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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

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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.

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