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Analytical Methods

Quantification of L-lysine in cheese by a novel amperometric biosensor

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A B S T R A C T

Lysine quantification in cheese by a novel, highly selective amperometric biosensor is reported. Based on L-lysine-α-oxidase immobilized by co-crosslinking onto Platinum (Pt) electrodes modified by overoxidized polypyrrole, the sensor proved almost specific to lysine, sensitive and stable. The pure enzymatic nature of current signals was confirmed by a control electrode modified without enzyme. The precision of the method showed relative standard deviations of 4.7% and 9.2% respectively for Parmigiano Reggiano and Grana Padano cheese ($n = 5$). The recovery data on various cheese, spiked with lysine at 50–100% of the measured content, ranged from 85% to 99%. Different types of cheese were analysed showing lysine concentrations related to the ripening time and the manufacture technology, in agreement with literature data. Within dairy products, no appreciable lysine was detected in yogurts. The method adopted revealed suited to satisfy the demands for precise and sensitive detection of lysine with minimal sample preparation and clean-up.

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1. Introduction

Foods differ enormously for their content in proteins and especially in amino acids. In general, products of animal origin contain in adjusted quantity all the essential amino acids. For instance, proteins of eggs and milk are used as reference of biological value, introducing all the essential amino acids in the ideal proportions. Contrarily, proteins of vegetable origin are relatively lacking in the essential amino acids lysine (cereals), methionine and cysteine (vegetables). Milk is therefore a food of remarkable importance in human feeding. Cheese, being derivatives of milk, also have a high nourishing value. Their principal components differ however from those of the raw material because of the modifications happening during the workmanship and the maturation. As an example, cheese proteins are comparable for quality to those of milk but are more digestible. This is due to the proteolysis process that happens during maturation upon the action of rennet, peptidases from starter bacteria, secondary micro flora and indigenous milk enzymes. Upon proteolysis, the enzymatic breakdown of long protein chains occurs with subsequent formation of low molecular weight peptides up to the release of free amino acids (Exterkate & Alting, 1995). The presence of free amino acids, apart from bringing nourishing value to cheese, confers them the desired flavour, texture and appearance. The released amino acids indeed are precursors for several amino acids not present in caseins (glutamic acid for α- and γ-amino butyric acid, arginine for ornithine and citrulline), biogenic amines (Urbach, 1993) and for volatile flavour compounds (i.e., aldehydes, ketones, short-chain fatty acids and alcohols). Various authors have reported valine, leucine, lysine and phenylalanine as the most representative free amino acids during the entire ripening period in different hard and semi-hard cheese (Barcina, Ibanez, & Ordonez, 1995; Frau, Massanet, Rossellò, Simal, & Canellas, 1997; Madrau et al., 2006). Lysine is one of the most abundant amino acids at the end of cheese ripening and its determination could provide an estimate of the ripening time and of the nutritional value of a cheese. With regard to this it is important to underline that the cheese-milk compartment is easily subject to the execution of frauds and that therefore it asks for a continuous studying and optimisation of analytical methods for their individualisation. It is often necessary to determine the correspondence of cheese products to how much is declared in the label. This is for example the case of the real age of average-long maturation cheese, manufactured so that it is not possible to reveal the crust. This problem has been examined by Italian researchers (Resmini, Hogenboom, Pazzaglia, & Pellegrino, 1993) that have noticed as during the maturation of cheese the proteolytic activity determines in the time a consistent and progressive increase of the content of free amino acids in the paste.

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During ripening, among all possible biochemical reactions, lactose hydrolysis to reactive reducing carbohydrates (galactose, glucose) also occurs. Accumulation of reactive reducing sugars as well as the formation of free amino acid may create a higher potential for the Maillard reaction. This is a non enzymatic browning or glycation reaction which start with the condensation between carbonyl groups of sugars and amino groups of free amino acids and peptides to yield Amadori products. This reaction can decrease the nutritional value of proteins, especially as concern lysine availability, and may lead to the development of potentially toxic substances (Mottram, Wedzicha, & Dodson, 2002). Available lysine content can hence be used as an indicator of both early and advanced Maillard reaction phases (Ferrer, Alegria, Faire, Abellan, & Romero, 1999).

There have been several studies on lysine loss due to heat treatment and storage of dairy products (Ferrer et al., 2003, Schwietzke, Malinowski, Zerge, & Henle, 2011). The most widely employed technique is High Performance Liquid Chromatography (HPLC) with pre- or post-columns derivatization and ultraviolet or fluorescence detection (Castillo, Sanz, Serrano, Hernandez, & Hernandez, 1997; Izco, Torre, & Barcina, 2000), or coupled to mass spectrometry (Hegele, Buetler, & Delatour, 2008). Lysine assessment in food is always complicated by time consuming methods involving sample pretreatment, analyte derivatization in the case of spectrophotometric detection or by the need of complex and expensive instrumentations. High resolution magic angle spinning NMR spectroscopy has been for example employed to evaluate amino acids profile in ripened cheese (Shintu & Caldarelli, 2005). This technique undoubtedly requires qualified personnel for analysis execution. In comparison to such methods, biosensors constitute an interesting alternative assuring important advantages such as simplicity of operation, low cost and short times of analysis. In this context in the present work an amperometric biosensor has been employed to selectively detect lysine in cheese by flow injection analysis. As previously stressed, lysine determination is indeed of notable relevance in order to assess cheese nourishing value, as it could be used as marker of both ripening period and heat treatment damage. Lysine, moreover, is the limiting essential amino acid in many foodstuffs. Narrowing the field of investigation only to lysine, without evaluating the entire amino acids composition, surely simplifies and shortens the protocol of analysis without lacking in information.

In the laboratory of the authors an immobilization protocol of lysine oxidase (LO) by co-crosslinking onto Pt electrode has been developed (Guerrieri, Cataldi, & Ciriello, 2007), which assured fast response time and notable long term stability. Then, such an enzymatic layer was combined with a permselective overoxidized polypyrrole film (PPy_{ox}), electrosynthetized on Pt electrode before enzyme immobilization, in order to allow an interference free determination of lysine in real matrices like pharmaceutical samples (Guerrieri, Ciriello, & Cataldi, 2013). A separation step from other amino acids was avoided upon optimisation of lysine oxidase recognition towards lysine by properly choosing the experimental conditions (Guerrieri et al., 2013). In the present work the application of such a biosensor has been extended to a detailed analysis of free lysine in cheese. In literature there are examples of biosensors for lysine detection almost in hydrolysate protein samples prepared from various foodstuffs e.g. milk, pasta (Chauhan, Singh, Narang, Dahiya, & Pundir, 2012; Divritsioti, Karalemas, Georgiou, & Papastathopoulos, 2003; Kelly, O'Connell, O'Sullivan, & Guilbault, 2000; Mazzei, Botrè, Favero, Podestà, & Botrè, 2009) whose performances in terms of easiness of electrode modification, stability, linear range and sensitivity were not as encouraging as in the case of the biosensor herein employed (Guerrieri et al., 2013).

2. Experimental

2.1. Materials and reagents

L-lysine and pyrrole were from Aldrich (Aldrich-Chemie, Steinheim, Germany). L-lysine-α-oxidase (EC 1.4.3.14, from Trichoderma viride, 20-40 units per mg protein), glutaraldehyde (grade II, 25% aqueous solution) and bovine albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pyrrole was purified by distillation under vacuum at 52 °C. All other chemicals were of analytical reagent grade. Pyrrole solutions were prepared just before their use. L-lysine stock solutions were stored at 4 °C.

2.2. Apparatus

A Gilson (Gilson Medical Electronics, Villiers-Le-Bel, France) Minipuls 3 peristaltic pump and a seven port injection valve (Rheodyne mod. 7725, Cotati, CA, USA) equipped with a 20 μl sample loop were used for the flow-injection experiments. A PEEK tubing (0.25 mm ID, 150 cm length) was used to connect the sample injection valve to the electrochemical cell. An EG&G Model 400 electrochemical detector (Princeton Applied Research, Princeton, NJ, USA) was used. The detector included a thin-layer electrochemical cell with a dual Pt disk (3 mm diameter) working electrode and an Ag/AgCl, 3 M NaCl reference electrode. The dual electrodes were employed in parallel configuration with respect to running electrolyte flow. Two thin layer flow cell dual gaskets (Bioanalytical Systems, Inc., West Lafayette, IN, USA) of 0.004 inch thickness were used. To record flow injection signals a Kipp & Zonen (Delft BV, Holland) mod. BD 11 E Flatbed Yt recorder was used.

Controlled electrochemical deposition of polypyrrole films was carried out using an EG&G model 263 A potentiostat/galvanostat equipped with a M270 electrochemical research software (EG&G) version 4.23 for data acquisition.

2.3. Biosensor preparation

Each electrode modification has been preceded by a cleaning procedure consisting in dipping the Pt working electrode in hot nitric acid and then in polishing it by alumina (0.05 μm particles) mechanical abrasion, extensive washing and sonication in double distilled water. The electrode was afterwards immersed in a 0.5 M sulphuric acid solution and its potential cycled between −0.255 and +1.225 V versus SCE at 100 mV/s until a steady-state cyclic voltammogram was obtained.

Of the two working electrodes, one was modified by overoxidized polypyrrole and then by lysine oxidase co-crosslinked with Bovine Serum Albumin (BSA) (Pt/PPyox/LO) while the other one by overoxidized polypyrrole and then by only BSA (Pt/PPyox/BSA) according to the following protocol.

The electropolymerization of polypyrrole films (PPy) (Guerrieri et al., 2013) was performed at a constant potential of +0.7 V versus SCE, until a deposition charge of typically 300 mC/cm^2 was achieved, by using a solution of 0.4 M pyrrole in 0.1 M KCl supporting electrolyte. The Pt/PPy modified electrode was overoxidized at +0.7 V versus SCE in a phosphate buffer (pH = 7.0, I = 0.1 M) until a steady-state background current was obtained (at least 7 h). Overoxidised Pt/PPy electrodes were then washed and air-dried at room temperature. Lysine biosensors were prepared by following the procedure elsewhere developed by the authors (Guerrieri et al., 2007). 25 units of lysine oxidase (approximately 1 mg) were dissolved into 250 μL of 0.1 M phosphate buffer, pH 7.4: 50 μL of the enzymatic solution were used to dissolve 2.6 mg of BSA, and

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