Deproteinization Techniques for HPLC Amino Acid Analysis in Fresh Pork Muscle and Dry-Cured Ham

María-Concepción Aristoy and Fidel Toldrá*

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Jaime Roig 11, 46010 Valencia, Spain

Different deproteinization techniques have been tested for free amino acids analysis in fresh pork muscle and dry-cured ham. These techniques include chemical precipitants (trichloroacetic acid, perchloric acid, sulfosalicylic acid, phosphotungstic acid, picric acid, and acetonitrile) and ultrafiltration through 10 000 and 1000 M_r cutoff membranes. Phenyl isothiocyanate amino acids were analyzed by reverse-phase HPLC. Good amino acid recoveries (more than 90%) from a standard amino acid solution were obtained except when sulfosalicylic acid, phosphotungstic acid, and ultrafiltration through 1000 M_r cutoff membrane were used, which gave important losses of some amino acids. Similar results were obtained for both meat and dry-cured ham samples. However, an interfering peak that coelutes to arginine was observed. Only phosphotungstic acid completely removed that peak, but it gave important losses in dibasic amino acids.

INTRODUCTION

Free amino acids may play an important role in meat flavor (Kato et al., 1989). However, the analysis of free amino acids in meat may be problematic, especially due to the high protein and peptide contents (Pearson and Young, 1989). Thus, it is obvious that an effective deproteinization technique is necessary. The most widely used methods include acid precipitation, ultrafiltration, and precipitation with organic solvents.

The use of acids as protein precipitants has been extensively studied in cheese (Jarret et al., 1982; Kuchroo and Fox, 1982a,b; Fox, 1989) and plasma (Blanchard, 1981). The most usual concentrations of acid are 2–12% trichloroacetic acid (TCA) or 5% phosphotungstic acid (PTA) in the case of cheese (Reiter et al., 1969; Gripon et al., 1965; Visser, 1976; Kuchroo and Fox, 1982b; Hickey et al., 1983) or 2.5–3.5% sulfosalicylic acid (SSA) in the case of cheese and plasma (Reiter et al., 1969; Godel et al., 1984; Ramos et al., 1987; Hubbard et al., 1988; Fürst et al., 1989). Other usual precipitants include perchloric acid (PCA) (Ali Qureshi et al., 1984), picric acid (PA), which is supposed to be the most discriminating precipitant (Reville and Fox, 1978; Fox, 1989), and organic solvents such as acetonitrile with worts (Bidlingmeyer et al., 1987) or organs and physiological fluids (Schuster, 1988; Sarwar and Botting, 1990).

Ultrafiltration is a physical deproteinization technique which is becoming usual, especially through the 10 000 M_r cutoff membrane (Cohen and Strydom, 1988; Moughan et al., 1990). The use of 5000 (Schuster, 1988) and 1000 M_r cutoff membranes (Aston and Creamer, 1986) has been also reported.

Although there are some works dealing with deproteinization techniques, there is scarce information about their application to animal tissues, particularly meat and meat products. Thus, the goal of the present work is to compare the main chemical and physical deproteinization methods to optimize the HPLC analysis of free amino acids in meat and meat products.

EXPERIMENTAL PROCEDURES

Materials. Muscle biceps femoris from 6-month-old pigs, 20–30 h post-mortem, and from 8-month-dry-cured ham, which had undergone the typical Spanish dry-curing process, were used as samples.

Reagents. Amino acid standards were obtained from Sigma (St. Louis, MO). Acetonitrile and methanol were of HPLC grade; sodium acetate and phosphate, glacial acetic, hydrochloric, trichloroacetic, perchloric, sulfosalicylic, phosphotungstic, and picric acids were of reagent grade. Triethylamine (TEA) and phenyl isothiocyanate (PITC) were obtained from Fluka. High-purity water was supplied by a Milli-Q purification system (Millipore, Bedford, MA).

A standard solution was made by dissolving 21 amino acids (listed in Tables I and II) to a final concentration of 1 mM each in 0.1 N hydrochloric acid. Another solution containing 5 mM α-aminobutyric acid was used as internal standard. Both solutions were stored at −30 °C until needed.

Preparation of Muscle Extracts. Three solvents were tested for amino acid extraction: (1) 0.03 M phosphate buffer, pH 7.4, (2) 0.05 M acetate buffer, pH 4.5, and (3) 0.1 N hydrochloric acid. Two different homogenizations were tested for each solvent: (i) three strokes, 20 s each, with a Polytron (Kinemmatica GmbH) and (ii) different times (2, 4, 8, and 16 min) in a Stomacher homogenizer (Seward Laboratory). Except when indicated, the amino acid extraction was performed as follows: 8 g of fresh muscle or 4 g of dry-cured ham was diluted 1:5 or 1:10, respectively, with 0.1 N hydrochloric acid. Samples were then homogenized at 5 °C with a Stomacher homogenizer for 8 min and centrifuged at 10000*g* for 20 min. Supernants were filtered through glass wool and collected for further processing.

Parallel assays with the standard solution and samples were performed in triplicate for each deproteinization technique.

(*a*) *Chemical Deproteinization.* The following precipitating agents were used: 5% (w/v) perchloric acid (PCA), 5% (w/v) sulfosalicylic acid (SSA), 5% (w/v) phosphotungstic acid (PTA), 1% (w/v) picric acid (PA), and acetonitrile (ACN). Two milliliters of the standard solution or samples was mixed with 2 mL of acid precipitants or 4 mL in the case of acetonitrile. The mixtures were allowed to stand for 30 min before centrifuging at 10000*g* for 15 min; 500 μL of each supernant (750 μL in the case of acetonitrile) was mixed with 100 μL of internal standard solution previously diluted 1:1 with water (1:3 in the case of fresh muscle), and then 50 μL (75 μL when acetonitrile was used) was taken for derivatization.

(*b*) *Physical Deproteinization.* The standards and samples were filtered through polysulfone membranes, 10 kDa cutoff (type centrifugal ultrafree, Millipore) by centrifugation at 2000 rpm for 30 min (UF_{10000}), and through cellulose membranes, 1 kDa cutoff (disk type, Millipore) by pressure (UF_{1000}). In all cases, filters were previously washed by sonication in distilled water to remove any filter contaminant. Ten milliliters of standard or sample solutions was mixed with 2 mL of internal standard (1 mL in the case of fresh ham) and filtered through the 10-kDa membrane, yielding approximately 1 mL of ultrafiltrate; 50 μL

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Table I. Percent Recovery of Standard Amino Acids after Deproteinization Pretreatment

	amino acid	chemical precipitants						ultrafiltration	
		PCA	TCA	PTA	SSA	PA	ACN	10 kDA	1 kDa
1	Asp	99.5	97.0	52.7	70.0	98.0	97.7	97.0	97.1
2	Glu	99.1	99.0	77.6	73.5	98.3	99.2	97.7	98.8
3	Asn	101.0	102.3	71.4	100.9	101.8	97.3	99.3	95.3
4	Ser	98.5	94.5	92.4	95.0	99.3	100.6	101.2	98.7
5	Gln	98.6	96.0	81.4	92.3	101.2	97.2	98.2	94.5
6	Gly	103.6	104.3	96.5	113.1	103.2	100.4	101.2	105.7
7	His	96.7	92.2	93.1	89.7	99.7	100.0	100.0	55.2
8	Tau						101.3	99.1	101.6
9	Arg	90.8	92.1	35.4	83.3	100.3	99.0	103.5	69.5
10	Thr	97.1	96.4	95.5	97.9	100.2	98.5	98.3	94.6
11	Ala	103.8	104.5	100.5	96.4	102.2	100.7	100.9	99.5
12	Pro	104.5	108.2	104.2	150.0	104.2	100.0	99.3	102.1
13	Tyr	92.8	98.2	102.8	96.2	101.5	101.6	102.9	104.7
14	Val	97.5	98.5	98.5	89.2	100.0	98.1	98.8	98.8
15	Met	103.6	106.2	91.5	106.7	105.3	101.2	99.5	100.4
16	Ile	98.5	97.9	95.7	88.7	99.8	98.7	101.7	98.7
17	Leu	98.6	97.3	101.8	84.5	96.1	100.0	103.9	98.3
18	Phe	99.3	99.4	97.6	92.9	106.0	99.4	100.0	101.4
19	Trp	72.9	92.8	83.7	97.5	100.8		98.5	103.5
20	Orn	95.5	94.1	38.7	96.2		97.5	101.2	34.3
21	Lys	99.9	96.6	10.4	88.0	102.1	98.6	99.2	51.6

Table II. Amino Acid Concentrations in Pork Fresh Muscle and Dry-Cured Ham

	amino acid	mg/100 g in fresh muscle[a,b]	mg/100 g in dry-cured ham[a,b]
1	Asp	0.82	252.74
2	Glu	4.37	543.60
3	Asn	1.50	74.02
4	Ser	2.90	233.20
5	Gln	22.87	43.10
6	Gly	21.42	211.45
7	His	1.92	184.92
8	Tau	31.25	134.06
9	Arg		
10	Thr	7.06	220.32
11	Ala	13.48	387.00
12	Pro	5.30	211.66
13	Tyr	4.08	126.16
14	Val	3.56	253.88
15	Met	1.72	98.74
16	Ile	2.69	195.53
17	Leu	3.90	340.91
18	Phe	2.92	190.18
19	Trp	0.88	14.43
20	Orn	1.07	19.21
21	Lys	4.65	649.28

[a] The moisture contents of both fresh muscle and dry-cured ham were 75.1% and 63.5%, respectively. [b] Samples were deproteinized with acetonitrile.

was directly derivatized while the rest was applied to the 1-kDa membrane, yielding around 0.1 mL of ultrafiltrate (1-kDa fraction). Fifty microliters of the ultrafiltrate was also derivatized.

Amino Acid Derivatization. Standards and samples were dried under vacuum in conical shaped tubes at 38 °C and derivatized according to the method of Bidlingmeyer et al. (1987). Twenty microliters of methanol–1 M sodium acetate–TEA (2:2:1) was added to each tube and dried again under vacuum. Derivatization took place by addition of 20 μL of methanol–water–TEA–PITC (7:1:1:1) to the tube samples, which were then sealed and left to stand for 20 min at room temperature before drying. Both reagent mixtures were made fresh daily. Once derivatized, 500 μL of 5 mM sodium phosphate, pH 7.6, containing 5% acetonitrile was added as diluent. TEA and PITC should be carefully handled.

Chromatography. Samples were analyzed on a Hewlett-Packard Model 1090 A liquid chromatograph equipped with an autosampler and a HP-1040 diode array detector (254 nm) and controlled with a HP-85 B microprocessor. The column was a Supelcosil LC-18 DB, 25 cm × 4.6 mm (5-μm particle size)

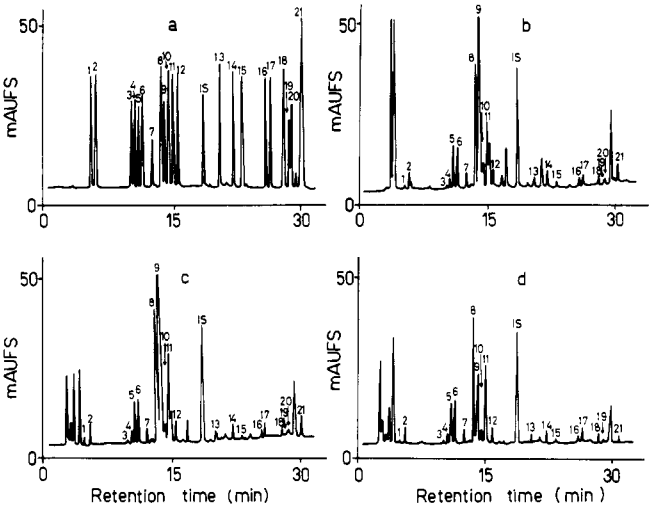

Figure 1. Chromatograms of phenyl isothiocyanate free amino acids from a standard solution containing 250 pmol of each component (a) and from fresh pork meat extracts deproteinized with perchloric acid (b) or by ultrafiltration through 10 000 (c) and 1000 (d) M_r cutoff membranes. Chromatographic conditions are described in the text. The numbering corresponds to that used in Tables I and II. IS, internal standard (α-aminobutyric acid).

protected with a LC-18 Pelliguard packed guard column (2 cm × 2.1 mm) from Supelco (Bellefonte, PA). The temperature was controlled to 40 °C within ±1 °C with a column heater (Hewlett-Packard).

The solvent system consisted of two eluents: (A) 0.14 M sodium acetate containing 0.5 mL/L of TEA and adjusted to pH 6.4 with glacial acetic acid; (B) 60:40 acetonitrile–water. To achieve the amino acid separation, the flow rate was set to 0.8 mL/min and the following gradient was performed: Initial 10% B; 6 min linear change to 12.5% B; 32 min linear to 58% B; 33 min step to 100% B; wash for 8 min and reequilibrate at 10% B during 20 min before a new injection. Twenty microliters of standards or samples, previously filtered through a 0.45-μm membrane filter (Millipore) was injected into the system.

RESULTS AND DISCUSSION

The chromatogram of the standard solution is shown in Figure 1a. As can be observed, all of the amino acids were well resolved. α-Aminobutyric acid, which was used as internal standard, eluted in a region of the chromatogram free of other peaks. All amino acids in samples were identified by comparison with the relative retention times of standard amino acids.

Three different solvents were assayed for amino acid extraction from pork muscle: 0.03 M phosphate buffer, pH 7.4; 0.05 M acetate buffer, pH 4.5; and 0.1 N hydrochloric acid. The highest amino acid recoveries were obtained by using the 0.1 N hydrochloric acid, so it was used for further amino acid extractions. Other authors have also used this acid for free amino acid extraction from brain tissue (Cohen and Strydom, 1988) and mammalian organs (Sarwar and Botting, 1990). Polytron and Stomacher homogenizers were also compared in their effectiveness for amino acid extraction. As also observed by Kuchroo and Fox (1982) with cheese, the homogenization in Stomacher was better than that in Polytron. In our case, homogenization for 8 min was enough to get the maximum yield.

To obtain a deproteinized extract with minimal amino acid losses, different chemical (TCA, PCA, SSA, PTA, PA, and ACN) and physical (UF through 10 000 and 1000 M_r cutoff membranes) precipitation techniques were assayed. The percent recoveries from a standard amino acid solution are shown in Table I. Although all of these

techniques are very effective for sample deproteinization (Blanchard, 1981; Deyl et al., 1986; Hubbard et al., 1988), poor amino acid recoveries can be obtained with some of them. These losses could be due to the deproteinization method or to the PITC derivatization procedure. However, amino acid analysis by liquid chromatography of precolumn phenyl isothiocyanate derivatives has been successfully validated by the ion-exchange chromatographic methods in other research centers (Beaver et al., 1987; Bidlingmeyer et al., 1987; Sarwar et al., 1988; Fürst et al., 1989), so that a comparison of the liquid chromatography method with an established classical method of amino acid analysis was not considered necessary in this investigation.

Losses of amino acids should be then due to the particular deproteinization technique. So, sulfosalicylic acid (SSA) gave especially poor recovery of aspartic and glutamic acids, histidine, arginine, valine, isoleucine, leucine, and lysine. Deyl et al. (1986) and Cohen and Strydom (1988) also reported losses in SSA-prepared samples. Curiously, SSA is one of the most common precipitants used for biological samples (Godel et al., 1984; Hubbard et al., 1988; Fürst et al., 1989) and cheese (Ramos et al., 1987). Another relevant fact is the abnormally high proline (around 150%) and glycine (around 110%) recoveries, although no interfering peaks were observed in the SSA blank chromatogram. Phosphotungstic acid (PTA) gave poor recoveries of aspartic and glutamic acids, asparagine, glutamine, and tryptophan. Arginine, ornithine, and lysine were very poorly recovered as was also observed by Jarret et al. (1982). Very good amino acid recoveries, higher than 90% (see Table I), were obtained with the rest of the chemical precipitating agents (PCA, TCA, PA, and ACN). Only tryptophan recovery was poor (around 73%) in the presence of perchloric acid (PCA). A special disadvantage of both PCA and TCA is the longer time spent drying the samples under vacuum. Cohen and Strydom (1988), using organic solvents such as methanol at concentrations of 70–90%, observed poor recoveries of polar amino acids. However, amino acid recoveries higher than 97% were obtained when 66.6% ACN was added as deproteinizing agent (see Table I).

Ultrafiltration through membranes (10 000 and 1000 M_r cutoff) was assayed as a physical deproteinization technique. The same amino acid standard solution was filtered through the two membranes. More than 95% recovery was obtained with the 10 000 Da cutoff membranes (see Table I). However, Cohen and Strydom (1988) detected low yields of certain amino acids such as γ-aminobutyric acid, β-alanine, and threonine using an Amicon membrane. They also found filter contaminants interfering with analysis even after extensive washings of the filter. In our case, a previous washing with water was enough for an effective elimination of the contaminants. There were important losses of dibasic amino acids when the 1000 M_r cutoff membrane filter was used (see Table I). In this case, a 3-min sonication in water was enough for a complete removal of filter contaminants.

The effectiveness of these chemical and physical deproteinizing techniques was tested with both fresh and dry-cured meat samples. Examples of amino acid chromatograms from both samples, deproteinized with PCA (Figures 1b and 2a, respectively) and through 10 000 M_r cutoff ultrafilter (Figures 1c and 2c, respectively), are shown. An interfering peak appeared around 14 min of retention time, making quantitation of arginine, and in some cases taurine and/or threonine, difficult. This zone of the chromatogram was similar with all of the deproteinizing agents except when PTA was used (see Figure 2b). In this case, there was a complete elimination of the interfering peak but with very important losses in dibasic amino acids. Because this reagent has an approximately 600 M_r cutoff (Jarret et al., 1982), it could be assumed that the interfering peak might be either peptides greater than 600 Da or basic compounds. The ultrafiltration through 1000 M_r cutoff membrane also gave a considerable decrease of the interfering peak (see Figures 1d and 2d), more noticeable in the case of meat. As in the case of PTA precipitation, there were losses in dibasic amino acids, although to a lesser extent.

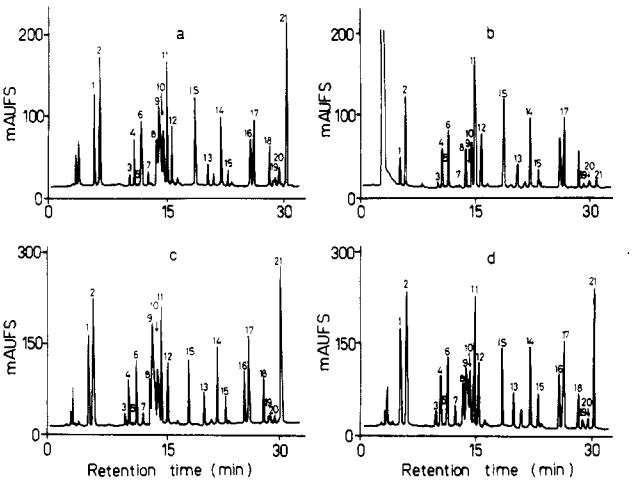

Figure 2. Chromatograms of phenyl isothiocyanate free amino acids from dry-cured meat extracts deproteinized with perchloric acid (a) or phosphotungstic acid (b) or by ultrafiltration through 10 000 (c) and 1000 (d) M_r cutoff membranes. Chromatographic conditions are described in the text. The numbering corresponds to that used in Tables I and II. IS, internal standard (α-aminobutyric acid).

It is clear that free amino acids in fresh meat and dry-cured ham can be effectively analyzed after deproteinization with PCA, TCA, PA, ACN, and UF_{10000}. Free amino acid contents in both fresh meat and dry-cured ham are reflected in Table II. In both cases, samples were deproteinized by ACN addition. A very relevant fact is the high amino acid concentrations in the dry-cured hams. There is still an unsolved problem in the analysis of arginine and, sometimes, taurine and/or threonine. Future work will focus on trying to solve this problem by changing the selectivity and/or gradient conditions of the chromatographic separation for the displacement of the interfering peak to a zone of the chromatogram free of amino acids.

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Registry No. Asp, 56-84-8; Glu, 56-86-0; Asn, 70-47-3; Ser, 56-45-1; Gln, 56-85-9; Gly, 56-40-6; His, 71-00-1; Tau, 107-35-7; Arg, 74-79-3; Thr, 72-19-5; Ala, 56-41-7; Pro, 147-85-3; Tyr, 60-18-4; Val, 72-18-4; Met, 63-68-3; Ile, 73-32-5; Leu, 61-90-5; Phe, 63-91-2; Trp, 73-22-3; Orn, 70-26-8; Lys, 56-87-1; trichloroacetic acid, 76-03-9; perchloric acid, 7601-90-3; sulfosalicylic acid, 97-05-2; phosphotungstic acid, 12067-99-1; picric acid, 88-89-1; acetonitrile, 75-05-8.