Selective Molecularly Imprinted Polymer Obtained from a Combinatorial Library for the Extraction of Bisphenol A

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Abstract: In the present work, an analytical methodology based on molecularly imprinted solid-phase extraction (MISPE) has been developed for the determination of bisphenol A (BPA) in environmental and food samples. In order to select the optimum material, a combinatorial library of molecularly imprinted polymers in small-scale (mini-MIPs) was prepared using BPA as template. Different monomers (methacrylic acid or 4-vinylpyridine), crosslinkers (ethylene glycol dimethacrylate or trimethylolpropane trimethacrylate) and porogens (methanol, acetonitrile or toluene) were used leading to 24 different polymerisation mixtures. After BPA removal, the ability of mini-MIPs to recognise BPA was evaluated by equilibrium rebinding-elution experiments. The copolymer of 4-vinylpyridine (4-VP) and trimethylolpropane trimethacrylate (TRIM) prepared in toluene showed the higher affinity for the template. Subsequently, a scaled-up version of the optimum polymer was prepared and used in the development of MISPE procedures for the extraction of BPA. The optimised MISPE protocols were successfully applied to the selective extraction of BPA from soils and aqueous canned peas samples.

Keywords: Bisphenol A, molecularly imprinted polymer, combinatorial library, soils, aqueous canned foods.

INTRODUCTION

Bisphenol A (4,4'-isopropylidenediphenol, commonly named BPA) is widely used in the manufacture of chemical products including both epoxy and polycarbonate resins and flame retardants. Consequently, it is present in pipes, adhesives and coatings of cans for foodstuff packaging purposes among other items [1]. Its high production, widespread use and ubiquitous occurrence in the environment as well as its endocrine-disrupting properties are of concern for regulatory agencies [2]. In this sense, a Specific Migration Limit (SML) in food and food simulants has been set by the European Union to 3 mg/kg for BPA [3]. In addition, it has been predicted that ~50% of BPA in the environment has the potential to bind to sediments and soils [4] making necessary the evaluation of its concentration in those environmental compartments in order to perform a correct risk assessment.

Different analytical methods have been reported for the determination of BPA in different kinds of samples such as river waters [5, 6], river sediments [6], aqueous foods [7] and milk [8], among others, by gas or liquid chromatography coupled to mass and ultraviolet or fluorescence detectors, respectively. Such methods require the previous extraction and clean-up of BPA from samples by liquid-liquid or solid-phase (micro)extraction, which are not always successful due to the inherent lack of selectivity of these extraction methodologies. During last years, molecularly imprinted polymers (MIPs) have been proposed as useful materials in analytical chemistry [9]. Especially, they have been recognised as suitable selective sorbents to be used in solid-phase extraction [10-13], namely molecularly imprinted solid-phase extraction (MISPE).

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MIPs are synthetic polymers obtained by polymerising a monomer with a cross-linker around a template molecule in the presence of a suitable solvent. After polymerisation, the template is removed by washing, leaving cavities in the polymeric matrix complementary in size and shape to the template. Thus, theoretically, the imprinted polymer will be able to rebind selectively the analyte (the template) in certain experimental conditions. There are several variables, such as kind and amount of monomer or nature of cross-linker and solvent, which affect the final characteristics of the obtained materials in terms of capacity, affinity and selectivity for the target analytes. Thus, the optimisation of the MIP might take several weeks of trial-and-error experiments using different formulations, which has provoked an overuse of certain standard formulations [14]. Therefore, the optimisation of MIP formulations is an ideal candidate for a combinatorial approach making easier and faster the screening of various formulations.

However, in spite of the mentioned advantages, few attempts dealing with the use of combinatorial approaches in the molecular imprinting field have been made [15-18]. Thus, the aim of this paper is the preparation of an optimum MIP able to selectively recognise BPA following a combinatorial approach reducing the time devoted to the synthesis and the subsequent evaluation of materials. Besides, the optimum MIP will be used in the development of analytical methodologies for the determination of BPA in soils and aqueous canned peas samples.

EXPERIMENTAL

Reagents

Bisphenol A (BPA), methacrylic acid (MAA), 4-vinylpyridine (4-VP), trimethylolpropane trimethacrylate (TRIM), ethylene glycol dimethacrylate (EDMA) and 2,2´-azobis methylbutyronitrile (AIMN) were purchased from Sigma-Aldrich (Madrid, Spain). All other used chemicals

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were of analytical reagent grade obtained from Scharlab (Barcelona, Spain).

Preparation of Small-Scale MIPs

A previously reported procedure [16] for the synthesis of the small-scale MIP layers was adapted for the present work. Firstly, two pairs of mother solutions (with and without template) were prepared by mixing 943 μL of EDMA (5 mmol) or TRIM (3 mmol) and 12.5 mg of AIMN. Both template mother solutions also contained 0.25 mmol of BPA. Then, a volume of 20 μL of each mother solution, 45 μL of porogen (methanol, acetonitrile or toluene) and 1 mmol of monomer (MAA or 4-VP) were added into the corresponding 1.5 mL glass vials leading to 24 different polymerisation mixtures. Subsequently, the vials were sealed under nitrogen with silicon caps and placed in a water bath at 65 ºC for 8 h. The obtained thin polymer layers were repeatedly washed with methanol/acetic acid (9:1; v/v) until the template could not be detected in the extraction solvent. Typically, 4 washing cycles were enough to remove BPA completely and unreacted monomers from the mini-MIPs. Finally, the ability of mini-MIPs to recognise BPA was evaluated by equilibrium rebinding-elution experiments. Table **1** summarises the different polymerisation mixtures (monomer/crosslinker/porogen) used and the corresponding control and imprinted polymers (CPs and MIPs, respectively) evaluated in the present study.

Table 1. Small Scale Polymers Evaluated

Polymer	Template	Monomer	Cross-Linker	Porogen
CP1	---	MAA	EDMA	Toluene
CP2	---	MAA	EDMA	Acetonitrile
CP3	---	MAA	EDMA	Methanol
CP4	---	4-VP	EDMA	Toluene
CP5	---	4-VP	EDMA	Acetonitrile
CP6	---	4-VP	EDMA	Methanol
CP7	---	MAA	TRIM	Toluene
CP8	---	MAA	TRIM	Acetonitrile
CP9	---	MAA	TRIM	Methanol
CP10	---	4-VP	TRIM	Toluene
CP11	---	4-VP	TRIM	Acetonitrile
CP12	---	4-VP	TRIM	Methanol
MIP1	BPA	MAA	EDMA	Toluene
MIP2	BPA	MAA	EDMA	Acetonitrile
MIP3	BPA	MAA	EDMA	Methanol
MIP4	BPA	4-VP	EDMA	Toluene
MIP5	BPA	4-VP	EDMA	Acetonitrile
MIP6	BPA	4-VP	EDMA	Methanol
MIP7	BPA	MAA	TRIM	Toluene
MIP8	BPA	MAA	TRIM	Acetonitrile
MIP9	BPA	MAA	TRIM	Methanol
MIP10	BPA	4-VP	TRIM	Toluene
MIP11	BPA	4-VP	TRIM	Acetonitrile
MIP12	BPA	4-VP	TRIM	Methanol

Preparation of Normal-Scale Optimum MIP

Template molecule (BPA, 1 mmol), functional monomer (4-VP, 4 mmol) and 150 ml of dry toluene were placed into a 250 mL round-bottomed flask and the mixture was left in contact for 10 min. Subsequently, TRIM (12 mmol) and AIMN (0.88 mmol) were added. The flask was sealed and the mixture was purged with nitrogen for 15 min. Polymerisation took place in a water bath at 65 ºC for 12 h. Finally, the template was removed by Soxhlet extraction with methanol/acetic acid mixture (9:1; v/v) for 8 h, and the obtained polymer particles (10-50 μm) were air dried before storage in a glass container at room temperature. The corresponding control polymer (CP) was prepared as described above but without the addition of template.

Sample Preparation

The extraction of BPA from soil samples was carried out by sonicated-assisted extraction in small columns. This methodology was developed by Sánchez-Brunete, *et al*., and successfully employed for the extraction of pesticides in soils [19, 20]. Briefly, 5 g of soil was placed in a glass column equipped with a polyethylene frit. Fortified samples were obtained by adding a small volume of BPA standard solution to reach a final concentration of 40 ng/g. Then, soil samples were extracted with 5 mL of acetonitrile for 15 min in an ultrasonic water bath at room temperature. After extraction, columns were placed on a multiport vacuum manifold where the solvent was filtered and collected for further MISPE as described below. Regarding the aqueous canned food samples, just 1 mL of the aqueous phase in contact with canned foods (peas) was filtered through a 0.45 μm nylon syringe filter before the MISPE procedure.

Molecularly Imprinted Solid-Phase Extraction (MISPE) of Samples

The optimised molecularly imprinted polymer (100 mg) was placed in an empty solid-phase extraction cartridge and properly conditioned depending on the kind of sample to be analysed. Table **2** shows the entire MISPE process employed for the analysis of soil sample extracts and aqueous canned food samples. The final extracts were directly analysed by HPLC according to the procedure described below.

Table 2. Optimum Molecularly Imprinted Solid-Phase Extraction Procedure Followed for the Analysis of Soil and Aqueous Food Samples

	Soil Sample Extracts	Aqueous Canned Food Samples
Conditioning	10 ml of ACN	10 ml of ACN + 5 ml of water
Loading	5 ml of sample extract in ACN	1 ml of filtered aqueous sample
Washing	3 ml of ACN	5 ml of water + 5 ml of ACN
Elution	4 ml of 2% Ethanolamine in MeOH	4 ml of 2% Ethanolamine in MeOH

Chromatographic Analysis

HPLC measurements were made using a Hewlett-Packard 1100 Series HPLC instrument equipped with a qua-

ternary high-pressure pump, an autosampler and a fluorescence detector. A sample volume of 100 μL was injected into a Kromasil 5 ODS (150 mm x 4.6 mm i.d.) analytical column, and BPA was separated from other matrix components using a mobile phase consisting of acetonitrile/water (40:60; v/v) at a flow rate of 1 mL/min. BPA was monitored by fluorescence (λ_{exc} = 275 nm; λ_{em} = 320 nm) and quantified by external calibration using peak area measurements.

RESULTS AND DISCUSSION

Evaluation of Mini-MIPs

It is known that the template, monomer(s), cross-linker and solvent (porogen) are the key parameters for the obtainment of a successful selective MIP. Since all the parameters mentioned have a strong influence on the overall performance of MIPs in terms of affinity, selectivity, loading capacity, etc. their proper selection will ensure that polymers with the appropriate properties are obtained for a particular application. Thus, in order to identify the best reagents mixture, a series of mini-MIPs were prepared containing different combinations of functional monomers (MAA or 4-VP), cross-linkers (EDMA and TRIM) and porogen (methanol, acetonitrile and toluene) and subsequently, evaluated during equilibrium rebinding-elution experiments.

Initially, after removal of the template, 1 mL of a standard solution of BPA (1 mg/L) in acetonitrile was added to each vial. After incubation for 18 h at room temperature, the vials were directly placed in the autosampler and the amount BPA unbound to the polymers was determined by HPLC according to the procedure described in Experimental section. From the results obtained in this first series of experiments shown in Table **3**, it was already possible to discard those polymers prepared with combinations of MAA and EDMA since no recognition was observed in any of the imprinted (MIP1-MIP3) and control (CP1-CP3) polymers assayed. The remaining 18 polymers were able to interact with BPA regardless of whether imprinted or control polymers were used.

Table 3. Percentage of BPA Bound to Each Polymer After Incubation of 1mL of BPA Standard Solution (1 mg.l^{-1} in Acetonitrile) for 18 h at Room Temperature

Control Polymer	% Bound	Imprinted Polymer	% Bound
CP1	< 1	MIP1	< 1
CP2	< 1	MIP2	< 1
CP3	< 1	MIP3	< 1
CP4	17.0	MIP4	12.0
CP5	17.8	MIP5	11.6
CP6	19.3	MIP6	13.0
CP7	17.3	MIP7	10.0
CP8	18.4	MIP8	11.6
CP9	16.3	MIP9	13.1
CP10	11.5	MIP10	10.9
CP11	9.3	MIP11	14.0
CP12	9.2	MIP12	9.8

Next, the former solution was removed from the vials, and 1 mL of acetonitrile was added where it remained in contact with the polymeric layer for 24 h at room temperature in order to remove non-specific interactions. As shown in Table **4**, it is clear that the amount of BPA removed from CPs and MIPs for the systems based on VP-EDMA (CP4-CP6 and MIP4-MIP6) and MAA-TRIM (CP7-CP9 and MIP7-MIP9) was similar. These results suggest that the binding of BPA with these polymers was based on non-specific interaction, and thus the control polymers CP4-CP9 and the imprinted polymers MIP4-MIP9 were excluded from further experiments. However, according to the results shown in Table **4**, BPA was more strongly retained by the imprinted 4-VP-TRIM-based polymers (MIP10-MIP12) than by the corresponding CPs (CP10-CP12), due to the polarity of the porogen used in its synthesis. In this sense, although similar binding to the MIPs was obtained, lower non-specific binding was observed when toluene was used as porogen. These results can be attributed to the strong interaction of the phenol groups of BPA with two pyridyl groups by hydrogen bonding in apolar media during the pre-arrangement step of polymer preparation as suggested previously [21]. However, the results suggest that interaction BPA-MAA is not strong enough (even using toluene as porogen) to allow the creation of imprinted sites.

Table 4. Percentage of BPA Removed with 1 mL of Acetonitrile from Polymers After Incubation for 24 h at Room Temperature*

Control Polymer	% Removed	Imprinted Polymer	% Removed
CP4	35.7	MIP4	34.0
CP5	39.3	MIP5	40.6
CP6	37.2	MIP6	35.8
CP7	15.6	MIP7	17.3
CP8	14.4	MIP8	13.0
CP9	13.8	MIP9	13.2
CP10	77.4	MIP10	47.3
CP11	57.2	MIP11	45.1
CP12	47.2	MIP12	35.2

*Polymers were previously incubated with 1 ml of BPA standard solution (1mg.l^{-1}) in acetonitrile for 18 h at room temperature.

Besides this, the better performance of the TRIM-based polymers suggests a better accessibility of BPA to the binding sites likely due to the higher degree of cross-linking provided by TRIM. From these results, it is clear that the cross-linker is a key element in the preparation of MIPs. Thus, the imprinted polymer MIP10 (based on the combination of 4-VP as functional monomer, TRIM as cross-linker and toluene as porogen) was chosen as optimum for preparing the MIP on a large scale for subsequent experiments.

It is important to stress that the preparation and evaluation of the 24 polymers were performed in only 5 working days. If traditional methodologies of preparing MIPs in large scale had been followed, no less than 30 working days would

have been necessary to perform polymer synthesis and the corresponding evaluation. In this regard, it is evident that the use of a combinatorial approach reduced to a large extent the time devoted to synthesis and evaluation of MIPs.

Optimisation of MISPE Procedures

The aim of this work was the extraction and clean-up of BPA from complex samples by a MISPE protocol. As in other SPE procedures, the common steps of conditioning, sample loading, washing and elution had to be optimised. In general, loading solvent is chosen in order to stabilise analyte-monomer interaction, allowing rebinding of the analyte to specific sites, whereas the elution solvent should be optimised taking into account its ability to disrupt such interaction. However, it is also important to point out that the loading solvent has to be optimised in each application in order to minimise sample handling and to prevent non-specific interactions. Taking these comments into account as well as the fact that molecular recognition is often more efficient in the solvent used as porogen during polymer preparation, toluene, acetonitrile and water were evaluated as loading solvents. Table **5** shows the recoveries and breakthrough data for BPA obtained during the loading and subsequent washing steps of 1mL of a solution of BPA (1 mg/L) in the different solvents assayed both onto the imprinted and controlled polymers. According to these results, the amount of BPA remaining bound to the MIP after the corresponding washing steps is always higher than that bound to the CP. Thus, it is clear that selective molecular recognition takes place regardless of the solvent used during the loading step. However, the amount of non-specific interactions is dependent on the loading solvent and such interactions are more important if water is used. It seems clear that the extraction of BPA from water is predominantly dominated by hydrophobic interactions which are partially disrupted by acetonitrile (Washing 2 in Table **5**) in CP, whereas BPA remains bound to the MIP to a larger extent. This result demonstrates that analyte is able to diffuse from non-specific locations to the imprinted sites by a solvent switch in the MIP and opens the possibility of direct MISPE of BPA from aqueous canned food samples. On the other hand, as expected, BPA is strongly retained mostly through specific interactions in both toluene and acetonitrile since BPA-4-VP interactions are clearly stabilised in such media. However, the use of toluene required the inclusion of long drying steps in order to remove toluene traces completely that otherwise disrupted the final detection of BPA. Subsequently, toluene was discarded and acetonitrile was selected for the MISPE of BPA from soil sample extracts.

Once the loading and washing conditions were established, it was necessary to find out the optimum elution conditions to allow quantitative elution of BPA. Accordingly, methanol, acetonitrile/acetic acid, methanol/acetic acid, acetonitrile/ethanolamine, and methanol/ethanolamine were evaluated for their ability to disrupt BPA-4-VP interactions present in the MIP. In this study, it was observed that only those mixtures with ethanolamine in their composition were able to quantitatively elute BPA. However, only a 2 % solution of ethanolamine in methanol was able to elute BPA in a low volume of elution solvent (4 mL) preventing further sample dilution. Any attempt to reduce the elution volume by increasing the amount of ethanolamine in the mixture was unsuccessful, and thus the above mentioned mixture was chosen as optimum for subsequent studies.

MISPE of BPA from Different Samples

As stated above, one of the objectives of this study was the evaluation of a MISPE procedure for the extraction of BPA from both environmental and food samples due to the environmental and health risk associated with the widespread use of BPA. Subsequently, the developed MISPE procedures were evaluated for the cleanup of soil sample extracts (organic media) and canned peas and olives (aqueous media) at low concentration levels.

Fig. **1** shows the chromatograms obtained with and without MISPE of spiked (40 ng/g) soil sample extracts. As can be observed, the detection of BPA without clean-up is not possible due to interferences appearing in the chromatograms which produced serious signal suppression at the retention time of BPA. However, BPA was quantitatively recovered (80 ± 12 %, n = 3) and easily determined free of co-extractives at a very low concentration level after clean-up of sample extracts by the proposed MISPE procedure. Based on this preliminary evaluation, it seems clear that the developed MISPE procedure is an appropriate method for the selective monitoring of BPA in soil samples at low concentration levels.

In parallel, the imprinted polymer was also evaluated for the extraction of BPA from aqueous canned foods by the developed MISPE procedure for the analysis of aqueous samples. Fig. **2** shows the chromatograms obtained with and without MISPE of spiked aqueous phase sample in contact

Table 5. Recoveries of BPA (1 µg) Obtained in the Breakthrough and Washing Fractions in Both Imprinted and Control Polymers Regarding the Used Loading Solvent

Loading Solvent	Recovery (%)							
	Control Polymer				Imprinted Polymer			
	Breakthrough	*Washing 1*[a]	*Washing 2*[b]	*% Bound*	*Breakthrough*	*Washing 1*[a]	*Washing 2*[b]	*% Bound*
Toluene	6	16	65	13	n.d.[c]	2	10.9	87.1
Acetonitrile	6.5	71	---	22.5	3.2	21	---	75.8
Water	n.d.[c]	0.6	39.8	59.6	n.d.[c]	n.d.[c]	18.9	81.1

[a]Washing 1 was performed with 5 ml of the same solvent used for loading.
[b]Washing 2 was performed with 5 ml of acetonitrile.
[c]not detected.

with canned peas. It can be easily observed that the huge peak appearing at the beginning of the chromatogram without MISPE is almost completely eliminated after clean-up allowing BPA to be recovered (78 ± 10%, n = 3) and determined at a concentration of 1 mg/kg, which is 3 times lower than the SML established by the European Union [3].

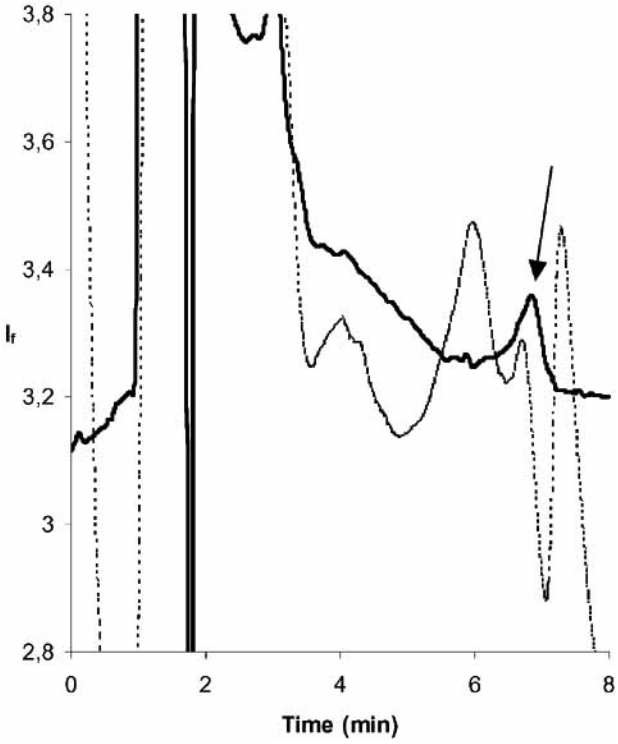

Fig. (1). LC-FLD chromatograms obtained without (dotted line) and with (solid line) MISPE of soil sample extracts spiked with BPA (40 ng/g). Arrow indicates the peak corresponding to BPA. Chromatographic conditions were as given in the Experimental section.

CONCLUSIONS

In this work, the potential of combinatorial chemistry for the optimisation of MIP formulations has been demonstrated resulting in the selection of a 4-VP-TRIM-based imprinted polymer as optimum for the extraction and clean-up of BPA from complex samples. The developed MISPE protocols were applied successfully to the selective extraction of BPA from soils and aqueous canned peas prior to its final determination by HPLC with fluorescence detection at low concentration levels. Consequently, further research is underway in our laboratory in order to extend the field of application of the proposed methodology to other complex samples as well as to BPA derivatives.

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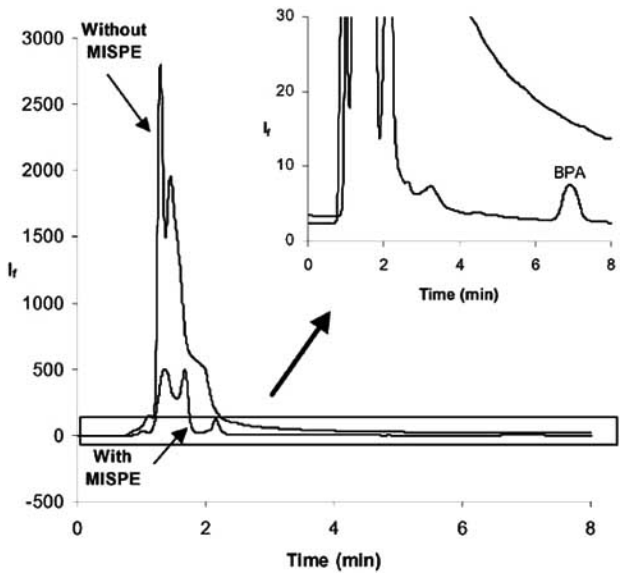

Fig. (2). LC-FLD chromatograms without and with MISPE of canned peas spiked with BPA (1 mg/kg). Graph insert shows the same chromatograms with different scale. Chromatographic conditions were as given in the Experimental section.

REFERENCES

[1] Dermer, O.C. In *Encyclopedia of Chemical Processing and Design;* McKelta, J.J.; Cunningham, W.A., Eds.; Marcel Dekker: New York, **1977;** Vol.4, 406.
[2] Kuiper, G.G.J.M.; Carlsson, B.; Grnadien, K.; Enmark, E.; Haggblad, J.; Nilsson, S.; Gustafsson, J.-A. *Endocrinology*, **1997**, *138*, 863.
[3] European Commission Directive 90/128/EEC, *Off. J. Eur. Commun.*. **1990**, L75, 19.
[4] Staples, C.A.; Dorn, P.B.; Klecka, G.M.; O'Block, S.T.; Harris, L.R. *Chemosphere*, **1998**, *36*, 2149.
[5] Suzuki, T.; Nakagawa, Y.; Tacano, I.; Yaguchi, K.; Yasuda, K. *Environ. Sci. Technol.*, **2004**, *38*, 2389.
[6] Patrolecco, L.; Capri, S.; De Angelis, S.; Polesello, S.; Valsecchi, S. *J. Chromatogr. A*, **2004**, *1022*, 1.
[7] Nerin, C.; Philo, M.R.; Salafranca, J.; Castle, L. *J. Chromatogr. A*, **2002**, *963*, 375.
[8] Casajuana, N.; Lacorte, S. *J. Agric. Food Chem.*, **2004**, *52*, 3702.
[9] Mayes, A.G.; Mosbach, K. *Trends Anal. Chem.*, **1997**, *16*, 321.
[10] Martin-Esteban, A. *Fresenius J. Anal. Chem.*, **2001**, *370*, 795.
[11] Masque, N.; Marce, R.M.; Borrull, F. *Trends Anal. Chem.*, **2001**, *20*, 477.
[12] Lanza, F.; Sellergren, B. *Chromatographia*, **2001**, *53*, 599.
[13] Chapuis, F.; Pichon, V.; Hennion, M.-C. *LC-GC Europe*, **2004**, *17*, 408.
[14] Batra, D.; Shea, K.J. *Curr. Opin. Chem. Biol.*, **2003**, *7*, 434.
[15] Takeuchi, T.; Fukuma, D.; Matsui, J. *Anal. Chem.*, **1999**, *71*, 285.
[16] Lanza, F.; Sellergren, B. *Anal. Chem.*, **1999**, *71*, 2092.
[17] Takeuchi, T.; Fukuma, D.; Matsui, J.; Mukawa, T. *Chem. Lett.*, **2001**, 530.
[18] Dirion, B.; Cobb, Z.; Schillinger, E.; Andersson, L.I.; Sellergren, B. *J. Am. Chem. Soc.*, **2003**, *125*, 15101.
[19] Sanchez-Brunete, C.; Pérez, R.A.; Miguel, E.; Tadeo, J.L. *J. Chromatogr. A*, **1998**, *823*, 17.
[20] Castro, J.; Sánchez-Brunete, C.; Tadeo, J.L. *J. Chromatogr. A*, **2001**, *918*, 371.
[21] Haginaka, J.; Sanbe, H. *Chem. Lett.*, **1999**, *8*, 757.

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