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Analytical Methods

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

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ABSTRACT

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.

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

then carefully mixed with 5 μL of 5% glutaraldehyde solution (25% glutaraldehyde solution diluted 1:5 with phosphate buffer). Four to five microliters of the resulting solution were carefully pipetted onto the Pt/PPy_{ox} working electrode surface, avoiding air bubble formation, and carefully spread out to cover the electrode surface completely. The modified electrode was then air-dried at room temperature for few minutes. Pt/PPy_{ox}/LO modified electrodes were preliminarily soaked in the background electrolyte for a few minutes just to removing any weakly bound or adsorbed enzyme and to permit swelling of the enzyme layer. When not in use, the enzyme electrode was stored in phosphate buffer, pH 7.4, I 0.1 M, at 4 °C in the dark. Control modified electrodes (Pt/PPyox/BSA) were prepared in absence of lysine oxidase by dissolving 2.6 mg of BSA in 50 μL of 0.1 M phosphate buffer (pH 7.4) and then following the same scheme just reported for Pt/PPyox/LO.

2.4. Electrochemical measurements

All the electrochemical measurements were performed using a detection potential of +0.7 V versus Ag/AgCl/NaCl (3 M) in 0.1 M acetate buffer (pH 5.0). Solutions and carrier stream were air saturated and the temperature was ambient. The flow rate in flow injection experiments was 0.6 ml/min.

2.5. Samples preparation

Cheese samples, i.e., "Mozzarella" (Pace e Becce, Italy), "Certosa" (Galbani, Italy), "Camembert" (Président, France), "Taleggio" (Brescialat Spa, Italy), "Gorgonzola" (Invernizzi, Italy), "Fontina" (Marienberger Mila, Italy), "Provolone" (Auricchio, Italy), "Pecorino Romano" (Brunelli, Italy), "Pecorino Sardo" (Sepi, Italy), "Pecorino Filiano" (Piaceri Lucani, Italy), Emmenthal (Switzerland), Gruyère (Switzerland), Parmigiano Reggiano (Consorzio del Parmigiano Reggiano, Italy) and "Grana Padano" (Consorzio Tutela Grana Padano, Italy) were obtained from a local grocery store. Cheese samples were grated, dissolved in distilled water (2 g of cheese in 20 ml of water) and sonicated for half an hour (sonication frequency 35 kHz). The suspension was filtered by passage through filter paper (cut-off 16-23 μm), diluted 1:100 with the supporting electrolyte and then injected.

The yoghurt samples analysed, obtained as well from a local grocery store, were: "LC1" from Nestlè (milk 1.1% of fat, 9.3% sugar, *Lactobacillus Johnsonii LA1*, flavourings, coloring E150d, sodium ascorbate, vitamin B6), "Activia" from Danone (skimmed milk fermented with *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Bifidobacterium*), "Mio" from Nestlè (*L. bulgaricus* and *S. thermophilus*, 11% apple puree, sugar, milk minerals concentrate, pectin thickener, xanthan gum, natural flavours, vitamin D, sugar 6%), "Muller" (whole milk and live lactic acid bacteria, sugar, cream (3.6%), grape sugar (1.9%), flavouring), "Parmalat" (yoghurt, sugar, vanilla extract, 1%, thickener: tapioca starch E1442, flavourings), "KYR" from Parmalat (*Lactobacillus delbrueckii var bulgaricus*, *S. thermophilus*, *Lactobacillus acidophilus*, *Bifidobacterium Bb12*, vitamins C, E, D3). The same preparation procedure of cheese was adopted in the case of yoghurt samples.

3. Results and discussion

3.1. Performances of Pt/PPyox/LO biosensor

The amperometric biosensor employed in the present work has been realised electrosynthesizing a permselective film on the electrode surface and therefore immobilizing on the modified electrode L-lysine-α-oxidase by co-crosslinking with bovine serum albumin and glutaraldehyde. The optimisation of enzyme immobilization procedure, carried out by exactly evaluating the concentration values of enzyme, inert protein and co-crosslinker, allowed the realisation of an enzyme layer characterised by high biocomponent stability and good mechanical properties (Guerrieri et al., 2007). The overoxidized polypyrrole film realised on Pt bare electrode, before enzyme immobilization, assured notable rejection characteristics of faradic interferences. In order to improve enzyme specificity towards lysine a careful control of the kinetic behaviour of the device was assured by optimising pH of running electrolyte and flow rate (Guerrieri et al., 2013). Lysine oxidase indeed is known to catalyse in solution the oxidation of other amino acids than lysine to varying extents (Kusakabe et al., 1980). The representative linear part of the calibration curves of Pt/PPy/LO electrodes by flow injection of lysine is presented in Fig. 1. The experimental points and the relevant error bars represent the mean values with the standard deviations of the current signals acquired, for each lysine concentration, on three different biosensors realised in different days on a Pt electrode. The small error bars testify the effectiveness of the biosensor realisation protocol optimised by the authors. Response was linear from 0.02 to 2 mmoL/L (regression equation: $y = (0.377 \pm 0.006) \cdot x + (0.014 \pm 0.006)$, $r = 0.9985$). The detection limit was found to be 4 μmol/L (S/N = 3), which corresponded to 80 pmol of lysine for a 20 μL sample injection.

The amperometric biosensor developed and optimised by the authors proved quite promising in terms of sensitivity, stability and specificity and was therefore employed in the present work for L-lysine detection in food.

3.2. Application to real samples

3.2.1. Method validation

Before discriminating various kinds of cheese depending on the different content of lysine, the reproducibility and therefore the validity of the extraction procedure has been verified. A first screening has been carried out by extracting lysine twice in a day from the following cheese: mozzarella, crescenza, pecorino romano, gorgonzola, emmenthal, provolone and gruyère. Whereas for mozzarella and crescenza no lysine was detected in both extractions, for the other analysed cheese the difference related to the two extractions was not superior to 1–2 μM for concentrations values

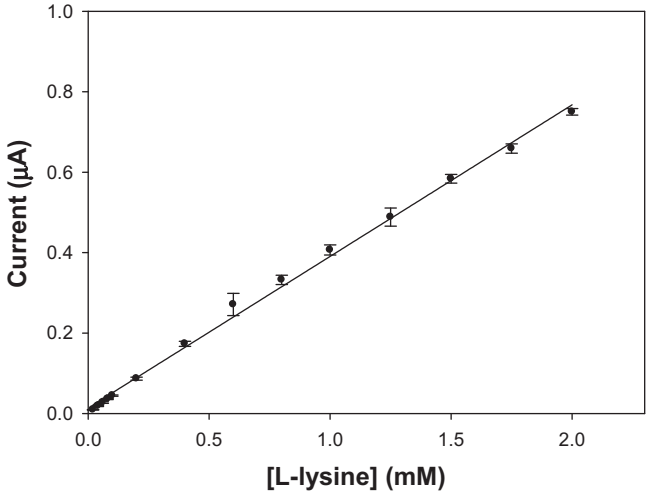

Fig. 1. Linear part of the calibration curve relative to successive injections of lysine standard solutions of increasing concentration at three different Pt/PPYox/LO biosensors. For each lysine concentration the mean value is reported with the standard deviation (error bar). Flow cell: thin layer configuration. Flow rate: 0.6 ml/min. Supporting electrolyte: acetate buffer pH = 5.0, $I = 0.1$ M; injection volume: 20 μl.

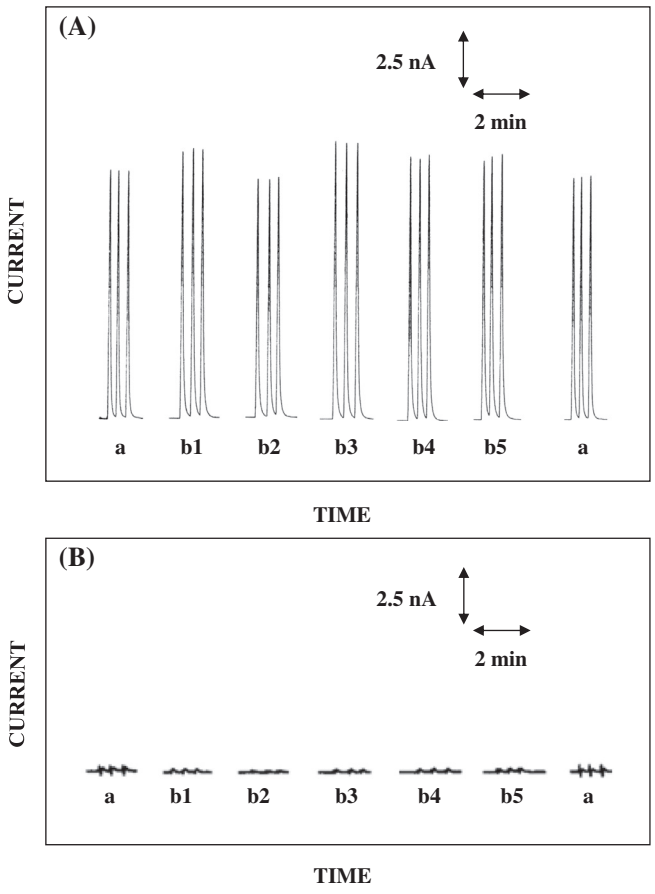

Fig. 2. Typical flow-injection peaks for triplicate injections respectively of L-lysine standard 0.06 mM (A) and five different solutions (b1 ... b5), realised by as many extractions from Parmigiano Reggiano cheese, at Pt/PPyox/LO (A) and at Pt/PPyox/BSA (B) modified electrodes. Flow cell: thin layer configuration with working electrodes parallel with respect to flow direction. Flow rate: 0.6 ml/min. Supporting electrolyte: acetate buffer pH = 5.0, I = 0.1 M; injection volume: 20 μl.

ranging from 9 to 41 μM (see Table S1 of supporting information for detailed values). A systematic study on the reproducibility of the extraction method has then been carried out using Parmigiano Reggiano cheese. Fig. 2A shows the flow injections peaks relevant to five solutions obtained by extracting lysine several times from such cheese at a Pt/PPyox/LO electrode. The current values related to the peaks, converted in terms of concentrations, furnished a mean value and a standard deviation of 0.064 ± 0.003 mM. Similar results were obtained when the experiment was carried out by using Grana Padano cheese (0.065 ± 0.006 mM, n = 5).

In order to confirm the purely enzymatic origin of current signals, the experiment was simultaneously carried out on the control working electrode, Pt/PPyox/BSA, realised without employing lysine oxidase (see the experimental Section 2.3). The relative current signals, depicted in Fig. 2B, are almost indistinguishable from the background noise thus confirming the capability of PPyox to remove any faradic interference. Moreover, considering the selectivity of the enzymatic catalysis towards lysine achieved upon optimisation of the kinetic control of the biosensor (Guerrieri et al., 2013), the signals coming from cheese analysis can be undoubtedly attributed to lysine.

Accuracy of the developed method and interference of matrix components were checked by performing analytical recovery experiments for more than a kind of cheese. Exactly, each matrix was spiked with lysine at the two concentrations level of 50% and then 100% of the lysine concentration previously calculated for that particular cheese. Extractions were performed in triplicate (n = 3). The comparison between the found and the spiked concentrations was done with a Student's t-test, comparing the experimental t value with the tabulated one for $v(n-1) = 2$ and $(1-\alpha) = 0.95$. The corresponding results are listed in Table 1. All experimental t values were lower than t tabulated (4.301) with satisfactory analyte recoveries for every cheese analysed.

3.2.2. Analysis of lysine content in different cheese

Once verified the reproducibility and accuracy of recovery, a detailed study has been carried out in order to detect lysine content in various cheese which differ for both ripening time and applied manufacture technology. Fig. 2 shows, by way of example, the current peaks recorded after triplicate injections of a lysine standard solution and of solutions obtained by extracting lysine from numerous cheese varieties. Also in this case no appreciable current signals were detected on the control working electrode (Pt/PPyox/BSA) (data not shown). The lysine concentration mean values, coming from the injection of four replicates samples, along with the associated standard deviations are listed in Table 2. As milk does not contain an appreciable quantity of free lysine, the different content determined for the various cheese analysed seems to be due to the employment of microorganisms with a more or less marked proteolytic power and to the conditions and the period of ripening.

In the case of fresh cheese (i.e. Mozzarella, peaks (a) in Fig. 3), of soft cheese without crust (i.e. Crescenza, peaks (b)) and with crust (i.e. Camembert, peak (c)) the current signals corresponding to the relevant flow injections peaks are so low that it is impossible to make any appreciable quantification. Indeed, the production of such cheese is based on the employment of starters characterised by a poor proteolytic activity. Moreover the ripening period is

Table 1
Recovery assay of lysine for various cheese with different lysine content.

Cheese	[Lysine] (mM) before spiking	Spiked level (mM)	Total found[c] (mM)	t[d]	Recovery (%)
Emmenthal	0.014	–	–	–	–
Emmenthal + Lys 50%[a]	0.014	0.007	0.020 ± 0.003	0.58	85
Emmenthal + Lys 100%[b]	0.014	0.014	0.027 ± 0.004	0.43	91
Pecorino	0.042	–	–	–	–
Pecorino + Lys 50%[a]	0.042	0.021	0.055 ± 0.005	2.77	84
Pecorino + Lys 100%[b]	0.042	0.042	0.075 ± 0.006	2.60	89
Parmigiano	0.049	–	–	–	–
Parmigiano + Lys 50%[a]	0.049	0.024_5	0.070 ± 0.007	0.87	83
Parmigiano + Lys 100%[b]	0.049	0.049	0.103 ± 0.011	0.79	99

[a] Sample was spiked at the 50% of the concentration of naturally occurring lysine.
[b] Sample was spiked at the 100% of the concentration of naturally occurring lysine.
[c] Total found (average value ± SD of 3 determinations) includes before spiking content plus spiked level.
[d] t = 4.303 for $v(n-1) = 2$ and $1-\alpha = 0.95$.

Table 2
Lysine concentration found in several cheese typologies.

Cheese	Lysine content[a]	
	mM[b]	μmol/g[c]
Mozzarella	n.d.[d]	n.d.
Certosa	n.d.	n.d.
Camembert	n.d.	n.d.
Taleggio	n.d.	n.d.
Gorgonzola	1.8 ± 0.2	18 ± 2
Fontina	0.76 ± 0.08	7.6 ± 0.8
Provolone	2.3 ± 0.3	23 ± 3
Pecorino Romano	3.2 ± 0.7	32 ± 7
Pecorino Sardo	2.0 ± 0.3	20 ± 3
Pecorino Filiano	2.7 ± 0.4	27 ± 4
Emmenthal	2.2 ± 0.4	22 ± 5
Gruyère	3.7 ± 0.6	37 ± 6
Parmigiano Reggiano	6.4 ± 0.3	64 ± 3
Grana Padano	6.5 ± 0.6	65 ± 6

[a] mean ± Standard Deviation of four determinations.
[b] concentrations have been corrected for the dilution factor (1:100).
[c] μmol of lysine/g of cheese.
[d] not detected.

completely absent in the case of Mozzarella whereas in the case of Crescenza it is of 10 days, time insufficient to cause the release of an appreciable quantity of lysine.

Gorgonzola is an internally mould ripened cheese. The free L-lysine content of 18 ± 2 μmol/g of cheese estimated in the case of Gorgonzola is due to the proteolytic capacity of *Penicillium Roqueforti*. A content of free lysine of 7.6 ± 0.8 μmol/g was observed for Fontina whereas in the case of Provolone, a semi-hard paste cheese, lysine concentration is relatively higher and equal to about 23 ± 3 μmol/g. This is due to the quite long ripening period, which is declared not inferior to 6 months for Provolone, and to the intense proteolytic activity of *Lattobacilli caseis*, *brevis* and *cellobiosus*. For the three different kinds of Pecorino cheese analysed, which differ for the place of origin, a content of free lysine of 32 ± 7 μmol/g (Romano Pecorino), 20 ± 3 μmol/g (Sardo Pecorino) and 27 ± 4 μmol/g (Filiano Pecorino) was detected. In order to attribute such differences to a different processing technology, it would be appropriate to exclude any possible variation in the ripening period. More information about the three samples would therefore be necessary. Madrau et al. (2006) evidenced for Pecorino Sardo a lysine content of 256.58 mg/100 g of total solid (circa 18 μmol/g) for 210 days of ripening time, and then not significantly different from the value herein found according to a paired Student's *t*-test evaluated for $1 - \alpha = 0.95$ (t calculated was 1.333, *t* tabulated for $v(n-1) = 3$ was 3.182).

Interestingly, Emmenthal and Gruyère, both belonging to the same class of cheese and similarly produced, gave lysine contents significantly different (22 ± 5 μmol/g for Emmenthal and 37 ± 6 μmol/g for Gruyère). This difference could be attributed to some peculiarities in the related production. The Gruyère cheese suffers a greater salting and its maturation happens at inferior temperatures in comparison to Emmenthal cheese. The crust of Gruyère is continually humidified allowing the growth of microorganisms such as *Micrococcus*, *Staphilococcus* and *Brevibacterium linens* which accelerate the maturation unlike the Emmenthal crust that remains almost dry. The lysine content found in Emmenthal is not significantly different from the value of 29 μmol/g reported by Casella, Gatta, and Cataldi (2000) employing liquid chromatography, according to a paired Student's *t*-test evaluated for $1 - \alpha = 0.95$ (*t* calculated was 2.8, *t* tabulated for $v(n-1) = 3$ was 3.182).

Among cheese showing a high lysine content is, as to expect, Parmigiano Reggiano. Samples of such cheese of 24 months ripening time showed a lysine content of 64 ± 3 μmol/g. The employment of *Pediococchi*, microorganisms characterised by a strong proteolytic activity, in the manufacture technology justifies the high lysine content found in the case of Parmigiano Reggiano. It is important to outline that the ripening time for cheese like Parmigiano Reggiano and Grana Padano is the longest one. A lysine content of circa 65 μmol/g (spring production season) and circa 66 μmol/g (winter production season) comes from values reported by Careri, Spagnoli, Panari, Zannoni, and Barbieri (1996). In that case a 24 months ripened Parmigiano Reggiano cheese was analysed by using reversed phase liquid chromatography equipped with a fluorescence detector. The authors have noticed a significant difference in samples produced during autumn and summer seasons.

3.2.3. Influence of the ripening time on the lysine content

Parmigiano Reggiano and Grana Padano are extra-hard, long ripened cheese made in Northen Italy from raw cows' milk and registered at European Union level as Protected Designation of Origin (PDO) cheese in accordance with Council regulation (2006). After a careful inspection, cheese wheel are marked with a quality label thus guaranteeing conformity with the commercial standard. The economic importance of such cheese has stimulated many studies on their composition in order to establish the nutritional properties, but also to define chemical and physical parameters for an objective evaluation of their standard features and quality (Cattaneo et al., 2008). Among cheese authenticity issues, assessment of ripening time has become of great interest. Cheese

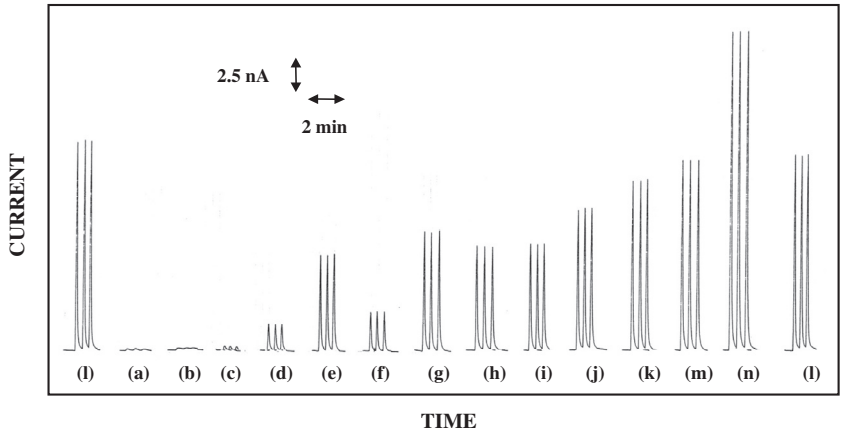

Fig. 3. Typical flow-injection peaks for triplicate injections respectively of L-lysine standard 0.04 mM (l), Mozzarella (a), Crescenza (b), Camemberth (c), Taleggio (d), Gorgonzola (e), Fontina (f), Provolone (g), Emmenthal (h), Pecorino Sardo (i), Pecorino Filiano (j), Pecorino Romano (k), Gruviera (m), Parmigiano Reggiano (n) at a Pt/PPyox/LO biosensor. Flow cell: thin layer configuration. Flow rate: 0.6 ml/min. Supporting electrolyte: acetate buffer pH = 5.0, *I* = 0.1 M; injection volume: 20 μl.

wheel are marked after 9 months of ripening in the case of Grana Padano and 12 months in the case of Parmigiano Reggiano, which is generally submitted to a longer maturation period. Even if the minimum time is 12 months, additional value is placed on cheese aged over 18 (red seal), 22 (silver seal) and 30 months (gold seal). Parmigiano Reggiano fully matures at around 24 months producing its best nutritional values, taste and aroma. The product specification of Grana Padano instead has only recently introduced 2 new categories: "GP ripened over 16 months" and "GP Riserva", ripened over 20 months.

The influence of the ripening period on the content of free lysine in Parmigiano Reggiano and Grana Padano has been analysed by the present biosensor. The samples investigated were 24 and 36 months ripened Parmigiano Reggiano and 18 and 24 months ripened Grana Padano. The choice of the ripening period was dictated by the commercial availability of cheese samples. Fig. 4 shows the current peaks relevant to the injections of such samples at a Pt/PPy_{ox}/LO modified electrode. Both cheeses evidenced an high lysine content for longer ripening period (+38% for Grana Padano, +32% for Parmigiano Reggiano). The reproducibility of data showed in Fig. 4 has been tested by duplicating the experiment using different samples of Parmigiano Reggiano and Grana Padano, at the ripening months above specified. The entity of lysine increase has been confirmed just in the case of Parmigiano Reggiano (+33%). For Grana Padano a less increment of lysine was detected (+16%). Resmini et al. (1993) found great differences in free amino acids levels among patterns of Grana Padano sample with the same ripening, whereas in the case of Parmigiano Reggiano a high reproducibility was observed at least in the first 12–15 months. It was therefore possible to state Parmigiano Reggiano age with a maximum error of ±1.5 months by deriving linear regression functions for individual free amino acids (Resmini, Pellegrino, Pazzaglia, & Hogenboom, 1985). In both cases the release of free amino acids flattens with ripening time. As an example, the accumulation rate of free amino acids in Grana Padano is rapid during the first 10 months (18.3% on cheese protein) while subsequently their content increases slightly by passing from 13–14 (19.6%) to 19–20 (22.16%) months of ripening (Resmini et al., 1993). Similar results were showed by Masotti, Hogenboom, Rosi, De Noni, and Pellegrino (2010) who evidenced a rapid increase only during the first 12 months of ripening. Also

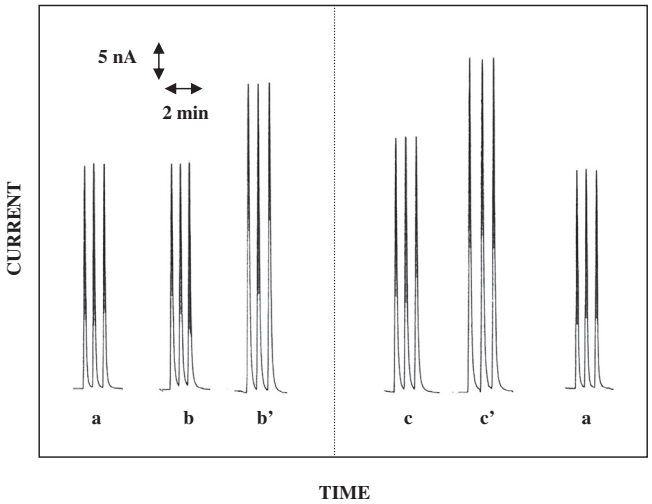

Fig. 4. Typical flow-injection peaks for triplicate injections respectively of L-lysine standard 0.08 mM (a) 18 months (b) and 24 months ripened Grana Padano (b′), 24 months and 36 months ripened Parmigiano Reggiano (c′) at a Pt/PPyox/LO biosensor. Flow cell: thin layer configuration. Flow rate: 0.6 ml/min. Supporting electrolyte: acetate buffer pH = 5.0, I = 0.1 M; injection volume: 20 μl.

in the case of Parmigiano Reggiano, after a certain period, the amount of free amino acids levels off with time whereas the "relative" content of some amino acids continues to vary significantly (Resmini et al., 1985). Such a period is generally longer for Parmigiano Reggiano than for Grana Padano. This could be justified considering that after 13 months of ripening the total content of free amino acids is equal to the 90% of the maximum value for Grana Padano while the corresponding value reached in Parmigiano Reggiano is 75%. We have to consider, however, that 9 and 12 months are the maturation periods certifying DOP denomination and that in this period the increase and therefore the variation of free amino acid is notable. In light of such consideration, the analysis of lysine with the present method could identify products with a maturation period inferior to the minimum one necessary to confer them the protected designation.

It is important to point out that differences in amino acids contents may be due not only to the different ripening time but also to other important factors such as farm, season and orographic zone of production. Therefore, at a parity of ripening time, cheese samples produced in different seasons can show significant differences with regard to free amino acids content such as aspartic and glutamic acids, serine, arginine and lysine (Careri et al., 1996). The results herein showed are therefore only preliminary and need a more systematic investigation in order to exclude the influence of other parameters on the increase of lysine content with ripening time.

3.2.4. Analysis of yoghurt samples

The so called fermented milks are dairy products obtained after milk coagulation by microorganisms of the acidic or acid-alcoholic fermentation. Milk is homogenised, pasteurized, assembled up to the 14% of the dry residue (to confer to the product a certain consistence) and inoculated with lactic bacteria for some hours, until a pH value of 4 is reached. The obtained product can be added of fresh or frozen fruit (with addition of saccharose). The fermented milks can be grouped in two great categories: the acidic milks also known as yogurt; the acid-alcoholic milk also known as kefir. In the present work yogurts of different brands have been analysed hypothesising the presence of an appreciable quantity of free amino acids because of the low pH characterising such products. The yoghurt samples have been submitted to the same pretreatment adopted in the case of cheese. From the flow injections peaks relevant to the yogurt varieties analysed it was no possible to determine an appreciable content of free lysine (see supporting information, Fig. S1). Indeed this result can be explained if we consider that in the yoghurt production lactic ferments such as *S. thermophilus* and *L. bulgaricus* are used which are characterised by an high acidic power but by a reduced or completely absent proteolytic power. The principal action of such bacteria is to turn lactose into lactic acid, which is the major end product of lactic acid bacteria. Our finding is in agreement with Izco, Tormo, and Jimenez-Flores (2002) who evidenced no lysine content in yougurt samples by capillary electrophoresis with DAD detector.

4. Conclusions

The production of cheese specialties with traditional manufacturing and informative labelling is quite expensive and therefore requires protection against cheaper industrial imitation products. As it was previously stressed, the content of free amino acids in cheese is tightly connected to the technology of cheese processing and is enormously influenced by the times and the conditions of maturation.

The aim of the present work was to propose a new approach for the detection of free lysine in cheese by an amperometric biosensor. The method revealed rapid and precise with a minimal

sample pretreatment and a satisfactory recovery of the analyte. The different lysine contents found for various cheese typologies has been justified in terms of the more or less pronounced proteolytic activity associated to the particular production technology and maturation period. For some cheese a significant agreement has been found, at a confidence level of 95%, with lysine content elsewhere reported by employing different methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.07.141.

References

Barcina, Y., Ibanez, F. C., & Ordonez, A. I. (1995). Evolution of free amino acids during Idiazábal cheese ripening. *Food Control, 6*(3), 161–164.

Careri, M., Spagnoli, S., Panari, G., Zannoni, M., & Barbieri, G. (1996). Chemical parameters of the non-volatile fraction of ripened Parmigiano-Reggiano cheese. *International Dairy Journal, 6*, 147–155.

Casella, I. G., Gatta, M., & Cataldi, T. R. I. (2000). Amperometric determination of underivatized amino acids at a nickel-modified gold electrode by anion-exchange chromatography. *Journal of Chromatography A, 878*, 57–67.

Castillo, G., Sanz, M. A., Serrano, M. A., Hernandez, T., & Hernandez, T. (1997). An isocratic high performance liquid chromatographic method for determining the available lysine in foods. *Journal of Chromatographic Science, 35*, 423–429.

Cattaneo, S., Hogenboom, J. A., Masotti, F., Rosi, V., Pellegrino, L., & Resmini, P. (2008). Grated Grana Padano cheese: New hints on how to control quality and recognize imitations. *Dairy Science & Technology, 88*(4/5), 595–605.

Chauhan, N., Singh, A., Narang, J., Dahiya, S., & Pundir, C. S. (2012). Development of amperometric lysine biosensors based on Au nanoparticles/multiwalled carbon nanotubes/polymers modified Au electrodes. *Analyst, 137*, 5113–5122.

Council Regulation (EC) No 510/2006 of 20 March 2006 (2006). On the protection of geographical indications and designations of origin for agricultural products and foodstuffs. *Official Journal of the European Union of 31.3.2006, L 93*, 12–25.

Divritsioti, M. H., Karalemas, I. D., Georgiou, C. A., & Papastathopoulos, D. S. (2003). Flow injection analysis system for L-lysine estimation in foodstuffs using a biosensor based on lysine oxidase immobilization on a gold-poly(m-phenylenediamine) electrode. *Analytical Letters, 36*(9), 1939–1963.

Exterkate, F. A., & Alting, A. C. (1995). The role of starter peptidases in the initial proteolytic events leading to amino acids in Gouda cheese. *International Dairy Journal, 5*, 15–28.

Ferrer, E., Alegria, A., Faire, R., Abellan, P., & Romero, F. (1999). Review: Indicators of damage of protein quality and nutritional value of milk. *Food Science and Technology International, 5*(6), 447–461.

Ferrer, E., Alegria, A., Faire, R., Abellan, P., Romero, F., & Clemente, G. (2003). Evolution of available lysine and furosine contents in milk-based infant formulas throught the shelf-life storage period. *Journal of the Science of Food and Agricultural, 83*, 465–472.

Frau, M., Massanet, J., Rossellò, C., Simal, S., & Canellas, J. (1997). Evolution of free amino acid content during ripening of Mahan cheese. *Food Chemistry, 60*(4), 651–657.

Guerrieri, A., Cataldi, T. R. I., & Ciriello, R. (2007). The kinetic and analytical behaviours o fan L-lysine amperometric biosensor based on lysine oxidase immobilized onto a platinum electrode by co-crosslinking. *Sensors and Actuators B, 126*, 424–430.

Guerrieri, A., Ciriello, R., & Cataldi, T. R. I. (2013). A novel amperometric biosensor based on a co-crosslinked L-lysine-α-oxidase/overoxidized polypyrrole bilayer for the highly selective determination of L-lysine. *Analitica Chimica Acta, 795*, 52–59.

Hegele, J., Buetler, T., & Delatour, T. (2008). Comparative LC–MS/MS profiling of free and protein bound early and advanced glycation induced lysine modifications in dairy products. *Analytica Chimica Acta, 617*, 85–96.

Izco, J. M., Torre, P., & Barcina, Y. (2000). Ripening of Ossau-Iraty cheese: Determination of free amino acids by RP-HPLC and of total free amino acids by the TNBS method. *Food Control, 11*, 7–11.

Izco, J. M., Tormo, M., & Jimenez-Flores, R. (2002). Rapid simultaneous determination of organic acids, free amino acids, and lactose in cheese by capillary electrophoresis. *Journal of Dairy Science, 85*, 2122–2129.

Kelly, S. C., O'Connell, P. J., O'Sullivan, C. K., & Guilbault, G. G. (2000). Development of an interferent free amperometric biosensor for determination of L-lysine in food. *Analytica Chimica Acta, 412*, 111–119.

Kusakabe, H., Kodama, K., Kuninaka, A., Yoshino, H., Misono, H., & Soda, K. (1980). A new antitumor enzyme, L-lysine-α-oxidase from *Trichoderma viride*: Purification and enzymological properties. *Journal of Biological Chemistry, 255*, 976–981.

Madrau, M. A., Mangia, N. P., Murgia, M. A., Sanna, M. G., Garau, G., Leccis, L., et al. (2006). Employment of autochthonous microflora in Pecorino Sardo cheese manufacturing and evolution of physicochemical parameters during ripening. *International Dairy Journal, 16*, 876–885.

Masotti, F., Hogenboom, J. A., Rosi, V., De Noni, I., & Pellegrino, L. (2010). Proteolysis indices related to cheese ripening and typicalness in PDO Grana Padano cheese. *International Dairy Journal, 20*, 352–359.

Mazzei, F., Botrè, F., Favero, G., Podestà, E., & Botrè, C. (2009). Partially disposable biosensor for the quick assessment of damage in foodstuff after thermal treatment. *Microchemical Journal, 91*, 209–213.

Mottram, D. S., Wedzicha, B. L., & Dodson, A. T. (2002). Acrylamide is formed in the Maillard reaction. *Nature, 419*, 448.

Resmini, P., Pellegrino, L., Pazzaglia, C., & Hogenboom, J. A. (1985). Free amino acids in the quality control of Parmigiano-Reggiano cheese and particularly grated cheese. *Scienza e tecnica lattiero-casearia, 36*(6), 557–592.

Resmini, P., Hogenboom, J. A., Pazzaglia, C., & Pellegrino, L. (1993). Gli amminoacidi liberi nella caratterizzazione analitica del formaggio Grana Padano. *Scienza e tecnica lattiero-casearia, 44*(1), 7–19.

Schwietzke, U., Malinowski, J., Zerge, K., & Henle, T. (2011). Quantification of Amadori product in cheese. *European Food Research and Technology, 233*(2), 243–251.

Shintu, L., & Caldarelli, S. (2005). High-resolution MAS NMR and chemometrics: Characterization of the ripening of Parmigiano Reggiano cheese. *Journal of Agricoltural and Food Chemistry, 53*, 4026–4031.

Urbach, G. (1993). Relations between cheese flavour and chemical composition. *International Dairy Journal, 3*, 389–422.