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Multidimensional analysis of denatured milk proteins by hydrophobic interaction chromatography coupled to a dynamic surface tension detector

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Abstract

Multidimensional analysis of denatured milk proteins is reported using high-performance liquid chromatography (HPLC) combined with dynamic surface tension detection (DSTD). A hydrophobic interaction chromatography (HIC) column (a TSK-Gel® Phenyl-5PW column, TosoBiosep), in the presence of 3.0 M guanidine hydrochloride (GdmHCl) as denaturing agent is employed as the mobile phase. Dynamic surface tension is measured through the differential pressure across the liquid–air interface of repeatedly growing and detaching drops. Continuous surface tension measurement throughout the entire drop growth (50 ms to 4 s) is achieved, for each eluting drop of 4 s length, providing insight into both the kinetic and thermodynamic behavior of molecular orientation processes at the liquid–air interface. An automated calibration procedure and data analysis method is applied with the DSTD system, which allows two unique solvents to be used, the HIC mobile phase for the sample and a second solvent (water for example) for the standard, permitting real-time dynamic surface tension data to be obtained. Three-dimensional data is obtained, with surface tension as a function of drop time first converted to surface pressure, which is plotted as a function of the chromatographic elution time axis. Experiments were initially performed using flow injection analysis (FIA) with the DSTD system for investigating commercial single standard milk proteins (α-lactalbumin, β-lactoglobulin, α-, β-, κ-casein and a casein mixture) denatured by GdmHCl. These FIA–DSTD experiments allowed the separation and detection conditions to be optimized for the HIC–DSTD experiments. Thus, the HIC–DSTD system has been optimized and successfully applied to the selective analysis of surface-active casein fractions (αs1- and β-casein) in a commercial casein mixture, raw milk samples (cow's, ewe's and goat's milk) and other diary products (yogurt, stracchino, mozzarella, parmesan cheese and chocolate cream). The different samples were readily distinguished based upon the selectivity provided by the HIC–DSTD method. The selectivity advantage of using DSTD relative to absorbance detection is also demonstrated.

Keywords: Hydrophobic interaction chromatography; Dynamic surface tension detection; Detection, LC; Proteins

1. Introduction

Proteins exhibit a high propensity to migrate and adsorb to air/water and oil/water interfaces and decrease the interfacial tension because of their amphiphilic structure [1–3]. This property of proteins allows the stabilization of colloidal dispersions which are thermodynamically unstable and which otherwise quickly phase separate. Thus, proteins play a key role in the stabilization of foams, emulsions, and composite systems in food products and in cosmetics [4–10]. In addition to lowering interfacial tension, adsorbed proteins can form a strong viscoelastic film via intermolecular interactions which can withstand thermal and mechanical perturbations [11,12]. The latter property makes proteins more desirable than low-molecular-weight amphiphiles as surfactants in emulsion- and foam-type food products.

Proteins differ significantly in their surface activity and in their ability to stabilize colloidal systems due to differences in structural properties (size, shape, charge distribution) and stability and flexibility [7,13]. The surface activity of proteins has been shown to vary with changes in these properties [14–16], e.g., by applying denaturing conditions (pH changes, temperature increase or chemical modification

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with denaturing agents) [17–22], by chemical modification (e.g., succynilation, disulfide reduction, enzymatic hydrolysis) [23], or by interaction with other compounds (e.g., sugars) [24].

Milk proteins in soluble and dispersed form are widely valued as food ingredients with excellent surface-active and colloid-stabilizing characteristics [6–27]. Caseins represent about 80–85% of milk proteins, and they play a key role in human nutrition, in dairy industry and as additives in food, paints and glues because of their emulsifying properties [6,26,27–29]. Caseins are classified as α_{s1}-, α_{s2}-, β- and κ-caseins, representing about 38, 10, 36 and 13%, respectively, of the casein fraction. The B variant of α_{s1}-casein is predominant, containing 8 mol phosphate/mol protein, and it is characterized by largely hydrophobic amino acidic residues [30]. α_{s2}-Casein is characterized by a high content of phosphate (about 10–13 mol of phosphate/mol protein) and by two –SH groups, being the most hydrophilic of the α-casein fraction [30]. The most common A variant of β-casein is characterized by 5 mol phosphate/mol protein, all located in the hydrophilic N-terminal region of 47 amino acids, and by a hydrophobic tail. This division of two functional domains confers to β-caseins surfactant properties. κ-Casein is characterized by 1 mol phosphate/mol protein, a hydrophilic C-terminal peptide with a high negative charge density and a predominantly hydrophobic remainder that has a small positive charge at near-neutral pH. The reciprocal ratio between these four fractions strongly affects micelle size, size distribution and, in general, the chemical-physical properties of milk. It is known, e.g., that the proportion of κ-casein increases as the average size of the micelles decreases, as well as the proportion of α_{s2}-casein increases significantly with ease of sedimentation [30].

As many of these properties are fundamental in milk processing and in the dairy-food industry, development of methods for fast separation and determination of α_{s1}-, α_{s2}-, β- and κ-casein fraction is of interest. In addition, the development of novel instrumental devices for the knowledge of the surface active properties of milk proteins associated to specific fractions separated by high-performance liquid chromatography (HPLC) is critically important in fundamental as well as applied problems.

In previous studies, we have developed a detector capable of measuring dynamic surface tension, i.e., a dynamic surface tension detection (DSTD) system. The DSTD system has evolved from an optical measurement-based instrument to a pressure sensor-based instrument, and has been used in conjunction with flow injection analysis (FIA) and HPLC [31–38]. Most recently, we have shown that real time dynamic surface pressure values are easily obtained when the calibration process is utilized in conjunction with pneumatic drop detachment [36,37]. The initial version of the calibration procedure utilizes the differential pressure signals from three drop profiles, namely the solvent (water or the eluent buffer of HPLC, standard in the solvent, and sample solution containing the analyte in the solvent) obtained from the same experimental run [36]. More recently, a novel DSTD calibration procedure is presented and applied, based on a dual-mobile phase calibration procedure that allows the analyst to apply different mobile phases for the analyte (e.g. denatured protein) and the calibration standard [39]. This is a very important achievement in the evolution of the DSTD technique. The dual-mobile phase calibration permits more types of samples to be examined in complicated or unusual sample matrices, while utilizing a convenient standard. Further details on the data processing using the dual-mobile phase calibration procedure are reported in Section 2.

The aim of this manuscript is to report the DSTD system as a specific detector for hydrophobic interaction chromatography (HIC) for the selective detection and identification of proteins based on their surface-active properties. The unique form of chemical selectivity provided by DSTD enables one to obtain analyte-specific information, with potential use in formulation science and quality control applications. Despite the various studies reported in the literature regarding the surface activity and properties of casein at interfaces [40,41], no data have been reported for the hyphenation between a separation technique and a surface activity-based detector for selective protein analysis. In a previous paper, in fact, DSTD coupled to hydrophobic interaction chromatography was utilized for the selective measurement and analysis of surface active impurities in protein formulations, where proteins were not surface active [35].

In this paper, instead, the HIC–DSTD technique is applied for the direct measurement of the surface activity of eluting caseins in milk and diary samples. Denaturants both for the solubilization of dairy raw samples [3.0 M guanidine hydrochloride (GdmHCl)] and in the eluent buffers of HIC separations (3.0 M GdmHCl) are employed, based on previous successful applications of denaturants in HIC [42–45]. First, experiments were performed using the FIA–DSTD system for investigating and optimizing the surface activity of commercial single standard milk proteins (α-lactalbumin, β-lactoglobulin, α-, β-, κ-casein and a casein mixture) denatured by GdmHCl. Thus, the HIC–DSTD system has been optimized and successfully applied to the selective determination and quantitation of the detectable surface active casein fractions (αs1- and β-casein) in a commercial casein mixture, raw milk samples (cow's, ewe's and goat's milk) and other diary products (yogurt, stracchino, mozzarella, parmesan cheese and chocolate cream). The HIC–DSTD configuration provides additional analyte selectivity over traditional detection methods such as absorbance detection. The advantage of the HIC–DSTD configuration in the food analysis application is illustrated.

2. Theory

DSTD is based upon a growing drop method, implementing a pressure sensor where the pressure signal is dependent

upon surface tension properties of a given sample. The pressure sensor, mounted in the side arm off the main flow of a capillary, measures the internal pressure of the growing drops relative to atmospheric pressure. The mobile phase flows through a short capillary, forming drops at a specially designed sensing capillary tip. The drops subsequently formed either can be blown off via an air burst supplied by a solenoid valve (pneumatic detachment) or fall off when the force of gravity overcomes the attractive forces between the tip and the drop (gravity detachment). During pneumatic detachment mode, drops are blown off the sensor tip at a preset rate, well before the drops would fall due to gravity, resulting in a higher data density than the gravity detachment mode (generally about a five-fold faster drop rate in this work). In addition, using pneumatic detachment, the data is much easier to process into a usable form, i.e., surface pressure or surface tension. Because all the drop pressure data profiles are the same length in time, the start of drop growth is easily determined, objectively arranged, and easily and quickly processed via the following procedure.

Pressure measurement is made throughout the growth of each drop forming at the capillary tip. The liquid–air surface tension, γ, for a drop of radius r, is related to the pressure signal P (pressure relative to atmospheric pressure), described by Eq. (1), a time dependent Young–Laplace equation [36].

$$P(t)_{\mathrm{X,Mi}} = \frac{2\gamma(t)_{\mathrm{X,Mi}}}{r(t)} + P_{\mathrm{C,Mi}} \tag{1}$$

where the P, γ, and r are all a function of time, t, during drop growth, the X represents sample, Mi mobile phase and P_{C} accounts for the viscosity-based pressure drop in the capillary tubing and the relative position of the sensor from the capillary tip and is essentially time independent [36]. The $P(t)$ are simply the individual drop profile pressure data measured by the pressure sensor in the DSTD. The dynamic surface pressure of a given sample in mobile phase, $\pi(t)_{\mathrm{X,Mi}}$, can be defined as the surface tension of the sample in mobile phase, $\gamma(t)_{\mathrm{X,Mi}}$, subtracted from the surface tension of the mobile phase itself, $\gamma(t)_{\mathrm{Mi}}$.

$$\pi(t)_{\mathrm{X,Mi}} = \gamma(t)_{\mathrm{Mi}} - \gamma(t)_{\mathrm{X,Mi}} \tag{2}$$

The subtraction of the two surface tension values removes the pressure offset (P_{C}) from the surface pressure term, with P_{C} assumed to be constant for a given mobile phase. For the current work, the sample mobile phase will be the denaturing agent GdmHCl in phosphate buffer and the mobile phase for the standard will be water. The following will derive the calibration procedure used in the current study utilizing these two distinct mobile phases for the sample and standard.

First, the dynamic surface pressure of an analyte (A) in mobile phase one (M1), GdmHCl–phosphate buffer, can be expressed as function of the drop radius and pressure signals of mobile phase and the sample via the combination of Eqs. (1) and (2). A similar equation can be derived for the calibration standard (S) in mobile phase one.

$$\pi(t)_{\mathrm{A,M1}} = \frac{r(t)}{2}[P(t)_{\mathrm{M1}} - P(t)_{\mathrm{A,M1}}] \tag{3}$$

$$\pi(t)_{\mathrm{S,M1}} = \frac{r(t)}{2}[P(t)_{\mathrm{M1}} - P(t)_{\mathrm{S,M1}}] \tag{4}$$

By combining Eqs. (3) and (4), the dynamic surface pressure of the analyte in the denaturing agent is given by:

$$\pi(t)_{\mathrm{A,M1}} = \pi(t)_{\mathrm{S,M1}}\left[\frac{P(t)_{\mathrm{M1}} - P(t)_{\mathrm{A,M1}}}{P(t)_{\mathrm{M1}} - P(t)_{\mathrm{S,M1}}}\right] \tag{5}$$

where a standard value for $\pi(t)_{\mathrm{S,M1}}$ would need to be obtained from literature or measured in the laboratory. However, the surface pressure of the standard in mobile phase one (GdmHCl–phosphate buffer) is not readily available and a measurement in the laboratory is undesirable. Therefore it would be advantageous to have the standard in a water sample matrix for which a literature value is easily obtained. To obtain the surface pressure of the standard in the denaturing agent ($\pi(t)_{\mathrm{S,M1}}$), from Eq. (5), the following theory is useful and readily applied.

Using a standard in a water mobile phase (M2), the surface pressure ($\pi(t)_{\mathrm{S,M2}}$) can be defined as the following,

$$\pi(t)_{\mathrm{S,M2}} = \frac{r(t)}{2}[P(t)_{\mathrm{M2}} - P(t)_{\mathrm{S,M2}}] \tag{6}$$

Combining Eq. (6) with Eq. (4) and solving for $\pi(t)_{\mathrm{S,M1}}$, yields Eq. (7).

$$\pi(t)_{\mathrm{S,M1}} = \pi(t)_{\mathrm{S,M2}}\left[\frac{(P(t)_{\mathrm{M1}} - P(t)_{\mathrm{S,M1}})}{(P(t)_{\mathrm{M2}} - P(t)_{\mathrm{S,M2}})}\right] \tag{7}$$

Substitution of $\pi(t)_{\mathrm{S,M1}}$ from Eq. (7) into Eq. (5), followed by factoring out $(P(t)_{\mathrm{M1}} - P(t)_{\mathrm{S,M1}})$ yields:

$$\pi(t)_{\mathrm{A,M1}} = \pi(t)_{\mathrm{S,M2}}\left[\frac{(P(t)_{\mathrm{M1}} - P(t)_{\mathrm{A,M1}})}{(P(t)_{\mathrm{M2}} - P(t)_{\mathrm{S,M2}})}\right] \tag{8}$$

where the time-dependent surface pressure of an analyte (e.g., protein) in mobile phase one, a denaturing agent system, is determined using the surface pressure reference value of a standard in water utilizing the four $P(t)$ drop profiles in Eq. (8). This calibration procedure allows the proteins to be chemically modified with various denaturing agents (mobile phase one) and a typical standard to be run in water (mobile phase two) and permits the DSTD system to be easily implemented on-line. Thus, the surface pressure of the standard in water, $\pi(t)_{\mathrm{S,M2}}$, is then readily available from the literature and is independent of time and a constant at a given concentration. This derivation is an important step in the evolution of DSTD; it allows samples to be utilized in many solvent matrices, i.e., HPLC mobile phases, without changing the standardization step of the calibration, utilizing a standard in a water sample matrix. In fact, it guarantees a correct calculation of the analyte surface pressure whatever elution buffer is used in the chromatographic experiment and during gradient elution in which analytes

are eluted in a mobile phase with continuous changes in composition.

3. Experimental

3.1. Materials

β-Lactoglobulin (L-3908) and α-lactalbumin from bovine milk (L-6010) were purchased from Sigma (St. Louis, MO, USA). α-, β- and κ-caseins and a casein mixture (product No. 22078) were purchased from Fluka (Buchs, Switzerland) (22084 α-caseins ≥90% M_r 23 500, 22 086 β-caseins ≥80% M_r 24 000, 22 087 κ-caseins ≥70% M_r 19 000). The buffer solutions were prepared from monobasic monohydrate sodium phosphate, dibasic anhydrous sodium phosphate (BDH, Poole, UK), ammonium sulfate (Bio-Rad Labs., Hercules, CA, USA) and guanidine hydrochloride, abbreviated as GdmHCl (Sigma). The buffer solutions contained 0.1 M phosphate at a pH of 7.2 (PBS). Water deionized with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout and degassed prior to use. A TSK-Gel® Phenyl-5PW column (TosoBiosep, Stuttgart, Germany), porosity 1000 Å, with dimensions of 7.5 cm × 7.5 mm i.d. was used for all the experiments.

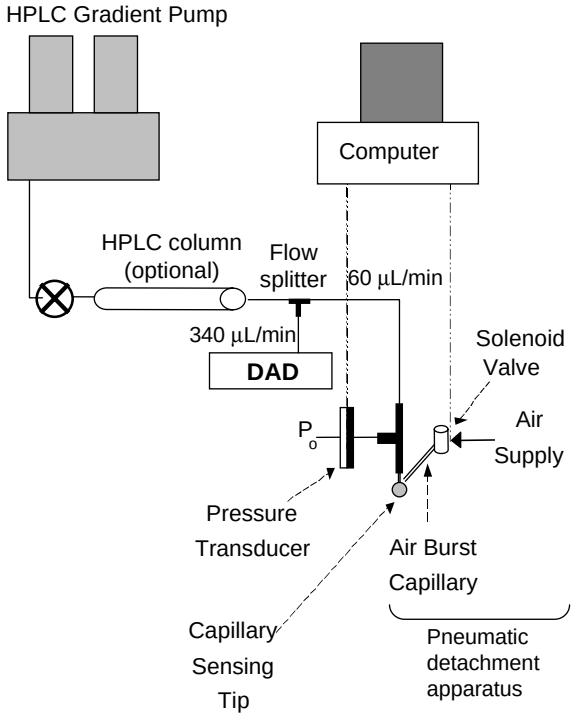

Fig. 1. HPLC–DSTD instrument configuration. The flow splitter delivers 60 µl/min to the DSTD system and 340 µl/min to the diode array absorbance detection (DAD) system. The differential pressure, $P(t)$, across the liquid–air interface of each eluting drop, is measured with respect to the atmospheric pressure, P_0 by a pressure transducer on a side arm of the DSTD capillary sensor tip. A small capillary directs short burst of air regulated to 25 psi at ∼45° angle via a computer-controlled solenoid valve and pneumatically detaches the eluent from the sensor tip.

3.2. Working solutions

Native protein solutions were prepared by dissolving lyophilized powder in PBS at the concentration indicated. Denatured protein working solutions were prepared freshly by dissolving protein lyophilized powder in PBS, containing GdmHCl at the indicated concentration. The concentrations of stock solutions of proteins were determined spectrophotometrically [46,47]. Samples were injected at $t \geq 1$ h after preparation, without significant variations of their surface tension value during the working day. Unprocessed milk samples from cow, goat and ewe were obtained from a farmer in Toscany (Italy), diluted in PBS, 3.0 M GdmHCl and injected. Parmesan, mozzarella and stracchino cheese stock solution was prepared solubilizing chopped cheese in PBS, 3.0 M GdmHCl, centrifuged and diluted in the same buffer before the injection. Chocolate cream was prepared using the same procedure, centrifuged, diluted and injected. Yogurt was diluted 1:1 in PBS, 3.0 M GdmHCl and injected.

3.3. Instrumentation

A detailed schematic of the DSTD configuration with pneumatic detachment is shown in Fig. 1. A narrow bore

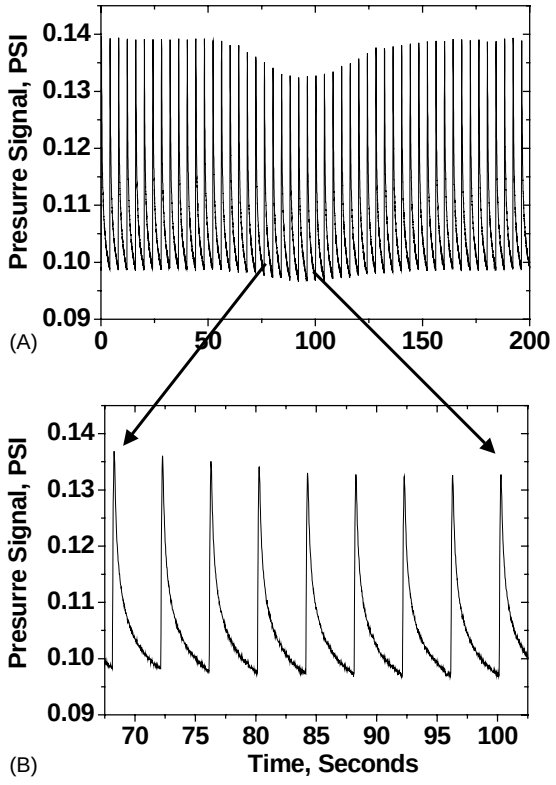

Fig. 2. (A) Raw pressure data observed from the DSTD system. Injection plug shown is 5% acetic acid sample with 4 µl drops (i.e., 4 s each) controlled via pneumatic detachment. (B) The steady-state region of the injected sample plug is shown for clarity. Inspection reveals reproducible drops and no observable disturbance due to the pneumatic detachment process.

HPLC gradient pump (P2000, ThermoQuest) equipped with a mechanical degassing system (SC1000, ThermoQuest) was connected to a Diode Array Detector (UV6000, ThermoQuest). A total flow of 0.4 ml/min was split in order to provide 340 μl/min to DAD and 60 μl/min to DSTD. Samples were introduced via a ten-port injection valve (Rheodyne PR700-102-1 Cotati, CA, USA). A poly(etheretherketone) (PEEK) injection loop of 85 μl of tubing (Upchurch, Oak Harbor, WA) was used for all experiments. The pneumatic detachment apparatus for the DSTD, shown in Fig. 1, consists of a small capillary directing a repeated short burst of in-house air at 45° angle to the capillary sensing tip via a computer controlled solenoid valve to detach drops from the sensor tip capillary at controlled time intervals. Pneumatic detachment was performed at a rate of 0.25 Hz, corresponding to 4 μl drops at 60 μl/min, using a solenoid valve (MBD002, Skinner Valve, New Britain, CT, USA). The pressure sensor (Validyne P305D-20-2369, Northridge, CA, USA) was configured with a sensing membrane (Validyne diaphragm 3–36) that was optimized for the response time of the DSTD measurements of interfacial kinetics. The sensor capillary tip was made from a short piece of polyether ether ketone (PEEK) tubing, 457 μm i.d. × 635 μm o.d. All data were collected at 20 kHz with a personal computer (850 MHz Pentium, Intel, Santa Clara, CA, USA) equipped with a data acquisition card (AT-MIO-16XE-50, National Instruments, Austin, TX, USA). The data were averaged to 50 points/sec prior to saving. Data collection was completed using LABVIEW (Version 5, National Instruments) with programs written in-house. Subsequent data analysis was performed using MATLAB 5.0 (MathWorks, Natick, MA, USA). The DSTD configuration is essentially identical to that detailed in previous work [36–39,48]. Absorbance measurements for the determination of protein concentration were performed using a Varian DMS 300 spectrophotometer (Varian, Palo Alto, CA, USA).

3.4. Calibration procedure

Typical raw pressure data from the DSTD are shown in Fig. 2. Inspection of Eq. (8) reveals four drop profiles are necessary in the dual-mobile phase calibration to obtain the dynamic surface pressure of a protein sample in GdmHCl: $P(t)_{M1}$, $P(t)_{A,M1}$, $P(t)_{M2}$ and $P(t)_{S,M2}$. The drop profiles are extracted from data such as in Fig. 2. Fig. 3A shows drop profiles of 6.0 M GdmHCl ($P(t)_{M1}$) and 1500 mg/l commercial casein mixture in 6.0 M GdmHCl ($P(t)_{A,M1}$), upper and lower profiles, respectively. Similarly, Fig. 3B shows drop

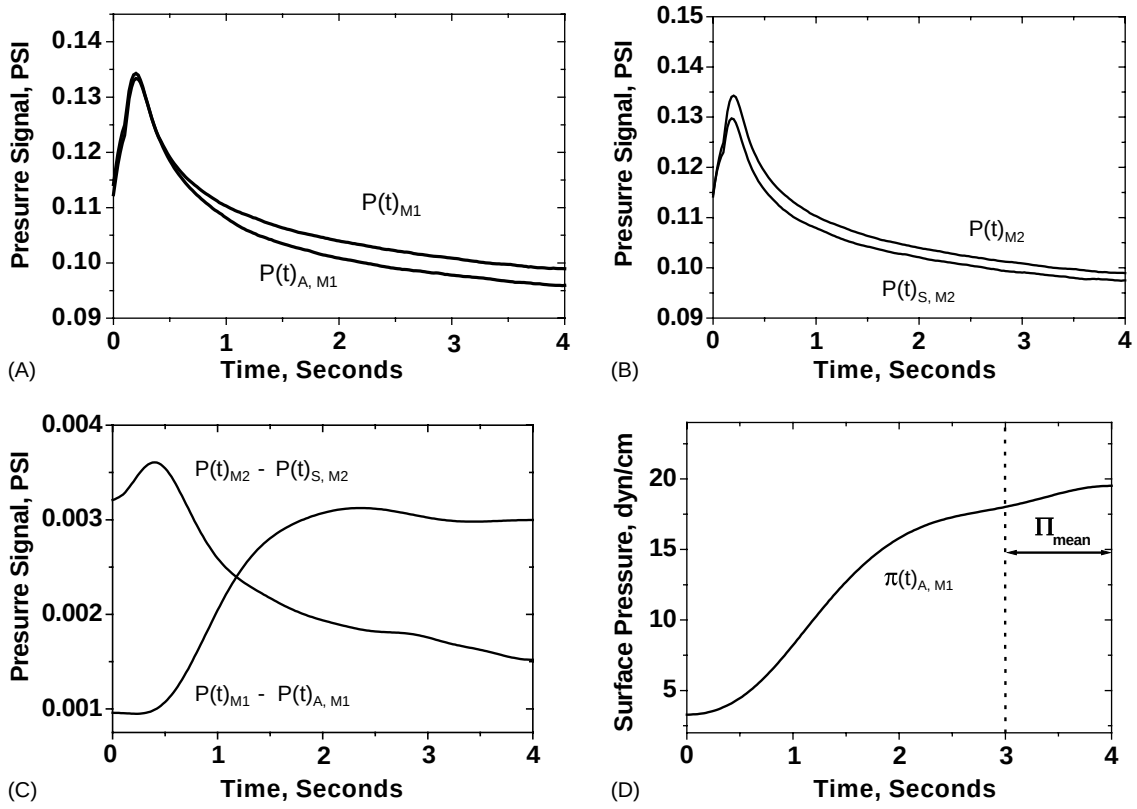

Fig. 3. The dual-mobile phase calibration procedure is demonstrated using four drop profiles along with Eq. (8). (A) Drop profiles for 6.0 M GdmHCl ($P(t)_{M1}$) and 1500 mg/l commercial casein mixture in 6.0 M GdmHCl ($P(t)_{A,M1}$). (B) Drop profiles for water ($P(t)_{M2}$) and 5% acetic acid standard in water ($P(t)_{S,M2}$). (C) Analyte and standard drop profiles subtracted from their respective mobile phases, with drop profiles shown in (A) and (B), respectively. (D) The resulting dynamic surface pressure (π) plot for 1500 mg/l casein mixture in 6.0 M GdmHCl utilizing Eq. (8). Π_{mean} represents the surface pressure signal averaged over the last 50 points (1 s equivalent) at the end of the 4 s drop (average values from 3.0 to 4.0 s from each surface pressure plot).

profiles of water ($P(t)_{M2}$) and the standard, 5% acetic acid in water ($P(t)_{S,M2}$), upper and lower profiles, respectively. Fig. 3C shows the result of the subtraction of the two pair of drop profiles in the denominator and the numerator of Eq. (8), $P(t)_{M1} - P(t)_{A,M1}$ and $P(t)_{M2} - P(t)_{S,M2}$, respectively. The resultant dynamic surface pressure plot, $\pi(t)_{A,M1}$, for 1500 mg/l casein mixture in 6.0 M GdmHCl/0.1 M phosphate buffer at pH 7.2 is seen in Fig. 3D. The four drop profiles from Fig. 3A and B and the surface pressure of the 5% acetic acid standard in water (10.3 dyn/cm) were applied using Eq. (8) to obtain Fig. 3D. In Fig. 3D is also indicated the Π_{mean} value, defined as the surface pressure signal averaged over the last 50 points (1 s equivalent) at the end of the 4 s drop (average values from 3.0 to 4.0 s from each surface pressure plot).

3.5. Chromatographic conditions

In all the experiments, 3.0 M GdmHCl was kept constant in the mobile phase in order to prevent casein aggregation. The elution conditions were the following: 3 min isocratic elution followed by 25 min linear salt gradient from 100% high salt buffer (PBS, 1.8 M ammonium sulfate, 3.0 M GdmHCl) to 100% low salt buffer (PBS, 3.0 M GdmHCl) at 20 ± 1 °C. A flow rate of 0.4 ml/min in the column was used. The mobile phase was filtered by a 0.45 μm cellulose acetate filter (Millipore) and degassed (10 min) before using.

4. Results and discussion

Experiments were initially performed using a FIA–DSTD system for investigating and optimizing the surface activity of commercial single standard milk proteins, α-lactalbumin, β-lactoglobulin, α-, β-, κ-casein and a casein mixture, denatured by GdmHCl. Fig. 4 shows the surface pressure data at the end of the 4 μl drop (Π_{mean}) of α-, β-, κ-casein (1000, 500 and 1000 mg/l, respectively) and their commercial mixture (500 mg/l) (A) and the two major whey proteins (B), α-lac (1000 mg/l) and β-LG (1000 mg/l) solutions as a function of the GdmHCl concentration in PBS 0.1 M pH 7.2 employed for dissolving the sample (denaturation curves). In the same figures the trend of surface pressure signal of GdmHCl (d) as a function of its concentration is shown.

The surface-active behavior of the two denatured globular whey proteins, as the concentration of GdmHCl increases, is substantially different than observed for α-, β- and κ-casein. As the denaturant concentration increases, α-lac and β-LG increase their surface activity at liquid/air interface. These data are consistent with literature data [1]. As denaturation of proteins is more effective, typical events occur which increase their surface activity: unfolding, increased flexibility, exposure of hydrophobic patches. For the caseins, while taking in account their concentration, in the absence of the denaturant (native conditions) the surface pressure signal for β-casein is about twice that of α-casein and 8-fold

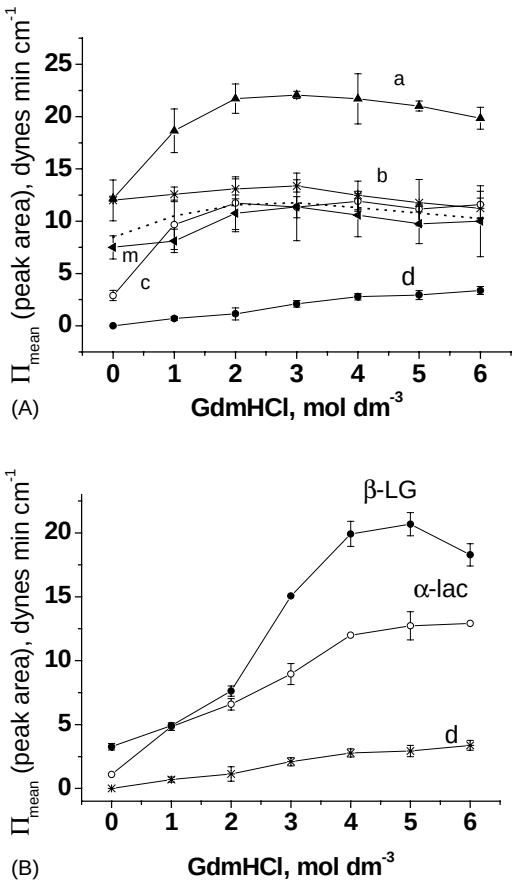

Fig. 4. Surface pressure of milk proteins as a function of increasing concentration of guanidine hydrochloride. The peak area of FIA–DSTD data is reported after processing the three-dimensional data as Π_{mean} vs. elution time plot. (A) Milk caseins in PBS and GdmHCl: (a) α-casein 1000 mg/l; (b) β-casein 500 mg/l; (c) κ-casein 1000 mg/l; (d) casein mixture 500 mg/l. (B) Milk whey proteins caseins in PBS and GdmHCl: β-lactoglobulin 1000 mg/l and α-lactalbumin 1000 mg/l. In both the plots the data obtained by the injection of the denaturant GdmHCl in PBS, are reported (d curve).

greater than for κ-casein. This is in agreement with several studies that indicate β-casein is more surface active than α_{S1}-casein, α_{S1}-casein representing the 80–85% of α-casein fraction [49,50]. No data are available regarding the surface activity of κ-casein. From the literature it is known that both α- and β-casein are random-coil type flexible proteins with few elements of a α- and β-structure [51] and that they experience no conformational constraints to unfold/spread at the liquid/air interface. However, the differences in surface activity between the two caseins have been ascribed to different structures, net charge, and mean residue hydrophobicity [52]. α-Casein is considered to be more ordered (≈10% α-helix and 20% β-sheet) than β-casein (≈7% α-helix and 13% β-sheet), and whereas it has a net charge of −21 at pH 7.0, β-casein is only about −13.9 net charge. Also, the mean residue hydrophobicity of α-casein is lower (1170 cal/mol) than that of β-casein (1330 cal/mol) [53].

As the GdmHCl concentration increases, the β-casein surface pressure signal is independent of denaturant

concentration; α- and κ-casein show, instead, an increase of their surface pressure signal, reaching a plateau value for GdmHCl concentrations ≥2.0 M. In the GdmHCl concentration range of 2.0–6.0 M the surface pressure signal of α-casein is about the same as β-casein; in the same range the κ-casein surface pressure is one-half of that one of β-casein.

In Fig. 4B the experimental results obtained for the commercial casein mixture are reported and compared with the theoretical trend (dotted line) resulting from the linear combination of the Π_{mean} values of the pure milk casein constituting the mixture, taking into account that the mixture contains about 49% α-casein, 45% β-casein and 6% κ-casein [44]. It is worth noting that the experimental and the theoretical curves do not differ significantly, showing an additive effect of α-, β- and κ-casein to the surface pressure signal both in native and denaturant conditions.

Following the DSTD calibration procedure described by Eq. (8) in Section 2, the dynamic surface pressure responses for α-, β-, κ-casein and casein mixture solutions were determined. Fig. 5 shows the adsorption and diffusion kinetics at the water/air interface of caseins during the drop time in native (Fig. 5A) and denatured conditions (6.0 M GdmHCl, Fig. 5B), observed at identical sample concentration (85 μg injected). Fig. 5 shows also the kinetics of the commercial casein mixture (m curve, continuous line) compared with the theoretical trend resulting from the linear combination of the Π_{mean} values of the pure milk casein constituting the mixture, taking into account the mixture composition (dotted line). Again, the good agreement of the two trends shows an additive effect of the surface pressure of the single caseins in the mixture.

The change in the surface pressure signal (kinetic signature) over the growth of a single drop is attributed to the finite time required for diffusion, adsorption and molecular arrangement of the protein at the liquid–air interface, as has been discussed in another study [54]. Despite the similar molecular masses of α-, β- and κ-casein of 23.5, 24.0 and 19.0 kDa, respectively, the kinetic responses are very different for the three caseins in native conditions, because

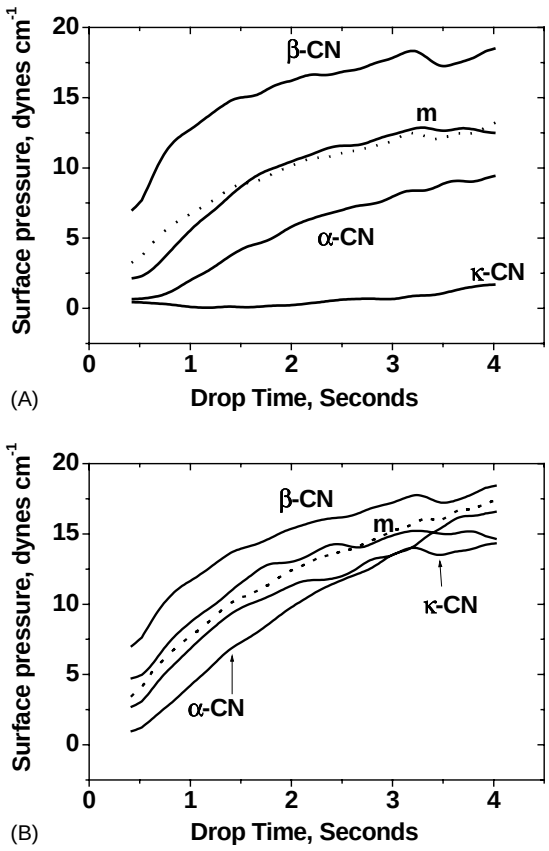

Fig. 5. Dynamic surface pressure plots for α-casein, β-casein, κ-casein and casein mixture (1000 mg/l) in (A) 0.1 M PBS pH 7.2 (native conditions) and (B) PBS, 6.0 M GdmHCl (denaturing conditions).

of differences in their structure, net charge and hydrophobicity [30]. GdmHCl, by breaking hydrogen bonds of the secondary structures and by the specific binding of Gdm^+ cations [55], changes significantly the characteristics of the DSTD kinetics due to the flexible polypeptide chains of α-, β- and κ-casein [56]. An overall increase in detection sensitivity for α- and κ-casein is observed.

Table 1
Results of FIA–DSTD calibration experiments for standard solutions of α-lactalbumin, β-lactoglobulin, α-, β-, κ-casein and casein mixture in native (0.1 M PBS, pH 7.2) and denatured conditions (0.1 M PBS, pH 7.2, 6.0 M GdmHCl)

Protein	Sensitivity (dyn min l cm^{-1} mg^{-1}) ± DS	*N*	*R*	Dynamic linear range (mg/l)
β-LG in PBS	0.0016 ± 0.0002	5	0.9861	500–1600
β-LG in PBS, 6.0 M GdmHCl	0.0200 ± 0.0005	6	0.9989	300–1000
α-Lac in PBS	0.0027 ± 0.0003	5	0.9860	800–1800
α-Lac in PBS, 6.0 M GdmHCl	0.0112 ± 0.0006	5	0.9950	200–800
α-CN in PBS	0.018 ± 0.0003	4	1	300–800
α-CN in PBS, 6.0 M GdmHCl	0.019 ± 0.0004	6	0.9990	300–1200
β-CN in PBS	0.034 ± 0.003	6	0.9867	300–1000
β-CN in PBS, 6.0 M GdmHCl	0.034 ± 0.0019	7	0.9923	300–1000
κ-CN in PBS	0.007 ± 0.0005	4	0.9963	500–1000
κ-CN in PBS, 6.0 M GdmHCl	0.026 ± 0.001	4	0.9970	500–800
CN mixture in PBS	0.031 ± 0.0007	4	1	300–800
CN mixture in PBS, 6.0 M GdmHCl	0.034 ± 0.0012	4	0.9987	400–1000

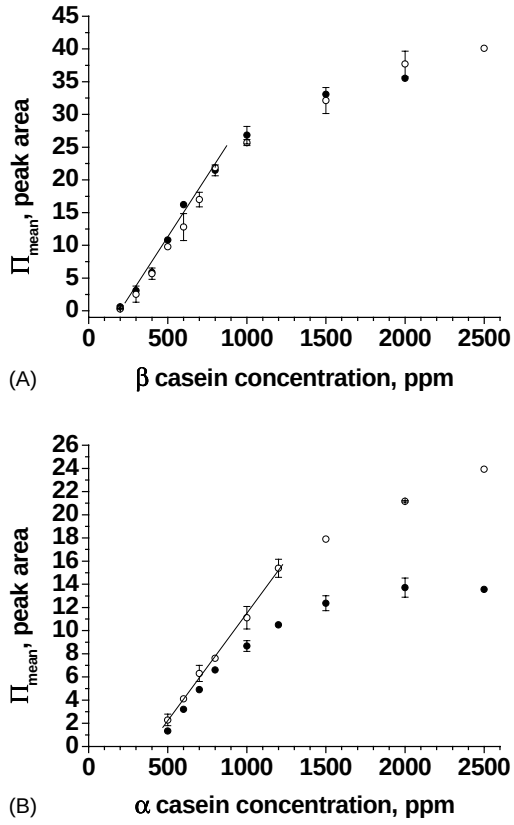

Fig. 6. Calibration plot for surface pressure data of β-casein (A) and α-casein (B) in PBS (native conditions) (solid circles) and PBS, 6.0 M GdmHCl (denaturing conditions) (open circles). A significant enhancement of the surface pressure of α-casein due to the denaturant is observed. The peak area of FIA–DSTD experiments is reported after processing the three-dimensional data as Π_{mean} vs. elution time plot.

While the growing drop technique is key for the observation of the kinetic response of proteins at air/water interfaces, the overall increase in detection sensitivity provided by the employment of protein denaturing agents is a very important finding for the analytical applications and employment of the DSTD as an HPLC detector. Table 1 shows the results of calibration experiments performed by the FIA–DSTD system on standard solutions of milk whey proteins and caseins in native (0.1 M PBS, pH 7.2) and denatured conditions (0.1 M PBS, pH 7.2, 6.0 M GdmHCl). Calibration curves have been obtained by plotting the surface pressure data at the end of the 4 μl drop (Π_{mean}) (peak area of FIA experiments) as a function of the concentration of untreated (native) and 6.0 M GdmHCl denatured caseins. Fig. 6 shows, for example, the calibration curves of α- and β-casein. From the ratio of the slopes of the calibration curves it can be calculated that the GdmHCl denaturation enhances sensitivity by about 12.5- and 4.1-fold for native β-LG and α-lac, respectively. For the caseins, a significant enhancement of 3.7-fold is observed only for κ-casein. For α-casein, however, the employment of GdmHCl improves the dynamic linear range. Taking in account the flow splitting in the apparatus of Fig. 1, with only 15% of the effluent delivered to the detector, the mass limit of detection (LOD) ranges between 0.05–0.2 μg of injected proteins and 8–30 ng of protein at the DSTD. The mass LOD values were calculated as the concentration corresponding to three times the standard deviation of the background, on the basis of the sensitivity factors reported in Table 1. On the basis of these results, we can assess that the employment of DSTD coupled with GdmHCl as denaturant offers a novel tool for a sensitive detection of proteins utilizing surface activity.

The DSTD system has been optimized and successfully coupled to HPLC, specifically HIC, for the selective determination of surface active proteins in a commercial casein mixture, milk raw samples (cow's, ewe's and goat's milk) and other diary products (yogurt and stracchino, mozzarella and parmesan cheese and chocolate cream). A TSK gel Phenyl HIC column was employed in the presence of 3.0 M GdmHCl in the mobile phase buffer. A 3.0 M GdmHCl concentration was chosen in order to enhance surface activity while avoiding a substantial increase in viscosity, adsorption phenomena and salt crystallization at the capillary tip. Fig. 7A shows a three-dimensional (3D) plot of the separation of the commercial casein mixture (297.5 μg injected) in a TSK Gel Phenyl HIC column by the HIC–DSTD apparatus. In the plot the surface pressure values are reported as a function of the drop time and the elution time. The data of Fig. 7 have been obtained after the subtraction of the baseline 3D data matrix (chromatographic run without sample injection). The same separation of the caseins shown in Fig. 7A is presented in Fig. 7B in the form of a topographical contour plot. In the contour plot the higher surface pressures values are depicted as darker shades; the baseline due to the mobile phase is the white background.

In Fig. 7C the two-dimensional plot of Π_{mean} processed from three-dimensional data, as a function of the elution time is shown and compared with the typical UV absorbance chromatogram at 280 nm simultaneously acquired by the diode array absorbance detector (Fig. 7D). In particular, the Fig. 7C was obtained by averaging along the drop time axis the surface pressure over the last 50 points (1 s equivalent) at the end of each 4 s drop (Π_{mean} defined in Fig. 3D) and by plotting the Π_{mean} value versus elution time. Despite the complex UV chromatogram (Fig. 7D) due to the co-elution of casein fractions with other unknown interferences, both the 3D and the 2D surface pressure plots (Fig. 7A–C) selectively indicate two surface-active components readily discernible. The two peaks have been assigned to α- and β-casein by injecting in the same operating conditions the standard solutions of the two proteins. κ-Casein was not detectable in this mixture due to being at a low concentration [43,44]. As with the surface pressure plots (Fig. 5B) obtained from individual drop profiles, the difference in surface pressure response between the two caseins is readily discernible. As discussed above, the measurable surface pressure difference is due to the kinetics of the protein interaction at the liquid–air interface.

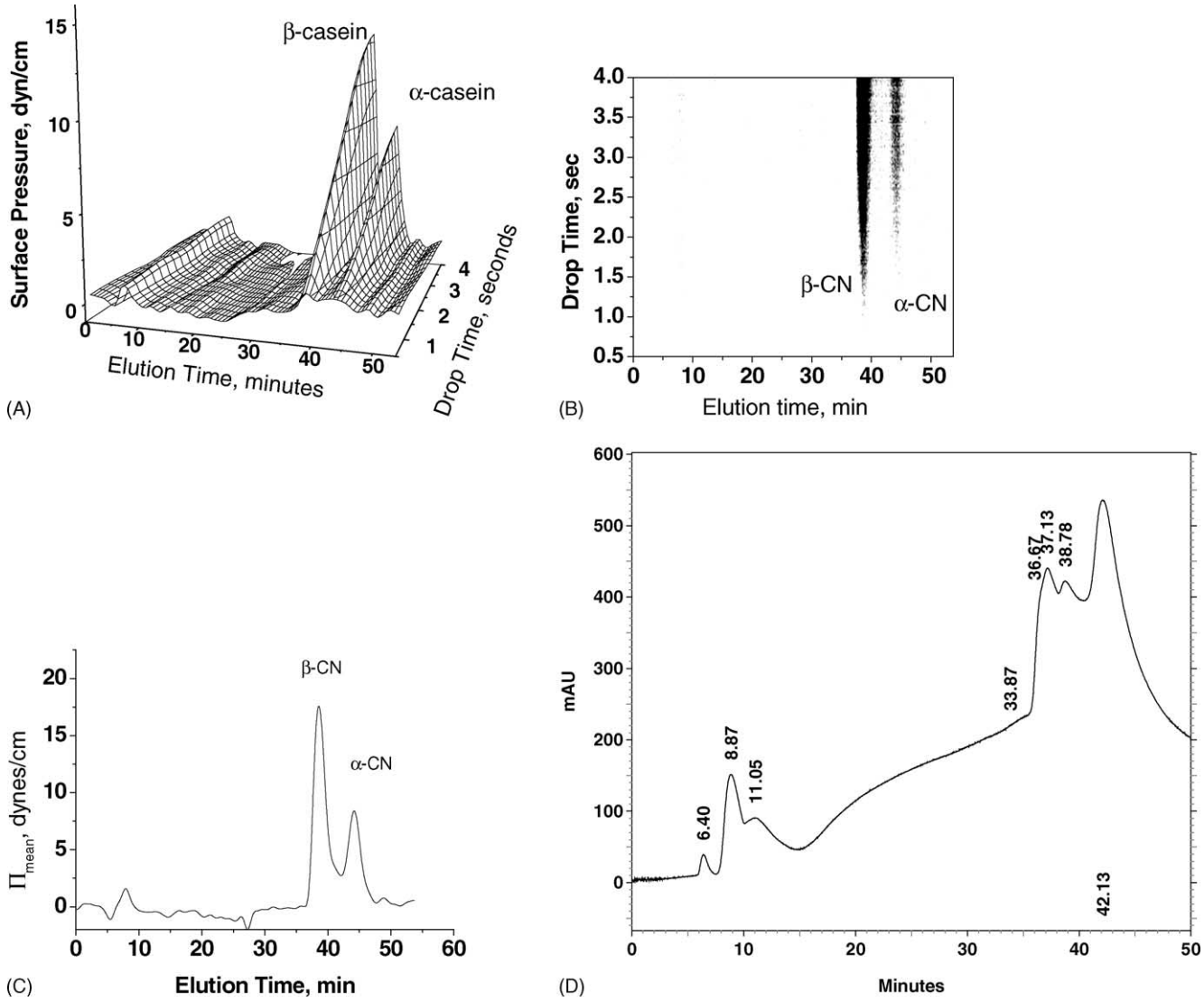

Fig. 7. HIC–DSTD separation of the commercial casein mixture (297.5 μg injected). (A) Surface pressure three-dimensional plot: surface pressure values are reported as a function of drop time and elution time. (B) Topographical contour plot of the same separation. (C) Π_{mean} vs. elution time plot processed from three-dimensional data. (D) UV absorbance chromatogram at 280 nm of the same separation acquired simultaneously by diode array detector. Chromatographic conditions: 3 min isocratic elution followed by 25 min of linear gradient from PBS, 1.8 M ammonium sulphate, 3.0 M GdmHCl to PBS, 3.0 M GdmHCl. Flow = 0.4 ml/min.

In addition to the information obtained from eluting chromatographic peaks (i.e., concentration and retention volume), the dynamic surface pressure is obtained for each of the eluting analytes. This added selectivity of the HIC–DSTD configuration is one of the advantages characteristic of a good hyphenated system. The selectivity of DSTD with respect to UV absorbance detection, for example, can provide unique information (i.e., surface activity) of individual analytes in complex protein mixtures. Figs. 8–10 show the HIC–DSTD chromatograms (part A) of cow's, ewe's and goat's raw milk samples, respectively, compared with the UV absorbance chromatogram at 280 nm (part B). HIC–DSTD chromatograms of the other diary products examined (yogurt, stracchino, mozzarella, parmesan cheese and chocolate cream), not shown here for brevity, present, as well, the two surface active components assigned to α- and β-casein. In all the real samples examined both whey proteins and κ-casein are not detectable in the surface pressure chromatogram because of their low concentration [43,44].

The application of the HIC–DSTD method to diary products clearly shows how this approach provides the analyst a selective view of the surface-active components, that differ significantly for each sample examined. It is worth noting, e.g., that the number and the concentration of surface-active proteins in cow's, ewe's and goat's milk samples (Figs. 8–10) are substantially different. In goat's milk, α-casein component is absent and the eventual detection of this component in the HIC–DSTD chromatogram of a caprine cheese sample could be directly correlated with the fraudulent addition of cow's milk to the more expensive

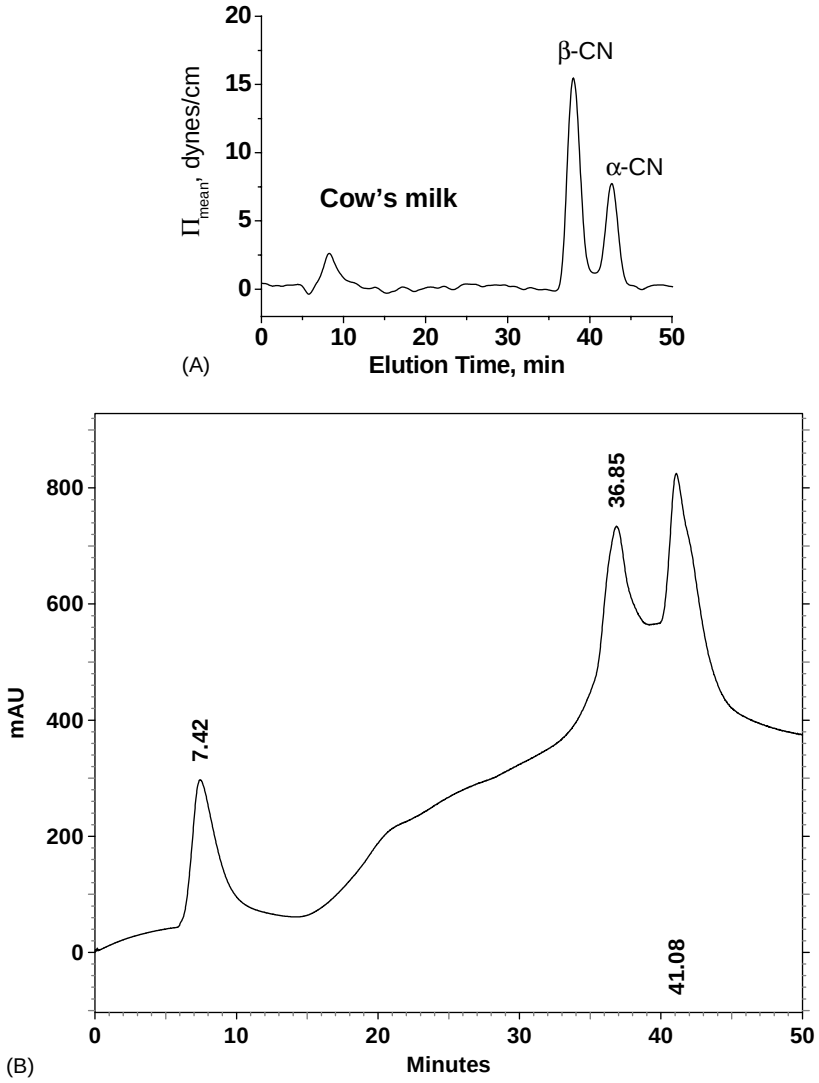

Fig. 8. HIC–DSTD of the cow's milk (diluted 1:10 with PBS, 3.0 M GdmHCl). (A) Π_{mean} vs. elution time plot processed from 3D data. (B) UV absorbance chromatogram at 280 nm of the same separation acquired simultaneously. Chromatographic conditions: 3 min isocratic elution followed by 25 min of linear gradient from PBS, 1.8 M ammonium sulphate, 3.0 M GdmHCl to PBS, 3.0 M GdmHCl. Flow = 0.4 ml/min.

goat's milk. In more general applications, the detection of the number and concentration of surface active proteins in real samples can be fundamental for the quality control during all the steps of food processing (from the raw materials to the processed foodstuff), in pharmaceutical industry and in environmental chemistry.

On the basis of the peak area of the two-dimensional surface pressure plots (Π_{mean} versus elution time, Fig. 7C) and the calibration data reported in Fig. 6 and Table 1, the quantitative analysis of α- and β-casein in the commercial casein mixture and in the examined real samples was performed. Table 2 shows a summary of the quantitative results. Recoveries of α- and β-casein, calculated taking into account their quantitation in the same samples performed in previous studies by HIC [42–45], range between 74 and 105% and 70 and 113%, respectively. It should be noted that the raw samples in this study are solubilized in 3.0 M GdmHCl instead 4.0 M GdmSCN as in the previous studies [42–45], and that elution buffers contain 3.0 M GdmHCl instead of 8.0 M urea. Since GdmSCN is the most effective denaturant and solubilizing agent for proteins, it is possible that the yield of casein extraction from raw samples is lower in this study with respect to the previous ones. For this reason, the recovery values have to be considered indicative. The effect of the denaturant employed in the sample solubilization is described by the results obtained on ewe's milk sample for which the recovery is higher in the milk sample solubilized in 4.0 M GdmSCN (c sample) instead of 3.0 M GdmHCl (b sample). In the particular case of complex food samples such as several diary products (mozzarella, parmesan cheese and chocolate cream), the effect of the low recovery of casein due to the minor solubilization effectiveness of 3.0 M GdmHCl with respect to 4.0 M GdmSCN is more important, showing a recovery of α- and β-casein of about 10%. At

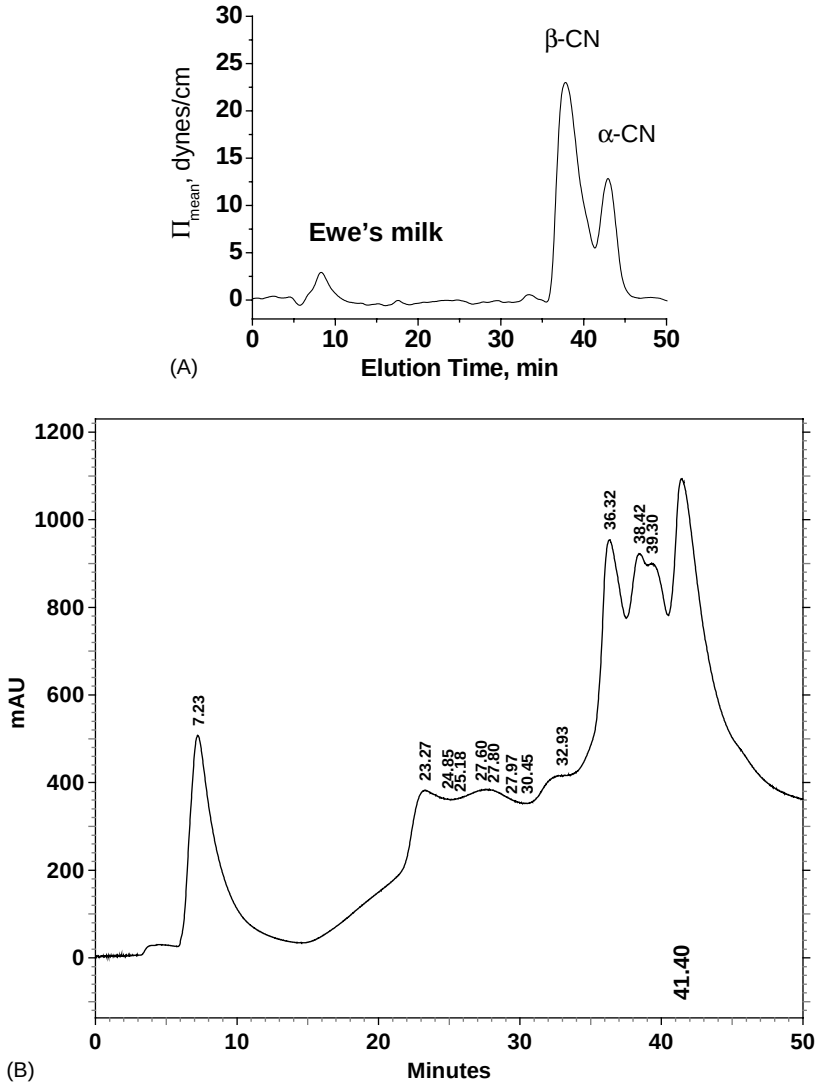

Fig. 9. HIC–DSTD of the ewe's milk (diluted 1:10 with PBS, 3.0 M GdmHCl). (A) Π_{mean} vs. elution time plot processed from 3D data. (B) UV absorbance chromatogram at 280 nm of the same separation acquired simultaneously. Chromatographic conditions: 3 min isocratic elution followed by 25 min of linear gradient from PBS, 1.8 M ammonium sulphate, 3.0 M GdmHCl to PBS, 3.0 M GdmHCl. Flow = 0.4 ml/min.

Table 2
Quantitative results performed by HIC–DSTD, using the peak area values of the Π_{mean} signal vs. elution time and the calibration data from Table 1

Sample	$[\beta CN]_{inj}$ (mg/l)[a]	$[\alpha CN]_{inj}$ (mg/l)[a]	[βCN] found (mg/l)	[αCN] found (mg/l)	βCN recovery (%)	αCN recovery (%)
Casein mixture	1334	1554	1276.47	1440	96	93
Cow's raw milk	915	809	848.53	595.26	93	74
Goat's raw milk	2685	0	1877.65	–	70	–
Ewe's raw milk[b]	1761	1608	1525.88	1331.58	87	83
Ewe's raw milk[c]	1761	1608	1985.29	1694.74	113	105
Yogurth	1568	1056	1655.88	900	106	85
Stracchino cheese	2174	2536	2247.06	2363.16	103	93

Chromatographic conditions: HIC TSK Gel Phenyl 5PW column 7.6 cm × 7.6 mm; 3 min isocratic elution followed by a 25 min linear gradient from 100% PBS 0.1 M pH 7.2, 3.0 M GdmHCl, 1.8 M ammonium sulfate to 100% PBS 0.1 M pH 7.2, 3.0 M GdmHCl.

[a] Recoveries of α- and β-casein have been estimated on the basis of the data available in previous studies obtained by HIC analysis in denatured conditions of the same samples [43–45].

[b] The stock solution of the ewe's milk sample is diluted in 3.0 M GdmHCl.

[c] The stock solution of the ewe's milk sample is diluted in 4.0 M GdmSCN.

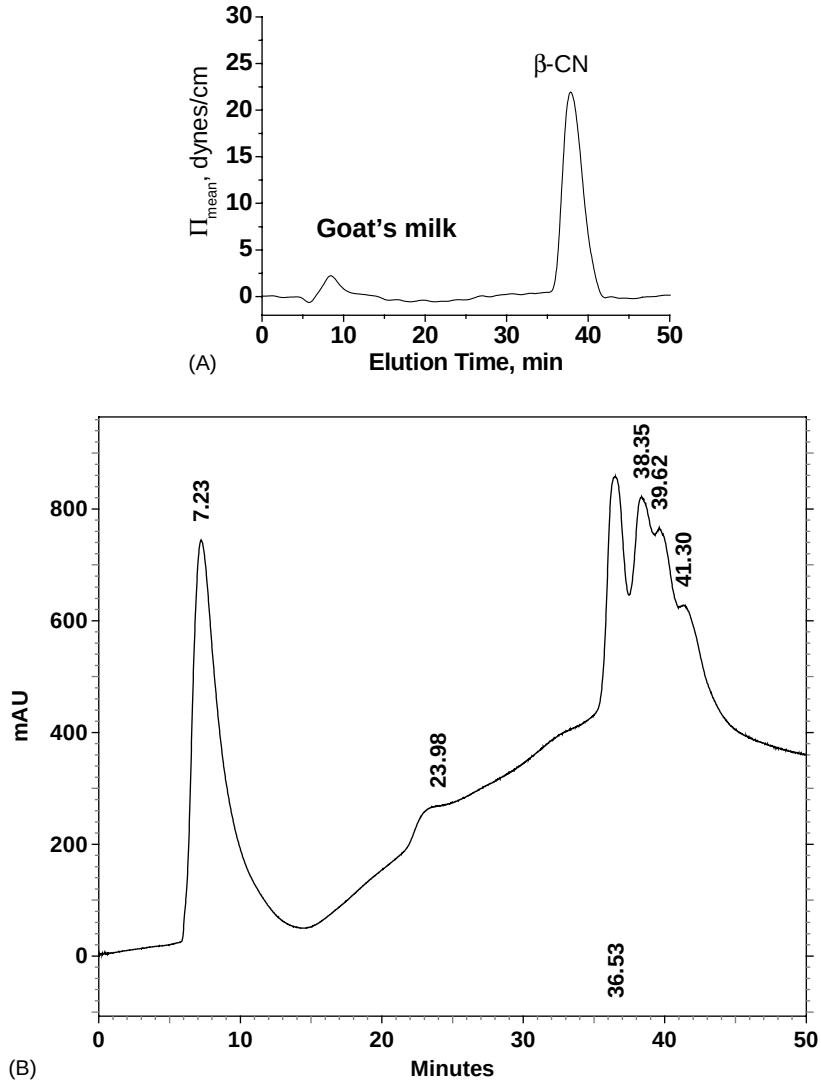

Fig. 10. HIC–DSTD of the goat's milk (diluted 1:10 with PBS, 3.0 M GdmHCl). (A) Π_{mean} vs. elution time plot processed from 3D data. (B) UV absorbance chromatogram at 280 nm of the same separation acquired simultaneously. Chromatographic conditions: 3 min isocratic elution followed by 25 min of linear gradient from 0.1 M PBS pH 7.2, 1.8 M ammonium sulphate, 3.0 M GdmHCl to PBS, 3.0 M GdmHCl. Flow = 0.4 ml/min.

present, the employment of GdmSCN in the mobile phase buffer for HIC–DSTD experiments is in progress.

5. Conclusions

The DSTD coupled to HIC provides for the selective determination and quantitation of surface active proteins, α- and β-caseins, in complex food mixtures (raw milk and cheeses). In the specific case of caseins, as they play a key role in dairy industry and as additives in food, paints and glues because of their emulsifying properties [6,26–29], this technique has promise to assist in the evaluation of important properties of raw and processed samples. In more general applications, in addition to the information obtained from eluting chromatographic peaks (i.e., concentration and retention volume), the selective nature of DSTD allows the identification and concentration of surface-active proteins in complex real matrices. This represents a potentially useful approach for the quality control in all steps of food processing, in the pharmaceutical industry, as well as in environmental chemistry.

Although a chromatographic separation is often essential to solve many analytical problems, the straightforward application of FIA–DSTD can in principle give reliable information, thanks to the selectivity of DSTD, once suitable operating conditions are chosen for the analysis. In the specific case of milk proteins in diary samples, the analysis of the samples in native conditions, where only caseins are surface active, and in denaturing conditions (e.g., 5.0 M GdmHCl), where casein *and* whey proteins are surface active, could provide a fast, quantitative measure of the casein/whey protein ratio, a fundamental parameter in the basic process of the preparation of infant formulae from cow's milk [57].

The calibration procedure reported herein is an important step in the evolution of the DSTD, allowing samples to be utilized in many solvent matrices, using gradient elution, without changing the standardization step of the calibration. This improvement makes the hyphenation of DSTD potentially applicable to other HPLC techniques and promising for the study of surface-active proteins, as well as other surface compounds.

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References

[1] D. Mobius, R. Miller (Eds.), Proteins at Liquid Interfaces, Elsevier, Amsterdam, 1998, p. 498.
[2] J.L. Brash, P.W. Wojciechowski, Interfacial Phenomena and Bioproducts, Marcel Dekker, New York, 1996, p. 510.
[3] V.N. Izmailova, G.P. Yampolskaya, Z.D. Tulovskaya, Colloids Surfaces A 160 (1999) 89.
[4] M.E. Mangino, in: N.S. Hettiarachchy, G.R. Ziegler (Eds.), Protein Functionality in Food Systems, Marcel Dekker, New York, 1994 pp. 147–179.
[5] J.B. German, L. Phillips, in: N.S. Hettiarachchy, G.R. Ziegler (Eds.), Protein Functionality in Food Systems, Marcel Dekker, New York, 1994, pp. 181–208.
[6] L.G. Phillips, D.M. Whitehead, J. Kinsella, Structure Function Properties of Food Proteins, Academic Press, London, 1994, p. 271.
[7] S. Damodaran, in: S. Damodaran, A. Paraf (Eds.), Food Proteins and their Applications, Marcel Dekker, New York, 1997, p. 57.
[8] N. Garti, Colloids Surfaces A 152 (1999) 125.
[9] E. Dickinson, An Introduction to Food Colloids, Oxford University Press, Oxford, UK, 1992.
[10] P.J. Halling, CRC Crit. Rev. Food Sci. Nutr. 13 (1981) 155.
[11] S. Damodaran, K.B. Song, Biochim. Biophys. Acta 954 (1988) 253.
[12] M.C. Phillips, Food Technol. 35 (1981) 50.
[13] S. Damodaran, L. Razumovsky, Am. Chem. Soc. Symp. Ser. 708 (1998) 2.
[14] J.R. Clarkson, Z.F. Cui, R.C. Darton, J. Colloid Interface Sci. 215 (1999) 333.
[15] J.R. Clarkson, Z.F. Cui, R.C. Darton, J. Colloid Interface Sci. 215 (1999) 323.
[16] K.D. Schwenke, in: S. Magdassi (Ed.), Surface Activity of Proteins: Chemical and Physiochemical Modifications, Marcel Dekker, New York, 1996, p. 61.
[17] D.E. Graham, M.C. Phillips, J. Colloid Interface Sci. 70 (1979) 427.
[18] S. Damodaran, K.B. Song, in: N. Parris, R. Barford, (Eds.), Interactions of Food Proteins, ACS Symposium Series, No. 454, American Chemical Society, Washington, DC, 1991, pp. 104–121.
[19] L.P. Voutsinas, E. Cheung, S. Nakai, J. Food Sci. 48 (1983) 26.
[20] R.D. Waniska, J.K. Shetty, J.E. Kinsella, J. Agric. Food Chem. 29 (1981) 826.
[21] A. Kato, S. Nakai, Biochim. Biophys. Acta 624 (1980) 13.
[22] J.Y. Lee, M. Hirose, Biosci. Biotechnol. Biochem. 56 (1992) 1810.
[23] S. Arai, M. Watanabe, in: E. Dickerson, G. Stainsby (Eds.), Advances in Food Emulsions and Foams, Elsevier, 1988, pp. 189–200.
[24] A.S. Antipova, M.G. Semenova, L.E. Belyakova, Colloids Surfaces B 12 (1999) 261.
[25] H.E. Swaisgood, in: P.F. Fox (Ed.), Developments in Dairy Chemistry, Part I, Proteins, Applied Science Publishers, London, 1982, pp. 1–60.
[26] P. Walstra, R. Jenness, Dairy Chemistry and Physics, Wiley/Interscience, New York, 1984.
[27] D.W.S. Wong, W.M. Camirand, A.E. Pavlath, Crit. Rev. Food Sci. Nutr. 36 (1996) 807.
[28] E. Dickinson, J. Food Eng. 22 (1994) 59.
[29] P.W.J.R. Caessens, H. Gruppen, C.J. Slangen, S. Visser, G.J. Voragen, J. Agric. Food Chem. 47 (1999) 1856.
[30] C. Holt, Adv. Prot. Chem. 43 (1992) 63.
[31] L.R. Lima, D.R. Dunphy, R.E. Synovec, Anal. Chem. 66 (1994) 1209.
[32] L.R. Lima, R. Synovec, J. Chromatogr. A 691 (1995) 195.
[33] T.E. Young, R.E. Synovec, Talanta 43 (1996) 889.
[34] N.A. Olson, R.E. Synovec, W.A. Bond, D.M. Alloway, K.J. Skogerboe, Anal. Chem. 69 (1997) 3496.
[35] N.A. Olson, K.J. Skogerboe, R.E. Synovec, J. Chromatogr. A 806 (1998) 239.
[36] K.E. Miller, K.J. Skogerboe, R.E. Synovec, Talanta 50 (1999) 1045.
[37] K.E. Miller, R.E. Synovec, Anal. Chim. Acta. 412 (2000) 149.
[38] K.E. Miller, E. Bramanti, B.J. Prazen, M. Prezhdo, K.J. Skogerboe, R.E. Synovec, Anal. Chem. 72 (2000) 4372.
[39] W.W.C. Quigley, E. Bramanti, B.A. Staggemeier, K.E. Miller, A. Nabi, K.J. Skogerboe, R.E. Synovec, J. Anal. Bioanal. Chem. (2003) in press.
[40] S. Xu, S. Damodaran, J. Colloid Interface Sci. 159 (1993) 124.
[41] K. Anand, S. Damodaran, J. Agric. Food Chem. 44 (1996) 1022.
[42] E. Bramanti, F. Ferri, G. Raspi, L. Lampugnani, M. Spinetti, K.E. Miller, R.E. Synovec, Talanta 54 (2001) 343.
[43] E. Bramanti, C. Sortino, G. Raspi, R.E. Synovec, Analyst 126 (2001) 995.
[44] E. Bramanti, C. Sortino, G. Raspi, J. Chromatogr. A 958 (2002) 157.
[45] E. Bramanti, C. Sortino, M. Onor, F. Beni, G. Raspi, J. Chromatogr. A 994 (2003) 59.
[46] W.N. Eigel, J.E. Butler, C.A. Ernstrom, H.M. Farrel, V.R. Harwarkar, R. Jenness, R. Whitney, J. Dairy Sci. 67 (1984) 1599.
[47] http://www.expasy.ch/tools/protparam.html.
[48] W.W.C. Quigley, A.J.P.B. Nabi, N. Lenghor, K. Grudpan, R.E. Synovec, Talanta 55 (2001) 551.
[49] J. Mitchell, L. Irons, G.J. Palmer, Biochim. Biophys. Acta 200 (1970) 138.
[50] E. Dickinson, D.J. Pogson, E.W. Robson, G. Stainsby, Colloids Surfaces 14 (1985) 135.
[51] J.F. Farise, P. Cayot, J. Agric. Food Chem. 46 (1998) 2628.
[52] T. Sengupta, L. Razumovsky, S. Damodaran, Langmuir 16 (2000) 6583.
[53] H.E. Swaisgood, in: Advanced Dairy Chemistry, Elsevier, Amsterdam, 1992, pp. 63–110.
[54] V.N. Izmailova, G.P. Yampolskaya, in: D. Mobius, R. Miller (Eds.), Protein at Liquid Interfaces, Elsevier, Amsterdam, 1998. p. 103.
[55] J.C. Lee, S.N. Timasheff, Biochemistry 13 (1974) 257.
[56] H.E. Swaisgood, in: P.F. Fox (Ed.), Development in Dairy Chemistry, vol. 1, Elsevier, London, 1982, pp. 1–58.
[57] A.C. Goedhart, J.B. Bindels, Nutr. Resev. Rev. 7 (1994) 1.