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**Selective determination of lysine in dry-cured meats using a sensor
based on the chemistry immobilization of the lysine- α -oxidase on a
nylon membrane**

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Abstract

An enzymatic sensor employing lysine oxidase (LOx) with the immobilized enzyme system by crosslinking with glutaraldehyde using an immunodyne ABC nylon membrane, in combination with an oxygen electrode has been optimised to determine the lysine content in dry-cured ham and dry-fermented sausage at different cured times. The amperometric signal obtained due to the oxygen depletion (consumed oxygen) during the lysine oxidation was recorded at 200 s in the immobilised enzyme sensor at 5 s and the reaction rates (slope) were related to the lysine content. A linear relationship between the consumed oxygen as a function of time ($\text{mg O}_2/\text{L}\cdot\text{s}^{-1}$) and the lysine concentration in the range 10-250 μM ($R^2=0.9946$) for the immobilised enzyme system were found. The immobilised enzyme sensor showed a high specificity and sensibility. Nevertheless, the stability of the immobilized enzyme at the assay temperature was very poor and thus a new membrane was required for each analysis. The analysis of lysine with the immobilized enzyme system in cured meat samples revealed very good agreement with the determination performed through standard HPLC methodology, which validated the use of this sensor as an alternative technique to evaluate cured meat quality.

Keywords: Immobilized enzyme system, Lysine, L-lysine- α -oxidase, dry-cured ham, dry-fermented sausages.

1. Introduction

Large amounts of amino acids are generated during the proteolysis taking place in the processing of dry-cured meat products. This high generation of free amino acids is partially responsible for the typical and desirable sensory characteristics of this type of products (Toldrá, Aristoy, and Flores, 2000). Lysine is the amino acids generated in largest amount and thus, it could be used as a marker of quality in this type of food. Therefore, the analysis of the lysine content has been proposed as a rapid and simple method to determine the process time of cured meat products. In fact, liquid chromatographic methods have been traditionally used for the analysis of lysine in foods. In this sense, the use of biosensors as an alternative to conventional methods is being used in the food industry, for example for the analysis of histamine in prawns and fish samples (Keow, Abu Bakar, Salleh, Heng, Wagiran, Sidiquee, 2012; Pérez, Bartolí, Fábregas, 2012). The analysis of hypoxanthine, using an enzyme sensor with xanthine oxidase, was useful to control the time of meat ageing (Hernández-Cázares, Aristoy, Toldrá, 2010) or even the time of processing in dry-cured meats (Hernández-Cázares, Aristoy, Toldrá, 2011). Lysine has been analysed in several foods like cheese and yoghurt (Ciriello et al., 2015), as well as in hydrolysate food like pasta and wheat flour (Curulli et al., 1998; Divritsioti et al., 2003; Chauhan et al., 2012). The use of lysine decarboxylase combined with diamine oxidase and the horseradish peroxidase has been proposed for the analysis of free lysine in pet food (Bóka et al., 2015). In this context, the analysis of lysine in cured meat through the use of a biosensor could be a useful rapid method of process control.

The L-lysine- α -oxidase (LOx) catalyzes the oxidation of lysine in the presence of oxygen forming α -keto- ϵ -aminocaproate, hydrogen peroxide, and ammonia, according to the following scheme (Kusakabe et al., 1980):

This reaction can be monitored by measuring either the consumption of oxygen at negative potential (-500 to -700 mV) or the generated hydrogen peroxide at positive potential (600 – 650 mV). However high operation potential can cause oxidation of other electroactive compounds in real samples. The specificity can be improved by lowering the detection potential (Karalemas, Constantinos, and Papastathopoulos, 2000; Kelly, O'Connell, O'Sullivan, and Guilbault, 2000; Ricci et al., 2003). The enzyme used for the reaction can be free in solution but, most often, it is immobilised in a matrix for a better economic yield (Saurina, Hernández-Cassou, Fábregas, and Alegret, 1998; Guerrieri, Cataldi, Ciriello, 2007; Sahin, Gulce, and Gulce, 2012).

The main objective of this study is to evaluate the use of the lysine- α -oxidase (LOx) using a sensor to quantify the lysine in meat samples at different curing times. The immobilized enzyme system, have been optimized and evaluated for this use.

2. Materials and methods

2.1. Chemical and reagents

Lysine oxidase (LOx, E.C.1.4.3.14 from trichoderma viride, each vial contains 4.6 mg of protein with 20-60 units/mg protein), L-Lysine, L-phenylalanine, L-ornithine, L-arginine, tyrosine, L-histidine, L-leucine, glutaraldehyde (50%), *o*-phthaldialdehyde and 2-mercaptopropionic acid were from Sigma (Sigma-Aldrich, St Louis, MO, USA). Methanol, acetonitrile and tetrahydrofurane, grade HPLC were from Sigma-Aldrich.

123 Hydrochloric acid (37%), sodium phosphate, sodium acetate anhydrous, sodium
124 hydroxide, glacial acetic acid and acetone were from Panreac (Panreac Química,
125 Barcelona, Spain). Bidistilled milli-Q water was used throughout.

126 The preactivated immunodyne ABC membrane (Nylon 66, pore size 0.45 μm) was
127 supplied by Pall Europe (Porsmounth, United Kingdom).

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129 2.2. Preparation of the LOx solution

130 Commercial enzyme reagent (4.6 mg) was dissolved in 25 ml of 0.05 M
131 potasium phosphate buffer, pH 7.5, with an activity of 6.8 U/mL; aliquoted in 0.5 mL
132 eppendorf tubes and frozen stored at -80°C until use.

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134 2.3. Equipment

135 The enzymatic sensor consists of a platinum electrode (Rank Brothers,
136 Bottisham, Cambridge, England) upon which was used immobilised enzyme. The
137 electrode consists of a central 2 mm diameter platinum disk (working electrode), with a
138 surrounding silver ring (Ag/AgCl, reference electrode), a thermostated reaction cell and
139 an oxygen permeable Teflon membrane (12.7 μm). The current output was recorded by
140 a datalogger ADC-16 (Tip Technology Limited, St. Net. Cambridgeshire, United
141 Kingdom) and displayed as oxygen consumed (mg/L) during the Lys oxidation reaction.
142 The chromatographic analysis was performed in a 1100 Agilent liquid chromatograph
143 (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler and a
144 fluorescence detector.

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2.4. Sample preparation

Samples of muscle (*Biceps femoris*) from pork ham at different dry-curing times (0, 2, 5.5, 5, 6.5, 9, 10 months) and from dry-fermented sausage at different processing times (0, 15, 35, and 66 days) were obtained from a local dry-cured meat products industry. Ground sample (5 g) was homogenized with 0.01M hydrochloric acid (25 ml) for 8 min at 4 °C in a stomacher (IUL Instrument, Barcelona Spain). The homogenate was centrifuged at 10,000g at 4 °C and the supernatant filtered through glass wool. One volume of the extract was mixed with 2 volumes of acetonitrile, left to stand for 1 hour and centrifuged for 5 min again. The supernatant was used for the analysis of lysine by HPLC and by the enzyme sensor.

2.5. Biosensor preparation and mode of operation

A damp paper tissue with 3 M KCl covering the sensor electrodes to let their electrical communication and an oxygen permeable Teflon membrane over it was placed as described in Hernández-Cázares et al (2010). In this way, the electrochemical oxidation occurs in the platinum cathode with a potential of polarization fixed at -600 mV with respect to the Ag/AgCl reference electrode. The sensitivity of the equipment was adjusted by filling the reaction cell with bidistilled water saturated with oxygen, under stirring and the recorder corresponded to 100% of initial oxygen in the reaction media. The whole process takes about 1 minute.

2.5.1 Immobilised enzyme sensor

The immobilization process was based on that described in Hernández-Cázares et al. (2010) for xanthine oxidase but using 6 %, glutaraldehyde instead of 2%. Thus, a mixture (total of 50 µl) containing 15 µl of LOx solution (0.1U), 29 µl of 50 mM

sodium phosphate buffer, pH 7.6 and 6 μ l of 50 % glutaraldehyde solution, was placed drop by drop on a immunodyne ABC membrane (1 cm²) and then dried under air for 1 h in order to initiate the cross-linking. Once it was dried, interferences coming from matrix of ham were prevented by adding 5 μ l of 2% cellulose acetate in acetone solution (Qiong et al., 1998). The LOx membrane was attached onto the Teflon membrane in the oxygen electrode by an o-ring and clamped to the thermostated reaction cell.

After each analysis, the lysine membrane was replaced by another membrane and 3 min were left to reach the equilibrium before a new injection. The enzymatic rate ($\text{mg O}_2 \cdot \text{L}^{-1} \cdot \text{s}^{-1}$) value at 5 s was taken and extrapolated in the calibration curve to estimate the lysine content in meat samples. To build the calibration curve, lysine concentrations (0.01, 0.03, 0.05, 0.07, 0.1 and 0.25 mM) were used.

2.6. Actuating Mechanism of the lysine- α -oxidase onto lysine

In another study the effect of the lysine- α -oxidase on lysine was observed. In the presence of LOx 1500 μ L of the enzyme solution (10.2 U) plus 1500 μ l of a lysine standard solution 10 mM (1mM in the reactor) or meat extract (dilution 1/5) were brought to 15mL of potassium phosphate buffer, pH 7.6, 0.05 M and left to react at 37 °C under gently stirring. Every 40 seconds, 900 μ l aliquot was taken and immediately mixed with 100 μ l of hydrochloric acid (37%) to stop the reaction. Aliquots were centrifuged (12,000 rpm for 5 min at 4°C) and analysed by HPLC.

2.7. Description of the chromatographic method

The chromatographic analysis of the lysine content in meat products extracts and in the model systems was performed by after OPA derivatization. One micro liter of

sample was automatically derivatized RP-HPLC in the autosampler with OPA-mercaptopropionic acid as described in (Schuster 1988) with some modifications. Thus, 1 µL of sample was mixed with 5 µL of OPA reagent (50 mg OPA in 1 mL methanol plus 50 µL of 2-mercaptopropionic acid and taken to 10 mL with 0.04 M borate buffer, pH 10.4), wait for 2 min and injected in a Hypersil C18, (3.0 x 150) mm and 3 µm particle size, column (Agilent Technologies, Palo Alto, CA, USA) maintained at 45 °C. The separation was achieved by means of a gradient between two solvents: 20 mM sodium acetate buffer, pH 7.2 as solvent A, and 100 mM sodium acetate, pH 7.2: acetonitrile: methanol (20:40:40) (v:v) as solvent B. The gradient was linear from 0% to 60% of solvent B in 10 min and the flow rate was 1 mL*min⁻¹. Fluorescence detection at excitation and emission wavelengths of 230 nm and 455 nm, respectively were used. The quantification was performed by means of a calibration curve between 0.01 mM and 0.15 mM lysine.

2.8. Assay of validation

The linearity was obtained from a triplicate analysis of their respective standard concentration intervals: 0.01-0.25 mM for the immobilised enzyme system and applying the least- squares method. Dilute sample is necessary to avoid saturating the capacity of the enzyme and that the signal enter the range. Repeatability was assayed by 25 consecutive injections of 1 mM lysine standard or samples of meat products and analysed on the same day with the same equipment under the same conditions in the soluble enzyme sensor. In the immobilised enzyme sensor 0.1 mM lysine standard or meat extract were analyzed in the same day, but using a new membrane each time. The overall mean concentration and the coefficient of variation (%) were calculated.

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223 The stability of the membrane LOx immobilized, stored a different temperatures, was
224 evaluated.

225 For the stability under operation conditions, successive injections of 0.1 mM lysine
226 were made in a same membrane and the response recorded until a 50 % loss in the
227 activity was observed. For the storage stability several LOx membranes were
228 maintained at 4, 10, 20, 30, and 37 °C and then enzyme activity assayed as usually one
229 time for each membrane. The results were compared by plotting their response against
230 time for each temperature .

231 The correlation between samples (extracts meat) in different dilutions, were measures
232 with the sensor and HPLC.

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234 2.9. Specificity of the assay

235 The degree of specificity of LOx towards the lysine with respect to other amino
236 acids was studied by determining the relative rate of activity against each one. The
237 amino acids used were phenylalanine, histidine, arginine, ornithine, leucine and tyrosine
238 and were assayed by directly injection of 2.000 µL of each amino acid (0.01 mg/mL) in
239 the reaction chamber at 37°C onto the membrane. The rate of the consumed oxygen
240 ($\text{mgO}_2 \cdot \text{L}^{-1} \cdot \text{s}^{-1}$) in the oxidation of each amino acid was compared as a percentage of the
241 rate with lysine as a control (100%). The analysis was also assayed for each amino acid
242 with the HPLC method.

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248 2.10. Application of enzymatic sensor for analyzing samples of ham and dry-fermented
249 sausage

250 The application of the enzymatic sensor for the analysis of dry-cured ham samples was
251 evaluated by determining the content of lysine in the samples of muscle (*Biceps*
252 *femoris*) at different curing times (0,2,3.5,5,6.5,9,10 months). Similarly lysine content
253 was determined in dry-fermented sausage during the fermentation to (0,15,35, and 65
254 days). The method was validated by comparing the results with those obtained on the
255 same samples by HPLC.

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257 2.11. Statistical method

258 Linear correlation analysis between the lysine content measured by the enzyme
259 sensor and HPLC was carried out and the statistical analysis done by using Statgraphics
260 Plus (v5.1) software.

261

262 **3.Results and Discussion**

263 **3.1 Biosensor Optimisation**

264 The lysine biosensor is based on the immobilisation on a preactivated nylon
265 membrane by crosslinking with glutaraldehyde. The relevant parameters were
266 optimized and applied as follows.

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268 3.1.1 Effect of pH and Temperature

269 The effect of pH and the temperature on the biosensor response was studied. The pH
270 studied was in the range of 5.6-8.6 using phosphate buffer pH 7.6. Different
271 temperatures were studied (25, 27, 30, 37 and 43 °C) and the optimal temperature was
272 found at 37° C (Data not shown).

3.1.2 Optimal amount of enzyme

The amount of enzyme used in the immobilization process was optimized using 5, 7, 15 and 20 μL of the enzyme solution which contained 0.034, 0.047, 0.102 and 0.136 respectively U. Optimal activity was observed when taking 15 μL (0.102 U) of the enzyme solution. The amount of protein contained in 15 μL was of 0.00276 mg.

3.1.3 Optimal concentration of glutaraldehyde

Glutaraldehyde was chosen as the immobilising agent. However, high amounts of glutaraldehyde may modify the amino acid and thus affect in more or less extent the enzyme activity. This type of immobilization may produce losses in sensitivity and stability after use (Karalemas et al., 2000), and for this reason, it is important to determine the adequate quantity and concentration of glutaraldehyde to get the best response of the biosensor (Saurina et al., 1998; Betancor, López-Gallego, Hidalgo, Alonso-Morales, Dellamora-Ortiz, Mateo, Fernández-Lafuente, Guisán, 2006). So, different concentrations of glutaraldehyde ranging 0.15 to 8% were tested and the best response was observed when using 6% for a given LOx concentration fixed at 0.1 U (data not shown). Under such conditions, the specificity of the immobilised enzyme was improved. In fact, the apparent Michaelis-Menten constant ($K_{\text{M (app)}}$) of the immobilised enzyme system using the optimised assay conditions was 0.36 mM indicating a good affinity of the enzyme for the substrate. Similar values (333.33 μM) have been reported (Chauhan et al., 2013).

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Therefore it is important to use preactivated membranes that are incorporating amino groups in one molecule of glutaraldehyde (monomers), or two molecules of glutaraldehyde (dimers) per amino group. The use of nylon membranes as a support to immobilize LOx has been reported (Vrbová and Marek 1992; Saurina et al., 1999).

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299 3.2 Biosensor Characteristics

300 A typical calibration curve, obtained for repeated injections of increasing
301 concentrations of standard lysine solutions with the lysine biosensor under optimal
302 working conditions is shown in figure 1. The limit of detection for the immobilised
303 enzyme sensor was 10 μM confirming the high sensitivity of this system. The linear
304 measuring range was 10-250 μM ($R^2=0.9946$). Larger ranges were not possible probably
305 due to the high concentrations of lysine that may inhibit the enzyme by excess of
306 substrate.

307 The operational stability of the biosensor, tested by repeated measurements of 0.1 mM
308 lysine standard solution and meat samples as substrates, showed good values
309 confirming a valid response within the day, as well as low interferences from the
310 matrix. The values of the coefficients of variation of their responses were 7.6% for
311 lysine standard solutions and 8.99% for meat. The storage stability of the developed
312 lysine sensor was studied as well. The freshly prepared enzyme electrodes could be
313 stored in the refrigerator (4°C) for 7 days with only 10% loss of activity.
314 However, the operational stability of the immobilised enzyme was strongly dependent
315 on the temperature of use. Thus, at the temperature of the assay (37°C), the stability was
316 very low and forced to change the membrane before each new injection.

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318 3.3 Biosensor Selectivity

319 The specificity for lysine of this lysine oxidase sensor and the possible
320 interference of other amino acids were investigated because all these free amino acids
321 are present in meat products samples and, in general, in any protein food. These
322 interferences were investigated by other authors and phenylalanine, arginine, ornithine,

and tyrosine were reported as the most interfering amino acids (Saurina, et al 1998; Kelly et al, 2000; Matsuda, Asano, 2010; Guerrieri, Ciriello, and Cataldi, 2013). To overcome the poor enzyme specificity, some approaches were proposed like lowering the temperature of reaction, finely tuning the experimental parameters (Kelly et al., 2000; Guerrieri et al., 2013) or changing the enzyme source (Kelly et al., 2000). The oxidation rates of these amino acids, assayed individually with the soluble enzyme at 37 °C were evaluated by means of HPLC. The results indicate that arginine and phenylalanine are the most potential interfering amino acids, although the other assayed amino acids also gave a significant response as shown in Table 1. The test was also performed with the immobilised enzyme sensor and the obtained results were something different (Table 1) because in this case, only phenylalanine and ornithine gave some response. Apparently, all or some of these amino acids could produce some degree of interfering effect in the quantification of lysine but, in fact, only small if any variations in the response were observed when phenylalanine was added in equimolar amounts to 0.1 mM lysine solution (data not shown). Such result was explained by the higher affinity of the enzyme for lysine than for the rest of amino acids and confirms the fact that, in their presence, lysine will be degraded almost selectively (Saurina et al., 1998; Lukasheva, and Berezov, 2002).

3.4 Actuating Mechanism of the lysine- α -oxidase onto lysine

During the enzymatic oxidation of lysine the disappearance of the same occurs, this has been monitored with HPLC. The disappearance of lysine due to the oxidation reaction at 37 °C was analyzed during 10 minutes, time in which all substrate disappeared, using an initial lysine concentration of 0.1 mM (Figure 2A). The same

experience was repeated with a sample of 10 months dry-cured ham extract that was diluted to a content of 0.1 mM of lysine. The rate of lysine disappearance was very similar indicating a good catalytic affinity of the LOx to lysine in real cured meat samples with apparently very low interferences even though the response of the enzyme in the sensor system is influenced by the operational conditions (Figure 2B). (Endo, Hayashi, Kitani, Ren, Hayashi, Nagashima, 2008).

3.5 Analysis of Food Samples

The enzyme sensor was used to analyse lysine in dry-cured ham and dry-fermented sausages and verify its effectiveness to use the determined lysine as an index of ripening of dry-cured meat products. So, samples of dry-cured ham and dry-fermented sausage at different times of their respective processes were analysed. The results for the immobilised sensor (Fig 3A and 3B) show the high increase of the lysine content with time in both types of products and the similarity profile when compared with the results obtained with the HPLC method. Maximum content, near 1070 mg/100 g, were detected at 10 months of ham processing and in the case of dry-fermented sausages the amounts reached 36.5 mg/100g by the end of the process at 65 days. In this case, the proteolysis is much lower and this is the reason for the lower amounts of generated lysine. The amount of lysine in both types of products is in accordance with those reported in the literature (Flores, Sanz, Spanier, Aristoy, and Toldrá, 1998; Toldrá, et al 2000; Bolumar, Sanz, Flores, Aristoy, and Toldrá, 2006; Durá, Flores and Toldrá, 2004). Data from the enzyme sensor (immobilised) obtained with the dry-cured ham samples were compared to the data obtained when analyzing the same samples with the HPLC. These values confirm the usefulness of this sensor for an effective determination of lysine in meat products.

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374 **4. Conclusion**

375 Based on the obtained results, the sensor can be considered a valid alternative technique
376 for determining the amount of lysine in dry-cured meats. The sensor shows a good
377 sensitivity, repeatability and stability for the detection of lysine in such products and
378 low interference due to the good affinity of the lysine oxidase for the substrate despite
379 the presence of other amino acids in the meat sample.

380 The results are comparable to those values obtained with HPLC but the average
381 measurement time per sample is much shorter (<3 min). The use of immobilised
382 enzyme allows the possibility to keep the immobilised enzyme under refrigeration for
383 several days. Through the use of this sensor, it is possible to monitor the ripening of
384 different dry-cured meat products, resulting in a useful tool for the meat industry due to
385 its easy use, short time requested, good feasibility and low cost.

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387 **Acknowledgements**

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389 Grant Prometeo 2012/001 from Conselleria d'Educació of Generalitat Valenciana
390 (Valencia, Spain) is acknowledged. The scholarship from Secretaria de Educación
391 Superior, Ciencia, Tecnología e Innovación (SENESCYT) of Ecuador to F. A. Jadán
392 Piedra is also fully acknowledged.

393

394 **Funding:** This study was funded by Grant Prometeo 2012/001 from Conselleria
395 d'Educació of Generalitat Valenciana (Valencia, Spain) and the scholarship to F. A.
396 Jadán Piedra from Secretaria de Educación Superior, Ciencia, Tecnología e Innovación
397 (SENESCYT) of Ecuador

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Conflict of Interest: The authors Felipe Jadán, María-Concepción Aristoy, and Fidel Toldrá declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Not applicable.

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Figure Captions

Figure 1. Evolution of the lysine content determined through HPLC (●) and the immobilised lysine oxidase sensor (▲) during the processing of dry-cured ham (A) and dry- fermented sausage (B).

Figure 2. Correlation between lysine contents measured by enzyme sensor with soluble (A) and immobilised lysine oxidase (B) vs HPLC in dry-cured ham samples of 10 months. The study was done with six samples with six replicates each.

Figure 1.- Calibration curve obtained with the LOx biosensor at 37°C in phosphate buffer 0.05M pH 7.6.

Figure 2. Action of LOx soluble enzyme when incubated at 37°C and pH 7.6 using as substrate a standard 0.1 mM Lysine solution (A) and a ham extract (B).

Figure 3. Evolution of the lysine content measured by HPLC methodology (-) and with the immobilized lysine oxidase sensor (---) during the processing of dry-cured ham (A) and dry- fermented sausage (B).

Table 1.- Oxidation rate of the enzyme onto some free amino acids as measured with HPLC or with the immobilised enzyme biosensor.

Substrate	Oxidation rate (%) HPLC	Oxidation rate (%) Biosensor
Lysine	100.0	100.0
Arginine	25.0	0.0
Phenylalanine	18.8	53.8
Ornithine	9.4	9.1
Tyrosine	9.4	0.0
Histidine	6.3	0.0
Leucine	3.1	0.0