Thermal Denaturation of Milk Whey Proteins: Rapid Quantification

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Heat treatment of milk signifies a certain degree of protein denaturation, which modifies the functional properties of dairy products. Traditional methods for detecting and quantifying the denaturation of whey proteins are slow, complex and require sample preparation and qualified staff. The world's current trend is to develop rapid, real-time analytical methods that do not destroy the sample and can be applied on/in-line during processing.

Keywords: whey proteins ; denaturation ; heat treatment

1. Introduction

In the dairy industry, raw milk goes through several processes, including standardization and heat treatment. Pasteurization and ultrahigh-temperature (UHT) sterilization, the most commonly used heat treatments, affect the quality, safety and shelf life of milk and dairy products, inducing physical and chemical changes in protein structure and functionality [1][2]. Thermal processing above 65 °C produces the denaturation of whey proteins such as BSA, while the denaturation of β-lactoglobulin and α-lactalbumin occurs above 70–74 °C [3]. When whey proteins are denatured, their hydrophobic groups are exposed, leading to interactions with themselves, forming aggregates or binding to the casein micelles through irreversible disulphide bonds (e.g., between β-lactoglobulin and κ-casein on the surface of the casein micelle). If the heating process is intensified, irreversible denaturation of α-lactalbumin occurs, enhancing the formation of a complex with denatured β-lactoglobulin [3][4]. Consequently, heat denaturation of proteins induces changes in the functional properties of milk, which may be of significant impact on the dairy industry. In particular, changes in the structure of milk proteins have an significant effect on milk structure and stability [4], which might be undesirable or advisable depending on the dairy product being manufactured. For instance, extensive denaturation of whey proteins and subsequent interaction with caseins is pursued in several dairy products due to functional or yield advantages. This is the case on the intensive thermal stabilization of milk required to ensure good heat stability during sterilization of unsweetened condensed milk, as well as to withstand the evaporation processes in sweetened condensed milk.

Control and quantification of heat-induced protein denaturation are important in the dairy industry. There are different methods and techniques for determining proteins in milk and dairy products. The Kjeldahl, Dumas and Amido Black methods are three chemical procedures to quantify total protein content. The advantages of standard methods such as Kjeldahl or Dumas are that they are precise and have good reproducibility. However, when it comes to quantifying whey-protein content from milk, whey-protein precipitation becomes necessary since these methods quantify the total protein content from the sample. Consequently, this adds more time to the procedure and is not suitable for in-, on-, or at-line determination. The Kjeldahl method can be performed within 30 min to 2 h, while the modern Dumas instrument takes around 5 min [5]. In order to analyze the individual proteins, several classical techniques are used at the laboratory scale: electrophoresis, chromatography and immunochemistry. These techniques need complex sample preparations before carrying out the analysis. Previously, it is necessary to fractionate the caseins and whey proteins by precipitation, and then these must be filtered to obtain a pure preparation of protein [6]. Moreover, they also need rather expensive reagents and equipment, which means that they are slow protein-detection and quantification methods. Since classical techniques are time-consuming methods, new rapid technologies are being studied for in-, on-, or at-line determination and real-time process control in the dairy industry [7][8].

2. Rapid Methods for Determining the Thermal Denaturation of Whey Proteins in Milk

2.1. Biosensors, Nanosensors and Microchips

Biosensors are devices that detect or measure compounds using biological material, i.e., antibodies, enzymes, or whole cells. When the biosensor and the compounds contact, they produce a physical or chemical change, emitting a response transduced into a physical signal, which can be used by a control system [9].

The surface plasmon resonance (SPR) principle has been applied to assess specific soluble proteins directly in milk. The antigen–antibody binding produces changes in the refractive SPR angle which is proportional to the mass of bound material [10]. Dupont et al. [11] used this immunological system (Biacore® 3000 International AB, Uppsala, Sweden) for determining the denaturation index of α-lactalbumin in milk after heat treatment. They produced two specific monoclonal antibodies: Mab 20 and Mab 130. The antigen–antibody binding produces changes in the refractive index on a metal surface that is detected and quantified by an optical system in the form of changes in the reflectance angles, which are expressed as resonance units, represented in a displayed curve on a computer [10]. The study showed that it was possible to determine the heat treatment to which the sample had been subjected by quantifying both native and denatured α-lactalbumin and expressing the results as percentage of denatured α-lactalbumin. Although the denaturation index for sterilization differed drastically from that of pasteurization, 97.2–99.7% vs. 5.2–18.9%, it was harder to differentiate highly pasteurized milk from direct UHT. The same principle (Biacore® Q optical biosensor, GE Healthcare, Uppsala, Sweden) was used to assess the decrease in native BSA after four hours of heat treatment at 70 and 80 °C in diluted skim milk (1:10, v/v) [12]. Sheep polyclonal antibovine BSA was evaluated and selected from four anti-BSA antibodies due to its highest affinity to BSA. Results showed a decrease of 30 and 80% of native BSA, depending on the temperature. The SPR has shown to be very versatile for various purposes, namely clinical [10]. This technology is developing, meaning that investigation is necessary to use the body and antibody that best suits a specific sample. It was recently demonstrated that whey protein's evaluation is reliable, repeatable, automated, sensible and only requires dilution of the milk in a buffer [13]. These biosensors are faster (~4 min) than the traditional Dumas method, which requires whey separations plus 5 min of the procedure. In addition, biosensors could complement other analytical methods to determine the denaturation of proteins since these biosensors have low detection limits [12][13].

Nanosensors are another sensor type that convey information from nanoparticles [14]. Rezende et al. [15] used an aqueous suspension of vesicles (Polydiacetylene/triblock copolymer L64/cholesterol) to detect BSA. This colorimetric sensor changes the color intensity from blue to red according to the concentration of native BSA proteins due to the occurrence of intermolecular interactions between them (Figure 1). Diluted skim milk, without casein micelles, presented a colorimetric response (CR) average of 50 ± 2.5%, indicating 0.045 ± 0.03 mM of native BSA. The result was compared with the HPLC technique and was well-correlated. Likewise, denatured BSA showed a CR value of 5%. Furthermore, other proteins such as α-lactalbumin and β-lactoglobulin were also evaluated, but they showed unsatisfactory results. As the authors suggest, BSA denaturation may produce the exposure of hydrophobic amino-acid residues that creates a repulsive interaction between the BSA hydrophobic group and the hydrophilic segments of the nanosensor. This lack of blue-to-red transition in the presence of denatured protein allows for the discrimination of native from denatured BSA molecules. L64 nanosensor is a simple, fast (<1 min) and low-cost analytical tool since vesicles are easy to prepare and produce at a large scale [16]. However, milk caseins may cause interferences, and thus, precipitation and separation of micelles before the analysis may be required [17].

A microchip is a miniaturized microfluidic device that utilizes a variety of microchannels to assess a sample. A well-known method to separate and analyze milk proteins is the SDS-PAGE electrophoresis, where proteins move through an SDS-polyacrylamide gel according to their electrophoretic mobility as a function of their molecular weight [18]. This technique has been miniaturized by using a microfluidic chip commercialized in different "lab-on-a-chip" versions. It has been proven to separate and quantify whey proteins [19][20] in the presence or absence of reducing agents [21]. The Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), the ExperionTM Automated Electrophoresis System (Bio Rad, Hercules, CA, USA) or the LabChip® GXII TouchTM (Perkin Elmer, Waltham, MA, USA) are some examples of the commercialization of this technique. They include all necessary reagents/operations: gel matrix solution, protein-dye concentrate solution and marker-protein buffer solution. In addition, they are faster (~30 min) than the traditional method (between 45 to 60 min), automated, require small quantities of sample, consume fewer toxic chemicals and are a potential tool for quality control in the dairy industry [9][21].

Although biosensors and microchips have some advantages such as fast responses and real-time analysis, they may be of single use or prone to saturation after being used [21], and thus are not suitable for on-line monitoring of whey-protein denaturation.

2.2. Electrochemical Sensors

Electrochemical sensors are based on the measurement of electron transfer due to oxidation/reduction reaction of the analyte through electrodes. Changes in the electric signal are proportional to the concentration of the target molecule, and the analyte is consumed by the electrode [25][26]. There are different types of electrochemical transducers such as amperometric, conductimetric, impedimetric and potenciometric devices, with the amperometric being the most widely employed. These transducers maintain a constant potential to detect variations in the current [9][22][23].

Cosio et al. [24] proposed using of an amperometric sensor with a Tungsten electrode to evaluate the severity of heat treatment to which milk was subjected. This research was because the sulfhydryl groups become free and form thiols after heat denaturation of proteins. Thiols react with mercury ions and the electrode detects mercury ions that are left. Therefore, the more whey proteins are denatured, the more thiols can react with mercury ions, and less current is observed. This study was carried out with raw, pasteurized and UHT milk after casein precipitation at pH 4.6. The results showed that this method differentiates between raw milk or mild treatment and UHT milk. Although this method is fast (90 samples/h), simple and straightforward, a previous step to isolate whey proteins is compulsory. Thus, it is not suitable for on-line control of thermal denaturation.

The three-omega method measures thermal conductivity (k) [25]. It was tested with BSA [30] and β-lactoglobulin [29] solutions. These studies demonstrate that alteration in protein conformation, resulting from temperature change, can be correlated successfully with their thermal conductivity, which increased as temperature increased. The temperature of denaturation for BSA and β-lactoglobulin in solution (5% w/w, pH 7.00) started at 53 and 61 °C, respectively. The maximum k could be recorded at 68 and 71 °C. This method is under development; thus, more research is necessary for complex solutions such as milk or whey. This technique is fast (<1 min) and could be used to monitor protein denaturation in real time [25].

Electrochemical sensors are less popular than optical methods but they are used to identify and quantify pathogens, bacterial toxins and pesticides in food due to advantages such as rapid response, simple equipment required, small or moderate cost, miniaturization capabilities and high portability [22][23].

2.3. Spectroscopy

Spectroscopy, classified according to the spectrum used, is the study of phenomena such as absorption, emission or scattering of electromagnetic radiation caused by the interaction with matter to obtain both qualitative and quantitative information on molecules [23]. Spectroscopy techniques take less than 1 min to be performed.

2.3.1. Infrared Spectroscopy (from 780 to 50,000 nm)

Molecules that absorb the energy of infrared lights produce vibrations. Infrared spectroscopy (IR) expresses the vibration of covalent bonds in molecules that contains quantitative information about all the constituents that absorb IR radiation [8][26]. Infrared spectrometers consist of an interferometer and a detector. The interferometer produces infrared light over a range of wavelengths, and the detector registers the signals of vibrations. A Fourier mathematical function transforms these signals to convert data to a more meaningful absorbance/transmittance response over a frequency/wavelength form. These instruments allow for the measuring of all the frequencies simultaneously [11][32]. Thus, it is necessary to perform statistical analyses with multivariate statistical software and apply procedures to correlate changes in infrared waves with a concentration of target molecules.

Fourier transform near-infrared (FT-NIR) spectroscopy studies the molecules' absorption in the range of 780–2500 nm, whilst Fourier transform mid-infrared (FT-MIR) spectroscopy studies it in the range of 2500–30,000 nm [26][32]. NIR radiation penetrates more of the sample; therefore, it produces bands with less absorption than MIR radiation. Protein absorption is due to the combination of vibrations from C = O and N-H bonds of amide I and amide II with corresponding FT-NIR absorption bands at 2000 and 2222 nm, respectively [32]. However, FT-NIR spectra of food represent absorptions of other chemical bonds, e.g., C-H and O-H, overlapping in the spectrum, corresponding mainly to overtones and combinations of vibrational mode, which makes the association of frequencies with specific chemical groups difficult [27]. On the contrary, FT-MIR spectra can discriminate specific chemical compositions and associate frequencies to slight differences in sample composition, allowing for the determination of the composition and structure of chemical molecules. Peaks around 6100 and 6500nm correspond to amide I and amide II bands .

Infrared spectroscopy has been applied in the dairy industry as a tool to quantify most of the milk's components and establish milk quality or authenticity [8][28]. Due to the versatility of this technology, at an early stage, Pouliot et al. [29] used NIR to evaluate heat denaturation (80 °C, every 2 min up to 120 min) of skimmed whey-protein concentrate with a NIRS 6500 near-infrared spectrophotometer (NIR Systems Inc., Silver Spring, MD, USA) in the range of 1100–2500 nm.

Spectra of the different samples were calibrated to monitor the whey proteins' heat denaturation determined by the Kjeldahl method and expressed as a nitrogen-solubility index at pH 4.6. Obtained indexes varied from 95 to 45% after 120 min of treatment. More recently [30], with a FT-NIR model MB 154S (Bomem technologies, Quebec, Canada), used the whey-protein index to classify skim-milk power as low (70 °C 15 s), medium (85 °C 60 s) and high (>120 °C 4 min), obtaining nitrogen-index values of ≥6.00, 1.51–5.99 and ≤1.50 for each heat treatment, respectively. The NIR prediction of the soluble whey-protein index was adequate, and calibration with known nitrogen values through partial least square (PLS) showed good correlations. Briefly, these studies showed that NIR spectroscopy could determine the heat denaturation of whey proteins through calibrations.

Recent studies have focused in using FT-NIR to detect conformational changes in whey proteins due to heat treatment [3] [31]. Whey-protein isolate and solutions of α-lactalbumin and β-lactoglobulin, studied with a BrukerTensor 27 FTIR instrument (Bruker Optik, Ettlingen, Germany) [31] and a Nexus 670 spectrometer (Thermo Electron, Madison, WI, USA) [3], showed maximum peak intensities at ≈2940, 6040 and 6130 nm, for each type of sample. After heat treatment at temperatures of 50 to 90 °C for 5 min, the absorbance peaks of whey-protein isolate and β-lactoglobulin, which consists of 8% α-helix, 45% β-sheet and 47% random coil, decreased, with a concomitant increase in wave numbers by the formation of random coils [3][31].

In parallel, a calibration model was developed by Pabari et al. [32] to predict powdered infant formula changes in casein and whey-protein isolates due to heat (80 and 105 °C for 10 min and 24 h). A MultiEye®(Innopharma Labs, Dublin, Ireland) NIR spectrophotometer was used in the IR range of 1515–2200 nm (Figure 3). The analysis of the spectra variation obtained before and after thermal treatment through a principal-component analysis (PCA) and the partial least-square (PLS) calibration showed that NIR spectroscopy could be a good technique to determine protein stability after heat treatment. However, more data points were needed for the second statistical method.

In another study, Alvarez et al. [33] designed an empirical model for predicting protein aggregation of heat-denatured whey-protein concentrate, according to three factors: whey-protein concentration (8–12%), time (5–30 min) and temperature (85 to 100 °C) of heat treatment. The Box–Behnken-response surface method was used to relate them to the response of the amide I band with a FTIR Magna IR 560 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The resulting model was dominated by changes in processing temperature, while both heating time and protein concentration resulted in no significant model factors. However, the data exhibited a significant linear fit.

Infrared spectroscopy is accepted as an in-line/on-line detection method in food because it does not require any sample preparation (nondestructive). In addition, it is simple to use, rapid, specific, allows several constituents to be measured simultaneously and is reliable for monitoring chemical and physical parameters during a process [34][35]. Nonetheless, a reference-method-calibration analysis, covering any possible composition fluctuations, is necessary to determine and quantify components [29].

2.3.2. UV-Visible Spectroscopy

Ultraviolet and visible light absorptions by molecules excite electrons and produce electronic transitions. The molecules that absorb this radiation have a chromophore. The light that is not absorbed by chromophores is transmitted through the sample [23][36].

Light-scatter

Scatter light is a part of the light that is not absorbed or transmitted by the molecules. Milk has compounds such as casein micelles that scatter light, and therefore light backscatter is one of the most used techniques for developing optical sensors for food applications. In these light-scattering sensors, a diode (LED) emits radiation, which is conducted by an optic fiber through the sample, and the refracted backscatter light (180°) is received by a detector [37].

Lamb et al. [38] studied the relationship between changes in the size of casein micelles and the denaturation of milk whey proteins. This technique relies on variations of backscatter light intensity according to wavelength and particle diameter. After heat denaturation, β-lactoglobulin aggregates and binds to casein micelles, whose diameter increases. Although a priori, the wavelength intervals used varied from 200–1100 nm; the highest response was observed at wavelengths ranging 500–900 nm. This optical method proposed by [38] showed a proper correlation with data collected with currently used techniques (SDS-PAGE and differential scanning calorimetry). Nevertheless, the optical method tended to slightly overestimate the amount of protein denaturation. Furthermore, protein aggregates produced during denaturation may also affect this technique [2].

The binding of β-lactoglobulin strongly depends on pH, and the highest diameter of casein micelles was found at pH 6.3 [3]. Taterka and Castillo [37] studied the relation between light backscatter measurements and the size of casein micelles at pH 6.3, 6.7 and 7.1 after 10 min of heat treatment at 80 and 90 °C. On one hand, they found a positive correlation between treatment temperatures, the intensity of light backscatter and micelle size at pH lower than 7.1. On the other hand, at pH 7.1, denatured whey proteins tend to form aggregates, and the temperature treatment does not change micelle size.

The same authors recently studied the relation between light backscatter measurement and the size of the casein micelles, but as a function of whey-protein binding to the casein micelles and the formation of whey-protein soluble aggregates [42]. This study confirmed a positive correlation between bound whey proteins, micelle size and intensity of backscattered light. At pH lower than 7.1, a proportion of β-lactoglobulin forms aggregates and the rest binds at the casein micelle depending on pH. Consequently, the casein micelle size and the intensity of light backscatter also depend on the number of protein aggregates present in the heat-treated milk [37]. Optical sensors using a light backscatter are currently in development. Different factors that affect whey-protein denaturation must be considered (e.g., temperature, pH and the amount of whey-protein aggregates). However, this optical method could be used in-line in the dairy industry to evaluate heat-treated milk to make cheese or yoghurt depending on the level of denatured whey proteins, since there is no need to alter the milk sample and the measurement is provided in real time [38].

Fluorescence spectroscopy

Fluorescence occurs when specific molecules absorb photons from light, and they release the excess energy by emitting light at a longer wavelength [7]. Milk has intrinsic fluorescent components such as vitamin A, aromatic amino acids, NADH and some advanced Maillard compounds [39]. Whey proteins such as β-lactoglobulin and α-lactalbumin have at least one amino-acid tryptophan residue, responsible for whey proteins' intrinsic fluorescence [40]. Tryptophan fluorescence depends on the environment, and thus on milk characteristics [7].

Right-angle fluorescence spectroscopy

Right-angle fluorescence spectroscopy, using 90° configuration, requires dilution of milk samples because if the absorbance of the sample is higher than 0.1, reabsorption of emitted radiation occurs and decreases fluorescence intensity (inner-filter effect) [7].

Birlouez-Aragon et al. [41] evaluated milk heat treatment using the right-angle configuration on whey obtained by precipitation and separation of milk caseins at pH 4.6. The FAST method (Fluorescence of Advanced Maillard products and Soluble Tryptophan) was applied, where the FAST index was used as an indicator of the damage during a heat process. This method measured two indicators of heat treatment: fluorescence of advanced Maillard products (excitation/emission wavelengths of 330/420 nm) and soluble tryptophan (290/340 nm) to calculate the FAST index, which is obtained by dividing advanced Maillard fluorescence compounds by tryptophan fluorescence and multiplying the result by 100. The results of this study showed a good correlation between the FAST index and traditional protein-quantification methods such as the Lowry method and HPLC [41]. Later on, Birlouez-Aragon et al. [42] validated the FAST method with standardized HPLC methods and studied its capacity to discriminate milk heat treatments. The study showed that a milder heat treatment provoked lower denaturation with a concomitant higher tryptophan fluorescence intensity and a corresponding low FAST index. Tryptophan fluorescence allowed for the distinguishing of thermalized milk (68 °C, 24 s) from pasteurized (68 °C, 2 min and 72 °C, 2 min) milk. Furthermore, the FAST index could discriminate between direct, indirect and bottle treatments. Hence, it was proven that the FAST index allows for the estimation of the intensity of heat treatment in milk by quantifying protein denaturation through changes in fluorescence intensity.

Murillo et al. [40] evaluated total fluorescence spectra (bandpasses of 4 nm for both excitation and emission with 3.2 increments and 0.4 nm step size) of whey obtained from milk samples subjected to different treatments (raw, pasteurized and UHT). In the UV region, two fluorescent bands were obtained. The liquid chromatographic analysis with fluorescence detection showed that the two fluorescent amino acids, tyrosine and tryptophan, which were present at a soluble fraction of milk, were responsible for the intrinsic fluorescence of whey. Heat treatment did not modify the position of the two bands but did modify the fluorescence intensity with the smaller corresponding to UHT milk.

Not long ago, the research group of Birlouez-Aragon [43] implemented a fluorometric method in a compact and portable right-angle fluorometer named Amaltheys® (Spectralys Innovation, Romainville, France). This device is at-line and provides three key indicators on milk, powders and dairy derivatives with high accuracy: soluble proteins, whey-protein nitrogen index and FAST index. This tool is fast but needs calibration models to determine the amount of denatured whey proteins as a way to qualify the effect of heat treatment [43].

Even though devices based on the right-angle fluorescence spectroscopy are being developed to offer control of milk protein for the dairy industry at-line, they cannot be used on-line since they require either the dilution of milk samples or precipitation of caseins to analyze whey.

Front-face fluorescence spectroscopy

Front-face fluorescence spectroscopy uses an illumination angle between 30° and 60° [44]. This position minimizes light's reflection, scatter and polarization [7], decreasing the real intensity of fluorescence. Light does not go through the sample; it is recovered just from the sample's surface. As a result, front-face fluorescence can be used directly on turbid, solid, powder and concentrated samples [7][44].

Dufour and Riaublanc [7] studied the emission fluorescence of different molecules such as tryptophan in proteins to characterize the changes in fluorescence of raw and heat-treated milk. The authors used a 56° configuration and determined that the fluorescence properties were modified when applying a heat treatment (70 °C, 20 min). Furthermore, these authors suggested the use of a matrix with fluorescence results of tryptophan (excitation: 290 nm, emission: 305–400 nm) and others fluorescent compounds, such as retinol (excitation: 321 nm, emission: 260–350 nm), could improve discrimination between raw milk and heat-treated milk.

Fluorescence changes of whey protein after denaturation by several heat treatments were studied by Kulmyrzaev et al. [45]. In this study, together with the front face spectroscopy (56° configuration) and the statistical technique, principal-component regression was used to create regression models for both qualitatively and quantitatively evaluating the thermal denaturation of whey proteins. This study revealed that NADH and FADH spectra (excitation: 360 nm, emission: 460 and 518 nm, respectively) were good predictors of the content of the native β-lactoglobulin. Furthermore, front-face fluorescence correlated with immunological methods to determine residual native whey proteins subjected to different temperatures of low-pasteurization treatment.

Higher treatment temperatures (72–120 °C) were studied with front-face fluorescence excitation–emission matrices (60° configuration, excitation: 250–350 nm, emission: 260–500 nm) by Hougaard et al. [46]. A parallel factor analysis (PARAFAC) was used to estimate the different concentrations of the fluorophores of samples subjected to different heat treatments. Another model evaluated for heat treatment was the partial least-squares model used by Diez et al. [44]. This study used front-face spectroscopy (32° configuration) to obtain emission spectra of tryptophan (excitation: 290 nm, emission: 312-398 nm) and advanced Maillard products (excitation: 350 nm, emission: 402–498 nm), and to predict FAST index and soluble whey protein from heat-treated milk (72–115 °C) at six different times (from 2 min to 9.3 min). A good regression was obtained between the concentration of soluble whey protein measured with conventional methodologies and those predicted by partial least squares. The FAST index had a deviation at high temperatures due to the predominance of Maillard fluorescence compounds over tryptophan. The authors highlight the need for more samples with high heat charge to create another model and correctly predict the FAST index.

Ayala et al. [47] also studied the tryptophan fluorescence emission by using front-face fluorescence (56° configuration, excitation: 290 nm, emission: 300–450 nm) with heat-treated skim-milk powder. Significant changes were observed at 90 and 100 °C. A decrease in tryptophan fluorescence intensity and a red shift was found when the heat-treatment intensity increased. Principal-component analysis was performed and suggested using well-selected specific wavelengths or narrowband array.

Front-face fluorescence spectroscopy and multivariate statistical analysis have a great potential to become a method for distinguishing between milk samples subjected to different heat treatments based on the changes in fluorescence of intrinsic fluorophores of milk [7][39][44][45][46]. More studies and models are needed to validate the method to become a fast at-line or on-line method [10]. The advantages of this method are that no preliminary sampling is needed (nondestructive) since it can operate on opaque samples such as nondiluted milk, and it is fast, relatively inexpensive and sensitive [7][39][44].

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