Food Chemistry 176 (2015) 123–129

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Detection of melamine in milk using molecularly imprinted polymers–surface enhanced Raman spectroscopy

Yaxi Hu [a], Shaolong Feng [a], Fang Gao [a,b], Eunice C.Y. Li-Chan [a], Edward Grant [b], Xiaonan Lu [a,*]

[a] Food, Nutrition and Health Program, Faculty of Land and Food Systems, The University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada
[b] Department of Chemistry, The University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

ARTICLE INFO

Article history:
Received 14 June 2014
Received in revised form 5 November 2014
Accepted 13 December 2014
Available online 20 December 2014

Chemical compound studied in this article:
Melamine (PubChem CID: 7955)

Keywords:
Molecularly imprinted polymers
SERS
Raman spectroscopy
Melamine
Food adulteration
Chemometrics

ABSTRACT

A novel biosensor combining molecularly imprinted polymers and surface-enhanced Raman spectroscopy (MIPs–SERS) determines melamine in whole milk. MIPs were synthesized by bulk polymerization of melamine (template), methacrylic acid (functional monomer), ethylene glycol dimethacrylate (cross-linking agent) and 2,2′-azobisisobutyronitrile (initiator). Static and kinetic adsorption tests validated the use of MIPs to efficiently separate and enrich melamine from whole milk. Silver dendrite nanostructure served as SERS-active substrate for signal collection. Principal component analysis and hierarchical cluster analysis segregated Raman signatures of whole milk samples with different melamine concentrations. Regression models showed a good linear relationship ($R^2 = 0.93$) between the height of melamine SERS band (at 703 cm^{-1}) and melamine concentration in the range from 0.005 mmol L^{-1} to 0.05 mmol L^{-1}. The limit of detection and limit of quantification were 0.012 mmol L^{-1} and 0.039 mmol L^{-1}, confirming the high sensitivity of this biosensor to accurately determine melamine in whole milk. Simple sample pretreatment reduced full analysis time to determine melamine in whole milk to less than 20 min.

1. Introduction

Melamine, a triazine compound, is widely used for the industrial production of melamine resins. This substance is two-thirds nitrogen by weight, and as a result it has been added deliberately to processed foods to give a false appearance of high protein content. Melamine itself has low toxicity, but can cause renal failure by interaction with cyanuric acid in the body (Liu, Todd, Zhang, Shi, & Liu, 2012). The adulteration of pet food and infant formula has caused widely publicized cases of injury and death (Brown et al., 2007; Wu, Zhao, & Li, 2009). Although in principle melamine can transfer into food systems from packaging or other industrial sources, and trace levels are allowed in foods, it is universally banned as a substance to be used for intentional adulteration. In Canada, the maximum levels of melamine allowed in dried infant formula and other products containing milk are 0.5 ppm (ppm) and 2.5 ppm, respectively. Assured conformance with these standards requires an accurate and highly efficient approach for the detection and quantification of trace level of melamine in food systems.

The assured determination of melamine in agricultural and food products demands the combination of a separation technique and a detection tool. Commonly used approaches involve liquid chromatography (LC) or gas chromatography (GC) coupled with mass spectroscopy (MS), photodiode array detector (DAD) or ultraviolet (UV) detection. Although some of these methods can achieve a limit of detection (LOD) and limit of quantification (LOQ) as low as 0.3 ppb (Li, Qi, & Shi, 2009), they often require labor intensive and time consuming sample pretreatment, which interferes with high-throughput detection necessary for use in food industry.

Solid phase extraction (SPE) has been widely applied for analyte extraction and sample clean-up (Hennion, 1999). However, the sorbents used in traditional SPE columns often lack selectivity for a specific analyte in complex matrices, leading to low extraction efficiency. Molecularly imprinted polymers (MIPs) offer a promising alternative as a sorbent for SPE, and their use has been reported for the extraction of chemical hazards in foods (Wang, Liu, Fang, Zhang, & He, 2009). MIPs are constructed by the copolymerization of a functional monomer and cross-linker, to form a rigid complex around template molecules (Haupt, 2003). Removing template molecules by means of appropriate solvent exposes cavities with chemical and physical specificities. Thus, MIPs can serve as a "lock" to selectively recognize and bind a targeted analyte "key". MIPs

* Corresponding author. Tel.: +1 604 822 2551; fax: +1 604 822 5143.
E-mail address: xiaonan.lu@ubc.ca (X. Lu).

http://dx.doi.org/10.1016/j.foodchem.2014.12.051
0308-8146/

have been recognized as "artificial antibodies" and have high stability and broad-spectrum applicability to specifically recognize analytes (Ye & Haupt, 2004). Used as the sorbent in SPE, MIPs have higher extraction and enrichment effectiveness than traditional sorbents.

Raman spectroscopy has been recognized as a fast and nondestructive technique for the detection and quantification of chemicals and microbes (Choo-Smith et al., 2002; Li-Chan, 1996; Williams & Edwards, 1994). Surface enhanced Raman spectroscopy (SERS) has been widely investigated owing to its significant enhancement of faint Raman scattering signals. Noble metal (*e.g.*, silver, gold) nanostructures as SERS substrates generate a localized surface plasmon resonance, and this induces an electromagnetic field (Haynes, McFarland, & Duyne, 2005). Analytes approaching the surface of the substrate present significantly enhanced Raman cross sections, lowering detection limits to ppb or even at single molecule levels (Nie & Emory, 1997). This feature has contributed to the fast development and application of SERS in food safety inspection, such as the detection of foodborne pathogens (Lu et al., 2012), chemical contaminants (Costa, Ando, Camargo, & Corio, 2011) and food adulterants (Cheung, Shadi, Xu, & Goodacre, 2010).

SERS collects vibrational signals associated with numerous functional groups in molecules (Haynes et al., 2005). As a result, both targeted analytes and interferences derived from food matrices contribute to SERS spectra. Extensive sample pretreatment is therefore required to eliminate the interferences and obtain accurate and reliable results. Common methods for sample clean-up require large amounts of solvent and laborious extraction procedures (*e.g.*, liquid–liquid extraction). New approaches such as antibody-based separation and aptamer-based separation have been successfully used to enrich food chemical and microbiological hazards, such as ovalbumin (He, Rodda, et al., 2011), ricin (He, Lamont, et al., 2011), and bacterial spores (He, Deen, Pagel, Diez-Gonzalez, & Labuza, 2013), enabling the use of SERS for detection. However, only a small number of analytes can be isolated by such approaches owing to the limited availability of antibodies and aptamers. Our group recently developed MIPs-based SERS biosensors to detect α-tocopherol in vegetable oils (Feng et al., 2013) and chloramphenicol in honey and milk (Gao et al., 2014), and these studies offer promise for the general feasibility of this technique as a highly efficient and accurate alternative for the detection of chemicals in agricultural and food products.

The present study aims to fabricate an "artificial antibody" having specific affinity to melamine. With the involvement of SERS-active substrate, an innovative "two-step" MIPs–SERS biosensor for the separation and detection of melamine in whole milk was constructed. To the best of our knowledge, no previous work has demonstrated a MIPs–SERS biosensor for rapid detection and quantification of melamine in foods.

2. Materials and methods

2.1. Chemicals and reagents

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), 2,2′-azobis(isobutyronitrile) (AIBN), melamine, silver nitrite, and zinc plate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade), ethanol (HPLC grade), ammonium hydroxide and acetic acid were purchased from Thermo Fisher Scientific (Toronto, ON, Canada). Whole milk (3.25% fat) samples were obtained from local grocery stores. Melamine stock solution (1 mmol L^{-1}) was prepared in methanol, stored at room temperature, and diluted by methanol for further use as working solution.

2.2. Synthesis of MIPs

The synthesis of MIPs for melamine followed the procedures by Yang and others (2009) with modifications. Briefly, 0.5 mmol of melamine (template) was dissolved in 5 mL of ethanol and 1.5 mL of water (porogen). After adding 6 mmol of MAA (functional monomer), the mixture was magnetically stirred for 10 min. Then 30 mmol of EGDMA (cross-linker) and 0.25 mmol of AIBN were added, followed by purging with nitrogen for 10 min. The solution was sealed and incubated for 24 h at 60 °C in oil bath. The monolithic product was ground and sieved through a 200 mesh steel sieve to obtain the homogeneous fine particles. To remove the template, Soxhlet extraction was conducted using 250 mL of methanol/acetic acid (8:2, v/v) for 24 h, and another 250 mL of methanol was used for further extraction. Finally, the MIPs were dried for 12 h in a vacuum drying oven at 50 °C. The non-imprinted polymers (NIPs) was synthesized following the same process without the addition of template (*i.e.*, melamine).

2.3. Adsorption tests

The static adsorption capability for MIPs and NIPs was determined by the following procedure. Ten milligrams of MIPs or NIPs was mixed with 2 mL melamine standard solutions at selective concentrations ranging from 0.01 mmol L^{-1} to 0.06 mmol L^{-1}. The mixtures were horizontally shaken for 18 h at room temperature. After centrifugation at 12,000×*g* for 3 min, the supernatant was filtered through a 0.22-μm nylon syringe filter (Thermo Scientific, Rockwood, TN, the United states), and the final concentration of melamine was determined by high performance liquid chromatography (HPLC) with DAD set at a wavelength of 240 nm.

The kinetic adsorption test was conducted by mixing 10 mg MIPs or NIPs with 2 mL melamine solution (0.05 mmol L^{-1}). The mixtures were horizontally shaken for different time intervals ranging from 5 min to 240 min. After the centrifugation at 12,000×*g* for 3 min, the supernatant was treated and determined following the same procedures as for static adsorption.

2.4. Sample pretreatment

Whole milk was spiked with different contents of melamine ranging from 0.001 mmol L^{-1} to 0.05 mmol L^{-1}. Melamine was recovered following the procedures by Tran, Okoniewski, Storm, Jansing, and Aldous (2009). Briefly, 0.5 mL of milk was mixed with 4.5 mL of methanol. After shaking for 30 s, the mixture was ultrasonicated for 5 min and then centrifuged at 8000×*g* for 5 min. The supernatant was collected for further analysis.

2.5. HPLC conditions

HPLC analysis was conducted on an Agilent 1100 series HPLC system and DAD was set at a wavelength of 240 nm. Samples (0.2 μL) were injected into a hydrophilic interaction liquid chromatography (HILIC) column (Waters Atlantis HILIC silica, 5 μm, 2.1 mm × 150 mm, Milford, MA, USA) at 30 °C. Each sample was tested in triplicate. The mobile phase was 0.01 M ammonium acetate–acetonitrile (10:90, v/v) with the flow rate of 0.2 mL min^{-1}. The total running time for melamine standard solution was 10 min while for each milk sample was 22 min.

2.6. Molecularly imprinted solid phase extraction (MISPE)

Three hundred milligrams of synthesized MIPs or NIPs were packed into a 6 mL SPE cartridge with one PTFE frit at each side (Agilent, Santa Clara, CA, USA). The MISPE columns were conditioned with 2 mL water and then 2 mL methanol. Each sample

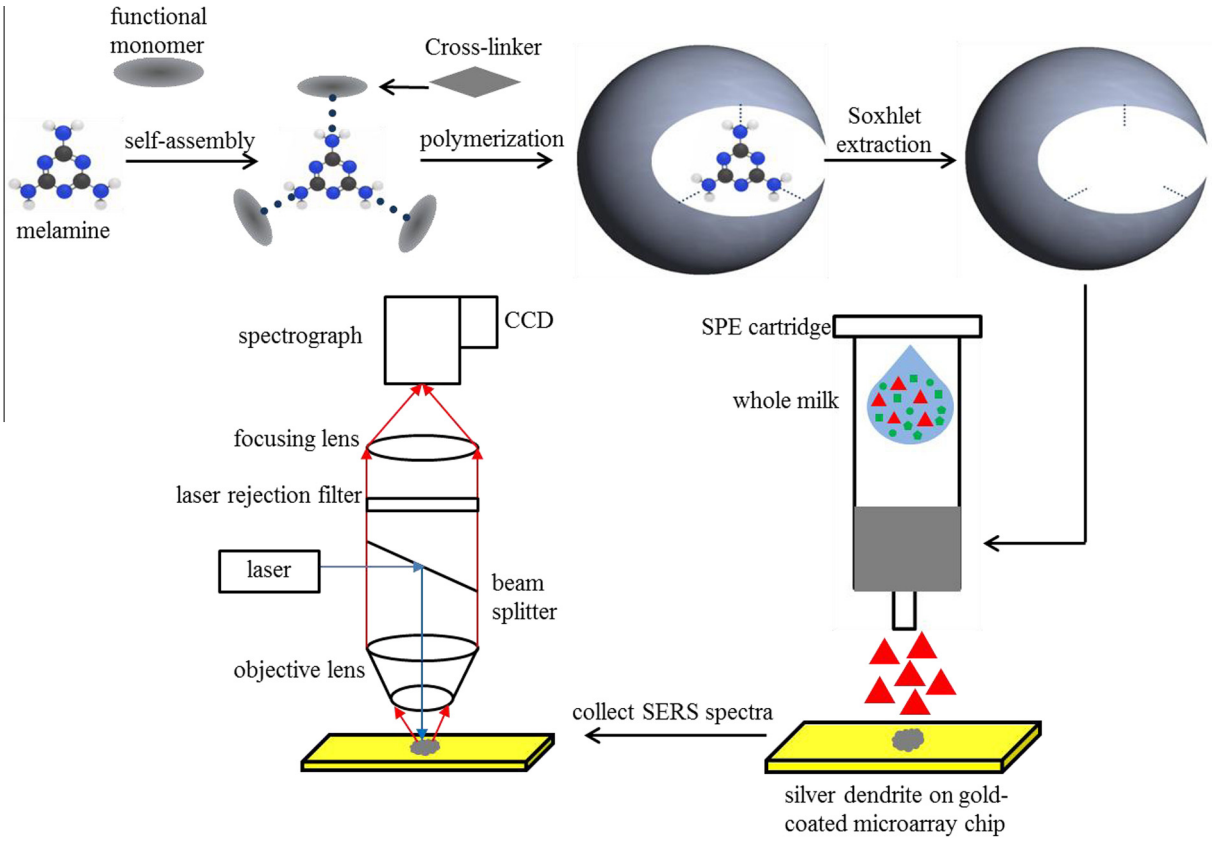

Fig. 1. Schematic illustration of MIPs–SERS biosensor for the detection of melamine in whole milk. CCD: charge coupled device; MIPs: molecularly imprinted polymers; SERS: surface enhanced-Raman spectroscopy.

(5 mL) was loaded onto the columns, followed by washing with 2 mL water and then 2 mL methanol. After drying the column, 3 mL methanol containing 5% ammonium hydroxide was used to elute the analyte (*i.e.*, melamine) adsorbed on the sorbent. The flow rate was 1.5 mL min^{-1} for each step. The eluent (2 μL) was blew to dryness under nitrogen, redissolved in 2 μL of 90% methanol, and then directly deposited onto silver dendrite SERS substrate for spectral collection. Duplicates were conducted for each concentration.

HPLC was conducted to determine the recovery of melamine. The eluted solution was evaporated to dryness and redissolved in 1 mL methanol for melamine standard solutions or 0.5 mL methanol for milk samples, followed by HPLC determination.

2.7. Synthesis of silver dendrite SERS substrate

Silver dendrite SERS substrate was synthesized by replacement reaction following the protocol by Feng and others (2013). The zinc foil (99.99% purity) was immersed into 0.02 mol L^{-1} hydrochloride acid and ultrasonicated overnight to remove the oxidative products and contaminations on the surface. After rinsing with distilled water, the pretreated zinc foil was reacted with 200 mmol L^{-1} $AgNO_3$ solution at room temperature for 1 min. Then, the silver dendrites were gently peeled off the zinc foil and rinsed with distilled water. Ultrasonic bath was used to break the silver dendrites into homogeneous nanostructure. Finally, these silver dendrite products were stored in a glass vial at room temperature and it can be used as SERS substrate for more than 6 months (Feng et al., 2013). For SERS spectral collection, 4 μL of silver dendrite was deposited onto a gold-coated microarray chip (Thermo Electron, Waltham, MA, USA) and blew dried with nitrogen.

2.8. Raman spectroscopic instrumentation

SERS spectra were collected using a confocal micro-Raman spectroscopic system coupled with a 785 nm near-infrared laser (0.25 mW incident laser power). The spectrometer (Renishaw, Gloucestershire, UK) has an entrance aperture of 50 μm, a focal length of 300 mm, and is equipped with a 1200 line mm^{-1} grating. The Raman signals were recorded by a 578- by 385-pixel charge coupled device (CCD) array detector, with a pixel size of 22 μm.

After mounting the SERS substrate onto the stage of Leica microscope (Leica Biosystems, Wetzlar, Germany), SERS spectra were collected using a 50× Nikon objective (NA = 0.75, WD = 0.37). The spectra of each sample were collected with an exposure time of 10 s. All experiments were conducted at least in triplicate.

2.9. Spectral analysis and chemometric models

Raw SERS spectra were analyzed by OMNIC software version 7.0 (Thermo-Nicolet, Madison, WI, USA). Automatic baseline correction and spectral smoothing (11-point Savitzky–Golay algorithm) were employed to reduce the spectral noise. Raman band at 1072 cm^{-1}, which was attributed to nitrate on silver dendrite SERS substrate, was used as internal standard to normalize SERS spectra (He, Rodda, et al., 2011).

By using Matlab, unsupervised principal component analysis (PCA) and hierarchical cluster analysis (HCA) were conducted to cluster and differentiate whole milk samples with different concentrations of melamine. Linear regression model was constructed to reveal the correlation between spiking melamine content and intensity of featured SERS band at 703 cm^{-1}, which is derived from melamine.

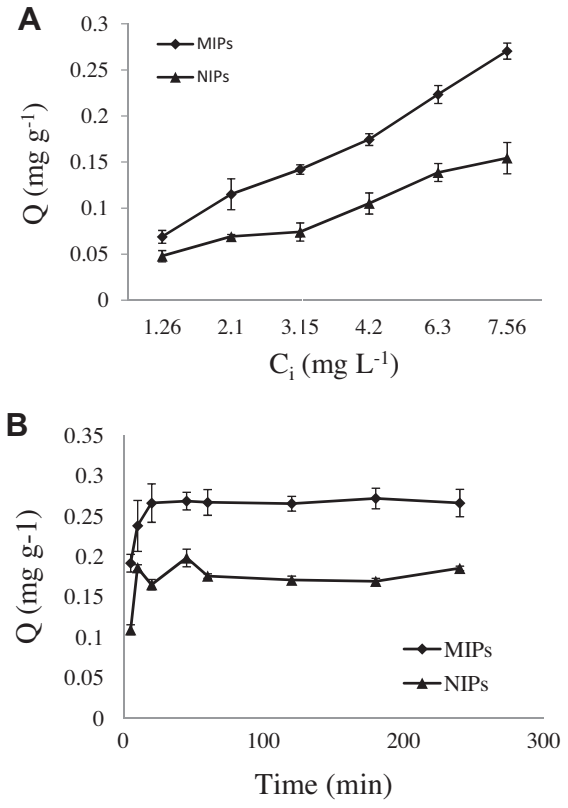

Fig. 2. (A) Static binding isotherm of molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) for melamine; (B) kinetic binding isotherm of MIPs and NIPs (C_i: 6.3 mg L^{-1}). C_i: initial concentration of melamine; Q: binding capacity; error bars indicated standard deviation ($N = 3$).

Table 1
Recoveries of melamine in whole milk by molecularly imprinted solid phase extraction (MISPE) and non-imprinted solid phase extraction (NISPE).

Concentration (mg L^{-1})	MISPE recoveries	NISPE recoveries
6.30	1.00	0.49
3.15	1.07	0.45
1.26	0.96	0.64
0.63	1.08	0.62
0.126	N/A*	N/A

* The recovered concentration of melamine spiked at 0.126 mg L^{-1} was too low to be detected by HPLC-DAD.

3. Results and discussion

3.1. Synthesis and characterization of MIPs

The schematic illustration of the fabrication of MIPs–SERS biosensor is shown in Fig. 1. MIPs for melamine were prepared by bulk polymerization. Previous studies have confirmed the feasibility of MIPs synthesized for the separation and enrichment of melamine, using techniques that include bulk polymerization (Curcio et al., 2010), precipitation polymerization (Zhang, Cheng, Zhang, Wang, & Li, 2012), and polymer films (Pietrzyk et al., 2009). Among these methods, bulk polymerization has successfully yielded a variety of templates (Baggiani, Anfossi, & Giovannoli, 2007). It requires no sophisticated instruments and the synthesis procedure is fast and simple (Baggiani et al., 2007).

To evaluate the rebinding properties of the synthesized MIPs, both static adsorption and kinetic adsorption were tested on MIPs and NIPs. The selectivity of MIPs toward analyte molecules is derived from complementary cavities and specific bonds. The cavities for template molecules are produced during polymerization process and remain constant, but specific binding sites may fail to interact with analyte in some circumstances because solvent used in the rebinding experiments can influence binding between analyte and polymers. Generally, the solvent used as porogen for the synthesis of MIPs provides the highest specificity in rebinding experiments (Pichon, 2007). However, in the current study, when melamine was dissolved in ethanol–water (10:3, v/v), no differences in the adsorption capacity were observed between MIPs and NIPs. This result may be attributed to the fact that water in the solvent increases its polarity and consequently interferes with the formation of hydrogen bonds between the MIPs and analyte.

In accordance with the study by Yang and coworkers (2009), we selected methanol as the solvent for adsorption tests. Fig. 2(A) illustrates the static adsorption isotherm of MIPs and NIPs. The adsorption capacity (Q) was calculated by the following formula: $Q = ((C_i - C_f) \times V/W)$ (Qian, Fang, He, Pan, & Wang, 2010), where C_i and C_f represent the initial and final concentration of melamine in the solution, respectively. V is the volume of the solution, and W is the mass of the polymer. Regardless of the initial concentration of melamine, MIPs yield higher Q values than NIPs. Q_{MIPs} and Q_{NIPs} increase with the increase of melamine concentration, and reach 0.27 mg g^{-1} and 0.15 mg g^{-1}, respectively, at a melamine concentration of 0.06 mmol L^{-1}. Due to the small size and simple structure of the melamine molecule, it adsorbs easily on rough materials. Therefore, the differences in Q values between MIPs and NIPs are not as significant for melamine as some other analytes (Gao et al., 2014).

We conducted kinetic adsorption tests to assess the rate of development of specific bonds between analyte and MIPs. If the time required to equilibrium is short, specific bonds can be formed rapidly, indicating that MIPs are ideal for rapid separation of targeted analyte. Fig. 2(B) shows the equilibration isotherm of 10 mg of MIPs and NIPs with 2 mL of melamine (0.05 mmol L^{-1}) for different time intervals at room temperature. MIPs reached to binding equilibrium within 20 min, which was faster than NIPs. This confirms the potential of MIPs for fast and specific separation and enrichment of melamine in complicated food matrices.

3.2. MISPE for melamine spiked whole milk

In the preliminary experiment, the flow rate of MISPE procedure was optimized using a melamine standard solution. With a slow flow rate, more melamine was flushed out of the NIPs column before eluting, resulting in a lower recovery of NIPs. However, one of the objectives in the current study was to create a biosensor with high efficiency. To reduce operating time, we increased flow rate, which led to an increase in the recovery of NIPs. By setting the flow rate at 1.5 mL min^{-1}, the time required for SPE operation and the differences in recoveries of MIPs and NIPs reached a desirable balance. The recovery of MIPs using a standard melamine solution of 0.05 mmol L^{-1} was 106%, while the corresponding recovery of NIPs was 50.5%.

After the optimization of MISPE procedures, we evaluated the recovery of melamine from whole milk samples. Before loading onto the MISPE column, these samples required a simple pretreatment to remove a variety of macromolecules, including proteins, fats. After spiking with melamine at concentrations from 0.001 mmol L^{-1} to 0.05 mmol L^{-1}, milk samples were deproteinized and defatted with methanol. The pretreated milk samples were then directly loaded onto the MISPE column following the same procedure as the melamine standard solution. The recovery of melamine in whole milk by MIPs and NIPs ranged from 96% to 108% and 45% to 64%, respectively, as shown in Table 1.

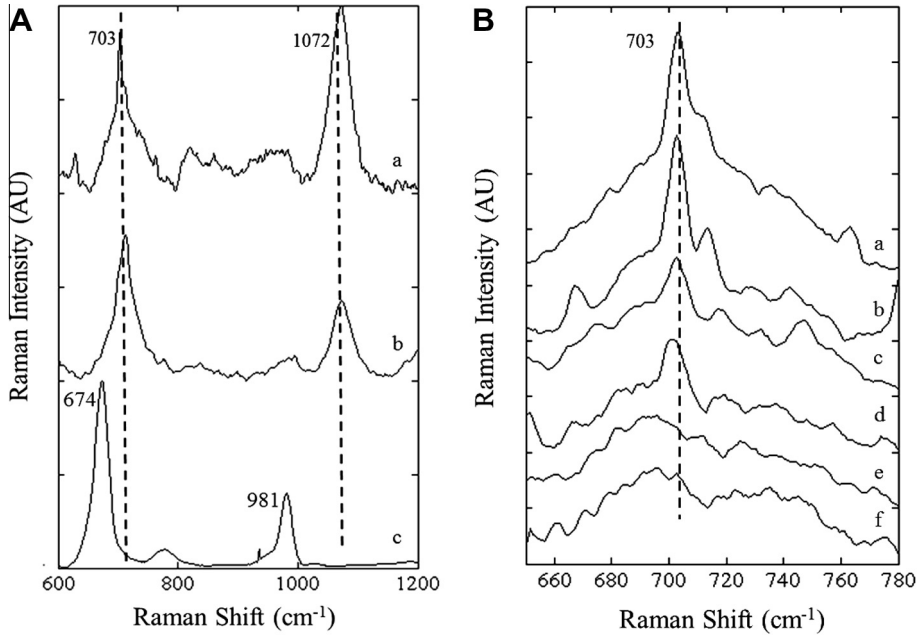

Fig. 3. (A) Representative spectral features of melamine with (a) MIPs–SERS of whole milk sample spiked with 6.3 mg L^{-1} melamine, (b) SERS of standard melamine solution (63 mg L^{-1}), and (c) normal Raman of melamine crystal. (B) Representative MIPs–SERS spectra of whole milk samples spiked with different concentrations of melamine: (a) 6.3 mg L^{-1}, (b) 3.15 mg L^{-1}, (c) 1.26 mg L^{-1}, (d) 0.63 mg L^{-1}, (e) 0.126 mg L^{-1}, and (f) 0 mg L^{-1}.

Traditional methods for extraction and recovery of melamine from milk samples took over an hour (Health Canada, 2008c). Hydrochloric acid and dichloromethane were used to remove proteins and fats, followed by loading the supernatant onto silica SPE column for further separation of melamine from remaining milk residues. Pretreatment of milk samples is not only time-consuming. It involves a large amount of organic solvent, which is not environmental friendly. A previous study applied trichloroacetic acid to extract melamine from milk. After neutralizing pH, the supernatant was percolated through the MISPE column. The time required for sample pretreatment was over 30 min and the flow rate for MISPE was 1 mL min^{-1} (Wang, Fang, Liu, & Chen, 2012). In the current study, the time for sample pretreatment was only 10 min and the flow rate was higher (1.5 mL min^{-1}). The complete recovery of melamine from MISPE procedures validated the feasibility to use methanol for the extraction of melamine and MISPE for sample clean up.

3.3. Determination of melamine in whole milk by SERS

We have selected silver dendrite as a SERS-active substrate for its easy fabrication and reliable SERS enhancement factor ($\sim 10^4$) (Feng et al., 2013). Fig. 3(A) shows SERS spectra collected after depositing 2 μL milk samples eluted from MISPE onto silver dendrite. These average spectra ($N = 8$) show features of melamine determined by normal Raman, SERS, and MIPs–SERS. Each band in the Raman spectrum represents a specific vibrational mode derived from the molecules present, and thus reflect the structural features of the analytes (Lu, Al-Qadiri, Lin, & Rasco, 2011) and interferents. We assign the distinct bands at wavenumbers of 674 cm^{-1} and 981 cm^{-1} in the normal Raman spectrum to ring-breathing mode II, involving the in-plane deformation of the triazine ring and the triazine ring-breathing mode I, respectively (Koglin, Kip, & Meier, 1996). In the SERS and MIPs–SERS spectra, the band that occurs at 674 cm^{-1} in the normal Raman spectrum shifts to 703 cm^{-1}, which can be attributed to a SERS effect (Haynes et al., 2005). When target molecules adsorbed onto the surface of SERS-active substrate, some molecules interact with the noble-metallic nanostructures, resulting in the changes in dipole of the molecules and subsequent shifts in the location of SERS spectral bands. In the MIPs–SERS spectra, the band at 703 cm^{-1} shows a minor blue shift compared to SERS spectrum of melamine in a standard solution. This blue shift may arise owing to the presence of Ca^{2+}, Fe^{2+} and Zn^{2+} in whole milk (Chen & Liu, 2011). We use the band at 1072 cm^{-1}, derived from nitrate residues on silver dendrite, as an internal standard. Although a SERS-active substrate generally acts to significantly increase Raman signal intensity, the band observed at 981 cm^{-1} in the normal Raman spectrum does not appear in SERS and MIPs–SERS spectra because the surface of the silver dendrite orients the polarization tensor of the melamine molecule orthogonal to polarization of the light field. The uniformity of the bands in SERS and MIPs–SERS demonstrate the effectiveness of MIPs for the separation and enrichment of melamine from whole milk.

MIPs–SERS spectra of whole milk spiked with different levels of melamine were collected, and the raw spectra were normalized based upon the band at 1072 cm^{-1} and are shown in Fig. 3(B). The intensity of band at 703 cm^{-1} decreases with the decrease of melamine concentration in whole milk samples. The band intensity for whole milk samples spiked with melamine at the level of 0.001 mmol L^{-1} (equivalent to 0.126 mg L^{-1}) shows no features that can be assigned to melamine.

Fig. 4(A) represents a two-dimensional PCA model that differentiates whole milk samples spiked with different levels of melamine using features at wavenumbers of 650 cm^{-1} to 750 cm^{-1}. Most of the samples were tightly clustered and clearly separated on the basis of different spiked levels of melamine. However, cluster overlap appeared between groups with a melamine spike of 0.001 mmol L^{-1} and samples with no added melamine, indicating that the LOD of our MIPs–SERS biosensor is 0.005 mmol L^{-1} (equivalent to 0.63 mg L^{-1} or 630 ppb). The segregation by PCA model was further confirmed by the development of an HCA model, as shown in Fig. 4(B).

Fig. 5 shows a linear regression model constructed to correlate the concentration of melamine using the intensity of the Raman band at 703 cm^{-1} ($N = 6$). The model shows a good linear relationship for concentration of melamine in whole milk between

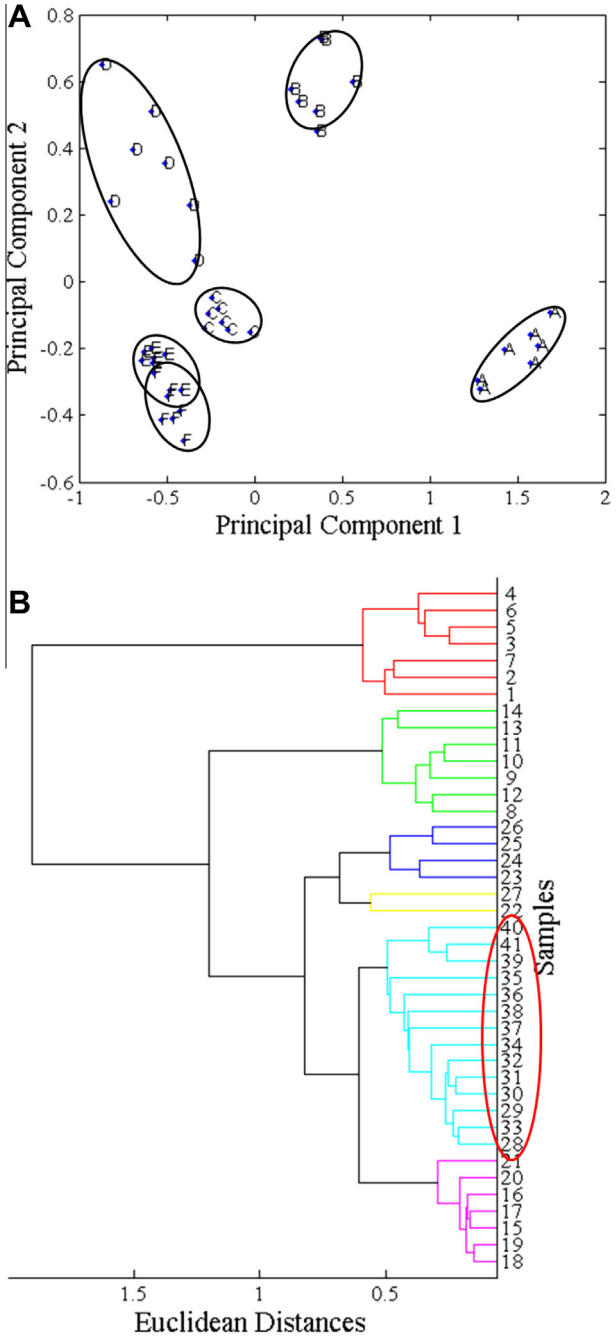

Fig. 4. (A) Representative two-dimensional principal component analysis for whole milk samples spiked with different contents of melamine. Principal component (PC) 1 represents 70.4% variances and PC2 represents 16.3% variances. Letters from A to F denote whole milk samples spiked with melamine at the concentrations of 6.3, 3.15, 1.26, 0.63, 0.126 and 0 mg L^{-1}, respectively ($N = 7$). (B) Representative hierarchical cluster analysis (HCA) for the classification of whole milk spiked with different levels of melamine. Euclidean distance was employed to calculate the distances between samples. Different colors represent samples with distances larger than 0.6. Numbers 1–7, 8–14, 15–21, 22–27, 28–34, 35–41 represent melamine concentrations of 6.30, 3.15, 1.26, 0.63, 0.126, and 0 mg L^{-1}, respectively. Samples from 28 to 41 cannot be grouped into different clusters ($N = 7$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.005 mmol L^{-1} and 0.05 mmol L^{-1}. The coefficient of determination (R^2) of this regression model is 0.93. However, R^2 decreased to 0.89 with the range of melamine content extended from 0.001 to 0.05 mmol L^{-1} (data not shown). We thus conclude that this regression model is applicable to accurately predict the content

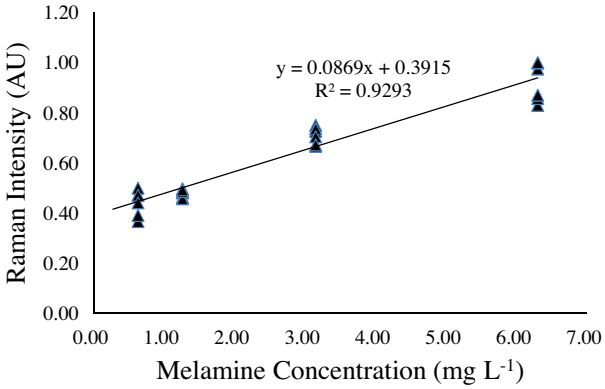

Fig. 5. Linear relationship between the intensity of SERS band at 703 cm^{-1} and the spiked levels of melamine in whole milk samples ($N = 6$).

of melamine in an unknown whole milk sample. Taken together, our MIPs–SERS biosensor can be used for quantification when the concentration of melamine in whole milk sample falls between 0.005 mmol L^{-1} and 0.05 mmol L^{-1}.

The LOD (0.012 mmol L^{-1}) and LOQ (0.039 mmol L^{-1}) calculated by the three times and ten times of standard deviation of the Raman intensity at 703 cm^{-1} of non-spiked samples (Giovannozzi et al., 2014) are higher compared to those calculated by PCA (0.005 mmol L^{-1}) and linear regression models (0.005 mmol L^{-1}). However, because of the dilution during the sample pretreatment and MISPE, the real concentrations of melamine deposited onto the SERS-active substrate (from 0.00017 mmol L^{-1} to 0.0083 mmol L^{-1}) were lower than the spiked amount. The corresponding LOD and LOQ are 0.0021 mmol L^{-1} and 0.0069 mmol L^{-1}, demonstrating high sensitivity of the SERS-active substrate employed in the current study. These results validated that if the procedures for sample pretreatment and MISPE could be optimized, lower LOD and LOQ could be achieved.

HPLC-DAD conducted in the current study required 22 min for the detection of melamine in whole milk, not to mention a long time for sample pretreatment. In comparison, SERS spectral collection significantly shortened the detection time (51 s per spectrum). The overall analysis time for our MIPs–SERS biosensor is about 18 min, including sample pre-treatment. A recent study demonstrated that the LOD and LOQ of melamine in milk was 0.17 mg L^{-1} and 0.57 mg L^{-1} (equivalent to 0.0013 mmol L^{-1} and 0.0045) by using gold nanoparticles as SERS-active substrate, and the total analysis time was 30 min (Giovannozzi et al., 2014). Owing to the efficient separation and enrichment of melamine by the incorporation of MIPs, the LOD and LOQ of this MIPs–SERS biosensor meets the detection requirements for melamine in dairy products and infant formula demanded by Health Canada regulations (2008a, 2008b), which are 2.5 ppm (equivalent to 0.02 mmol L^{-1} in whole milk) and 0.5 ppm (equivalent to 0.0165 mmol L^{-1} when 12.6 g formula redissolved in 3 mL water), respectively.

4. Conclusion

A "two-step" MIPs–SERS biosensor offers a means for efficient separation, enrichment and accurate detection and quantification of melamine in whole milk. Used as a sorbent in SPE, MIPs achieved effective clean-up of whole milk samples rapidly. SERS, applied for rapid and precise detection of melamine, provides LOD and LOQ values of 0.012 mmol L^{-1} and 0.039 mmol L^{-1} melamine in whole milk, respectively. In summary, this innovative biosensor shows great promise for use both in government and food industry laboratories where high-throughput and trace level detection of food chemical hazards is required.

Acknowledgments

The authors thank Huey Kuan and Benny Chan for technical support. This study was supported by funds award to X.L. by Natural Sciences and Engineering Research Council of Canada.

References

Baggiani, C., Anfossi, L., & Giovannoli, C. (2007). Solid phase extraction of food contaminants using molecular imprinted polymers. *Analytica Chimica Acta, 591*(1), 29–39.

Brown, C. A., Jeong, K.-S., Poppenga, R. H., Puschner, B., Miller, D. M., Ellis, A. E., et al. (2007). Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. *Journal of Veterinary Diagnostic Investigation, 19*(5), 525–531. http://dx.doi.org/10.1177/104063870701900510.

Chen, L.-M., & Liu, Y.-N. (2011). Surface-enhanced Raman detection of melamine on silver-nanoparticle-decorated silver/carbon nanospheres: Effect of metal ions. *ACS Applied Materials & Interfaces, 3*(8), 3091–3096.

Cheung, W., Shadi, I. T., Xu, Y., & Goodacre, R. (2010). Quantitative analysis of the banned food dye Sudan-1 using surface enhanced raman scattering with multivariate chemometrics. *The Journal of Physical Chemistry C, 114*(16), 7285–7290. http://dx.doi.org/10.1021/jp908892n.

Choo-Smith, L. P., Edwards, H. G. M., Endtz, H. P., Kros, J. M., Heule, F., Barr, H., et al. (2002). Medical applications of Raman spectroscopy: From proof of principle to clinical implementation. *Biopolymers, 67*(1), 1–9. http://dx.doi.org/10.1002/bip.10064.

Costa, J. C. S., Ando, R. M. A., Camargo, P. H. C., & Corio, P. (2011). Understanding the effect of adsorption geometry over substrate selectivity in the surface-enhanced Raman scattering spectra of simazine and atrazine. *The Journal of Physical Chemistry C, 115*(10), 4184–4190. http://dx.doi.org/10.1021/jp112021j.

Curcio, M., Puoci, F., Cirillo, G., Iemma, F., Spizzirri, U. G., & Picci, N. (2010). Selective determination of melamine in aqueous medium by molecularly imprinted solid phase extraction. *Journal of Agricultural and Food Chemistry, 58*(22), 11883–11887. http://dx.doi.org/10.1021/jf102936p.

Feng, S., Gao, F., Chen, Z., Grant, E., Kitts, D. D., Wang, S., et al. (2013). Determination of α-tocopherol in vegetable oils using a molecularly imprinted polymers–surface-enhanced Raman spectroscopic biosensor. *Journal of Agricultural and Food Chemistry, 61*(44), 10467–10475.

Gao, F., Feng, S., Chen, Z., Li-Chan, E. C. Y., Grant, E., & Lu, X. (2014). Detection and quantification of chloramphenicol in milk and honey using molecularly imprinted polymers-Canadian penny based SERS nano-biosensor. *Journal of Food Science, 79*, N2542–N2549.

Giovannozzi, A. M., Rolle, F., Sega, M., Abete, M. C., Marchis, D., & Rossi, A. M. (2014). Rapid and sensitive detection of melamine in milk with gold nanoparticles by surface enhanced Raman scattering. *Food Chemistry, 159*, 250–256. http://dx.doi.org/10.1016/j.foodchem.2014.03.013.

Haupt, K. (2003). Molecularly imprinted polymers: The next generation. *Analytical Chemistry, 75*(17), 376A–383A. http://dx.doi.org/10.1021/ac031385h.

Haynes, C. L., McFarland, A. D., & Duyne, R. P. V. (2005). Surface-enhanced Raman spectroscopy. *Analytical Chemistry, 77*(17), 338A–346A.

He, L., Deen, B. D., Pagel, A. H., Diez-Gonzalez, F., & Labuza, T. P. (2013). Concentration, detection and discrimination of *Bacillus anthracis* spores in orange juice using aptamer based surface enhanced Raman spectroscopy. *Analyst, 138*(6), 1657–1659.

He, L., Lamont, E., Veeregowda, B., Sreevatsan, S., Haynes, C. L., Diez-Gonzalez, F., et al. (2011a). Aptamer-based surface-enhanced Raman scattering detection of ricin in liquid foods. *Chemical Science, 2*(8), 1579–1582.

He, L., Rodda, T., Haynes, C. L., Deschaines, T., Strother, T., Diez-Gonzalez, F., et al. (2011b). Detection of a foreign protein in milk using surface-enhanced Raman spectroscopy coupled with antibody-modified silver dendrites. *Analytical Chemistry, 83*(5), 1510–1513.

Hennion, M.-C. (1999). Solid-phase extraction: Method development, sorbents, and coupling with liquid chromatography. *Journal of Chromatography A, 856*(1), 3–54.

Koglin, E., Kip, B. J., & Meier, R. J. (1996). Adsorption and displacement of melamine at the Ag/electrolyte interface probed by surface-enhanced Raman microprobe spectroscopy. *The Journal of Physical Chemistry, 100*(12), 5078–5089. http://dx.doi.org/10.1021/jp953208t.

Li-Chan, E. C. Y. (1996). The applications of Raman spectroscopy in food science. *Trends in Food Science & Technology, 7*(11), 361–370. http://dx.doi.org/10.1016/S0924-2244(96)10037-6.

Li, J., Qi, H.-Y., & Shi, Y.-P. (2009). Determination of melamine residues in milk products by zirconia hollow fiber sorptive microextraction and gas chromatography–mass spectrometry. *Journal of Chromatography A, 1216*(29), 5467–5471. http://dx.doi.org/10.1016/j.chroma.2009.05.047.

Liu, Y., Todd, E. E., Zhang, Q., Shi, J. R., & Liu, X. J. (2012). Recent developments in the detection of melamine. *Journal of Zhejiang University Science B, 13*(7), 525–532. http://dx.doi.org/10.1631/jzus.B1100389.

Lu, X., Al-Qadiri, H., Lin, M., & Rasco, B. (2011). Application of mid-infrared and Raman spectroscopy to the study of bacteria. *Food and Bioprocess Technology, 4*(6), 919–935. http://dx.doi.org/10.1007/s11947-011-0516-8.

Lu, X., Huang, Q., Miller, W. G., Aston, D. E., Xu, J., Xue, F., et al. (2012). Comprehensive detection and discrimination of campylobacter species by use of confocal micro-Raman spectroscopy and multilocus sequence typing. *Journal of Clinical Microbiology, 50*(9), 2932–2946. http://dx.doi.org/10.1128/jcm.01144-12.

Nie, S., & Emory, S. R. (1997). Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. *Science, 275*(5303), 1102–1106. http://dx.doi.org/10.1126/science.275.5303.1102.

Pichon, V. (2007). Selective sample treatment using molecularly imprinted polymers. *Journal of Chromatography A, 1152*(1), 41–53.

Pietrzyk, A., Kutner, W., Chitta, R., Zandler, M. E., D'Souza, F., Sannicolo, F., et al. (2009). Melamine acoustic chemosensor based on molecularly imprinted polymer film. *Analytical Chemistry, 81*(24), 10061–10070.

Qian, K., Fang, G., He, J., Pan, M., & Wang, S. (2010). Preparation and application of a molecularly imprinted polymer for the determination of trace metolcarb in food matrices by high performance liquid chromatography. *Journal of Separation Science, 33*(14), 2079–2085. http://dx.doi.org/10.1002/jssc.200900877.

Tran, B. N., Okoniewski, R., Storm, R., Jansing, R., & Aldous, K. M. (2009). Use of methanol for the efficient extraction and analysis of melamine and cyanuric acid residues in dairy products and pet foods. *Journal of Agricultural and Food Chemistry, 58*(1), 101–107.

Wang, S., Liu, L., Fang, G., Zhang, C., & He, J. (2009). Molecularly imprinted polymer for the determination of trace ractopamine in pork using SPE followed by HPLC with fluorescence detection. *Journal of Separation Science, 32*(9), 1333–1339.

Wang, X., Fang, Q., Liu, S., & Chen, L. (2012). The application of pseudo template molecularly imprinted polymer to the solid-phase extraction of cyromazine and its metabolic melamine from egg and milk. *Journal of Separation Science, 35*(12), 1432–1438.

Williams, A. C., & Edwards, H. G. M. (1994). Fourier transform Raman spectroscopy of bacterial cell walls. *Journal of Raman Spectroscopy, 25*(7–8), 673–677. http://dx.doi.org/10.1002/jrs.1250250730.

Wu, Y.-N., Zhao, Y.-F., & Li, J.-G. (2009). A survey on occurrence of melamine and its analogues in tainted infant formula in China. *Biomedical and Environmental Sciences, 22*(2), 95–99. http://dx.doi.org/10.1016/S0895-3988(09)60028-3.

Yang, H.-H., Zhou, W.-H., Guo, X.-C., Chen, F.-R., Zhao, H.-Q., Lin, L.-M., et al. (2009). Molecularly imprinted polymer as SPE sorbent for selective extraction of melamine in dairy products. *Talanta, 80*(2), 821–825.

Ye, L., & Haupt, K. (2004). Molecularly imprinted polymers as antibody and receptor mimics for assays, sensors and drug discovery. *Analytical and Bioanalytical Chemistry, 378*(8), 1887–1897. http://dx.doi.org/10.1007/s00216-003-2450-8.

Zhang, Z., Cheng, Z., Zhang, C., Wang, H., & Li, J. (2012). Precipitation polymerization of molecularly imprinted polymers for recognition of melamine molecule. *Journal of Applied Polymer Science, 123*(2), 962–967.