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Rapid detection of milk adulteration using intact protein flow injection mass spectrometric fingerprints combined with chemometrics

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ABSTRACT

Flow injection mass spectrometry (FIMS) combined with chemometrics was evaluated for rapidly detecting economically motivated adulteration (EMA) of milk. Twenty-two pure milk and thirty-five counterparts adulterated with soybean, pea, and whey protein isolates at 0.5, 1, 3, 5, and 10% (w/w) levels were analyzed. The principal component analysis (PCA), partial least-squares-discriminant analysis (PLS-DA), and support vector machine (SVM) classification models indicated that the adulterated milks could successfully be classified from the pure milks. FIMS combined with chemometrics might be an effective method to detect possible EMA in milk.

1. Introduction

Milk is one of the most likely adulterated foods (Moore, Spink, & Lipp, 2012). Protein powders such as soybean and pea protein isolates are among possible adulterants because of their low prices (Luykx et al., 2007; Maraboli, Cattaneo, & Giangiacomo, 2002). Consuming milk adulterated with other powders may cause health problems such as allergies (Morr, 1979). Therefore, detecting possible protein adulterants in milk is significant in protecting consumer's interest and the public welfare. In addition, adulteration of raw milk hurts not the consumer, but diary manufacturers as well. Particularly, to prevent possible EMA, analyses of raw milk in every batch from all suppliers is challenging due to large sample size. As a result, a rapid adulteration detection method is highly demanded.

Mass spectrometry is an important and widespread analytical approach in food authentication. Milk adulteration detection based on liquid chromatography-mass spectrometry (LC-MS) have been extensively studied (Cordewener et al., 2009; Scholl, Farris, & Mossoba, 2014). However, a typical LC-MS run of milk took about 1 h (Cordewener et al., 2009; Luykx et al., 2007). In comparison, flow injection mass spectrometry (FIMS) was demonstrated to analyze many food ingredients such as peppermints and Chinese wolfberries in less than 2 min for each sample (Gao et al., 2012; Lu et al., 2014), a more than 30-fold efficiency increment. FIMS fingerprints combined with principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA) showed promising results in the quality assurance of functional foods and spices (Chen, Harnly, & Lester, 2010; Gao et al., 2012; Lu et al., 2014; Zhao et al., 2013) without time-consuming sample pretreatment methods, such as borate enrichment and proteolytic digestion (Cordewener et al., 2009). Therefore, FIMS may be suitable for rapid detection of adulterated milk.

Chemometrics play an increasingly important role in the untargeted detection of adulteration nowadays. For example, chromatographic fingerprints combined with PCA and PLS-DA showed promising results in the detection of food adulteration without identifying specific compounds (Rodríguez, Ortiz, Sarabia, & Gredilla, 2010); Soft independent modeling of class analogy (SIMCA) classification models, support vector machine (SVM), and partial least-squares regression (PLSR) were used to detect and quantify milk adulteration by mid-infrared (MIR) spectrometry (Bassbasi, Platikanov, Tauler, & Oussama, 2014; Santos, Pereira-Filho, & Rodriguez-Saona, 2013); PCA using the mass spectral intensities of selected peptide fragments from LC-MS analyses was able to detect milk adulterated with non-milk proteins (Cordewener et al., 2009). Compared to LC-MS, because FIMS fingerprints of milk yield no chromatographic separation and minimal sample pretreatment, it is inherently complicated with multiply-charged protein peak series and large amounts of unrelated noise. Consequently, chemometrics may especially be useful for FIMS to extract relevant information from

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complex fingerprints.

This work aimed to detect adulterated milk using FIMS combined with chemometrics. FIMS fingerprints of pure milks and milks adulterated with soybean, pea, and whey protein isolates were measured. The chemometric adulterant detection approaches were compared to direct visual inspection approaches using both raw and charge state deconvoluted FIMS spectra. Specifically, PCA was applied to examine whether there were significant differences between pure and adulterated FIMS fingerprints. Additionally, PLS-DA and SVM classification were evaluated for their ability to automatically classify pure and adulterated milks. This work may provide a novel perspective about whether FIMS with chemometrics could be a rapid, suitable, and reliable milk quality control tool.

2. Materials and methods

2.1. Materials and reagents

Twenty-two raw milk samples were provided by Direct Supplier Farmers of Nestlé Qingdao factory, located in Shandong Province, China. Milks were immediately stored at −20 °C upon reception, and lyophilized by a Labconco freeze dryer (Kansas City, Missouri, USA). Seven protein powders obtained from different suppliers in China were selected as potential protein adulterants. Specifically, soybean protein isolates 1 and 2 were offered by Yuwang Food (Yucheng, Shandong, China); pea protein isolates 1 and 2, and whey protein isolates 1, 2, and 3 were offered by Shuangta Food (Yantai, Shandong, China).

Ultrapure water was obtained using a Milli-Q Advantage A10 system (EMD Millipore, Darmstadt, Hesse, Germany). Mass spectrometric grade acetonitrile and HPLC grade formic acid were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and used as the solvent phases. All other chemical reagents were of analytical grade and used without further purification.

2.2. Sample preparation

Twenty-two pure and thirty-five adulterated samples were prepared. All samples were prepared in triplicates. For pure milks, 50 mg milk powder was dissolved by 10 mL 70 °C water. The solution was immediately vortexed for 5 min and centrifuged for 10 min at 13400*g* at 4 °C. Then, 200 μL supernatant was collected as the reconstituted milk solution at 5 mg/mL for each FIMS analysis.

For adulterated milks, adulterant stock solutions were prepared first, following the same method for reconstituted milk solution. Subsequently, 0.05, 0.1, 0.3, and 0.5 mL adulterant stock solutions were respectively added to 0.995, 0.99, 0.97, and 0.95 mL reconstituted milk solutions to prepare 0.5, 1, 3, 5, and 10% (w/w) adulterated milks. Afterwards, the mixture was vortexed for 2 min and centrifuged for 10 min at 13400*g* at 4 °C. Then, 200 μL supernatant was collected for FIMS analysis.

2.3. Instrumentation

An ACQUITY ultra-performance liquid chromatography combined with a Xevo G2 quadrupole time-of-flight mass spectrometer (UPLC-Q-TOF-MS, Waters, Milford, Massachusetts, USA) was used for FIMS analysis. The chromatographic column was not installed, an ACQUITY UPLC Protein BEH C4 VanGuard precolumn (2.1 mm i.d. × 5 mm, 1.7 μm, Waters) was connected instead as an additional online filter. The mobile phase was 0.1% formic acid in 50% acetonitrile (v/v). The flow rate was 0.5 mL/min and the injection volume was 2 μL. Mass spectra were collected from 0.05 to 1.00 min at m/z from 500 to 2500. The detection parameters were as follows: ionization temperature, 120 °C; desolvation temperature, 450 °C; capillary voltage, 3 kV; cone voltage, 30 V; cone gas flow, 50 L/h; desolvation gas flow, 800 L/h; collision energy, 6.0 eV.

2.4. Data preprocessing

Raw data were collected by MassLynx version 4.1 (Waters) and exported into MATLAB R2013b (The MathWorks, Natick, Massachusetts, USA). Each FIMS spectra was binned to a 0.1 m/z increment. The data matrix consisted of 171 instances by 20,001 variables, including 66 and 105 spectra of pure and adulterated counterparts, respectively. The preprocessing by normalization (scaling the maximum intensity of each spectra to unity) and subsequent autoscaling (mean-centering and scaling to unit variance) was applied for PCA, and normalization was applied for PLS-DA and SVM.

2.5. Charge state deconvolution

The molecular weight determination (MoWeD) algorithm (Pearcy & Lee, 2001) was used as the charge state deconvolution method for preliminary investigation. The MoWeD routine were calculated using an in-house MATLAB routine. The deconvolution was performed on the high-resolution raw spectra before 0.1 m/z peak binning. The maximum possible charge was set to +30. After deconvolution, the spectra were transformed to zero-charged spectra with mass error of 10 ppm. The amino acid sequences of milk proteins were obtained from the Universal Protein Knowledgebase (The UniProt Consortium, 2017). The protein theoretical average masses were calculated by the Compute pI/Mw tool in the Expert Protein Analysis System Bioinformatics Database (Artimo et al., 2012; Gasteiger et al., 2005).

2.6. Chemometrics modeling

The PCA and PLS-DA routines were all written in-house using MATLAB. LIBSVM software package version 3.21 with MATLAB interface (Chang & Lin, 2011) was used for SVM calculations. For classification models, the prediction accuracy, i.e., percentage correctly classified samples in the test set were used for performance evaluation. The bootstrapped Latin partitions (BLP) statistic with 10 bootstraps and 5 Latin partitions was applied for PLS-DA to determine the optimal number of latent variables (Harrington, 2006). In SVM modeling, obtaining the optimal parameters is a critical step to achieve the expected performance. The radial basis function (RBF) was selected as the kernel function of SVM training with hard penalty. A grid-search algorithm by 3-fold cross-validation was used to determine the optimal parameters (Ward, McGuffin, Buxton, & Jones, 2003). The penalty (C) and kernel radii (γ) were optimized based on the lowest cross validation error. A series of values were searched in the grid as follows: $C = 2^0, 2^1, 2^2, \ldots, 2^{10}$, $\gamma = 2^{-8}, 2^{-7}, 2^{-6}, \ldots, 2^0$. All other parameters were kept unchanged.

3. Results and discussion

3.1. Spectral characteristics of pure and adulterated milks

The FIMS spectra of pure milk, and milk adulterated with 10% (w/w) soybean protein isolate were demonstrated in Fig. 1. Major peaks observed were m/z = 533.3, 707.2, 1148.7, 1225.2, 1312.7, 1413.5, etc., and many minor peaks were observed between m/z = 600 and 1700. In Fig. 1, intensities of many minor peaks were presented in the adulterated milk with respect to pure milk. However, no significant observable marker peaks in FIMS spectra between pure and adulterated counterparts were present even at 10% (w/w). Therefore, determination of adulterated samples by solely inspecting specific peaks of the original spectra was not possible. This was consistent for all adulterated samples at other concentration levels and adulteration proteins (data not shown). Because FIMS measure all components at once, many components were at lower signal-to-noise ratio or missing in the spectra, compared with previous LC-MS studies (Cordewener et al.,