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Chapter 22

Innovative and Rapid Procedure for 4-Hydroxyproline Determination in Meat-Based Foods

Maria Cristina Messia and Emanuele Marconi

Abstract

This report describes a rapid and innovative microwave procedure for protein hydrolysis coupled with high performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD) to quantify the amino acid 4-hydroxyproline in meat and meat-based products. This innovative approach was successfully applied to determine collagen content (4-hydroxyproline×8) as the index quality of meat material used in the preparation of typical meat-based foods.

Key words: 4-Hydroxyproline, Collagen, Microwave protein hydrolysis, HPAEC-PAD

1. Introduction

The addition of low value meat is generally considered to be the most frequent adulteration of meat-based products, such as sausages, fresh filled pasta, hamburger, etc. A suitable marker for identifying the quality of raw material used for meat-based preparations is collagen content which can be calculated by the concentration of the imino acid 4-hydroxyproline (1–3).

The method most commonly used for 4-hydroxyproline analysis is the colorimetric method based on hydrochloric acid or sulphuric acid hydrolysis of meat sample, oxidation of 4-hydroxyproline with chloramines-T and spectrophotometric measurement at 560 nm of red-purple colour formed. Although this method is very specific for 4-hydroxyproline, it is difficult to control oxidation, colour formation, and the hydrolysis step is very time consuming (16–24 h).

One of the most significant and recent developments in the performance of compositional food analysis is the use of microwave radiation energy for protein hydrolysis (4, 5). The procedure has been successfully used for fast protein hydrolysis (6) in determining

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natural as well as non-natural single amino acids, such as tryptophan (7), lysine (8), furosine (9, 10), *meso*-diaminopimelic acid (11), and total amino acids (4, 12–15).

Several analytical techniques have been developed to detect total and single amino acids. An alternative to derivatization systems (16) is the electrochemical detection (17, 18), and in particular integrated amperometry (HPAEC-PAD) (19, 20) which allows the direct detection of amino acids on a platinum or a gold electrode after the separation by anion exchange.

In this chapter, we describe the use of microwave hydrolysis coupled to high performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD) for 4-hydroxyproline analysis in meat-based products. This combined approach allows the reduction of protein hydrolysis time to 20 min and the direct 4-hydroxyproline detection without pre- or post-column derivatization.

2. Materials

2.1. Chemicals

1. Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C).
2. NaOH 50% (*p*/*v*) (Mallinckrodt Baker B.V., Deventer, Holland).
3. 4-Hydroxyproline standard (Sigma Chemical Co., St. Louis, MO, USA).
4. HCl: Approximately 6N HCl. Add 250 ml H_2O to 1-L volumetric flask. Slowly add, 500 ml of HCl (solution at 37.5%, density: 1.186). Cool to room temperature and dilute to volume with water.
5. Hydroxyproline standard solution: Stock solution (13 mg/ml). Dissolve 1.31 g of hydroxyproline in water in 100-ml volumetric flask. Dilute to volume with water. Solution is stable ca. 2 months at 4°C.
6. All other chemicals and reagents of HPLC grade.
7. Samples of meat-based foods are chopped and then homogenized with a steel blade homogenizer, for no longer than 15–20 s to minimize any increase in temperature.

2.2. Microwave Apparatus

Microwave Digestion System, Mod. MDS 2000 (CEM Corporation, Mattews, NC, USA). This microwave oven has a maximum power of 630 ± 50 Watts and a magnetron frequency 2,435 MHz. The system is equipped with probes to detect and control the pressure and temperature inside the sealed vessel. The oven is equipped with a 12-positioned turntable (3 rpm) and a mode stirrer to prevent the uneven distribution of microwaves in the hydrolysis vessels (Fig. 1).

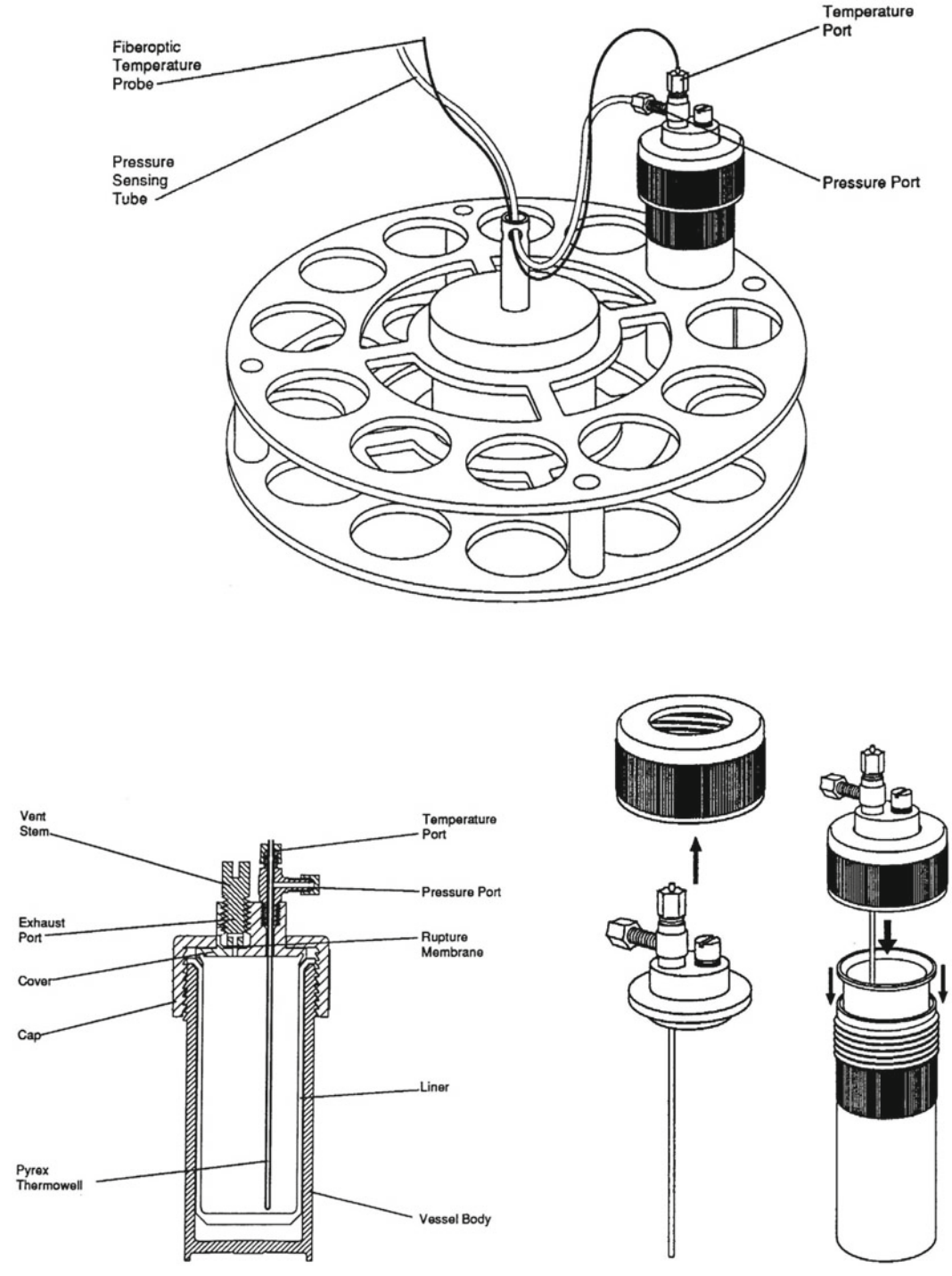

Fig. 1. Cross section of lined digestion vessel for pressure and temperature control and turntable collection vessel (MDS-2000 Operative manual, CEM Corporation, Mattews, NC, USA).

2.3. Chromatographic Apparatus

1. 1 Dionex DX500 Ion Chromatograph (Dionex Corporation, Sunnyvale, CA, USA) composed of a gradient pump (mod GP50) with an online degasser and electrochemical detector (model ED40).
2. Instrument control, data collection, and total quantification are managed using Chromeleon chromatography software.
3. The flow-through electrochemical cell consists of a 1 mm diameter gold working electrode, a pH reference electrode, and a titanium body of the cell as the counter electrode.
4. A controlled Rheodyne injector (Rheodyne L.P., CA, USA) with a 25 µl sample loop is used for sample injection.
5. Aminopac PA10 analytical column 250×2 mm, with 8.5 µm particle size (Dionex Corporation).

3. Methods

3.1. Microwave Protein Hydrolysis (see Ref. 21)

1. Microwave hydrolysis is carried out using a microwave digestion system designed for laboratory use.
2. The sample, corresponding to 25 mg of protein (see Note 1), is placed into four Teflon PFA digestion vessels (see Note 2) and 8 ml 6N HCl are added (see Note 3).
3. The vessel cup is screwed manually; the pressure and fibre optic probes are connected to the vessel with the triple ported cap.
4. After the irradiation cycles (Table 1), the vessels are cooled (see Note 4) and then removed.
5. The hydrolysates are filtered (see Note 5) by Whatman paper n.1.
6. The filtered samples are evaporated to dryness by a rotary evaporator (bath temperature 40°C, pressure 25 mbar) and then redissolved with a precise volume of 0.1N HCl.
7. Before the analysis, samples are diluted 1:50–1:100 with ultrapure water, filtered through 0.20 µm filter and then injected in the chromatographic system.

3.2. Chromatographic Separation (see Ref. 21)

1. Quantitative determination is carried out at a flow rate of 0.25 ml/min using a mobile phase of water (eluent E1) (see Note 6), 250 mM sodium hydroxide (eluent E2) (see Note 7) and 1.0 M of sodium acetate (eluent E3) (see Note 8) as shown in Table 2 and an optimized time-potential waveform as shown in Table 3.
2. 4-Hydroxyproline identification and quantification is carried out by means of external amino acid standard (see Note 9).

Table 1
Microwave protein hydrolysis conditions in meat-based foods

	1st Cycle	2nd Cycle
Power (% 630 Watts)	85	85
Time (min)	1	5
Temperature (°C)	100	155
Pressure max (psi)	100	130

Table 2
Gradient conditions for anion-exchange separation of 4-hydroxyproline

Time (min)	Eluent 1 water (%)	Eluent 2 NaOH (%)	Eluent 3 sodium acetate (%)
0.0	80	20	0
2.0	80	20	0
12.0	80	20	0
16.0	68	32	0
24.0	36	24	40
40.0	36	24	40
40.1	20	80	0
42.1	20	80	0
42.2	80	20	0
62.0	80	20	0

3. Figure 2 shows an HPAEC-PAD chromatogram of meat-based food hydrolysed by microwave procedure. The retention time of 4-hydroxyproline is 9 min. The same HPAEC-PAD chromatographic run can also permit the identification and quantification of all other amino acids.

3.3. Calculation of 4-Hydroxyproline Content

1. Calculate 4-hydroxyproline content (g/100 g) as follows:

$$\text{4-Hydroxyproline(g/100g)} = ((\text{SA} \times \text{Std})/\text{StdA}) \times \text{MW} \times \text{D} \times 100/\text{SW},$$

where: SA = 4-hydroxyproline sample area; Std = 4-hydroxyproline standard concentration; StdA = 4-hydroxyproline standard

Table 3
Integrated amperometry waveform used to detect 4-hydroxyproline

Time (s)	Potential (V)	Integration
0.00	+0.13	
0.04	+0.13	
0.05	+0.28	
0.11	+0.28	Begin
0.12	+0.60	
0.41	+0.60	
0.42	+0.28	
0.56	+0.28	End
0.57	–1.67	
0.58	–1.67	
0.59	+0.93	
0.60	+0.13	

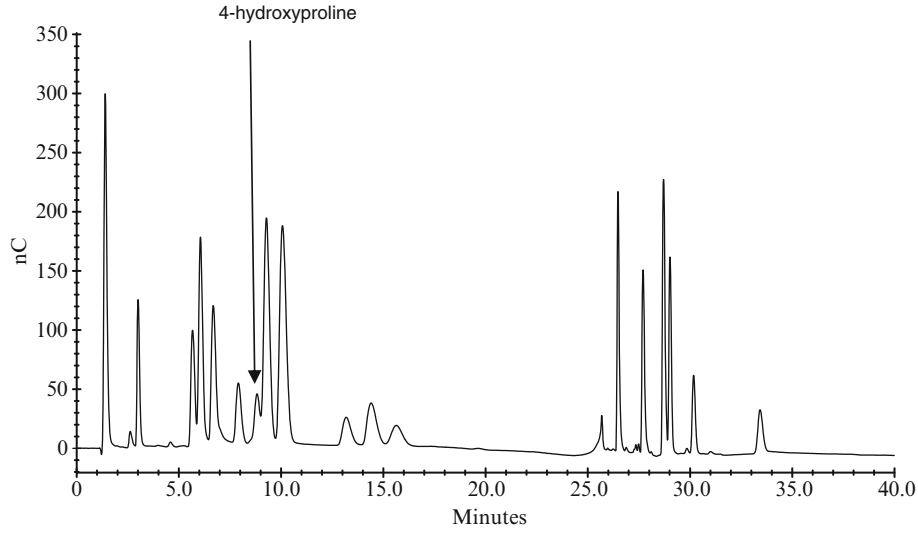

Fig. 2. HPAEC-PAD chromatogram of meat-based food after microwave hydrolysis.

area; MW = 4-hydroxyproline molecular weight; D = dilution factor which consider the final volume of hydrolysate; the volume of hydrolysate dried by rotary evaporator; the volume of 0.1 N HCl used to resume the sample after drying with rotary

evaporator; the sample dilution (1:50 or 1:100) before the injection in the chromatographic system; and SW = sample weight.

2. The collagen content can be estimated by multiplying the 4-hydroxyproline content (g/100 g) by 8 as provided by AOAC method 990.26 (22) (see Note 9)

4. Notes

1. Protein content ($N \times 6.25$) was determined according AOAC method 928.08 (22).
2. The sealed vessels used consist of perfluoroalkoxy (PFA) sample liner (volume 50 ml) surrounded by an advanced composite sleeve.
3. Each cycle of hydrolysis has to be carried out using same number of vessels (four), same typology and quantity of samples in order to allow a uniform microwave distribution and absorption.
4. The turntable is cooled into an ice bath until the pressure is equal to atmospheric pressure (about 5 min).
5. The sample is filtered into 100-ml volumetric flask and diluted to mark with deionized water.
6. Eluent E1: Deionized water. Filter the pure deionized water through 0.2 μm nylon filters and then transfer into a DX500 eluent bottle. Seal the filtered water immediately. Remember, that atmospheric carbon dioxide adsorbs even into pure water, albeit at much lower levels than in alkaline solutions. Minimize the contact time of water surface with the atmosphere (Dionex DX500 Operative Manual).
7. Eluent E2: 250 mM Sodium hydroxide. The first step in the preparation of sodium hydroxide eluent is filtration of a water aliquot (typically 1.0 L). Hermetically seal the filtered water immediately after filtration while preparing a disposable glass pipette and a pipette filler. Using a pipette filler, draw an aliquot of 50% sodium hydroxide into pipette (13.1 ml), unseal the filtered water, and insert the full pipette approximately 1 inch. below the water surface and release the sodium hydroxide. If done properly and without stirring, most of the concentrated sodium hydroxide stays at the lower half of the container and the rate of carbon dioxide adsorption is much lower than that of a normally prepared 250 mM sodium hydroxide solution. Seal the container immediately after sodium hydroxide transfer is complete. Remember to put the screw cap back on the 50% hydroxide bottle immediately as well. Mix the content of the tightly sealed container holding the 250 mM hydroxide.

Unscrew the cap of the eluent bottle E2 attached to the chromatographic system. Allow the helium or nitrogen gas to blow out of the cap. Unseal the bottle holding 250 mM sodium hydroxide and immediately, without delay, start the transfer into the eluent bottle E2. Try to minimize the carbon dioxide absorption by holding the gas orifice of the bottle cap as close as possible to the 250 mM hydroxide during the transfer. With the inert gas still blowing, put the cap on the eluent bottle. Allow the pressure to build up inside the bottle and reopen the cap briefly several times, to allow trapped air to be gradually replaced by inert gas (Dionex DX500 Operation Manual).

8. Eluent E3: 1.0 M Sodium acetate. Dissolve 82.04 g of anhydrous sodium acetate in approximately 750 ml water. Seal the container during the dissolution step. Make up to 1.0 L with water and filter through a 0.2 μm nylon filter. Transfer the filtered sodium acetate eluent into the eluent bottle E3 of the chromatographic system, using the same procedure as for the sodium hydroxide (Dionex DX500 Operation Manual).
9. Collagen connective tissue contains 12.5% 4-hydroxyproline if the nitrogen-to-protein factor is 6.25.

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