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Analytical Methods

Rapid chromatographic determination of caseins in milk with photometric and fluorimetric detection using a hydrophobic monolithic column

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ABSTRACT

Reverse-phase liquid chromatographic methods using a hydrophobic C_{18} monolithic column and on-line photometric and fluorimetric detection for the determination of the major casein (CN) proteins in milk are presented. The separation of αs_1-CN, αs_2-CN, β-CN and κ-CN was achieved in only five minutes. Fluorimetric detection enabled better analytical results than photometric detection. Thus, the dynamic ranges of the calibration graphs and detection limits obtained using fluorimetric detection were (mg mL^{-1}): αs_1-CN (0.74–10.0, 0.22), αs_2-CN (0.15–10.0, 0.045), β-CN (0.68–10.0, 0.20) and κ-CN (0.21–10.0, 0.06). The analytical features of the photometric method, which does not allow the quantification of β-casein, were (mg mL^{-1}): αs_1-CN (1.5–9.0, 0.45), αs_2-CN (1.4–10.0, 0.43) and κ-CN (0.4–9.0, 0.12). Precision data, expressed as relative standard deviation, ranged between 0.6% and 5.3% for the fluorimetric method and between 2.4% and 6.2% for the photometric method. Both methods were applied to the analysis of three different milk samples, obtaining recoveries in the ranges of 86.6–103.2% and 92.0–106.5% using fluorimetric and photometric detection, respectively.

1. Introduction

Quality control of milk and its derivatives is a very demanding field that requires the availability of economic and time-saving accurate methods, as these food products constitute a major source of proteins for human consumption (Thompson, Boland, & Singh, 2009). Bovine milk contains 3–3.5% (w/v) of proteins, which are usually separated into casein (CN) (80%) and whey proteins (20%), according to their different solubility behaviours. CN contains αs_1-CN, αs_2-CN, β-CN and κ-CN fractions, which are insoluble at their isoelectric point (pH 4.6 at 20 °C), while whey proteins are soluble at this pH value. CN has been the subject of intensive research, in an effort to unravel its structure and the composition of CN micelles (Farrell et al., 2004; Fox & Brodkorb, 2008).

The interest in the CN determination in milk and dairy products justifies that several electrophoretic and liquid chromatographic (LC) methods have been described. Adulteration and authenticity studies of cheese (Veloso, Texeira, Peres, Mendonca, & Oliveira, 2004) and yoghurt (Kaminarides & Koukiassa, 2002) have been carried out using urea–polyacrylamide gel electrophoresis (urea–PAGE). Sodium dodecyl sulfate–PAGE electrophoresis (SDS–PAGE) has been used in a multi-laboratory assay to study the digestion of β-CN under simulated conditions (Defernez, Mandalari, & Mills, 2010). Also, several methods involving capillary electrophoresis and UV detection have been described for CN determination in milk and dairy products (Miralles, Krause, Ramos, & Amigo, 2006; Miralles et al., 2001; Rehder-Silinski & McGown, 2003).

Although some LC methods for CN determination involve the use of ion-exchange columns (Holland, Yazdi, Titapiccolo, & Corredig, 2010; Plank, Andres, Krause, & Winter, 2008), reverse-phase-LC (RP-LC) has been more extensively used (Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008; Bonizzi, Buffoni, & Feligini, 2009; Bordin, Cordeiro-Raposo, de la Calle, & Rodriguez, 2001; Veloso et al., 2004; Wang, Zhang, Wang, & Li, 2009). Most of these RP-LC methods require between 35 and 45 min to obtain CN separation using a flow rate in the range of 0.25–1 mL min^{-1} and UV detection. Although these methods reach a high resolution, the availability of methods allowing faster separations is desirable for quality control in the dairy industry.

The advantages of monolithic columns over packed particle columns for the separation of large biomolecules have been widely described (Guiochon, 2007; Jungbauer, 2005; Samanidou & Karageorgou, 2011; Van de Meent & de Jong, 2011). The continuous and biporous structure of these columns, with macropores (2.0 μm) that permit high flow-rates with low back-pressure, and mesopores (13.0 nm) that provide a high surface area, enables fast

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and efficient biomolecules separations using conventional LC pumps. Other properties of these columns are high permeability and low mass transfer resistance, which are suited to the separation of analytes with low diffusion constants, such as proteins and peptides. In contrast to packed particle columns in which both convective (laminar and turbulent) and diffusive mass transport are present, only laminar convective mass transport is characteristic for monolithic columns (Albreht & Vovk, 2012).The potential of monolithic columns for protein and peptide separation by capillary liquid chromatography has been recently described (Liang, Zhang, & Zhang, 2013; Van de Meent & De Jong, 2009; Van de Meent & De Jong, 2011).

In spite of the analytical interest in monolithic columns, they have been scarcely used in milk analysis up to now. These columns have been mainly applied to the determination of antibiotic residues in milk (Samanidou & Karageorgou, 2011), although a method for the separation and isolation of major whey proteins has been recently proposed (Albreht & Vovk, 2012). This method provided the separation of β-lactoglobulin, bovine serum albumin and α-lactoalbuminin in 4 min, but it was not applied to the analysis of real samples. Also, a miniaturised multi-channel LC system for high-throughput analysis using monolithic silica capillary columns and β-CN as analyte model has been described (Shintani et al., 2005).

The study described here shows the usefulness of a reverse-phase octadecyl-silica monolithic column for the separation in only five minutes of the major CN proteins as a primary contribution to the innovation in CN determination and fast milk analysis. In addition to the use of UV detection, the usefulness of fluorimetric detection, which is based on the measurement of the native fluorescence of the protein tryptophan residue, using 280 and 340 nm as excitation and emission wavelengths, respectively, has been demonstrated.

2. Materials and methods

2.1. Apparatus and instruments

An Agilent 1200 series liquid chromatography system composed of a quaternary pump, a degasser unit, a vial autosampler, a thermostated column compartment and two on-line detectors, a diode array detector (DAD) and a fluorescence spectrometer (FLD) was used. The chromatographic separation was performed using an Onyx monolithic C_{18} column (Phenomenex, Torrance, CA), 100 × 4.6 mm i.d., pore sizes: mesopores (13 nm), macropores (2 μm).

2.2. Reagents

All chemicals used were of analytical reagent grade. The solutions were prepared freshly with distilled water and filtered through a 0.45-μm filter by using a glass Millipore filtration unit (Millipore, Billerica, MA). Purify proteins were obtained from Sigma–Aldrich (Madrid, Spain). Stock solutions (15 mg mL^{-1}) of caseins were prepared as follows: a premixed standard containing all of the casein proteins was prepared by dissolving them in the minimum volume of a denaturing solution containing 8 M urea, 165 mM Tris, 44 mM sodium citrate and 0.3% β-mercaptoethanol. Stock and intermediate solutions were stored at 4 °C in the dark and were stable for at least two weeks. Working standard solutions were prepared from intermediate solutions by their dilution in a blank solution constituting a mixture of 70:30 (v/v) of the two solvent chromatographic solutions used (designed as A and B).

The mobile phase used for the chromatographic separation constituted solvent A, 0.1% trifluoroacetic acid (TFA, 99.9%; Sigma–Aldrich) in water, and solvent B, acetonitrile/TFA/H_2O (95:0.1:4.9, v/v/v), which were mixed in gradient mode during the chromatographic separation.

2.3. Manifold and procedure

Standard or diluted sample solutions (10 μl), containing the analytes at concentrations within their corresponding dynamic ranges, were injected into the column. The mobile phase was pumped at 3 mL min^{-1} and the system operated under a linear gradient starting at 2.5% of solvent B and ending at 10% of the same solvent. Two chromatograms were obtained by monitoring the variation of the absorbance with time at 220 nm in the DAD and by measuring the native fluorescence of the protein tryptophan residue at λ_{ex} 280 nm, λ_{em} 340 nm in the FLD. The time necessary to achieve the corresponding chromatograms under the selected conditions was 5 min, with a delay of 0.15 min between the photometric and fluorimetric chromatograms. Then, clean-up and conditioning steps were applied to have the chromatographic system ready for the next injection after 30 s.

Chromatograms were taken using the original software supported for both DAD/FLD detectors and the raw data of signal intensity and time were exported and treated using adequate software packages for the estimation of the peak areas and the main chromatographic parameters (retention and half-width time, theoretical plate number, efficiency, selectivity and resolution factor).

2.4. Analysis of samples

Three bovine milk samples (whole, skimmed and fortified), which were purchased from local market, were analysed. These samples were frozen at −20 °C until analysis. Prior to the analysis, 2 mL of each sample were treated at pH 4.3 and 20 °C with 1 mM acetic acid/sodium acetate buffer solution to precipitate CN and centrifuged at 3200 rpm for 10 min at 4 °C. CN was dispersed in the same buffer solution and centrifuged again under the same conditions. This procedure was repeated twice, in order to separate the fat layer. The residue was treated with 1.6 mL of a denaturing solution containing 8 M urea, 165 mM Tris, 44 mM sodium citrate and 0.3% β-mercaptoethanol, and incubated for 30 min. An appropriate volume of this denaturing sample solution was diluted in the blank solvent solution described in Section 2.2. The diluted sample was filtered through a 0.45-μm cellulose membrane and directly injected into the chromatographic system following the procedure indicated above. Each determination was the mean of three measurements.

2.5. Results and discussion

2.5.1. Study of the chromatographic separation using photometric and fluorimetric detection systems

Chromatographic separations of CN using conventional packed column and photometric detection are usually monitored at 220 or 280 nm. Both wavelengths were assayed for the separation of these proteins using the monolithic column, but the chromatographic peaks obtained at 280 nm showed absorbance values

Table 1
Optimisation of variables.

Variable	Range studied	Value chosen
Mobile phase: Solvent A (TFA:H_2O%)	–	0.1:99.9
Solvent B (ACN:TFA:H_2O%)	–	95:0.1:4.9
Flow-rate, mL min^{-1}	0.5–4	3
Injection volume, μL	5–100	10
Column temperature, °C	25–50	40