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Determination of free amino acids in coated foods by GC-MS: optimization of the extraction procedure by using statistical design

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SHORT RUNNING HEAD

Optimization of amino acids extraction

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ABSTRACT

The development and validation of an extraction procedure for quantification of free amino acids in coated products by MTBSTFA derivatization and GC-MS detection is described. The extraction method entailed the sample homogeneization with HCl by stirring at 40 °C followed by two centrifugation steps. The optimum combination of the extraction variables was achieved by response surface methodology. HCl concentration and volume and stirring time influenced free amino acid extraction yield. At five grams of sample were added 7.5 ml of 0.1N HCl and stirred during 90 min, these conditions were selected as optimal extraction conditions. Consistency between predicted and experimental values as well as in the quality parameters was observed. The calibration curves were linear within the range 5−100 μg ml^{-1} with correlation coefficient values (R^2) higher than 0.99. Detection and quantification limits of the analytical procedure ranged 2.10^{-5} to 18.10^{-2} μg μl^{-1} and from 8.10^{-5} to 60.10^{-2} μg μl^{-1}, respectively. Precision was 0.20-12.59 % for run-to-run and 3.38-17.60% for day-to-day. The accuracy varies between 82.99 and 115.77%. Nineteen amino acids were analysed in frozen-thawed and deep-fried coated products from different origin, with cysteine being the most relevant.

KEY WORDS

Free amino acids; extraction procedure optimization; response surface methodology; coated foods.

INTRODUCTION

Free amino acids (AA) play important roles in foodstuffs. Some of them have an important effect in food flavour[1], and influence its palatability[2]. They also contribute to the formation of amines and volatile compounds throughout decarboxylation and Maillard reactions[3]. In addition, changes in the content of free AA are used for measuring proteolytic and hydrolytic activity throughout the processing of some products such as cheese, dry-cured ham, or fish[4-9], and have also been used for identification food origin in honey, wine and cheese[10].

Amino acid analysis has been originally carried out by ion-exchange chromatography followed by post-column derivatization with ninhydrin and ultraviolet detection, which has gradually been supplanted by faster, more sensitive and versatile methodologies[11], like gas chromatography (GC) and high-performance liquid chromatography (HPLC). Probably, the most used current methods to determine AA in food matrices is reverse phase HPLC with a pre-column derivatization step. However, GC presents some advantages, given its higher resolution, speed of analysis and even its instrumental cost which is one third of that of HPLC[12]. GC coupled with mass spectrometry (MS) can be a useful alternative to other methods of AA analysis, especially when sample amounts are limited and high sensitivity is required[13]. However, the two time consuming and tedious derivatization steps (esterification+acylation), required for GC analyses of AA, influenced negatively a wide acceptation of this method. Consequently, the simultaneous silylation of the amino- and carboxyl groups, in a single step, with bis-trimethylsilyltrifluoroacetamide (BSTFA), was very welcomed and promising, although trimethylsilylation has not been realized in practice, because scientists searched for are agent of more general application. N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide, MTBSTFA, gave more stable AA derivatives under convenient reactionconditions[14].

The extraction of free AA from the food matrix is a required step previous to the derivatization and chromatographic procedures. Several extraction methods for free AA from food products have been described in literature. Most of them used HCl or perchloric acid diluted in water or in ethanol[4,12,15-19], although other authors have also described the use of water:acetonitrile (50:50, v/v)[20], petroleum ether[21], ethanol[22] and 0.1% (v/v) formic acid in 20% (v/v) methanol[23], among others. The homogeneization step has been done by using magnetic stirring[12], ultraturrax[16,17], stomacher[17], omnimixer[4], rotatory mixer at 50 °C[22], vortex[23] and a heating block at 40 °C with stirring[18]. The next step in general is centrifugation, and supernatant collection[21]. In some cases filtration through glass wool[4,15], nylon membrane[23] or Whatman 42 paper[17] or pass through a cartridge and elution[12,22] have been also carried out.

Most studies on AA analysis have been focused on development of accurate derivatization and chromatographic determinations, while the extraction procedure has received less attention[11,15]. To our knowledge, there are not works based on optimizing the extraction of AA from food samples. Method optimisation is usually performed by univariate strategies which could generate some limitations since the interactions betweenfactors are not considered. The response surface methodology (RSM) explores the relationship between several explanatory variables and one or more response variables by means of a mathematical model able to properly predict the values of the response. This is a very useful tool for selecting the optimum conditions when there are interactions between variables[24,25].

In the scientific literature, no data was found documenting the AA content in coated products. This type of foodstuff is composed by a food matrix covered by a layer made with flour, oil, water, starch, salt, and spices among others. The coated deep-fried products are highly appreciated and consumed by the young population. In addition, these products are easily and quickly prepared. The aim of this study was to develop and validate an AA extraction procedure with MTBSTFA

derivatization and GC-MS analysis for the quantification of these compounds in coated products from diverse source.

MATERIAL AND METHODS

Chemical and samples

Hydrochloric acid (HCl), 32% extra pure, and MTBSTFA were purchased by Sigma–Aldrich (St Louis, MO, USA). Acetonitrile of HPLC-gradient grade was provided by Panreac (Barcelona, Spain). Dichloromethane was supplied by Merck (Darmstadt, Germany) and ultrapure water (0.055 µS/cm) was obtained by using a Serial Milli-Q system for Millipore (Supor DCF, Gelman Sciences, Chentelham, Australia). Standard AA (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 and tryptophan. DL-Norleucine (Sigma-Aldrich) was used as internal standard (IS). Frozen coated products, with 30 g on average, were obtained from a local store. Plain sunflower oil used for frying was also from a local recognized brand.

Stock solution of DL-norleucine (IS) at 5 µg ml^{-1} was prepared in 0.1 N HCl. An initial standard solution at 200 µgml^{-1} of each individual AA was also prepared in 0.1 N HCl and seven increasing dilutions of this solution were made (150, 100, 50, 25, 10, 5, and 1 µg ml^{-1}).

Sample preparation

This study was developed with frozen coated products from different sources (hake, shrimps, vegetal, chicken and beef-pork). Of each type of product, six samples were thawed at 4 ºC during 16 h and analysed. Other six sticks of each batch of products were deep-fried individually in sunflower oil using a domestic deep-fryer (KENWOOD DF-150, 1l) at 180 ºC during 4 min, according to recommended preparation

conditions given by the manufacturer. Deep-fried coated products were slightly drained after frying, placed on paper towel to remove external excess oil, and grinding by using a device named "masticator shears straight" (BUENO HERMANOS, S.A., La Rioja, Spain, ISO 9001-2000 Quality Certified Company) which simulates the chewing process. The oil was replaced every 6 frying sessions. All samples were processed individually.

Free amino acid extraction and derivatization

The optimized extraction procedure for free AA analyses in the coated food products was as follows: sample (1 g) was weighed into a 15 ml flat-bottom glass vial, and 7.5 mL of 0.1 N HCl were added. After a stir bar had been inserted, the vial was sealed with a screw-top cap and stirred at high speed 90 min at 40 °C. The mixture was then centrifuged (5000 rpm, 30 min), and the supernatant was transferred into another 15 ml graduated vial, flushed until 10 ml with distilled water and centrifuged again (10000 rpm, 15 min). 1 ml of the supernatant were collected and stored at - 80 °C until analysis.

Free AA deproteinization and derivatization was carried out as previously described[15] with slight modifications. To 100 µl of the extract, 250 µl of acetonitrile were added to deproteinize the samples. Tubes were subsequently centrifuged at 8000 rpm for 5 min. From this point, standard solutions and food samples followed the same process. Then, 100 µl of the supernatant (or the standard solution) were transferred to heat-resistant tubes, and 100 µl of IS solution (5 $\mu g ml^{-1}$) was added. Tubes were dried under nitrogen. 50 µL of dichloromethane were added to the dried samples, and again evaporated under nitrogen. Finally, 50 µl of the derivatization agent (MTBSTFA) and 50 µL of acetonitrile were added to the dried tubes, which were shaken and subsequently incubated at 100°C for 60 min to induce the derivatization reaction to occur.

GC–MS conditions

The chromatographic analysis was carried out in Agilent 6890 gas chromatograph (Agilent, Avondale, PA, USA) coupled to a MS detector (Agilent 5973). The MS system was routinely set in selective ion monitoring (SIM) mode and each compound was quantified based on peak area using one target and one or two qualifier ions. Table 2 summarizes the target free amino acids with respective time windows (TWs), retention time (t_R) and ions selected in SIM mode.

One µL portion of the derivatized extract was injected in splitless mode onto the column. The column used was a 58 m × 0.32 mm i.d., 0.05 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 held for 16 min. Mass spectra were obtained by electronic impact at 70 eV, with a multiplier voltage of 2056 V. The transfer line to the mass spectrometer program was as follows: 280 °C for 35 min, 0.5 °C/min ramp to 290 °C, collecting data over the m/z range 30–600. Total run time was 55.75 min. Free AA were identified from Nist 98 data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.) and using both their retention time and by comparison of their characteristic m/z ions with those published in the literature[14].

Free AA quantification in coated samples was carried out in the SIM mode by external calibration curve method and by standard addition method. For each AA a calibration curve (quantification ion AA peak area/ quantification ion IS peak area versus AA amount/IS amount) was constructed, obtaining R^2 values of 0.9999. The final results, expressed in µg per g dry weight, take into account the moisture content and the exact weight of the sample.

Quality control

Quality control of the GC-MS analysis was performed through the routine analysis of procedural blanks and quality control standards and samples to ensure the absence of contaminants and the possible carryover between samples and to assess the quality of the results.

Limit of detection (LOD) and quantification (LOQ) based on a signal-to-noise ratio of 3:1 and 10:1, respectively, were determined using standard solutions (n=5). For calculating the relative standard deviation (RSD) run-to-run and day-to-day, five replicate analyses of samples were analysed in one day and in three different days, respectively. In order to study the percentage recovery of each AA, coated samples were spiked with appropriate amounts of each AA (7.5–40 µg) and were extracted using the established conditions.

Experimental design

The conditions for extraction of free AA from coated products were optimized by RSM. A full-factorial central composite design (CCD) was applied. It consisted of a complete 2^3-factorial design with six centre points and two axial points on the axis of each design variable at a distance of a = 1.682 from the design centre, resulting 20 combinations, including six replicates of the centre point. Experiments were carried out with HCl concentration, HCl volume and stirring time at 40 °C, varying from 0.05 to 0.15 N, from 2.5 to 7.5 ml and from 30 to 90 min, respectively. The response was the sum of each quantification ion AA peak area/ quantification ion IS peak area versus AA amount/IS amount). Table 1 shows the complete experimental design. Data analysis, response surfaces and contour diagrams, was performed by Design Expert trial-Version 7 (Stat-Ease, Inc., Minneapolis, MN).

RESULTS AND DISCUSSION

Selection of the AA extraction procedure

First assays were performed applying some of the methods previously described in literature[15,18,23], obtaining the better results when

samples (1g) were mixed with 5 ml of 64% ethanol in 0.5 M HCl in a 15 ml flat-bottom glass vial, which was placed in a heating block and was stirred at high speed for 1 h at 40 °C. The mixture was then centrifuged at 5000 rpm during 30 min. The supernatant (100 µl) was deproteinized, derivatized and analysed. Under these conditions, AA peaks were obtained, but many contaminant peaks appeared in the chromatogram. Moreover, this AA extraction procedure only allowed qualitative analyses. Consequently, improvements were made based in this methodology. In order to be able to quantify the extracted AA, the supernatant was transferred into another 15 ml graduated vial and flushed until 10 ml with distilled water and a second centrifugation (10000 rpm, 15 min) was included to improve clean up. The use of 0.01N, 0.1N and 0.5N HCl as extraction solvent was tested, by taking into account scientific literature[4,12,15-17]. Results from these tests showed chromatograms with minimum contaminant peaks and highlighted the influence of HCl concentration on AA extraction, the highest response was obtained when 0.1N HCl was used.

Typical chromatograms obtained after derivatization of twenty-one free AA are presented in Figure 1. Figure 1A corresponds to a standard solution of 100 µg/ml of each amino acid.

Model Prediction Adequacy

Three variables of the AA extraction procedure previously tested, HCl normality and volume and time of stirring at 40 °C, were optimized for free AA quantification in coated products. The full-factorial CCD involved 20 experiments, which include six replicates of the centre points for verifying any change in the estimation procedure and measuring the precision property.

Results on analysis of the variance by means of Fisher's *F* test are shown in Table 3. The model *F* value was 5.59, indicating the significance of the model and that there is only a 0.64 % chance that a so large model *F* value could occur due to noise. Lower values than 0.05 were

found for Prob> F in X_2, X_3, X_1^2 and X_3^2, which indicates that they are significant terms. The lack of fit *F* value of 0.57 is good and shows that the model is valid for the present study[24,26]. There is a 71.85 % chance that a so large lack of fit *F* value could take place due to noise. Moreover, the obtained R_{pred}^2 and R_{adj}^2 (0.6215 and 0.6852, respectively) were in concordance, and a ratio of 7.886 was attained, which indicates an adequate signal. Thus, the response surface quadratic model is adequate and significant.

Selection of the optimum conditions

The surface and contour plots on the response function (sum of each characteristic ion AA peak area/characteristic ion peak area IS) as affected by the three studied variables (HCl concentration and volume and stirring time) can be seen in Figure 1. Two variables are varying with time, while the third is keeping constant. Figure 2A shows the contour map of the combined effect of HCl volume and concentration in the response. The combined effect of stirring time with HCl concentration and volume is shown in Figure 2B and 2C, respectively. Within the ranged tested, the three variables seem to effect AA extraction, with HCl volume and stirring time being so influencing variables. The sum of each characteristic ion AA peak area/characteristic ion peak area IS increased as the HCl volume and stirring time increased and when the HCl concentration is around 0.1N. As a result, the optimum combination of the three studied variables, which should provide the maximum free AA quantity in coated products, consists of 7.5 ml of HCl 0.1N and 90 min of stirring time at 40 °C (Table 4). To our knowledge, this is the first work studying the effect of the extraction parameters and selecting the optimum combination of variables in order to develop and validate a method for the free AA quantification.

Method validation and confirmation

Observed and predicted values of the sum of each characteristic ion AA peak area/characteristic ion peak area IS under the optimum combination of HCl concentration and volume and stirring time are shown in Table 4. It can be observed the consistency between the experimental and predicted values (1.199 *vs.* 1.185, respectively).

To examine the performance of the proposed methodology, quality parameters for each individual AA were determined. Good linearity was obtained for the range 5−100 μg ml^{-1} for the twenty-one AA, with correlation coefficients higher than 0.99. LODs and LOQs of the analytical procedure ranged from 2.10^{-5} to 18.10^{-2} μg μl^{-1} and from 8.10^{-5} to 60.10^{-2} μg μl^{-1}, respectively, with threonine and phenylalanine obtaining the highest limits, which were lower in comparison to other studies[12,20]. Adequate precision was achieved with a RSD of 0.20-12.59 % for run-to-run and 3.38-17.60% for day-to-day. Generally, the accuracy (% recovery) was good, varying between 82.99 and 115.77 %. The free AA quantification was carried out by external calibration curve method and by standard addition method. Paired Student's *t*-test showed no significant differences between the two methods (p = 0.995, n = 5). Moreover, higher standard deviation was found when applying the standard addition method, which indicates the higher suitability of external calibration for quantifying free AA in coated products.

Moreover, in order to confirm the validity of the experimental model, frozen-thawed and deep-fried coated products from different sources (hake, shrimps, vegetal, chicken and beef-pork) were analysed using the selected optimum conditions. Nineteen AA were quantified: alanine, glycine, valine, leucine, isoleucine, proline, methionine, threonine, phenylalanine, aspartic acid, cysteine, glutamic acid, arginine, asparagine, lysine, glutamine, histidine, tyrosine and tryptophan, being serine lower than LOQ and hidroxyproline lower than LOD. In addition, threonine and glutamic acid were lower than LOQ in some of the analysed samples. Figure 1B illustrates the typical chromatograms of extracted ions in a frozen-thawed beef-pork coated

sample, exemplifying the success of current method for the determination of AA. One peak was observed for 17 of the 19 free AA detected in coated samples, whereas for phenylalanine and histidine two peaks were obtained. In fact, the formation of extra derivatives for amino acids when using MTBSTFA as derivative agent has been previously reported[14,15].

Table 5 shows the quantity of free AA in frozen-thawed and deep-fried coated products from different sources. Cysteine was the major AA, followed by phenylalanine and aspartic acid, while the others showed minor content. There were significant differences in the content of all AA between frozen-thawed coated products, with the highest levels of total AA found in vegetal samples. A similar trend was observed in deep-fried coated products. Deep-frying in sunflower oil influences AA quantity. Thus, sum of total AA significant increases from frozen-thawed to deep-fried samples in all coated products, mainly due to changes in cysteine, which mainly comes from proteolysis[4].

CONCLUSIONS

This paper reported the development and validation of a method, based on a simple extraction, MTBSTFA derivatization and GC-MS analysis, for the quantification of free AA in coated products. The use of CCD allows reaching the optimum combination of analytical variables within the extraction procedure. The HCl concentration and volume and the stirring time at 40 ºC influenced amino acid yield.

The proposed methodology for the determination of free AA can be used in both frozen-thawed and deep-fried coated products.

REFERENCES

1. P.D. Chiang, C.T. Yen, and J.L. Mau, Food Chem. 101, 932 (2007).
2. N. Barylko-Pikielna, and E. Kostyra, Food Qual. Pref. 18, 751 (2007).
3. M. Cornet, and J. Bousset, Meat Sci. 51, 215 (1999).
4. T. Perez-Palacios, J. Ruiz, J.M. Barat, M. Aristoy, and T. Antequera, MeatSci. 85, 121 (2010).

5. E.F. Kechaou, J. Dumay, C. Donnay-Moreno, P. Jaouen, J.P. Gouygou, J.P. Bergé, and R.B. Amar, J. Biosci Bioeng. 107, 158 (2009).
6. I.M.P.L.V.O. Ferreira, C. Veiros, O. Pinho, A.C.A. Veloso, A.M. Peres, and A. Mendonça, J. Dairy Sci. 89, 2397(2006).
7. S.D. Curtis, R. Curini, M. Delfini, E. Brosio, F. D´Ascenzo, and B. Bocca, Food Chem. 71, 495 (2000).
8. J. Ruiz, C. Garcia, M.D. Diaz, R. Cava, F.J. Tejeda, and J. Ventanas, Food Res. Int. 32, 643 (1999).
9. A. Gonçalves, M.L. Nunes, and I. Batista, In Process Optimisation and Minimal Processing of Foods, Leuven, Belgium, 1997.
10. V. Pereira, M. Pontes, J.S. Camara, and J.C. Marques, J. Chromatogr. A, 1189, 435 (2008).
11. R.W. Peace, and G.S. Gilani, J. AOAC Int. 88, 877(2005).
12. B.M. Silva, S. Casal, P.B. Andrade, M.S. Seabra, M.B. Oliveira, and M.A. Ferreira, Anal. Sci. 19, 1285 (2003).
13. M.W. Duncan, and A. Poljak, A. Anal. Chem. 70, 890 (1998).
14. Zs. Molnfir-Perl, and E. Katona, Chromatographia Supplement, 51, 228 (2000).
15. E. Jiménez-Martín, J. Ruiz, T. Pérez-Palacios, A. Silva, T. Antequera, and J. Agric. Food Chem. 60, 2456 (2012).
16. O. Özden, and N. Erkan, British Food J. 113, 457 (2007).
17. M. Erbas, M.F. Ertugay, O. Erbas, and M. Certel, Int. J. Food Sci. Nutr. 56, 349 (2007).
18. A. Becalski, and B.P-Y. Lau, D. Lewis, S.W. Seaman, S. Hayward, M. Sahagian, M. Ramesh, Y. Leclerc, J. Agric. Food Chem. 52, 3801 (2004).
19. O. Pinho, I.M.P.L.V.O. Ferreira, E. Mendes, B.M. Oliveira, and M.A. Ferreira, Food Chem. 75, 287 (2001).
20. V. Gökmen, A. Serpen, and B.A. Mogol, Anal. Bioanal. Chem. 403, 2915 (2012).
21. R. Pätzold, and H. Brückner, Amino Acids. 31, 63 (2006).
22. Mustafa, P. Aman, R. Andersson, and A. Kamal-Eldin, Food Chem. 105 317 (2007).
23. M.S. Nimbalkar, S.R. Pai, N.V. Pawar, D. Oulkar, and G.B. Dixit, Food Chem. 134, 2565 (2012).
24. C. Pinho, A. Melo, C. Mansilha, and I.M.P.L.V.O. Ferreira, J. Agric. Food Chem. 59, 50 (2011).
25. R. Leardi, Anal. Chim. Acta. 652, 161 (2009).
26. D.C. Montgomery, Design and Analysis of Experiments, fifth ed., Wiley, New York, 2000.

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Caption Figure 1. A GC–MS separation of free AA after derivatization in selected monitoring ion mode (each AA was at the concentrations level of 100 µg/ml, see Table 2 for the legend of peaks). **B** GC–MS chromatogram of frozen-thawed beef-pork coated sample with extracted ion chromatograms in SIM mode of proline (ions 184, 286, 258) and histidine (ions 196, 489, 440, 338).

Caption Figure 2. Response surface plots on the sum AA peak area/DL-norleucine peak area in coated products as affected by HCl volume and normality and stirring time at 40 °C. **A** HCl volume and normality at constant stirring time (60 min), **B** stirring time and HCl normality at constant HCl volume (5 ml), and **C** stirring time and HCl volume at constant HCl normality (0.1 N).

Figure 1
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Figure 1.

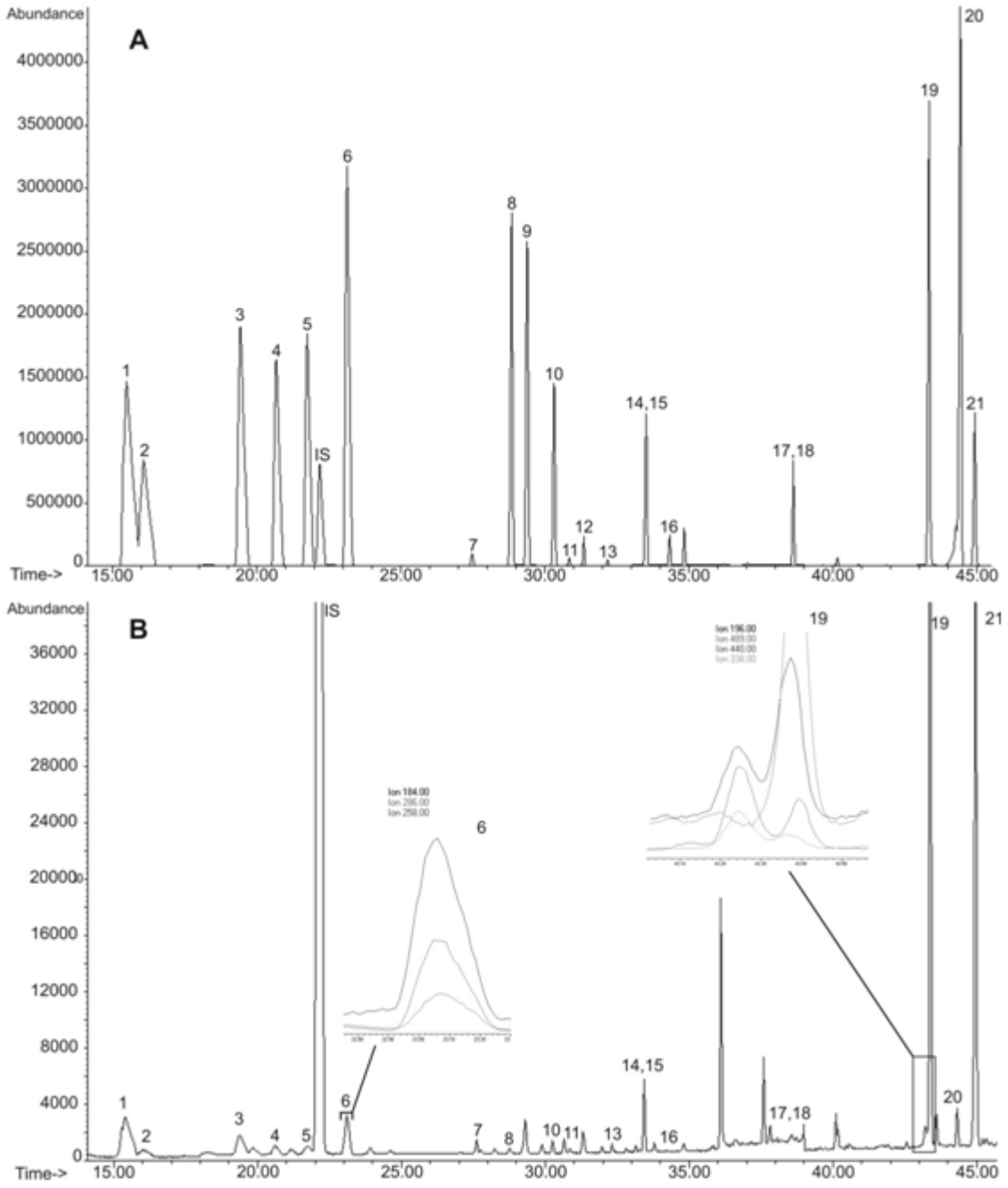

Figure 2
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Figure 2.

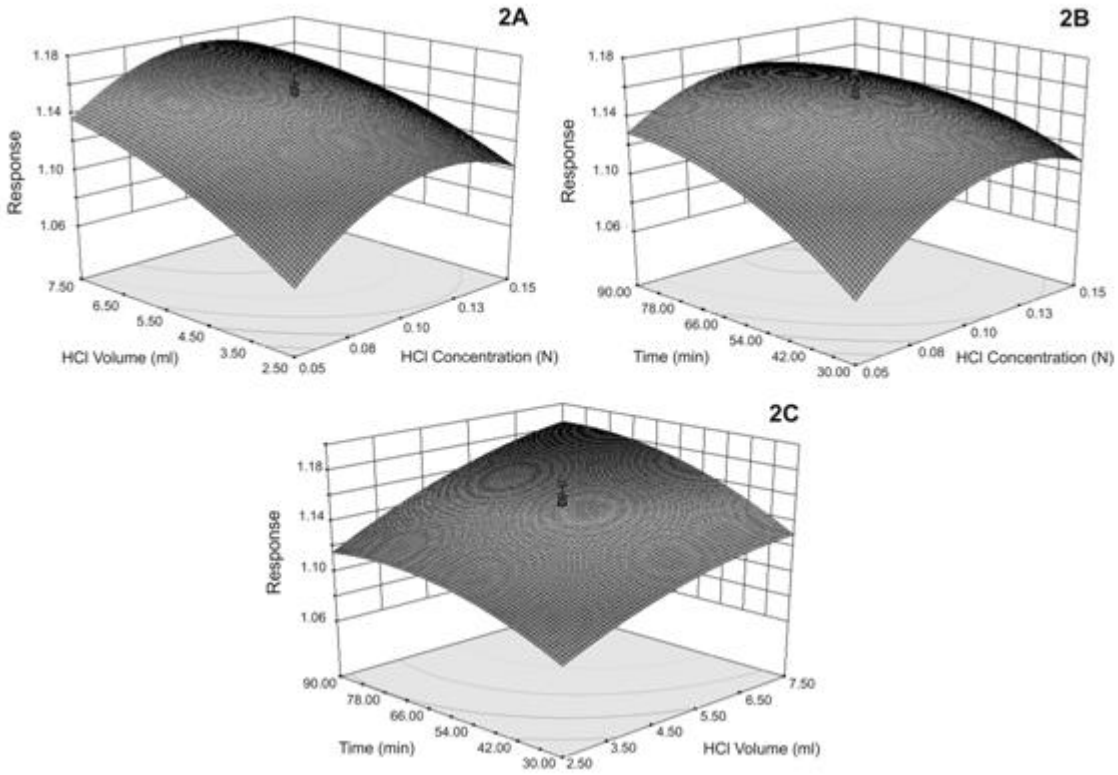

Table 1
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Table 1. Coded and uncoded values of the independent variables of the central composite design for the free AA extraction method optimization.

	Independent variables					
	CODED			UNCODED		
Standar order	X_1 HCl concentration (N)	X_2 HCl volume (ml)	X_3 Stirring time (min)	X_1 HCl concentration (N)	X_2 HCl volume (ml)	X_3 Stirring time (min)
1	-1	1	1	0.05	7.50	90
2	1	-1	1	0.15	2.50	90
3	-1	-1	1	0.05	2.50	90
4	-1.682	0	0	0.02	5.00	60
5	0	0	-1.682	0.10	5.00	10
6	0	0	0	0.10	5.00	60
7	0	-1.682	0	0.10	0.80	60
8	0	0	0	0.10	5.00	60
9	0	-1.682	0	0.10	9.20	60
10	-1	-1	-1	0.05	2.50	30
11	1	1	1	0.15	7.50	90
12	0	0	0	0.10	5.00	60
13	0	0	-1.682	0.10	5.00	110
14	0	0	0	0.10	5.00	60
15	-1	1	-1	0.05	7.50	30
16	0	0	0	0.10	5.00	60
17	1	1	-1	0.15	7.50	30
18	1	-1	-1	0.15	2.50	30
19	-1.682	0	0	0.18	5.00	60
20	0	0	0	0.10	5.00	60

Table 2
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Table 2. MS conditions for the analysis of free AA and norleucine (IS) after derivatization with respective time windows (TW), ions selected in SIM mode (quantification ions in bold) and retention time (t_R).

Peak	Amino Acid	Ions	t_R (min)	TW
1	Alanine	**158**, 260, 232	15.4	1
2	Glycine	**218**, 246	16.0	1
3	Valine	**186**, 288, 260	19.4	2
4	Leucine	**200**, 302, 274	20.6	2
5	Isoleucine	**200**, 302, 274	21.7	2
	Norleucine (IS)	**200**, 147, 274	22.1	2
6	Proline	**184**, 286, 258	23.1	2
7	Phenylalanine	**336**, 302, 234	27.6	3
8	Methionine	**218**, 320, 292	28.8	3
9	Serine	**362**, 390	29.3	3
10	Treonine	**404**, 376, 303	30.3	3
11	Asparticacid	**316**, 418, 390	30.9	4
12	Hydroxyproline	**388**, 416, 314	31.3	4
13	Cysteine	**406**, 378	32.3	4
14	Glutamicacid	**432**, 330, 272	33.4	5
15	Arginine	**286**, 474	33.5	5
16	Asparagine	**417**, 302	34.3	5
17	Lysine	**300**, 431, 329	38.6	6
18	Glutamine	**329**, 431, 357, 338	38.6	6
19	Histidine	**196**, 489, 440, 338	43.3	7
20	Tyrosine	**466**, 438, 364, 302	44.4	7
21	Tryptophan	**244**, 489, 302	44.9	7

Table 3
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Table 3. Analysis of Variance for Response Surface Model for the sum of each characteristic ion AA peak area/characteristic ion peak area IS in coated products.

Source	Sum of Squares	Degrees of freedom	Mean Square	*F* value	Prob > *F*	Remarks
Model	**0.03748901**	**9**	**0.00416545**	**5.59575102**	**0.0064**	**significant**
X_1	**0.00210591**	**1**	**0.00210591**	**2.82903151**	**0.1235**	
X_2	**0.01163314**	**1**	**0.01163314**	**15.6276632**	**0.0027**	**significant**
X_3	**0.00666204**	**1**	**0.00666204**	**8.94961153**	**0.0135**	**significant**
$X_1 X_2$	**0.00030012**	**1**	**0.00030012**	**0.40318014**	**0.5397**	
$X_1 X_2$	**0.00103512**	**1**	**0.00103512**	**1.39056007**	**0.2656**	
$X_2 X_3$	**0.00021013**	**1**	**0.00021013**	**0.28227647**	**0.6068**	
X_1^2	**0.01276534**	**1**	**0.01276534**	**17.14862**	**0.0020**	**significant**
X_2^2	**0.00143054**	**1**	**0.00143054**	**1.92174724**	**0.1958**	
X_3^2	**0.00343701**	**1**	**0.00343701**	**4.61719239**	**0.0472**	**significant**
Residual	**0.00744394**	**10**	**0.00074439**			
Lack of Fit	**0.00680861**	**7**	**0.00136172**	**0.57487523**	**0.7412596**	**not significant**
Pure Error	**0.00063533**	**5**	**0.00012707**			
Total	**0.04493295**	**19**				

Table 4
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Table 4. Model Validation

NaCl concentrarion (N) X_1	HCl volumen (ml) X_2	Stirring time (min) X_3		Response[a]	
			Desirability	Experimental	Predicted
0.1	7.5	90	0.98	1.199	1.185

[a] sum of each characteristic ion AA peak area/characteristic ion DL-norleucine peak area

Table 5
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Table 5. Amino acid content (mg g^{-1} sample dry matter) in frozen-thawed (FT) and deep-fried (DF) coated products from different sources[a].

	hake		shrimps		vegetal		chicken		beef/pork		*p*
	FT	DF	FT	DF	FT	DF	FT	DF	FT	DF	
Alanine	0.42±0.01bc	0.50±0.11bc	0.26±0.01d	0.39±0.02c	0.62±0.16b	0.41±0.05bc	0.43±0.03bc	1.09±0.02a	0.31±0.02d	0.27±0.00d	< 0.001
Glycine	0.40±0.01d	0.49±0.05c	0.35±0.00de	0.61±0.05b	0.31±0.02ef	0.23±0.00fg	0.35±0.01de	0.84±0.03a	0.25±0.01fg	0.20±0.00fg	< 0.001
Valine	0.41±0.02b	0.37±0.01c	0.31±0.00de	0.32±0.01d	0.47±0.00a	0.37±0.03c	0.35±0.00c	0.42±0.00b	0.29±0.01e	0.24±0.00f	< 0.001
Leucine	0.14±0.00b	0.14±0.01b	0.11±0.00c	0.14±0.01b	0.15±0.00b	0.11±0.01bc	0.15±0.01b	0.22±0.01a	0.09±0.00de	0.08±0.00e	< 0.001
Isoleucine	0.15±0.00b	0.13±0.01bc	0.12±0.00de	0.13±0.01cd	0.17±0.00a	0.13±0.01cd	0.14±0.01bc	0.17±0.01a	0.10±0.00e	0.11±0.01e	< 0.001
Proline	0.35±0.00b	0.33±0.01b	0.25±0.01d	0.28±0.01c	0.33±0.02b	0.23±0.01de	0.28±0.00c	0.37±0.01a	0.21±0.00e	0.18±0.00f	< 0.001
Methionine	0.30±0.01a	0.26±0.01bc	0.24±0.01de	0.23±0.01def	0.27±0.00b	0.22±0.01ef	0.25±0.00cd	0.30±0.02a	0.21±0.01f	0.18±0.00g	< 0.001
Threonine	< LOQ	2.16±0.36b	< LOQ	0.65±0.08c	2.67±0.54b	< LOQ	0.74±0.09c	9.34±0.47a	< LOQ	< LOQ	< 0.001
Phenilalanine	2.96±0.08e	13.04±2.24c	10.63±2.40cd	4.82±1.17de	0.54±0.02e	20.01±5.80b	1.89±0.70e	41.65±4.75a	1.27±0.21e	40.44±1.56a	< 0.001
Asparticacid	3.22±0.19c	6.14±0.26a	2.14±0.43d	4.74±0.90b	4.14±0.41bc	0.27±0.00e	1.78±0.12e	0.29±0.01e	4.09±0.96bc	0.24±0.00e	< 0.001
Cysteine	19.15±0.13d	46.01±7.39cd	26.66±1.77d	113.04±11.17c	209.57±11.10b	647.95±100.53a	21.16±1.06d	90.23±3.44cd	23.09±4.36d	245.46±13.04b	< 0.001
Glutamicacid	0.08±0.01f	< LOQ	0.05±0.00f	0.50±0.14c	0.38±0.07d	0.90±0.58b	0.27±0.07d	2.66±0.13a	0.13±0.12e	< LOQ	< 0.001
Arginine	0.37±0.01cd	0.31±0.04cde	0.26±0.01e	0.40±0.01bc	0.46±0.03b	0.34±0.08cde	0.35±0.00cde	0.66±0.02a	0.28±0.00de	0.31±0.01de	< 0.001
Asparagine	0.47±0.01c	0.39±0.01cd	0.37±0.01d	0.33±0.01e	0.40±0.01c	0.32±0.00e	0.38±0.02cd	0.53±0.01a	0.35±0.00e	0.28±0.00f	< 0.001
Lysine	0.37±0.01c	0.42±0.06b	0.25±0.00ef	0.25±0.01ef	0.33±0.00cd	0.23±0.01g	0.30±0.01de	0.53±0.05a	0.23±0.01g	0.19±0.00g	< 0.001
Glutamine	0.40±0.01c	0.45±0.03b	0.29±0.00c	0.25±0.01c	0.31±0.01bc	0.26±0.01a	0.33±0.00bc	0.62±0.03a	0.27±0.00fc	0.22±0.00bc	< 0.001
Histidine	0.39±0.01c	0.41±0.03c	0.28±0.00c	0.29±0.01c	0.37±0.01c	0.60±0.10b	0.31±0.00c	0.54±0.08a	0.26±0.01c	0.38±0.05c	< 0.001
Tyrosine	0.08±0.00b	0.13±0.00c	0.07±0.00c	0.18±0.01bc	0.20±0.00a	0.39±0.18b	0.14±0.01c	1.09±0.07a	0.05±0.00d	0.10±0.00c	< 0.001
Triptophan	0.55±0.01c	0.78±0.08c	0.40±0.03c	1.34±0.14c	1.82±0.64c	4.29±0.81b	0.48±0.03c	1.96±0.35c	0.87±0.01c	10.79±1.59a	< 0.001
∑ AA	30.20±0.22h	72.47±10.69f	43.05±2.00g	128.89±13.28e	223.54±0.27c	677.27±94.96h	30.08±0.54d	154.01±8.61d	32.36±1.18h	299.68±16.15b	< 0.001

[a] Values are expresses as mean ± standard deviation.

Means with different letters differ significantly.