Integrated photografted molecularly imprinted polymers with a cellulose acetate membrane for the extraction of melamine from dry milk before HPLC analysis

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In this study, a new separation technique based on membrane extraction is described for the determination of melamine in dry milk. The water-compatible cellulose acetate membrane, which is photografted by melamine imprinted nanospheres, was prepared by placing the membrane into the polymerization solution containing methacrylic acid as a functional monomer, ethylene glycol dimethacrylate as cross-linker, acetonitrile as porogen, and melamine as the template molecule. The characterization of the polymeric membrane was performed by Fourier transmission infrared spectroscopy and scanning electron microscopy. This integrated composite membrane was used as a solid-phase extraction medium for the extraction of melamine from dry milk samples. Various parameters affecting the extraction efficiency of the membrane were evaluated. The results showed higher binding capacity for melamine imprinted membranes in comparison with the nonimprinted membranes. High-performance liquid chromatography analysis showed that the extraction of melamine from dry milk by the photografted cellulose acetate membrane had a linear calibration curve in the range of 0.02–11.80 μg/mL with an excellent precision of 2.73%. The limit of detection and quantification of melamine was 0.007 and 0.020 μg/mL, respectively. The recoveries of melamine were in the range of 88.7–94.8%.

KEYWORDS
cellulose acetate, dry milk, imprinted polymers, melamine, membrane solid-phase extraction

1 | INTRODUCTION

Nowadays, developing new extraction and analytical methods for emerging concerns in food safety as well as accreditation and validation of analytical results are mandatory for national and reference food control laboratories. Establishment of this system is crucial for acceptance of test results and very beneficial in supporting food health in societies.

However, establishing this system requires simple, fast, efficient, and reliable sample preparation procedures for analysis of desired compounds in food samples [1]. Melamine (C₃H₆N₆; Fig. 1) is a potentially hazardous compound, which is illegally added into the milk-based foodstuffs to increase its apparent protein content. There are several health concerns about consuming melamine containing foodstuffs. Besides its nephrotoxicity, the illegal addition of melamine especially into the infant milk formula caused illness and even deaths of humankind, primarily as a result of the accumulation of melamine–uric acid crystals in the urinary tract [2]. According to the World Health Organization criteria, the tolerable daily intake value for melamine

Abbreviations: AIBN, 2,2′-azobis isobutyronitrile; CA, cellulose acetate; EGDMA, ethylene glycol dimethacrylate; MAA, methacrylic acid; MIP, molecularly imprinted polymer; NIP, nonimprinted polymer

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has been set at 0.2 mg/kg of body mass [3]. So there is an urgent need for developing efficient and reliable analytical procedures for extraction and determination of melamine in dry milk and any other foodstuffs, which are formulated by dry milk. Several extraction, enrichment, and clean-up methods have been reported for improving melamine detection in different matrices using solvent extraction by polar solvents as water, acetonitrile, and diethylamine [4]; SPE by commercial cartridges [5,6]; or treatment with acids, buffers, or reagents [7,8]. But many of these methods have been hindered by matrix and memory effects, the solubility of melamine–cyanuric acid complex, and background contamination that reduce the selectivity and lead coelution of interferences. Generally, the complexity of sample matrix and very little amount of analyte are two main drawbacks in the analysis of melamine in dry milk, which contain considerable amount of protein, carbohydrate, fat, and minerals. There are several reports with a relatively new improvement in selectivity for extraction of melamine from milk by using molecularly imprinted polymers (MIPs) [9,10]. MIPs are synthetic polymers with specific recognition sites for target analytes. They are synthesized by cross-linking complexes of template molecules and functional monomers. After removal of the template molecules, binding sites are exposed that are complementary to the template in size, shape, and position of functional groups. Due to their excellent molecular recognition capability and chemical and mechanical stability, MIPs are more and more investigated for extraction and
preparation of photografted membrane

2.1 | Chemicals and reagents

All standards and samples were prepared with 18 MΩ cm deionized water (Millipore, Le montsur-Lausanne, Switzerland). All solvents used in chromatography were of HPLC grade and obtained from Merck (Darmstadt, Germany). Melamine reagent (99.0%) was acquired from Sigma–Aldrich (Louis, USA). The melamine stock solution was prepared in methanol at concentration of 1000 µg/mL and stored at 4°C until analysis. Working standard solutions in different concentrations (20–12 000 µg/mL) were obtained by diluting appropriate amount of stock solution with the chromatographic mobile phase. Hydrophilic CA membranes with low protein binding capacity and 50 mm id and 0.45 µm pore size were obtained from Macherey-Nagel (Düren, Germany). Ethylene glycol dimethacrylate (EGDMA) and 2,2’-azobis isobutyronitrile (AIBN) from Sigma–Aldrich (Steinheim, Germany) were of reagent grade and used without any further purification. Methacrylic acid (MAA) from Merck (Darmstadt, Germany) was distilled in a vacuum before use to remove the stabilizers.

2.2 | Apparatus

A DIONEX HPLC instrument was used for chromatographic analysis of melamine. This chromatographic system was composed of a multisolvent gradient pump, a UVD 170U detector, and an on-line degasser. A Rheodyne model 7725i injector with a 20 µL loop was used to inject the extracted samples. Chromatographic separations were achieved on a Nucleosil-NH2, 4.6 × 250 mm, 5 µm analytical column. For the mobile phase, a degassed mixture of acetonitrile/phosphate buffer (0.01 M) (80:20) was prepared and delivered in isocratic mode at a flow rate of 0.8 mL/min. All of the analyses were carried out at 220 nm and HPLC data were acquired and processed using a PC and Chromeleon Ver. 6.60 chromatography manager software. In all solutions, the pH was adjusted by digital Metrohm pH meter (model 744) equipped with a combined glass–calomel electrode. The characterization of the CA–MIP membrane was performed by transmission FTIR spectroscopy and SEM. FTIR spectra of optimized unleached and leached CA–MIP were recorded on a Shimadzu FTIR 4300 spectrometer (186; Shimadzu, Kyoto, Japan) using KBr pellets in the range of 400–4000 cm⁻¹. SEM (Philips XL30 scanning microscope, Philips, Netherlands) was employed to determine the shape and surface morphology of the photografted polymer particles in the CA network. The membrane was sputter coated with gold before the SEM measurement.

2.3 | Preparation of photografted membrane

MIPs were grafted into the circular hydrophilic CA membrane by placing the membrane into the 20 mL acetonitrile/water (80:20, v/v) polymerization solution containing 31.53 mg (0.25 mmol) melamine as template, 0.129 mL (1.5 mmol) MAA as functional monomer, and 2.6 mL (14.0 mmol) EGDMA as cross-linker. Then, the mixture was degassed in ultrasonic bath (Model 55743-Fritsch, Germany) for 10 min. After sonication, it was purged with N₂ for 5 min and the vessel was sealed under this atmosphere. Then, the reaction initiator AIBN (120 mg, 0.82 mmol) was added. After thoroughly sink of the CA membrane in the above solution, it was taken from the mixture and placed between two glass plates and compressed to remove any trapped gas bubbles. A UV cabinet (CAMAG REPROSTAR 3, Switzerland) equipped with 300 W UV lamp, which provides illumination of 254 nm UV light, was employed to illuminate the membrane for 6 h at about 25°C. After soaking CA membrane in the polymerization solution, MAA polymerization starts at some potentially active sites by creating free radicals on the CA network in the presence of AIBN and UV irradiation. By this, self-assembly complexes, which were already loaded into the pores of the CA membrane, create photografted nanospheres of MIPs in the CA membrane network. After the glass plates were separated, to extract the nongrafted polymers, the excess of
template and polymerization constituents from the membrane networks and creating a porous surface, the unleached MIPs were washed three times with methanol containing 10% acetic acid v/v, until no template could be detected in the washing solvent by spectrometric measurement (at \( \lambda = 220 \text{ nm} \)) (Scinco S-3100, Korea). The membrane finally washed with the same volume of deionized water and acetone, and the resulted leached membrane was dried in an oven (Memmert INB 400, Germany) at 50°C overnight. The CA–NIP (where NIP is nonimprinted polymer) membranes were prepared in the same way, but without the addition of the imprint molecule melamine and worked-up by the same procedure.

2.4 Sample preparation

Exactly 1.0 g of thoroughly homogenized dry milk without melamine addition was transferred to a 10 mL volumetric flask and 5.0 mL aqueous trichloroacetic acid and 5.0 mL acetonitrile successively were added and solution was mixed for 5 min and ultrasonicated for 10 min at 25°C. This led to precipitate proteins and fat portion [8]. The mixture was centrifuged in 4°C at 10 000 rpm for 5 min, and the supernatant was collected and spiked with melamine. Spiked samples were prepared by the addition of 2, 4, and 8 \( \mu \text{L} \) from stock standard solution into centrifuge test tube containing 5.0 mL supernatant solution and then shaken for 10 min. All of the prepared blank and spiked samples were loaded on CA–MIP and CA–NIP membranes after pH adjustment to 6.5 with phosphate buffer. For this, a lab-made membrane holder (stainless-steel frit, id = 50 mm) was considered in a scaled barrel syringe and the CA–MIP/CA–NIP membranes were placed into the holder (Fig. 1). Exactly 5.0 mL of melamine solutions was loaded on the membranes, and then 20 \( \mu \text{L} \) of the eluted solutions were injected into the HPLC system.

3 RESULTS AND DISCUSSION

3.1 Characterization studies

3.1.1 Morphology of the photografted imprinted polymers in membrane network

Figure 2A–C shows the SEM images of blank CA membrane, CA–MIP membrane, and CA–NIP membrane, respectively. The uniformly sized nanospheres of MIPs were photografted into the CA membrane network when the membrane was soaked into the acetonitrile/water (80:20 v/v) solution. This resulted in the formation of some self-assembly complexes from MAA monomers, which were loaded into the pores of the CA membrane. High dilution conditions of monomer, appropriate amount of template/functional monomer/crosslinker (molar ration of 1:6:56), and gentle mixing during the polymerization made such a smooth and uniform distribution of grafted nanospheres. After polymerization and removal of the template molecules, numerous imprinted polymeric nanospheres attached to the CA membrane network was achieved. However, it was observed that the modified membrane was smooth and flexible, and there was no obvious difference in micrograph backbone between blank CA, CA–MIP, and CA–NIP membrane. But, obvious differences can be observed in the SEM images (Fig. 2). Blank CA membrane has a regular fibrous structure (Fig. 2A), while a significant change was observed in its morphology after it interacted and grafted by MIPs. Very tiny white spots in the SEM image (Fig. 2B) showed that grafting MIP into the CA membrane was really reliable. There were no substantial differences in the morphology of imprinted polymer and NIP. However, the size of the NIP-grafted particles was larger than the MIP particles, suggesting that the melamine had a profound influence on particle nucleation and growth during the precipitation
polymerization. This effect may not be surprising given that the functional monomer MAA existed in different forms in the two reaction systems [23]. In the absence of melamine, MAA can form hydrogen-bonded dimers in the nonimprinted system. The prepolymerization solution contains both free MAA and MAA dimers. In the imprinted system, there is an additional molecular interaction between MAA and melamine, which might somehow affect the growth of the cross-linked polymer nuclei, so that it results in smaller polymer particles, which were grafted into the CA membrane network. These particles preferred over irregularly shaped particles for most applications and in particular for those involving HPLC and SPE. However, more increase in the size of the grafted particles in CA–NIP membrane refers to the swelling of the polymer particles in the acetonitrile media before the morphologic analysis by SEM.

3.1.2 IR spectra

The IR absorption spectra of the imprinted poly(MAA-co-EGDMA) photografted in the CA membrane are shown in Supporting Information Fig. S1. The IR spectra of leached CA–MIP and unleached CA–MIP membrane displayed similar characteristic peaks, indicating the similarity in the backbone structure of the different polymers. The O–H stretching and bending and the C=O stretching vibrations in the leached CA–MIP are seen at 3408, 1422, and 1712 cm⁻¹, whereas these peaks in the unleached CA–MIP are seen at 3440, 1454, and 1729 cm⁻¹, respectively. This displacement toward lower frequencies is due to the hydrogen bonding of the O–H and the C=O groups from MAA with amine groups in melamine in the unleached CA–MIP. Other absorption peak that is observed in both leached and unleached membranes is a relative wide band at 2950 cm⁻¹ for stretching of aliphatic C–H bond and one sharp band with low relative intensity at 1130 cm⁻¹. There was also other difference between IR spectra of the leached and unleached CA–MIPs in stretching vibration of residual vinylic C=C bonds. In the unleached polymer, there was one sharp band with low relative intensity at 1630 cm⁻¹ that appeared at 1619 cm⁻¹ in the corresponding leached MIP. Other absorption peaks match peaks of MIP and NIP: 988 cm⁻¹ (out-of-plane bending vibration of vinylic C–H bond in CA), which was shifted to the 967 cm⁻¹ in the leached membrane. The status of other peaks in each of the two types of membranes is similar.

3.2 Optimization of formulation in the photografted membrane

There are several variables, such as amount of monomer or nature of cross-linker and solvent, that affect the final mechanical and adsorption characteristics of the obtained photografted CA–MIP membrane in terms of affinity, capacity, and selectivity for the melamine. Primary experiments revealed that the imprinted polymers prepared in 80:20 acetonitrile/water show better molecular recognition ability in aqueous extraction media than MIPs prepared in chloroform and obtained well-controlled physical forms for the CA membrane and its photografted MIPs nanospheres. As can be seen in Fig. 1, the structure of the melamine owns basic amino groups that make it an ideal compound to interact with MAA monomers in the polar solvent. Using these monomers resulted in higher recognition ability to target molecule with stronger electrostatic and hydrophobic interactions with the template in the polar solvent. Thus, in acetonitrile different formulations for the obtaining of photografted MIPs with improved molecular recognition capabilities in CA network have been used (Table 1). Proper molar ratios of functional monomer to template are very important to enhance specific affinity and number of MIPs recognition sites in the membrane. High ratios of functional monomer to template result in high nonspecific affinity, while low ratios produce fewer complexations due to insufficient functional groups [24]. Five molar ratios of the monomer to template of 2, 3, 4, 5, and 6 were used in the experiments. The optimum ratio of functional monomer to template for the specific rebinding of melamine was 6:1, which had the best specific affinity and the highest recovery of 93.69% while that of the corresponding NIPs was low at 23.01%. The specific adsorption recovery obtained from difference between recoveries for CA–MIP and corresponding CA–NIP of melamine at 6:1 was 70.68% while those at 2:1, 3:1, 4:1, and 5:1 were 36.95, 51.47, 53.11, and 57.35%, respectively. With increasing the ratio of functional monomer with respect to the melamine yielded higher specific affinity due to increase in functional groups. Therefore, the typical 1:6:56 template/monomer/cross-linker molar ratio was used for further studies (MIP5; Table 1).

3.3 Effect of pH and optimization of adsorption capacity

The pH of the melamine solution in loading phase and its adsorption into the membrane is one of the most important variables affecting retention behavior and selectivity of the CA–MIP WAS membrane as a consequence of hydrophobic interactions. The effect of pH on the rebinding efficiency of melamine was investigated by varying the solution pH from 2.0 to 10.0, as shown in Fig. 3A. Batch experiments were performed as triplicate by loading 5.0 mL of extracted dry milk sample containing 0.05 μg/mL of melamine on CA–MIP/NIP membranes under the desired range of pH. It was observed in Fig. 3A that melamine underwent complete rebinding/elution at pH 6.5. The lower responses observed at lower and higher pHs may be due to the protonation of the amine groups of melamine and deprotonation of carboxyl groups of the photografted polymers, respectively. The capacity of the membrane for adsorption of analyte is a crucial factor that
TABLE 1 Compositions and comparisons of the extraction of melamine from melamine standard solution (5 mL, 0.05 μg/mL) using integrated CA–MIP/CA–NIP membranes at pH 6.5, elute: 2.0 mL methanol/acetic acid (9:1, v/v)

<table>
<thead>
<tr>
<th>MIP/NIP</th>
<th>MAA (mmol)</th>
<th>Melamine (mmol)</th>
<th>EGDMA (mmol)</th>
<th>AIBN (mmol)</th>
<th>Extraction (%) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP1</td>
<td>0.50</td>
<td>0.25</td>
<td>14.00</td>
<td>0.82</td>
<td>45.81 (±3.05)</td>
</tr>
<tr>
<td>MIP2</td>
<td>0.75</td>
<td>0.25</td>
<td>14.00</td>
<td>0.82</td>
<td>58.90 (±2.85)</td>
</tr>
<tr>
<td>MIP3</td>
<td>1.00</td>
<td>0.25</td>
<td>14.00</td>
<td>0.82</td>
<td>60.63 (±2.90)</td>
</tr>
<tr>
<td>MIP4</td>
<td>1.25</td>
<td>0.25</td>
<td>14.00</td>
<td>0.82</td>
<td>80.32 (±2.20)</td>
</tr>
<tr>
<td>MIP5</td>
<td>1.50</td>
<td>0.25</td>
<td>14.00</td>
<td>0.82</td>
<td>93.69 (±2.02)</td>
</tr>
<tr>
<td>NIP1</td>
<td>0.50</td>
<td>–</td>
<td>14.00</td>
<td>0.82</td>
<td>8.86 (±1.72)</td>
</tr>
<tr>
<td>NIP2</td>
<td>0.75</td>
<td>–</td>
<td>14.00</td>
<td>0.82</td>
<td>7.43 (±2.05)</td>
</tr>
<tr>
<td>NIP3</td>
<td>1.00</td>
<td>–</td>
<td>14.00</td>
<td>0.82</td>
<td>7.52 (±2.32)</td>
</tr>
<tr>
<td>NIP4</td>
<td>1.25</td>
<td>–</td>
<td>14.00</td>
<td>0.82</td>
<td>22.97 (±3.33)</td>
</tr>
<tr>
<td>NIP5</td>
<td>1.50</td>
<td>–</td>
<td>14.00</td>
<td>0.82</td>
<td>23.01 (±2.13)</td>
</tr>
</tbody>
</table>

aAverage of three determinations.

FIGURE 3 pH effect (A) and optimization of adsorption capacity (B) for CA–MIP and CA–NIP membranes in batch experiments: sample volume: 5.0 mL; melamine concentration: 0.05 μg/mL at pH 6.5

determines how much of the target analyte can be extracted before breakthrough occurs. This is a general indication for the presence of cavities and porosities in MIP-based sorbents. This evaluation was performed through measuring the membrane capacity to adsorb melamine from aqueous solution during extraction with the membrane. For this, 5.0 mL of melamine solution at the concentration of 50–600 μg/mL was loaded on the CA–MIP membrane (75 mg weight) in optimum extraction condition. Once the system has attained equilibrium, the remained melamine in the filtrate was determined by HPLC–UV to evaluate the amount of adsorbed template. In this case, the isothermal adsorptions were plotted in Fig. 3B. According to these results, the maximum amount of melamine that can be adsorbed by CA–MIP membrane was found to be 279 mg/g (2212 μmol/g) at pH 6.5. This value is more than previous data reported for surface-coated MIPs on CA membrane rather than photografting a numerous MIPs nanospheres into the CA network in a dilute solvent in this study [25]. For higher melamine amounts, a slight increase in retained melamine was observed on CA–MIP capacity curve. Moreover, to evaluate the binding properties of the CA–MIPs, the imprinting factor (IF) was used by equation (IF = Q_{CA–MIP}/Q_{CA–NIP}), where Q_{CA–MIP} and Q_{CA–NIP} are equilibrium capacity of CA–MIP and CA–NIP for melamine, respectively. An IF of 6.2 was achieved, which is among the best values reported for membrane imprinting.

3.4 Choice of loading, washing, and eluent solution

Actually, both the CA and its photografted membranes have binding ability with specific and nonspecific interactions. The specific interactions may be mainly due to the imprinted polymers, which produced selective recognition sites in the CA membrane. The nonspecific interactions were evaluated by measuring the binding of the nonimprinted photografted CA membrane. Due to the hydrophilic property of the unmodified CA membranes, efficient rebinding is possible with modified
CA-imprinted membranes in aqueous solutions. At first, the CA–MIP membranes were conditioned with 1.0 mL methanol followed by 1.0 mL of deionized water and 1.0 mL phosphate buffer (0.02 M, pH 3.0) at a flow rate of 1.0 mL/min. Aqueous medium was employed for the loading of melamine similar to the extracted solutions prepared from dry milk at the same flow rate. Then, the washing procedure was evaluated by obtaining maximum recovery of the analyte using several solvents and binary mixtures including acetonitrile, methanol, deionized water, acetone, DMF, tetrahydrofuran, acetonitrile/methanol, acetonitrile/acetone, methanol/acetone, dichloromethane/acetone, dichloromethane/acetonitrile, and methanol/water. To investigate the efficiency of washing step, 5.0 mL of 0.05 μg/mL of melamine solution (pH 6.5) was loaded on the CA–MIP/CA–NIP membranes individually, followed by desorption with the washing solvent. The results showed that washing the photografted CA membrane with 2 mL of tetrahydrofuran and dimethyl formamide had no significant effect on the retention of melamine on both MIP- and NIP-modified CA membranes. In contrast, polar solvents, such as deionized water, acetonitrile, methanol, and acetone, had noticeable effect on the retention of melamine on both CA–MIP and CA–NIP membranes. It was found that with methanol/acetone (1:1 v/v), the recovery of melamine in CA–NIP membrane was decreased to 12%, while the recovery of melamine by CA–MIP membrane was not reduced (93%). An extra wash step was necessary for dry milk samples. In this case, by using 1.0 mL hydrochloric acid (0.01 M), 1 mL methanol/acetone (1:1 v/v), and following 1.0 mL dichloromethane, the recovery of melamine in CA–NIP membrane was decreased to 9% while its recovery by CA–MIP membrane was not reduced noticeably (92%). In all cases, a wash step with hydrochloric acid is necessary for the removal of metal ions, which were retained due to minerals present in dry milk. To avoid contaminating the membranes in the extraction process and to recovery of strongly bonded melamine, the membranes were eluted with 5.0 mL of methanol/acetic acid (9:1 v/v) after each extraction. Moreover, increasing of the sample volume up to 100 mL only had a minor effect on the extraction of melamine.

3.5 | Melamine assay and validation in dry milk

To demonstrate the potential of photografted CA–MIP membrane for the selective separation of analyte, the CA–MIP/CA–NIP membranes were applied to the extraction of melamine in spiked dry milk samples, which were pretreated before loading on the membranes. The results for the preclean-up method display that the procedure can elute interferences from complex dry milk matrices and avoid contaminating HPLC column using methanol/acetic acid (9:1 v/v). The chromatograms obtained for eluted solutions of CA–MIP/CA–NIP membranes spiked with 5.0 μg/mL solution of melamine are depicted in Fig. 1. For comparison, a HPLC chromatogram obtained for blank sample is also shown in the figure. Notably, the method permitted obviously clean extracts in the retention area for melamine and interfering peaks arising from the complex dry milk matrices to be suppressed. The response of the 5.0 μg/mL standard solution for melamine was also recorded after it was prepared in the presence of matrix components. The recorded response was not different from that obtained directly in the calibration by the standard solution. This indicates that determination of melamine by this protocol is free from any interferences due to matrix components. Results from the HPLC analyses and correlation between the melamine concentration and detector response as mAU-min showed that the calibration curve of melamine for dry milk are linear in the range of 0.02–11.80 μg/mL ($r^2 > 0.9997$). The recovery percentages of the melamine with the MIP and NIP are shown in Supporting Information Table S1. The results showed that the recoveries for dry milk samples were between 88.7 and 94.8%. Typical chromatograms presented in Fig. 1 reveal that the CA–MIP membranes have a good potential for cleanup and when it was used, a broad peak at retention of about 10 min was thoroughly omitted. The LOD and LOQ for melamine in dry milk samples were 0.007 and 0.02 μg/mL. The reproducibility and repeatability of the method were evaluated from run-to-run CA–MIP experiments (5.0 μg/mL melamine standard solution, $n = 6$), and different batch experiments (ten batches) and RSDs of 2.73 and 5.44% for the extraction run of melamine were obtained, respectively.

4 | CONCLUDING REMARKS

In this work, molecular imprinting was applied to create a large number of selective recognition sites for melamine in hydrophilic CA membranes with low protein binding capacity. The MIPs as tailor-made polymer materials, which were photografted into the CA network, are practically applied in the separation of melamine from dry milk samples. The high affinity of MIP modified CA membrane to melamine, and its water compatibility in aqueous extraction together with its long lifetime in extractions provides a suitable basis for the development of applications of CA–MIP membranes in the separation and detection of melamine in dry milk.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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