

Detection of plant protein adulterated in fluid milk using two-dimensional gel electrophoresis combined with mass spectrometry

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Abstract The illegal or unlabelled addition of plant protein in milk can cause serious anaphylaxis. For sustainable food security, it is therefore important to develop a methodology to detect non-milk protein in milk products. This research aims to differentiate milk adulterated with plant protein using two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry. According to the protein spots highlighted on the gel of adulterated milk, β -conglycinin and glycinin were detected in milk adulterated with soy protein, while legumin, vicilin, and convicilin indicated the addition of pea protein, and β -amylase and serpin marked wheat protein. These results suggest that a 2-DE-based protein profile is a useful method to identify milk adulterated with soy and pea protein, with a detection limit of 4% plant protein in the total protein.

Keywords Milk adulteration · Plant protein · Two-dimensional gel electrophoresis · Mass spectrometry

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Introduction

Milk adulteration with exogenous nitrogen-rich components leads to increased apparent protein content, which is used, along with fat content, to define the price of milk. Due to their low cost and abundant sources, vegetable proteins are potential adulterants for dairy products (Haasnoot et al. 2001). Compared with whey, plant proteins in food such as soy protein and wheat gluten would induce a lower postprandial insulin response in consumers (Chalvon-Demersay et al. 2017). However, such plant proteins are also identified food allergens, which can result in hypotension and anaphylaxis (Nakamura and Teshima 2013). The undeclared addition of these proteins into dairy products may therefore cause serious health risks. Consequently, the development of analytical methods to detect vegetable proteins in milk products is of paramount importance.

In recent years, a variety of targeted analytical methods have been used to detect the addition of plant proteins (mainly soy, pea, and wheat) in dairy products. Most recent studies of milk adulteration with plant protein have focused on milk powder. Added soy, pea, and soluble wheat protein in milk powder could be detected through immunological tests. Development of polyclonal antibodies in immunoassays has made it possible to detect these adulterants in a range of 1–5% of plant protein in the total milk protein content, although the results seem to be significantly affected by ultra-high temperature (UHT) treatment applied to the samples (Haasnoot et al. 2001; Sanchez et al. 2002). Compared with Enzyme-Linked Immunosorbent Assay (ELISA), the results of vegetable protein percentages obtained from Sodium Dodecyl Sulfate-Capillary Electrophoresis (SDS-CE) had a higher rate of accuracy. However, due to its poor reproducibility, SDS-CE requires

improvement in terms of instrument and operation standardisation before large-scale use is feasible (Sanchez et al. 2002). The application of mass spectroscopy (MS) allowed the identification of plant protein added to milk powder. With tetraborate- ethylene diamine tetraacetic acid (EDTA) extraction and trypsin digestion, the peptides from soy and pea proteins present in skimmed milk powder could be identified by quadrupling time-of-flight MS followed by High Performance Liquid Chromatography (HPLC) (Luykx et al. 2007). Untargeted MS was also developed for screening soy and pea protein mixed into skimmed milk powder. The comparative Liquid Chromatography-MS approach enabled unequivocal discrimination between skimmed milk powder (SMP) containing 5% soy or pea protein and unadulterated skimmed milk powder (Cordeuener et al. 2009).

Only a few studies have developed detection of the adulterated plant proteins in fluid milk. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) has been employed to detect the presence of soy milk in cow's milk. Based on the infrared absorption of peaks of amide, α -34 tocopherol, and soybean kunitz trypsin inhibitor, the spectra of soy milk and control milk adulterated with soy milk presented significant differences to that of control milk (Jaiswal et al. 2015). Calibrated by the external standard method, a reversed phase HPLC method was validated to be good enough in terms of detection performance (such as robustness, reproducibility, accuracy, and precision). The reversed phase HPLC method was used to quantify soy protein in milk, and had both a rapid separation run (11 min) and low detection limit (13 μ g/g of bovine milk) (Krusa et al. 2000).

Thanks to the application of high-resolution spectrometers and bioinformatic tools, two-dimensional gel electrophoresis (2-DE) is used to separate protein mixtures in proteomic studies (Pomastowski and Buszewski 2014). Combined with isoelectric focusing and SDS-polyacrylamide gel electrophoresis (PAGE) in two vertical directions, 2-DE was expected to separate foreign protein from milk protein in adulterated samples with a low detection limit. When compared with control samples, foreign protein spots in the adulterated samples would be visible in the gel map, which is helpful for the detection of exogenous protein in milk products. Coupled with mass spectrometry, 2-DE gel showed the different protein spot distribution of milk from different species, with several unique spots of casein and whey protein serving as markers to differentiate milk adulteration (Yang et al. 2014). These results mean that this method has the potential to detect vegetable proteins in adulterated milk, given different 2-DE gel maps for the major protein spots of animal and plant sources (Sirtori et al. 2012; Yang et al. 2014; Zarkadas et al. 2007).

We assumed that plant protein in fluid milk would be separated from milk protein in the 2-DE gel, and that the foreign protein spots, identified by MS, would indicate the potential adulteration of milk. The objective of this study is to present the gel map of milk adulteration with soy, pea, and wheat protein at low levels (below 8% of total protein) using 2-DE, and to find the marker proteins that can serve as an indicator, identified via matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF-MS).

Materials and methods

Sample preparation

Soy protein isolate (Nature's Bounty, Inc, Bohemia, NY, USA), pea protein isolate (LifeTime Nutritional Specialties, Inc. Orange, CA, USA), and wheat protein isolate (Honeyville Food Products, Salt Lake City, UT, USA) were used in this study. Raw cow's milk was obtained from a herd located in Beijing (China). Known amounts of plant protein isolate samples were dissolved in phosphate buffer solution (PBS, 0.1 mol/L, pH 7.2) and vortexed for at least 3 min. Then the mixtures were sonicated for 30 min and stirred by magnetic stirrer overnight. Vegetable protein solutions were prepared after centrifugation at 3000 g for 10 min. The protein contents of the raw milk and vegetable protein solution were determined using the Kjeldahl method (KjelROC Analyzer, Furulund, Sweden). Finally, different amounts of plant protein solution were added to raw milk in order to comprise 2, 4, and 8% of the total protein. Skimmed milk samples were prepared by centrifugation at 3000 g for 10 min to remove milk fat and stored at -20 °C until further electrophoresis separation.

Separation by 2-DE

The protein concentration in thawed samples was determined using bicinchoninic acid (BCA) assay kits (P0010S, Beyotime Institute of Biotechnology, China) before isoelectric focusing. Samples of a total of 250 mg protein mixed with rehydration buffer (8 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT), 0.4% immobilised pH gradient (IPG) buffer and trace bromophenol blue) were loaded onto 17 cm pH 4–7 IPG strips (Bio-Rad), as described by Yang et al. (2014). Isoelectric focusing was carried out at 20 °C. The IPG strips were rehydrated overnight and a series of focusing steps were performed as follows: desalting at 50 V for 2 h, 50–100 V for 30 min, 100–500 V for 1 h, 500–1000 V for 1 h, 1000–9000 V for 5 h, and then 9000 V for

80,000 V h. Before the second separation, IPG strips were immersed in 2% (w/v) dithiothreitol, 0.05 mol/L Tris–HCl pH 8.8, 6 mol/L urea, 30% (v/v) glycerol, and 2% (w/v) sodium dodecyl sulphate (SDS) and shaken gently at room temperature for 12 min, followed another incubation in 2.5% (w/v) iodoacetamide, 0.05 mol/L Tris–HCl pH 8.8, 6 mol/L urea, 30% (v/v) glycerol, and 2% (w/v) SDS for 12 min. Subsequently, strips were transferred to 12% polyacrylamide gels and sealed with 0.5% (w/v) low-melting-point agarose. The electrophoresis conditions used were 50 V for 30 min and 220 V to the end. Then the gels were stained with 0.12% Coomassie Brilliant Blue G-250 solution overnight, and destained with distilled water. Each sample was repeated three times. The gel images were scanned using a GS800 calibrated densitometer (Bio-Rad, USA) and exported to PDQuest 8.0 (Bio-Rad, Hercules, CA, USA) for protein spot analysis. To compare the differences between gels, protein spots were automatically matched and manually compiled, with ‘all or none’ as the determining criterion. Finally, protein spots detected only in adulterated milk, when compared with pure milk, were selected.

In-gel digestion, protein identification, and database search

As reported in the existing literature (Yang et al. 2014), the selected protein spots were cut manually from the gels and washed three times in acetonitrile/water (v/v, 50:50). After de-staining and drying, the chopped pieces were incubated with a volume of digestion buffer containing 5 ng sequence-grade trypsin solution for 20 h at 37 °C. Finally, the digestion was stopped through the addition of 100 µL 60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The digested samples were then analysed using a 5800 Plus MALDI TOF Analyzer (Applied Biosystems, Foster City, CA, USA). Protein identification was performed using MASCOT (Matrix Science) to search the uniprot database (<http://www.uniprot.org/>). PDQuest 8.0 (Bio-Rad Laboratories, CA, USA) was employed to find the unique spots that appeared in adulterated milk in contrast to pure milk.

Results and discussion

In the current study, 2-DE maps were constructed for cow’s milk, soy, pea, and wheat plant protein extracted by PBS, and cow’s milk adulterated with 2, 4, and 8% soy, pea, and wheat protein. The well-resolved and repeatable 2-DE gel maps of cow’s milk, plant proteins, and milk adulterated with plant protein at the 4% level are presented in Fig. 1. There were no significant differences between raw milk and milk adulterated with plant protein at the 2% level.

Typical vegetable protein spots were observed for milk adulterated with 4 and 8% pea or soy protein, with only a few foreign protein spots being detected in milk adulterated with 4 and 8% wheat protein. The foreign protein spots detected only in adulterated milk were selected as marker proteins. The relative intensity and identification of these spots is listed in Table 1.

Due their potential to be food allergens (Nakamura and Teshima 2013), the detection of plant proteins in adulterated milk is an important matter for public health. Therefore, the hypothesis of this research was to use 2-DE as a method to differentiate plant proteins from milk proteins, with a low detection limit (2% plant protein), in fluid skimmed milk spiked with soy, pea or wheat protein. For that, two sub-objectives must be fulfilled. Firstly, the 2-DE method must detect the presence of plant proteins in adulterated milk, and secondly, this detection must be reliable.

Sample preparation

The solubility of vegetable protein in skimmed milk and its availability for isoelectric focusing electrophoresis produced a good separation of plant protein via the 2-DE method in this study. PBS was used to prepare a plant protein solution in our experiment. Moderate pH values in PBS promoted the dissolution to aqueous solution of β -conglycinin and glycinin from soy-source protein, and legumin and vicilin from pea-source protein (Samoto et al. 2007). Tetraborate-EDTA buffer was an effective tool for extracting plant protein from milk powder, as 2% of milk protein, 94% of soy-source protein, and 87% of pea-source protein were retrieved in the pellet after centrifugation (Luykx et al. 2007; Scholl et al. 2014). Moreover, protein extraction at pH 8.3 via tetraborate-EDTA buffer did not support isoelectric electrophoresis in this study (Scholl et al. 2014).

Detection of plant protein

Protein spots which were only detected in milk adulterated with soy protein were labelled S1–S12 (Fig. 1, Table 1). Gel maps containing soy protein showed soy protein spots related mainly to β -conglycinin and glycinin. β -conglycinin contained α (such as spots S9–S12) and β (such as spots S1–S3) subunits. Their identified molecular weight ranged from 63 to 72 kDa and from 48 to 50 kDa, respectively. Their isoelectric points ranged from 4.92 to 5.50 and from 5.67 to 6.14 for α and β subunits, respectively. The molecular weight and isoelectric point of glycinin identified in this study (spots S7–S8) were between 59 and 60 kDa and between 5.52 and 5.79. Our results were similar to those previously reported (Zarkadas et al.

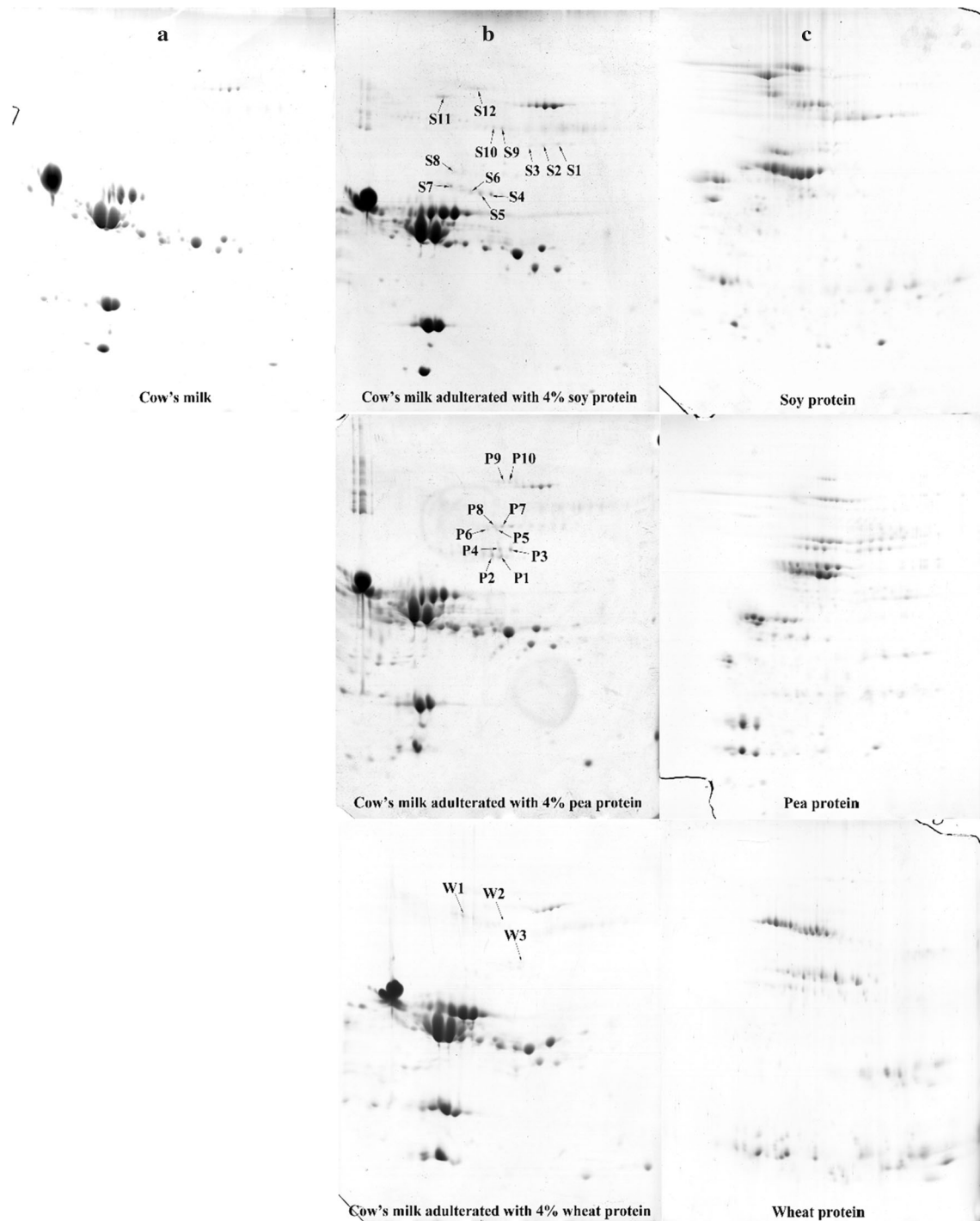


Fig. 1 Two-dimensional electrophoresis maps of protein spots of cow's milk (**a**), cow's milk adulterated with 4% plant protein (**b**), and plant protein (**c**). Plant protein sources are soy, pea, and wheat.

Protein spots labelled with arrow are only detected in adulterated milk compared with cow's milk

2007). Although three additional spots detected in soy protein adulterated milk (spots S4–S6) were not characterised by the Uniprot database, they have a location comparable to acidic subunits of glycinin observed by Zarkadas et al. (2007). Based on their acidic isoelectric

point, spots S7 and S8 were likely to also be acidic subunits of glycinin. Disulfide bonds linking the subunits of glycinin were broken by the DDT used in the sample preparation and, therefore, the acidic subunits with a molecular weight of about 35 kDa would be dissociated from the basic

Table 1 Identification and optical density of protein spots only detected in spiked milk compared with control milk on the gels, analysed by Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALTI-TOF MS)

Protein spot	Protein name (organism)	Uniprot IDs ^a	Molecular mass (kDa) ^a	Isoelectric point ^a	Score	Number of matched peptides	Average relative intensity	Coefficients of variance of relative intensity (%)
S1	β -conglycinin β subunit (<i>Glycine max</i>)	F8WQS1	50.010	6.14	644	26	72.1	16.5
S2	β -conglycinin β subunit (<i>Glycine max</i>)	F7J077	50.468	5.88	592	26	105.4	37.4
S3	β -conglycinin β subunit (<i>Glycine max</i>)	Q50JD8	48.358	5.67	483	24	30.8	17.5
S4	Uncharacterized protein (<i>Glycine max</i>)	I1LST1	88.639	5.10	6	40	246.6	15.3
S5	Uncharacterized protein (<i>Glycine max</i>)	I1L939	71.254	5.23	3	43	254.2	6.8
S6	Uncharacterized protein (<i>Medicago truncatula</i>)	G7I2I6	6.749	5.14	3	47	211.1	12.1
S7	Glycinin G2 (<i>Glycine soja</i>)	A0A0B2PSP9	59.640	5.79	350	14	287.0	8.4
S8	Glycinin A3B4 subunit (<i>Glycine max</i>)	Q7GC77	58.608	5.52	132	10	96.4	23.5
S9	β -conglycinin α subunit (<i>Glycine max</i>)	Q94LX2	70.549	5.12	628	20	94.4	55.2
S10	β -conglycinin α subunit (<i>Glycine max</i>)	O22120	63.184	4.92	725	23	87.9	55.7
S11	β -conglycinin α subunit (<i>Glycine soja</i>)	A0A0B2Q6W9	70.521	5.09	262	18	345.0	2.8
S12	β -conglycinin α prime subunit (<i>Glycine max</i>)	Q4LER6	72.469	5.50	233	22	244.8	5.3
P1	Legumin A (<i>Pisum sativum</i>)	P15838	59.633	6.21	295	15	361.3	8.2
P2	Legumin A (<i>Pisum sativum</i>)	Q9T0P5	59.153	6.16	137	12	387.7	6.2
P3	Legumin A (<i>Pisum sativum</i>)	P15838	59.633	6.21	305	11	214.4	10.9
P4	Legumin A (<i>Pisum sativum</i>)	P15838	59.633	6.21	241	14	253.4	5.5
P5	P54 protein (<i>Pisum sativum</i>)	O49927	55.027	6.05	445	18	60.4	51.3
P6	P54 protein (<i>Pisum sativum</i>)	O49927	55.027	6.05	521	18	57.5	18.5
P7	Vicilin (<i>Pisum sativum</i>)	P13918	52.257	5.39	211	18	128.8	9.6
P8	Vicilin (<i>Pisum sativum</i>)	P13918	52.257	5.39	670	26	116.2	24.2
P9	Convicilin (<i>Pisum sativum</i>)	Q9M3X6	72.134	5.50	427	23	65.1	3.2
P10	Convicilin (<i>Pisum sativum</i>)	Q9M3X6	72.134	5.50	643	26	81.4	13.3

Table 1 continued

Protein spot	Protein name (organism)	Uniprot IDs ^a	Molecular mass (kDa) ^a	Isoelectric point ^a	Score	Number of matched peptides	Average relative intensity	Coefficients of variance of relative intensity (%)
W1	β -amylase (<i>Triticum aestivum</i>)	W5EK10	61.360	5.00	938	16	228.4	20.4
W2	β -amylase (<i>Triticum aestivum</i>)	W5C8P9	57.105	5.29	368	6	97.1	9.8
W3	Serpin 3 (<i>Triticum aestivum</i>)	C0LF32	43.227	5.56	1010	13	97.8	38.3

^aID, Molecular mass and isoelectric point derived from the uniprot database

subunits (Nishinari et al. 2014). This explains why the molecular weight of spots S4–S8 identified in our 2-DE gels was around 36–37 kDa. Results for the optical intensity for the spots S4–S7 and S11–S12, the relative density with a value above 200, and coefficients of variation (CV) ranging from 2.8 to 15.3%, suggest high β -conglycinin and glycinin content in soy protein adulterated milks. This was confirmed in a study by Zarkadas et al. (2007), where α and α' subunits of conglycinin, as well as acidic subunits of glycinin, accounted for 21–33 and 35–45% of the soy protein content. The differentiation of soy protein spots compared to milk protein spots can be explained by the difference in molecular weight between these different proteins. Indeed, the identified soy protein spots (for example, S1–S4 and S7–S12) have a higher molecular weight than casein and whey protein, whose molecular weight is below 35 kDa. In conclusion, β -conglycinin and glycinin are identified in this study as the indicator of milk adulteration with soy protein. Peptides originating from these proteins were also detected by mass spectrometry in reports on milk powder adulteration (Cordewener et al. 2009; Luykx et al. 2007).

Protein spots (P1–P10) detected in milk spiked with pea protein (Fig. 1), were identified as legumin A, vicilin, convicilin, and P54 protein from peas in the uniprot database (Table 1). Pea protein spots identified as having higher relative intensities (from 214 to 387) were P1–P4. Their CV ranged from 55 to 10.9%. The relative intensity and CV for the rest of the identified pea protein spots (P5–P10) were 60–129 and 3.2–51.3%, respectively. The molecular weight of these proteins varied from 52 to 72 kDa and their isoelectric point ranged from 5.39 to 6.21. The higher molecular weight of these proteins compared to milk proteins explains the observed separation on 2-DE gels. Meanwhile, it was observed that some of the extracted pea protein fractions were covered by casein and whey protein and therefore were not visible in the 2-DE gels of adulterated milks. The observed high relative intensity of

spots P1–P4 and the moderate relative intensity of spots P7 and P8 suggest high legumin contents and moderate vicilin contents in pea protein. This accords with previous findings (Sirtori et al. 2012), which also stated that the soluble globulin in pea seeds accounted for approximately 70% of the total protein; the two major proteins were vicilin and legumin, with a minor protein, convicilin. Tzitzikas et al. (2006) suggested that the ratio of legumin to vicilin ranged from 2 to 4 in pea globulin. Similar to glycinin in soy protein, legumin subunits (around 60 kDa) consisted of one acidic α (35–43 kDa) and one basic β (19–23 kDa) polypeptide, linked together via a disulfide bridge (Gatehouse et al. 1980). Legumin, identified in this study, was also found to be a major protein in kidney beans (Parmar et al. 2014). Based on their location on the gel, spots P1–P4 in our 2-DE gels were more likely to be the acidic α polypeptides of legumin. P54 protein detected in our study was mentioned by Wang et al. (2012) as an important storage protein in peas. As the major seed protein of peas, peptides from legumin and vicilin were also detected in adulterated milk powder (Cordewener et al. 2009; Luykx et al. 2007).

The gel map of milk adulteration with wheat protein highlighted W1–W3 as marker proteins (Fig. 1), which were characterised as beta-amylase and serpin 3 from wheat in the uniprot database. Fewer spots with high intensities for significant proteins were observed in wheat protein adulterated milk compared with those observed in soy and pea protein adulterated milks. This may pose some difficulty for unequivocal discrimination. The identified β -amylase (W1, W2) and serpin (W3) were shown to be wheat-source protein fractions in research performed by Becker et al. (2012). Only spot W1 indicated the existence of wheat protein in adulterated milk with high intensity (228.4), the other two spots showed low optical density (97). These spots also have a higher molecular weight (43–61 kDa) than major milk proteins. The wheat protein fraction extracted by PBS in this study was inconsistent

with KCl soluble/methanol-soluble fractions from wheat flour in previous reports, due to the absence of gliadins or glutenin subunits (Hurkman and Tanaka 2004). The protein in wheat seed is comprised of glutenins, gliadins, albumins, and globulins (Hurkman and Tanaka 2004). The insolubility of gliadin and aggregation of glutenin were likely to contribute to the absence of these proteins in the supernatant after centrifugation (Becker et al. 2012). Interestingly, analysis of pellets of skimmed milk powder adulterated with wheat protein isolate after tetraborate-EDTA extraction has also failed to identify wheat source proteins (Scholl et al. 2014).

Reliability

The separation of soy and pea protein adulteration in milk was more visible than in the case of wheat protein adulteration. Typical foreign protein spot groups for pea and soy proteins appeared in the gel map at a 4% level of adulteration. Marker proteins (S4–S7, S11, S12, P1–P4) with high intensity (211.1–387.7) could be considered as indicators of milk adulteration and showed good reliability (CV ranged from 2.8 to 15.3%). In contrast, only one spot (W1) detected for 4% wheat protein adulterated milk had an intensity above 200. Therefore, 2-DE used in our study was of limited use to detect the presence of wheat protein in milk.

In this study, the detection limit of 2-DE for soy and pea protein in milk adulteration was 4% (8 µg). No obvious soy and pea protein spot appeared in the 2% plant protein adulterated samples, and only appeared when the foreign protein level was more than 8 µg, as found in the 4% plant protein adulterated milk. Yang et al. (2014) observed around 1 µg of bovine α -lactalbumin and β -lactoglobulin in a gel of goat's and camel's milk samples adulterated with 2% cow's milk; however, this limit detection was not confirmed in this study. The poor detection limit found in the present study can be ascribed to the incomplete aqueous solubility of plant protein. In future research, low non-target protein preparation (ultracentrifugation) may improve the resolution and detection limit for plant protein with 2-DE (Yang et al. 2014). In addition, the application of fluorescence staining could allow a lower limit (1–2 ng) of protein detection than Coomassie Brilliant Blue staining (8–16 ng) for 2-DE detection, as applied in current study (Berggren et al. 2000).

2-DE separated soy protein β -conglycinin and glycinin, pea protein legumin, vicilin, and convicilin, as well as wheat protein β -amylase and serpin from milk casein and whey protein on the gel of adulterated milk. This not only highlights the exogenous plant protein from milk protein, but prevents the masking of the trace amounts of plant protein by highly abundant casein and whey proteins in the

successive identification by MS. Preliminary screening for manufactured skimmed milk powder containing 5% soy or pea protein isolates using MS showed few plant protein peptides were identified (Luykx et al. 2007). Similar peak profiles were also observed in the comparative LC–MS analysis between skimmed milk powder and skimmed milk powder adulterated with 5% soy protein isolates, and discrimination of these samples needs multivariable analysis by a post-alignment clustering procedure (Cordewener et al. 2009). From this point of view, 2-DE provided high sensitivity and specificity for the final identification of added plant protein, using MALDI-TOF MS.

Conclusion

The results demonstrate that 2-DE would be effective for screening milk adulterated with at least 4% soy and pea protein, with successive mass spectrometry analysis identifying several peptides: β -conglycinin and glycinin from soy and legumin, vicilin and convicilin from peas. For milk adulteration with wheat protein, only β -amylase and serpin were identified. As a potential detection method, 2-DE is robust for the validation of milk adulteration with soy and pea protein. Moreover, an improved electrophoresis procedure with special sample preparation and staining methods would reduce the limit of detection effectively.

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