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Rapid discrimination between buffalo and cow milk and detection of adulteration of buffalo milk with cow milk using synchronous fluorescence spectroscopy in combination with multivariate methods

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This research paper describes the potential of synchronous fluorescence (SF) spectroscopy for authentication of buffalo milk, a favourable raw material in the production of some premium dairy products. Buffalo milk is subjected to fraudulent activities like many other high priced foodstuffs. The current methods widely used for the detection of adulteration of buffalo milk have various disadvantages making them unattractive for routine analysis. Thus, the aim of the present study was to assess the potential of SF spectroscopy in combination with multivariate methods for rapid discrimination between buffalo and cow milk and detection of the adulteration of buffalo milk with cow milk. SF spectra of cow and buffalo milk samples were recorded between 400–550 nm excitation range with $\Delta\lambda$ of 10–100 nm, in steps of 10 nm. The data obtained for $\Delta\lambda = 10$ nm were utilised to classify the samples using principal component analysis (PCA), and detect the adulteration level of buffalo milk with cow milk using partial least square (PLS) methods. Successful discrimination of samples and detection of adulteration of buffalo milk with limit of detection value (LOD) of 6% are achieved with the models having root mean square error of calibration (RMSEC) and the root mean square error of cross-validation (RMSECV) and root mean square error of prediction (RMSEP) values of 2, 7, and 4%, respectively. The results reveal the potential of SF spectroscopy for rapid authentication of buffalo milk.

Keywords: Buffalo milk, cow milk, authenticity, synchronous fluorescence spectroscopy.

Buffalo milk, the second most widely produced milk in the world (IDF, 2014) is of great importance due to its nutritional and technological aspects. It differs from cow milk in terms of not only major components but also some minor components, some of which exhibit fluorescence (Karoui et al. 2005). Substantial valuable features of buffalo milk make it favourable in production of premium products such as Mozzarella, Ricotta, Yoghurt, Ghee or Dahi (Thomas, 2008). Because of the high price and limited availability of buffalo milk, these value-added dairy products generally have higher prices than their substitutes, which make buffalo milk and its products an attractive target for fraudulent activities. Addition of cow milk to buffalo milk, and using adulterated raw material in the production of original buffalo products is one of the most common examples of possible adulterations (Czerwenka et al. 2010).

Authenticity of dairy products is a crucial issue for consumers as well as for food processors, sellers, and food authorities. Various methods based on electrophoresis (Fuselli et al. 2015) immunochemistry (Hurley et al. 2004), chromatography (Enne et al. 2005), mass spectrometry (Czerwenka et al. 2010) and biomolecular techniques (Agrimonti et al. 2015) have been proposed for determining the authenticity of buffalo milk and its products. These methods provide sensitive and reliable detection of possible adulterations. However, these are expensive and time-consuming methods. Some of them also involve usage of toxic chemicals, and require complicated instrumentation and skilled experts. These drawbacks make them unattractive for routine analysis. Hence, there is a need for detection of the authenticity of buffalo milk using a rapid, low-cost and safe method especially for routine control.

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Nowadays, spectroscopic methods have become popular in food research for determining authenticity (Boyaci et al. 2015). One of these methods, fluorescence spectroscopy, is considered as a promising tool in food quality analysis (Becker et al. 2003). It is a rapid, sensitive, and non-destructive technique offering spectral signatures of samples in a few seconds (Sadecka & Tothova, 2007). In both classical excitation and emission spectroscopy, a spectrum which is mainly characteristic of a specific fluorophore is generated (Blecker et al. 2012). However, synchronous fluorescence (SF) spectroscopy allows scanning both the excitation and emission monochromators simultaneously, usually maintaining a constant wavelength interval, $\Delta\lambda$, between excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}). This enables a simplified spectrum providing information on several fluorophores. SF spectroscopy also minimises spectral overlaps and has greater selectivity which make it an attractive method for the analysis of complex systems (Patra & Mishra, 2002; Sikorska et al. 2005; Sadecka & Tothova, 2007) This method was reported to work well in food classification, discrimination and analysis as well as detection of adulteration of food (Sikorska et al. 2005; Blecker et al. 2012; Dankowska et al. 2014, 2015; Liu et al. 2014). However, to the best of our knowledge, there is no study using SF spectroscopy for detecting the authenticity of buffalo milk. Thus, the aim of the present study was to determine the possibility of using SF spectroscopy in combination with multivariate methods for rapid discrimination between buffalo and cow milk samples and to detect the adulteration of buffalo milk with cow milk.

Materials and methods

Materials

The samples constituted two groups, which were cow milk and buffalo milk. A total of 20 milk samples (i.e. cow milk samples from ten different farms and buffalo milk samples from ten different farms located in Istanbul, Turkey, where each sample was pooled from the milk of 10 animals) were purchased. The samples were placed into glass bottles and kept refrigerated at 4 °C, and analysed immediately after they were delivered to the laboratory. The SF spectra of these 20 different milk samples were used in principal component analysis (PCA) for discrimination of cow and buffalo milk.

In order to be used in the adulteration study, ten cow milk samples were mixed to make the master sample for cow milk and ten buffalo milk samples were mixed to make the master sample for buffalo milk. Then the model adulterant mixtures were prepared by spiking buffalo master sample with cow milk master sample at levels ranging from 10–90% at 10% intervals. Two sets of these samples were prepared. The SF spectral data of the 22 samples (two different sets of nine adulterant mixture samples and master samples) were recorded immediately. These data were used to build the quantitative model for detection of adulteration level of buffalo milk using PLS.

Sulphuric acid (95–97%, w/w), amyl alcohol and NaOH used were purchased from Merck (Merck KGaA, Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) (99·9% minimum purity) was obtained from Sigma-Aldrich (Sigma-Aldrich St. Louis, MO, USA).

Chemical analysis of milk samples

Total protein content of the samples was determined with Dumas (IDF, 2002) method using Flash 4000 nitrogen/protein analyser (Thermo Fisher Scientific Inc., Waltham, MA USA). EDTA was used as the nitrogen standard. The conversion factor was used as 6.38 (IDF, 2002). Total fat contents of the samples were determined according to Gerber method (British Standards Institution, 1955) using Gerber centrifuge (Funke-Dr. N. Gerber Labortechnik GmbH, Berlin, Germany). Total solids content was determined using conventional oven-drying (60 °C for 2–3 h, then 100 °C for 6 h) of a 5 g sample. Solids non-fat content was calculated by the subtraction of total fat content from total solids content. pH measurements of the samples were carried out using MP220 Basic pH Meter (Mettler-Toledo, LLC, Parkway Columbus, OH, USA).

SF spectroscopy analysis

SF spectra of the samples in Quartz cuvettes of 10 mm pathlength were recorded at 25 °C in synchronous scanning mode on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, CA, USA) equipped with a Xenon flash lamp as the source of excitation. SF spectra were acquired through simultaneous scanning of both monochromators over 400–550 nm excitation range in 1 nm increments with $\Delta\lambda$ of 10–100 nm, in steps of 10 nm. Excitation and emission slit widths were set at 5 nm, and the scan rate was 600 nm/min. The acquisition interval and integration time were set at 1 nm and 0·1 s, respectively. The mean value of two parallel measurements of each sample was used. The total SF spectra were obtained in a way that y-axis represents the fluorescence intensity as a function of excitation wavelength (x-axis). The contour maps of the total SF spectra were plotted using Matlab® 7.5.0 (The Mathworks, Inc., Natick, MA, USA).

Chemometric analysis

Chemometric analyses of the SF data were carried out using PLS Toolbox (Eigenvector Research, Inc., Wenatchee, Washington, USA) in Matlab® 7.5.0. Pre-processing techniques were selected after applying various techniques (smoothing, first derivative and mean centering) for improving the performance of PCA and partial least square (PLS) methods. Initially, PCA was utilised to investigate the clustering of samples using SF data of the milk samples. PCA

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scores were plotted using OriginPro 7.5 (OriginLab Corp., Northampton, MA, USA).

PLS was performed for determining the level of cow milk addition to buffalo milk, quantitatively. For this purpose, spectral data of the milk samples belonging to buffalo, cow and adulterant mixtures with different ratios were used as the calibration and validation data sets. Data set assignment for calibration and validation was made using the Kennard–Stone algorithm (Kennard & Stone, 1969) available in Matlab®. The algorithm selects the data one by one which are farthest from each other in the data set. After selection of the first two data which are the farthest two data, the third one is selected according to the following procedure: (i) The distances between each data and the previously selected samples are calculated. (ii) The one having the shortest distance with the maximum value in the group is selected. The selection of the data is maintained until all the samples are selected for the reserved data set. As a result, uniform distribution for cluster points is provided. Percentage of the samples to be transferred from the calibration set to the validation set was chosen as 33%.

Coefficient of determination (R^2), and root mean square error of cross-validation (RMSECV), root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP) values were calculated. And they were used to evaluate the performance of the models proposed. Limit of detection value (LOD) was calculated based on the SD of the response and the slope of the calibration graph (Hubert et al. 2007). The statistical analysis was carried out using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All analyses were performed in duplicate, and the results are expressed as mean ± SE.

Results and discussion

Chemical analyses of milk samples

The results of the chemical analyses of the milk samples are given in online Supplementary Table S1. The average of protein, fat and solids non-fat contents of buffalo milk were 4·21 g/100, 6·95 g/100 and 9·55 g/100 g, respectively. These values for cow milk were determined to be 3·11 g/100, 3·29 g/100, 8·14 g/100 g, respectively, indicating the significant ($P < 0·01$) differences between two sample groups. These results showed the higher contents of protein, fat, and solids non-fat in buffalo milk than in cow milk.

Discrimination of milk samples using PCA

Using ten different $\Delta\lambda$, ten different SF spectra were obtained for each sample. The total SF spectra of cow milk and buffalo milk are given in Fig. 1a, c, respectively. Figure 1b, d also show the contour plots of the total SF spectra of cow and buffalo milk samples, respectively. SF spectra of cow milk obtained at $\Delta\lambda = 10$ nm, showed a prominent peak at about 410 nm (Fig. 1a). For $\Delta\lambda = 20$ nm, no clear peak could be observed within these wavelengths. In Fig. 1c the same pattern was also observed for the buffalo milk data with $\Delta\lambda = 10$–100 nm. However, the peak at about 410 nm, the prominent peak of cow milk, was less intense in buffalo milk than in cow milk for $\Delta\lambda = 10$ nm. Differences seen in cow milk and buffalo milk throughout the spectra obtained for $\Delta\lambda$ of 10 nm were more distinct in the areas close to 410, 480 and 520 nm. These differences can also be clearly seen in the contour plots (Fig. 1b, d). In the present study, SF spectra recorded at $\Delta\lambda = 10$ nm were used in PCA for the discrimination of milk samples, because this corresponding spectrum provided distinct identified bands without any noise. The first derivative and smoothing were selected as the pre-processing techniques. Two principal components (PC) were chosen with regard to their highest cumulative variance. Scores of the PCs were used to differentiate the samples. The first PC explained 99·71% of the total variance, and the second PC explained 0·20% of the total variance. It is clearly seen from the PCA score plot (Fig. 2) that cow milk and buffalo milk samples can be successfully discriminated using the spectral data. All the other $\Delta\lambda$ values also enabled successful discrimination (data not shown).

It is known that the fluorescence spectra of the samples give valuable information about the molecules containing conjugated double bonds. Because of the various molecules in milk such as aromatic amino acids and nucleic acids, vitamin A and riboflavin that contain these bonds (Karoui et al. 2005), fluorescence spectroscopy presents an alternative for the analysis of milk samples. Zaidi et al. (2008) used this method for discrimination between ewe milk samples produced from two different breeds. They reported aromatic amino acids and nucleic acid spectra recorded after excitation set at 250 nm as fingerprints allowing a good discrimination between samples. In another research by Hammami et al. (2010), front face fluorescence spectroscopy was employed, and a good discrimination of ewe milk samples from different feeding systems was reported. They also employed SF spectroscopy for this aim, and reported that discrimination of aforementioned milk samples could not be obtained. However, for discrimination of such complex samples, SF spectroscopy has been reported to work better than conventional fluorescence techniques (Sikorska et al. 2005). In the case of SF spectroscopy, the shape and intensity of spectra depends on the wavelength interval $\Delta\lambda$, which defines the overlap of the excitation and emission bands of various fluorescent molecules. This means that SF spectrum of a complex sample could give valuable information on more than one fluorophore in a single spectrum and may be considered as a characteristic fingerprint allowing the identification of the sample (Sikorska et al. 2005; Zaidi et al. 2008). SF spectroscopy have been employed in combination with statistical methods for discrimination of various complex systems such as milk (Blecker et al. 2012; Liu et al. 2014) and edible oils (Sikorska et al. 2005). In the study of Sikorska et al. (2005) the potential of SF

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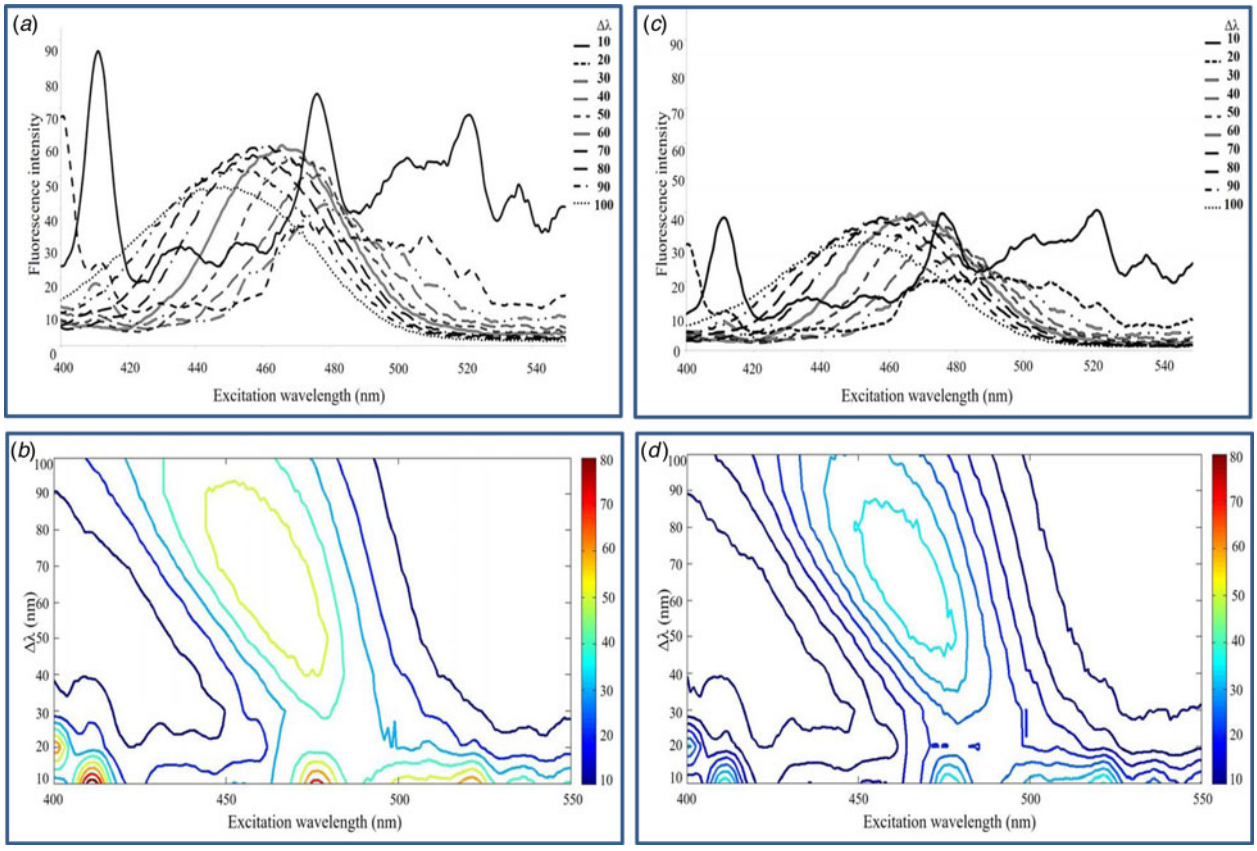

Fig. 1. Total synchronous fluorescence (SF) spectra of representative cow milk (a) and buffalo milk (c) samples recorded at $\Delta\lambda$ from 10 to 100 nm, and contour plots of total SF spectra of cow milk (b) and buffalo milk (d).

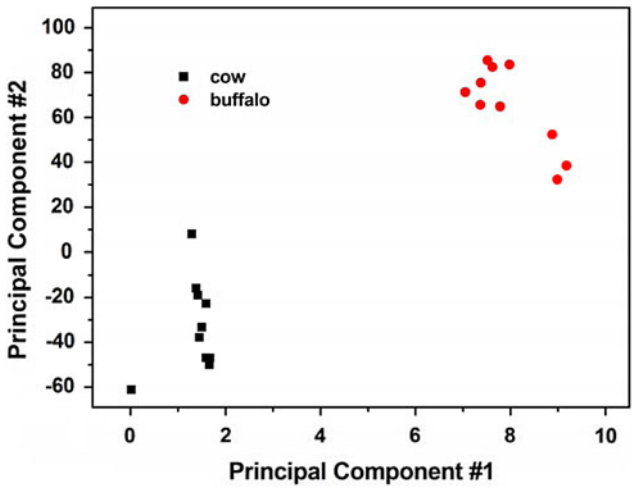

Fig. 2. PCA score plot of the differentiation of cow and buffalo milk samples.

spectroscopy for discriminating the edible oils was evaluated, and they acquired a successful classification with 99% accuracy. In a recent research conducted by Liu et al. (2014) SF spectroscopy was also used for qualitative detection of reconstituted milk adulteration in raw milk and pasteurised milk samples, and accurate discrimination was obtained. Similar to these previous studies, the present study also provided successful (100%) discrimination of milk samples by using SF spectroscopy in combination with PCA.

Quantitative prediction of adulteration of buffalo milk using PLS

The SF spectra of cow milk and buffalo milk obtained for $\Delta\lambda = 10$ nm (Fig. 1a–d) were used for determination of adulteration of buffalo milk with cow milk. The adulteration of buffalo milk resulted in visible changes in the spectra (Fig. 3) characterised by a continuous differentiation in fluorescence intensity of the peaks as the concentration of cow milk, the adulterant, increased. The PLS calibration models were generated between the level of adulteration and the SF spectral data recorded for $\Delta\lambda$ value of 10 nm. Smoothing was chosen as the pre-processing method which enabled the reduction of noise in the system measurements while keeping useful variation. Correlations between the spectral data and the level of adulteration were calculated. For $\Delta\lambda = 10$ nm, R^2 values for the calibration and validation data sets are 0·998 and 0·989,

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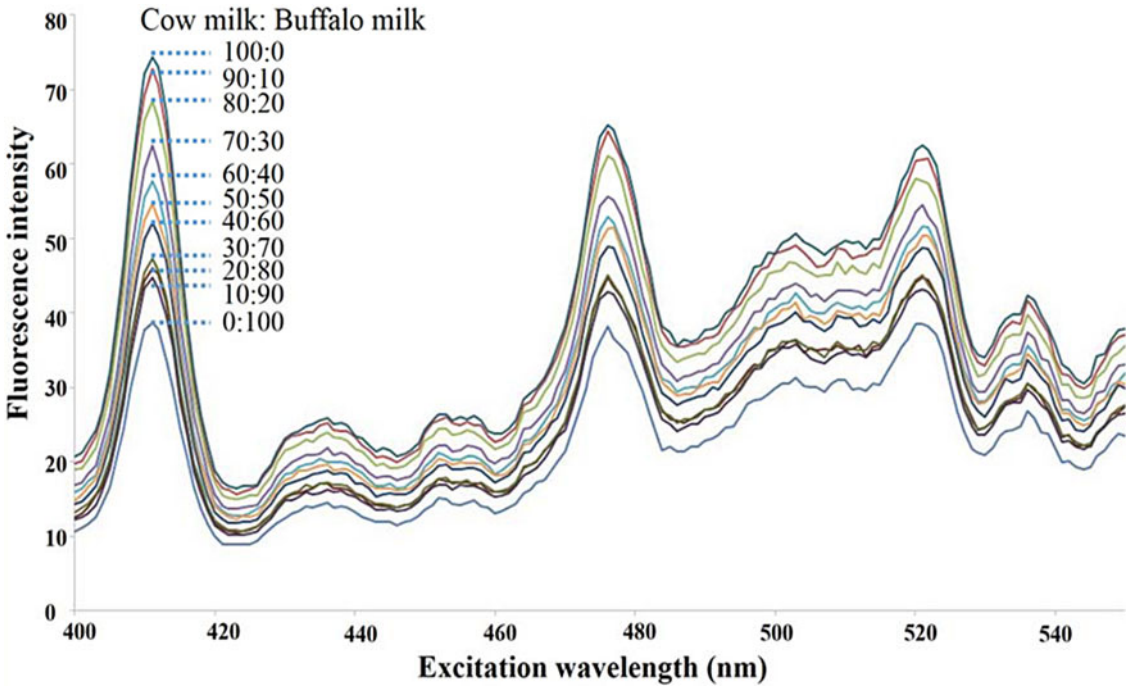

Fig. 3. The SF spectra of adulterated buffalo milk samples recorded at $\Delta\lambda$ of 10 nm.

respectively (online Supplementary Fig. S1a, b). This showed that there was a good correlation between the actual values of the level of buffalo milk adulteration and the values estimated using PLS regression for calibration and validation data sets. RMSEC, RMSECV and RMSEP values were determined as 2, 7 and 4%, respectively, indicating higher prediction errors than the values in previous findings.

Few studies have been conducted in which SF spectroscopy was used for detecting the level of adulteration in dairy products. In the aforementioned study of Liu et al. (2014), which also included the quantitative detection of reconstituted milk adulteration in raw milk and pasteurised milk samples, the correlation coefficients (r) of actual adulteration values *vs* predicted values using SF technique were reported to be 0·911 for both sample groups, corresponding to lower R^2 values than the values obtained in the present study. However, they achieved low RMSEC and RMSEP values. Dankowska et al. (2014) also used SF spectroscopy for detection of butter adulteration. They reported the lowest RMSEC of 3·8% and RMSEV of 3·9%, and obtained adjusted R^2 coefficients ranging from 0·91 to 1·00 (Dankowska et al. 2014). The same researchers also applied SF spectroscopy for detecting plant oil addition to cheese (Dankowska et al. 2015). They reported that the applied model could predict the level of adulteration with the RMSEP and RMSECV values of 1·5 and 1·8%, respectively (Dankowska et al. 2015).

A fraudulent addition of cow milk to buffalo milk might be present at a substantial level as lower levels of cow milk addition would not give any relevant commercial advantages (Czerwenka et al. 2010; Fuselli et al. 2015). LOD value of the present study (6%) is low enough to be used in the detection of possible adulteration that might be encountered in commercial practice. However, further studies would also be useful in order to improve LOD value. In previous studies, lower values were reported. Dankowska et al. (2014) reported the lowest LOD for adulteration of butter with palm and coconut oils as 5·5%. In their study about detecting plant oil addition to cheese using SF spectra, the lowest LOD values of 3·0 and 4·4% were reported for two different $\Delta\lambda$ values (Dankowska et al. 2015). In the light of the previous information, it was shown that SF spectroscopy worked well in predicting the level of detection of cow milk addition to buffalo milk with high R^2 values and acceptable prediction errors and LOD values.

In conclusion, buffalo milk and cow milk exhibit significant differences in SF spectral data in the range of 400–550 nm with $\Delta\lambda$ of 10 nm, enabling discrimination of buffalo milk and cow milk and detection of adulteration of buffalo milk with cow milk. This model study demonstrates the capability of SF spectroscopy for rapid authentication of buffalo milk, hence it can be implemented for routine analysis.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000073.

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References

Agrimonti C, Pirondini A, Marmiroli M & Marmiroli N 2015 A quadruplex PCR (qxPCR) assay for adulteration in dairy products. *Food Chemistry* **187** 58–64

Becker EM, Christensen J, Frederiksen CS & Haugaard VK 2003 Front-face fluorescence spectroscopy and chemometrics in analysis of yogurt: rapid analysis of riboflavin. *Journal of Dairy Science* **86** 2508–2515

Blecker C, Habib-Jiwan JM & Karoui R 2012 Effect of heat treatment of rennet skim milk induced coagulation on the rheological properties and molecular structure determined by synchronous fluorescence spectroscopy and turbiscan. *Food Chemistry* **135** 1809–1817

Boyaci IH, Temiz HT, Genis HE, Soykut EA, Yazgan NN, Guven B, Uysal RS, Bozkurt AG, Ilaslan K, Torun O & Seker FCD 2015 Dispersive and FT-Raman spectroscopic methods in food analysis. *Rsc Advances* **5** 56606–56624

British Standards Institution 1955 *Gerber Method for the Determination of Fat in Milk and Milk Products*. London: British Standards Institution

Czerwenka C, Muller L & Lindner W 2010 Detection of the adulteration of water buffalo milk and mozzarella with cow's milk by liquid chromatography-mass spectrometry analysis of beta-lactoglobulin variants. *Food Chemistry* **122** 901–908

Dankowska A, Malecka M & Kowalewski W 2014 Application of synchronous fluorescence spectroscopy with multivariate data analysis for determination of butter adulteration. *International Journal of Food Science and Technology* **49** 2628–2634

Dankowska A, Maecka M & Kowalewski W 2015 Detection of plant oil addition to cheese by synchronous fluorescence spectroscopy. *Dairy Science and Technology* **95** 413–424

Enne G, Elez D, Fondrini F, Bonizzi I, Feligini M & Aleandri R 2005 High-performance liquid chromatography of governing liquid to detect illegal bovine milk's addition in water buffalo Mozzarella: comparison with results from raw milk and cheese matrix. *Journal of Chromatography A* **1094** 169–174

Fuselli F, Deluca A, Montepeloso EA, Ibba G, Tidona F, Longo L & Marianella RM 2015 Detection of fraudulent addition of bovine whey in water buffalo ricotta cheese by isoelectric focusing. *Journal of the Science of Food and Agriculture* **95** 2757–2762

Hammami M, Rouissi H, Salah N, Selmi H, Al-Otaibi M, Blecker C & Karoui R 2010 Fluorescence spectroscopy coupled with factorial discriminant analysis technique to identify sheep milk from different feeding systems. *Food Chemistry* **122** 1344–1350

Hubert P, Nguyen-Huu JJ, Boulanger B, Chapuzet E, Chiap P, Cohen N, Compagnon PA, Dewe W, Feinberg M, Lallier M, Laurentie M, Mercier N, Muzard G, Nivet C, Valat L & Rozet E 2007 Harmonization of strategies for the validation of quantitative analytical procedures – A SFSTP proposal – Part II. *Journal of Pharmaceutical and Biomedical Analysis* **45** 70–81

Hurley IP, Coleman RC, Ireland HE & Williams JHH 2004 Measurement of bovine IgG by indirect competitive ELISA as a means of detecting milk adulteration. *Journal of Dairy Science* **87** 543–549

IDF 2002 *Milk and Milk Products: Determination of Nitrogen Content : Routine Method Using Combustion According to the Dumas Principle*. International Dairy Federation, Geneva, Switzerland

IDF 2014 *The World Dairy Situation 2014*, pp. 1–230, International Dairy Federation, Brussels, Belgium

Karoui R, Martin B & Dufour E 2005 Potentiality of front-face fluorescence spectroscopy to determine the geographic origin of milks from the Haute-Loire department (France). *Lait* **85** 223–236

Kennard RW & Stone LA 1969 Computer aided design of experiments. *Technometrics* **11** 137–148

Liu H, Han DH & Wang SP 2014 Potentiality of synchronous fluorescence technology for determination of reconstituted milk adulteration in fresh milk. *Spectroscopy and Spectral Analysis* **34** 2685–2689

Patra D & Mishra AK 2002 Recent developments in multi-component synchronous fluorescence scan analysis. *Trends in Analytical Chemistry* **21** 787–798

Sadecka J & Tothova J 2007 Fluorescence spectroscopy and chemometrics in the food classification – a review. *Czech Journal of Food Sciences* **25** 159–173

Sikorska E, Gorecki T, IV K, Sikorski M & Koziol J 2005 Classification of edible oils using synchronous scanning fluorescence spectroscopy. *Food Chemistry* **89** 217–225

Thomas CS 2008 *Efficient Dairy Buffalo Production*. Sweden: DeLaval

Zaidi F, Rouissi H, Dridi S, Kammoun M, De Baerdemaeker J & Karoui R 2008 Front-face fluorescence spectroscopy as a rapid and non-destructive tool for differentiating between sicilo-sarde and comisana Ewe's milk during lactation period: a preliminary study. *Food and Bioprocess Technology* **1** 143–151