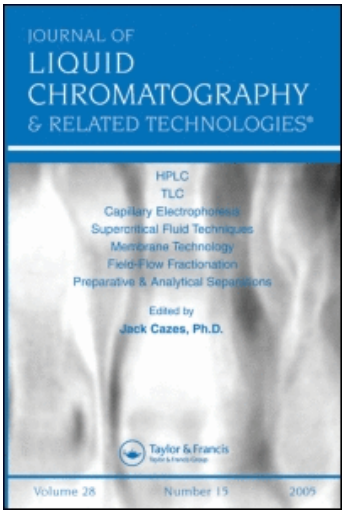

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Development and Validation of an HPLC/UV Method for Quantification of Bioactive Peptides in Fermented Milks

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Development and Validation of an HPLC/UV Method for Quantification of Bioactive Peptides in Fermented Milks

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Abstract: The simultaneous separation and quantification of two casein peptides (IPP, VPP) presenting potent inhibitory activity of angiotensin-converting-enzyme (ACE) and casein in fermented milks was developed. Gradient elution was carried out at a flow-rate of 1 mL/min, using a mixture of two solvents. Solvent A was 0.1% TFA in water and solvent B was acetonitrile-water-trifluoracetic acid 95:5:0.1. The effluent was monitored by UV detector at 214 nm. Calibration curves were constructed in the interval of 0.01–1.0 mg/mL for VPP, 0.005–1.0 mg/mL for IPP, and 0.05–3.0 mg/mL for casein. R^2 invariably exceeded 0.999. The detection limits were 0.004 for VPP, 0.002 mg/mL for IPP, and 0.02 mg/mL for casein. Repeatability of the method was evaluated by six consecutive injections of two standard solutions containing VPP, IPP, and casein. The RSD values for concentration were all below 5.08%. Recovery studies were carried out to determine the accuracy of the method. Recoveries

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ranged between 88 and 98.2%. The methodology was applied, not only, for the monitorization of VPP, IPP, and casein in commercial fermented milks labeled as presenting antihypertensive properties, but also, in milk with different degrees of fermentation by *L. Helveticus*, and in other commercial functional fermented milks, such as, those presenting cholesterol lowering properties.

Keywords: Fermented milks, ACE-inhibitory peptides, Casein, RP-HPLC

INTRODUCTION

During the past decade, many bioactive peptides derived from milk proteins were characterized. These bioactive peptides are latent in the primary sequences of proteins and may be released in the course of gastrointestinal like proteolysis and food processing such as milk fermentation. Studies have identified a great number of peptide sequences with specific bioactivities and the conditions for their release have also been reported.[1–5] Consequently, a variety of naturally formed bioactive peptides have been found in fermented milk, and there are already a few commercial dairy products supplemented with milk protein derived bioactive peptides whose health benefits have been assayed in clinical human studies. Special attention was given to dairy products containing ACE-inhibitory or antihypertensive peptides.

In fermented milk products, peptide activities depend on the type of bacterial starter cultures and degree of proteolysis. Milk fermented by several strains of *L. helveticus* showed antihypertensive activity. However, milk fermented by other species of lactic acid bacteria, i.e., *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. acidophilus*, *L. delbrueckii* subsp. *lactis*, *Streptococci thermophilus*, *L. lactis* subsp. *Lactis*, and *L. lactis* subsp. *cremoris* did not show significant antihypertensive activity.[6] The use of *L. Helveticus* bacteria increases the production of proline containing short peptides, such as Iso-Pro-Pro (IPP) and Val-Pro-Pro (VPP). These IPP and VPP peptides known to have a potent angiotensin converting enzyme (ACE) inhibitory activity, are derived from the hydrolysis of β-casein, f84–86 which corresponds to VPP, and f 74–76 which corresponds to IPP, and one from k-casein, f108–110, which corresponds to IPP.[4,6] The overall effect of an ACE inhibitor is the control of high blood pressure through dilation of blood vessels and its effect on blood volume. ACE has a role in regulating blood pressure by converting angiotensin I (Ang I) to angiotensin II (Ang II), which contracts the blood vessels.[7–10]

Thus, commercially fermented milk with antihypertensive effects can be obtained by the action of *L. Helveticus* bacteria or by the addition of bioactive peptides obtained through a patented process. These fermented milks have different degrees of protein hydrolysis that should be evaluated.

High performance liquid chromatography (HPLC), especially in the reverse phase mode (RP-HPLC), proved to be efficient to separate peptides from protein hydrolysates and also to give some indications about their hydrophilicity and hydrophobicity.[11] The use of a reversed-phase column that contains a polystyrene-divinylbenzene copolymer-based packing enables separation of peptides and proteins based on differences in surface hydrophobicity between the molecules. Peptide and protein retention is controlled by increasing the concentration of organic solvents like acetonitrile, hereby altering the polarity of the aqueous mobile phase.[12–14]

The development of HPLC methods that can be used in the monitorization of VPP, IPP, and casein fractions during milk fermentation and in the commercially fermented milks is useful for optimization and quality control of those functional foods. Thus, the aim of this work was to develop a RP-HPLC methodology with UV detection for simultaneous separation and quantification of most potent bioactive peptides inhibitors of angiotensin converting enzyme (ACE) and casein fractions in fermented milks.

EXPERIMENTAL

Instrumentation

The HPLC equipment consisted on a Gilson chromatograph (Gilson Medical Electronics) equipped with a type 302 pump, a type 305 pump, and a type 7125 Rheodyne Injector with a 20 μL loop. A Gilson 118 variable wavelength ultra violet detector was used. The equipment was controlled by Gilson 712 software that controlled the solvent gradient, data acquisition, and data processing. The column was a reversed-phase *Chrompack* P 300 RP column that contains polystyrene-divinylbenzene copolymer based packing (8 μm, 300 Å, 150 × 4.6 I.D.). A *Chrompack* P RP (24 × 4.6 mm I.D.) was used as a precolumn.

Separation Conditions

Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water and solvent B was acetonitrile-water-TFA (95:5: 0.1, v/v). Peptides and proteins were eluted with the following gradient increasing the proportion of solvent B, from 0 to 50% over 50 minutes: 0–5 min, 0% B; 5–9 min, 0–5% B; 9–15 min, 5–15% B; 15–25 min, 15–33% B; 25–31 min, 33–40% B; 31–37 min, 40% B; 37–45 min, 40–50% B; 45–50 min returning to initial conditions (50–0% B), in a total run time of 50 min. The flow-rate was

1.0 mL/min. The column was used at ambient temperature and detection at 214 nm.

Reagents and Protein Standards

All reagents used were of analytical grade purity. Eluents for HPLC were filtered through 0.22 μm NL 17 filters and degassed under vacuum for at least 15 min before use.

Synthetic VPP and IPP were supplied by Caslo Laboratory ApS and had a minimum purity of 99.55 and 95.1%, according to the respective Product Report. Peptide standard solutions were prepared by dissolution in water, the solutions were stored at −20°C until use.

Bovine milk casein, with a minimum purity of 75%, determined by the Bradford method,[15] as supplied by Sigma. Purified α-, β- and κ-casein were also obtained from Sigma Chemical Co., and had a minimum purity of 85, 90, and 80% (according to Sigma), respectively. Purified bovine standards of β-lactoglobulin (β-lg) and α-lactalbumin (α-la) were supplied by Sigma Chemical Co. Cn, α-la and β-lg standard solutions were dissolved in a mixture of 70% of water and 30% of acetonitrile (v/v). The solutions were stored at −20°C until use.

Sampling and Sample Preparation

Skim milk was prepared by separating the fat from the whole milk by centrifugation at 700 g, at 4°C, for 10 min, and stored at −20°C until use. Before HPLC analysis milk was diluted with water and filtered, this sample was coded as M1.

Two different brands of commercially fermented milks containing bioactive peptides ACE-inhibitors were analyzed. One of those brands (coded as A1) was produced from skim milk inoculated with *L. helveticus* and the other (coded as B1) was produced by the addition of bioactive peptides obtained through a patented process.

These two brands have been commercialized as functional food labeled as cholesterol lowering fermented milk that were also assayed and coded as A2 and B2, respectively.

Skim milk added to powdered milk was inoculated with a single strain of *L. helveticus* under aseptic conditions. The milk was fermented for 22 hours, at 37°C, in optimal growth conditions to reach a high proteolytic activity. A final pH of 4.0 was obtained. Aliquots were taken after 12 and 22 hours incubation, coded as C1 and C2, respectively, and hydrolysis stopped using low temperature (2°C).

Each sample (3 mL) was diluted with 2 mL of water and filtered before HPLC analysis.

Method Validation

Analyses were performed in several batches over a period of 2 weeks. Each batch consisted of replicate analyses of blanks (limit of detection), standard solutions (sensitivity, precision, and linear range), and both spiked and unspiked samples (recovery).

The detection limits (LOD) were calculated as the concentration corresponding to three times the background noise of the blank. A total of six analyses were performed for two standard solutions in the linear range, one near the upper and another near the lower limits of concentrations. Thus, a standard solution containing 0.020 mg/mL of VPP, 0.010 mg/mL IPP, and 0.1 mg/mL of total cn and a standard solution containing 0.80 mg/mL of IPP and VPP and 2.5 mg/mL of total cn were used to evaluate the relative standard deviation (RSD%) of the method.

The experimental recovery was obtained from difference between 2 measurements (sample and spiked samples), according to the following relationship:

$$\text{Recovery, \%} = (\text{total analyte found} - \text{analyte originally present}) \times 100/\text{analyte spike}$$

RESULTS AND DISCUSSION

Using the adopted separation conditions, VPP, IPP, and cn showed different retention times which allow their separation. Figure 1 shows a typical chromatogram obtained for a standard solution. With this separation method an appropriate separation was readily achieved, thus, VPP, IPP, and caseins can be assayed simultaneously.

Quantitative analysis of IPP, VPP, and cn in real samples has been performed by calibration curves of each peptide and protein. Table 1 show the equation curve parameters for IPP, VPP, and cn, obtained by plotting the integrated area of chromatographic peaks as a function of peptide or protein concentration. In the same table, the limit of detection (LOD) and RSD values are also reported.

Assignment of IPP, VPP, and casein fraction peaks has been done by applying the standard addition method, i.e., by adding a standard solution of the peptides and casein to the original sample (Figure 2a and b). It was also verified by standard addition, that native α-la eluted between k-cn and α and β-cn and native β-lg elute after α and β-cn peaks (results not shown). However, these proteins are not in native form in temperature treated milks, thus, they do not appear in the chromatogram of fermented milks.

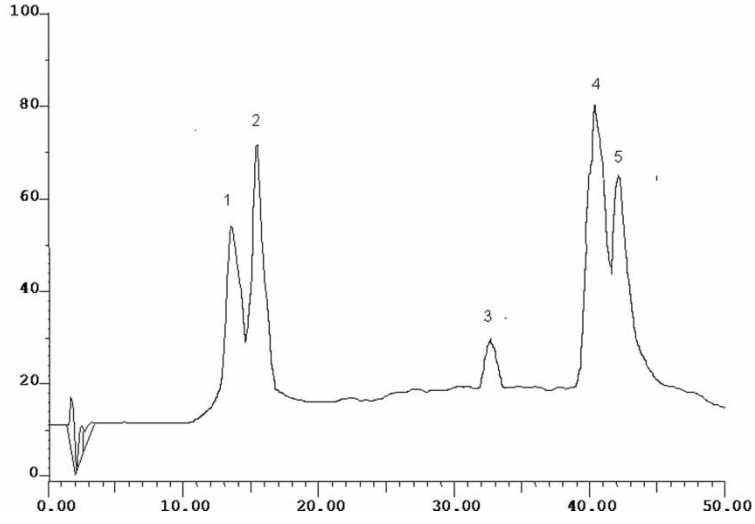

Figure 1. Typical chromatogram obtained for an aqueous standard solution containing 0.20 mg/mL of VPP, IPP and 0.4 mg/mL of total cn. 1–VPP; 2–IPP; 3–k-cn; 4–α-cn; 5–β-cn.

The reliability of the method was confirmed by two recovery experiments performed in two commercially fermented milk samples labeled as containing VPP and IPP. Table 2 presents the results for the recovery studies. Recoveries ranged between 88 and 98.2%.

Table 3 shows results of quantitative determination of IPP, VPP, and total cn present in skim milk (M1), commercial samples of fermented milks containing bioactive peptides ACE-inhibitors (A1, B1), commercial

Table 1. Results of linear fitting calibration data of VPP, IPP and total casein

Data	VPP	IPP	Caseins
Concentration range (mg/mL)	0.01–1.0	0.005–1.0	0.05–3.0
R^2 (number of points = 7)	0.9996	0.9996	0.9993
Slop (area units/mg)	1×10^7	2×10^7	3×10^6
RDS (%)[a]	5.08	3.12	3.61
RDS (%)[b]	2.21	1.52	0.93
LOD (mg/mL)[c]	0.004	0.002	0.02

[a]Average value of six replicate determinations for a standard solution containing 0.020 mg/mL of VPP, 0.010 mg/mL IPP and 0.1 mg/mL of total cn.

[b]Average value of six replicate determinations for a standard solution containing 0.80 mg/mL of IPP and VPP and 2.5 mg/mL of total cn.

[c]LOD calculated as the concentration corresponding to three times the background noise of the blank.

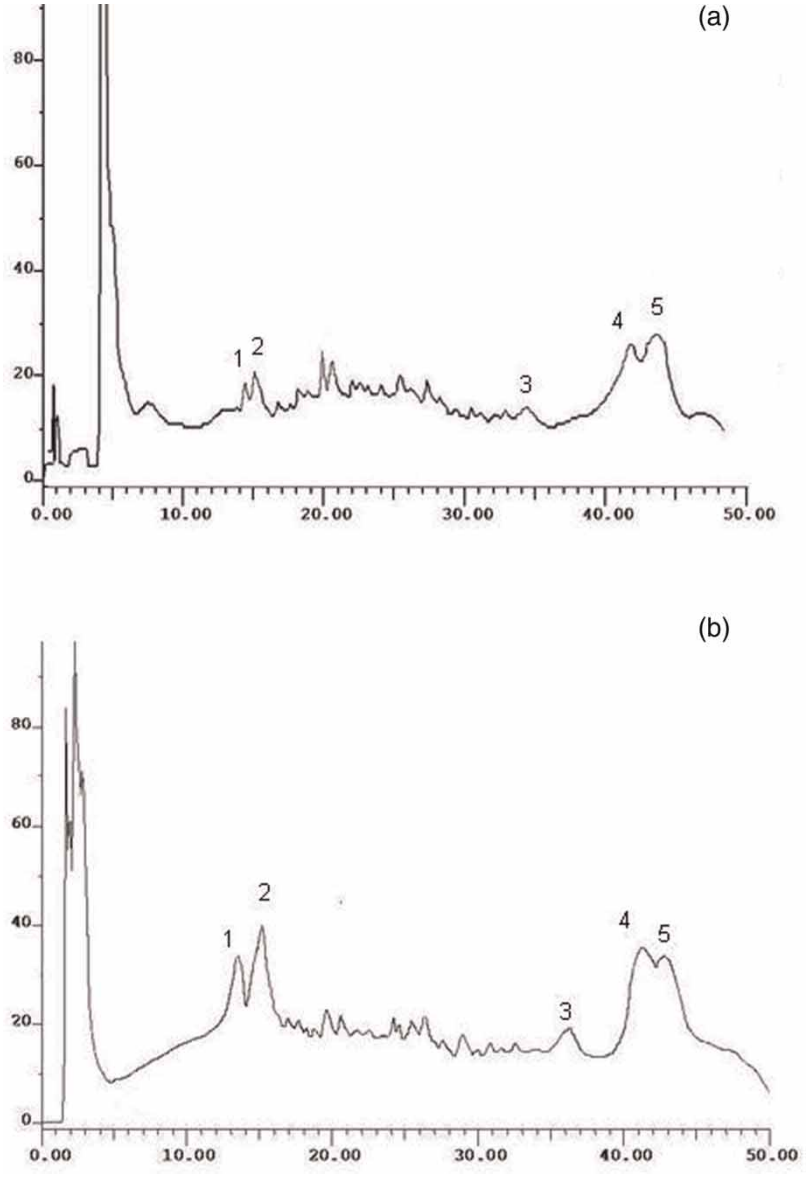

Figure 2. Chromatogram obtained for fermented milk A1 unspiked (a) and spiked (b) with VPP, IPP and casein. 1–VPP; 2–IPP; 3–k-cn; 4–α-cn; 5–β-cn.

samples of fermented milks containing phytosterols (A2, B2), and skim milk added to powdered milk and fermented with a single-strain of *L. helveticus*, during 12 hours and 22 hours (C1, C2, respectively). VPP and IPP were not detected in skim milk, neither in fermented milks with phytosterol, that was

Table 2. Results from recovery assays

Sample	Compound	Initial content (mg/5 mL)	Addition (mg/5 mL)	Measured content (mg/5 mL)	Recovery (%)
A1	VPP	0.0903	0.10	0.1869	98.2
	IPP	0.1131	0.20	0.302	96.5
	Casein	1.610	0.50	2.06	97.6
B1	VPP	0.0630	0.050	0.101	89.4
	IPP	0.0828	0.10	0.161	88.0
	Casein	8.330	2.00	10.11	97.8

Table 3. Results of quantitative determination of VPP, IPP and casein in milk and fermented milks

Compound	M1 (mg/mL)	A1 (mg/mL)	B1 (mg/mL)	A2 (mg/mL)	B2 (mg/mL)	C1 (mg/mL)	C2 (mg/mL)
VPP	n.d.	0.0301	0.0210	n.d.	n.d.	0.0145	0.0298
IPP	n.d.	0.0377	0.0276	n.d.	n.d.	0.0198	0.0324
Casein	20.7	0.54	2.77	1.79	3.22	2.52	1.73

M1-skim milk; A1, B1-commercial samples of fermented milks containing bioactive peptides ACE-inhibitors; A2, B2-commercial samples of fermented milks containing phytosterols; C1, C2-skim milk added of powdered milk and fermented with a single-strain of *L. helveticus*, during 12 hours and 22 hours, respectively.

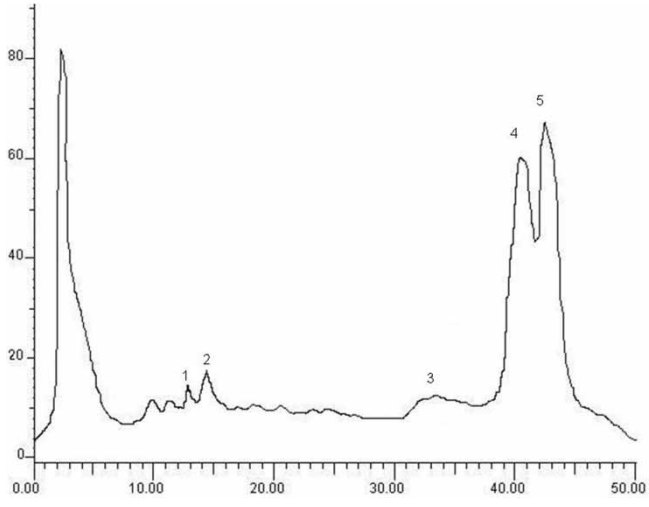

Figure 3. Chromatogram obtained for fermented milk B1. 1–VPP; 2–IPP; 3–k-cn; 4–α-cn; 5–β-cn.

not surprising since it is described that milk fermented by other species of lactic acid bacteria does not increase the production of VPP and IPP.[6] A higher degree of casein hydrolysis was observed in commercially fermented milk samples containing *L. helveticus* than in commercially fermented milk samples added to VPP and IPP (Figures 2a and 3). Good agreement was obtained between the levels of VPP and IPP in these two brands and respective labeling.

In conclusion, the described RP-HPLC method is suitable for routine assays of VPP, IPP, and cn, thus, it can be applied for optimization and quality control of functional fermented milks with antihypertensive properties. Furthermore, as casein is responsible for many milk intolerances, the monitorization of this protein during milk fermentation and in the final product is useful.

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