Talanta 136 (2015) 190–197

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Pulsed electromembrane extraction for analysis of derivatized amino acids: A powerful technique for determination of animal source of gelatin samples

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ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form
3 January 2015
Accepted 6 January 2015
Available online 28 January 2015

Keywords:
Pulsed electromembrane extraction
Amino acids
Gelatin
Porcine
Bovine
o-phthalaldehyde derivatization

ABSTRACT

Differentiation of animal sources of gelatin is required for many reasons such as some anxieties about bovine spongiform encephalopathy or a ban on consuming porcine gelatin in some religions. In the present work, an efficient method is introduced for determination of animal origin of gelatin samples. The basis of this procedure is the application of pulsed electric field for extraction, preconcentration, and analysis of derivatized amino acids in gelatin. To this end, after derivatization of amino acids of interest by means of *o*-phthalaldehyde (OPA) for enhancing their ultraviolet (UV) absorbance as well as increasing their lipophilicities, a 137 V electric field was applied for 20 min with 10 min^{-1} frequency to make the analytes migrate through a 200 μm organic liquid membrane into an aqueous acceptor phase. Finally, the acceptor phase was analyzed by HPLC-UV. The proposed technique offered a high efficiency for analysis of amino acids, regarding 43% and 79% as extraction recoveries and 25 ng mL^{-1} and 50 ng mL^{-1} as limits of detection (LODs) for asparagine and glutamine, respectively. Therefore, due to sample cleanup ability of the proposed method and obtained preconcentration factors (29 and 53 for asparagine and glutamine, respectively), it could be carried out for differentiation of animal origins of gelatin samples, even if only small amounts of samples are available or in complicated media of foodstuffs and medicament.

1. Introduction

Gelatin is a water-soluble biopolymer, which is obtained from partial hydrolysis of collagen. Gelatin is widely used in foodstuffs and medicament to improve the quality, and its applications in cosmetic, dairy, and meat products make it a popular polypeptide [1]. The main gelatin sources are bovine and porcine bones and skin. An alkaline treatment is employed in industries to form bovine gelatin, known as type B gelatin. Whereas, porcine or type A gelatin is gained by an acidic treatment [2]. One of these kinds of gelatin may be chosen by manufacturer, due to their possible diverse characteristics as a result of different production processes. Also, consumption of porcine gelatin is forbidden in some religions. Therefore, development of an experimental method for determination of the origin of gelatin seems to be necessary. However, high similarities in structures and physicochemical properties of gelatins with different origins make their differentiation difficult. There are few reports on differentiation of gelatin sources, such as chemometric techniques [3,4], analysis of marker peptides by means of high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) [5], and polyclonal anti-peptide antibodies in indirect and competitive indirect enzyme-linked immunosorbent assay (ELISA) [6]. Nevertheless, a simple, low cost, accurate, and practical method is needed for analysis of animal sources of gelatins in industries.

It was shown that the bovine and porcine gelatins have different amino acid compositions [2]. There are some reports for source differentiation of gelatin samples via analysis of their amino acids [7–9]. All of these works focused on the differences in concentrations of 15 amino acids whereas there are two marker amino acids which have not been investigated in any of these papers. It may be due to their relatively low concentrations or very hydrophilic natures which make their analysis difficult [2]. Porcine products contain two amino acids, asparagine (ASN) and glutamine (GLN), while bovine gelatin is free of them. Hence, a method for selective extraction, preconcentration and analysis of ASN and GLN in gelatins could be exploited for differentiation of sources.

It is obvious that the ionizable compounds migrate through an electric field. This is the basis of a microextraction method, called

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http://dx.doi.org/10.1016/j.talanta.2015.01.007
0039-9140/

electromembrane extraction (EME), which was originally utilized by Pedersen-Bjergaard and his research group [10]; this method has been associated with rapid progressing in recent years [11–18]. In this method, the desired analytes move across a relatively selective supported liquid membrane (SLM), and are extracted into the acceptor solution that is located in the lumen of a piece of hollow fiber membrane. Nonetheless, it was confirmed that the extraction of amino acids, especially ASN and GLN, via EME is very difficult owing to their hydrophilic nature [19]. For more hydrophilic analytes, hard conditions (e.g., high applied voltage, moderately long extraction time, and addition of some carriers to SLM) are required in EME because of the lower affinity of these analytes for organic liquid membrane. These conditions lead to system instability and cause the extraction of this kind of compounds to be rather difficult. Consequently, pulsed electromembrane extraction (PEME) was introduced by Yamini et al. [20]. It was revealed that the application of pulsed voltages instead of continuous voltage in EME significantly increases the system stability and the extraction efficiency [20–22]. Also, two-way PEME was employed for analysis of histidine, phenylalanine and tryptophan as model zwitterions and it was shown that this technique could be used for selective extraction of this kind of analytes [21].

The main aim of this work is to introduce a practical, simple and efficient technique for determination of animal origin of different gelatins. Therefore, PEME followed by HPLC-UV was developed and employed, for the first time, for analysis of ASN and GLN as marker amino acids. This work is the first attempt to analyze ASN and GLN with the aid of PEME technique. Since the two target amino acids had no UV absorbance, some derivatization procedures were conducted prior to their analysis. Up to now, different derivatization reagents have been used to derivatize amino acids prior to their HPLC analysis. These reagents include activated halides, acyl halides, OPA alone or in combination with different mercaptans, other dialdehydes except OPA, dabsyl, phenylthiocarbamyl, succinimidyl ester, cobalt with hydroxylamines and sulfonic acid with halides [23,24]. Among them, *o*-phthalaldehyde (OPA) was found as the most popular and efficient derivatization reagent [23,24]. Thus, OPA was used as derivatization reagent to enhance the hydrophobicity of the amino acids as well as their UV absorbance. The suggested method may be capable of determining the origins of all complex animal products, even if only small amounts of samples are available, due to large extraction recovery, high preconcentration factor, and high sample cleanup.

2. Experimental

2.1. Equipment for PEME

The equipment for PEME procedure is shown in Fig. 1. A glass vial with internal diameter of 10 mm and height of 8 cm was used. The electrodes used in this work were platinum wires with diameters of 0.2 and 0.5 mm for cathode and anode, respectively, which were obtained from Pars Platin (Tehran, Iran). The electrodes were coupled to a power supply model 8760T3 with a programmable voltage in the range of 0–600 V and a current output in the range of 0–500 mA from Paya Pajoohesh Pars (Tehran, Iran). A homemade pulse generator was used to set the pulse duration and outage period with a timer in the range of 1 s to 10 min. The schematic diagram of the pulse generator is shown in Fig. 2. During the extraction, the PEME unit was stirred at a stirring speed range of 0–1250 rpm by a heater-magnetic stirrer model 3001 from Heidolph (Kelheim, Germany) using a 5 mm × 2 mm magnetic bar.

2.2. Chemicals and materials

ASN, GLN, and OPA were acquired from Sigma (St. Louis, MO, USA). A stock solution, comprising 1 mg mL^{-1} of each analyte, was prepared in ultrapure water and stored at 4 °C, protected from light. Working standard solutions were prepared by dilution of the stock solution with water.

2-Nitrophenyl octyl ether (NPOE), tris(2-ethylhexyl) phosphate (TEHP), and di-(2-ethylhexyl) phosphate (DEHP) were purchased from Fluka (Buchs, Switzerland). 1-Octanol, methanol, ammonium acetate, di-sodium tetraborate, HCl, and NaOH were obtained from Merck (Darmstadt, Germany). All the chemicals used were of analytical-reagent grade. The porous hollow fiber, utilized for SLM, was a PPQ3/2 polypropylene hollow fiber from Membrana (Wuppertal, Germany) with the inner diameter of 0.6 mm, wall thickness of 200 μm, and pore size of 0.2 μm. Ultrapure water was produced by a Young Lin aquaMAx purification system 370 series (Seoul, Korea).

2.3. Preparation of real samples

Porcine gelatin sample was supplied by Fluka (Buchs, Switzerland). Bovine gelatins were kindly donated by Faravari Darooie Gelatin Halal (Qazvin, Iran). A 0.2 g of each solid sample was dissolved in 10 mL of a 0.5 M NaOH solution and heated at 90 °C for 15 min to hydrolyze the gelatin and release the free amino acids. Thereafter, excess amounts of OPA (10 mL, 40 mmol L^{-1}) were added and the obtained solution was heated again at 90 °C for 15 min to complete the derivatization process. The derivatization reagent was prepared by dissolving 0.027 g of OPA in 5 mL borate buffer (20 mmol L^{-1}, pH = 10.0). The OPA solution should be prepared daily and stored at 4 °C during the day. The sample, containing the amino acids and the derivatization reagent, was mixed (1:1) and placed in an oil bath for an optimized time to complete the reaction. After derivatization, the color of the solution changed to yellow. The pHs of 20-fold diluted final samples were adjusted to 3.0 by the addition of proper amounts of HCl solution and checking the pH values using a Metrohm 691 pH-meter (Zofingen, Switzerland) before PEME process.

2.4. PEME procedure

A 2.5 mL of the sample solution was transferred into the PEME vial. To impregnate the organic liquid membrane in the pores of hollow fiber wall, a 5.5 cm piece of the hollow fiber (which offers about 15 μL volume for housing the acceptor phase) was cut out and dipped into the solution for 5 s, and then the excess of the organic solvent was gently wiped away by blowing air with a Hamilton syringe. A solution of 100 mmol L^{-1} HCl (acceptor phase) was introduced into the lumen of the hollow fiber through a microsyringe, and then the lower end of the hollow fiber was mechanically sealed. A platinum cathode with the diameter of 0.2 mm was introduced into the lumen of the fiber. The fiber, containing the cathode together with the SLM and the acceptor solution, was afterwards directed into the sample solution. A platinum anode with the diameter of 0.5 mm was led directly into the sample solution. The electrodes were subsequently coupled to the power supply. The predetermined voltage (137 V) was turned on and the extraction was performed for 20 min while the pulse frequency was 10 min^{-1}. When the extraction was completed, the acceptor solution was collected by a microsyringe and injected into the HPLC instrument for further analysis.

2.5. HPLC conditions

Separation and detection of the target analytes were conducted by a Varian HPLC (Walnut Creek, CA, USA) system consisting of a 9012 HPLC pump, a six-port Cheminert HPLC valve from Valco Instruments (Houston, TX, USA) with a 15 μL sample loop, and a Varian 9050 UV–vis detector. Chromatographic data were recorded