Establishment and application of milk fingerprint by gel filtration chromatography

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ABSTRACT

Raw milk adulteration frequently occurs in undeveloped countries. It not only reduces the nutritional value of milk, but it is also harmful to consumers. In this paper, we focused on investigating an efficient method for the quality control of raw milk protein. A gel filtration chromatography (GFC) fingerprint method combined with chemometrics was developed for fingerprint analysis of raw milk. To optimize the GFC conditions, milk fat was removed by centrifugation, and GFC analysis was performed on a Superdex 75 10/300GL column (Just Scientific, Shanghai, China) with 0.2 M NaH₂PO₄-Na₂HPO₄ buffer (pH 7.0) as the mobile phase. The flow rate was 0.5 mL/min, and the detection wavelength was set at 280 nm. Ten batches of 120 raw milk samples were analyzed to establish the GFC fingerprint under optimal conditions. Six major peaks common to the chromatogram of each raw milk sample were selected for fingerprint analysis, and the characteristic peaks were used to establish a standard chromatographic fingerprint. Principal component analysis was then applied to classify GFC information of adulterated milk and raw milk, allowing adulterated samples to be effectively screened out from the raw milk in principal component analysis scores plot. The fingerprint method demonstrates promising features in detecting milk protein adulteration.

Key words: raw milk, gel filtration chromatography, fingerprint, application

INTRODUCTION

Dairy products represent one of the most important food groups in our daily life because they contain neces-

sary nutrients, especially protein (2.8–3.4% in milk), that play a role in meeting human nutritional requirements. However, milk safety has been a severe challenge in recent years (Zhang et al., 2014). Milk products are sometimes adulterated with water, glucose, neutralizers, and other substances (Borin et al., 2006) that alter milk composition and reduce milk nutritional quality. For example, in the case of the Chinese dairy safety scares in 2008, melamine was intentionally added to milk to boost the measured protein content (Balabin and Smirnov, 2011). This adulteration led to the deaths of 6 infants and caused serious kidney complications in more than 51,900 infants and young children (Xu et al., 2009b). Therefore, authenticating and identifying the characteristics of chemical compounds in milk and related products and conducting quality control are necessary (Ferreira and Caçote, 2003).

Protein is a complex nitrogenous organic compound, and different proteins vary from one another based on their structures and amino acid contents. The richer products are in proteins, the higher their nutrition and economic values (Marcó et al., 2002). Soybeans, as a cheap source of plant proteins, are added to feed and food products all over the world (Luykx et al., 2007). The usual method for determining the protein content in a product is the Kjeldahl method, which estimates the protein content through measuring nitrogen (Kamizake et al., 2003). The effectiveness of the Kjeldahl method is reduced when adulteration of a product involves nitrogen-rich compounds (Draher et al., 2014). Therefore, HPLC, liquid chromatography-tandem mass spectrometry (LC-MS/MS), GC-MS, and GC-MS/MS have been used extensively to detect milk protein adulteration. However, time-consuming and complex sample pretreatment procedures limit their use (Zhang et al., 2014).

As a comprehensive analytical method, the chromatographic fingerprint approach was introduced and accepted by the World Health Organization for evaluating herbal medicines, and it has commonly been used for quality control of dietary supplements, botanical

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materials, and foods in recent years (Arceusz and We-solowski, 2013; Zhao et al., 2013). The chromatographic fingerprint method can comprehensively reflect the species and quantities of chemical components and then be used to describe and evaluate the overall quality of a product (Xu et al., 2009a; Zhou et al., 2010; Gao et al., 2012, 2013). The obtained data may be analyzed by chemometrics, which has been applied in plant science, nutrition, traditional Chinese medicine, systemic biology, and other areas (Kannel et al., 2007; Yi et al., 2009; Hossain et al., 2011). Many potential analytical approaches exist for detecting adulteration, such as thin-layer chromatography (Marchand et al., 2008), HPLC (Xie et al., 2006), ultra-high performance liquid chromatography (Sun and Chen, 2012), and capillary electrophoresis (Yu et al., 2007). Compared with thin-layer chromatography, HPLC, and capillary electrophoresis, the GFC approach has many advantages, such as pretreatment being convenient and organic solvents not being needed. The GFC approach can also separate proteins and peptides effectively.

Casein and whey protein are the 2 major proteins in milk. Casein contains α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN), while whey protein is primarily composed of β -LG and α -LA (Bonfatti et al., 2013). Based on the molecular sieve effect of GFC (London et al., 2014), different milk protein molecules generate different chromatogram profiles, from which a milk protein fingerprint can be obtained.

Chemometric technique for data analysis plays a fundamental role in both food characterization and detection of adulteration. In particular, the chemometric analysis of chromatograms does not require the identification of all compounds detected in foods (Rodríguez et al., 2010). As a sophisticated technique, principal component analysis (PCA) is widely used to reduce the dimensions of multivariate data setting, while retaining its variation and developing inferential models for important unmeasured properties (Nelson et al., 2006). Some researchers have applied PCA to deal with data on the concentration of protein, fat, minerals, and trace elements in raw milk (Sola-Larrañaga and Navarro-Blasco, 2009). Pintus et al. (2012) predicted genomic breeding values for dairy traits in Italian Brown and Simmental bulls with PCA. Processing the chromatography fingerprint with PCA is simple and automatic, which avoids subjective decisions and provides visual patterns.

The objectives of this study were to develop a GFC fingerprint method combined with chemometrics for fingerprint analysis of raw milk protein and to use the standard chromatographic fingerprint along with PCA to detect protein adulteration in raw milk.

MATERIALS AND METHODS

Materials and Reagents

Ten batches of 120 raw milk samples were collected from the bulk tanks of 4 dairy farms in Guanzhong region of Shaanxi, China (every month from January to November 2014). Guanzhong region is located at 33.39° to 35.52° N and 106.56° to 110.22° E, with an area of 55,600 km². The temperature of the milk was maintained at 4 ± 2°C during transport and storage. The milk underwent analysis within 24 h after sampling. Three equal milk samples were taken from 3 bulk tanks on each dairy farm, and then all 12 samples were mixed to form 1 batch sample per farm. The composition of raw milk was 3.26 ± 0.10 g/100 g of milk fat, 3.04 ± 0.16 g/100 g of milk protein, and 4.41 ± 0.13 g/100 g of lactose. Soybeans, peanut protein powder, soybean protein powder, wheat gluten meal, and hydrolyzed animal protein (HAP) powder were purchased from a local supermarket. Melamine with 99% purity was purchased from Sigma-Aldrich Company (St. Louis, MO). All reagents used in this research were of analytical purity.

Selection of Defatting Method

Centrifuging. The raw milk sample (300 mL) was centrifuged at 2,500 × *g* at 4°C for 20 min, then the skim milk was filtered through a 0.22-μm poly(ethersulfone) syringe filter for analysis.

Röse-Gottlieb Method. The milk fat was extracted from raw milk by the Röse-Gottlieb method (Vlaeminck et al., 2005) with little modification. A 10-mL milk sample was put into an appropriate-sized volumetric flask and mixed with 1.25 mL of ammonia water, heated in a 60°C water bath for 5 min, and then shaken vigorously. Next, 10 mL of ethanol, 25 mL of diethyl ether, and 25 mL of petroleum ether were added sequentially and mixed gently. After the extraction step, the supernatant was transferred into fat-collecting vessels after phase separation (30 min).

Optimization of Chromatographic Conditions

To optimize chromatographic conditions, a Superdex 75 10/300GL column (10 × 300 mm, Just Scientific, Shanghai, China), Superdex 200 10/300GL column (10 × 300 mm, Just Scientific), and Superdex Peptide 10/300GL column (10 × 300 mm, Amersham Co., Uppsala, Sweden) were used for the separation of samples (300 μL). The mobile phase was 0.2 M NaCl-KCl-Na₂HPO₄ buffer (pH 7.0), 0.2 M NaH₂PO₄-Na₂HPO₄ buffer (pH 7.0), 2.5 mL/100 mL isopropanol-0.05 M phosphoric acid buffer (pH 7.0), and 2.5 mL/100 mL

acetonitrile-0.05 *M* phosphoric acid buffer (pH 7.0), respectively. The flow rate was 0.5 mL/min, and the detection wavelengths were 215, 254, and 280 nm. The injection volume was 200 μ L. The isolation was carried out at room temperature.

Preparation of Sample Solution

Six types of fake milk sample solutions (3.0 g/100 g protein) were prepared as follows. The melamine sample solution was prepared by using the filtrate from dissolving 0.7053 g of melamine in 100 mL of distilled water and filtering through a 0.22- μ m filter membrane. The total protein content was determined by using the Kjeldahl method (hereinafter the same). Preparations of the freshly ground soybean milk and peanut pulp were as follows. The dry soybeans (Jidou 12, 60 g) were soaked in 200 mL of distilled water at 20 to 25°C for 8 to 12 h. Defatted soybean milk was then obtained by centrifuging the mixture at $2,500 \times g$ at 4°C for 20 min, removing the soaking water, adding 480 mL of fresh water, pulping by soy milk machine (JYL-350, Joyoung Co. Ltd., Shandong, China), and filtering with a 200-mesh screen through a 0.22- μ m filter membrane. The peanuts (Jinhua No. 2, 50 g) were soaked in 300 mL of distilled water at 20 to 25°C for 8 to 12 h, and the sample was then prepared by the same procedure as the soybean milk. The aqueous solutions of soybean protein powder (90% protein), wheat gluten meal, and HAP powder were prepared using the same procedure. The powders were dissolved in 1.0 mL of 0.1 *M* NaOH and diluted with distilled water to 100 mL, and the solutions were then filtered through a 0.22- μ m filter membrane. All final sample protein concentrations were adjusted to 3.0 g/100 g.

Adulterated milk samples were prepared by adding fake milk sample solutions to raw milk. For all adulterants other than melamine, the concentrations in the adulterated samples were 0.1, 0.5, 1.0, 2.5, and 5.0 g/100 g; for melamine, the concentrations were 1.0, 1.5, 2.0, and 2.5 mg/kg. Adulterated milk samples were prepared in triplicate for each concentration. All of the sample solutions were stored in the refrigerator at 4°C until analysis, which was carried out within 24 h of sampling.

Validation of the GFC Method

The stability, precision, and repeatability were tested to validate the method. The sample for the precision test was from the same batch of mixed milk, and detection was conducted 6 times. Six samples for the repeatability test were from the same batch of mixed milk, but the sampling, treatment, and determination were

carried out independently. Samples for stability were from the same batch of milk and were detected at 0, 2, 6, 10, 14, and 24 h, respectively. Retention time and peak areas under common peaks on the chromatogram were used to calculate the value of relative standard deviation (**RSD**).

Similarity of the GFC Fingerprint of the Raw Milk

The correlation coefficient and angle cosine value were calculated based on the fingerprint information obtained by GFC (Cao et al., 2006).

Principal Component Analysis

As a nonparametric method of classification, principal components (**PC**) make no assumption about the underlying statistical data distribution (Kannel et al., 2007) and primarily concern the transformation of a large set of related variables into a new smaller set of uncorrelated variables. Because all the constructed PC are orthogonal, the object scores can be plotted against each other to present the distribution of the objects in the space (Zhao et al., 2013). In this study, PCA was used to classify the gel fingerprint chromatography information of 6 types of adulterated milk and raw milk.

Statistical Analysis

Statistical analysis of chromatographic data was carried out by using Unicorn 5.01 (GE Healthcare, Uppsala, Sweden) software. The similarities among the sample fingerprints were analyzed by PCA, which was set up by using the Minitab 15 software (Six Sigma Academy International, New York, NY). All other statistical analysis was conducted by using SPSS 17.0 (SPSS Inc., Chicago, IL) software. The data were expressed as mean $\pm$ standard deviations or relative standard deviations.

RESULTS AND DISCUSSION

Selection of Defatting Method

To identify the optimal fat removal conditions for sample pretreatment, defatting methods (centrifugation and Röse-Gottlieb method) were investigated. Centrifugation yielded a better peak shape (Figure 1a) and a larger peak area on chromatography, compared with the Röse-Gottlieb method (Figure 1b). The Röse-Gottlieb method had a negative effect on peak because ethanol could cause milk protein precipitation (Horne and Muir, 1990). In addition, diethyl ether has a stronger toxicity, which carries a severe safety risk and

Figure 1. Effect of defatting method on gel filtration chromatography profiles of milk protein: (a) centrifuging; (b) Röse-Gottlieb method. Color version available online.

represents a serious environmental hazard (Evers et al., 2000). Consequently, centrifugation was not only safer than the Röse-Gottlieb method, but it was also found to be more effective for isolating the fat from raw milk.

Optimization of GFC Conditions

To develop a chromatographic fingerprinting method of raw milk protein, an optimized strategy for GFC conditions was performed. The mobile phase, detector wavelength, and separation column were all investigated, which were the key factors for GFC analysis (Chen et al., 2010).

The effect of different milk protein contents on the types of GFC column was studied. Figure 2 shows that more symmetrical peaks and better separation were obtained by using the Superdex 75 10/300GL column compared with the Superdex 200 10/300GL and Superdex Peptide 10/300GL columns. Moreover, the former had a smoother baseline than the latter columns, and the molecular weight range of the Superdex G75 column was largely consistent with that of milk proteins.

The effects of mobile phase on chromatographic separation of the milk proteins were also investigated.

Figure 3 indicates that the separation of sample constituents was significantly influenced with 0.2 M NaCl-KCl- Na_2HPO_4 buffer (pH 7.0), 2.5 mL/100 mL isopropanol-0.05 M phosphoric acid buffer (pH 7.0), and 2.5 mL/100 mL acetonitrile-0.05 M phosphoric acid buffer (pH 7.0), respectively. High resolution and symmetrical peaks were obtained by using 0.2 M NaH_2PO_4 - Na_2HPO_4 buffer (pH 7.0).

The optimized GFC conditions were as follows: the separation was performed on a Superdex 75 10/300GL column using the 0.2 M NaH_2PO_4 - Na_2HPO_4 (pH 7.0) buffer as the mobile phase, the flow rate was 0.5 mL/min, and the detection wavelength was set at 280 nm.

Figure 2. Effect of chromatographic columns on gel filtration chromatography profiles of milk protein: (a) Superdex 75 10/300GL (Just Scientific, Shanghai, China); (b) Superdex 200 10/300GL (Just Scientific); (c) Superdex Peptide 10/300GL (Amersham Co., Uppsala, Sweden). Color version available online.

Figure 3. Effect of mobile phases on gel filtration chromatography profiles of milk protein: (a) NaCl-KCl- Na_2HPO_4 buffer; (b) isopropanol and phosphoric acid buffer; (c) NaH_2PO_4 - Na_2HPO_4 buffer; (d) acetonitrile and phosphoric acid buffer. Color version available online.

Establishment of GFC Fingerprint of Raw Milk and Similarity Evaluation

Determination of the Common Peaks of the Raw Milk Protein. Ten batches of raw milk samples were analyzed to establish the GFC fingerprint under the optimization conditions. The overlap chromatograms of the 10 batches of raw milk samples are shown in Figure 4. Six major peaks (peak no. 1, 2, 3, 4, 5, and 6) appeared in the chromatogram of each batch of milk samples, and the chromatographic patterns were generally consistent with each other. The total area of the 6 peaks was $94.5 \pm 3.2\%$ of the whole area, which was more than the criterion (90%; Yang et al., 2011), so the 6 peaks were assigned as the common peaks. The peak area rank was peak 1 > peak 5 > peak 6 > peak 2 > peak 3 > peak 4. The average ratio of peak areas was about 1.000:0.664:0.232:0.143:0.084:0.004 (peaks 1, 5, 6, 2, 3 and 4, respectively). In addition to the common peaks, 9 to 12 noncommon peaks appeared in different chromatograms, but the noncommon peak area was $5.0 \pm 3.3\%$, which was less than the national criterion (10%) (Pan et al., 2011). According to the molecular weight, the elution order of milk protein should be as follows: BSA, $\alpha_{\text{S}2}$ -CN, $\alpha_{\text{S}1}$ -CN, β -CN, κ -CN, β -LG, and α -LA (Kinghorn et al., 1995; Park and Jin, 1998; Wal, 2002; Javed et al., 2012). The overlapping of peaks suggests that the same peak would include different milk protein components. The specific peaks representing different components of milk proteins (Figure 4) did not play a major role for the purpose of this study. The area of the peaks and the proportional relationship among them were pivotal, which could be used to differentiate milk samples. In this study, the 6 common peaks were used to establish a standard GFC fingerprint of raw milk in Guanzhong region (Shaanxi, China).

Validation of the Method. The results of validation experiments of retention volume and peak area are shown in Tables 1 and 2, respectively. The RSD was taken as a measurement. Six independent samples from the same batch of milk were tested for stability. The RSD of the retention volume ranged from 0.88 to 2.40%, and the RSD of each peak area ranged from 3.32 to 4.88%. The results demonstrated that the GFC fingerprint method was found to be stable within 24 h for milk. The precision of the method was determined by successive injection of a sample of the same milk 6 times; the retention volume and peak area of each common peak RSD were below 5.0%. Six independently prepared sample solutions of the same batch of milk were analyzed to validate the repeatability of the method. The RSD of the retention volume ranged from 1.35 to 2.55%, and the RSD of each peak area ranged from 2.76 to 4.98%. Therefore, the precision, repeat-

Table 1. Validation of stability, precision, and repeatability of retention volume

Peak no.	Stability		Precision		Repeatability	
	Mean	RSD ¹ (%)	Mean	RSD (%)	Mean	RSD (%)
1	9.328	2.406	9.13	3.124	9.164	2.555
2	12.73	1.222	12.47	1.519	12.53	1.685
3	14.21	1.591	14.09	2.038	14.14	2.156
4	17.45	2.348	17.60	1.001	17.54	2.027
5	21.88	0.8856	21.12	1.410	21.09	1.356
6	28.13	2.096	28.75	1.857	28.64	1.606

¹Relative standard deviation.

ability, and stability of this method were successfully demonstrated. The GFC fingerprint of raw milk can be used to conduct quality control of milk protein.

Similarity of the GFC Fingerprint of Raw Milk.

The similarity of the GFC fingerprints of 10 batches of raw milk was calculated by correlation coefficients, and the results are shown in Table 3. The similarity value among 10 batches of milk samples was more than 0.96, and the correlation coefficient and angle cosine value of each chromatogram compared with the simulative mean chromatogram were 0.9761 ± 0.0119 and 0.9904 ± 0.0083 , respectively (Table 3), which indicated that the raw milk protein was extremely similar in the 10 batches. So, distinguishing the raw milk from adulterated protein milk could be conveniently done by comparing the common peaks of the GFC fingerprints.

Application of GFC Fingerprint of Raw Milk

Many methods exist for detecting milk protein adulteration. When we know what materials are added into milk (intentionally or unintentionally), the corresponding detection methods can be selected easily. If the identity of the materials or the presence of adulterant is unknown, finding the corresponding detection methods is very difficult. Under these circumstances, the GFC fingerprint method can be easily carried out to check the authenticity of milk (i.e., whether adulteration has occurred). For determining the species and quantity of

adulterants, the corresponding detection methods need to be selected accordingly.

When the milk contained the maximum concentration of 2.5 mg/kg melamine or 5.0 g/100 g of another adulterant (HAP powder, peanut milk, soybean milk, soybean protein powder, or wheat gluten meal), the difference between adulterants and raw milk protein could be easily found in chromatograms. However, for a low concentration of adulterant (melamine ranged from 1.0 to 2.0 mg/kg, and the other adulterants were from 0.1 to 2.5 g/100 g), the chromatograms hardly differed. If a small quantity is added, finding obvious differences in the chromatograms by the naked eye is difficult. Therefore, using PCA to classify the GFC information of adulterated milk and raw milk was necessary. The results revealed 2 PC (PC1 and PC2), with a cumulative variance contribution rate of 89.53% being found. Figure 5 shows a plot of PCA scores obtained for the analysis of the raw milk and the adulterated milk samples. The 6 adulterants at different concentration levels are obviously different from raw milk samples. The raw milk samples and the milk mixed with peanut milk or wheat gluten meal, could be easily distinguished from PC1. Moreover, it should be noted that the PC2 revealed the difference between the raw milk samples and the milk containing soybean protein powder, soybean milk, HAP powder, or melamine. The soybean milk, soybean protein powder, and wheat gluten meal samples were placed above zero on PC2, whereas

Table 2. Validation of stability, precision, and repeatability of peak area

Peak no.	Stability		Precision		Repeatability	
	Mean	RSD ¹ (%)	Mean	RSD (%)	Mean	RSD (%)
1	425.2	4.423	429.5	3.022	407.1	4.183
2	104.7	4.436	93.31	4.762	97.68	3.702
3	66.12	4.880	64.34	4.606	64.04	3.238
4	2.498	4.860	2.644	4.863	2.347	3.576
5	315.8	3.325	316.3	3.611	298.3	2.762
6	162.6	3.936	155.9	3.615	153.2	4.985

¹Relative standard deviation.

Figure 4. The gel filtration chromatography fingerprint of raw milk. Peaks 1 to 6 are the major peaks. Color version available online.

melamine, HAP powder, and peanut milk samples were positioned below zero on PC2. Raw milk was almost on the zero line on PC2. The PCA results indicated that the protein compositions of the 6 adulterants and raw milk differed, which could easily help to distinguish adulterated milk from raw milk. Some overlapping in fingerprint patterns was observed for soybean protein powder and soybean milk samples in our present work, and a strong need exists to obtain more representative results from more samples. Nevertheless, the results illustrated that the main chemical components in soybean protein powder and soybean milk samples were

similar, confirming that the relationship between the 2 materials was very close.

Melamine, HAP, and raw milk were all in PC1/PC2 quadrants, showing that great similarities existed in the chemical composition of the 3 substances. Raw milk and HAP powder both include animal protein and have high homology. Although the chemical composition of melamine had obvious differences compared with raw milk, the amount of melamine added into raw milk was very small. In addition, the main ingredients were very similar to the raw milk, so the scores were relatively close in the plot and could be easily confused. But, we can see from Figure 5 that raw milk was on the negative side of the PC1 axis and nearby area, HAP powder was located in the lower left portion of negative PC2 axis, and melamine was in the lower right portion. The boundaries among the 3 substances were relatively clear, so they could be differentiated. These results suggest that a standard chromatographic fingerprint combined with the chemometric method could be used to distinguish adulterated milk from raw milk.

CONCLUSIONS

This study successfully established the GFC fingerprint of raw milk protein. Six satisfactorily resolved characteristic peaks were identified in the fingerprint

Table 3. Similarity evaluation of 10 batches of raw milk protein fingerprints

Batch no.	Correlation coefficient	Angle cosine value
1	0.9845 ± 0.0148	0.9940 ± 0.0054
2	0.9955 ± 0.0043	0.9981 ± 0.0016
3	0.9956 ± 0.0041	0.9903 ± 0.0084
4	0.9694 ± 0.0142	0.9852 ± 0.0142
5	0.9514 ± 0.0132	0.9809 ± 0.0167
6	0.9665 ± 0.0151	0.9903 ± 0.0087
7	0.9420 ± 0.0154	0.9835 ± 0.0124
8	0.9903 ± 0.0079	0.9931 ± 0.0055
9	0.9804 ± 0.0144	0.9926 ± 0.0062
10	0.9854 ± 0.0152	0.9958 ± 0.0037
Total mean ± SD	0.9761 ± 0.0119	0.9904 ± 0.0083

Figure 5. Scores plot of the first 2 principal components (PC1 and PC2) from principal component analysis for milk and adulterant samples: ● = raw milk, × = hydrolyzed animal protein (HAP) powder, ▼ = peanut milk, + = soybean milk, ▲ = soybean protein powder, ◆ = wheat gluten meal, ■ = melamine.

profiles of 10 batches of 120 raw milk samples. The conditions of the 6 peaks were used to screen out the adulterants, using the plot of PCA scores. The GFC fingerprinting method combined with chemometrics is effective for differentiating milk adulteration. The GFC method is simple and easy, does not require organic solvents, and has high separation efficiency for milk protein and polymers. Different types of milk can be distinguished by associating GFC fingerprinting with PCA, which may be used for quality control of milk samples and dairy products.

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