Technical note: Nontargeted detection of adulterated plant proteins in raw milk by UPLC-quadrupole time-of-flight mass spectrometric proteomics combined with chemometrics

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ABSTRACT

We built and validated a chemometric model to detect possible milk adulteration with plant proteins. Specifically, we extracted proteins in raw milk, treated with tryptic digestion, and obtained peptide fingerprints by UPLC-quadrupole time-of-flight-mass spectrometry with proteomics to differentiate authentic milks from their counterparts adulterated with nonmilk proteins. This approach is able to detect soybean and pea powder-adulterated milks at as low as 1% (wt/wt). Additionally, we obtained the characteristic peptide sequences for milk authentication by principal component analysis. The prediction accuracies for milk authentication by partial least-squares-discriminant analysis were greater than 95%. These results indicated that peptide fingerprints with the chemometric analysis could be successfully applied for milk quality control.

Key words: milk adulteration, nontargeted fingerprinting, UPLC-qTOF-MS, chemometrics

Technical Note

Milk-related food fraud was ranked second in the food fraud and economic adulteration database in 2012 (Moore et al., 2012). A typical type of fraud is adulteration using inexpensive materials, such as soybean or pea powder, to raise the protein content of milk (Luykx et al., 2007). Alteration of protein composition in milk potentially threatens customers' well-being and food safety (Sanchez-Monge et al., 2004). Consequently, techniques to differentiate authentic milk from adulterated milk with nonmilk proteins is in high demand.

In recent years, various analytical methods, including chromatography, spectroscopy, and enzyme immunoas-

say combined with chemometric techniques, have been investigated in quality control of milk (Cheng et al., 2010; Mecker et al., 2012; Jablonski et al., 2014; Lu et al., 2015). For instance, Jablonski et al. (2014) used UPLC coupled with UV detector to detect soybean and pea proteins in skim milk powder above 3% (wt/wt). Lu et al. (2015) successfully applied a UPLC AA fingerprinting technique in differentiating milk and nonmilk proteins in our previous study. Near-infrared and Raman spectroscopy combined with principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA) were used to detect possible adulteration in liquid and powdered milk (Botros et al., 2013; Yang et al., 2013; Alves da Rocha et al., 2015; Nieuwoudt et al., 2016). However, these methods often offered little information about the identities of adulterated proteins, which limited the final precision for the identification of adulterated proteins. Mass spectrometric proteomics has been proven reliable in identification of proteins due to its high sensitivity and accuracy (Czerwenka et al., 2007; Luykx et al., 2007; Cordewener et al., 2009; Cunsolo et al., 2011; Nicolaou et al., 2011; Gao et al., 2014; Chen et al., 2016). The frequently used approach is bottom-up MS proteomics, in which peptide fragments are obtained by enzyme digestion (Baldwin, 2004).

Previously, Luykx et al. (2007) demonstrated that authentic milk and counterparts adulterated with numerous soy or pea storage proteins could be identified using liquid chromatography with tandem mass spectrometry in combination with a borate enrichment step. However, uncertainty of the adulterant in actual application will demand a nontargeted detection approach (i.e., an approach that statistically defines what signals should be included in authentic samples), and any samples showing abnormal signals are determined to be not authentic. Unlike a screening approach such as spectroscopy, the MS proteomics-based nontargeted detection model is more challenging because proteomics

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analysis by liquid chromatography-mass spectrometry (LC-MS) is more time-consuming.

Our study aimed to evaluate the suitability of nontargeted UPLC-quadrupole time-of-flight-mass spectrometric (UPLC-qTOF-MS) proteomics model for differentiating authentic and adulterated raw milk. Specifically, differentiation is performed on a relatively large batch of adulterated and unadulterated samples (84 samples) using proteomics and chemometrics without any specific standards to achieve a model with certain statistical confidence (Hiller et al., 2009; Robinson et al., 2011; Gao et al., 2012). In our study, PCA was used to explore the distribution of the data, and PLS-DA was used to construct chemometric models and validate the model automatically without human intervention. We demonstrated that nontargeted peptide fingerprinting is feasible to provide a foundation toward effective quality control of raw milk and its related products, such as infant milk powder.

We collected raw milk samples from local dairy farms in China. We gathered 64 milk samples, including 31 and 33 samples collected in Shandong and Shanghai, China, respectively. The samples were lyophilized using a freeze dryer (Labconco, Kansas City, MO) once transferred to the laboratory and stored at -20°C . Two pea proteins and 3 soybean proteins were included as potential adulterants. Pea protein 1 and 2 were from Shuangta Food (Yantai, Shandong, China). Soybean protein 1 was from Gushen Biotech (Dezhou, Shandong, China), soybean protein 2 and 3 were from Yuwang Food (Yucheng, Shandong, China).

Milk powder was reconstituted in water to 20 mg/mL (20 mg of milk powder per milliliter of solution). Samples were shaken in a water bath at 40°C for 30 min to dissolve, and then centrifuged at $3,900 \times g$ for 10 min at 4°C . Solution that contained soluble proteins was collected as the stock solution, and the undissolved fats and water-insoluble proteins were discarded. Subsequently, the stock solution was diluted twice with water to make the working solutions. The dilution ratios were chosen separately to reach the protein concentration of 2.0 ± 0.1 mg/mL, quantitated by the enhanced bicinchoninic acid protein assay kit (Beyotime Biotechnology, Shanghai, China) with a standard curve prepared using a 25 mg/mL BSA standard solution.

Adulterated milk simulants at 4 different levels were prepared, including 1, 2, 4, and 10% (wt/wt, weight percentages of nonmilk protein in total milk protein). One milk sample from Shanghai was randomly selected as an authentic milk standard. First, nonmilk protein powders were dissolved and then added to authentic milk at a certain proportion. Both working solutions of milk and foreign proteins were prepared to reach

Table 1. Preparation of adulterated milk

Item	Abbreviation	Nonmilk protein solvent (mL)	Milk (mL)
1% Soybean milk	1%S	0.1	9.9
2% Soybean milk	2%S	0.2	9.8
4% Soybean milk	4%S	0.4	9.6
10% Soybean milk	10%S	1.0	9.0
1% Pea milk	1%P	0.1	9.9
2% Pea milk	2%P	0.2	9.8
4% Pea milk	4%P	0.4	9.6
10% Pea milk	10%P	1.0	9.0

a final protein concentration of 2.0 ± 0.1 mg/mL for further analysis. The preparation of adulterated milk is outlined in Table 1. Specifically, a calculated volume of working solution of nonmilk protein was added to the milk working solution to achieve an expected adulterant level. Adulterated samples were vortexed immediately. Forty adulterated milk samples were prepared.

The samples were treated by trypsin (Sigma-Aldrich, St. Louis, MO) to convert proteins to peptide fragments according to previous tryptic digestion protocols (Zhang et al., 2012, 2014). First, 1 mL of working solution was added to 1 mL of 100 mM ammonium bicarbonate (Sigma-Aldrich); the mixtures were then vortexed. Afterward, the disulfide bonds were reduced with 10 μL of 1 M DL-dithiothreitol (Sigma-Aldrich) at 50°C in a water bath for 30 min. Alkylation for reduced cysteine side chains was carried out with 30 μL of 1 M iodoacetamide (Sigma-Aldrich) in the dark for 30 min. Subsequently, 20 μL of 2 mg/mL trypsin solutions were added and the mixtures were incubated at 37°C for 12 h. Tryptic hydrolysates were heated at 85°C for 10 min to terminate digestion. The digested mixtures were centrifuged at $16,110 \times g$ for 15 min at 4°C . The supernatants were stored at -20°C before UPLC-MS analysis. All samples were prepared in duplicate.

The UPLC-MS analyses were performed by a Waters Acquity H-class UPLC system coupled to a Xevo G2 quadrupole time-of-flight mass spectrometer (Waters, Milford, MA). A UPLC BEH C18 column (2.1 mm i.d. $\times$ 100 mm, 1.7 μm , Waters) with a VanGuard BEH C18 precolumn (2.1 mm i.d. $\times$ 5 mm, 1.7 μm , Waters) was used for separation. The column temperature was 30°C and the sample temperature was 10°C . The injection volume was 5.0 μL . The 0.1% (vol/vol) formic acid (Sigma-Aldrich) in Milli-Q deionized water (EMD Millipore, Darmstadt, Germany) and 0.1% (vol/vol) formic acid in acetonitrile (HPLC-grade, Merck, Darmstadt, Germany) were used as mobile phases. The total run time for each injection was 78 min. The long run time ensured clear separation of peptides. The linear elution gradient program is listed in Table 2. A blank

Figure 1. Base peak intensity chromatograms of peptides generated by milk and different nonmilk proteins: (A) milk, (B) soybean, (C) pea.

run (acetonitrile, HPLC grade, Merck) was injected between every 10 injections.

The mass spectrometer was operated with an electrospray ionization source under the positive ion mode. The capillary voltage was 3.0 kV; the sampling cone voltage was 40 V. The source temperature was 120°C and the desolvation temperature was 450°C. The cone gas flow was 50 L/h and the desolvation gas flow was 600 L/h. Two channels of MS signals were collected. Channel 1 was recorded at 6.0 eV of collision energy and channel 2 was recorded between 20 to 50 eV. The scanning speed was 0.3 s/scan; the mass spectra between 50 and 1,800 *m/z* were recorded.

Base peak intensity chromatograms of peptides generated by milk and different nonmilk proteins are given

in Figure 1. Base peak intensity chromatograms were similar to TIC chromatograms, except they contained only the intensity of the most intense mass spectral peak at each chromatogram scan, which greatly avoids interferences of the baseline noise (Murray et al., 2013). The elution pattern of plant peptides differs greatly from that of milk during 25 to 27, 27 to 32, 36 to 40, and 41 to 45 min due to the dissimilar polarity of peptides.

The UPLC-MS spectra were analyzed using Protein-Lynx Global Server (**PLGS**; version 3.0, Waters) to identify proteins in the samples. The PLGS parameters were lock mass window at 0.25 Da, low-energy threshold at 25 counts, the elevated energy threshold was 10 counts, the intensity threshold was 750 counts, and the peptide tolerance was 10 µg/mL. Matched protein fragments and their corresponding intensities were exported to a spreadsheet file. Although protein identification provides further information of the nonmilk proteins, it is not essential for a nontargeted adulteration detection. The identification of abnormal samples can be performed solely on peptide fragments or raw spectral data when handling unknown plant protein adulterants without sequencing data.

We identified 267 proteins in adulterated milk matched by PLGS. After manual comparison, the identities of 26 major relevant proteins were revealed (Table 3). Six major bovine milk proteins (α-LA, α_{S1}-

Table 2. Gradient elution program for UPLC¹

Time (min)	Flow (mL/min)	A (%)	B (%)
0.0	0.4	98	2
4.9	0.4	98	2
5.0	0.2	98	2
65.0	0.2	60	40
67.0	0.4	15	85
75.0	0.4	0	100
76.0	0.4	98	2
78.0	0.4	98	2

¹A = 0.1% (vol/vol) formic acid in deionized water; B = 0.1% (vol/vol) formic acid in acetonitrile.

α_{S2} - and β -CN, β -LG, and glycosylation-dependent cell adhesion molecule-1) were detected in both authentic and adulterated milk samples. Eleven soybean and 9 pea proteins in adulterated milk at the 10% adulterant level were identified, respectively. The identified soybean proteins included trypsin inhibitor, glycinin, conglycinin, and albumin, and the identified pea proteins included provicilin, vicilin, legumin, and convicilin. The numbers of identified proteins were less than the work by Luykx et al. (2007), in which a borate enrichment step before liquid chromatography with tandem mass spectrometry measurement could identify 12 soybean and 11 pea proteins in adulterated milk at 5% (wt/wt). However, only 3 bovine milk proteins were detected at the same time. Moreover, the detection limits of the UPLC method for foreign proteins in skim milk powder were above 3% (wt/wt, percentages over total weight) by Jablonski et al. (2014). In comparison, soybean protein (β -conglycinin, α and β subunit) and pea protein (provicilin type B and vicilin) could be detected at 1% (weight percentages of nonmilk protein over milk protein) in the current study, exemplifying the better sensitivity of our approach.

All data were then processed using MATLAB R2013b software package (The MathWorks, Natick, MA) for

chemometrics processing. The peptide intensities of the precursor ions were used for chemometrics modeling. The intensities were combined when peptides had different retention times within the same sequence. The autoscaling preprocessing was used before data modeling. Both PCA and PLS-DA were performed using in-house MATLAB programs. The fingerprint data set consisted of 169 objects with 1,799 variables (peptide sequences). These peptide sequences contained the peptides generated by milk and nonmilk proteins.

The data set was examined for its overall distribution using all available peptide information with exploratory data analysis by PCA. The principal component scores and loading plots for authentic and adulterated milks using the targeted method are displayed in Figure 2. The first 2 principal component scores are plotted in Figure 2A. The adulterated milk samples formed 2 clusters away from the authentic milk samples. Three separated clusters suggested the authentic and adulterated milks were clearly differentiated from each other. Milks mixed with different types of plant proteins behaved differently in PCA, which could be attributed to different mass fingerprints of plant protein (Cordewener et al., 2009). The loading plot yielded similar presentation of point distribution, except each point corresponded

Table 3. Identified proteins in adulterated milk¹

ID	Organism	Protein name	Mass (Da)	Coverage (%)	Score	Occurrence
P00711	<i>Bos taurus</i>	α -LA	16,247	38.73	7,196	All
P02662	<i>Bos taurus</i>	α_{S1} -CN	24,529	62.15	9,834	All
P02663	<i>Bos taurus</i>	α_{S2} -CN	26,019	23.87	2,917	All
P02666	<i>Bos taurus</i>	β -CN	25,107	21.88	2,245	All
P02754	<i>Bos taurus</i>	β -LG	19,883	79.21	18,920	All
P80195	<i>Bos taurus</i>	GlyCAM-1	17,152	23.53	1,737	All
P01070	<i>Glycine max</i>	Trypsin inhibitor A	24,005	31.02	450	10%S2;10%S3
P01071	<i>Glycine max</i>	Trypsin inhibitor B	20,041	24.31	292	10%S2;10%S3
P02858	<i>Glycine max</i>	Glycinin G4	63,587	31.32	625	10%S1;10%S2;10%S3
P04347	<i>Glycine max</i>	Glycinin	57,956	22.48	255	10%S3
P04405	<i>Glycine max</i>	Glycinin G2	54,931	31.13	1,437	4%-,10%S1;4%-,10%S2;4%-,10%S3
P04776	<i>Glycine max</i>	Glycinin G1	55,706	36.57	992	4%-,10%S1;4%-,10%S2;4%-,10%S3
P11827	<i>Glycine max</i>	β -Conglycinin, α' subunit	74,325	36.15	770	4%-,10%S1;10%S2;10%S3
P11828	<i>Glycine max</i>	Glycinin G3	54,242	5.82	388	10%S1;10%S2;4%-,10%S3
P13916	<i>Glycine max</i>	β -Conglycinin, α subunit	70,293	40.66	2,162	1%-,2%-,4%-,10%S1;4%-,10%S2;4%-,10%S3
P19594	<i>Glycine max</i>	2S albumin	18,460	34.18	449	10%S2
P25974	<i>Glycine max</i>	β -Conglycinin, β subunit	50,552	27.33	560	1%-,4%-,10%S1;10%S2;1%-,4%-,10%S3
P02854	<i>Pisum sativum</i>	Provicilin (type B)	46,385	26.83	2,212	1%-,2%-,4%-,10%P1;1%-,2%-,4%-,10%P2
P02855	<i>Pisum sativum</i>	Provicilin (type A)	31,540	49.45	1,745	4%-,10%P1;4%-,10%P2
P02856	<i>Pisum sativum</i>	Vicilin, 14 kDa component	14,040	37.1	2,130	4%-,10%P1;10%P2
P02857	<i>Pisum sativum</i>	Legumin A	58,805	17.21	230	10%P1
P13915	<i>Pisum sativum</i>	Convicilin	66,990	19.26	689	4%-,10%P1;4%-,10%P2
P13918	<i>Pisum sativum</i>	Vicilin	52,231	44.01	2,579	2%-,4%-,10%P1;1%-,4%-,10%P2
P13919	<i>Pisum sativum</i>	Convicilin	46,396	28.24	435	10%P1
P14594	<i>Pisum sativum</i>	Legumin B	38,990	21.89	1,153	4%-,10%P1;4%-,10%P2
P15838	<i>Pisum sativum</i>	Legumin A2	59,270	21.73	424	10%P1;4%-,10%P2

¹ID = protein accession number; coverage = percent protein sequence coverage; GlyCAM-1 = glycosylation-dependent cell adhesion molecule 1. All = detected in all samples; X%Y means sample adulterated with nonmilk protein Y at X% (wt/wt); S1, S2, and S3 were 3 soybean proteins. P1 and P2 were 2 pea proteins.

to a peptide fragment in the sample. Specifically, some characteristic peptide sequences for differentiating authentic and adulterated milks could be easily identified from the loading plot (Figure 2B). For instance, LQES-

VIVEISK, FYLAGNQEQEFLK, SQSDNFEYVSFK, and QIVTVEGGLSVISPK had high values in milks adulterated with pea proteins according to the loading of the first principal component. These peptide

Figure 2. Principal component (PC) analysis scores (A) and loading (B) plots for authentic and adulterated milk. The representative peptides are labeled.

sequence existed in glycinin G2 (P04405), glycinin G1 (P04776), β -conglycinin, α' subunit (P11827), glycinin G3 (P11828), and β -conglycinin, α subunit (P13916), as shown in Table 3. According to the second principal component, VEDGLHIISPELQEEEEQSHSQR was typically high in adulterated milk with soybean proteins, and legumin B (P14594) contained this peptide sequence. In addition, PQYLK and FLDDDLTDDIM-CVK accounted for the main part on both principal components in milk, which existed in α_{S2} -CN (P02663) and α -LA (P00711). This result was consistent with the previous PLGS analysis, which showed that all corresponding proteins could be identified in most adulterated milks (Table 3). Our finding is also in agreement with the previous study by Luykx et al. (2007), in which glycinin, β -conglycinin (soybean), and legumin (pea) could be identified in adulterated milks with plant proteins.

The PLS-DA was applied afterward as a fully automated multivariate classification method, and the model was validated by an independent set of samples. Specifically, the fingerprints were divided into a training set (75% samples) and a test set (25% samples) using the Kennard-Stone algorithm (Kennard and Stone, 1969). The bootstrapped Latin partition of 10 bootstraps and 5-fold Latin partition was used to select suitable numbers of latent variables, following the method reported by Lu et al. (2014). The prediction accuracies were 100% for both training and test set (latent variables = 3), respectively. This finding was consistent with the result of PCA, indicating peptide fingerprint combined with chemometrics successfully differentiated milk and adulterated milk.

In summary, we successfully differentiated authentic and adulterated milk by nontargeted UPLC-MS peptide fingerprints combined with chemometrics analyses. We detected soybean protein (β -conglycinin, α and β subunit) and pea protein (provicilin type B and vicilin) at the 1% adulteration level (percentage of foreign protein in milk protein). In an independent model validation using PLS-DA, all samples in the test set were correctly classified. The results indicated that UPLC-qTOF-MS peptide fingerprints with chemometrics were able to successfully differentiate authentic and adulterated milk. These findings may assist the quality control of raw milk and its related products in the future.

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