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Improvement of extraction and concentration of milk peptides with solid-phase cartridges for analysis by high-performance liquid chromatography

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ABSTRACT

Peptides were measured in skim milk by reversed-phase high-performance liquid chromatography after a solid-phase extraction step. Milk proteins were removed by precipitation and centrifugation after the samples were adjusted to pH 4.6. The peptides were then extracted from the supernatant using solid-phase cartridges, which had been treated to reduce irreversible non-specific adsorption of peptides. Different treatments using either α-lactalbumin or milk fractions to block the binding sites of the solid extractor have been compared. The treatment increased total peptide recovery and reduced cartridge-to-cartridge variability. Because this method effectively concentrates the peptides in a sample, it is applicable to peptide mapping of industrial milk samples.

INTRODUCTION

Peptides usually occurring in or produced by proteolysis of milk have potential applications in nutrition [1,2], flavouring [3–5] and bioregulation [6–8]. This last point has been extensively reviewed by Maubois and Leonil [9]. Functional properties of these peptides may also find applications in dairy technology [10–14]. Finally, as these peptides can serve as valuable tracers of milk proteolysis, they may be useful in the determination of quality standards.

In general, in dairy applications, the usual analytical methods are unsatisfactory in attempting to make correlations between proteolysis and specific technological parameters or sensory evaluation, owing to their lack of sensitivity and to interference of milk proteins. Typically, at present, methods for the analysis of the extent of proteolysis in skim milk or coagulum are based on trichloroacetic acid (TCA) precipitation of proteins followed by derivatization of primary amines with trinitrobenzene

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sulphonic acid, and finally measurement of optical density [4]. Until recently, chromatography of milk and milk products was restricted to separation by size exclusion, principally for the determination of caseinomacropeptide [15,16]. This peptide is purportedly a good tracer of milk proteolysis resulting from contamination of milk by psychotrophic bacteria. However, using reversed-phase high-performance liquid chromatography (HPLC), it was recently shown that caseinomacropeptide is only a minor product of the proteolysis of milk due to these bacterial contaminants [17].

Although HPLC is generally convenient for peptide analysis, it is of limited use in the case of milk products, owing to the presence of micellar proteins, lipids and other interfering material. These components are often irreversibly retained by the support, resulting in irreproducible analysis and short column life-time. For this reason, most of the published data on milk peptides obtained by reversed-phase HPLC analysis concerns peptides resulting from the action of purified enzymes on purified milk proteins [18,19]. As these proteolytic models are made in homogeneous solution and for an extended time, extraction and concentration of peptides before HPLC analysis is useless.

Thus, although these models may be useful, they are insufficient to account for the complex reactions that occur in milk. The aim of this work is to optimize the application of solid-phase extraction (SPE) techniques to the analysis of peptides in milk.

Indeed, SPE [20–24] appears to be a promising technique for peptide extraction, with application to milk product. One advantage of this technique is the concentration effect of the extraction process. This is especially important for milk peptides, which are normally present in low concentrations. A limitation of the technique is caused by the properties of solid-phase cartridges based on octadecylderivatized silica, which exhibit non-specific adsorption characteristics altering the reproducibility of measurements. The optimized protocol for the analysis of milk developed in this study includes a sample deproteinization step, followed by a peptide extraction step on treated solid-phase (TSP) cartridges obtained from different procedures, which have been compared.

EXPERIMENTAL

Materials

All chemicals and biochemicals were from Carlo Erba (La Défense, Paris, France) unless otherwise indicated. Milk was obtained from Gervais-Danone (Le Plessis-Robinson). Trifluoroacetic acid HPLC grade (TFA) and α-lactalbumin were from Sigma Chimie (La Verpillière, France). SPE cartridges (type 70206) and the extraction apparatus with manifold were from J. T. Baker (Sochibo, France). Other SPE cartridges were from Waters (ref. 20805, St Quentin en Yveline, France); Supelco (ref. 57054, St. Germain en Laye, France) and Alltech (ref. 205350, Templeur, France). Skim milk powder AFD from Prolait (Niort, France) is a reference material from Institut Technique du Gruyère (Rioz, France). Reference proteolytic hydrolysate, "Peptide N3", is from Armor Proteine (Cogles, France). The C_{18} analytical column (Nucleosil 255, 25 × 0.46 cm I.D.) was from SFCC (Neuilly Plaisance, France). The water used for HPLC analysis was produced on a Milli-RO–Milli-Q system (Millipore, St. Quentin en Yvelines, France).

The water used for the analysis was purified until the extract from 100 ml passed through an SPE cartridge produced no peak on HPLC analysis with high sensitivity UV detection (220 nm, 0.01 a.u.f.s.). As previously described [25,26], good water quality is critical, because of the concentration of its impurities during extraction on SPE-cartridges

Instrumentation

The HPLC system (Waters) consisted of a Wisp Model 710 B injector, linked to two Model 510 pumps with Model 490 UV detector and a Gilson Model 811 dynamic mixer. Gradient monitoring, data acquisition and processing were achieved on a Compaq 40 micro-computer with Maxima 820 software from Waters.

Sample processing before solid phase extraction

Solid phase extraction and HPLC analysis require the preliminary removal of casein micellea. Two methods to achieve this were compared: TCA precipitation, and casein precipitation at pH 4.6.

Protein precipitation by TCA. A 100-ml volume of 24% (w/v) aqueous TCA solution were added to 100 g of skim milk, then mixed for 30 min at 4°C. The mass was adjusted to 250 g with water, and the supernatant was collected by centrifugation at 2000 *g* for 20 min at 4°C.

Protein precipitation at pH 4.6. A milk sample was prepared by a procedure similar to Aschaffenburg method [27], as follows: to 100 g of skim milk were added 100 g of water, and 10 ml of 10% (w/w) acetic acid. The solution was mixed well for 10 min, at which time 10 ml of 1 *M* sodium acetate in water were added and mixed for 15 min. The mass was adjusted to 250 g with water. The precipitate was removed by centrifugation at 2000 *g* for 20 min at 5°C.

Extraction cartridge processing before peptide extraction

In addition to the conventional conditioning procedure, commercial SPE cartridges needed a treatment to enhance the yield and reproducibility of peptide extraction. The cartridges were first conditioned, using 12 ml of methanol followed by 12 ml of solution S1 (0.1% TFA by volume in water), and then treated by one of three different conditioning solutions: 50 ml of pH 4.6 soluble fraction of skim milk (prepared as described above) (TSP 1); or 50 ml of pH 4.6 soluble fraction of a 10% (w/w) water solution of skim milk powder (TSP 2); or 50 ml of a solution of α-lactalbumin (0.48 g/l) (TSP 3).

All treatments were performed under mild vacuum (0.5 bar), at a flow-rate of 5 ml/min. The cartridges were washed with 12 ml of solution S1, followed by 2 ml of solution S2 (acetonitrile–water–TFA, 90:10:0.1, v/v/v).

Finally, the cartridges were flushed with 18 ml of methanol and air-dried using vacuum suction (0.5 bar) for 10 min.

Dried TSP cartridges can be stored in air-tight containers for at least 2 weeks at 4°C without affecting performance.

Each treatment was performed on five cartridges. The relative standard deviation (R.S.D.), which is the standard deviation expressed as a percentage of the mean, was calculated for each chromatographic peak area (the seventeen major ones).

Skim milk peptide extraction

TSP cartridges were conditioned with 12 ml methanol followed by 12 ml of solution S1 immediately before use. Subsequently, 125 ml of the supernatant were passed through the cartridges, followed by 12 ml of solution S1. The peptides were eluted with 2 ml of solution S2. The resulting 2 ml of eluate were used for HPLC analysis.

HPLC analysis

The chromatographic conditions were: column, C_{18} Nucleosil; flow-rate, 0.8 ml/min; temperature, 20°C; mobile phase A, 0.1% (v/v) TFA in water; mobile phase B 0.1% TFA in acetonitrile–water (90:10, v/v); gradient elution, 0–47% B in 60 min, then 47–100% B in 5 min; isocratic elution, 100% solution B maintained until reaching (within 10 min) 0.122 absorbance (which is pure solvent B absorbance); injection volume, 20 μl; UV detection wavelengths, 220 nm and 280 nm in parallel, zero absorbance adjusted with 100% solution A.

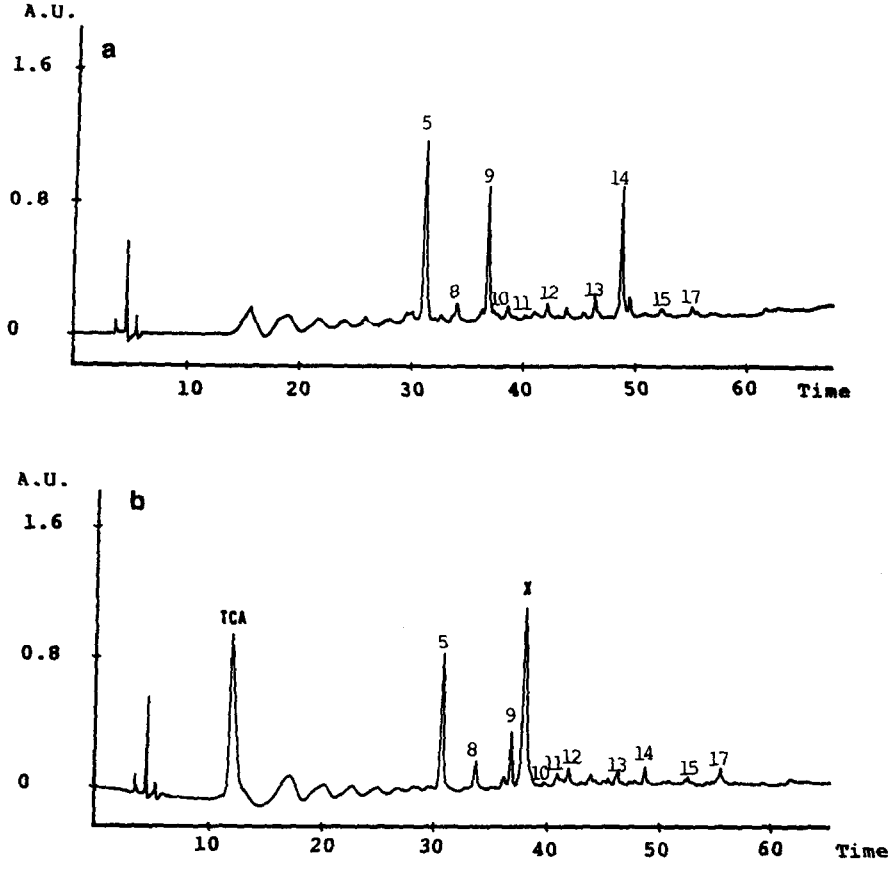

Fig. 1. HPLC analysis of peptides extracted from skim milk after various treatments for casein micellae removal. (a) Supernatant from protein precipitation at pH 4.6; chromatographic conditions as in Experimental (b) TCA supernatant (X = TCA impurity). Time in min.

RESULTS AND DISCUSSION

Milk sample pretreatment evaluation

In order to choose the optimum treatment of dairy skim milk for peptide extraction, two techniques that are common in dairy analysis and described above were compared (Fig. 1a and b). Peptide recovery using TCA (Fig. 1b) is poorer than with pH 4.6 treatment (Fig. 1a). In addition, contaminants in the TCA reagent interfere strongly in the subsequent chromatographic analysis.

The second method of casein removal based on pH 4.6 precipitation leads to the presence of hydrophobic and hydrophilic peptides (Fig. 1a).

This latter technique appears to be the better compromise as it allows the extraction and quantitation of most peptides.

Effects of SPE cartridge modification on extraction characteristics

The presence of strongly adsorbing sites in the beads of commercial cartridges leads to irreversible adsorption of several compounds contained in the milk samples. Therefore, incomplete recovery and variable yields are often observed with commercial cartridges. In order to overcome these drawbacks we have studied the effects of three different cartridge treatments by trying to saturate their binding sites as decribed in experimental. A typical chromatogram of an extract from milk processed on commercial SPE cartridges is shown in Fig. 2.

To evaluate the reproducibility of TSP 1, TSP 2, TSP 3 and commercial SPE cartridges, peptide extraction was carried out on five columns of each type. The

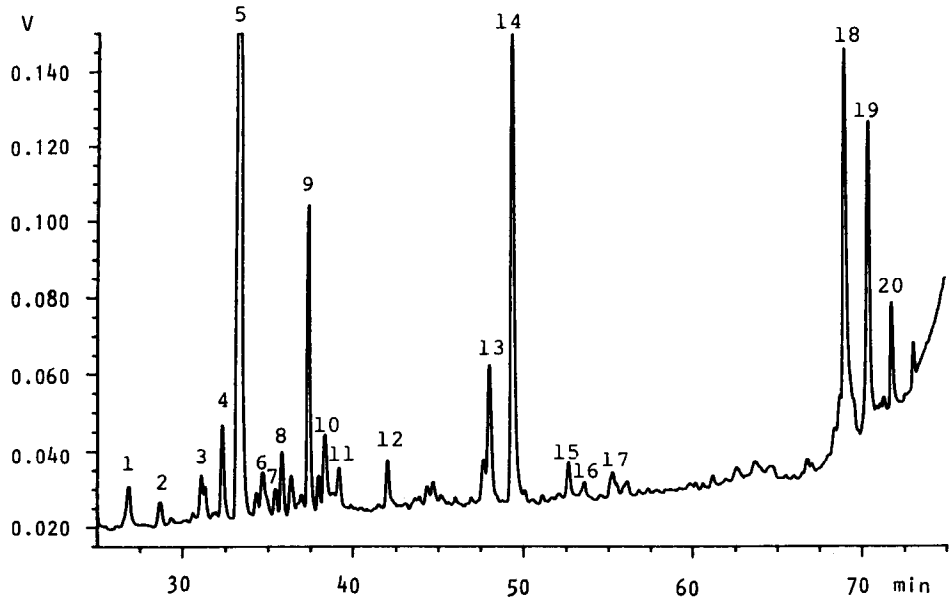

Fig. 2. Typical chromatogram of extracted peptides from dairy skim milk, after extraction on a commercial cartridge. Samples treatment and chromatographic conditions as Experimental; protein precipitation at pH 4.6, extraction of 125-ml sample on solid-phase cartridge, and injection in the HPLC system.

TABLE I

COMPARATIVE EVALUATION OF YIELD AND REPRODUCIBILITY USING TREATED (TSP) AND COMMERCIAL SPE CARTRIDGES FOR THE EXTRACTION OF MILK PEPTIDES

	Commercial	TSP 1	TSP 2	TSP 3
Lowest variation = peak 10; R.S.D. (%)	4.0	3.2	3.5	3.0
Highest variation = peak 1; R.S.D. (%)	23.2	15.0	12.8	3.8
Average yield ratio: TSP / commercial	1 (reference)	1.16	1.13	1.17

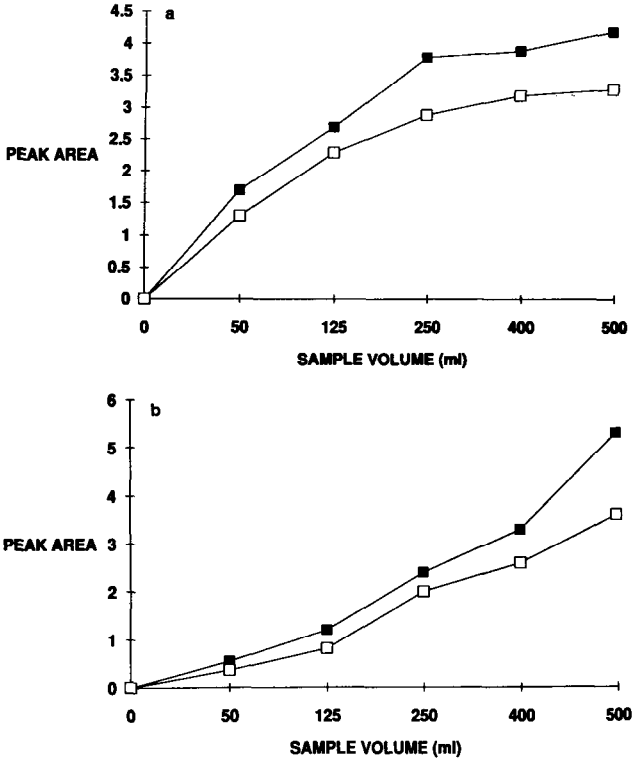

Fig. 3. Variation of HPLC peak area with extracted sample volume: Representative peaks were chosen from the chromatogram in Fig. 2. (a) Peak 13, typical of type I peptides. (b) Peak 18, typical of type II peptides. ■ = Samples extracted on TSP 3 cartridges. □ = Samples extracted on commercial cartridges. Sample treatment as in Experimental.

R.S.D. of the peak areas corresponding to the seventeen major peptides of each chromatogram were calculated as described in Experimental. The results (Table I) show that the peak areas are systematically larger and the R.S.D. smaller with the TSP cartridges, specially with TSP 3, than with commercial ones. This improvement of cartridge efficiency may be attributed to the saturation of irreversible adsorption sites (residual silanol groups) by the material present in the different conditioning solutions. In the subsequent extraction, peptide losses were minimized and cartridge capacity was standardized by using α-lactalbumin treatment (TSP 3).

Moreover, the average yield ratio calculated from the seventeen peak areas, compared with that obtained from commercial cartridges, is higher for all the treated cartridges.

The choice of α-lactalbumin (TSP 3) is based on the better control of this solution compared with the skim milk mixture. Moreover, this protein exhibits a high interaction with reversed-phase material [28,29] and its small size suggests complete access to the adsorbing sites present in the smaller pores.

In order to evaluate their effective capacities, five TSP 3 cartridges were compared with commercial ones by separately processing skim milk samples with volume 50–500 ml. The peak areas were plotted *versus* the sample volume processed in the extraction step (Fig. 3).

For all these experiments an increase in the yield of peptides extracted was observed with TSP cartridges compared with commercial SPE cartridges, and it appeared that a sample volume of 125 ml is enough for valuable analysis.

Two typical peptide retention profiles were observed. In type I, the curve plateaued after 125–250 ml of sample (Fig. 3a shows the capacity curve for peak 13), suggesting a limited number of fixation sites of the peptides considered. Type II exhibited a continuous, almost linear increase (Fig. 3b shows the capacity curve for peak 18).

This means that the difference observed between commercial and TSP cartridges is much higher than the R.S.D. and that, in all cases, TSP cartridges exhibit better performance than commercial ones. We chose peaks 13 and 18 to illustrate this effect because of their low R.S.D. deviation after five assays: peak 13 had an R.S.D. of 4.5% and 4.3% for commercial and TSP 3 cartridges, respectively, and peak 18 had an R.S.D. of 4.9% and 2.6%, respectively, measured for a volume of 125 ml.

Qualitative evaluation of the method efficiency on a milk peptide mixture

Peptide N3 (see Materials), a complex hydrolysate obtained from milk, was added to skim milk. Samples (0.16 g/l final concentration) were processed as described above with TSP cartridges (Fig. 4b). The peptide analysis was compared with direct HPLC analysis (Fig. 4a) of peptide N3 hydrolysate (4 g/l in water). Each chromatogram was run in triplicate and very similar results were obtained. Direct HPLC analysis shows at least 65 peptides of various hydrophobicities. The chromatogram from the milk extract is very similar to this reference chromatogram. Only the first five peaks, corresponding to the most hydrophilic peptides, were partially lost.

This model shows the efficiency of the method over a very large range of hydrophobicity, which allows the study of the very complex mixtures of peptides produced by the natural proteolysis of milk.

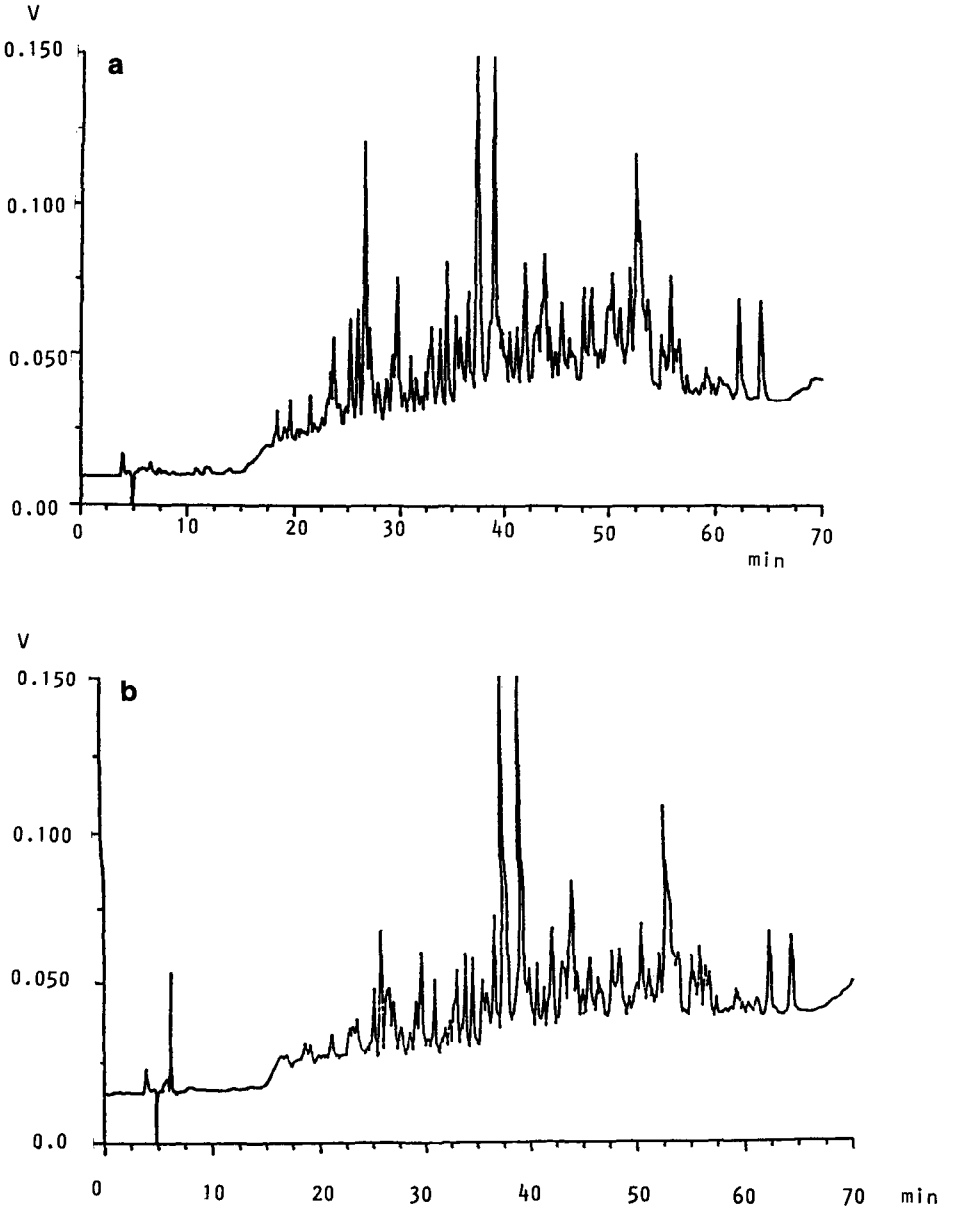

Fig. 4. HPLC analysis of peptide N3: (a) direct HPLC analysis of 4 g/l solution in water; (b) HPLC analysis of peptides extracted from peptide N3 added to skim milk at a final concentration, of 0.16 g/l.

Effects of extraction-cartridge processing on commercial SPE cartridges from different suppliers

The studies described above were all performed on commercial cartridges from J.T. Baker. The effects of treatment with α-lactalbumin (TSP 3) was also evaluated on

TABLE II

EFFECTS OF α-LACTALBUMIN TREATMENT ON CARTRIDGES FROM DIFFERENT SUPPLIERS

Supplier	R.S.D. for the total area of peaks (%)	
	Commercial cartridge	TSP 3 cartridge
J. T. Baker	11	3.6
Waters	15	6.7
Alltech	9.7	7
Supelco	11	6

cartridges from three other suppliers, Waters, Alltech and Supelco. In all the experiments the same skim milk was used. Samples were extracted on five untreated and five α-lactalbumin-treated cartridges, from each supplier. Table II sums up the results.

It appears that treatment with α-lactalbumin upgrades all four makes of cartridge, using coefficient of variation measurements and yields as comparison criteria.

CONCLUSIONS

The analytical method presented here affords a convenient way to evaluate the presence of peptides in milk and to follow the effect of proteolysis. Previously, only proteolysis by plasmin has been extensively analysed, and many products have been identified [30–41]. Some authors have reported the appearance order of peptides [41]. These data, obtained from model systems (pure proteic material solutions), do not account for the complex interactions occuring in milk or for the effective conformation of proteins in micellae.

The method described in this paper can be used to compare proteolysis occurring in real milk samples with that observed in models. It is based on HPLC evaluation of peptides in skim milk, following SPE.

The combination of protein precipitation at pH 4.6 with SPE on α-lactalbumin-treated commercial cartridges permitted the analysis of industrial milk samples with improved reproducibility. As SPE concentrates the peptides in a sample, only small volumes of milk (50 ml) are necessary. This suggests possible routine application of this method in milk quality-control laboratories.

This method has several advantages over other techniques. It gives a representative profile of the peptides in the sample, with most of the hydrophobic and hydrophilic peptides present. The reduction of non-specific adsorption on solid cartridges increases peptide recovery and significantly reduces measurement variability.

Work is currently underway to identify specific peptides that could serve as tracers of proteolysis.

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