J. Dairy Sci. 97:1–6
http://dx.doi.org/10.3168/jds.2013-7526

Short communication: Determination of lactoferrin in Feta cheese whey with reversed-phase high-performance liquid chromatography

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ABSTRACT

In the current paper, a method is introduced to determine lactoferrin in sweet whey using reverse-phase HPLC without any pretreatment of the samples or use of a separation technique. As a starting point, the most common HPLC protocols for acid whey, which included pretreatment of the whey along with a sodium dodecyl sulfate-PAGE step, were tested. By skipping the pretreatment and the separation steps while altering the gradient profile, different chromatographs were obtained that proved to be equally efficient to determine lactoferrin. For this novel 1-step reverse-phase HPLC method, repeatability was very high over a wide range of concentrations (1.88% intraday to 5.89% interday). The limit of detection was 35.46 μg/mL [signal:noise ratio (S/N) = 3], whereas the limit of quantification was 50.86 μg/mL (S/N = 10). Omitting the pretreatment step caused a degradation of the column's lifetime (to approximately 2,000 samples). As a result, the lactoferrin elution time changed, but neither the accuracy nor the separation ability of the method was significantly influenced. We observed that this degradation could be easily avoided or detained by centrifuging the samples to remove fat or by extensive cleaning of the column after every 5 samples.
Key words: whey, lactoferrin, reverse-phase HPLC, Feta cheese

Short Communication

During cheese making, after the casein curd separates from milk and following coagulation of the casein proteins through the action of chymosin (rennet) or organic or mineral acid, the remaining thin, watery liquid is called whey (Zall, 1992). Sweet whey with a pH between 5 and 6 contains valuable constituents. The complex of whey proteins is thought to be the most important due to its high biological and nutritional value (Madureira et al., 2007). Lactoferrin (**LF**), one of the minor whey proteins, has attracted a lot of scientific interest because of its unique properties.

Lactoferrin is a glycoprotein of the transferring family; it has a molecular weight of about 80 kDa (García-Montoya et al., 2012). Whereas it was identified as a milk protein in 1960 (Tomita et al., 2009), LF is also present in most biological fluids (exocrine secretions and neutrophil granules in mammals; Conesa et al., 2008). Its ability to bind iron eventually led to the discovery of its antibacterial activity (Jenssen and Hancock, 2009); furthermore, it interacts with molecular and cellular components of both hosts and pathogens (García-Montoya et al., 2012). Several studies have demonstrated the potential antiviral, antifungal, and antiparasitic activity of LF toward a broad spectrum of species (Wakabayashi and Takase, 2006). Lactoferrin is also considered to be an important host defense molecule during infant development (Jenssen and Hancock, 2009). During the last decade, it has become evident that oral administration of LF exerts several beneficial effects on the health of humans and animals, including antiinfective, anticancer, and antiinflammatory effects. This has enlarged the application potential of LF as a food additive (Wakabayashi and Takase, 2006).

For the determination or quantification of the LF included in food, several analytical methods have been suggested. The most commonly used methods are SDS-PAGE, HPLC, and MS. In most HPLC methods, an extensive preparation step of the samples is included to separate LF. The extraction of high-value dairy proteins normally requires extensive pretreatments of milk

Received September 26, 2013.
Accepted February 12, 2014.
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to remove fat and caseins by centrifugation, precipitation, Ca^{2+} chelation, or filtration. Such pretreatments can result in significant loss of protein yield or activity (Palman and Elgar, 2002).

Whey composition and sensory characteristics vary depending on the kind of the whey (acid or sweet), the origin of the milk (cow, sheep, goat, and so on) and the feed of the animal that produced the milk, the cheese-processing method used, the time of the year, and the stage of lactation. In the present study, it was decided to test sweet whey samples resulting from Feta cheese making. Feta cheese is made by minimum 70% ovine milk and maximum 30% caprine milk with the use of both lactic acid starter cultures and rennet. The yearly production of protected-designation-of-origin Feta cheese (produced only in parts of Greece) is 95,500 t, resulting in 300,000 t of sweet whey.

The objectives of the current paper were, first, to develop a reverse-phase (**RP**) HPLC method that allows the quantification of LF directly in sweet whey without any pretreatment of the samples or use of a separation technique. Second, as no studies are available about the amount of LF in Feta cheese or Feta cheese whey, to investigate the LF content in Feta cheese whey based on the developed method (and this during the peak Feta cheese-making period of January to June).

Samples and Standards

Whey was collected from Feta cheese-makers in the area of Tyrnavos, Greece, during the period from January to June 2010. Whey samples were immediately deep-frozen at −18°C and stored until the analysis. No pretreatment was applied before the analysis, apart from filtration with 25-mm filters and 0.45-μm Cellulose Acetate Blue Luer Lock filters (Restek, Bellefonte, PA). Lactoferrin from bovine milk (90% purity; Sigma-Aldrich, Milwaukee, WI) standards were used. Bovine whey was obtained after laboratory scale cheese-making. Two-percent commercial yogurt was added in pasteurized bovine milk at 32°C. After 20 min, 0.8% rennet, and 0.2% $CaCl_2$ was added. The cheese curd was cut in 2- × 2-cm cubes 30 min later and left to rest for 10 more minutes before whey was collected.

HPLC Separation

For the experiment, a VWR Hitachi module with a diode-array detector L-2455 Elite La Chrom (VWR International. Radnor, PA) was used. The column was a Zorbax SB 300-C8, 4.6 × 150 mm, 5-μm particle size (Agilent, Santa Clara, CA). Mobile phase A consisted of acetonitrile, water, and trifluoroacetic acid in a ratio of 50:950:1 (vol/vol/vol) and mobile phase B consisted again of acetonitrile, water, and trifluoroacetic acid in a ratio of 950:50:1 (vol/vol/vol). Linear gradient within a run time of 32 min and combination of flow rates from 1 to 1.5 mL/min were used: 0 to 10 min isocratic to 33% B, 10 to 20 min 33 to 38% B, 20 to 29 min to 38% B, 29 to 32 min to 39–33% B. The column temperature was to 50°C, whereas the injection volume was 10 μL. The detection was by absorbance at 205 nm.

SDS-PAGE Analysis

The SDS-PAGE was carried out as described by Laemmli (1970), using acrylamide-bis acrylamide 30% solution (mix 29:1; Sigma-Aldrich), TEMED (Acros Organics, ThermoFisher Scientific, Fairlawn, NJ), and ammonium persulfate (Merck, Darmstadt, Germany). A 4% stacking and a 16% separating gel were used. One hundred micrograms of each sample was incubated with the denaturing loading solution (12% glycerol, 1.2% SDS, 5.4% mercaptoethanol, saturated bromophenol blue) at 100°C for 5 min and then loaded onto the gel. Electrophoresis was performed in 160- × 180- × 0.5-mm polyacrylamide gel using SE600 Vertical Stab Gel Unit (Hoefer Scientific Instruments, San Francisco, CA) at 4 to 5°C with a constant voltage of 200 V. A solution of LF from bovine milk (90% purity; Sigma-Aldrich) in sterile distilled water was used as a control. Coomassie blue staining [0.1% Coomassie Brilliant Blue R 250 (BioRad, Cambridge, MA) in 50% methanol and 10% acetic acid] was carried out for 1 h, followed by an overnight gel-destaining step (10% acetic acid, 10% glycerol, and 80% methanol at 1:1 in water) at room temperature, and subsequently visualizing the protein bands on the gel. Proteins were identified on the basis of molecular weight with recombinant prestained protein standards (BioRad, Cambridge, MA) by comparing the migration pattern to the bovine LF. The Coomassie-stained bands were quantified by scanning the protein bands, followed by densitometric analysis using ImageJ software (http://imagej.nih.gov/ij/), with bovine LF as reference band of standard concentration (25 μg).

Method Development

When only LF standards were tested, the elution time of LF was 10.2 min (Figure 1) and its peak had a distinctive shape. When Feta cheese whey samples were tested under the same conditions the elution time of LF was at 10.1 min (Figure 2). Elution was monitored at the range of 200 to 400 nm and detection of LF was achieved at both 205 and 278 nm. At 205 nm, the detection of LF has the advantage that it improves the sensitivity of response, as the LF peak was better baseline resolved.

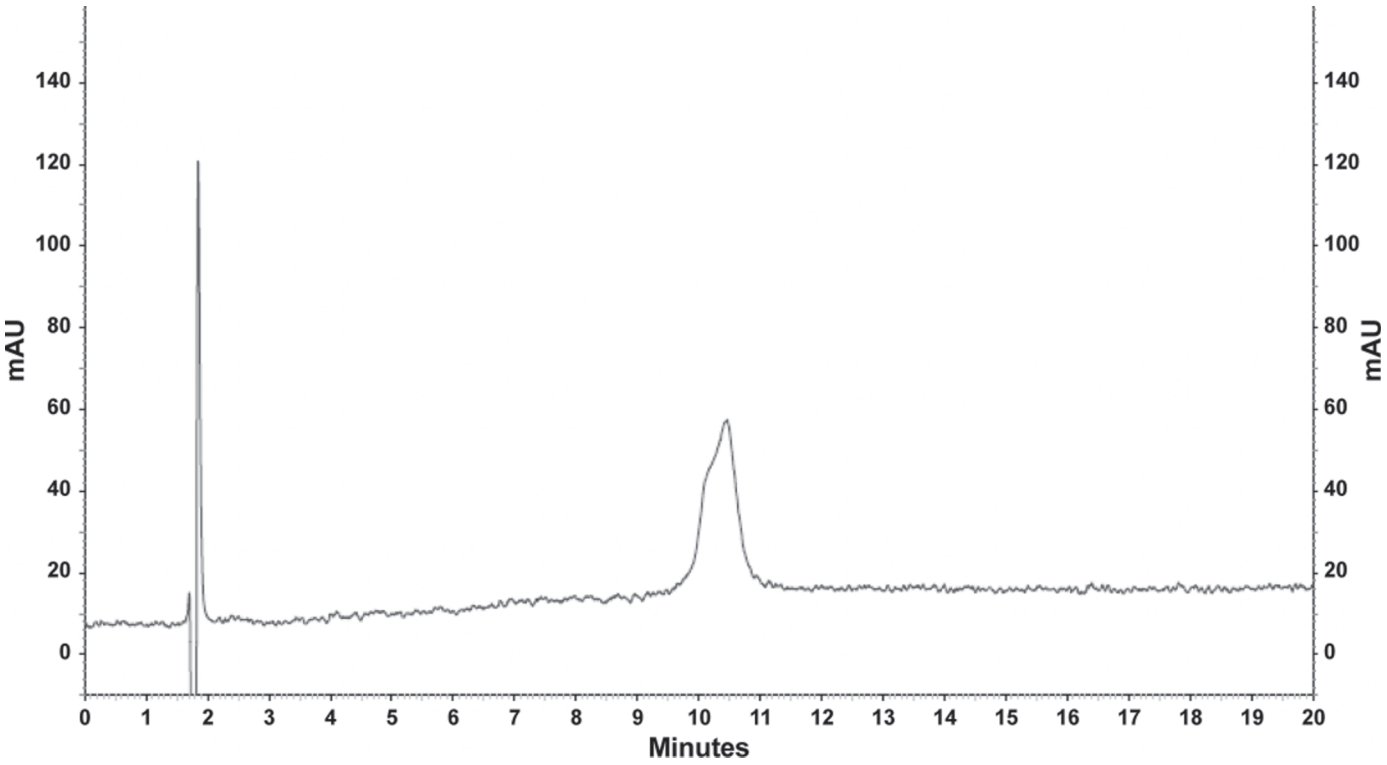

Figure 1. Chromatograph of lactoferrin 150 μg/mL (lactoferrin peak elution time = 10.2 min). Color version available in the online PDF.

Optimization of the HPLC method was carried out using standard solutions of LF. The calibration curve was extrapolated in the concentration range of 50 to 1,200 μg/mL (y = 49,509, x = 106, R^2 = 0.99). The sensitivity of the method is the slope of the calibration curve of LF. Repeatability was estimated by calculating the relative standard deviation (RSD). A standard LF sample of 300 μg/mL was measured 6 times on 3 different days. Intraday repeatability was found to be 1.88% RSD, whereas interday repeatability was found to be 5.89% RSD. The limit of detection for 3 S/N (signal:noise ratio) was found to be 35.46 μg/mL while the limit of quantification for 10 S/N was found equal to 50.86 μg/mL.

Palmano and Elgar (2002) reported an RP-HPLC method for the determination of LF. According to their results and in conditions similar to the present study, LF is eluting very close but ahead of BSA. These authors tested the assay ability of LF in the presence of very large BSA concentrations. Although it is possible to quantify very low LF levels using the RP-HPLC method, care should be taken in delineating LF from BSA (when present), whereas highly sensitive electronic data acquisition is necessary to ensure reliability. Furthermore, it was reported that reproducibility of measurements deteriorates when the ratio of LF to BSA decreases below 1:10.

By using the Palmano and Elgar (2002) RP-HPLC method to determine proteins in Feta cheese whey, Moatsou et al. (2003) reported that BSA has a retention time equal to that of bovine LF and similar to that of bovine α-lactalbumin. Lactoferrin was not taken into account during the calculations due to its very low concentration in the whey (compared with the BSA content).

In the present study, standards of BSA were used first to determine its elution time to ensure that the peak identified as LF did not include any BSA. Bovine serum albumin elutes close to but after LF (12.4 min), thus confirming the findings of Moatsou et al. (2003).

Table 1. Average amount of lactoferrin (LF) in Feta cheese whey for a 6-mo period from January to June 2010[1]

Item (μg/mL)	LF
January	302 ± 16
February	284 ± 19
March	263 ± 22
April	269 ± 22
May	267 ± 20
June	248 ± 13
Average	272 ± 24

[1]The highest amount of LF was obtained in January whereas the lowest in June; a slowly decreasing trend exists from January to June.

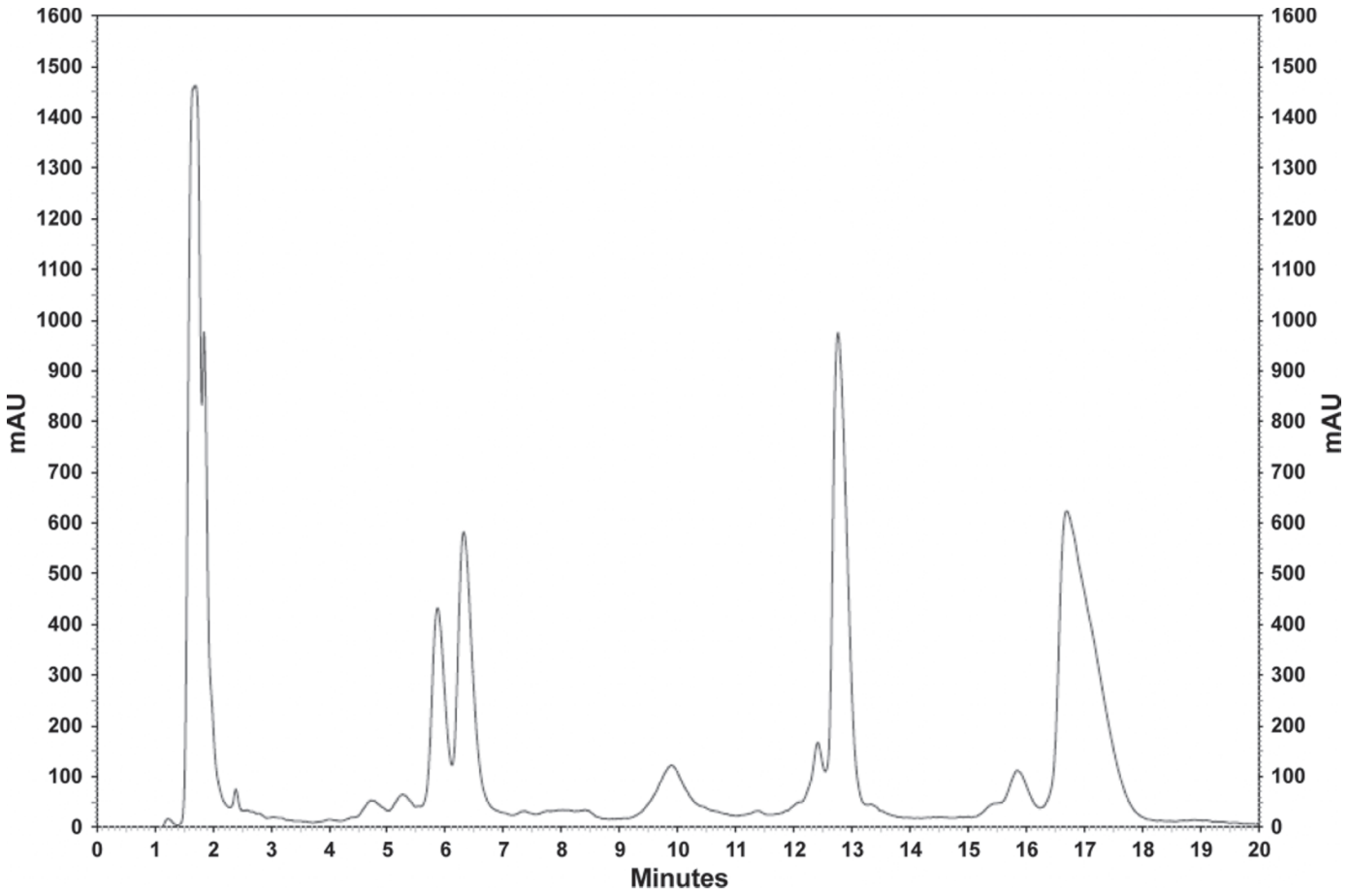

Figure 2. Chromatograph of Feta cheese whey (lactoferrin peak elution time = 10.1 min). Color version available in the online PDF.

Second, Feta cheese samples of whey were tested with the addition of BSA standards (0–250 μg/mL), and the resulting chromatographs indicated a small peak after the LF peak that was increasing with higher BSA concentrations. No alteration of the LF peak was observed during these tests. Finally, the samples of Feta cheese whey enriched with BSA were tested with addition of LF standard in different concentrations (0–250 μg/mL). In this case, the LF peak increased linearly with increasing LF concentration, whereas, again, no alteration of the BSA peak was observed.

Drakova et al. (2009) tested a single-step HPLC for the determination of LF in goat milk under similar conditions. In that study, milk was processed to obtain whey that was subsequently analyzed. The resulting whey chromatograph is much different from the one obtained in the present study, and, due to the limited information provided, the results of the 2 studies cannot be compared.

To validate that no cross contamination of the LF peak by other minor whey proteins occurred, an SDS-PAGE analysis was carried out (Figure 3). This analysis confirmed the presence of LF, and its concentration in given samples was found equal to 272 ± 17 μg/mL; whereas, with the 1-step RP-HPLC analysis, it was found to be 274 ± 19 μg/mL. As the results of the 2 methods match, the possibility of a cross contamination of the LF peak is annihilated.

Finally, the applicability of the method on bovine whey samples was tested as well. To minimize the dependency on the cheese-making method, white soft cheese was produced from bovine milk by following the Feta cheese-making process step-by-step. The resulting bovine cheese whey samples were analyzed with the novel 1-step RP-HPLC method and the obtained chromatographs were quite similar with the Feta cheese whey samples, indicating that determination of LF is also possible in bovine whey.

Case Study

The developed method was used to determine the amount of LF in Feta cheese whey throughout the peak Feta cheese-making period in Greece (January to June). To minimize variation of the parameters that influence the presence of LF, a specific area for sampling was

Table 2. Average amount of lactoferrin in Feta cheese whey from 5 producers for a 6-mo period from January to June 2010

Item (μg/mL)	January	February	March	April	May	June
Cheese-maker 1	298	314	267	289	290	260
Cheese-maker 2	279	291	261	239	271	229
Cheese-maker 3	304	277	251	184	236	255
Cheese-maker 4	323	274	297	290	271	241
Cheese-maker 5	306	264	239	265	271	257

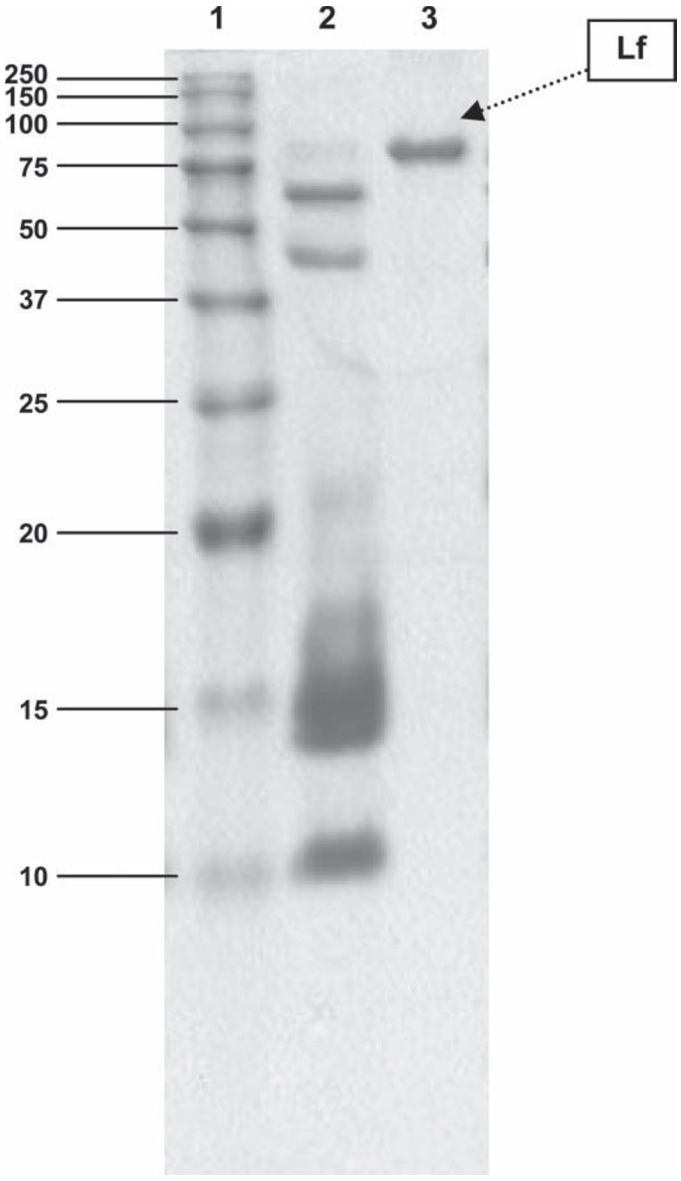

Figure 3. Results of SDS-PAGE with molecular weight markers (expressed in kDa; column 1), Feta cheese whey (column 2), and control bovine lactoferrin (Lf; column 3). Color version available in the online PDF.

chosen (Tyrnavos, Greece). The average pH value of all samples was equal to 6.37 ± 0.07. The concentration of LF was calculated by the method of standard curves. Different concentrations of commercial bovine LF (Sigma-Aldrich) were used, corrected by the given purity. All samples were examined twice, on different days and in duplicate each time. The average amount of LF found in Feta cheese whey throughout January to June 2010 is presented in Table 1.

A 2-factor variance analysis without replications was performed for the data of Table 2 to determine the possible dependency of the LF amount in Feta cheese on the cheese producer or the month of the year. It was concluded that the amount of LF present in the whey did not depend on the specific cheese producers; however, a dependency was found on the specific month of the year. In addition, no interaction between the 2 examined parameters could be found. This analysis was performed at a 99% confidence level.

The average amount of LF in Feta cheese, 272 ± 24 μg/mL, is much higher than that in bovine cheese whey, 100 μg/mL (Plate et al., 2006). This result was expected, as the concentration of LF depended on both the milk origin and the cheese-making process (Blaschek et al., 2007). The main material for Feta cheese is ovine milk (at least 70% of total milk used), which has a total whey protein of 17 g/L (Vincenzetti et al., 2008), which equals to almost 3 times of the total whey protein in cow's milk (6.3 g/L; de Wit, 1998). Goat milk (maximum 30% in Feta cheese making) has a whey protein content of 0.7 g/L. A greater amount of other whey proteins have been reported in Feta cheese whey when compared with bovine cheese whey. In particular, Moatsou et al. (2003) demonstrated that Feta cheese whey protein concentrates (WPC) had a much higher β-lactoglobulin and higher α-lactalbumin percentage than those reported for bovine WPC (Morr, 1985; de Wit et al., 1986; Kinghorn et al., 1995; Holt et al., 1999; Elgar et al., 2000). Casper et al. (1999) found a ratio equal to 4:1 in ovine WPC by SDS-PAGE.

Main Contributions

A novel 1-step RP-HPLC method for the determination of LF in whey was developed. The original aim of

investigating the results of skipping pretreatment and separation steps and experimenting only in modifying the gradient profile resulted in good LF peak resolution when whey was tested. This is a fast and accurate method—within its limits—that could be used either for everyday analysis or, in the worst case scenario, as a useful qualitative screen for presence or absence of LF. For the first time the average concentration of LF in Feta cheese whey was examined and it was found to be significantly higher than in bovine cheese whey. This fact, along with other studies about the nutritional value of Feta, can be a strong tool to support Feta's advantages, as a product with unique characteristics, and its further commercial exploration.

ACKNOWLEDGMENTS

This work was supported by project PFV/10/002 (Center of Excellence OPTEC - Optimization in Engineering) of the KU Leuven Research Council, projects G.0930.13 and KAN2013 1.3.189.13 of the Fund for Scientific Research-Flanders, and the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian Federal Science Policy Office. J. Van Impe holds the chair Safety Engineering sponsored by the Belgian chemistry and life sciences federation essenscia.

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