Soy milk protein detection in milk- A review

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Abstract: Milk being considered as a complete food can also be easily adulterated by a large number of adulterants. Such type of adulteration deteriorates its quality and can affect the health of the consumers. Processing such type of adulterated milk can affect certain processing parameters in the dairy industry thus affecting the quality of final products. A large number of adulterants in milk have been used worldwide and have been reported like water, urea, carbohydrates, foreign proteins, vegetable or animal fats, melamine and so on. Unscrupulous producers even add hazardous substances to gain profits. This review focuses on the detection of soy proteins by different methods like RP-HPLC, UHPLC, SDS-CE, SDS-PAGE, SDS-PAGE/MS, FPLC, NIR spectroscopy, FTIR, lateral flow assay, biosensors, LC/MS-MS that have been developed and used since last 25 years. From long and time-consuming electrophoretic to biosensor-based methods, this review will provide a clear understanding of the various assays.

Keywords: Adulteration, FTIR, Gold nanoparticles, Polyacrylamide gel electrophoresis, Reverse phase high-pressure liquid chromatography, Soyabean, Soy milk

Introduction

Based on various scientific literature on adulteration of food, milk is ranked next to olive oil as the most adulterated food commodity (Jablonski et al. 2014). In order to gain maximum profit and to bridge the gap between demand and supply of milk during lean season, the unscrupulous producers adulterate milk with a number of adulterants. Apart from its fat content, nitrogen content of milk is also used as a parameter for determining the price of milk (Gautam et al. 2017). United States Pharmacopeial Convention has advocated the use of improved methods to detect the economically motivated adulteration, as conventional nitrogen assays like Kjeldahl and Dumas are not much effective methods of detection (Moore et al. 2010). In 2002 and 2007, EU has reported use of plant proteins as adulterants for skimmed milk powder (SMP) (Scholl et al. 2014). The low prices of plant proteins make them potential adulterants in SMP. Soyabean is a cheap and easily accessible source of plant protein in contrast to milk proteins (Luykx et al. 2007). Soy milk added to bovine milk is being sold as fluid milk (Sharma et al. 2009). A variety of soyabean products like soyabean juices, soyabean flour, textured soyabean, soyabean infant formula are available (Saz & Marina, 2007). Soy protein is also used in milk mimetics like simulated yoghurt, coffee whiteners and frozen desserts, thus a major potent adulterant (Haasnoot et al. 2001). In spite of its nutritional properties, soyabean is considered as an allergen (Marina et al. 2006). It is considered as one of the major eight food allergens (Wang et al. 2015) and its presence in food poses potential risk to some allergic people especially infants (Poms et al. 2004, Azad & Ahmed, 2016) as their gut barrier is underdeveloped (Rozenfeld et al. 2002). Glycinin, a major soy protein has been reported to affect intestinal function, immune function and promote diarrhea (Wang et al. 2015). Soy milk contains anti-nutritional factors like trypsin inhibitor, proteinase inhibitor, tannins, alkaloids, so its absence is to be ensured to ascertain consumer health (Jaiswal et al. 2015). Soy protein is statutorily defined as allergens by FALCPA (Food Allergen Labeling and Consumer Protection Act) as per US law (Scholl et al. 2014). Keeping in view the interest and to protect the consumer, EU Directive (2007) instructed strict labeling of food commodities containing soyabean and its products regardless of its mass function in the product (Gautam

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et al. 2017). In spite of remarkable success achieved in the field of chemistry of major soy proteins, the quantitative determination of glycinin (11S) & β -conglycinin (7S) is a major problem especially when mixed in low proportions with other products (Tapia et al. 1999). In addition, processing conditions modifies the native state of the soy proteins thereby affecting their determination (Garcia & Marina, 2006). Processing of soymilk adulterated bovine milk can affect certain downstream processes in the dairy industry (Gautam et al. 2017). This review discusses in detail various analytical approaches employed to detect soymilk adulteration in milk.

Chromatographic methods

HPLC/UHPLC

Pure SMP was differentiated from SMP adulterated with soy protein isolates (SPI) using UHPLC-UV fingerprinting technique employing water + 0.1% trifluoroacetic acid (TFA) and acetonitrile + 0.1% TFA as mobile phase at a wavelength of 215 nm and a column temperature of 50°C. The samples were prepared by adding a denaturing buffer consisting of 6 M guanidine HCl, 20 mM dithiothreitol, 5 mM trisodium citrate followed by vortexing, dilution, refrigeration and centrifugation. The chromatogram for SMP used data points from the entire chromatogram excluding 0-3 min retention window because of very high solvent front peak. The retention window from 3-35 min was taken for further statistical analysis. SMP adulterated with SPI at 3% (w/w) was differentiated from point-by-point t-test comparison of the chromatogram, while adulteration up to 1% was detectable using Soft Independent Modeling of Class Analogy (SIMCA) analysis at 95% confidence levels. Using appropriate statistical tools (Q statistic and t-test), it was possible to correctly classify adulteration of SMP with SPI at 1 and 3% levels, respectively, while minimizing false classifications of authentic SMP (Jablonski et al. 2014). The presence of soybean sugars such as sucrose, raffinose, stachyose in suspected milk samples have been used for detection of soymilk in milk. The sugars from milk samples were extracted by using Carrez I, Carrez II and 20% acetonitrile followed by sonication of the filtrates for degassing. Use of acetonitrile facilitated protein precipitation so that a clear filtrate could be obtained for its further application in HPLC. The process for the extraction of sugars was not affected by the use of 20% acetonitrile. The sugars were separated based on their chemistry with NH_2 column using acetonitrile and water in 70:30 ratio as mobile phase at a flow rate of 1 ml/min using refractive index detector. The temperature of the column and RI was maintained at 40°C. Buffalo milk/ bovine milk exhibited a major peak corresponding to lactose which was very broad and thus masked the peaks of sucrose and raffinose. The peak for lactose was absent in pure soymilk. Raffinose, sucrose and stachyose peak were absent in bovine or buffalo milk. Sucrose and raffinose peak were masked by the peak of lactose in the case of adulterated

milk at 5% soymilk. However, well resolved stachyose peak (soymilk sugar) appearing at 12 min retention time can be used as a marker for detection of adulteration of milk with soymilk up to 5% levels (Fig 1) (Sharma et al. 2009). A linear binary gradient of acetonitrile-water-trifluoroacetic acid at a flow rate of 5 ml/min at 254 nm (because maximum absorption of phenylalanine is observed at this wavelength and it has been shown to be adequate for detection of soy proteins) (Garcia et al. 1998 & Garcia et al. 2000) was employed to detect soyabean proteins adulteration in cow, goat and ewe milk using perfusion reversed-phase high-performance liquid chromatography. In this method, SPI were used for sample preparation. Milk diluted with Milli-Q water was filtered through 0.45- μm pore size filters followed by mixing with SPI solutions. The spiked milk was centrifuged and the supernatant was stored at 4°C before using for HPLC analysis. The retention time for goat and ewe milk proteins was slightly higher than that for cow milk proteins. Soyabean proteins eluted in eight peaks but the last peak coeluted with the second peak of the milk proteins. Fresh whole and defatted cow milk showed similar recoveries. Milk adulterated with soyabean proteins upto 30 $\mu\text{g/ml}$ was detected using the above method within 2 min (Fig 2). Maximum matrix interferences were observed in case of fresh milk which was independent of the fat content and its source i.e. animal species, while liquid, defatted cow's milk and liquid semi defatted goat milk showed minimum matrix interference which was quantitatively evaluated by the recoveries obtained by using external standard and standard addition methods (Espeja et al. 2001). Perfusion chromatography enables separation of the biomolecules rapidly without affecting the resolution (Afeyan et al. 1990 & Fulton et al. 1991) as the stationary phase particles allow a better interaction with the solute/analyte molecules (Espeja et al. 2001). A method using reversed phase- perfusion chromatography was used to detect soyabean protein detection in milk, cheese and yoghurt. 0.1% TFA in water and 0.1% TFA in acetonitrile were used as mobile phase keeping the flow rate at 3 or 5 ml/min at a temperature of 60°C and detection carried at 254 nm. Using calibration curve for soy proteins, detection of soy proteins in adulterated dairy products could be quantified by interpolation of the area under the soy protein corresponding peaks. Least square regression analysis was done to obtain the linearity of the calibration curve. Sample for cheese was prepared by extracting the cheese spiked with SPI with water followed by homogenization, centrifugation and filtered. Soy proteins eluted at the start while cheese proteins eluted at the end. Soy proteins in fresh cheeses and in ripened cheeses were detected upto a level as low as 0.04% & 0.1% w/w, respectively. Results obtained for fresh or semi ripened cheese were free from matrix interferences unlike for soft and ripened cheese. Recoveries obtained for such type of cheeses were improved by standard additions calibration method. Powdered milks were adulterated with soyabean proteins from different sources at different levels. These milks were then further subjected to pasteurization and UHT treatment. The soy protein content determined in UHT milk was lower than that of pasteurized milk due to the intense heat treatment in case of the

Fig. 1 Separation of sugars from bovine, buffalo and soymilk using HPLC. (a) Bovine milk (b) buffalo milk (c) soymilk. a', b' and c' correspond to high-resolution chromatogram of bovine, buffalo and soymilk respectively. Peak 1- solvent; 2- fructose; 3- glucose; 4- galactose; 5- lactose; 6- raffinose and 7- stachyose. Adapted from Sharma et al. (2009) (Figure used with permission)

former. Different processing conditions used for the manufacture of cheese, yoghurt and enteral formula affected the determination of soy proteins (Garcia & Marina, 2006). Krusa et al. (2000) designed a RP-HPLC based method for quantification of soyabean proteins in bovine milk. A linear binary gradient of acetonitrile-water-0.1% TFA was used. The flow rate was maintained at 1 ml/min and the column temperature was maintained at 50°C. Standard solutions for SPI were prepared within range of 0.5-3.5 mg/ml. On comparing the chromatograms of the aqueous solutions of SPI, defatted bovine milk and bovine milk containing soy protein, it was observed that first peak of SPI overlapped with the peak of

bovine milk while the rest three peaks of SPI were separate, so these peaks were considered for quantitative analysis. The gradient was also shortened to reduce the analysis time. Effects of the matrix were studied for samples spiked with SPI and the results revealed that this method also suffered from matrix interferences. These effects became more noticeable when the milk concentration was increased. Limit of detection for this method was 13 µg of protein/g of bovine milk. Sensitivity for protein separations and their improved resolutions can be achieved by using µ-HPLC. In this method, Marina et al. (2006) used a capillary column having an internal diameter of 150 µm,

Fig. 2 Separation of cow milk and soy proteins using perfusion reverse phase high-performance liquid chromatography. Peak identification: 1-8, soyabean proteins; A-D, cow's milk proteins. Adapted from Espeja et al. (2001) (Figure used with permission)

which was filled by Genesis C18 stationary phase. Water and acetonitrile gradients coupled with TFA and acetic acid as ion pairing reagents were used and detection was carried out at 254 nm. The supernatant obtained after spiking of soy proteins in milk followed by centrifugation was injected in the chromatographic system. A number of gradients were used in order to decrease the analysis time from 42 min but it lead to the loss of resolution, thus water-acetonitrile-TFA gradient was used. The column used in the study could resolve the separation of soy proteins from milk proteins along with wheat, corn and rice proteins thus indicating good selectivity in separation. Also, this method indicated its efficacy for detecting soy proteins in commercial products.

Immunoassays

Hasnoot et al. (2001) developed a biosensor immunoassay for detecting non milk proteins in SMP. BIACORE 3000, an optical biosensor, based on the principle of surface plasmon resonance (SPR) was used in this method. Affinity purified antibodies against pea, soy and wheat proteins were immobilized on the biosensor chip. Labeling of the reagents was not required in this method, thus making it as a rapid detection technique, suitable for high-throughput screening. Nonmilk protein isolates (soy, pea, wheat) were spiked in skim milk at 0, 1, 2 and 5% levels. Spiked milk was then subjected to a number of treatments before pasteurization/UHT and spray drying. As low as 0.1% of plant proteins (soy, pea and wheat) in powdered milk were detectable within 5 min.

Results obtained for UHT milk differed from pasteurized milk as the heat treatment might have altered their antigenic properties or may have reduced their solubility. The antibodies got bound to other proteins like nuts, pumpkin seed, marrowfat, brown bean, rye, barley etc. This indicated that this method can be used to detect a wide range of non-milk proteins. The chip was reusable and worked even after 650 injections. An immuno-diffusion method which detected soymilk up to 1% within 12 h was developed using commercially available anti-soy antibodies. The anti-soy antibodies did not showed any cross-reactivity against milk proteins. Adulterated milk samples formed precipitating lines with anti-soy antibodies (Sharma et al. 2010). Using an ELISA based technique, the quantitative and qualitative detection of soy milk/protein in bovine milk was done. The soy proteins were slightly modified so as to react with the raised antibody. Caseins showed marked cross-reactivity against the anti soy antibody which made the application of this method slightly difficult in dairy products. The cross-reacting components were removed by partial purification of the anti soy antiserum by affinity chromatography. This method detects soy proteins up to 1% level in soy milk adulterated milk (Hewedy & Smith, 1990). Haasnoot & Jolanda (2007) developed a luminex based triplex immunoassay which involved use of affinity purified polyclonal antiserum against soy proteins. Inhibition assay was used for the detection as it was much easily distinguishable than sandwich method. Soya proteins, if present interacted with the antibodies immobilized on the sensor surface which were further detected. The time required for analysis was 5 min, and its limit of

Fig. 3 Detection of soymilk in whole milk spiked with soymilk at various concentrations (%) using lateral flow assay. C, control line; T, test line. Adapted from Gautam et al. (2017) (Figure used with permission)

detection in milk powder was reported to be below 0.1%. When compared to ELISA, this technique was more reproducible and less time consuming with a wide range of measurement (Dasso et al. 2002). At present the lateral flow based assay has become an attractive tool in the field of analytical chemistry. This method has been used to detect soymilk adulteration in milk (Gautam et al. 2017). Glycinin being a predominant soy protein, monoclonal and polyclonal antibodies against glycinin were raised in mouse and rabbit, respectively and the former exhibited high specificity for glycinin. Gold-labeled monoclonal antibodies were applied on conjugate pad, polyclonal antibody was applied on test line while control line had anti-mouse antibody. Milk samples having minimum 0.69 mg/kg of glycinin produced red color/ band on the test line while its absence indicated below detection levels (Wang et al. 2015). Another method was developed based on lateral flow

technique by Gautam et al. (2017) in which the commercially available rabbit anti-soy antibodies raised in rabbit was conjugated with gold nanoparticles (AuNPs). In this method, the test and control lines were immobilized by soymilk and anti-rabbit antibody, respectively. AuNPs prepared by citrate reduction method were analyzed for their stability and size. They have the property of exhibiting plasmon resonance peak and the same property was reflected by the prepared AuNPs at 520 nm. The size of AuNPs used for lateral flow assay was 28.59 nm and their zeta potential was -38.3 mV. The results indicated that the AuNPs were quite stable and thus suitable to be used in lateral flow assay. The AuNPs were conjugated to the anti-soy in borate buffer. Antibody conjugated AuNPs were applied on the conjugate pad and dried before application of the sample. Appearance of red band on the test line indicated pure milk while

Fig. 4 Two-dimensional electrophoresis maps of protein spots of cow's milk (a), cow's milk adulterated with 4% soymilk (b), Soy protein (c). Adapted from Yang et al. (2018) (Figure used with permission)

its absence indicated milk adulterated with soy. The presence of red band on the control line indicates validation of the assay. The sample applied on the sample pad travelled due to the capillary action so in order to obtain a better capillary action in case of whole milk, EDTA was added to the milk for disruption of casein micelles. The soymilk detection limit was 1.75% in whole milk (Fig 3).

Electrophoresis based methods

The property of milk proteins being soluble and plant proteins being insoluble in the tetraborate-EDTA buffer was exploited for the detection of soy proteins adulteration by Sodium Dodecyl Sulphate- Capillary Gel Electrophoresis. Treatment with tetraborate-EDTA buffer ensured selective removal of milk proteins thus enhancing the detection even at low proportions of plant proteins. The precipitated plant proteins were dissolved in Tris-HCl buffer in the presence of sodium dodecyl sulphate (SDS) and a reducing agent to disrupt and uncoil the proteins. The electrophoresis was carried at 25°C, with increasing the voltage linearly from 2 kV to 7 kV within a period of 1.7 min followed by a constant voltage of 7 kV. The detector was set at 214 nm. The peaks which corresponded to the subunits of glycinin a major soy protein were used for quantitation as these peaks were identified by Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) pattern of soy proteins in previous work (Molina et al. 1998 & Perez et al. 2000). A LOD of 1% soy protein in total protein was established in low heat milk powder. As the study conducted was interlaboratory, so Cochran and Grubbs test was applied to the obtained data. Since certain milk processing treatments like homogenization, heat treatment, spray drying dramatically reduced recovery of milk and plant proteins as proper separation between plant and milk proteins was not achieved, so capillary electrophoresis being

a sensitive method was used for analysis (Manso et al. 2002). Another method used for detection of soy protein in milk involved resolving of milk and soy proteins on 12.5% acrylamide gel using Laemmli buffer. The proteins of cow and soy differed in their molecular weight. Certain characteristic bands of proteins having molecular weight of about 20, 50 and 95 kDa suggested that these proteins were present in soymilk but absent in cow milk. The presence of these characteristic bands of soy proteins were used as a marker for the detection of soymilk in whole milk for up to 5 % levels (Sharma et al. 2010). Tapia et al. (1999) detected soy proteins in milk protein up to 1% level. The results indicated that the detection could be made on the basis of the basic subunits of glycinin and α and α' subunits of β -conglycinin. So they can be used as the markers for the detection of soy proteins in milk as the acid subunits of glycinin and the γ , β subunits of β -conglycinin coincided with caseins because of their similar molecular weights. The use of tetraborate-EDTA buffer minimized the interferences caused by milk proteins by removing them and the losses occurred during extraction of soy proteins was negligible. Similar electrophoretic patterns were observed in all of the commercial soy protein preparations except in the case of hydrolyzed products. Thus, it can be concluded that the main soy protein indicators can be detected by this method regardless of their composition and processing conditions. This method was less time consuming than SDS-PAGE but gave poor resolution. Urea alkaline polyacrylamide gel electrophoresis and SDS-PAGE were used for qualitative and quantitative detection of soymilk in pasteurized bovine milk by Hewedy and Smith (1989). Soymilk spiked bovine milk was freeze dried and the samples were mixed in the buffer. Fast protein liquid chromatography (FPLC) technique was employed for the detection purpose by maintaining a flow rate of 1 ml/ min and monitoring the eluate at 254 nm using a gradient of loading buffer and sodium chloride as a mobile phase. FPLC reduced the LOD of soymilk in pasteurized

Fig. 5 Detection of soymilk in milk using FTIR, spectra of milk, soymilk (SM) and adulterated milk (10% soymilk) in the wave number range of 4000–700 cm^{-1} . Adapted from Jaiswal et al. (2015) (Figure used with permission)

bovine milk from 5% as detected by SDS-PAGE to 1%. Results obtained by SDS-PAGE were difficult to quantify and the analysis was time-consuming, thus FPLC was employed for detection as it is a fully automatic technique with a greater and faster rate of analysis. Two-dimensional gel electrophoresis coupled with mass spectroscopy was used to detect soy proteins in milk. Milk samples were spiked with soy proteins at 2, 4 and 8% of total proteins. The stained gels were destained, dried and were subjected to tryptic digestion. The digested samples were subjected to and were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) for the identification of marker proteins to be used for adulterant detection. On analyzing the gel maps with successive mass spectroscopy containing soy proteins showed spots corresponding to β -conglycinin and glycinin. The identified soy protein spots had a higher molecular weight than milk proteins. β -conglycinin and glycinin were identified as the markers for milk adulteration with soy proteins. Soymilk added up to 4% in milk can be detected by this method (Fig 4) (Yang et al. 2018).

Spectroscopic methods

Optical property of sugars present in soymilk and whole milk were exploited for detection of presence of soymilk in milk. Lactose is the principle sugar in bovine and buffalo milk (Fox et al. 1998). Stachyose, raffinose and sucrose are present in soymilk and absent in buffalo and bovine milk (Wang et al. 2003). These sugars present in whole milk and soymilk are capable of rotating the plane polarized light clockwise or anticlockwise depending

on the functional groups attached to the chiral carbon. Protein free filtrates of bovine, buffalo, soymilk and milk adulterated with soymilk were analyzed using 200 mm polarimeter tube at a wavelength of 589.3 nm at 20°C. Soymilk indicated a lower optical rotation than cow and buffalo milk whereas there was no significant difference in the optical rotation of cow and buffalo milk. Results showed that the decrease in the optical rotation was directly proportional to the increase in the soymilk content in cow or buffalo milk. Soymilk adulterated up to 10% can be detected by this method in 45 minutes (Sharma et al. 2010). Near Infrared Spectroscopy being a quick and rapid technique was used for the detection of soy, pea and wheat isolates in milk powder. This technique was advantageous over electrophoretic and immunological methods as it requires no sample preparation and is convenient to use. The spectra were taken at room temperature and results were processed by a suitable statistical tool (sesame software) for the selection of the most significant wavelengths. Data pretreatments were also applied and measured by Partial Least Squares Regression, Principal Component Regression and Multiple Linear Regression. Skim milk was spiked with two different SPI followed by homogenization and pasteurization or UHT treatment followed by spray drying. These spray-dried samples were used for further analysis. Multiple Linear Regression was applied to the first derivative of the NIR absorbance values showed best relationship characterized by low SEC value and minimum outliers. A range of 2000-2400 nm showed most imperative differences amongst the samples and no sample overlapping was observed (Maraboli et al. 2002). Fourier Transform Infrared (FTIR) spectroscopy, a rapid technique

used for accessing quality of milk has been used for detection of soymilk in milk. Samples of milk adulterated with pasteurized unflavored soymilk were prepared at different levels and were stored at temperatures ranging from 15-30°C. The spectra were taken in the wave number ranging from 4000-500 cm^{-1} (Fig 5). Clear differences were observed in the spectra of milk and soymilk in the regions of 1639-1613 cm^{-1} , with soymilk showing higher absorption than milk because of presence of a higher amount of secondary structures in soy protein. Milk showed higher absorption values than soymilk in the spectral range of 1089-1058 cm^{-1} . These differences were due to the presence of phosphate groups covalently bound to the caseins in milk. Amide-I, II and III were present in higher amount in soymilk than milk and exhibited a higher absorption values in the spectral range of 1680-1631 cm^{-1} , 1560-1553 cm^{-1} and 1472-1239 cm^{-1} respectively. Samples of adulterated milk stored at 20°C exhibited better results as it had lowest particle depression index. SIMCA class projection helped in differentiating between pure and adulterated milk on the basis of the spectra generated by FTIR. Except at 6% level, correct classification was done at different levels of soymilk in the range of 1639-1635 cm^{-1} . The ATR-FTIR method was able to detect soymilk in milk as low as 2% level ($p < 0.05$) with minimum sample preparation and data treatments (Jaiswal et al. 2015). UPLC-quadrupole time-of-flight-mass spectrometric (UPLC-qTOF-MS) proteomics model combined with chemometrics was used for in the detection of raw milk adulterated with plant proteins. The extracted proteins from the adulterated milk samples were treated by trypsin to convert proteins to peptides. This digested solution was injected to UPLC-MS and analyzed over a period of 78 min. The elution pattern of plant peptides could be distinguished from that of milk from 25 to 27, 27 to 32, 36 to 40, and 41 to 45 min due to the dissimilar polarity of peptides. Protein-Lynx Global Server (PLGS) was used to analyze UPLC-MS spectra to identify proteins in the samples. Twenty six major proteins were identified out of which six were milk proteins and eleven were of soyabean origin (trypsin inhibitor, glycinin, β -conglycinin and albumin) at 10% adulteration level. β -conglycinin, α and β subunits were identified as the markers for the detection of adulterated milk as they could be detected at 1% level. Principal component analysis helped in clearly differentiating between pure and adulterated milk (Lu et al. 2017). Plant proteins were detected in adulterated skim milk powder by LC-MS/MS analysis on a quadrupole time-of-flight MS instrument. Milk samples were adulterated at 1 & 5% levels of SPI. Enrichment of the plant proteins was done by mixing the adulterated samples with borate buffer and the supernatant obtained after centrifugation was directly applied to reversed phase perfusion column. Modified porcine trypsin was added to the enriched adulterated samples for their proteolytic digestion. The identified peaks obtained from liquid chromatography were analyzed by using Q-TOF instrument. Specific proteins in the samples were identified using PLGS and NCBI NR database. Soy proteins glycinin (types G1-G5), β -conglycinin (α and β subunit), soy lipoxygenase-1, 2 & 3, alcohol dehydrogenase and glucose

binding protein were revealed with glycinin being the predominant one. This method could detect soy proteins at concentrations below 1% as majority of the plant proteins were retrieved in the borate buffer thus improving the efficiency of the method (Luykx et al. 2007). Cordwener et al. (2009) developed LC-MS method using a quadrupole TOF-MS instrument to detect soy protein adulteration in SMP. Plant proteins were easily detected in the adulterated SMP samples by LC-MS analysis of the corresponding total tryptic digests. Subsequent LC-MS/MS of the differential peptides successfully identified the adulteration of soy and pea proteins. Glycinin and β -conglycinin from soyabean were detected. The approach was advantageous as compared to other methods as the adulterations can be detected without any enrichment procedure. Flow injection mass spectrometry (FIMS) in combination with chemometrics is a handy tool for the rapid detection of soy protein adulteration in milk. The principal component analysis, partial least-squares-discriminant analysis and support vector machine classification models successfully differentiated between pure and adulterated milk. FIMS in combination with chemometrics detected adulteration by soyabean protein in milk within 1 min per sample at levels as low as 0.5% (w/w). FIMS coupled with chemometrics may prove to be an effective tool to detect possible economically motivated adulteration in milk (Du et al. 2018).

Miscellaneous methods

As milk proteins can be coagulated at a certain pH, the principle was used for detection of soymilk adulteration in milk. The pH range of 5.4 to 6.0 can be used to precipitate the major soy proteins (7S & 11S) while the major milk proteins remained soluble. Coagulation was done by acid and rennet. 0.1 N HCl was used for adjusting the pH of milk to 5.7 and incubated at 50°C. Visible coagulation was observed after the incubation within 10 min in case of milk samples containing 10% soymilk. No visible coagulation was witnessed even after extending the incubation for 30 min in case of milk containing 5% soymilk. The rennet whey obtained samples were also incubated at 50°C. The coagulation occurred within 10 min for samples containing 10% soymilk while a visible coagulum was observed after an extended incubation up to 20 min in rennet whey samples containing 5% soymilk (Sharma et al. 2010). Turbidimetric detection of plant protein involves the extraction of these plant proteins from SMP samples. In this method, SMP samples were spiked with 0.03-3.0% by weight of plant protein isolate powders i.e. soy, wheat, rice and pea isolates. These samples after mixing with sodium tetraborate-EDTA buffer were loaded in a 96 well plate column. The pellets obtained after diluting and centrifugation were resuspended in sodium tetraborate-EDTA buffer and the absorbance was measured at 360 ± 2 nm (as it gave the strongest signal) at 20°C. Limit of detection ranged from 0.1-0.2% weight of plant protein to SMP, generally considered satisfactory for economically motivated adulteration. EDTA was used to remove the turbidity and opacity of adulterated milk. This method is a

semi-targeted approach as it is not plant specific. Other plant proteins like zein, millet, sorghum, lupin can yield similar results due to chemical similarities among their storage proteins. The sensitivity of this method is comparable or better than that of other methods like ELISA, SDS-PAGE/CE. The simplicity of this test makes it suitable for use in poor resource environments (Scholl et al. 2014). A simple microchip-CE device was developed to detect soyabean proteins adulteration in milk. The method included on-chip protein fractionation, concentration and detection within a pI of 5.5-7.0. Most of the milk proteins are soluble at this pH as compared to soy proteins which made the detection easier. The method was able to detect 0.1% of soyabean proteins in total milk proteins within 10 min. As compared to other methods, this method was beneficial and time-saving as it involved no sample pretreatment, lesser amount of reagents were required and was less expensive (Wu et al. 2014). Dielectric property is the intrinsic property of every material/ food commodity. It is influenced by numerous factors like chemical composition, the temperature of materials and frequency of the applied electric field. This property was utilized for detecting the adulteration of soy protein in raw goat milk. The adulterated milk samples were subjected to discrete frequencies ranging from 20-4500 MHz at a temperature range of 5-75°C. Regression models were applied to co-relate permittivity with temperature and adulterated soy protein content in raw goat milk. Results indicated that dielectric constant was inversely related to the applied frequency at any temperature with a greater decrease in its value at a higher temperature than at lower one. Also, an inverse relationship was found between dielectric constant and temperature at a given frequency. The dielectric constant of milk showed a negative relation with the frequency when it was increased from 20-4500 MHz, furthermore it decreased, either on increasing the temperature or decreasing the soy protein content. A linear relationship was observed between permittivities and soy protein content. Permittivities as a function of soy protein content and temperature were explained by second-order polynomials (Zhu & Kang, 2015).

Future prospects

In future emphasis should be laid on not only improving the existing analytical methods but also simplifying the sample preparation without which it would be difficult to automate. Further, stress should be laid on maintaining the quality of milk by using rapid and automated methods, possibly coupling with the separation techniques and eliminating or simplifying the sample preparation step. More than one analyte should be targeted for detection so as to improve the reliability of the tests and prevent false positives. Methods in which the results are independent of the processing treatment given to the product, its physicochemical and intrinsic property, minimal matrix interference should be developed. Extensive validation of the existing methods is to be done. Novel markers should be discovered to strengthen the field of analytical chemistry deployed

for detecting adulteration. In the future, it is expected that the advancement in the field of analytical chemistry for milk testing will be based on modern instrumental techniques. Electronic nose technology could play an important role at industrial level to ensure product safety and quality assurance. The major obstacle faced in tackling the problem of adulteration is the failure to implement the existing methods at field level as these methods are time-consuming, expensive and requires skilled personnel to perform the tests. So a relatively cheap, quick method should be developed so that it can be applicable at the grass root level to curb adulteration. If such obstacles can be overcome, then surely we will be heading towards building a healthy society.

Conclusions

Detection of adulteration in milk is complex as the markers of adulteration in milk can vary in quantity due to various biological, climatic and agronomic factors. Moreover, processing parameters can modify or alter the natural composition of the final product. Further, its intrinsic properties can pose difficulty for setting specification ranges for various methods. Therefore, stringent specification may increase the number of false-positive results. The unscrupulous manufacturers or vendors engaged in adulteration of foods have developed the art of blending food with adulterants in such a way that they can pass the adulteration detection methods. The development of simple and economical techniques for detecting adulteration in milk having high degree of repeatability and precision, is the need of the hour.

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