Molecularly Imprinted Polymer for Solid Phase Extraction of Nicotinamide in Pork Liver Samples

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Received 12 April 2010; accepted 12 August 2010
DOI 10.1002/app.33267
Published online 1 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Nicotinamide (NAM) is often added in fortified infant and various food products to ensure an adequate consumption of vitamin. Thus, a proper monitoring of NAM content in foods can be important. In this study, a selective molecularly imprinted polymer as sorbent for solid phase extraction of NAM in animal sources was successfully developed. The molecularly imprinted polymer was synthesized by bulk polymerization technique. The performances of this polymer as sorbent were investigated in NAM standard solutions. One hundred milligrams of polymer was able to retain up to 244 μg of NAM with recovery >80% when chloroform was used as loading and washing solvent and ethanol as eluting solvent. Other solvent mixtures were also tested. The optimal molecularly imprinted solid phase extraction protocol was defined and used for the clean-up of NAM in pork liver samples. Moreover, the performances of the imprinted polymer were compared with that of nonimprinted polymer and with conventional reversed-phase C18 performances. Pork liver samples spiked with 49 μg mL^{-1} gave a good percentage of recovery of 87%, with relative standard deviation of 8% for imprinted polymer, whereas only 12% of recovery for nonimprinted polymer and 14% of recovery for reversed-phase C18 sorbent were found. J Appl Polym Sci 120: 1634–1641, 2011

Key words: molecular imprinting; templates; molecular recognition; nicotinamide; solid phase extraction

INTRODUCTION

Nicotinamide (NAM) is both a food nutrient and a drug. It is one of the two principal forms, with nicotinic acid, of the water-soluble vitamin B3 also called vitamin PP or niacin. NAM is also part of NAM adenine dinucleotide and NAM adenine dinucleotide phosphate coenzymes. Deficiency in niacin is related to a disease called "pellagra." NAM has been related to a myriad of effects. It acts as an antioxidant, anti-inflammatory agent, and immunomodulator. Niacin plays an important role in the production of energy and in the activity of the nervous and digestive systems, and it takes part in the synthesis of sex and adrenal hormones.[1–5]

High amount of vitamin PP are contained in yeast, meat and offal (especially liver), fish, and cereals, whereas fruits, vegetables, and eggs have lower amount. NAM is often added in fortified infant and various food products such as infant milk or cereals to ensure an adequate consumption of vitamin.[6] Thus, a proper monitoring of NAM content in foods and dietary supplements is important.

Conventional methods to quantify niacin in foods are microbiological and chemical assays,[7,8] which are time-consuming and expensive liquid chromatography procedures[9–11] and capillary electrophoresis.[12,13] Reversed-phase (RP) technique has been generally adopted in high-performance liquid chromatography (HPLC) analysis. In foodstuffs, "total NAM" or "free NAM" can be estimated depending on the extraction procedure selected.[14]

In complex real samples, purification procedure can be difficult, and an additional purification step by solid phase extraction (SPE) could be necessary. Molecularly imprinted polymers (MIPs) are synthetic molecular recognition systems able to specifically interact with a selected target molecule named template. Preparation of the MIP occurs according to the general procedure reported in Figure 1. First, the complexation of functional monomers with the target molecule (template) in a porogen solvent takes place, and then the polymerization in the presence of a cross-linker occurs around the complexes. Finally the template, added during the polymerization process, is removed by washing procedures, leaving definite binding sites in the polymer network. In this way, the polymer can exhibit high affinity toward the template molecule, which can be selectively re-bound to the specific sites.[15–20]

The following main approaches can be adopted to molecular imprinting: (a) the self-assembling approach, where the prearrangement between the template and the functional monomers is formed by noncovalent or metal coordination interactions, and

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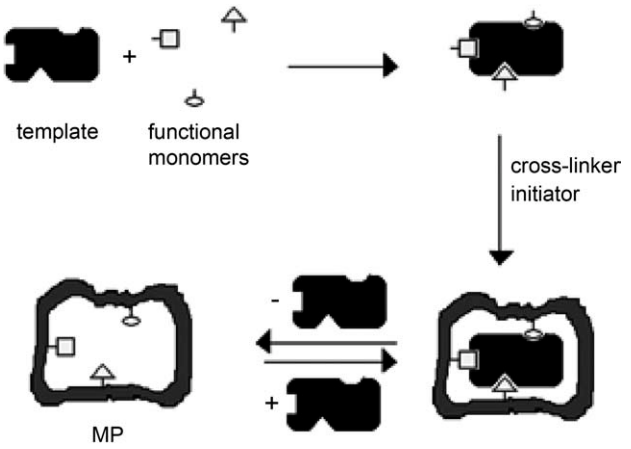

Figure 1 Scheme of molecular imprinting.

(b) the preorganized approach, where complexes in solution before polymerization are maintained by reversible covalent bonds.

Nowadays, MIPs have important applications in various areas, such as solid-phase extraction,[21–30] chromatography,[31–33] assays and sensors,[34,35] catalysis,[36,37] chemical traps, and drug delivery systems.[17,38,39]

One of the traditional applications of MIPs is in the area of SPE as highly selective and specific sorbents. The first molecularly imprinted SPE (MISPE) was reported by Sellergren.[40] Relevant advantages of MISPE are not only in terms of preconcentration and cleaning of the sample but also for a selective extraction of the target analyte, which is important, particularly when the sample is complex and impurities can interfere with quantification.

The objective of this study was to develop a tailored sorbent able to specifically interact with NAM analyte. Thus, a bulk imprinted polymer was synthesized using NAM as template, methacrylic acid (MAA) as functional monomer, and ethylene glycol dimethacrylate (EGDMA) as cross-linker. An optimization of the extraction procedure by an off-line MISPE technique was investigated in NAM standard solutions. A preliminary evaluation on the use of the developed MISPE as a clean-up step in the analysis of NAM in pork liver sample was also done. Finally, a comparative study for NAM extraction in NAM standard solutions and pork liver samples by using imprinted polymer, nonimprinted polymer (NIP), and a conventional sorbent was also described.

EXPERIMENTAL

Materials and instruments

NAM, MAA, EGDMA, and azobis-isobutyronitrile were obtained from Aldrich (Milwaukee, WI). Ultrapure water was obtained from a model New Human Power I ultrapure water system from Human Corp (Korea).

Dry chloroform (Aldrich, ≥99.8% A.C.S. reagent) was passed through a column of basic alumina (Aldrich, standard grade) before use. All other solvents (Baker, The Netherlands, analyzed grade or HPLC-grade) were used without further purification.

Conventional RP-C18 SPE cartridges (GracePure™ C18-low) were purchased from Grace Davison Discovery Sciences (Columbia, MD). Sonication was carried out using a Sonorex RK 102H ultrasonic water bath from Bandelin Electronic (Berlin, Germany). Centrifugation was achieved with a PK121 multispeed centrifuge from Thermo Electron Corp (Château Gontier, France). T18 basic ULTRA-TURRAX immersion homogenizer was obtained from IKA (Staufen, Germany). Absorbances were measured by the UV–vis spectrophotometer Cary 100 scan (Varian, Walnut Creek, CA). HPLC analyses were performed using an Agilent 1100 Series HPLC system with a diode array detector (Agilent, Santa Clara, CA). Chromatographic separation was carried out using a thermostated 150 mm × 4.6 mm inner diameter, 5-μm SS Wakosil C18 column.

Synthesis of NAM-imprinted polymer

NAM-imprinted polymer (N-MIP) was prepared by bulk method. Briefly, 1.60 mmol of NAM and 6.4 mmol of MAA were dissolved in 20 mL of dry chloroform, and then 25.6 mmol of EGDMA and 0.54 mmol of azobis-isobutyronitrile were added to the solution. The reaction mixture was saturated with nitrogen and heated at 60°C for 20 hr. The resultant bulk rigid polymer was crushed, ground into powder, and sieved repeatedly, collecting particles with diameter 20–70 μm. The template was removed by washing several times with 30 mL of ethanol/acetic acid (8/2 v/v) solution. Final washings with ethanol were done to remove the acetic acid present. N-MIP polymer obtained with 52% of yield was stored under vacuum and used for SPE experiments. As a control, a NIP in the absence of template was also prepared and treated in an identical manner of N-MIP.

Preparation of MISPE cartridges and optimization with standard solutions

A suspension of 100 mg of dry N-MIP or NIP in 2 mL of acetonitrile was packed into an empty 3-mL polypropylene SPE cartridge secured by polyethylene frits. Before use, each cartridge was activated by treatment with the same solvent used in the loading step.

Stock solution of NAM was prepared by dissolving 61.1 mg of NAM powder in 50 mL of chloroform to obtain a 1221 μg mL^{-1} solution. Six dilutions, from 733 to 9.7 μg mL^{-1}, were prepared from stock

solution. Each of these solutions was loaded onto MISPE cartridges.

In a typical MISPE protocol, the conditioning step between samples consisted of 2 × 3 mL of ethanol and 2 × 3 mL of chloroform. Next, 1 mL of each of standard solution was loaded onto the MISPE cartridge, the polymer was washed three times each with 1 mL of chloroform solvent, and the analyte was eluted three times each with 1 mL of ethanol. To remove residual solvent, MISPE was dried before to apply a different solvent. A flow rate of 0.5 mL min^{-1} was used. The fractions collected after loading (LF), washing (WF), and elution (EF) steps were dried and redissolved in 1 mL of water for HPLC analysis.

To obtain high extraction efficiency, the extraction conditions such as loading and washing solvents were optimized by using the protocol above described and loading 1 mL of 122 μg mL^{-1} NAM standard solution. In particular, to find the suitable loading condition, chloroform and four solvent mixtures of $CHCl_3$/EtOH (80 : 20, 90 : 10, 95 : 5, and 99 : 1) were examined. Chloroform and the mixture of $CHCl_3$/EtOH 90 : 10 were also tested as washing solvents.

A comparison of N-MIP, NIP, and RP-C18 cartridges was also carried out loading 1 mL of 122 μg mL^{-1} NAM standard solution and using chloroform as loading and washing solvent and ethanol as eluting solvent. The experiments were repeated twice.

Pork liver sample preparation

NAM was extracted from fresh pork liver according to Takatsuki et al.'s method,[41] modified in this work for our purpose, to obtain spiked pork liver sample. Thus, 10 g of shredded sample was homogenized, and then 30 mL of water and 6.1 mg of NAM, to obtain a concentration of 49 μg mL^{-1} in the final solution, were added. The solution was transferred into a 100-mL volumetric flask, and it was brought to volume with water. After centrifugation, the supernatant was filtered using a filter paper. Twenty milliliters of the filtered extract was transferred into a 25-mL volumetric flask, and, to precipitate the proteins, 1 mL of saturated zinc acetate solution and 1 mL of 1*M* NaOH solution were added successively; the extract was brought to volume with water. The mixture was filtered and then stored at 4°C. A similar procedure was used for blank pork liver sample, without addition of NAM powder. The experiments were repeated twice.

MISPE of pork liver samples

One milliliter of spiked pork liver sample was applied to N-MIP, NIP, and conventional RP-C18 cartridges. SPE extraction conditions and HPLC analysis were optimized according to standard solutions tests as described in the previous paragraph. One milliliter of spiked pork liver sample (previously dried, redissolved in 1 mL of $CHCl_3$/EtOH 90 : 10, and heated at 50°C) was loaded onto the cartridge. Conditioning step between samples loading consisted of ethanol and chloroform, whereas washing and eluted solvents were chloroform and ethanol, respectively.

The same procedure was followed for blank pork liver sample. The experiments were repeated three times.

Quantitative analysis

Quantification of NAM was performed by the external standard method using a six-point regression graph of the absorbance areas at 262 nm obtained with HPLC-diode array detector analysis. The NAM standard solutions were prepared (see Preparation of MISPE Cartridges and Optimization With Standard Solutions section) to cover the linear concentration range, with lowest value close to its limit of quantification (LOQ). The limit of detection (LOD) was determined from the amount of analyte required to give a signal-to-noise ratio of 3, and the LOQ was defined as the lowest concentration giving a signal-to-noise ratio of 10.[42]

The resulting absorbance areas of each standard were plotted against concentration for the calibration curves. The NAM contents of the sample extracts were obtained by interpolation on the standard curve. The experiments were repeated three times.

The percentage of NAM collected in loading, washing, and elution fractions (LF, WF, and EF, respectively) was calculated according to the following formula: NAM% = absorbance area of NAM in XF/[absorbance area of NAM in (LF + WF + EF)] × 100, where XF can be LF, WF, or EF. The accuracy of SPE with N-MIP, NIP, and RP-C18 sorbents for real sample was evaluated as recovery percentage, which was calculated according to the following formula: Recovery (%) = $(C_{ss} - C_{bs})/C_{NAM} \times 100$, where C_{ss} is the concentration of NAM measured in EF in cartridges loaded with spiked pork liver sample; C_{bs} is the concentration of NAM measured in EF in cartridges loaded with the blank pork liver sample; and C_{NAM} indicates the concentration of NAM added into the spiked pork liver sample.

HPLC analysis

The mobile phase consisted of methanol and 0.05*M* potassium dihydrogen phosphate buffer (pH 3.6), in a ratio of 7.5 : 92.5. An aliquot of 10 μL of sample was injected by an autosampler, at a flow rate of 1.2

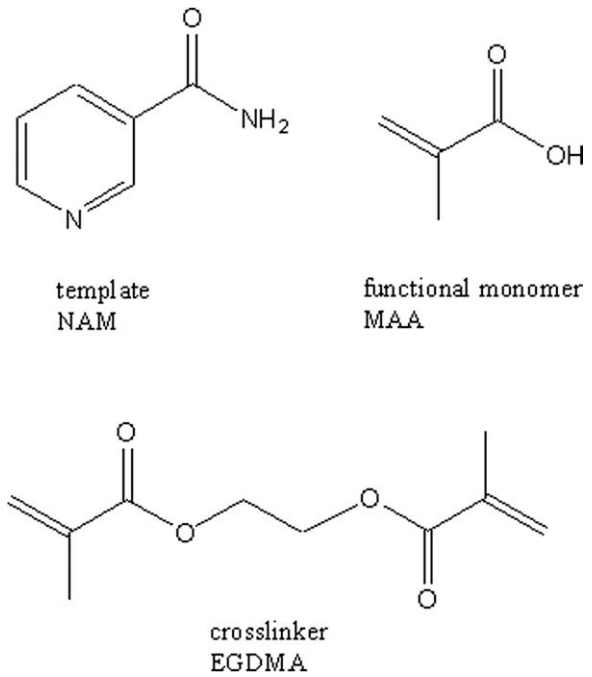

Figure 2 Structure of compounds used for NAM-imprinted polymer synthesis.

mL min^{-1}, and NAM analyte was detected at 262 nm. Retention time of the NAM was approximately 5 min. To evaluate the amount of template extracted, calibration curve was prepared reporting area versus template concentration as described in the previous paragraph.

RESULTS AND DISCUSSION

NAM as template for MIPs and its quantification by HPLC analysis

In this work, we used bulk polymerization because it is the most popular method for preparing MIPs suitable for MISPE following the procedure given in the experimental section. Moreover, a self-assembling approach, which represents the most common one for MIPs, was adopted by a noncovalent interaction between NAM and MAA (Fig. 2).

Synthesis of the NAM-imprinted polymer was carried out following a method described in our previous study,[43] with slight variations. The difference was the use of a bulk polymerization technique instead of a precipitation polymerization one, to obtain a suitable sorbent for SPE application.

Quantification of NAM was performed by the external standard method. The regression equation was $Y = 12.68\ X + 25.579$ ($R^2 = 0.9998$), and linear range was 5–731 μg mL^{-1}, with LOD of 0.0176 μg mL^{-1} and LOQ of 5 μg mL^{-1}.

Optimization of MISPE procedure

The empty cartridges were packed with 100 mg of the imprinted polymer or NIP as described in the Experimental section. Before carrying out the experiments on real samples, a study devoted to evaluate performances of MISPE, based on NAM standard solutions, was performed, which is discussed in this section.

A selective adsorption approach was thought in this work. Thus, NAM should be selectively retained in the cartridge by noncovalent interactions with N-MIP, whereas interferences not retained should be eliminated during loading and washing steps. Then, NAM should be selectively eluted in the final step, by using a solvent with a higher ionic strength. For this purpose, ethanol was used as eluting solvent in all the experiments.

In a typical experiment, the cartridge was first conditioned by using ethanol, and then loading solvent and 1 mL of sample solution were added in sequence. Then, 3 mL of washing solvent and 3 mL of eluting solvent were used.

At the beginning, chloroform was selected as loading and washing solvent because it was the solvent used in the imprinted polymer synthesis. In fact, it is known that the ideal solvent for selective re-binding to the imprints is the same as the porogen.[24,43–45]

The N-MIP cartridge was loaded with different amounts of NAM to calculate the specific loading capacity. Chloroform solutions of NAM in the range of 4.9–611 μg mL^{-1} were considered. In Figure 3 is reported the percentage of NAM collected in loading, washing, and eluting fractions for each experiment. It is worth noting that when 1 mg of NAM solution at lower concentrations than 366 μg mL^{-1} is used, a negligible percentage of NAM (<1%) is released in loading and washing fractions from 100 mg of N-MIP stationary phase. On the other hand,

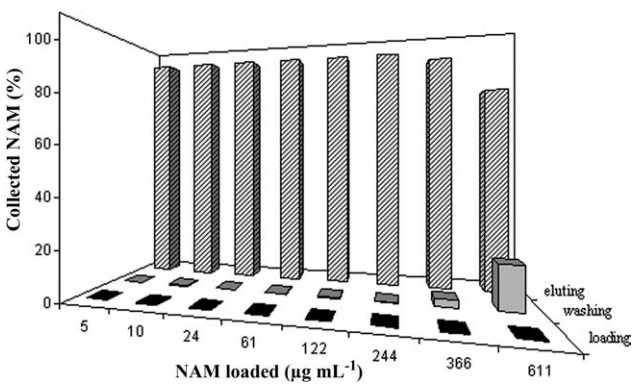

Figure 3 Mean of percentage of collected NAM in loading, washing, and eluting fractions versus concentration of NAM loaded. One milliliter of each NAM solution in chloroform was loaded onto a N-MIP cartridge, washed with chloroform, and eluted with ethanol ($n = 2$).

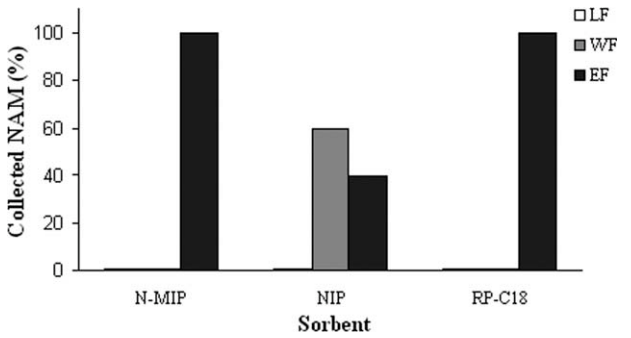

Figure 4 Mean of percentage of collected NAM in loading, washing, and eluting fractions using different SPE sorbents. One milliliter of a $CHCl_3$ solution of 122 μg mL^{-1} NAM was loaded, washed with chloroform, and eluted with ethanol ($n = 2$).

3% and 17% of loaded NAM is found in washing fraction when 366 and 611 μg mL^{-1} are loaded, respectively. Thus, it can state that 100 mg of N-MIP is able to retain up to 244 μg of NAM.

Percentages of recovery versus concentration of NAM loaded were also calculated, and, in each experiment, a recovery >80% was found. According to loading capacity results, in all the following experiments, 1 mL of a standard solution at a concentration of 122 μg mL^{-1} of NAM was chosen as model loading sample.

It was also tried to increase the chloroform volume up to 9 mL in the washing step, and no loss of MISPE activity was observed. This means that interactions between NAM and polymer in chloroform are strong enough to avoid release of NAM even when high volumes of chloroform need to be used.

A comparison with other sorbents was carried out to evaluate the effectiveness of MISPE performance. For this purpose, the NIP and a conventional RP-C18 were taken into consideration (Fig. 4) by using exactly the same protocol in each experiment.

It is worth noting that the cartridge prepared with the NIP sorbent gave a complete retention of analyte in the loading step, whereas washing with chloroform caused a significant loss of NAM (60%). This result is in agreement with the low binding capacity of NIP as found for similar polymeric systems in previous studies.[43] Thus, the effectiveness of molecular imprinting recognition of N-MIP cartridge was confirmed. On the other hand, the use of NIP for NAM extraction in real sample, which will be discussed in the next paragraph, seems not to be achievable.

A comparison with a conventional SPE sorbent was also carried out. Generally, total content of niacin is determined, converting NAM in nicotinic acid and eventually purifying and concentrating the extract by using a strong cationic-exchange cartridge.[10,11] Free NAM can be determined by extraction with a 0.1*M* acid solution and purified on a reversed-solid phase cartridge, typical for neutral polar compounds. Because NAM is the analyte under investigation, a conventional RP-C18 was used, and it was found that the performance is similar to MISPE cartridge (Fig. 4). In fact, also in this case, analyte is completely retained in loading and washing steps, giving a 100% of collected NAM in eluting fraction. This result is not unexpected because RP-C18 was chosen like a suitable sorbent for NAM extraction. It will be interesting to evaluate and compare the performance of N-MIP and RP-C18 when a real sample is loaded, because interferences in complex matrices could get worse the efficiency of traditional SPE.

Although chloroform demonstrated to be a proper solvent in loading and washing steps, for the aim of this work, it is important to select a solvent or a mixture of solvents more suitable than chloroform to extract NAM in pork liver samples. Pork liver is a complex matrix, and polar solvents, such as water or alcohols, are usually used in extraction procedures.[46,47] Moreover, NAM is not highly soluble in chloroform, and, although real extracts contain NAM concentrations below the limit of chloroform solubility, NAM could be prevented from the extraction in chloroform for the presence of interferences. Thus, in the second part of optimization of MISPE procedure, loading and washing solvents were changed, and the response of MISPE was analyzed to evaluate the possibility to use a more polar system.

When a small volume of a polar solvent was added to the loading and washing solution, moving to $CHCl_3$/EtOH 90 : 10, the percentage of NAM collected in the elution step was strongly decreased from 100% to 5% (Fig. 5). As shown in Figure 5, the most of NAM was released in the washing step, whereas only a small percentage was found in the

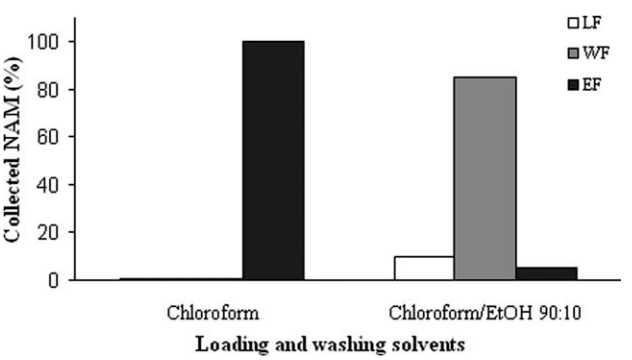

Figure 5 Mean of percentage of collected NAM in loading, washing, and eluting fractions versus different loading and washing solvents. One milliliter of a 122 μg mL^{-1} NAM solution was loaded and washed with chloroform or with a mixture of chloroform/EtOH 90 : 10 and eluted with ethanol ($n = 2$).

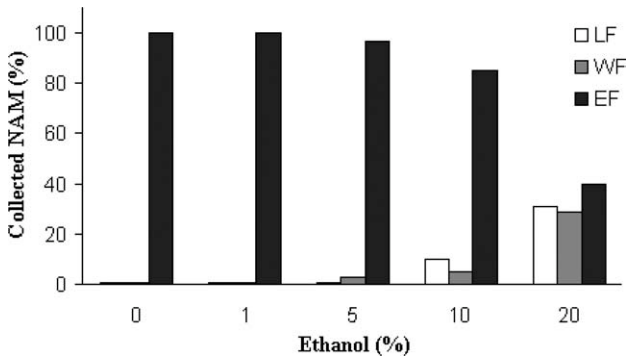

Figure 6 Mean of percentage of collected NAM in loading, washing, and eluting fractions (LF, WF, and EF) versus different percentages of ethanol in chloroform as the loading solvent. One milliliter of a 122 μg mL^{-1} NAM solution was loaded, washed with chloroform, and eluted with ethanol ($n = 2$).

loading fraction. In view of this result, and taking into consideration that this change was made with the intent to improve the extraction solvent, various attempts were next done only changing the loading solvent and maintaining chloroform as washing solvent.

An increasing percentage volume of ethanol in chloroform, from 1% to 20%, was used only in loading step. NAM collected in loading, washing, and eluting fractions is shown in Figure 6 as a function of the percentage of ethanol present in loading solution. Very little loss of NAM during loading and washing steps was observed when 1% and 5% of ethanol was used. Ten percent of ethanol resulted in an increase in loss of analyte, but the percentage of NAM collected in the elution step was still high (>85%), whereas a significant loss of NAM was observed with 20% of ethanol, and the percentage of analyte in the elution step fell to 40%.

Considerable improvements were obtained when 10% of ethanol was used only in loading solvent, 85% of collected NAM in EF (Fig. 5) compared with the trial in which 10% of ethanol was used also in washing step, 5% of collected NAM in EF (Fig. 6).

As a result, $CHCl_3$/EtOH 90 : 10 as the selective loading solvent, chloroform as washing solvent, and ethanol as eluting solvent were finally chosen as optimum conditions for MISPE procedure and used on pork liver extracts. Finally, it is important to point out that all MISPE experiments in this work were carried out with the same cartridge without present loss of performance.

MISPE of pork liver samples

Once the optimal MISPE protocol for the extraction of NAM was established, the developed procedure was used to clean-up NAM from pork liver samples. Free NAM was extracted from homogenized fresh pork liver samples. A spiked pork liver sample was prepared adding NAM at a 49 μg mL^{-1} concentration, whereas a blank pork liver sample was prepared without adding NAM analyte.

Other substances such as water-soluble vitamins are present in pork liver extracts.[48,49] A certified reference material, called CRM 487, is available on the market. It is a lyophilized pork liver powder, which is certified for total content of vitamin B1, B2, B6, B12, and folate.[50]

In complex real samples, the purification procedure is often difficult. Various compounds are present and detected together with NAM, and HPLC analysis is necessary to distinguish NAM from the other compounds. Sometimes, an additional purification step by RP-SPE could be considered.

In our trials, 1 mL of spiked and blank extracts were loaded onto N-MIP cartridge, and the developed protocol was applied. The eluting fractions were analyzed to calculate the percentage of recovery as reported in Table I. Repeatability was measured based on three experiments. A good percentage of NAM recovery in pork liver samples after MISPE protocol of 87%, with an RSD of 8% was found.

Pork liver samples were also loaded onto NIP cartridge to verify the effectiveness of molecular imprinting recognition of MISPE on these real samples. In agreement with the results obtained on standard solutions studies, NIP cartridge does not work well as N-MIP cartridge, showing very low recovery (12%) and confirming the hypothesis moved in the preview paragraph.

To evaluate the advantages of N-MIP over conventional sorbents, a comparison with a RP-C18 cartridge was carried out. A recovery of 14% was measured when pork liver sample was loaded onto RP-C18 cartridge (Table I). It is interesting to observe that RP-C18 showed good NAM extraction properties in standard solution (Fig. 4), whereas a great loss of efficiency was found when a complex matrix was loaded. On the contrary, N-MIP cartridge keeps its high efficiency also in pork liver sample because of the presence of specific NAM-sorbent interactions. From these results, it can be hypothesized that MISPE technique could be advantageous especially for complex matrices analysis.

TABLE I
Recoveries of NAM in Spiked Pork Liver Samples Using Different SPE Sorbents ($n = 3$, Spiked Level, 49 μg mL^{-1})

SPE sorbent	Recovery (%)	RSD (%)
N-MIP	87	8
NIP	12	5
RP C18	14	6

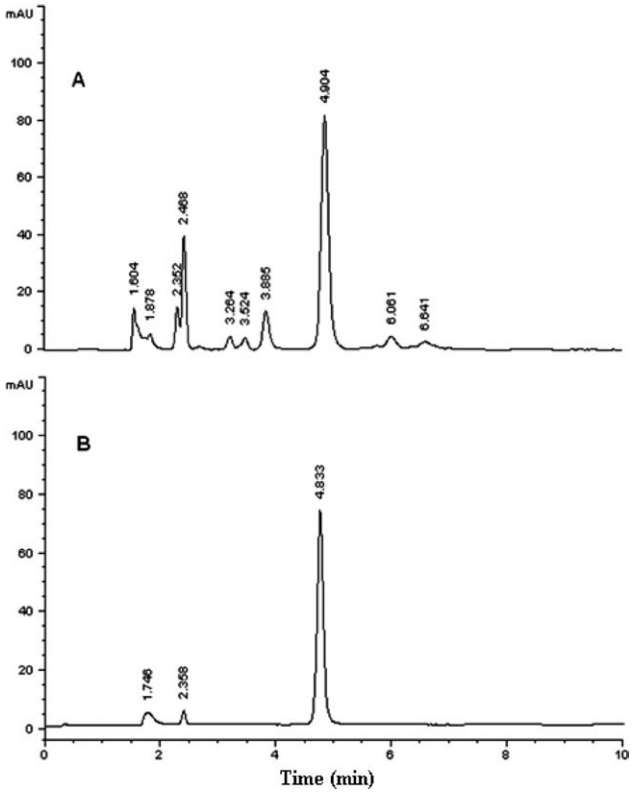

Figure 7 Comparison of chromatograms of (A) direct injection of spiked pork liver sample and (B) the eluting fraction of spiked pork liver sample after MISPE protocol.

The successful clean-up of pork liver with MISPE protocol is pointed out in Figure 7 where chromatograms, obtained with and without MISPE of spiked sample, are shown. Chromatogram of spiked extract, directly injected in HPLC, is reported in Figure 7(A). NAM signal at around 5 min is in a clean region and can be quantified. Spiked extract was passed through a N-MIP cartridge, and the chromatogram of the eluting fraction obtained is reported in Figure 7(B). The main advantage of N-MIP extraction can be explained in terms of a clean-up process because a satisfactory clean-up of the sample was achieved after the passage onto N-MIP cartridge. In fact, comparing chromatograms A and B in Figure 7, NAM quantification is achievable in both cases, but in the second one, most of the other signals were removed, leaving mainly the NAM signal.

From our point of view, it can be developed as a new way to determine NAM concentration by an easier and cheaper technique than HPLC. The solution of the pork liver extract, after MISPE protocol, could be directly analyzed with a UV detector at 262 nm for NAM quantification. To this aim, the eluting fractions obtained by N-MIP cartridge purification of the spiked and blank pork liver samples were also analyzed by using a UV spectrophotometer. A calibration curve was prepared reporting absorbance at 262 nm versus NAM concentration in chloroform/EtOH 90 : 10 and used to determined NAM concentration in the eluting fractions. Recovery percentage was calculated similarly to that described for HPLC method and compared with the percentage of recovery previously determined from HPLC analysis (Table I) giving comparable results. Even if the analytical method needs to be validated (LOD, relative standard deviations, and linear range) to demonstrate its applicability, these preliminary evaluations are promising, and N-MIP polymer is under further investigation in our laboratory to develop MISPE method for a direct spectrophotometrical determination of NAM in food samples.

CONCLUSIONS

The MIP synthesized in this work showed specific NAM recognition properties and was able to provide an efficient extraction method and clean-up of NAM in pork liver samples. The cartridge was prepared by packing 100 mg of N-MIP particles, and its performances were investigated in NAM standard solutions. Finally, chloroform/ETOH 90/10 in loading step, chloroform as washing solvent, and ethanol for the elution of NAM were selected.

The optimization of MISPE protocol allowed to obtain a good percentage (around 87%) of recovery of NAM from pork liver with RSD of 8%, whereas a great loss of recovery was observed when NIP or RP-C18 sorbent were used.

Further, these preliminary evaluations are promising for a future development of an easier and cheaper technique than HPLC analysis. Therefore, after a MISPE clean-up step on complexes real matrices, the amount of NAM could be determined directly by using a UV spectrophotometer.

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