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Luminex-Based Triplex Immunoassay for the Simultaneous Detection of Soy, Pea, and Soluble Wheat Proteins in Milk Powder

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An automated fluorescent microsphere-based flow cytometric triplex immunoassay, using the Luminex 100 flow analyzer with MultiAnalyte Profiling (xMAP) technology, was developed for the simultaneous detection of proteins from three vegetable sources as potential fraudulent adulterants in milk powder. In the final triplex inhibition immunoassay, soluble wheat proteins (SWP) and proteins from soy and pea were coupled to three different microsphere sets. A mixture of these microsphere sets was transferred to a microtiter plate well together with the sample and a mixture of three affinity-purified polyclonal antibodies raised against the proteins and labeled with a fluorophore (Alexa 532). After incubation for 1.5 h at room temperature in the dark, the fluorescence intensities on the microspheres were directly measured (no wash procedure) in the Luminex during 10 s per well (100 microspheres per set). The sensitivities of the three assays for plant protein extracts were determined as 0.5−0.6 μg/mL at 50% inhibition. For the detection of the vegetable proteins in milk powder, the samples were dissolved in buffer (0.1 g in 10 mL) and further diluted (20 times) to create a 50% inhibition at approximately 0.5% of the vegetable proteins in the total protein content of milk powder. With the help of calibration standards, prepared under conditions comparable to those for sample materials, the triplex immunoassay proved to be quantitative above 0.1%, although concentrations in high-heated milk powders were underestimated. Due to the xMAP technology, in which 100 different microsphere sets can be distinguished, this triplex immunoassay can easily be extended to detect other possible adulterants.

KEYWORDS: Luminex; multiplex; immunoassay; vegetable proteins; soy; pea; soluble wheat proteins; milk powder; adulteration

INTRODUCTION

The low prices of some vegetable proteins might make them attractive as potential adulterants in milk powders and other dairy products. In 1998 a European Project eiyhin the framework of the Standards, Measurements and Testing Programme (SMT4-CT97-2205) was granted. The major objective of this project was to develop and validate reliable methods for the detection and quantification of nonmilk proteins in milk powder and other dairy products (*1*). Soy protein was selected as a major potential adulterant because several preparations of soy proteins, such as flours, textured flours, protein concentrates, isolates, and hydrolysates, were commercially available at a low price. For comparable reasons, pea proteins and soluble wheat proteins (SWP) were selected in that project also.

Electrophoretic [sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and SDS−capillary gel electrophoresis (SDS-CGE (*2*)] and immunochemical methods [enzyme-linked immunosorbent assays (ELISAs)] were optimized and compared for the detection of such adulterations, and SDS-CGE and ELISA were selected for validation through collaborative trials. Levels of adulteration of soy and pea proteins between 1 and 5% were quantified in low-heat milk powders using both methods. SDS-CGE provided a slightly better accuracy, but ELISA presented the advantage of being suitable for samples containing SWP, which could not be detected by SDS-CGE. ELISA also allowed the detection of adulterations in ultrahigh-temperature (UHT) treated milk powders, although the quantitative data obtained were much lower than the actual values, probably due to the partial heat denaturation of the antigens (*1*). However, to detect the proteins from the three plant sources, three different ELISAs had to be performed. For the simultaneous immunochemical detection of the three plant proteins, an optical biosensor (Biacore 3000) with four serially connectable flow channels (Fcs) was used to develop a fast (5 min per sample) and combined biosensor immunoassay (BIA) (*3*). The limits of detection in milk powder were below 0.1% of plant protein in the total milk protein content. However, for the

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simultaneous detection of more than three protein sources (e.g., maize, bean, rice, potato, etc.), the application of this biosensor is limited by its four Fcs.

A fairly new and more multiplex-based immunotechnology uses the combination of the Luminex 100 flow analyzer and the MultiAnalyte Profiling (xMAP) technology. This microsphere-based flow cytometric system was developed by Luminex (Austin, TX) and involves covalent coupling of the antigen or antibody on carboxylated polystyrene microspheres (5.6 μm beads) internally dyed with a red and orange fluorophore (*4*). By varying the ratio of the two fluorophores, up to 100 different color-coded bead sets can be distinguished, and each bead set can be coupled to a different biological probe. Detection and quantification of the immunocomplex is obtained via a reporter molecule [a fluorophore (i.e., Alexa) coupled to the antibody or antigen]. A dual laser instrumentation system allows both the identification of the color-coded bead set by its characteristic color and the quantification of reporter molecules bound to the beads. Thus, this combination makes it possible to simultaneously measure up to 100 different biomolecular reactions in a single well. In addition, a no-wash assay format is feasible because the reporter molecule is measured only at the surface of the bead. Any residual (e.g., unreacted) reporter molecule will remain in solution and will not contribute to the assay value, simplifying the assay protocol relative to that of a typical ELISA.

This technology was compared with ELISA for the quantification of immunoglobulins in human serum and stool samples and, although the ELISA was more sensitive, the Luminex was more reproducible, had a greater dynamic range of measurement, and took considerably less preparation time than the ELISA (*5*). An increased dynamic range and lower limits of detection were seen during the comparison of the Luminex system with a microarray system for the detection of bacterial and viral proteins (*6*). Compared with ELISAs for the detection of antibodies to different foot-and-mouth disease nonstructural proteins, the Luminex-based technology was shown to be a sensitive and efficient method providing both time and cost savings to the laboratory (*7*). Compared with sandwich ELISAs for the detection of two isoforms of human growth hormone in serum, a better sensitivity (detection at the picograms per milliliter level) for the Luminex technology was observed (*8*). The successful application of the Luminex xMAP technology for the simultaneous, rapid, sensitive, and specific nucleic acid detection is reviewed (*9*), and the technology is qualified as capable to meet the current and future requirements of the molecular laboratory for high-throughput nucleic acid detection.

The above-described applications triggered us to investigate the use of this multiplex technology in food control starting with the simultaneous detection of proteins from different plant sources in milk powder at the fraud (percent) level. Using polyclonal antisera raised against soy, pea, and SWP, Luminex-based immunoassays in the sandwich and inhibition assay formats were compared. The triplex inhibition format was used to analyze different sample materials with known concentrations of the plant proteins prepared under different conditions (low- and high-heated). Finally, the assay was used during a small survey in which 260 milk and buttermilk powder samples obtained from the Dutch General Inspection Service were analyzed.

MATERIALS AND METHODS

Instruments and Reagents. The Luminex 100 IS 2.2 system [consisting of the Luminex 100 analyzer, Luminex XY Platform (programmed to analyze a 96-well plate), with the StarStation System control software], different sets of carboxylated microspheres (beads) and sheath fluid were obtained from Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, U.K.). Alexa 532 was purchased from Molecular Probes Europe (Leiden, The Netherlands). Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES) were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). CNBr-Sepharose and Sephadex G-50 Superfine were obtained from Amersham Biosciences AB (Uppsala, Sweden). Acrodisc CR 25 mm syringe filters with 0.45 μm PTFE membrane were from Pall Corp. (Ann Arbor, MI). The BCA protein assay and *N*-hydroxysulfosuccinimide (sulfo-NHS) were supplied by Pierce (Rockford, IL). Flat-bottom 96-well microplates were from Greiner Bio-One International AG (Frickenhausen, Germany) and 96-well filter-bottom microplates (1.2 μm hydrophil low protein binding) and Centricon centrifugal filter devices were from Millipore Corp. (Bedford, MA). The Snijder test tube rotator was obtained via Omnilabo Int. BV (Breda, The Netherlands) and the REAX 2 head-over-head rotator was from Heidolph Instruments (Schwabach, Germany). The Eppendorf centrifuge 5810R, dimethylformamide (DMF), and all other reagents were obtained from VWR International (Amsterdam, The Netherlands) unless otherwise stated.

Preparation of Plant Protein Extracts. The previously applied (*3*) and at that time commercially available plant protein products [Supro 500 E soy protein isolate (A) from Anvisa (Madrid, Spain), Europroducts 595 soy protein isolate (K) from Europroducts (Milan, Italy), pea protein isolate (Pisane HD) from Cosucra SA (Fontenoy, Belgium), and soluble wheat protein (SWP100) from the Amylum group (Belgium)] were used in this study. For the extraction of proteins, 1 g of the plant product was mixed with 20 mL of PBS (pH 7.4). After 30 min of mixing (magnetic stirrer) and centrifugation (5 min at 5000*g*), the supernatants were filtered (0.45 μm). The protein contents of the filtrates were 4.2, 9.0, and 15.5 mg/mL for soy, pea, and SWP, respectively, as determined by the BCA protein assay with BSA as a standard. The plant protein extracts were stored at −20 °C until further use.

Antibodies. The preparation and affinity purification of the rabbit polyclonal antisera raised against soy, pea, and SWP were described previously (*3*).

Protein Coupling to Beads. For the sandwich assay format, the three affinity-purified antisera were coupled to three different bead sets. For the inhibion assay format, SWP and proteins from soy and pea were coupled to three other bead sets. For the coupling, each stock bead suspension (1.25×10^7 beads/mL) was resuspended by vortexing for approximately 5 min. Of the suspension, 200 μL (2.5×10^6 beads) was transferred to an Eppendorf tube in which the beads were pelleted by centrifugation (3 min at 8000*g*) and the supernatant was removed. The pelleted beads were resuspended in 100 μL of water by vortex (2 min), and after centrifugation (3 min at 8000*g*), the supernatant was removed. The pelleted beads were resuspended in 80 μL of activation buffer ($NaHCO_3$, pH 6.3) by vortex (20 s), and 10 μL of Sulfo-NHS (50 mg/mL in water) was added; after gentle mixing by vortex, 10 μL of EDC (50 mg/mL in water) was added. After gentle mixing by vortex and an incubation in the dark at room temperature for 20 min under mixing by rotation in the test tube rotator, the activated beads were pelleted by centrifugation (3 min at 8000*g*). The supernatant was removed, and the beads were resuspended in 250 μL of 50 mM MES (pH 5.0) by vortex (20 s). The beads were pelleted by centrifugation (3 min at 8000*g*), and the supernatant was removed. This wash procedure with 50 mM MES was repeated twice.

To the pellet of beads was added 0.5 mL of the antibody or plant protein solution (100 μg/mL in 50 mM MES, pH 5.0). After mixing by vortex, the bead suspension was incubated for 2 h in the dark at room temperature and under mixing by rotation using the test tube rotator. The coupled beads were pelleted by centrifugation (3 min at 8000*g*), the supernatant was removed, and the beads were resuspended in 500 μL of blocking buffer (PBS containing 0.1% BSA, 0.02% Tween-20, and 0.05% sodium azide) by vortex (20 s) and incubated for 30 min in the dark. After this blocking, the beads were pelleted by centrifugation (3 min at 8000*g*) and resuspended in 300 μL of blocking buffer. The wash procedure was repeated twice with 300 μL of blocking

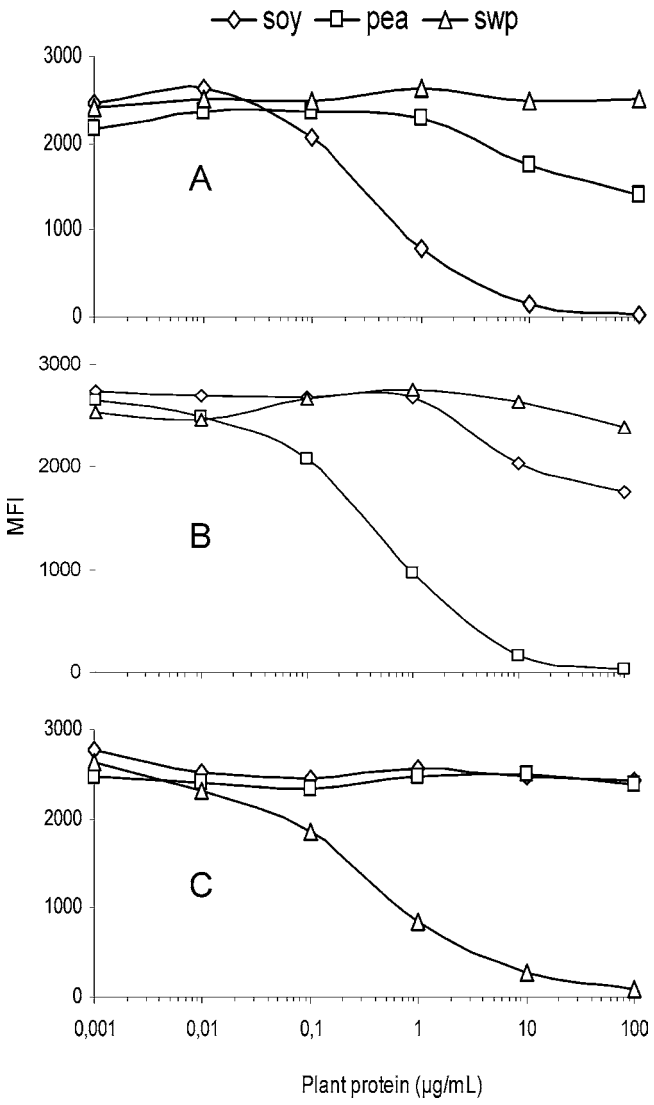

Figure 1. Calibration curves, obtained with proteins dissolved in buffer, of the immunoassays for soy (**A**), pea (**B**), and SWP (**C**) in the triplex inhibition assay (TA) format.

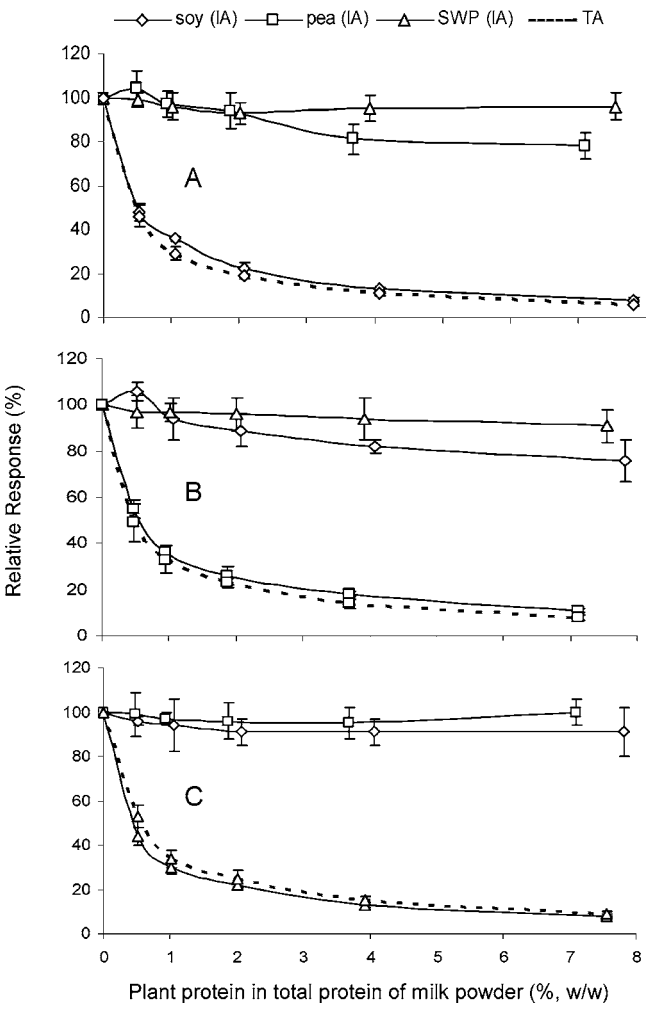

Figure 2. Calibration curves, obtained with the calibration standards of the three plant proteins in milk powder, of the immunoassays for soy (**A**), pea (**B**), and SWP (**C**) in the individual inhibition assay (IA) and triplex inhibition assay [(TA) dotted lines] format. Extracts were 20 times diluted in buffer.

buffer. The pelleted beads were resuspended in 300 μL of blocking buffer and stored refrigerated at 2−8 °C in the dark.

Coupling of Alexa to the Antibodies. The affinity-purified polyclonal antibodies against soy, pea, and SWP were labeled with Alexa Fluor 532, which is a reactive dye with a succinimidyl ester moiety that reacts efficiently with primary amines of proteins to form stable dye−protein conjugates. To 1 mL of the affinity-purified polyclonal antibody solution (4.0, 4.1, and 6.9 mg/mL PBS for soy, pea, and SWP, respectively) were added 0.1 mL of 1 M $NaHCO_3$ (pH 8.4) and 35 μL of Alexa Fluor 532 solution (1 mg dissolved in 110 μL of DMF). After mixing by vortex, the antibody−Alexa solution was incubated for 1 h in the dark at room temperature under mixing by rotation in the test tube rotator. The coupled fraction was separated from the free Alexa by gel filtration on a G-50 Sephadex column (30 cm column with a diameter of 1 cm) with PBS containing 0.02% sodium azide as buffer. The first pink fraction (about 2.5 mL) was collected, homogenized, and stored at 2−8 °C in the dark. The degree of labeling was determined according to the manufacturer's instructions (*10*), and the molar ratios of Alexa/antibody were determined as 1.7, 1.8, and 2.2 for anti-soy, -pea, and -SWP, respectively.

Sandwich Immunoassay. Three different bead sets coupled with the affinity-purified polyclonal antibodies raised against the three plant proteins were used.

In the individual sandwich assay format, each bead set (10 μL containing 1000 beads) was added to the sample (100 μL) in a 96-well filter-bottom microplate, and after a 30 min incubation at room temperature, the beads were washed two times with 200 μL of PBS per well by filtration to remove the nonbound proteins. The detector antibodies (110 μL of diluted solutions of the Alexa-labeled antibodies) were added, and after the second incubation (1.5 h at room temperature and in the dark), the complexes were directly detected (no wash step) in the Luminex [about 10 μL/well was measured in 10 s (about 100 events per bead set)].

In the triplex sandwich assay format, the three antibody-coated bead sets (10 μL each) were added to 100 μL of the sample, and after the incubation and washing, 130 μL of a mixture of the three Alexa-labeled antibodies was added, which was followed by the procedure described above.

Inhibition Immunoassay. The three different plant proteins were coupled to three different bead sets. During the coupling procedure, the process of pelleting and resuspending of the beads was repeating 10 times, and this caused bead recoveries of 44, 36, and 40% for the soy-, pea-, and SWP-coated beads, respectively.

In the individual inhibition assay (IA) format, each bead set (10 μL containing about 1000 beads) was added to the diluted sample (100 μL), together with the corresponding diluted Alexa-labeled antibodies (5 μL), and after incubation (1.5 h at room temperature in the dark), the beads were directly detected (no wash step) in the Luminex.

In the triplex inhibition assay (TA) format, the three plant protein-coated bead sets were combined, and 30 μL (containing 1000 beads per set) was added to the diluted sample (100 μL) together with 15 μL

Table 1. Pasteurized Milk Powders with Added Concentrations of Vegetable Proteins (VP) and Average Concentrations Found with the Luminex Assay Using the Calibration Graphs Prepared from the Nonheated Milk Powder with Known Concentrations of the Vegetable Proteins in the Individual Inhibition Assays (IA), Using Individual Calibration Graphs, and in the Triplex Inhibition Assay (TA) in Which Combined Calibration Graphs Were Used for the Calculations[a]

VP adulterated milk powder using	VP added (%)	individual assay (IA)		triplex assay (TA)		TA/IA
		VP found (%)	recovery (%)	VP found (%)	recovery (%)	
soy A	0.99	1.01 ± 0.10	102	0.77 ± 0.07	78	0.76
soy A	1.96	1.90 ± 0.13	97	1.43 ± 0.29	73	0.75
soy A	4.76	5.42 ± 0.83	114	3.99 ± 0.30	84	0.74
soy K	0.99	1.00 ± 0.12	101	0.77 ± 0.15	78	0.77
soy K	1.96	2.09 ± 0.27	107	1.51 ± 0.11	77	0.72
soy K	4.76	5.09 ± 0.54	107	3.78 ± 0.35	79	0.74
pea	0.99	0.95 ± 0.10	96	0.80 ± 0.18	81	0.85
pea	1.96	2.17 ± 0.29	111	1.81 ± 0.29	93	0.84
pea	4.76	7.11 ± 0.50	149	5.03 ± 0.40	106	0.71
SWP	0.99	1.00 ± 0.37	101	1.06 ± 0.39	107	1.06
SWP	1.96	1.88 ± 0.44	96	1.80 ± 0.36	92	0.95
SWP	4.76	4.54 ± 0.75	95	4.61 ± 0.51	97	1.01
av			106 ± 15		87 ± 12	0.82 ± 0.12

[a] Extracts of the milk powders were analyzed on five separate days.

Table 2. UHT Milk Powders with Added Concentrations of Vegetable Proteins (VP) and Average Concentrations Found by the Luminex Assay Using the Calibration Graphs Prepared from the Nonheated Milk Powder with Known Concentrations of the Vegetable Proteins in the Individual Inhibition Assays (IA), Using Individual Calibration Graphs, and in the Triplex Inhibition Assay (TA) in Which Combined Calibration Graphs Were Used for the Calculations[a]

VP adulterated milk powder using	VP added (%)	individual assay (IA)		triplex assay (TA)		TA/IA
		VP found (%)	recovery (%)	VP found (%)	recovery (%)	
soy A	0.99	0.99 ± 0.10	100	0.75 ± 0.03	76	0.75
soy A	1.96	1.75 ± 0.28	89	1.23 ± 0.18	63	0.71
soy A	4.76	4.14 ± 0.58	87	3.02 ± 0.33	63	0.73
soy K	0.99	1.32 ± 0.11	133	0.96 ± 0.15	97	0.73
soy K	1.96	2.35 ± 0.77	120	1.71 ± 0.54	87	0.73
soy K	4.76	4.52 ± 0.49	95	3.31 ± 0.27	69	0.73
pea	0.99	1.15 ± 0.19	116	1.02 ± 0.10	103	0.89
pea	1.96	2.01 ± 0.15	102	1.67 ± 0.21	85	0.83
pea	4.76	4.35 ± 0.53	91	3.47 ± 0.32	73	0.80
SWP	0.99	0.60 ± 0.07	61	0.70 ± 0.14	71	1.16
SWP	1.96	1.21 ± 0.35	62	1.27 ± 0.35	65	1.05
SWP	4.76	2.01 ± 0.42	42	1.91 ± 0.40	40	0.95
av			92 ± 26		74 ± 17	0.84 ± 0.15

[a] Extracts of the milk powders were analyzed on five separate days.

of a mixture of the three Alexa-labeled antibodies. After incubation (1.5 h at room temperature, in the dark and under mild shaking), the beads were directly detected (no wash step) in the Luminex [about 10 μL/well were measured in 10 s (ca. 100 events per bead set)].

Sample Materials. Soy protein isolate, pea protein isolate, and soluble wheat protein (SWP100) were used to prepare calibration standards in nonheated freeze-dried milk powder and to prepare adulterated milk powder samples, which were heat treated (pasteurized and UHT) prior to spray-drying. The preparation of these materials by NIZO Food Research (Ede, The Netherlands) was previously described (*1*), and they were stored at −20 °C before analysis.

Calibration Standards. The concentrations of vegetable protein (% w/w, vegetable protein/total protein) in the soy standards were 0.52, 1.05, 2.08, 4.07, and 7.82%; in the pea standards 0.48, 0.94, 1.87, 3.69, and 7.11%; and in the SWP standards 0.51, 1.01, 2.01, 3.93, and 7.56%. Extra milk proteins were added to achieve the desired vegetable protein to total protein content in a small total volume in order to facilitate freeze-drying. The average total protein content of these samples was determined as 57%.

Adulterated Skimmed Milk Powders. Skimmed milk was adulterated with each plant protein [two soy protein isolates (A and K), pea protein isolate, and SWP100] at levels of 0, 1, 2, and 5% (percentage of plant protein in total protein content). As part of the spray-drying process, adulterated samples were subsequently pasteurized at 74 °C for 10 s to obtain low-heat milk powders and UHT treated (130 °C for 1 min) to obtain high-heated milk powder samples. The average protein content of these milk powders was 37%.

Unknown Milk Powders. During a survey, 260 powder samples [178 milk powder samples (80 taken in 2006 and 98 in 2005), 12 buttermilk powder samples (5 taken in 2006 and 7 in 2005), and 70 powdered samples of artificial milk replacer (taken in 2006)], all obtained from the Dutch General Inspection Service (Kerkrade, The Netherlands), were analyzed in the triplex assay.

Alpro Soy-Spiked (Butter)milk Samples. For the preparation of another set of spiked milk powder and buttermilk powder samples, used as unknown controls during the survey, 1 L of UHT-treated Alpro soy milk (Alpro Soya Nederland B.V., Breda, The Netherlands), containing 6.6% dry matter and 3.7% of protein, was obtained from the local market. The soy milk was freeze-dried, mixed, and homogenized in a blender. This soy powder, of which the protein content was calculated as 56%, was added to a buttermilk powder and a skimmed milk powder in five concentrations (0.5, 1.0, 2.0, 5.0, and 10%, w/w). The percentages of soy protein in the (butter)milk powders were calculated as 0.28, 0.56, 1.12, 2.8, and 5.6%. The percentages of soy protein in the total protein content (about 37%) were calculated as 0.75, 1.5, 3.0, 7.5, and 15%.

Sample Preparation. Due to the higher protein content (57%) of the calibration standards, 60 mg was weighed in a plastic tube, and 9.9 mL of PBS was added. From the adulterated and unknown milk powder samples (protein content ∼ 37%), 100 ± 5 mg was weighed

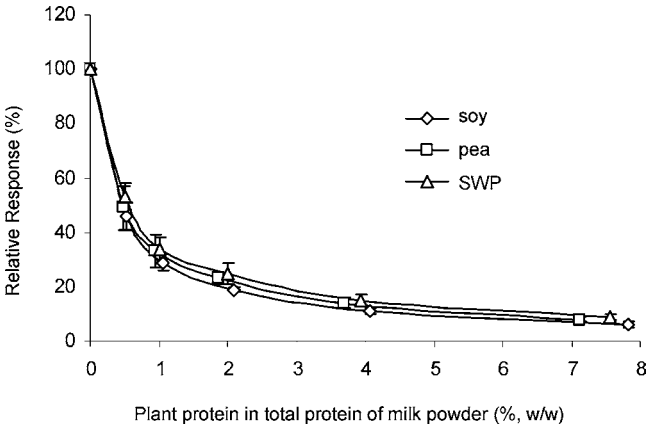

Figure 3. Calibration curves obtained with the calibration standards of the three plant proteins in milk powder in the triplex inhibition assay (TA) format.

in a plastic tube and 9.9 mL of PBS was added. After 10 s of vigorous mixing (vortex), the samples were mixed head over head for 30 min at room temperature. These standards and samples were 20 times diluted by pipetting 12 μL into a microtiter plate well and adding 228 μL of PBS containing 1% BSA and by shaking for 20 s. Of the diluted sample, 100 μL was pipetted into a microtiter plate well (inhibition assay) or into the 96-well filter-bottom microplate (sandwich assay).

RESULTS AND DISCUSSION

Comparison of Immunoassay Formats. The Luminex sandwich immunoassay format was tested first. After optimization, calibration curves, in which the responses increased with increasing concentrations (data not shown), with measurement ranges between 0.1 and 10 μg/mL (50% binding at 0.5−5 μg/mL) were obtained with high (up to 20000) maximum mean fluorescence intensities (MFI). Due to the necessary separation step, to remove access of sample material, this assay was performed in expensive 96-well filter-bottom microplates. The applied filtration steps (twice) and the double incubation (2 h in total) made this sandwich format relatively labor and time intensive. In the individual sandwich assays, the cross-reactivities for the other proteins were determined as 15 and 0% for pea and SWP in the soy assay, respectively; 25 and 0% for soy and SWP in the pea assay, respectively; and 50 and 20% for soy and pea in the SWP assay. Due to these cross-reactivities, the reagents in the triplex sandwich assay format (three antibody-coated bead sets and three Alexa-labeled antibodies) interacted with each other, and this format was found to be not distinguishable enough for the quantification of these individual vegetable proteins.

In the inhibition assay format, the protein-coated beads were mixed with the sample together with the fluorescent-labeled antibodies and, after one incubation, the beads were directly measured in the Luminex. The assay could be performed without a washing procedure because the fluorophores are measured only at the surface of the beads and unreacted fluorophores will remain in solution and will not contribute to the assay value. Therefore, compared with the sandwich assay format, the inhibition assay format is much less time- and labor-consuming, and this format was used during the following experiments in which the individual inhibition assay (IA) was compared with the triplex inhibition assay (TA).

For each IA, the optimum amount of labeled antibodies was determined, using a fixed amount of protein-coated beads (1000 beads per set per well) and a fixed time (10 s) for the measurement. The fast measurement (10 s/well) was chosen to avoid differences in incubation time and response when measuring 96 wells consecutively. The total time for measuring these 96 wells was 16 min. This is fast compared to the previously described fast BIA (*3*), which took 5 min per sample but 8 h for a complete 96-well plate.

Aiming for robust assays with a maximum MFI of >2000 and applying an incubation of 1.5 h at room temperature, the labeled antibody fractions (about 2.5 mL after gel filtration) could be diluted 10 times for the anti-pea and 20 times for the anti-soy and anti-SWP.

In the TA, the Alexa-labeled anti-soy, -pea, and -SWP were mixed in a ratio of 1:2:1 (v/v/v), and this mixture was 20 times diluted in PBS, of which 15 μL was pipetted per well. After optimization of this TA in PBS, the maximum responses varied per assay between 2400 and 2800 MFI (see **Figure 1**), and the measurement ranges were between 0.1 and 10 μg/mL with 50% inhibition values of about 0.5 μg/mL. Small cross-reactivities were seen for pea in the soy assay and for soy in the pea assay (**Figure 1**).

Calibration Curves in Milk Powder. The calibration standards were prepared in nonheated freeze-dried skimmed milk powder in the concentration ranges from 0 to 8% of the plant proteins in the total protein content, and they were dissolved in PBS (60 mg in 9.9 mL). At first, these standards were applied in the IA to find the optimum dilution to detect at least a concentration of 0.5% of the three plant proteins. As shown in **Figure 2**, the 20 times dilutions of the sample extracts resulted in the desired sensitivity. The similarity in sensitivity of the three assays was obvious, although differences were observed in the solubility of the proteins during the preparation of the plant protein extracts (8, 18, and 31% of the soy and pea isolate and SWP, respectively, were dissolved as proteins). This might be caused by the application of less sample material [0.1 g of milk powder (containing, e.g., 1% plant protein = 1 mg of plant protein)] in a larger volume (20 mL of buffer). As shown in **Figure 2A**, the effect of SWP standards in the soy assay was minimal, but a small effect (decrease of relative response with increasing concentration) was seen with the pea standards. This had a small influence (reduction of response) on the soy calibration curve in the TA in which the three standards were combined (dotted line in **Figure 2A**). A comparable effect was seen with soy protein in the pea assay (**Figure 2B**) and on the pea calibration graph in the TA (dotted line in **Figure 2B**). The SWP assay (**Figure 2C**) was less influenced by the other protein sources.

Quantification of Vegetable Proteins in Adulterated Milk Powders. The calibration standards in milk powder (0 to 8%) were used to create calibration graphs in the IA and in the TA, and they were used to calculate the percentage of plant proteins in the adulterated milk powders dissolved in PBS (100 mg in 9.9 mL) of which extracts were analyzed on five separate days. As shown in **Table 1**, the calculated average concentrations in the pasteurized milk powders were accurate in the IA with average recoveries 106 and 19% lower in the TA. In the UHT-treated milk powders (**Table 2**) the average concentration was lower, and this was mainly caused by the low concentrations found with the SWP adulterated samples. This was seen before in the BIA (*3*) and was blamed on denaturation of the proteins during the heating process. Also here, the TA resulted in 18% lower concentrations compared to the IA. As shown by the TA/IA ratios in both tables, the soy assay was most influenced in the triplex format (average ratio of 0.74) followed by the pea assay (average ratio of 0.82), and the SWP assay was not influenced (average ratio of 1.03).

The concentrations found in the blank milk powder samples (pasteurized and UHT) were <0.1%, and this was considered as the limit of detection (LOD), although the LOD could easily be improved by applying less diluted samples. The action level of the assay was established at 0.5% because a lower percentage of adulteration would not be of commercial interest.

Survey. As shown in **Figure 3**, the calibration curves obtained with the three plant protein standards in the TA showed comparable sensitivities (50% inhibition at 0.5%). These curves were used during the survey. None of the 260 powder samples obtained from the Dutch General Inspection Service was found to be positive in the TA (<0.5% of the three vegetable proteins in the total protein content). During that survey, the 10 with Alpro soy-spiked milk powder samples (containing 0.75, 1.5, 3.0, 7.5, and 15% of soy protein in the total protein content, see Materials and Methods) were analyzed as unknowns, and all samples ($n = 8$) spiked at or above 1.5% [1% soy powder in the (butter)milk powder] were found to be positive (>0.5%) for soy (ranging from 0.7 to >8% of soy protein in the total protein content). This soy milk was UHT treated and, just as with the UHT-treated milk powders (see **Table 2**) in which recoveries down to 63% were found, this might explain the lower concentrations (average recovery of approximately 55%) in the control samples compared to the calibration curve prepared from nonheated milk powders.

The samples spiked at a high level (15% soy) were also reactive in the pea assay in which concentrations of 0.5 and 0.6% (pea protein equivalents) were found. This indicates a cross-reactivity of soy protein in the pea assay of 7% (considering a recovery of 55%).

In conclusion, the Luminex proved to be a suitable system for the simultaneous immunochemical detection of the three plant proteins. Using polyclonal antisera raised against these proteins, the inhibition assay format was more distinguishable than the sandwich assay format. Another advantage of the inhibition assay format was that a wash procedure could be avoided. In buffer, protein concentrations could be measured in the range of 0.1−10 μg/mL. This was a narrow range compared to a direct BIA (*3*), showing a measurement range between 0.1 and 1000 μg/mL. However, the sensitivity and range of detection were more than enough to analyze milk powder samples for the possible presence of these protein adulterants. An extra 20 times dilution of the sample extract was necessary to obtain suitable curves with calibration standards in the range of 0.5−8% plant protein in the total milk powder protein content. Pasteurization of the adulterated milk, as part of the spray-drying process, had a limited effect on the quantification of the three vegetable proteins. The UHT treatment had a significant effect, especially with the SWP contaminated milk powders. This effect was previously observed in the BIA (*3*) and with ELISAs (*1*). Therefore, proper quantification of UHT-treated samples could only be possible with calibration standards prepared under the same conditions as used for the sample materials. However, if the sample treatment is unknown, quantification is limited because of denaturation and differences in solubility of the proteins. Another option might be the application of antibodies raised against heat-treated plant proteins. Compared with the fast BIA and for large-scale analyses (e.g., 96 wells), the total time of analysis for the Luminex (90 min of incubation plus 16 min for the measurements) is faster than the fast BIA [96 × 5 min/sample = 480 min (*3*)].

Over the years, about 500 Dutch milk powders were analyzed with different assays (ELISA, BIA, and Luminex) for SWP and proteins from soy and pea, and positives were never found. This indicates that the suggested fraud is minimal in The Netherlands. Enlargement to the detection of other vegetable proteins (maize, rice, bean, etc.) is ongoing. Other applications for the multiplex detection of vegetable proteins might be adulterations of meats and sausages.

ABBREVIATIONS USED

SWP, soluble wheat proteins; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CGE, capillary gel electrophoresis; ELISAs, enzyme-linked immunosorbent assays; Fc, flow channel; IgG, immunoglobulin G; BIA, biosensor immunoassay; xMAP, MultiAnalyte Profiling; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHS, *N*-hydroxysulfosuccinimide; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; UHT, ultrahigh temperature; MES, 2-(*N*-morpholino)-ethanesulfonic acid; DMF, dimethylformamide; IA, individual inhibition assay; TA triplex inhibition assay; VP, vegetable protein; LOD, limit of detection.

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