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Molecularly imprinted solid-phase extraction for cholesterol determination in cheese products

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Abstract

A new sorbent for molecularly imprinted solid-phase extraction (MISPE) to detect cholesterol (CHO) in food matrices able to work in an aqueous media was synthesized.

The proposed MISPE protocol could overcome the limitations of traditional detection methods, which require pre-treatments of the samples, such as saponification, extraction with organic solvents, etc. The possibility to obtain selective recognition of CHO from food matrices in an aqueous mixture without hydrophobical non-specific interactions is the main advantage of these materials. Another important advantage of our procedure is the direct HPLC analysis of eluate without any treatment.

In this study, three different MIPs, owing different hydrophilic characteristics were synthesized using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as crosslinking agent. After the evaluation of the selectivity of the CHO imprinted polymers, the performance of these materials as Solid-Phase Extraction (SPE) sorbents was investigated. Good recoveries were obtained in the evaluation of the MISPE–HPLC procedure, which can be successfully used for the determination of CHO in Calabrian pecorino cheese.

Keywords: Molecularly imprinted polymers; Solid-phase extraction; Cholesterol; Cheese products

1. Introduction

Low amounts of cholesterol (CHO) are essential for the human body because it takes part in several important biologic functions such as the manufacture of hormones (Adanyi & Varadi, 2003; Rozner & Garti, 2006). However, high level of blood cholesterol increases the risk of heart diseases (Okazaki et al., 2006). CHO, indeed, is involved in the atherosclerosis development and in heart degenerative processes and it is well-established that the drastic lowering of blood cholesterol concentration is followed by a reduction of clinical events and mortality (Sellergren, Wieschemeyer, Boos, & Seidel, 1998; Yetley & Park, 1995).

Cholesterol is also a constituent of animal foods such as eggs, meat and dairy products (Adanyi & Varadi, 2003). Determination of cholesterol content in food is of primary importance to select a diet with low intake of cholesterol. In the analytical practice, well known GC and HPLC methods are used for the cholesterol measurement, but a very intensive pre-treatment of the samples is needed (Zhang, Li, Liu, Chen, & Rao, 1999).

In the last few years a lot of research has gone into establishing rapid routine methods for the fast determination of cholesterol.

The most common and cheapest purification techniques are thin-layer chromatography (TLC) and Solid-Phase Extraction (SPE) (Boselli, Caboni, & Lerker, 1997).

This work, preliminarily presented at MIPs 2006 Conference (Puoci et al., 2006), reports on a novel approach

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based on the molecularly imprinted polymers (MIPs) for the clean-up and preconcentration of CHO from a complex food matrix and its HPLC detection.

MIPs are synthetic materials with high recognition properties for a target molecule named template. These specific binding properties must be attributed to specific interactions between the template and the functional groups in the polymeric network (Cheong et al., 1997; Wulff, 1995; Cunliffe, Kirby, & Alexander, 2005).

The technique to produce MIPs involves arranging the functional monomers around the template to form a prepolymerization complex by both covalent and non-covalent interactions. This complex is subsequently radically copolymerized in a solution containing a high ratio of a suitable crosslinker. After copolymerization, the template is removed from the polymer, leaving its imprint in the polymeric structure (Puoci et al., 2006; Ye, Yu, & Mosbach, 2001; Puoci et al., 2004).

As reported in the literature (Caro, Marce, Cormack, Sherrington, & Borrull, 2006), coupling MIPs and SPE is possible to combine the advantages of both molecular recognition and traditional separation methods. Molecularly imprinted solid-phase extraction (MISPE) presents the high specificity, selectivity and sensitivity of the molecular recognition mechanism and the high resolving power of separation methods (Puoci et al., 2007).

In our study, CHO imprinted polymers were synthesized by a non-covalent approach (Mosbach & Ramstrom, 1996; Alexander et al., 2006) using methacrylic acid (MAA) as a the functional monomer and ethylene glycol dimethacrylate (EGDMA) as a crosslinking agent.

Although a recent article (Shi et al., 2006) reported an MISPE procedure for the detection of CHO using no water–miscible organic solvents, a very time consuming procedure in the pre-treatment of the sample is required and an expensive derivatizing agent for GC analysis of eluate is needed.

On the contrary, we realized MIPs were able to recognize CHO in water–organic solvents mixtures and developed a very straight-forward protocol, involving only the crushing and filtration steps for the pre-treatment of the food samples, and the direct HPLC analysis of eluate solutions without any derivatization.

After the evaluation of the recognition properties of the materials by performing binding experiments in an aqueous media, MISPE cartridges were packed and their ability to selectively absorb cholesterol was studied by using two molecules similar to the template: in particular progesterone (PROG) and hydrocortisone (HY) were used for this purpose.

Finally, the ability of the MISPE cartridges to selectively absorb CHO from food matrices was investigated. This procedure allows to concentrate CHO and, after its elution for the cartridges, to immediately analyze the concentrated eluate by HPLC without any drying and derivatization process.

2. Materials and methods

2.1. Reagents and standards

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2,2′-azoisobutyronitrile (AIBN), cholesterol, progesterone and hydrocortisone were obtained from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO). All solvents were reagent grade or HPLC-grade and used without further purification and were provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

2.2. Preparation of molecularly imprinted polymers

The MIP stationary phase was prepared by bulk polymerization. Methacrylic acid was used as functional monomers to prepare the MIP by the non-covalent imprinting method. Briefly, template cholesterol, MAA, EGDMA and AIBN were dissolved in chloroform in a thick-walled glass tube. The tube was purged with nitrogen, sonicated for 10 min, and then photo-polymerized for 24 h with 360 nm light at 4 °C. After the photolysis, the tubes were incubated at 60 °C for 24 h (Schmidt, Belmont, & Haupt, 2005). The resultant bulk rigid polymer was crushed, grounded into powder and sieved through a 63 nm stainless steel sieve. The sieved MIP materials were collected and the very fine powders, suspended in the supernatant solution (acetone), were discarded. The resultant MIPs polymeric particles were soxhlet extracted with 200 ml of an acetic acid:tetrahydrofuran (1:1) mixture for at least 48 h, followed by 200 ml of tetrahydrofuran for another 48 h. The extracted MIPs materials were dried in an oven at 60 °C overnight and checked to be free from cholesterol and any other compound by HPLC analysis.

The formulations used for the preparation of the different matrices (MIP-1, MIP-2, MIP-3) are shown in Table 1. Blank polymers (to act as a control) were prepared under the same conditions without using the template.

2.3. Binding experiments

The binding experiments were performed in an acetonitrile:water mixture (7:3 v/v). The polymer particles (20 mg) were mixed with 1 ml cholesterol solution (0.2 mM) in a 1 ml eppendorf and sealed. Samples were shaken in a water bath for 6 h, centrifuged for 10 min (10 000 rpm) and the cholesterol concentration in the liquid phase was measured by HPLC. The amount of cholesterol bound by the polymer was obtained by comparing its concentration in the MIPs samples to the NIPs samples.

The same experiments were performed using progesterone and hydrocortisone solutions (Table 1).

Experiments were repeated five times.

Table 1
Polymers composition and percentage of bound analytes after 6 h

Polymers	CHO (mmol)	MAA (mmol)	EGDMA (mmol)	% Bound			α			ε	
				CHO	PROG	HY	CHO	PROG	HY	PROG	HY
MIP-1	1	8	25	30 ± 1	10 ± 0.4	3 ± 0.3	1.8	0.9	1.0	3.0	10.0
NIP-1	–			17 ± 0.7	11 ± 0.8	3 ± 0.2				1.5	5.7
MIP-2	1	12	25	30 ± 1	3 ± 0.5	3 ± 0.4	2.3	0.75	0.5	10.0	10.0
NIP-2	–			13 ± 0.5	4 ± 1	6 ± 0.7				3.3	2.2
MIP-3	1	16	25	28 ± 1	2 ± 0.7	3 ± 0.6	4.7	0.5	0.3	14.0	9.3
NIP-3	–			6 ± 0.5	2 ± 0.3	2 ± 1				3.0	3.0

All polymers were synthesized in 5.25 ml of chloroform using 0.045 g of AIBN.

2.4. Molecularly imprinted solid-phase extraction conditions

Five hundred milligrams of dry polymeric particles were packed into 6.0 ml polypropylene SPE columns. The columns were attached with a stop cock and a reservoir at the bottom end and the top end, respectively. The polymer was rinsed with chloroform, acetonitrile and then with the loading solvent.

CHO was dissolved in the loading solvent to the final concentration of 0.2 mM. After conditioning, dry MISPE columns were loaded with CHO standard solution. After column drying, washing solvent was passed through the cartridges and finally elution solvent was applied to perform the complete extraction of CHO. The loading, washing and eluting fractions were analysed by HPLC to detect the CHO amount. The MISPE protocol was optimized (Table 2) and the best conditions were.

Loading step: 2 ml of acetonitrile/water mixture (7/3 v/v); washing step: 7 ml of acetonitrile/water mixture (7/3 v/v); eluting step: 4 ml of hot acetonitrile (50 °C).

In order to evaluate the selectivity of the MIPs, optimized protocol was also applied using the PROG and HY solutions.

Experiments were repeated five times.

2.5. Cholesterol determination in Calabrian pecorino cheese

A Calabrian pecorino cheese sample and 200 mL of a choroform/methanol mixture (2/1 v/v) mixture were mixed and maintained under magnetic stirring for 2 h. After filtration, the solvent phase was washed twice with 90 mL of a KCl aqueous solution (0.9%) and 90 ml of distilled water, respectively, filtered through anhydrous Na_2SO_4 and evaporated under vacuum. The residue was reconstituted to solution by adding acetonitrile and the sample was analysed by HPLC.

2.6. Molecularly imprinted solid-phase extraction of food sample extracts

Twenty-five grams of the cheese was extracted with 150 ml of acetonitrile. The obtained solution was filtered and water was added to raise the loading solution concentration (acetonitrile/water 7/3 v/v). Eight millilitre of this solution was used to load the MISPE column. Two washing step were performed: 7 ml of an acetonitrile/water mixture (7:3 v/v) and 5 ml of an acetonitrile/water mixture (9:1 v/v). Finally, 4 ml of hot acetonitrile (50 °C) were used as elution fraction.

All the solutions were analysed by HPLC.

Experiments were repeated five times.

2.7. Instrumentation

The liquid chromatography consisted of a Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 208 nm for cholesterol and at 268 nm for progesterone and hydrocortisone. A 25 × 0.4 mm C4 Kromasil column, particle

Table 2
% of collected CHO in the loading, washing and elution fractions

Loading			Washing			Eluting CH_3CN (50 °C)	
CH_3CN/H_2O	MIPs	NIPs	CH_3CN/H_2O	MIPs	NIPs	MIPs	NIPs
10/0	4 ± 2.4	8 ± 2.1	10/0	55 ± 3.1	89 ± 3.3	41 ± 3.4	3 ± 1.1
			9/1	48 ± 2.2	90 ± 3.7	48 ± 2.4	2 ± 0.7
			8/2	44 ± 3.2	91 ± 3.1	52 ± 1.7	1 ± 0.9
			7/3	41 ± 1.7	88 ± 2.9	55 ± 2.2	4 ± 1.3
9/1	3 ± 1.2	7 ± 1.1	9/1	50 ± 2.7	86 ± 2.1	47 ± 3.6	7 ± 1.2
			8/2	43 ± 2.4	87 ± 2.8	54 ± 3.3	6 ± 1.6
			7/3	40 ± 2.8	84 ± 2.6	57 ± 2.8	9 ± 2.1
8/2	1 ± 0.4	5 ± 2.7	8/2	37 ± 1.9	85 ± 2.2	62 ± 3.3	10 ± 1.8
			7/3	31 ± 1.4	81 ± 2.1	68 ± 2.8	14 ± 2.6
7/3	1 ± 0.7	3 ± 1.4	7/3	24 ± 1.1	80 ± 2.0	75 ± 1.7	17 ± 1.1

size 5 μm (Teknocroma, Barcellona, Spain) was employed. The mobile phase was acetonitrile containing 0.5% of water for cholesterol and acetonitrile/water mixture (7/3 v/v) for progesterone and hydrocortisone. The flow rate was 1.0 ml/min.

The shaker and centrifugation systems consisted of a wrist action shaker (Burrell Scientific) and an ALC micro-centrifugette 4214, respectively.

2.8. Analytical parameters

Calibration curve and detection and quantitation limits (LOD and LOQ) were determined using CHO spiked Calabrian pecorino cheese. 0.050 g of cheese was spiked with 0.03, 0.06, 0.10, 0.30, 0.75, 1.5, 7.5, 15, and 30 mg of CHO. The samples were extracted with 150 ml of acetonitrile and then water was added to raise the right loading solution composition (acetonitrile/water 7/3 v/v), extracted using the MISPE protocol and analyzed by HPLC. Detection and quantification limits were calculated as the concentration corresponding to a signal 3 and 10 times the standard deviation of the baseline noise (American Chemical Society, 1980).

Repeatability of the MISPE method was evaluated by performing five repetitive analyses. The intraday and interday precisions of the relative peak areas were calculated as RSDs for five measurements.

3. Results and discussion

3.1. Preparation of the imprinted polymers

MIPs for selective detection of cholesterol were synthesized by using methacrylic acid as the functional monomers and ethylene glycol dimethacrylate as the crosslinker (Spizzirri & Peppas, 2005).

In order to maximize the interactions between the functional monomers and template in the prepolymerization complex, a photo-polymerization procedure was employed. The formation of the complex is a dynamic process and, when a template with poor functional groups like cholesterol is used, a low temperature is needed to reduce the kinetic energy of the system. In this case, indeed, a high temperature could drive the equilibrium away from the template-functional monomer complex toward the unassociated species (Cheong et al., 1997).

After UV irradiation at 4 °C for 24 h, the performance of the initially formed polymer was improved by thermal stabilization at 60 °C (Sellergren & Shea, 1993).

In the literature, many different ratios of template and functional monomer were used (Kempe & Mosbach, 1995). Our purpose was the selective extraction of CHO from food matrices with different water percentages. Although some MIPs exhibit moderate recognition properties under aqueous conditions, current technology could often fail to generate MIPs for use in the aqueous environment (Dirion, Cobb, Schillinger, Andersson, & Sellergren, 2003). Thus, we synthesized polymers at various molar ratios of methacrylic acid (Table 1) to carry out MIPs with increased hydrophilicity and better imprinting efficiency due to the reduction of the non-selective hydrophobic interactions.

3.2. Evaluation of the imprinting effect

The imprinting effect was initially evaluated by performing binding experiments in which amount of polymeric particles were mixed with both template and its analogues solutions. In Table 1, the percentage of CHO, PROG and HY bound by the imprinted and non-imprinted polymers after 6 h incubation was shown.

For each polymer the binding percentage and the binding efficiency, α, were calculated. α was calculated as the ratio of percentage of the bound template or analogues by MIPs and percentage of the bound analytes by NIPs.

As it is possible to note in Table 1, all the synthesized MIPs are able to rebind more template than the corresponding NIPs, confirming the presence of the imprinted cavities in their structure. Moreover, an increase of the amount of the functional monomer carries out an increase of the α value for CHO: the most effective polymers were the MIP-3 ones, and a considerable reduction of the corresponding percentage of bound CHO of NIP-3 was observed. The reduction of non-specific interactions was ascribable to the increased hydrophilicity of the polymers with the highest content of MAA. The binding percentages of the two analogues are much lower than that of CHO, confirming the selectivity of the imprinted cavities.

The experiments were performed in the same condition used for cholesterol. For each polymer ε values were calculated as the ratio of percentage of the bound template and percentage of the bound analogues by MIPs.

3.3. Optimization of the molecularly imprinted solid-phase extraction procedure

The most effective MIPs (MIP-3) as sorbents for SPE of cholesterol were investigated and a general procedure for a generic SPE (conditioning, loading, washing, and eluting) was employed (Puoci et al., 2005).

The cartridges were packed with 500 mg of the polymer and the loading and the washing steps were optimised. Different acetonitrile/water mixtures (10/0, 9/1, 8/2 and 7/3 v/v) were employed in the loading and washing step and hot acetonitrile in the eluting one (Table 2).

The best results were obtained when in the loading step an acetonitrile/water mixture (7/3 v/v) was employed. Both imprinted and non-imprinted polymers retain all the CHO loaded.

In order to eliminate the aspecific component of the interaction between the cholesterol and polymeric matrices

a washing step is needed and it was performed using an acetonitrile/water mixture (7/3 v/v).

The optimized elution was obtained employing hot acetonitrile. In the eluting fraction of MIPs cartridges, 76% of the loaded CHO was detected, while in the NIPs cartridges CHO was only 20%.

The selectivity of the packing cartridges was evaluated by using PROG and HY solutions in the same condition tested for CHO (Table 3).

For PROG solution, in the loading step, MIPs cartridges retain 95% of PROG, while the NIPs cartridges retain 81%. In the washing fraction of MIPs cartridges, 85% of PROG was detected, while in the NIPs samples only 57% of the analyte was recovered. In all the two steps, NIPs materials were found to retain much more PROG than MIPs ones, probably because of the presence of more non-specific interaction between the analyte and the polymeric matrices. In the eluting fractions, 10% and 24% of PROG were detected for the MIPs and NIPs cartridges, respectively.

For the HY solution, in the loading step, the MIPs cartridges retains 30% of HY, while the NIPs cartridges retain 27%. In the washing fraction of the MIPs cartridges, 25% of HY was detected, while in the NIPs samples 20% of the analyte was recovered. In the eluting fractions, 5% and 7% of HY were detected for the MIPs and NIPs cartridges respectively.

The last two experiments clearly showed the high selectivity of the synthesized materials. In the elution fractions, indeed, 76% of the loaded CHO was detected for the MIPs cartridges, while for the same cartridges, this value was only 10% for PROG and 5% for HY.

3.4. Molecularly imprinted solid-phase extraction of food sample extracts

Food samples are complex chemical matrices and in the chemical identification of their components, an intensive pre-treatment of the samples is always required. The use of selective sorbents such as MIPs can be very useful to obtain cleaner HPLC chromatograms (Caro, Marcé, Cormack, Sherrington, & Borrull, 2005).

In our experiments, Calabrian pecorino cheese was mixed with hot acetonitrile and crushed by stirring. After filtration, water was added to the acetonitrile to obtain the loading solution (acetonitrile/water (7/3 v/v)) and it was analyzed by HPLC (Fig. 1).

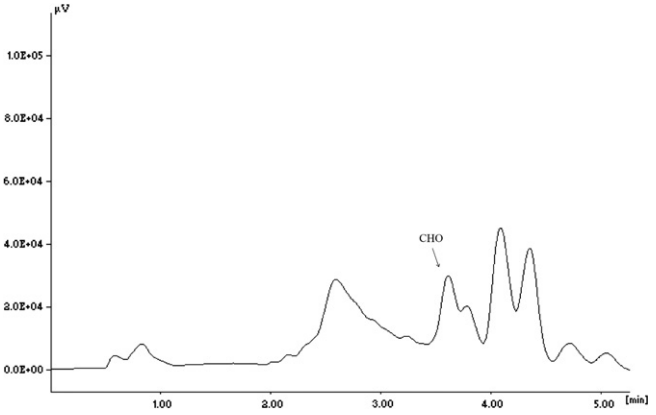

Fig. 1. Chromatogram of the food extract.

Food extract solution was loaded to the polymeric cartridges and the complete retention of CHO by the MISPE cartridges was raised (Fig. 2a). Two washing steps were performed: acetonitrile/water mixture (7/3 v/v) and acetonitrile/water mixture (9/1 v/v) respectively. In the washing fraction not so relevant peaks were observed at the retention time of CHO (Fig. 2b and c). Furthermore, a clean-up of the matrix was obtained as shown by the presence of several peaks referable to other compounds of the food sample. Finally, CHO was selectively eluted with hot acetonitrile.

As it is possible to note in Fig. 3, a selective recovery of CHO was obtained: 80% of the loaded CHO, confirming that the compounds, which co-eluted with CHO in the starting solution were completely removed during the MISPE procedure. The quantification of the CHO content in cheese was performed by a methodology reported in the literature (Contarini, Povolo, Bonfitto, & Berardi, 2002) with small modifications.

By performing the same experiments using the NIPs cartridges, in the elution fraction no relevant amount of CHO was detected.

3.5. Analytical parameters

Detection and quantitation limits (LOD and LOQ) correspond to 5.94×10^{-7} mol l^{-1} and 1.84×10^{-6} mol l^{-1}, respectively. The calibration curves were linear with correlation coefficients of $R^2 = 0.9982$.

The intraday precisions of the CHO relative peak areas were between 2.3% and 3.5%; the interday precisions were between 6.8% and 8.2%.

Table 3
% of collected PROG and HY in the loading, washing and elution fractions

Loading CH_3CN/H_2O 7/3				Washing CH_3CN/H_2O 7/3				Eluting hot CH_3CN (50 °C)			
PROG		HY		PROG		HY		PROG		HY	
MIPs	NIPs	MIPs	NIPs	MIPs	NIPs	MIPs	NIPs	MIPs	NIPs	MIPs	NIPs
5 ± 1.1	19 ± 1.2	70 ± 2.2	73 ± 2.7	85 ± 3.1	57 ± 2.9	25 ± 1.8	20 ± 2.3	10 ± 1.7	24 ± 2.2	5 ± 1.3	7 ± 1.2

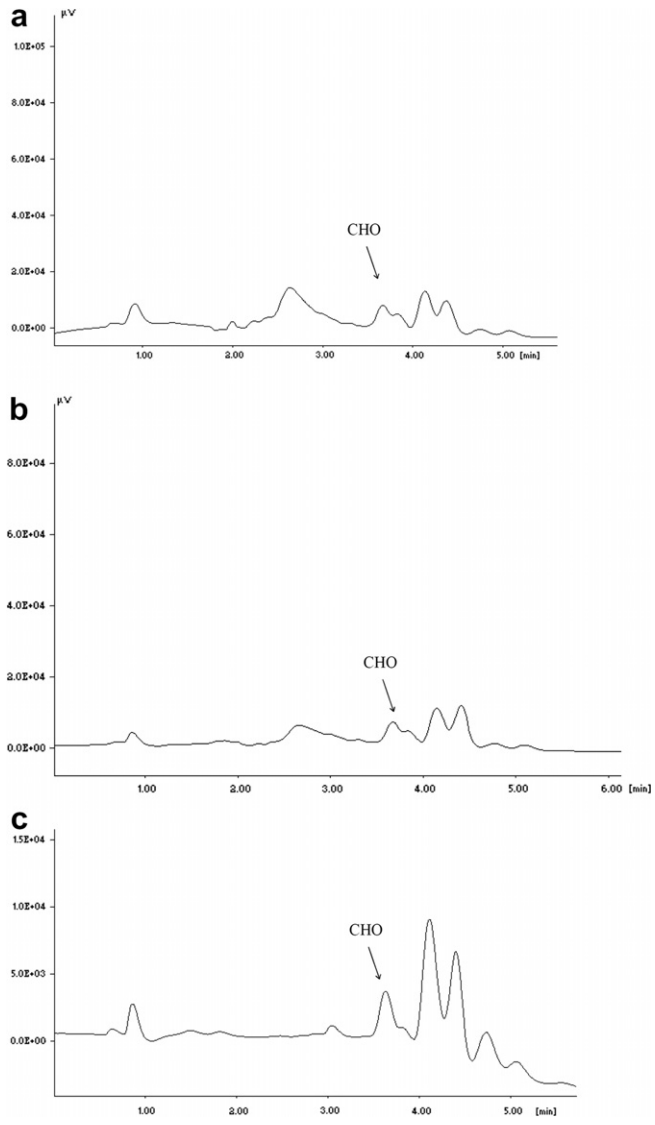

Fig. 2. Chromatograms of MISPE loading (a) and washing steps (b,c).

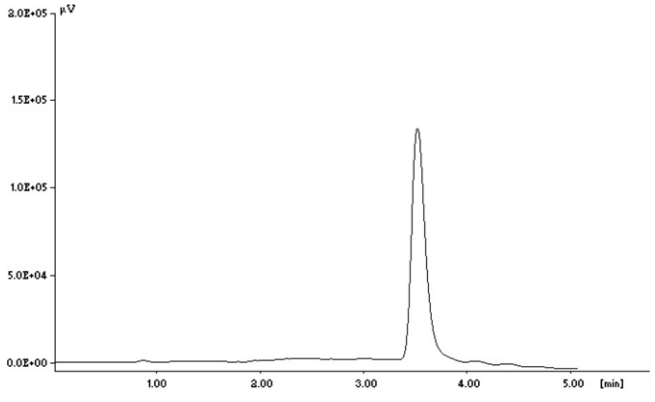

Fig. 3. Chromatogram of MISPE eluting step.

4. Conclusion

In this work, molecularly imprinted polymers have been synthesized using CHO as the template, and the obtained materials were applied as SPE selective materials. The imprinting effect and selectivity of the MIPs were evaluated by performing binding experiments in which CHO, PROG and HY solutions were employed. The MIPs were found to be highly selective for CHO. After the optimization of the MISPE protocols using standard solutions for all the analytes, the application of MISPE in complex food samples was demonstrated.

These new sorbents based on molecularly imprinted solid-phase extraction (MISPE) are able to work in an aqueous media and to clean/concentrate CHO in food matrices without any relevant pre-treatment of the sample. We used a cheese extract in acetonitrile to load the MIPs cartridges obtaining a selective purification and concentration of CHO in the elution step. In this fraction of MIPs cartridges, 80% of cholesterol was detected and the corresponding chromatogram shows only a peak at the retention time of the template.

These relevant results show that this method could be successfully applied for the determination of CHO in food samples.

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