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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* NaH_2PO_4-Na_2HPO_4 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 necessary 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 Wesolowski, 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 km^2. 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-Na_2HPO_4 buffer (pH 7.0), 0.2 *M* NaH_2PO_4-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