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Use of fluorometry for determination of skim milk powder adulteration in fresh milk*

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Abstract: A FAST (fluorescence of advanced Maillard products and Soluble Tryptophan) method for identification of reconstituted milk made from skim milk powder in the fresh milk was developed. Considering milk and skim milk powders variations from different seasons and countries, milk was collected from different dairy farms in different seasons and skim milk powders were collected from different countries to measure the Tryptophan (Trp), advanced Maillard products (AMP) fluorescence values. The results showed that there were differences (P<0.01) between raw and reconstituted milk. The plot of values in each mixed level of raw and reconstituted milk had a correlation coefficient >0.97. The FAST method is a simple, rapid, low-cost and sensitive method enabling the detection of 5% reconstituted milk in fresh milk. The measurement of the Trp, AMP fluorescence values and calculation of the FAST index is a suitable method for large-scale monitoring of fresh milk samples.

Key words: Adulteration, Skim milk powder, Advanced Maillard products, Tryptophan, Fluorescence value, FAST index
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INTRODUCTION

Adulteration of fresh milk by adding reconstituted milk or the selling of reconstituted milk as the fresh product can be economically advantageous when either a surplus of milk powder exists or in countries where the importation of dried milk powder is subsidized. Adulteration malpractice creates unfair competition, could lead to market distortions, which in turn may impact the local or even the international economy. When legislation prohibits such practice, the detection of reconstituted milk depicted as fresh milk or mixed with fresh milk, becomes an analytical problem.

Methods of detection have depended on changes brought about by heat treatment and drying of milk powder in the molecular structure of milk constituted. Some of these methods include: the determination of ultra violet and visible spectra (700 to 240 nm) (Madkour and Moussa, 1989), the detection of furosine as an indicator of presence of reconstituted milk powder in raw and in pasteurized milk by HPLC (Resmini *et al*., 1992), the detection of hydroxymethylfurfural as an indicator of dried powder in liquid milk (Rehman *et al*., 2000). In addition, measurements of δD and ^{18}O stable isotope ratios have been used to distinguish reconstituted milk (Lin *et al*., 2003). However, the methods above require tedious pretreatment procedures, are time consuming and expensive.

The FAST method, which has been described elsewhere (Birlouez-Aragon *et al*., 1998; 2002) and is a global and effective approach for measuring two complementary heat-induced phenomena using fluorescence: (1) overall whey protein denaturation, followed by means of tryptophan (Trp) fluorescence

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value (F_{Trp}) at excitation/emission of 290/340 nm on the pH 4.6-soluble supernatant (F_{Trp} is a rapid approach to measure the protein concentration in supernatant containing non-denatured whey proteins), and (2) the Maillard reaction, globally quantified by the fluorescence (F_{AMP}) of the advanced Maillard products (AMP), such as pyrrole and imidazole derivatives, at excitation/emission of 330/420 nm. The FAST index is obtained by dividing F_{AMP} by the F_{Trp} fluorescence and multiplying the resulting quotient by 100 ($100\times F_{AMP}/F_{Trp}$). Advanced Maillard products and Soluble Tryptophan fluorescence values and FAST index have been used to evaluate the intensity of heat treatment applied to milk (Birlouez-Aragon *et al*., 2002). Therefore, the measured fluorescent values of AMP and Trp and the calculated FAST index provide a basis to distinguish fresh milk from reconstituted milk made from skim milk powder.

The purpose of this study was to develop a rapid, simple and precise method to detect the presence of reconstituted milk. Fresh samples of milk were collected from different dairy farms and samples of skim milk powder were collected from different countries to see whether there are relationships between raw milk and reconstituted milk. Raw milk was mixed with reconstituted milk from the laboratory and the manufacturer in different ratios to evaluate the detection capability. Furthermore, large quantities of sample were collected to check whether there is any correlation or difference in the raw milk from different dairy farms in different seasons in China.

MATERIALS AND METHODS

Reagents and solutions

All solutions were prepared using Milli-Q water and analytical reagent grade chemicals.

Sodium acetate solution of 0.1 mol/L was prepared from sodium acetate (27.218 g dried for 2 h at 95 °C to 100 °C) in water. Acetic acid solution of 0.10 mol/L was prepared from acetic acid in water. pH 4.60 sodium acetate buffer of 0.10 mol/L was prepared from 0.10 mol/L sodium acetate solution and 0.10 mol/L acetic acid solution. One g/L bovine serum albumin stock solution was prepared from 0.50 g bovine serum albumin in water. One mg/L bovine serum albumin solution was obtained by appropriate dilution of stock solution with water.

Instrumentation

A LS-50B (Perkin Elmer, USA) fluorimeter equipped with a Xenon lamp and excitation and emission monochromators; MilkoScan FT120 (FOSS, Denmark) FTIR instrument designed for the analysis of dairy products; Delta 320-S (Mettler Toledo, Switzerland) pH meter, BioSafe Avanti J-10 centrifuge (Beckman, USA) with rotary speed reaching up to 17700 g. Millex-HA (Millipore, USA) filter. C5 (Avestin, Canada) High Pressure Homogenizer.

Sample description

Twenty raw milk samples were collected from different dairy farms in different seasons in Hangzhou City, China. Twenty skim milk powders and ten anhydrous milk fats were obtained from the market and manufacturers in Hangzhou City, China.

Reconstituted milk samples from the manufacturer were prepared at the same ratio and the same reconstitution process. This reconstituted milk prepared was the same as that normally sold in the market. Different levels of reconstituted milk were mixed into raw milk. These samples were kept for 1 d at 4 °C for equilibration. Reconstituted milk samples were prepared with skim milk powder and water. The milk was pasteurized for 20 s after the central temperature reached 90 °C. A high-pressure homogenizer was used to make the adulterated raw milk sample.

We repeated the detection capability test, with different levels of reconstituted milk mixed into raw milk. These samples were kept for 1 d at 4 °C for equilibration.

Sample treatment

One ml milk sample was measured and 6 ml sodium acetate buffer, 0.10 mol/L, pH 4.60 was added. The mixture was vigorously shaken and left for 5~10 min at room temperature. Then the solution was centrifuged at 4000 g for 10 min. Finally, the supernatant was filtered through a 0.45 μm pore filter and the filtrate was diluted 50 times in distilled water in order to avoid light scattering. Each sample was prepared and analyzed in triplicate.

Trp and AMP fluorescence measurement

Fluorescence was measured in counts of photons

emitted per second, Trp fluorescence was measured at 290/340 nm. Fluorescence of advanced Maillard products was measured at 330/420 nm.

Determination

The fluorescence values were given in equivalent 1 mg/L proteins by using 1 mg/L bovine serum albumin as external calibration. The FAST index was calculated by dividing F_{AMP} fluorescence by F_{Trp} and multiplying by 100.

RESULTS AND DISCUSSION

In our pretest, we assumed that if skim milk powders were used for preparing reconstituted milk, the content of the non-denatured whey proteins and the advanced Maillard products should be different between raw milk and reconstituted milk. As far as we know, skim milk powder was submitted to heat-treatments and pressure-treatments during processing, milk underwent chemical and biochemical changes that affect different components, mainly proteins, carbohydrates. It resulted in interactions between the amino acid lateral groups, degradation reactions of lateral chains of the proteins, insolubilization of whey proteins, and interactions between κ-casein and β-lactoglobulin, interactions with lipids, and interactions between carbohydrates and proteins (Pompei *et al*., 1988). Based on the intensity of the thermal treatment of milk, denaturation of whey proteins takes place in various degrees (Kolosta *et al*., 2004). Thus, in contrast to the native fraction, these denatured whey proteins precipitate together with caseins when the milk pH is lowered to 4.6 (Dalgleish, 1990; Corredig and Dalgleish, 1999). During heat treatment, drying and storage of dairy products, the relatively high concentration of the reducing carbohydrates (lactose) and lysine-rich proteins in milk make it especially sensitive to these thermally induced nonenzymatic reactions (Maillard reaction) (Henle *et al*., 1991; Erbersdobler, 1994; Leclère and Birlouez-Aragon, 2001). As mentioned previously, the Trp, AMP fluorescence values and FAST index in reconstituted milk should be related to skim milk powder. More precisely, the FAST index and AMP fluorescence values of reconstituted milk should be higher than that of raw milk but the Trp fluorescence values of reconstituted milk should be lower than that of raw milk.

After the pretest, we found that there was significant difference between raw milk and reconstituted milk made from skim milk powder. So we further conducted a sample survey to see if there was a difference between raw milk and reconstituted milk made from the skim powders that came from different countries.

Table 1 presents the Trp, AMP fluorescence values and FAST index of the raw milk from five dairy farms in different seasons and reconstituted milk from ten countries. These data were collected during the pretest period within 1 year. Data showed that the values in the same column were significantly different (P<0.01), it appears that the skim milk powders of different countries do not affect the significant difference (P<0.01) between raw milk and reconstituted milk made from skim milk powder. The measurement of milk FAST index presented the best repeatability among the three values, whereas the milk Trp fluorescence values showed the worst repeatability. The results indicated that there are significant difference (P<0.01) of the Trp, AMP fluorescence values and FAST index between the raw milk of different seasons and reconstituted milk from different countries.

Table 1 AMP and Trp fluorescence values and FAST index of raw milk and reconstituted milk (equivalent mg/L)

	F_{AMP}	F_{Trp}	FAST index
Raw milk	1.72±0.11[a]	20.15±1.06[a]	8.55±0.38[a]
Reconstituted milk	2.28±0.08[b]	7.22±0.27[b]	31.55±1.10[b]

[a] The values in the same column with the different letter are significantly different (P<0.01)

The results suggested that the non-denatured whey proteins and AMP of raw milk differ significantly from that of reconstituted milk due to the processing effects of skim milk powder (P<0.01). Therefore, the Trp, AMP fluorescence values and FAST index could serve as an indicator for monitoring the quality of raw milk. The Trp fluorescence values were more significantly different than AMP fluorescence values. The variation of whey protein was larger than that of AMP during heat and pressure treatment, because β-lactoglobulin is the major pro-

tein in whey protein of milk, whose denaturation is usually induced by heat and hydrostatic pressure (Valente-Mesquita *et al*., 1998; Botelho *et al*., 2000). FAST index had the most significantly difference among them because of the different accumulation of the Trp, AMP fluorescence values.

Table 2 presents the Trp, AMP fluorescence values and FAST index for raw milk from five dairy farms in the four seasons. Data showed that there was no significant difference (P>0.05) between Trp, AMP fluorescence values and FAST index of milk from different dairy farms and different seasons, it is concluded that the effect of two factors are neglectable.

Table 2 AMP and Trp fluorescence values and FAST index for raw milk from the same dairy farm in different seasons (equivalent mg/L)

	F_{AMP}	F_{Trp}	FAST index
Spring	1.73±0.15[a]	20.36±1.05[a]	8.48±0.66[a]
Summer	1.74±0.11[a]	20.13±1.03[a]	8.66±0.30[a]
Autumn	1.73±0.08[a]	20.35±1.22[a]	8.50±0.22[a]
Winter	1.69±0.09[a]	19.77±1.01[a]	8.54±0.23[a]

[a] The values in the same column with the different letter are significantly different (P<0.05)

The FAST index had the least coefficient of variation. However, the coefficients of variation of AMP fluorescence values being the largest could be attributed to the temperature variations among seasons. As far as we know, the Maillard reaction that occurs even at low temperatures, is relatively slow in high-moisture foods, but it is the predominant reaction at ambient temperatures in foods with low moisture content, such as milk or whey powders (Labuza and Saltmarch, 1981).

An experiment was conducted to measure the detection capability of adulteration. Figs.1, 2 and 3 show the plots of the Trp, AMP fluorescence values and FAST index of raw milk and reconstituted milk made by laboratory mixed in different ratios. It is shown that the correlation coefficients for Trp, AMP fluorescence values and FAST index were greater than 0.98. According to previous work, hydroxymethylfurfural was the main product of Maillard reaction, hydroxymethylfurfural values have been used to detect adulteration of raw, pasteurized and Ultra High Temperature (UHT) liquid milk with dried skim milk powder (Rehman *et al*., 2000). By using the ratio of β-casein to α-lactalbumin, the adulteration of fresh milk with 25% or more of skim milk powder could be detected (Chen and Zang, 1992). Analysis of those results indicated that the FAST index and Trp,

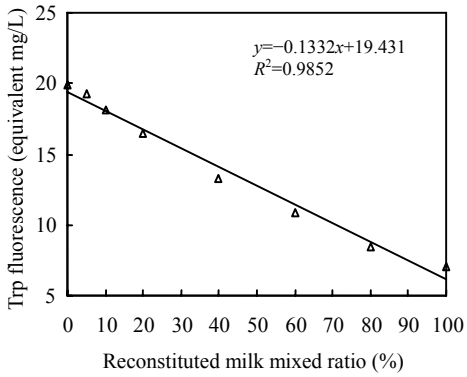

Fig.1 Trp fluorescence values of raw milk and reconstituted milk from laboratory mixed in different ratios

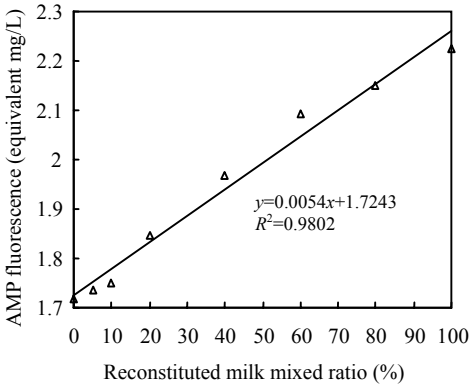

Fig.2 AMP fluorescence values of raw milk and reconstituted milk from laboratory mixed in different ratios

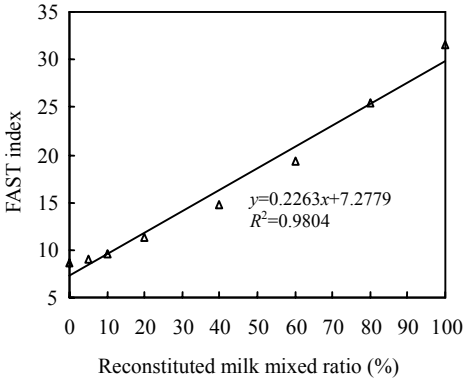

Fig.3 FAST index of raw milk and reconstituted milk from laboratory mixed in different ratios

AMP fluorescence values are suitable indicators of skim milk powder adulteration in raw milk. Furthermore, the FAST index had the most significant difference among them because the FAST index integrated the Trp, AMP fluorescence difference into one cumulative result. The FAST index is more sensitive to heat treatment than furosine determination which is an indicator for detection of reconstituted milk in raw and in pasteurized milk (Resmini *et al*., 1992; Birlouez-Aragon *et al*., 2002).

Figs.4, 5 and 6 show the plots of Trp, AMP fluorescence values and FAST index for different mixed levels of fresh milk and reconstituted milk made by manufacturers. As mentioned before, this reconstituted milk was prepared and pasteurized by the manufacturers as normally sold in the market. It is shown that the correlation coefficients for Trp, AMP fluorescence values and FAST index are greater than 0.98, 0.97 and 0.97, respectively. It is clear that the AMP fluorescence values and FAST index of pasteurized milk increased moderately while the Trp fluorescence values of pasteurized milk decreased a little. The most reliable index of pasteurized milk thermal damage is the ratio of soluble whey proteins to total whey proteins (Resmini *et al*., 1989; Pagliarini *et al*., 1990). Pasteurization significantly decreased the levels of pH 4.6-soluble protein (Rynne *et al*., 2004). Similarly, Maillard Reaction is characterized by complex chemical reactions that usually occur during processing and storage of foods containing reducing sugars and amino groups (Jing and Kitts, 2002). The heat treatment induced increase of the advanced Maillard products fluorescence values in dairy products (Birlouez-Aragon *et al*., 2002). These results may not be simply applied to every manufacturer because different manufacture may apply different heating procedure during pasteurization or different mixing steps during homogenization; but it is reasonable to conclude that the Trp, AMP fluorescence values in reconstituted milk were related to skim milk powder.

It was shown that the AMP fluorescence values were related to skim milk powder and could be differentiated from fresh milk, but the statistical difference and correlation coefficient of advanced Maillard products fluorescence values could only be used to detect the presence of large amounts of skim milk powders in fresh milk. The Trp fluorescence values

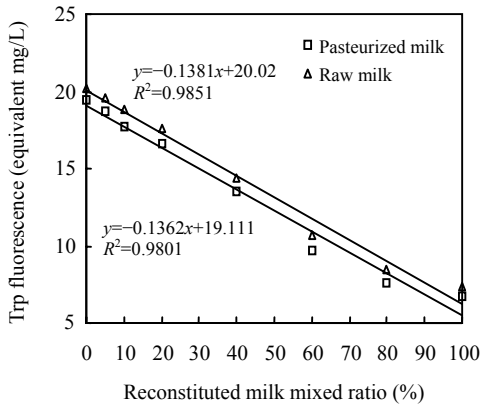

Fig.4 The plots of Trp fluorescence values in each level from raw milk and pasteurized milk adulterated with reconstituted milk made by manufacturer

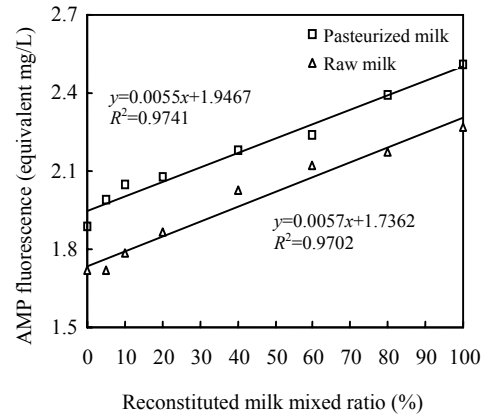

Fig.5 The plots of AMP fluorescence values in each level from raw milk and pasteurized milk adulterated with reconstituted milk made by manufacturer

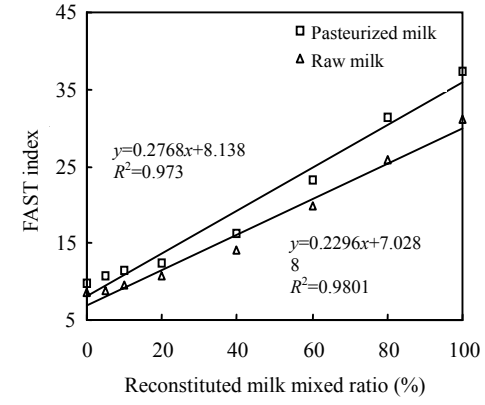

Fig.6 The plots of FAST index Trp fluorescence values in each level from raw milk and pasteurized milk adulterated with reconstituted milk made by manufacturer

and FAST index can be used to detect the presence of 10% and 5% of skim milk powders in fresh milk, respectively. If a databank of Trp fluorescence value and FAST index of each kind of skim milk powder and raw milk from different areas and season were set up, these data could serve as adulteration control for fresh milk.

CONCLUSION

The results of this study showed that the FAST method is a suitable, simple and rapid method for large-scale monitoring of raw milk samples. The measured Trp, AMP fluorescence values and the calculated FAST index in raw milk and reconstituted milk can be used to detect and monitor whether milk adulteration has occurred.

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