Synthesis of a Molecularly Imprinted Polymer for Melamine Analysis in Milk by HPLC-DAD

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Abbreviations:
AIBN - azobisisobutyronitrile; CL - cross-linker; EDMA - ethylene glycol dimethacrylate; K_d - dissociation constant; MAA - methacrylic acid; MIDE - molecularly imprinted dispersive extraction; MIP - molecularly imprinted polymer; NIP - non-imprinted polymer; PVA - polyvinyl alcohol; Q_max - maximum binding concentration; R - recovery; T - template.

Keywords:
Molecularly imprinted polymer (MIP), matrix effect, molecularly imprinted dispersive extraction coupled with high-performance liquid chromatography.
Abstract

A molecularly imprinted polymer for melamine was prepared by suspension polymerization, using melamine as template, methacrylic acid as functional monomer and ethylene glycol dimethacrylate as cross-linker.

The optimal template/monomer and monomer/cross-linker ratios were obtained, being defined as 1/15 and 1/12, respectively. The template removal was performed by Soxhlet extraction with methanol containing 10% of acetic acid, in two cycles of 24 h each. Molecular recognition properties and binding capacity towards melamine were evaluated by adsorption tests. Compared with the non-imprinted polymer (NIP), the imprinted polymer (MIP) showed higher affinity for melamine with a dissociation constant ($K_d$) and a maximum binding concentration of $568.4 \pm 7.4 \ \mu\text{mol/L}$ and $96.8 \pm 2.3 \ \mu\text{mol/g}$, respectively.

Under optimal conditions the imprinted polymer was used as a dispersive extraction sorbent to extract melamine from different milk samples, before HPLC-DAD analysis. The validated method presented a limit of detection of 0.6 mg/kg. From the analysis of six different milks, melamine was detected in two of the considered samples in a maximum concentration of $9.3 \pm 0.3 \ \text{mg/kg}$. 
1. Introduction

The 1,3,5-triazine-2,4,6-triamine, also known as melamine, is a polar and hydrophilic compound with high content in nitrogen, mainly used in plastic industry [1]. It has been detected in milk [2], as result of a fraudulent addition, with the purpose of increasing the apparent protein level [3]. Melamine, when combined with cyanuric acid (hydrolysis product) precipitates, leading to the formation of kidney stones, which can be lethal [4]. Therefore, efforts to detect melamine in various food products, particularly milk and dairy products, resulted in the development of several analytical methods in the last few years [5].

Literature describes solid phase extraction (SPE) as typical procedure for melamine extraction in food matrices [5]. However this technique may present lower selectivity towards specific compounds. In order to achieve higher levels of selectivity, new sorbents among which molecularly imprinted polymers, have been developed. Those may provide more specificity, rapidity and less laborious extraction techniques, allowing the application to a wide variety of food matrices, and also large monitoring schemes to assess the human exposure [6].

Molecular imprinting technique aims to produce materials with specific cavities that are able to recognize and retain the target analyte. Regarding MIP synthesis, template and functional groups of a monomer can interact through covalent [7] or non-covalent bonds [8]. Then, in the presence of a cross-linker agent, the complex is polymerized by thermal or photo-initiation. The template is afterwards removed from the polymer matrix, by extensive washing steps, resulting in the formation of cavities that are complementary to the template in terms of size and shape, allowing an optimal configuration for the analyte rebinding [9, 10].
Concerning the final application of the synthesized polymers, these are mostly applied in SPE procedures (MISPE). The high selectivity and affinity for the analyte have made MISPE one of the most important extraction techniques for the analysis of biological, pharmaceutical and environmental samples [11].

Recently, MIPs have been successfully applied in a matrix solid-phase dispersion extraction (MSPD), which is especially advantageous for solid matrices. In fact, the solvent extraction and clean-up steps take place at the same time, reducing significantly the time of the analysis. MIP-MSPD for selective determination of β-estradiol on goat milk [12], fluoroquinolones in eggs and tissue [13], chloramphenicol from soils [14] and fish [15], olaquindox in chicken [16], auxins in orange samples [17] and triazines in soil, fruit and vegetables [18] have been reported.

MIPs have also been reported in the analysis of melamine in milk samples, mainly by the MISPE methodology [19-22]. Table 1 resumes the polymerization conditions considered in the synthesis of melamine selective polymers. Bulk polymerisation [21-23] stands out as the most common procedure, however this methodology leads to irregular particles in size and shape as a consequence of the mechanical procedures applied to the resulting polymer [10]. In order to overcome these drawbacks some authors followed the suspension protocol which allows the synthesis of homogeneous beads with controlled size [19, 20]. In addition, methacrylic acid and ethylene glycol dimethacrylate were selected as functional monomer and cross-linker respectively and concerning to the imprinting molecule, melamine and cyromazine were the employed templates.

In 2012 a new MIP-MSPD for melamine extraction in bovine milk has been proposed. Yan et al [23] followed the bulk polymerisation protocol using cyromazine as template to prepare the MIPs.
This work intends to be an alternative to the drawbacks of bulk technique purposing a methodology to produce controlled and homogeneous molecular imprinted particles by suspension polymerisation. The analysis of the extent of the interaction between template and monomer, through the evaluation of imprinted template in different formulations is a contribution to the empirical optimization of MIPs.

Moreover, the synthesized MIPs were used as a new selective sorbent for a matrix solid-phase extraction of melamine from milk samples, prior to HPLC-DAD analysis.

This method intends to be low-cost, but fit-for-purpose, in order to enable the future screening of a large number of milk and related products, to evaluate the extent of contamination.

2. Materials and methods

2.1. Materials

Melamine, methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), azobisisobutyronitrile (AIBN), sodium 1-heptanesulfonate, polyvinyl alcohol (PVA) (87-89% hydrolysed with average molecular weight 130 000) were purchased from Sigma Aldrich (St. Louis, MO, USA) . Acetic acid, chloroform and citric acid were acquired from Merck (Darmstadt, Germany). Acetonitrile and methanol of HPLC grade were from VWR (West Chester, USA).

A total of 6 milk samples were purchased in a local market (4 from China and the other 2 of national provenance).
2.2. HPLC-DAD analysis of melamine

The chromatographic analysis of melamine was performed by a system Merck Hitachi Elite LaChrom (Darmstadt, Germany) equipped with a pump L-2130, an autosampler L-2200, a diode-array detector and an acquisition and data treatment.

The analysis were performed at room temperature, using an injection volume of 100 μL. A C18 reverse-phase Purospher®STAR end capped column (250 mm x 4 mm; 5 μm) combined with a pre-Purospher®STAR column (4 mm x 4 mm; 5 μm) from Merck (Darmstadt, Germany) was used.

The mobile phase consisted of acetonitrile (10%) and 10 mM of sodium 1-heptanesulfonate with 10mM of citric acid (90%) at flow rate of 1 mL/min. Data was acquired in a wavelength range between 220 and 440 nm although the absorbance of melamine was measured at 240 nm.

2.3. Standards preparation

An individual stock standard solution of 500 mg/L of melamine was prepared by weighing the appropriate amount of the standard into a 100 mL volumetric flask and brought to volume with distilled water. The standards for calibration were prepared by the addition of defined volumes of the stock standard solution and diluted with water. Eight standards were prepared in a concentration range of 0.05 to 20 mg/L.

2.4. MEL-MIP synthesis

The MEL-MIP particles were prepared by suspension polymerization. Initially 1 mmol of melamine (solid) and 15 mmol of MAA were added to 5.2 mL of porogenic agent (chloroform). Then 30 mmol of EDMA, 40 mg of initiator (AIBN) and 400 mg of PVA
were added to the previous mixture. All reagents were dispersed in 50 mL of distilled water.

The reaction occurred in a 250 mL volumetric flask equipped with dual manifold, one with a rubber cap and another with a thermometer. The flask was connected to a condenser. The solution was saturated with a gentle flow of nitrogen for 10 minutes before starting the reaction. The polymerization was then started using a thermostatic bath at 60 °C for a period of 24 h with a constant stirring of 400 rpm. The resulting particles were filtered and the template was extracted by Soxhlet with a mixture of methanol:acetic acid (9:1, v/v) for two periods of 24 h. Non-imprinted particles (NIPs) were prepared in the same way as MIPs, without the addition of the template.

2.5. Optimization of the MEL-MIP synthesis

The optimization of the MEL-MIP was evaluated by the percentage of the imprinted melamine in different formulations of MIP by varying the molar ratios of template/monomer (T/M) and monomer/cross-linker (M/CL).

After filtering the polymer particles, the amount of melamine present in the liquid phase was determined by HPLC-DAD. The difference between the template available for reaction and the one measured in liquid phase after polymerisation allows the determination of the amount of melamine bounded to the MIP. Therefore the percentage of melamine imprinted in each MIP was obtained by:

\[
\text{\% Imprinted template} = \frac{w_{\text{melamine initial}} - w_{\text{melamine filtered}}}{w_{\text{melamine initial}}} \times 100
\]  

(1)
2.6. Extraction optimization

The process of template removal was performed in a Soxhlet apparatus. Polymer beads previously transferred to a porous cartridge were placed in the extraction chamber of the Soxhlet. The extraction was accomplished by a mixture of methanol:acetic acid (9:1, v/v), with a reference volume of 100 mL of solvent per g of MIP [24]. Different times of extraction and solvent renewal were tested in order to achieve the best conditions for template removal.

Samples were collected during the extraction process, evaporated with N₂ and reconstituted in water before HPLC-DAD analysis.

The extraction efficiency was calculated according to:

\[
\% \text{ Extraction efficiency} = \frac{w_{\text{melamine}_{\text{extraction solvent}}}}{w_{\text{melamine}_{\text{imprinted}}}} \times 100
\]  

(2)

2.7. Morphological characterization of MIP and NIP

The morphological characterization of both MIP and NIP particles was performed by scanning electron microscopy (SEM) with an Quanta 400 FEG ESEM/EDAX Genesis X4M without gold coating application. The diameter of particles was measured in a Beckman Coulter LS230 laser diffraction particle size analyser.

2.8. Adsorption experiments

The binding capacity of the optimized MEL-MIP was investigated by performing static and dynamic adsorption tests carried out in aqueous solution. Initially 20 mg of MIP and NIP particles were weighed into a tube to which 5 mL of melamine standard solutions were subsequently added in concentrations between 1 and 120 mg/L. After incubation for
24 h at controlled temperature (20 °C) the samples were filtered and injected on HPLC-DAD to determine the concentration of free melamine.

Dynamic tests were performed in a similar procedure, although the concentration of the standard in contact with MIP was maintained constant (10 mg/L), and the samples were collected in different periods of incubation (1, 5, 10, 20, 40 and 60 min).

The Scatchard plot was constructed according to the Eq (3), where Q is the amount of melamine bounded to the polymer and \( C_{\text{free}} \) is the free concentration of melamine at the equilibrium. The constant of dissociation (\( K_d \)) and the maximum binding amount (\( Q_{\text{max}} \)) were obtained from the slope and intercept of the linear line plotted in \( Q/C_{\text{free}} \) vs Q.

\[
\frac{Q}{C_{\text{free}}} = \frac{Q_{\text{max}}-Q}{K_d}
\]

(3)

2.9. Method Validation

2.9.1. Calibration

A matrix-matched calibration was employed after different amounts of a melamine stock solution (500 mg/L) being added to a fluid milk (medium fat) previously analysed in order to prepare spiked samples with final concentrations between 0.5 and 20 mg/L.

In a centrifuge tube, 2.5 mL of each spiked solution was added along with 2.5 mL of acetonitrile. The mixture was stirred for 1 min, and then 5 mL of the same solvent was added. The precipitated proteins were separated from the liquid by centrifugation (10 min at 4000 rpm).

Into another tube, containing 200 mg of selective polymer (MIP), the previous liquid phase was added. This mixture was stirred for 10 min to allow the diffusion of analyte into the cavities. Further centrifugation, during 10 min at 4000 rpm, was applied, allowing the phase separation.
Elution of the analyte was carried out with 6 mL of methanol:acetic acid (7:3, v/v) added to the mixture. MIP and solvent were also stirred for 10 min and then separated by centrifugation (10 min at 4000 rpm). In the end, 500 μL were collected from the liquid and analysed in HPLC-DAD after being evaporated and reconstituted in 250 μL of distilled water.

2.9.2. Validation parameters

In the validation procedure, linearity, precision and accuracy were considered. Limits of detection and quantification were determined from the calibration curve using an S/N ratio of 3 and 10 respectively.

Precision was determined by intermediate precision expressed by the coefficient of variation from replicate measurements. Recovery assays were performed to determine the accuracy of the method.

2.10. Analysis of milk samples by MIDE-HPLC-DAD

Milk samples from Portuguese and Chinese origin were acquired from local markets and stored in the refrigerator at 4 ºC. For each sample 2.5 mL were collected and placed in a 15 mL centrifuge tube. All samples were extracted by the procedure above described.

3. Results and Discussion

3.1. MIP synthesis

In MIP synthesis, the interaction between monomer and template is defined as an equilibrium process. Based on the mechanism responsible for the formation of binding sites each singular cavity results from the association of functional monomers with a single template molecule. Therefore the complexation between these two species can be
increased with higher amounts of functional monomer [25]. Concerning to the cross-linker a thumb rule is that the molar ratio between this specie and functional monomer should be in an interval between 1 and 4 [26].

In this work, 6 different formulations were considered in order to study the effect of molar ratios between template, monomer and cross-linker in the amount of melamine imprinted on the polymeric matrix. The percentage of imprinted template was determined for each MIP and results were presented in Table 2. The results show that when ratios of 1/15 (T/M) were used a higher amount melamine is imprinted on the polymers. As for the ones prepared with ratios of 1/12, slightly lower values of impression are obtained. Moreover the higher amount of imprinted template is obtained when 67% of cross-linker is used (formulation 1).

Regarding the optimization of melamine selective polymers, He et al [22] tested different types of monomers, cross-linkers and porogenic solvents and based their analysis on molecular recognition ability, flexibility and surface area of the prepared MIPs. The ratios between these species were maintained constant, 1/8 between template (melamine) and monomer (MAA) and 1/3 between monomer and cross-linker (EDMA), using a mixture of methanol/water as porogenic solvent. Li et al [20] investigated the performance of 8 different formulations of MIPs and analysed melamine’s adsorption of both MIP and NIP under the same concentration of analyte. In this study they concluded that MIPs with molar ratios of 1/6 for melamine/MAA and 1/5 for MAA/EDMA, respectively, using acetonitrile as porogenic solvent, provided materials with best affinity to melamine.
3.2. Extraction optimization

In order to achieve the best conditions for template extraction, three independent MIPs (A, B and C) were synthetized under previous optimized ratios (T/M= 1/15 and M/CL=1/2) and then extracted in a Soxhlet apparatus with a mixture of methanol:acetic acid (9/1, v/v). The influence of different times of extraction and solvent renewal in the efficiency of extraction was analysed. MIP A was submitted to a total of 40 h extraction with solvent renewal every 4 h. For MIP B, solvent was replaced after 12 h and the process was carried out until 24 h. Finally in MIP C, a portion of a fresh solvent was applied after 24 h, and extraction was stopped after 48 h. In each extraction, samples were collected after solvent renewal allowing the monitoring of the extraction efficiency.

The representation of the efficiency values as a function of extraction time (Figure 1) shows final efficiencies of 97.5, 51.9 and 76.4% for MIPs A, B and C respectively. These results show that none of the employed methodologies achieve a full template removal. This confirms the existence of compact areas in the polymeric network limiting the access to template molecules by the solvent which can’t be suppressed even with extensive periods of extraction combined and successive solvent renewals.

Despite the highest extraction efficiency observed with the procedure applied to MIP A the high consumption of solvent that results from a large number of renewals turns unacceptable to implement this method. Therefore the extraction conditions tested in MIP C (48 h of process with solvent renewal after the first 24 h of extraction) were adopted. After extraction all the produced MIPs were washed with methanol in order to remove the acid that remained in the polymeric structure.

Analysing the recent studies with MIPs applied to melamine analysis it was verified that most of imprinted polymers were extracted by Soxhlet technique. Yang et al [27] and Wang et al [21], describe an extraction in a Soxhlet apparatus for 48 hours with a mixture
of methanol:acetic acid (9:1, v/v), however nothing is said about solvent regeneration nor
determination of the amount template that is being extracted. Meanwhile, Wang et al [19]
used a mixture of methanol:acetic acid (7:3, v/v) with control of the released template by
UV spectrophotometry at a wavelength of 240 nm.

3.3. Morphological characterization of MIP and NIP

The particles of both MIP and NIP obtained by suspension polymerization exhibit
diameters between 63 and 150 µm. Contrarily to the polymers prepared by bulk
polymerisation typically amorphous as a result of the grinding process [28], we were able
to produce particles with smoother surface (Figure 2). Although, from a lower
magnification (a1 and b1), imprinted and non-imprinted particles show no morphological
difference between them, it can be observed at higher magnifications (a2 and b2) that the
imprinted polymer has an irregular and rough morphology with a presence of small
cavities probably created by the removal of the template molecules allowing the
formation of specific binding sites.

3.4. Equilibrium rebinding

The data of the dynamic and static adsorption presented in Figure 3 show a higher
affinity from MIP toward the analyte. The curve of the dynamic adsorption (Figure 3a)
demonstrates that the polymer particles of both MIP and NIP reach the equilibrium after
40 min of contact with the standard of melamine in water.

With regard to static experiment, an incubation time of 24 h was defined, in order to
ensure the saturation of all active sites formed during the process of MIP synthesis. The
adsorption data was subsequently processed using the Scatchard format which is a
linearized version of the Langmuir isotherm model [29]. For homogeneous binding sites
the experimental data is adjusted by a straight line. On the other hand a curved Scatchard
plot is an evidence for binding site heterogeneity. If the curve falls on two different straight lines, two classes of binding sites can be derived (high and low affinity sites).

The results shown in Figure 3c point out to homogeneous binding sites, however other data was obtained (not shown because it was considered not significant) that might question if two types binding sites still exist. Nevertheless, from the slope and intercept of the presented data, the values obtained for $K_d$ and $Q_{\text{max}}$ were $568.4 \pm 7.4 \, \mu\text{mol/L}$ and $96.8 \pm 2.3 \, \mu\text{mol/g}$, respectively.

Analysing the studies with MIPs for melamine analysis, Yan et al [23] and Wang et al [21] described the preparation of imprinted polymers with two distinct groups of binding sites. High affinity sites showed dissociation constants of 1140.0 and 9.2 $\mu\text{mol/L}$ and maximum binding capacities of 160 and 7.3 $\mu\text{mol/g}$, respectively (values calculated from data shown). For the lower affinity sites, $K_d$ values of 5500.0 and 79.4 $\mu\text{mol/L}$ were obtained. As for $Q_{\text{max}}$ authors described maximum binding capacities of 430 and 32.4 $\mu\text{mol/g}$ in low affinity binding sites.

Still, homogeneous binding sites have been reported in imprinted polymers for melamine’s extraction. Yang et al [27], Li et al [20] and Wang et al [19] obtained polymeric particles exhibiting one group of binding sites with dissociation constants of 13.5, 90.5 and 37.6 $\mu\text{mol/L}$. The maximum binding capacities registered were 2.7, 53.2 and 30.9 $\mu\text{mol/g}$, respectively. From the comparison with these reports, our results are closer to those who claim heterogeneous binding sites, specifically the ones of higher affinity.

3.5. Method Validation

A matrix effect study was performed to evaluate possible changes in melamine’s analytical response due to high degree of complexity exhibited by milk samples. Water
and milk after previous spiking with melamine were extracted by a dispersive methodology with MIP. From the HPLC-DAD analysis it was verified that despite the good selectivity of the sorbent toward the analyte there’s still some interference in the analytical response caused by the presence of undetected compounds.

In order to compensate this effect a matrix matched calibration was applied to milk samples. For that purpose 7 "standards" of spiked milk (A) were prepared with concentrations between 0.5 and 20 mg/L. Samples were deproteinized and then extracted by a dispersive methodology (MIDE).

The performance of the MIP was investigated by the analysis of a different type of milk (B) spiked at the same levels that were used for calibration. Milk A and B had different formulations, low fat (A) and special calcium (B). No differences were detected between melamine’s recovery in both milk which allows us to say that the selectivity of the MIP is independent from the type of milk that is used. This result avoids the need to perform calibration according to the type of milk in analysis.

It must be empathized that the use of MIPs imprinted with the analyte may induce some bleeding problems, which may affect the calibration. In fact bleeding experiments were performed, using water without addition of melamine. The sample was treated with acetonitrile, and applied to 200 mg of MIP in a tube. After extraction a sample was injected in HPLC-DAD. This assay was performed in triplicate and an average bleeding level of 0.3 mg/kg was obtained.

The proposed method was validated in terms of precision and accuracy by the determination of CV for standards of a known concentration. Precision was evaluated in spiked samples at three concentration levels applied to three different MIP’s. The results varied from 2.8 to 12.0% indicating that the method is precise.
The accuracy, expressed as the observed value divided by the expected value after standard addition was determined by 3 independent extractions at 3 levels of spiking, the same ones used for precision’s investigation. High recoveries are obtained for spike levels of 10 and 20 mg/L with 94.9 and 99.7%, respectively.

3.6. Analysis of milk samples

To prove the stability and the performance of the described MIP six different brands of fluid milk (two national and four Chinese) were acquired from local markets and treated with the procedure above described and then melamine was extracted by MIDE. The extracts were analyzed by HPLC-DAD to determine the levels of melamine present in samples.

From the analyzed samples (data not shown) there was no evidence of melamine’s presence in the national samples, although with concern to the Chinese milks, one sample showed the presence of melamine with a maximum concentration of 9.3 mg/kg, which is above the safety limits set by European Union at 2.5 mg/kg [30].

For other food products, the research team had already examined a group of 20 samples (soy milk powder, milk powder, soybean powder, cookies and biscuits). In this study it was verified the presence of melamine in 11 samples (8 of Chinese origin, and 3 of national origin) with a maximum concentration of 3.4 mg/kg [1].

4. Concluding Remarks

A melamine imprinted polymer was successfully produced by suspension polymerization method. Template impression and extraction efficiency were monitored by HPLC-DAD in order to achieve the optimal synthesis conditions. Characterization experiments based on dynamic and static adsorptions showed that MIP particles have
higher affinity to analyte than the non-imprinted polymer (NIP). It was also revealed the presence of one type of binding sites in MIPs with a dissociation constant ($K_d$) of $568.4 \pm 7.4 \mu$mol/L and a maximum binding concentration ($Q_{\text{max}}$) of $96.8 \pm 2.3 \mu$mol/g.

The use of MIP particles as a selective dispersive sorbent proved to be a precise and accurate method for the extraction of melamine in spiked milk samples.

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The authors have declared no conflict of interest.

**5. References**

Table 1. MIP for the extraction of melamine in food products with respective analytical methods.

<table>
<thead>
<tr>
<th>Polymerization method</th>
<th>T/M/CL</th>
<th>Melamine extraction</th>
<th>Samples</th>
<th>Chromatographic technique</th>
<th>LOD/LQ</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk</td>
<td>Cyromazine/MAA/EGDMA</td>
<td>MSPD</td>
<td>Bovine milk</td>
<td>HPLC-UV</td>
<td>LOD – 0.05 µg/g</td>
<td>[23]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LQ – 0.16 µg/g</td>
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</tr>
<tr>
<td>Bulk</td>
<td>Cyromazine/MAA/EGDMA</td>
<td>MISPE</td>
<td>Feed and milk</td>
<td>HPLC</td>
<td>-</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>samples</td>
<td>GC-MS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suspension</td>
<td>Cyromazine/MAA/EGDMA</td>
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<td>Feed and milk</td>
<td>GC-MS</td>
<td>LOD – 0.01 µg/mL</td>
<td>[20]</td>
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<td></td>
<td></td>
<td></td>
<td>samples</td>
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<td>LOQ – 0.05 µg/mL</td>
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<tr>
<td>Bulk</td>
<td>Cyromazine/MAA/EGDMA</td>
<td>MISPE</td>
<td>Egg and milk</td>
<td>HPLC</td>
<td>-</td>
<td>[21]</td>
</tr>
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<td>samples</td>
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<tr>
<td>Bulk</td>
<td>Melamine/MAA/EGDMA</td>
<td>MISPE</td>
<td>Dairy products</td>
<td>HPLC</td>
<td>LQ – 0.5 µmol/L</td>
<td>[27]</td>
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<tr>
<td>Suspension</td>
<td>Melamine/MAA/EGDMA</td>
<td>MISPE</td>
<td>Milk samples</td>
<td>HPLC-UV</td>
<td>-</td>
<td>[19]</td>
</tr>
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</table>
Table 2. Composition of the prepared MIP’s and respective percentage of imprinted template.

<table>
<thead>
<tr>
<th>MIP</th>
<th>Template / Monomer (molar ratio)</th>
<th>Monomer / Cross-linker (molar ratio)</th>
<th>Imprinted template (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>1/2</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>1/15</td>
<td>1/4</td>
<td>32</td>
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<td>20</td>
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<td>4</td>
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<td>1/2</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>1/12</td>
<td>1/4</td>
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</tr>
<tr>
<td>6</td>
<td></td>
<td>1/6</td>
<td>22</td>
</tr>
</tbody>
</table>

Figure 1. Evolution of the amount of template extracted during the extraction time. The points show the moment at which the extraction solvent was renewed.
Figure 2. SEM micrographs of imprinted polymer after Soxhlet extraction at 200 x (a1) and 20000 x (a2) and non-imprinted polymer at 200 x (b1) and 20000 x (b2).
Figure 3. Dynamic adsorption (A) binding isotherm (B) of MIP’s and NIP’s for melamine and (C) Scatchard plot of MIP.