Determination of the Antihypertensive Peptide LHLPLP in Fermented Milk by High-Performance Liquid Chromatography–Mass Spectrometry

A. Quiro´ s,* M. Ramos,* B. Muguerza,† M. A. Delgado,† P. J. Martı´n-Alvarez,* A. Aleixandre,‡ and I. Recio*†

*Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Cientı´ficas (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain
†Department of Research and Development, Grupo Leche Pascual, Ctra. Palencia s/n, 09400 Aranda de Duero Burgos, Spain
‡Instituto de Farmacologı´a y Toxicologı´a (CSIC), Facultad de Medicina, Universidad Complutense de Madrid, Avda. Complutense s/n, 28040 Madrid, Spain

ABSTRACT

Among different lactic acid bacteria isolated from raw milk, 4 Enterococcus faecalis strains have stood out as producers of fermented milk with potent antihypertensive activity. The peptide β-casein f(133–138), LHLPLP, was identified as one of the major peptides responsible for the activity of these fermented milk products. A simple method was developed to quantify this peptide in fermented milk using high-performance liquid chromatography coupled in line with mass spectrometry. This procedure does not require any previous sample fractionation or extraction, and direct analysis of the water-soluble extract obtained from the fermented milk can be performed. Validation studies showed sufficient specificity, reproducibility, linearity, and recovery, demonstrating that this method can be used for the routine quantification of LHLPLP during the production of fermented milk products. The developed method was readily applied to quantify the peptide LHLPLP under different fermentation conditions and with different aromatized products.

Key words: antihypertensive peptide, Enterococcus faecalis, quantification, mass spectrometry

INTRODUCTION

Bioactive peptides derived from food proteins have been shown to play different physiological roles in the organism (Silva and Malcata, 2005). Among these, angiotensin-converting enzyme (ACE)–inhibitory peptides are of particular interest because of their capacity to prevent hypertension. These peptides can be used as ingredients in functional foods, representing a healthier and natural alternative to ACE-inhibitory drugs (Li et al., 2004). Fermented milks containing a particularly high number of peptides, among them many ACE-inhibitory and antihypertensive peptides, have been produced using proteolytic strains of different lactic acid bacteria (Nakamura et al., 1995; Rokka et al., 1997; Gobbetti et al., 2000; Leclerc et al., 2002; Fuglsang et al., 2003; Vermeirssen et al., 2003; Hernández-Ledesma et al., 2004; Robert et al., 2004; Quiro’s et al., 2005). Our research group has just demonstrated the ability of 4 different Enterococcus faecalis strains, isolated from raw milk, to produce fermented milk with potent ACE-inhibitory and antihypertensive activity in spontaneously hypertensive rats (SHR) (Muguerza et al., 2006). The administration of a fermented product produced with this microorganism exerted a significant blood pressure-lowering effect in these animals after short- and long-term intake (Miguel et al., 2005; Muguerza et al., 2006). The peptide β-CN f(133–138), with sequence LHLPLP, has been identified as one of the major peptides responsible for the antihypertensive activity of the fermented milk produced with these E. faecalis strains (Quiro’s et al., 2006).

A few studies have also demonstrated the hypotensive activity of peptides on hypertensive animals and in humans (Hata et al., 1996; Sipola et al., 2001; Seppo et al., 2003). In some of these studies, the antihypertensive activity has been shown to be highly dependent on the peptide dosage. Most studies are performed with hydrolysates or fermented products containing the peptides IPP and VPP, 2 of the most potent antihypertensive peptides derived from milk proteins. A clinical study with mildly hypertensive volunteers has shown that volunteers consuming a daily 1.8-mg dose of IPP and VPP exhibited a significant decrease in systolic blood pressure after 6 wk, but in the group receiving doses of 2.5 or 3.6 mg, this benefit was already recorded after 3 wk (Mizuno et al., 2005). Therefore, quantification of the active components in functional foods is essential to ensure the activity of the final product.

Quantitative determination of active peptides in foods generally requires an extensive time-consuming sample preparation with laborious fractionation and purification steps. Antihypertensive peptides have been quantified by enzyme immunoassay methods (Walsh et al., 2004), but these methods are limited by the cost of the assays, the size of the peptide to be detected and the
specificity. Some methods based on HPLC have also been applied for quantification of these peptides. For instance, quantification of the antihypertensive peptide YP, identified in a yogurt-like product fermented by Lactobacillus helveticus CPN4, required different steps for purification by HPLC (Yamamoto et al., 1999). Mass spectrometry provides a specificity that HPLC with conventional detectors does not, and several methods based on HPLC coupled with MS have recently been developed to quantitate bioactive peptides in protein hydrolysates or fermented products. An HPLC method coupled in line with tandem mass spectrometry (HPLC–MS/MS) has been developed to quantify the ACE-inhibitory and antihypertensive peptide LKPNM in bonito muscle hydrolysates (Curtis et al., 2002). Similarly, the peptides VPP and IPP have been determined in an Aspergillus oryzae hydrolysate by an HPLC–MS method (Matsuraa et al., 2005). However, both HPLC–MS methods used a solid-phase extraction step of the hydrolysate prior to analysis as a cleanup procedure.

The aim of this study is to develop a robust analytical method for rapid determination of the antihypertensive peptide LHLPLP, with minimal sample treatment or prefractionation of the fermented milk to be used for the routine determination of this peptide in fermented products. We evaluated the use of HPLC–MS or HPLC–MS/MS to quantify this bioactive peptide, and the method was validated by determining its repeatability, reproducibility, linearity, and recovery. Finally, this method was applied to different milk products obtained by milk fermentation with E. faecalis strains under different conditions.

MATERIALS AND METHODS

Production of Fermented Milk

The strains of E. faecalis used in this study were isolated from raw milk and belong to the Grupo Leche Pascual S.A. culture collection. Fermented milk was prepared as described previously (Muguerza et al., 2006). Briefly, precultures of E. faecalis strains CECT 5727, 5728, 5826, and 5827 were prepared in sterile reconstituted 10% (wt/wt) skim milk powder incubated overnight at 30°C. Three percent (vol/vol) of the corresponding preculture was added to reconstituted skim milk powder and fermentation was carried out for different periods of time (from 0 to 48 h) at 30°C. Bacterial growth through fermentation was determined by plate count on M17 agar after incubation at 30°C for 48 h. The fermentation process was stopped by pasteurization of the fermented milk at 75°C for 1 min. Alternatively, the preculture was used to inoculate a milk formulation prepared with sweetened milk (10% sugar) standardized to a certain protein content with a commercial milk protein concentrate. The resultant fermented milk was analyzed as such or after blending with variable quantities of a sweetened aromatized commercial yogurt drink (Miguel et al., 2005).

The preparation of water-soluble extracts of the fermented milks was carried out by centrifuging at 20,000 × g for 10 min at 10°C and filtering through a Whatman no. 40 filter.

Preparation of Standard Solutions

The peptides LHLPLP and GHLPLP (evaluated as internal standards) were prepared by the conventional 9-fluorenylmethyloxycarbonyl solid-phase synthesis method with a 431A peptide synthesizer (Applied Biosystems Inc., Überlingen, Germany). Their purity was higher than 90%. Stock solutions of 1 mg/mL for LHLPLP and of 1.6 mg/mL for GHLPLP were prepared.

Six-point calibration curves (250, 125, 62.5, 31.25, 15.62, and 7.81 μg/mL) of the synthetic peptide LHLPLP were prepared in Milli-Q deionized water (Millipore, Bedford, MA). The same calibration curve as described above was prepared in the water-soluble extract obtained from a commercial fermented product in which these peptides were absent.

HPLC–MS/MS Analysis

The HPLC analyses were performed on an Agilent HPLC system connected on line to an Esquire 3000 quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). The HPLC system was equipped with a quaternary gradient pumping system, an on line degasser, a variable-wavelength detector set at 220 nm, and an automatic injector (all 1100 series; Agilent Technologies, Waldbronn, Germany). Chromatographic separations were performed with a Widepore C18 column (250 mm × 4.6 mm × 5 μm particle size; Bio-Rad, Richmond, CA). Solvent A was a mixture of water:trifluoroacetic acid, 1,000:0.37, and solvent B was a mixture of acetonitrile:trifluoroacetic acid, 1,000:0.27. The water-soluble extracts of fermented milks were eluted by using a linear gradient of solvent B in A going from 0 to 45% in 60 min at a flow rate of 0.8 mL/min. The flow was split postdetector by placing a T-piece (Valco, Houston, TX) connected with a 75-μm i.d. peak outlet tube of an adjusted length to give approximately 20 μL/min of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as the nebulizing and drying gas, and the ion source parameters were nebulizer pressure, 60 psi; drying gas, 8 L/min; and dry temperature, 350°C. The capillary was held at 4 kV. For HPLC–MS, spectra were recorded over the mass-to-charge (m/z) range of 100 to 1,000 with a target mass of 689 m/z and
an average of about 5 spectra. The signal threshold to perform MS/MS analyses was 10,000 and the precursor ion 689.4 was isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.30 to 2 V. All the analyses were carried out using an injection volume of 50 μL; triplicate injections were done for each point on the calibration curves and duplicate injections were done for samples.

The quantifications for HPLC–MS and for HPLC–MS/MS were performed using the software QuantAnalysis 1.3 (Bruker Daltonik). Plots were made of the HPLC–MS peak area for the precursor m/z 689.4 and their sodium and potassium adducts m/z 711.1, 727, and 733. The determination by HPLC–MS/MS was based on the peak area of the most abundant product ions from fragmentation of the molecular ion of LHLPLP with m/z 251, 364.2, 439.2, and 574.3.

Statistical Methods

Linear and polynomial regression for the calibration curves and nonlinear regression for studying the evolution of the concentration with the time of fermentation were calculated with Statgraphics Plus for Windows 5.1 (2000; Statistical Graphics Corporation, Manugistics Inc., MD, www.statgraphics.com) and GraphPad Prism for Windows 4.02 (2004; GraphPad Software, San Diego, CA; www.graphpad.com).

RESULTS AND DISCUSSION

Selection of Analysis Conditions

A milk sample produced by fermentation for 24 h with *E. faecalis* CECT 5728 was selected to optimize the analytical conditions for determination of the peptide LHLPLP. The water-soluble extract of this sample had previously demonstrated in vitro ACE-inhibitory activity (IC₅₀ 59.6 μg/mL) and antihypertensive activity when administered to SHR at a dosage of 5 mL/kg of BW (Muguerza et al., 2006). The HPLC analysis of this water-soluble extract resulted in a very complex profile (Figure 1A). The peptide of interest, LHLPLP, eluted between 44.3 and 47.3 min together with other peptide fragments. The signal obtained from the mass spectrometer (total ion current) in this zone of the chromatogram also revealed a complex mixture of peptides (Figure 1B). Approaches to improve the resolution of the HPLC method by modifying the solvent gradient did not permit the peptide LHLPLP to be resolved in a single peak, and analysis by MS revealed the coelution of the peptide of interest with other peptides with m/z values of 962.2 and 838.4. In addition, ions corresponding to sodium and potassium adducts of the peptide (i.e., ions with m/z 711.4 and 727.4) were observed (Figure 2A).

A significantly better specificity was obtained by extracting the molecular ion of the peptide LHLPLP (ion with m/z 689.4; Figure 1C), where a single high-intensity peak with a retention time of 45.8 min was obtained. Although the selectivity and intensity of the MS signal were sufficient to perform a quantitative determination of the peptide, the response obtained by performing MS/MS was also evaluated. With this aim, optimization of the fragmentation of the peptide of interest was performed by direct infusion, at 4 μL/min, of a solution of the synthetic peptide (10 μg/mL) in acetonitrile:water (50:50) to which 0.3% formic acid was added. As expected, because of the presence of Pro residues, major fragment ions were observed at m/z 364.2 and 574.3, which were identified as b-type ions adjacent to Pro, in particular b₃ and b₅, respectively (Papayannopoulos, 1995; Figure 2B). In addition, this cleavage is especially favored when the previous residue N-terminal to Pro is Leu (Breci et al., 2003). The presence of His as the second N-terminal residue can explain the higher intensity observed for the b-type fragment ions vs. the y-type ions, with the exception of fragment y₄, which corresponded to an especially favored cleavage. Extraction of the main fragment ions (i.e., 574.3, 439.2, 364.2, and 251.0) resulted in 2 peaks (Figure 1D), one corresponding to LHLPLP and the other identified by MS/MS as LHLPLPL, which has some fragment ions in common with LHLPLP.
Figure 1. (A) Analysis of the water-soluble extract of milk fermented with Enterococcus faecalis CECT 5728 obtained by HPLC with UV detection at 214 nm; (B) total ion current chromatogram; (C) HPLC–mass spectrometric (MS) analysis, showing the extracted ion chromatogram of the molecular ion of peptide LHLPLP with \( m/z \) 689.4; and (D) HPLC–tandem MS (MS/MS) analysis, showing the extracted ion chromatogram of fragment ions with \( m/z \) 574.3, 439.2, 364.2, and 251.0. For experimental conditions see the Material and Methods section.
Figure 2. (A) High-performance liquid chromatograph–mass spectrum of the peptides eluting between 44.3 and 47.3 min. (B) Tandem mass spectrum of the singly charged ion $m/z$ 689.4. The sequence of this peptide is displayed with the fragment ions observed in the spectrum. Fragment ions are labeled according to the nomenclature proposed by Roepstorff and Fohlman (1984).

**Linearity.** A linear regression analysis of the peak area vs. concentration of the standard peptide dissolved in water or the water-soluble extract of a commercial fermented milk product was calculated by using 3 replicates at 6 different concentrations in the range indicated in Table 2 and analysis by HPLC–MS and HPLC–MS/MS. In the MS/MS dimension, 3 different curves were obtained taking into account 4, 3, or 2 fragment ions, respectively. To judge the adequacy of the linear models, the $F$-ratio for lack of fit was calculated (Massart et al., 1990), and when significant results were obtained, a second-degree polynomial regression was used. Figure 3 shows the peak area vs. concentration of the peptide for HPLC–MS and for HPLC–MS/MS using different fragment ions. Regression parameters and statistical properties can be found in Table 2. All the values of the coefficients of the table are significantly different from zero ($P < 0.05$). As shown in Table 2, the results obtained for all HPLC–MS/MS analyses corresponded to linear equations, whereas for HPLC–MS the result corresponded to a second-degree polynomial equation. The values for the coefficient of determination ($R^2$ in Table 2) were higher than 0.99 and indicated that the fits were acceptable, with a standard deviation of residuals, expressed as a percentage of the mean value of the response (CV) $\leq 6\%$ for all cases. Recovery has been estimated as
(the amount found in the spiked sample – the amount found in the sample) × 100/the amount added (Massart et al., 1990). The mean values were calculated from the recovery experiments by using 2 different concentrations of LHLPLP (0.1 and 0.2 mg/mL) added to E. faecalis-fermented milk, and triplicate injections of each point were performed. Better recovery values were achieved when using the calibration curves with the standard peptide dissolved in water in comparison with those obtained with the peptide dissolved in a water-soluble extract of the commercial fermented milk product. Therefore, the calibration curves obtained with the peptide dissolved in water were used for further calculations.

Recovery ranged from 87% for the HPLC–MS analysis to 94% for the HPLC–MS/MS analysis using 3 of the most abundant fragment ions (m/z 251, 364.1, 574.3), which were significantly different from 100 (P < 0.05). The mean recovery values for the analyses by HPLC–MS/MS using 2 fragment ions (m/z 364.1, 574.3) were not significantly different from 100 (P > 0.05), and the RSD of the areas were below 4%. Therefore, any of these 2 linear regressions can be used to calculate the concentration of LHLPLP.

**Detection and Quantification Limits.** The calibration curve obtained by HPLC–MS/MS using 3 fragment ions (m/z 251, 364.1, 574.3) was used for quantification. The detection limit and quantification limit were estimated as the peptide concentration giving a signal equal to the blank signal plus 3 and 10 standard deviations of the blank, respectively (Miller and Miller, 2000). The values obtained were 7 μg/mL for the detection limit and 25 μg/mL for the quantification limit. This detection limit can easily be improved by increasing the flow that was passed to the electrospray source (from 20 to 300 μL/min), but higher flows also resulted in the formation of a deposit on the cone of the mass spectrometer that should be removed periodically to avoid deterioration of the response.

**Analysis of Fermented Milks.** Using the linear regression obtained by HPLC–MS/MS with 3 fragment ions (m/z 251, 364.1, 574.3), we determined the concentrations of the antihypertensive peptide LHLPLP in the

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**Table 1.** Repeatability, as estimated by the relative standard deviation of the peak areas of peptide LHLPLP for 6 consecutive analyses of the water-soluble extract from milk fermented with Enterococcus faecalis CECT 5728, and reproducibility, as estimated by the relative standard deviation of the areas for 10 analyses of the fermented milk realized on 2 different days separated by 1 wk

<table>
<thead>
<tr>
<th>Selected ions</th>
<th>Repeatability, %</th>
<th>Reproducibility, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS (m/z 689.4; 711.1; 727; 733)</td>
<td>3.4</td>
<td>4.0</td>
</tr>
<tr>
<td>MS/MS (m/z 364.1; 574.3)</td>
<td>3.9</td>
<td>3.6</td>
</tr>
<tr>
<td>MS/MS (m/z 251; 364.1; 574.3)</td>
<td>2.6</td>
<td>2.3</td>
</tr>
<tr>
<td>MS/MS (m/z 251; 364.1; 439.2; 574.3)</td>
<td>3.9</td>
<td>3.6</td>
</tr>
</tbody>
</table>

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**Table 2.** Polynomial regression (y = a + bx + cx²) for area vs. concentration of standard solutions of LHLPLP in water obtained for HPLC–mass spectrometry (MS) and for HPLC–tandem mass spectrometry (MS/MS) using the ions indicated

<table>
<thead>
<tr>
<th>Selected ions</th>
<th>Range, mg/mL</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>R²</th>
<th>s</th>
<th>CV, %</th>
<th>P-value</th>
<th>n</th>
<th>Recovery, 1 mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS² (m/z 689.4; 711.1; 727; 733)</td>
<td>0 to 0.25</td>
<td>-2.16584E6</td>
<td>7.93250E8</td>
<td>-7.22586E8</td>
<td>0.997</td>
<td>2.86970E6</td>
<td>5.7</td>
<td>0.08</td>
<td>18</td>
<td>86.95*</td>
</tr>
<tr>
<td>MS/MS² (m/z 364.1; 574.3)</td>
<td>0 to 0.25</td>
<td>-1.04283E6</td>
<td>1.34205E8</td>
<td>—</td>
<td>0.999</td>
<td>0.34966E6</td>
<td>3.5</td>
<td>0.69</td>
<td>18</td>
<td>94.39</td>
</tr>
<tr>
<td>MS/MS² (m/z 251; 364.1; 574.3)</td>
<td>0 to 0.25</td>
<td>-1.38708E6</td>
<td>1.63390E8</td>
<td>—</td>
<td>0.999</td>
<td>0.41551E6</td>
<td>3.5</td>
<td>0.19</td>
<td>18</td>
<td>92.26</td>
</tr>
<tr>
<td>MS/MS² (m/z 251; 364.1; 439.2; 574.3)</td>
<td>0 to 0.25</td>
<td>-1.55363E6</td>
<td>1.78949E8</td>
<td>—</td>
<td>0.998</td>
<td>0.56676E6</td>
<td>4.3</td>
<td>0.37</td>
<td>18</td>
<td>92.31*</td>
</tr>
<tr>
<td>MS³ (m/z 689.4; 711.1; 727; 733)</td>
<td>0 to 0.25</td>
<td>-0.92200E6</td>
<td>9.40727E8</td>
<td>-1.00653E9</td>
<td>0.998</td>
<td>2.31252E6</td>
<td>4.2</td>
<td>0.27</td>
<td>18</td>
<td>92.31*</td>
</tr>
<tr>
<td>MS/MS³ (m/z 364.1; 574.3)</td>
<td>0 to 0.25</td>
<td>-0.20506E6</td>
<td>1.46935E8</td>
<td>—</td>
<td>0.997</td>
<td>0.65996E6</td>
<td>5.6</td>
<td>0.16</td>
<td>18</td>
<td>86.21*</td>
</tr>
<tr>
<td>MS/MS³ (m/z 251; 364.1; 574.3)</td>
<td>0 to 0.25</td>
<td>-0.28507E6</td>
<td>1.75606E8</td>
<td>—</td>
<td>0.998</td>
<td>0.64992E6</td>
<td>4.6</td>
<td>0.13</td>
<td>18</td>
<td>85.94*</td>
</tr>
<tr>
<td>MS/MS³ (m/z 251; 364.1; 439.2; 574.3)</td>
<td>0 to 0.25</td>
<td>-0.06945E6</td>
<td>1.92306E8</td>
<td>—</td>
<td>0.996</td>
<td>0.96292E6</td>
<td>6.1</td>
<td>0.04</td>
<td>18</td>
<td>86.08*</td>
</tr>
</tbody>
</table>

1Recovery corresponds to the mean of the individual values obtained from the recovery experiments by using 2 different concentrations of standard LHLPLP (0.1 and 0.2 mg/mL) added to Enterococcus faecalis-fermented milk. R² = coefficient of determination; s = residual standard deviation; CV (%) = (s/mean) × 100; residual standard deviation expressed as a percentage of the mean value; P-value = determined from the lack-of-fit test for the regression; n = number of used points; recovery = (amount found in the spiked sample – amount found in the sample) × 100/amount added. All regression coefficients (a, b, c) presented are significantly different from zero (P < 0.05).

2Standard solution of LHLPLP in water.

3Standard solution of LHLPLP in fermented milk.

4Significantly different from 100 (P < 0.05).
QUANTIFICATION OF PEPTIDE LHLPLP 4533

Figure 3. Calibration curves of the peptide LHLPLP in water obtained after HPLC–mass spectrometric (MS) analysis and extraction of the ion m/z 689.4 and its corresponding adducts (○) and after HPLC–ion trap tandem MS (MS/MS) analysis and extraction of different fragment ions (□, △, ▽).

fermented milk with *E. faecalis* CECT 5727 and *E. faecalis* CECT 5728 during fermentation (0, 3, 6, 9, 24, and 48 h; Figure 4). The mathematical model chosen to describe the evolution of the concentration with the fermentation time was as follows:

\[
Y = A + (B - A) \times [1 - \exp(-K \times t)]
\]

where \(Y\) represents the concentration of the peptide (in milligrams per milliliter), \(t\) is the time (in hours), \(A\) is the bottom, \(B\) is the top, and \(K\) is the velocity constant. The results from nonlinear regression analyses are also shown in Figure 4: the best-fit parameter values, as well as the statistical values to judge the goodness of fit (coefficient of determination, \(R^2\), and the standard deviation of the residuals, \(S_y\),). The amount of LHLPLP was higher when the strain CECT 5728 was used for milk fermentation. In both cases, after 20 h the concentration of LHLPLP reached a plateau; thus, it would be unnecessary to continue fermentation for longer times to obtain the maximal amount of peptide.

The concentration of peptide produced after fermentation with *E. faecalis* CECT 5728 was also evaluated in CN-enriched milk that had been standardized to different protein contents (Table 3). A protein content of 5.5% resulted in higher amounts of LHLPLP compared with that obtained in milk with 4.3% protein. Again, the concentration of the peptide increased with the fermentation time up to 24 h, and longer fermentation times seemed to be unnecessary to increase the production of LHLPLP. Other authors have previously demonstrated a higher ACE-inhibitory activity in CN-enriched milk fermented with *L. helveticus* compared with the activity found in reconstituted skim milk powder (Leclerc et al., 2002). Therefore, caseinate addition to milk could be of interest to increase the production of certain biologically active peptides during milk fermentation.

This method was also applied to quantify the amount of peptide after blending the resultant fermented milk with *E. faecalis* CECT 5728 after fermentation for 24 h with variable quantities of a sweetened aromatized commercial yogurt drink to give a more palatable product. In addition, one batch of fermented milk was enriched with Ca (2 g/L). These samples were prepared in different batches and were used in a long-term study to evaluate the antihypertensive effect of these fermented products (Miguel et al., 2005). The milk after fermentation contained 0.234 mg/mL of the peptide, and after
blending the amount of peptide was between 0.138 and 0.164 mg/mL, depending on the batch considered. The presence of Ca in the sample did not affect quantification of the peptide. These products caused a significant decrease in the systolic and diastolic blood pressure of SHR, and this effect was slightly more accentuated when the Ca-enriched products were used (Miguel et al., 2005).

Table 3. Quantification of the peptide LHLPLP in a water-soluble extract of milk standartized to different protein contents during fermentation with Enterococcus faecalis CECT 5728

<table>
<thead>
<tr>
<th>Fermentation time, h</th>
<th>Protein, %</th>
<th>LHLPLP, mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>4.3</td>
<td>0.258</td>
</tr>
<tr>
<td>26</td>
<td>4.3</td>
<td>0.243</td>
</tr>
<tr>
<td>28</td>
<td>4.3</td>
<td>0.247</td>
</tr>
<tr>
<td>31</td>
<td>4.3</td>
<td>0.226</td>
</tr>
<tr>
<td>34</td>
<td>4.3</td>
<td>0.240</td>
</tr>
<tr>
<td>24</td>
<td>5.5</td>
<td>0.301</td>
</tr>
<tr>
<td>26</td>
<td>5.5</td>
<td>0.266</td>
</tr>
<tr>
<td>28</td>
<td>5.5</td>
<td>0.265</td>
</tr>
<tr>
<td>31</td>
<td>5.5</td>
<td>0.281</td>
</tr>
<tr>
<td>34</td>
<td>5.5</td>
<td>0.298</td>
</tr>
</tbody>
</table>

1Medium value of duplicate analyses.

CONCLUSIONS

This report proposes a simple and rapid HPLC–MS/MS method for quantitative determination of the antihypertensive peptide LHLPLP in fermented milk products. The use of MS or MS/MS produced very clean chromatograms for this peptide, with a negligible contribution from the fermented milk background. The method had variability with RSD lower than 4% and good linearity in the range from 7 to 250 μg/mL required for the direct determination of this peptide in milk fermented with E. faecalis. The analysis was performed without any sample pretreatment or concentration and can be readily applied to optimize the fermentation process and the final products containing different ingredients.

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