (Hewlett-Packard, Barcelona, Spain) coupled to a mass selective detector (MSD) model 5973 (Agilent, Barcelona, Spain). A 1 μL portion of the derivatized extract was injected in splitless mode onto the column. The column used was a 50 m × 0.32 mm i.d., 1.05 μm, HP-5 (Hewlett-Packard), being a 5% phenyl-methyl polysiloxane bonded phase fused silica capillary column. Column head pressure was 12.8 psi, resulting in a flow of 1.2 mL/min at 280 °C. The oven program was as follows: 170 °C for 5 min, 4 °C/min ramp to 200 °C, held at 200 °C for 3 min, 4 °C/min ramp to 290 °C, held at 290 °C for 1 min, 20 °C/min ramp to a final temperature of 325 °C, and held for 15 min. The transfer line to the mass spectrometer program was as follows: 280 °C for 35 min, 10 °C/min ramp to 320 °C. Total run time was 55.75 min. Free amino acids were identified using both their retention time and by comparison of their characteristic m/z ions with those published in the literature.[19] The quantitation was carried out in the total ion chromatogram (TIC) mode, with the exception of hydroxyproline, arginine, and tyrosine, which were quantitated in the selected ion monitoring (SIM) mode with the extraction of the ions 314, 286, and 466, respectively.

Quality Control. Quality control (see the Supporting Information) of the GC-MS method was performed through the routine analysis of procedural blanks and quality control standards and samples to ensure the absence of contaminants and possible carry-over between samples and to assess the quality of the results. The relative standard deviation (%RSD) run-to-run was determined with five replicate analyses of samples in 1 day. Detection (LOD) and quantitation (LOQ) limits were calculated on the basis of signal-to-noise ratios of 3:1 and 10:1, respectively, using standard solutions (n = 3) with the following equations: LOD = SD/b and LOQ = 10SD/b, where, for each free amino acid, SD is the standard deviation of the average of the signal obtained for the calibration solution of lowest concentration (0.1 mg/100 mL) and b is the slope of the analytical curve calculated with the calibration solutions.[25]

RESULTS AND DISCUSSION

Free Amino Acid Analysis and Method Validation. The developed method allowed the detection and quantitation of all 22 free amino acids assayed. All of the free amino acids were separated in approximately 51 min. Retention times of the derivatives are shown in Table 1, and their average reproducibility, expressed as relative standard deviation (% RSD), was 0.04%. Figure 1 shows a standard chromatogram. Free amino acid silyl derivatives were observed in the chromatograms (Figures 1− 3) as sharp chromatographic zones, with no significant evidence of peak tailing, resolving sufficiently to permit the accurate quantitation, as expected when the analysis of silyl derivatives is performeed on polysiloxane stationary phases.[15] In the case of arginine, the derivative eluted just nearly after the glutamic acid derivative peak, although no coelution was observed. In addition to the peaks corresponding to the free amino acid derivatives, "extra" peaks can be observed in the food sample chromatograms (Figures 2 and 3), as is common in the analysis of such complex matrices. They are negligible and did not interfere in the free amino acid identification and quantitation. Only one peak was observed for 19 of the 22 free amino acids, whereas for glutamine, arginine, and tryptophan, extra derivatives were obtained. This is in agreement with the results obtained by other authors, who reported the formation of multiple derivatives for the majority of the amino acids when using MTBSTFA as derivative agent under different reaction conditions.[19] These extra derivatives were not taken into account for the quantitation process.

Given that for the range 0.10−20.0 mg/100 mL linearity was poor, the 20.0 mg/mL solution was kept out of the calculation of the calibration curve equations. Table 1 shows equations of standard curves for each of the 22 free amino acids analyzed. Good linearity was obtained for the range 0.10−15.0 mg/100 mL. The correlation coefficients were >0.99, except for tyrosine (R^2 = 0.9891). These results could be explained, to a great extent, with the use of norleucine, which may be considered a good internal standard for the control of the derivatization reaction of all the free amino acids. The values of the origin ordinates in all calibration curves were nearly 0, indicating low

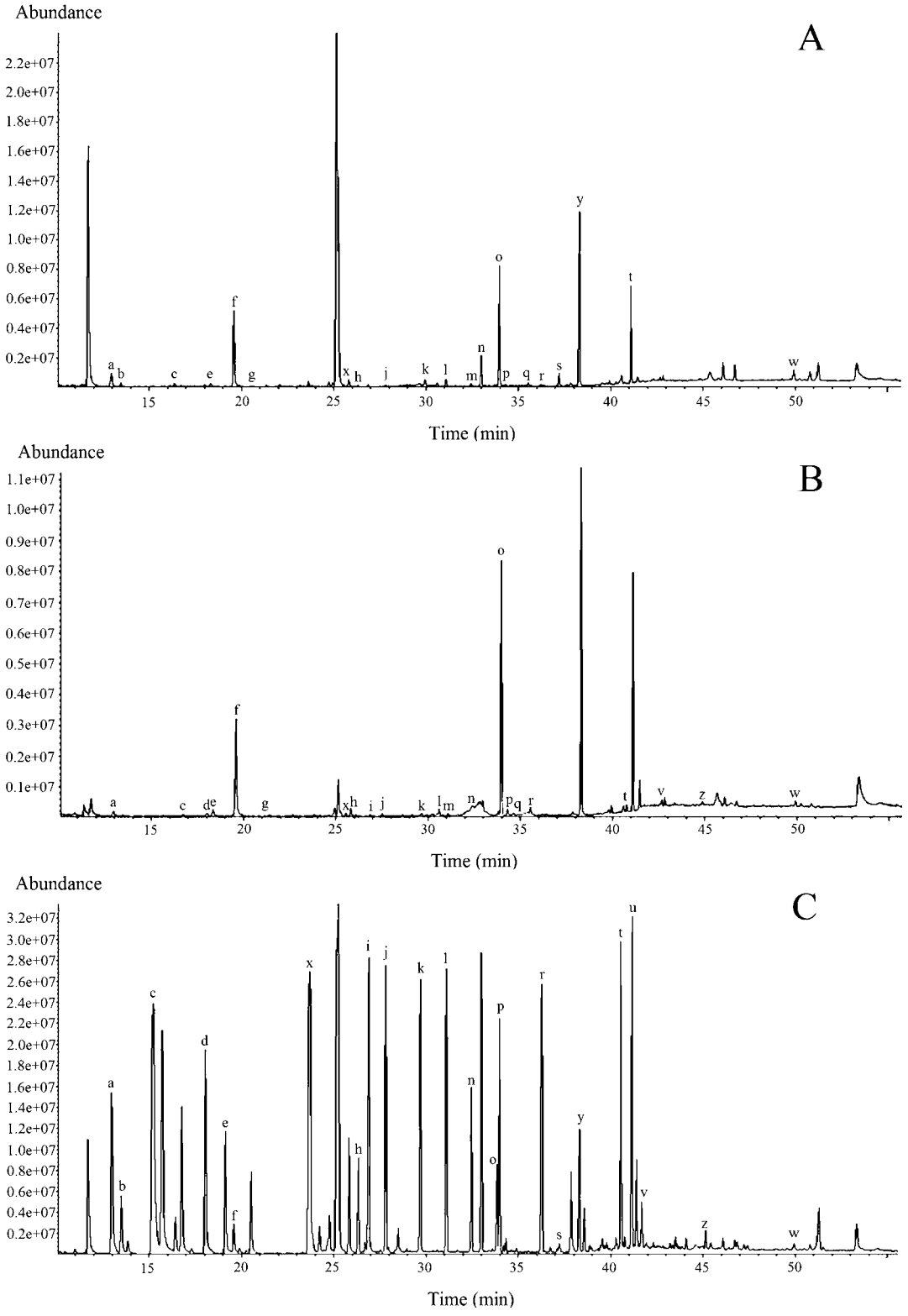

Figure 2. GC-MS chromatographic separation in (A) lean pork, (B) chicken stock, and (C) Iberian dry-cured ham, of the free amino acids assayed: alanine (a), glycine (b), valine (c), leucine (d), isoleucine (e), norleucine internal standard (f), proline (g), methionine (h), serine (i), threonine (j), phenylalanine (k), aspartic acid (l), hydroxyproline (m), cysteine (n), glutamic acid (o), arginine (p), asparagine (q), lysine (r), glutamine (s), histidine (t), tyrosine (u), tryptophan (v), and cystine (w). Extra derivatives were detected for glutamine (x), arginine (y), and tryptophan (z).

noise-signal on the chromatography of the free amino acid peaks when no concentration of the analytes was assayed. This could be due, in part, to the quick ramp to 325 °C programmed after the elution of the cystine derivative that allowed the cleaning of the column after each analysis was performed. Slope values were similar in all cases except for hydroxyproline, cysteine, glycine, arginine, and tyrosine, which obtained lower slope values, indicating less increasing rate in relation to the

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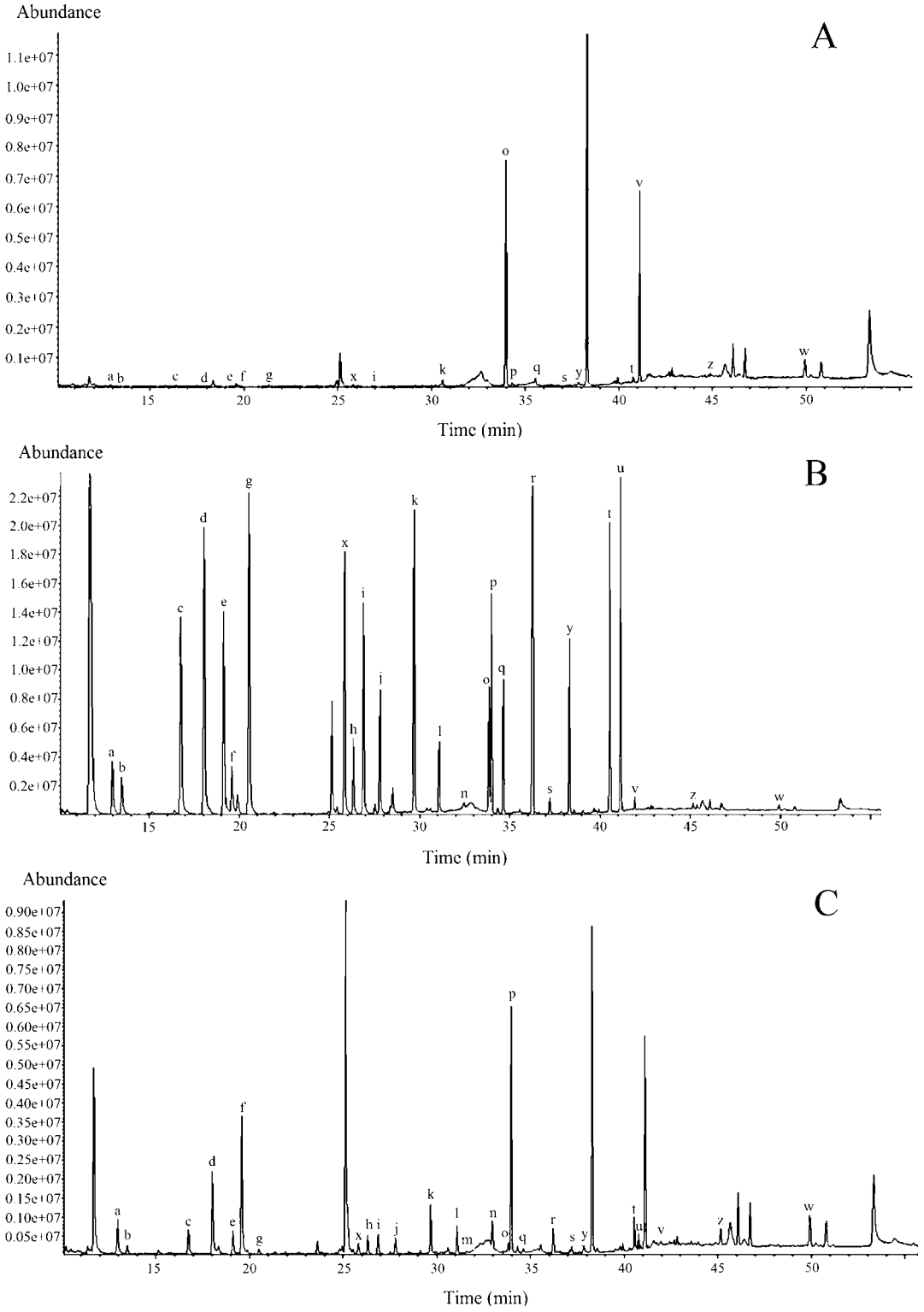

Figure 3. GC-MS chromatographic separation in (A) fresh cheese, (B) ripened cheese, and (C) dry salted sardine of the free amino acids assayed: alanine (a), glycine (b), valine (c), leucine (d), isoleucine (e), norleucine internal standard (f), proline (g), methionine (h), serine (i), threonine (j), phenylalanine (k), aspartic acid (l), hydroxyproline (m), cysteine (n), glutamic acid (o), arginine (p), asparagine (q), lysine (r), glutamine (s), histidine (t), tyrosine (u), tryptophan (v), and cystine (w). Extra derivatives were detected for glutamine (x), arginine (y), and tryptophan (z).

concentration level than the rest of the analyzed free amino acids.

The relative standard deviation (%RSD) run-to-run of the quantitation method was in the range of 1.9−12.2%, being <5.0% for 15 of the 22 free amino acids assayed, indicating excellent reproducibility of the method. The most elevated % RSD values were obtained for arginine, hydroxyproline, lysine, tryptophan, histidine, cystine ,and glutamine. These values could be due, in part, to the extra derivatives obtained for arginine, tryptophan, and glutamine. The derivatization process

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Table 2. Free Amino Acids Quantitated in Food Samples[a]

	lean pork	chicken stock	Iberian dry-cured ham	fresh cheese	ripened cheese	dry salted sardine
moisture (%)	71.6 ± 0.0	97.3 ± 0.0	49.0 ± 0.00	60.5 ± 0.0	34.2 ± 0.0	48.9 ± 0.0
alanine	3.9 ± 0.1	10.5 ± 0.5	44.0 ± 2.9	9.2 ± 0.2	11.9 ± 0.9	2.2 ± 0.1
glycine	0.3 ± 0.0	nd	14.9 ± 2.9	0.8 ± 0.0	8.2 ± 0.5	0.3 ± 0.0
valine	0.9 ± 0.0	10.2 ± 0.2	38.6 ± 1.4	2.4 ± 0.1	42.6 ± 1.5	2.3 ± 0.2
leucine	<LOQ	7.1 ± 0.3	70.0 ± 3.2	2.3 ± 0.1	64.1 ± 3.5	5.0 ± 0.4
isoleucine	1 ± 0.0	12.0 ± 0.8	35.1 ± 1.2	2.7 ± 0.1	42.6 ± 2.0	2.3 ± 0.0
proline	1.4 ± 0.0	14.9 ± 0.4	21.8 ± 2.0	2.0 ± 0.1	61.9 ± 4.4	1.1 ± 0.1
methionine	0.9 ± 0.0	9.2 ± 0.7	15.0 ± 0.7	nd	11.3 ± 0.6	1.6 ± 0.1
serine	<LOQ	4.0 ± 0.2	47.2 ± 9.8	1.8 ± 0.1	18.4 ± 0.9	0.8 ± 0.0
threonine	0.7 ± 0.0	5.6 ± 0.4	41.9 ± 8.3	nd	17.0 ± 0.8	1.2 ± 0.1
phenylalanine	6.6 ± 0.0	9.0 ± 0.6	54.3 ± 2.6	6.0 ± 0.2	40.5 ± 2.2	3.0 ± 0.2
aspartic acid	0.8 ± 0.0	2.8 ± 0.2	56.1 ± 3.6	nd	5.4 ± 0.5	1.0 ± 0.1
hydroxyproline	0.2 ± 0.0	1.7 ± 0.0	0.1 ± 0.0	nd	nd	0.1 ± 0.0
cysteine	8.2 ± 0.5	125.7 ± 13.4	52.3 ± 13.8	nd	6.3 ± 0.3	2.6 ± 0.1
glutamic acid	88.3 ± 0.4	246.9 ± 178.3	289.7 ± 13.4	21.1 ± 1.3	69.0 ± 4.9	2.8 ± 0.2
arginine	3.1 ± 0.3	26.1 ± 1.30	145.5 ± 20.1	2.4 ± 0.2	61.7 ± 1.8	2.8 ± 0.2
asparagine	0.2 ± 0.0	2.6 ± 0.1	<LOQ	4.0 ± 0.3	13.5 ± 1.2	0.2 ± 0.0
lysine	2.9 ± 0.2	25.4 ± 1.3	120.3 ± 15.8	nd	68.8 ± 5.2	2.9 ± 0.2
glutamine	4.1 ± 0.4	nd	1.5 ± 0.2	3.3 ± 0.5	4.8 ± 0.3	nd
histidine	2.1 ± 0.1	10.6 ± 0.7	40.0 ± 8.9	3.2 ± 0.0	22.9 ± 1.2	1.0 ± 0.1
tyrosine	<LOQ	nd	33.8 ± 3.8	nd	17.1 ± 1.1	1.0 ± 0.1
tryptophan	nd	15.7 ± 0.7	1.6 ± 0.0	4.6 ± 0.0	1.8 ± 0.1	1.0 ± 0.0
cistine	2.3 ± 0.0	39.2 ± 1.4	1.1 ± 0.0	37.9 ± 4.8	1.1 ± 0.0	1.2 ± 0.1
∑free amino acids	128 ± 30	579 ± 24	1125 ± 76	104 ± 10	591 ± 35	37 ± 2

[a]Results are expressed as mg of free amino acid/100 g of dry weight. nd, free amino acid was not detected; <LOQ, free amino acid was detected in lower quantity than the corresponding limit of quantitation.

of these free amino acids could have led to a slight run-to-run variation on the proportion of each of the two derivatives formed in the reaction. In addition, as explained above, hydroxyproline and arginine resolutions were poorer than those of the rest of the free amino acids. However, the results obtained are considered to be acceptable for quantitation purposes, not exceeding the recommended limit value (%RSD = 15.0%) for samples with concentration levels >0.001 mg/100 g.[26]

LODs and LOQs of the analytical procedure ranged from 0.01 to 0.46 mg/100 g and from 0.02 to 1.55 mg/100 g, respectively. The free amino acids that obtained higher LODs and LOQs were, in decreasing order, leucine, cysteine, aspartic acid, histidine, phenylalanine, serine, and tyrosine. This shows lower sensitivity of the developed method for these free amino acids, which did not allow the quantitation of some of these free amino acids in samples (Table 2) in which their levels were low (as is the case of leucine and serine in lean pork, tyrosine in chicken stock, and aspartic acid in fresh cheese). Nevertheless, the rest of the free amino acids obtained values of LOD and LOQ close to the lowest values mentioned above. Similar sensitivity has been reported in the quantitation of amino acids in food by GC-MS with other derivatization agents.[13] However, higher sensitivity has been obtained when amino acids in food were analyzed by HPLC, with detection limits <1 pmol.[13] Neverthless, in general, the sensitivity obtained with our method was adequate for the accurate quantitation of the free amino acids in the analyzed food samples.

Free Amino Acids in Animal Source Food Products. Table 2 shows the free amino acid content of the six animal source food products of this study. As can be observed, the content of these compounds among the analyzed products was, as expected, very variable. Iberian dry-cured ham was the food with the highest content of free amino acids (1125 mg/100 g dw), followed by ripened cheese (591 mg/100 g dw) and chicken stock (579 mg/100 g dw), lean pork (128 mg/100 g dw), fresh cheese (104 mg/100 g dw), and, finally, dry salted sardine (36 mg/100 g dw), which was surprisingly the food sample with the lowest total amount of free amino acids.

The highest number of free amino acids was found in the Iberian dry-cured ham sample, in which all 22 free amino acids assayed were detected, although in the case of asparagine it was not possible to carry out the quantitation because its quantity was lower than the corresponding LOD. The next samples with higher number of free amino acids were ripened cheese and dry salted sardine, with 21 free amino acids detected. On the other hand, fresh cheese was the sample with the lowest number of detected free amino acids, because only 15 of the 22 free amino acids assayed were detected.

Iberian dry-cured ham's high content of free amino acids is due to the proteolytic process produced during the drying and salting processes. In this work, we found an elevated amount of glutamic acid (289.7 mg/100 g dw), followed by relatively high quantities of arginine (145.5 mg/100 g dw), lysine (120.3 mg/100 g dw), and leucine (70.1 mg/100 g dw). Some authors have reported these free amino acids as the major ones in Iberian dry-cured ham.[4,6,7] Cordoba et al.[4] obtained similar values of lysine (156.2 mg/100 g dw) and arginine (145.5 mg/100 g dw) in Iberian dry-cured ham analyzed by HPLC. On the other hand, the studies analyzing this product by HPLC have reported very variable values of glutamic acid such as 1140.0 (7), 947.4 (6), 587.4 (5), and 551.5 (10) mg/100 g dw, all higher than the quantity reported in our sample. Recently, Perez-Palacios et al.[8] analyzed Iberian dry-cured ham by HPLC,

obtaining a value for glutamic acid (371.1 mg/100 g dw) near that quantitated in our sample. It should be noted that the processing conditions (time and temperature) for Iberian dry-cured ham in all of these studies are variable, which could partly explain such different results. The lower quantities obtained for basic free amino acids, such as arginine and lysine compared with that of the acidic free amino acids, such as glutamic acid, could be related to the implication of basic free amino acids in the reactions that produce volatile compounds and amines.[4] Although other authors have found amounts of phenylalanine, glutamine, and alanine near 200 mg/100 g dw[9] or higher than 1000 mg/100 g dw[8] in Iberian dry-cured ham, in our study the quantities of these free amino acids are lower (54.3, 1.5, and 44.0 mg/100 g dw, respectively).

Ripened cheese was the second highest sample in free amino acid content. The more abundant free amino acids in this sample were glutamic acid (69.0 mg/100 g dw, 11.7% of the total amount of free amino acids), lysine (68.8 mg/100 g dw, 11.6%), leucine (64.1 mg/100 g dw, 10.8%), and proline (61.9 mg/100 g dw, 10.5%). In a study carried out on the same type of cheese[10] similar proportional values were obtained for glutamic acid (15.3%), lysine (13.6%), leucine (9.2%), and proline (8.4%). These results are due to the proteolysis and metabolic processes that take place during the ripening of this product. In contrast, the main free amino acid reported in fresh cheese was, surprisingly, cysteine (37.9 mg/100 g dw, 36.5% of the total amount of free amino acids), followed by glutamic acid (21.1 mg/100 g dw, 20.3%), alanine (9.2 mg/100 g dw, 8.9%), phenylalanine (6.0 mg/100 g dw, 5.8%), and tryptophan (4.6 mg/100 g dw, 4.4%). Similar results were previously reported by other authors in fresh and semisoft cheeses for glutamic acid and phenylalanine, both being the major free amino acids in these studies, with values higher than 300 and 500 mg/100 g of cheese, respectively.[27,28] We have not found reports of the amount of cysteine in fresh cheese to be compared with the high value obtained in our study.

Moderate quantities of cysteine, cystine, arginine, and lysine were shown in chicken stock (125.7, 39.2, 26.1, and 25.4 mg/100 g dw, respectively). However, a high amount of glutamic acid was quantitated (246.9 mg/100 g dw, 42.6% of the total free amino acids), in accordance with other studies carried out on similar products such as chicken broth and chicken broth cubes with quantities of glutamic acid representing 13.5 and 52.9% of the total amount of free amino acids, respectively.[1,2] The quantity of glutamic acid in our chicken stock sample seems to come from chicken and meat extract, both being ingredients of this source animal product. Meat extracts, usually made of beef, are characterized by a high concentration of glutamic acid,[29] the main amino acid responsible for the umami flavor.[2]

Lean pork also showed high amounts of glutamic acid (88.3 mg/100 g dw) and moderate quantities of cysteine (8.2 mg/100 g dw), phenylalanine (6.6 mg/100 g dw), and glutamine (4.1 mg/100 g dw). Leucine, serine, and tyrosine quantities were lower than the LOQ calculated for these free amino acids. In addition, tryptophan, which has been proven to be in very low concentration in raw pork,[30] was not detected in our sample. Although in other studies glutamic acid has been shown to be present in moderate quantities (12−25% of the total of the free amino acids) in fresh pork meat,[3,24,30] the unusually high amount we found in our sample (69% of the total of the free amino acids) could be explained only by the degradation of glutamine, which is usually the major free amino acid in fresh pork meat,[3,24] in glutamic acid by means of a deamidation reaction.

In dry salted sardine, leucine (5.0 mg/100 g dw), phenylalanine (3.0 mg/100 g dw), lysine (2.9 mg/100 g dw), arginine (2.8 mg/100 g dw), and glutamic acid (2.8 mg/100 g dw) were the predominant free amino acids. Other authors[12] have also reported high quantities of leucine (0.281 mmol/mg dw), lysine (0.107 mmol/mg dw), and glutamic acid (0.515 mmol/mg dw) in dry salted sardine, as a consequence of the activity of proteolytic enzymes, resulting in the development of the typical sensorial characteristics of this product.[11]

In conclusion, the proposed GC-MS method for the determination of free amino acids in animal source foods can be used routinely for both analytical and research purposes.

■ ASSOCIATED CONTENT

Supporting Information

Additional table providing quality parameters of each individual free amino acid detected with the developed GC-MS method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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