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Determination of polybromodiphenyl ethers (PBDEs) in milk cream by gas chromatography-mass spectrometry

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Abstract

A method to analyse polybromodiphenyl ethers (PBDEs) in milk cream has been optimized. The six PBDEs targeted by the method were chosen on criteria of toxicity and occurrence in environmental matrices. Three methods of extraction were tested and compared in terms of lipid recovery yields and repeatability. The selected sample preparation process includes two steps: extraction by accelerated solvent extraction (ASE) and purification by solid phase extraction (SPE). The preferred method of extraction used a hexane / methylene chloride / methanol (5:2:1, v:v:v) solvent mixture. Three extraction cycles were carried out per sample at a temperature of 80°C and a pressure of 1500 psi. The method was validated on milk cream samples spiked with the PBDEs of interest. The recoveries for the whole sample preparation process (extraction and cleanup) for cream samples spiked at 10 ng/g and 100 ng/g were greater than 80 % (ranging from 81 to 106 %) at both concentrations for BDE-99, -100, -153 and 154. Recoveries were lower (ranging from 65 to 75 %) for BDE-28 and BDE-47. PBDEs were quantified by GC/MS detection with selected ion monitoring (SIM) using three ions formed by electron capture. The method was successfully tested on real samples.

Keywords:

Polybromodiphenylethers, milk cream, gas chromatography, mass spectrometry, accelerated solvent extraction, solid phase extraction

Introduction

Polybromodiphenylethers (PBDEs) are widely used as flame retardants in many industrial products; they are added to polymers which are used in plastics, TV, computers, building materials and textiles to slow down or inhibit fires (Alaee et al. 2003; De Wit 1999). They are of environmental concern because of their high lipophilicity and high resistance to degradation processes; they readily bioaccumulate and have potential endocrine disrupting properties; they can induce genetic recombination, which causes a number of diseases including cancer (Rahman et al. 2001; Helleday et al. 1999). The main findings concerning toxicology of PBDEs are changes in liver weight accompanied by histological alterations in animals (Fernlof et al. 1997). *In vitro* studies have shown that PBDEs can induce an estrogenic activity (Meerts et al. 2001). Recent studies indicate that the OH and H₃CO-PBDEs main metabolites of PBDEs can interfere with CYP17, an enzyme which catalyzes an important step in sex steroidogenesis and is responsible for the biosynthesis of dehydroepiandrosterone (Rocío et al. 2006).

During the last few years, PBDEs have been detected in environmental matrices such as soil, sludge (Eljarrat et al. 2004) and sediment (De la Cal et al. 2004; Zegers et al. 2003) as well as in biological matrices: human serum (Covaci et al. 2005), adipose and organ tissue (Saito et al. 2004; Li et al. 2005). Much research interest has recently been focused on the occurrence of PBDEs in mother's milk of countries such as Japan (Akutsu et al. 2003; Eslami et al. 2005), Sweden (Lind et al. 2003), Norway (Thomsen et al. 2002), Turkey (Erdoğan et al. 2004) and Czech Republic (Kazda et al. 2004). A European Commission Directive has prohibited the use of PBDEs in new electric and electronic components (Commission Directive RoHS 2002/95/EC) since July 1st 2006. The detection of PBDEs is traditionally performed by GC/MS.

The aim of the present study was to develop a method for PBDEs detection in milk cream, a raw material potentially higher in contamination than the finished product due to the lipophilic property of PBDEs. It was decided to validate the method on cream so that it could be used for the analysis of a variety of dairy products. Special emphasis was given to sample preparation since the development of GC-MS methods (Pirard et al. 2003; Thomsen et al.

2002) as well as the influence of chromatographic injection parameters (Tollbäck et al. 2003; Björklund et al. 2004) have been the object of several recent studies.

PBDEs have 209 possible congeners, depending on the number and positions of the bromine atoms on the two phenyl rings. Although the fully brominated congener deca-BDE is, by far, the main PBDE incorporated into manufactured products, the majority of PBDEs detected in biological samples are lower brominated compounds; this is assumed to result of the very poor capacity of highly halogenated compounds to accumulate in biological tissues (Darnerud et al. 2001, Jianwen et al. 2007). Six congeners were retained for this study because they are the most widely encountered in environmental matrices (Darnerud et al. 2001) and they are also suspected to be among the most toxic PBDEs (Rahman et al. 2001): the tri-BDE-28, tetra-BDE-47, penta-BDEs-99 and -100, hexa-BDEs-153 and -154 congeners.

Materials and methods

Standards and samples

BDE-28 (2,2',4-tribromodiphenyl ether), BDE-47 (2,2',4,4'-tetrabromodiphenyl ether), BDEs-99 (2,2',4,4',5-pentabromodiphenyl ether) and 100 (2,2',4,4',6-pentabromodiphenyl ether), BDEs-153 (2,2',4,4',5,5'-hexabromodiphenyl ether) and 154 (2,2',4,4',6,6'-hexabromodiphenyl ether) and 4,4'-dibromodiphenyl (Purity > 99%) were purchased from Cambridge Isotope Laboratories (Andover – USA). Hexane, nonane, acetone, methanol, acetonitrile, diethyl ether and methylene chloride were purchased from Sigma-Aldrich (Saint Quentin Fallavier - France); all were at least 99.9 % pure. Anhydrous Na₂SO₄ was purchased from Merck (Fontenay Sous Bois, France) and "hydromatrix" (cleaned and sieved diatomaceous earth) from Varian (Les Ulis – France). The samples of milk cream were extracted from milk batches delivered from different regions. The analyzed cream cheese is a commercial product. Toxitubes[®] A and B and C18 cartridges for solid phase extraction (SPE) were purchased from Varian (Les Ulis, France).

Development of the sample preparation process

The sample preparation process requires two steps. The first one, referred as "extraction", consists in extracting the lipid content from the cream; the second one, referred as "cleanup", consists in separating PBDEs from the fat.

Extraction

Three extraction methods were tested and compared in terms of lipid recovery yields: liquid-liquid extraction (LLE), accelerated solvent extraction (ASE) and extraction with toxitubes A and B.

Liquid-liquid extraction was carried out in a 100 mL separatory funnel. To 1 g of cream were added 10 mL of methanol and 10 mL of the extraction solvent: pentane (method 1) and hexane (method 2) were tested. The separatory funnel was manually agitated and the organic phase was transferred into a tube. 3 mL of a saturated solution of potassium oxalate were added drop to drop to break the emulsion. The methanolic phase was extracted again with 10 mL of the extraction solvent and the second organic phase was added to the first one.

ASE extractions were carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA) fitted with 11- mL ASE cells. Two protocols were tested. Each one used 5 g of cream thoroughly mixed with 3 g of Hygromatrix. The first protocol was proposed by Saito et al. to extract lipids from human adipose tissue (Saito et al. 2004). It uses a binary methylene chloride / acetone (1:1, v:v) mixture at a temperature of 100°C and a pressure of 1500 psi; the ASE cell is purged during 60 s with 5.5 mL of solvent. This first protocol involves two extractions per sample. The second protocol was derived from an application note by Dionex to extract PBDE from human breast milk (Dionex application note 2004). It uses a tertiary hexane, methylene chloride, methanol (5:2:1, v:v:v) mixture at a temperature of 80°C and a pressure of 1500 psi; the ASE cell is purged during 240 s with 6.0 mL of solvent. The second extraction protocol includes three extractions per sample.

Extraction with toxitubes was performed according to the following protocol. 4 mL of water were added to x g of cream; three values of x were tested: 0.10, 0.25 and 0.60. The mixture was transferred into a toxitube B, automatically mixed by inversion before being centrifuged at 3500 g during 15 min at 25 °C.

In all extraction processes, the resulting extract was dried with anhydrous Na₂SO₄. The Na₂SO₄ residue was rinsed with two aliquots of 2 mL of the extraction solvent. The organic extract was then taken using a 20 mL syringe and filtered on two 25 mm Acrodisc® syringe filters (Pall Life Sciences - Ann Arbor - USA). The mass of extracted fat was determined by gravimetry, according to the following procedure. Before extraction, a collecting vial was dried in an oven at 100°C for 30 min, cooled in a desiccator for 1 hour and weighted. After

extraction, 1 mL of extract was added in the vial; the solvent was evaporated under a N₂ stream at 35°C. The residue was then dried at 100°C for 1 hour, cooled in a desiccator for 1 hour and weighed to determine the exact mass of extracted fat.

Cleanup

The cleanup involves a SPE protocol which has been derived from the one described by Bordet et al. for the cleanup of polychlorinated biphenyl, organochlorine pesticides and pyrethrinoids from fatty food (Bordet et al. 2003). A C18 cartridge (cartridges containing 2 g and 5 g of phase were tested) was conditioned with 5 mL of diethyl ether, then 5 mL of acetone, then 10 mL of methanol and allowed to dry. The extracted fat was solubilized in 2 mL of acetonitrile which were loaded onto the cartridge. The tube was rinsed either with 20 mL of acetonitrile or with four aliquots of 5 mL of acetonitrile (recovery yields are compared in the results and discussion part). The elution was carried out at about one drop per second. The solvent was then evaporated under a N₂ stream at 45 °C. 100 µL of nonane were added for GC-MS analysis.

The retained sample preparation process

Figure 1 summarizes the experimental protocol used to develop the sample preparation process. It shows that ASE has been preferred to the other extractions modes; the ASE protocol number 2 has been retained. The cleanup of the ASE extract was finally performed on the 5g-C18 SPE cartridge. The detailed results of method development are given in the "results and discussion" part.

Please insert Figure 1 near here

GC-MS analysis

All analyses were carried out on a Varian "CP3800" gas chromatograph fitted with a "CP8400" autosampler and connected to a Varian "1200" triple stage quadrupole mass spectrometer. The chromatographic separation was performed on a 60 m "Factor four VF-XMS" capillary column (internal diameter: 0.25 mm, film thickness: 0.25 µm) from Varian. Helium was used as the GC carrier gas and the flow-rate was maintained constant at 1.4 mL/min owing to an electronic flow controller. All experiments were performed by automatically injecting the sample in the splitless mode at a rate of 50 µL/sec. 1 µL of sample was injected in the experiments concerning the development of the method but a value of 1.5

μL has been finally retained for the validation procedure according to the detection thresholds required. The injector temperature was 280°C. The split valve opened after 0.8 min, with a split ratio of 35/100. The capillary column was ramped from an initial temperature of 100°C, held for 1 min, increased at 20°C/min up to 300°C where it was held for 14 min. The total duration of GC analysis was 25 min. The transfer line was maintained at 300°C. The ion source and quadrupole temperatures were 150°C and 40°C, respectively. Tuning of the mass spectrometer was automatically performed using the ions resulting from ionization of perfluorotributylamine. Electron capture ionization was performed using methane as thermalization gas at a pressure of 800 Pa. The filament emission current and the electron energy were set to 150 μA and 150 eV, respectively. The multiplier voltage was automatically set to 1200 V. All experiments were performed in the negative mode, with a scan time of 0.4 sec/spectrum.

Results and discussion

GC-MS

In the electron attachment mode, all PBDEs dissociate to provide only Br⁻ and HBr₂⁻ product ions, in agreement with results of previous work. Under electron attachment, 4,4' – dibromodiphenyl mainly provides molecular ions M⁻ and Br⁻ ions. A SIM (Selected Ion Monitoring) method has thus been developed. Three ions are monitored for PBDEs (m/z 78.9, m/z 80.9 and m/z 160.8) and for the internal standard (m/z 78.9, m/z 80.9 and m/z 311.9). The main disadvantage of this method is its lack of specificity since some brominated compounds may interfere with PBDE peaks in the chromatogram but several studies showed that electron attachment is the ionization mode, which provides by far the lowest detection thresholds for PBDEs. The analytical performance of the GC/MS method for solutions of standards are summarized in Table 1. Limits of quantification (LOQ) were determined for a signal-to-noise ratio of 10; they range from 10 pg/mL for tri and tetra-BDE to 80 pg/mL for hexa-BDE. Coefficients of variation were determined for ten injections at two concentrations: 2.5 and 30.0 ng/mL and ranged between 0.5 and 17.7 %. For all the PBDEs which were investigated, the coefficient of correlation (r²) was greater than 0.995 for a concentration range from the LOQ to 50 ng/mL.

Please insert Table 1 near here.

Extraction

All the extraction protocols described in the experimental part were tested; they are compared in Figure 2 in terms of lipid recovery yields and repeatability. The liquid-liquid extraction process using pentane as the extraction solvent gave the greater recovery yields, with a mean value for ten extractions of 90.6 % (with a CV of 4.3 %). ASE gave a mean value of 82.6 % (CV = 5.3 %) for the method using a tertiary mixture of solvents. Two kinds of toxitubes are available on the market: toxitubes A for the extraction of alkaline compounds and toxitubes B for acidic compounds. Both toxitubes were tested to extract PBDEs from milk cream. Adding cream into a toxitube A led to a strong emulsion which was impossible to break. The extraction protocol using toxitubes B was tested with three amounts of cream: 0.10, 0.25 and 0.60 g (referred as tests 1, 2 and 3, respectively, in figure 2). In all cases, it provided lower recovery yields (with a maximum value of 70 %) with coefficients of variation ranging from 5 to 15 %. Given the slight difference (8 %) between the recovery yields obtained with ASE and those obtained with “classical” LLE extraction, ASE was retained for further experiments because it is much less solvent consuming and fully automatizable. Furthermore, ASE permits the processing of several samples (up to 24 with the apparatus used in this study) at the same time.

Please insert Figure 2 near here

Cleanup

We estimated the selectivity of the cleanup process by determining the amount of fat eluted through the SPE cartridge as a function of the amount of sample deposited into it. Four amounts were tested: 50, 250, 500 and 1500 mg. Fat recovery yields are compared in Figure 3. The uncertainties displayed on it were determined as follows. M_{fe} and M_{fp} being the mass of fat in the sample after extraction and the mass of fat recovered after purification, respectively, the recovery yield of fat (Γ_f) is defined as $\Gamma_f = (M_{fp} / M_{fe}) \times 100$ and the associated uncertainty is $\ln(\Gamma_f) = \ln(M_{fp}) - \ln(M_{fe}) + \ln(100)$. $\Delta(\Gamma_f) = \Gamma_f \times [\Delta(M_{fp}) / M_{fp} + \Delta(M_{fe}) / M_{fe}]$. The uncertainty on the weighting of fat is 0.1 mg. Figure 3 shows that fat is well retained on the C18 phase. Even with a sample amount of 1500 mg, the fat was barely eluted (only about 4 %) and the cartridge was not plugged. The successive use of two cartridges may thus be considered where cleanup of large amounts of sample is required. A loading of 500 mg provided a fat recovery of 0.7 %, meaning that 99.3 % of the fat was

retained on the C18 phase. This value of 500 mg has been retained for further optimization of the purification step.

Please insert Figure 3 near here

Figure 4 shows two chromatograms of the same extract of cream spiked at 0.5 ng/g for each PBDE. The upper chromatogram shows the results from an extract purified on a SPE cartridge containing 2 g of C18 phase; it is very noisy and shows evidence of insufficient selectivity of the cleanup using the 2g cartridge. The lower chromatogram shows the results from the same extract purified on a SPE cartridge containing 5g of C18 phase; it permits the detection of the six analytes. 5g-cartridges have thus been retained for further experiments.

Please insert Figure 4 near here

The recovery from the SPE cleanup step was determined for each PBDE using the GC/MS method described above. A cream extract was divided into six aliquots of 500 mg; 30 ng of each PBDE were added to each aliquot. Cleanup was carried out from each spiked sample according to the procedure described in the experimental part. The tube containing the ASE extract was rinsed with 20 mL of acetonitrile. Mean recovery yields from the cream extract ranged from 43 % for BDE-154 to 79 % for BDE-28 and the associated coefficients of variation for n=6 ranged from 6 to 26 %. Considering the poor recovery yields and the large values of the corresponding CV, the influence of the rinsing of the tube containing the ASE extract was studied. The SPE process was applied to extract a sample spiked in PBDEs at 50 ng/g of fat. The tube was rinsed eight times with 5 mL of acetonitrile. After each rinsing, the rinsing solution was deposited onto the cartridge and eluted. Each corresponding collected fraction was analyzed by GC/MS. In Figure 5 the recovery yields are plotted as a function of the number of 5 mL-eluted fraction(s) collected. For all PBDEs, the recovery increased with the number of collected fractions to reach a maximum for 5 fractions collected. Under these conditions, recoveries were between 90 and 96 %, according to the PBDE considered. The CV on cleanup recovery yields for five extractions ranged from 1.0 to 5.4 % according to the individual PBDE. This experiment showed the importance of carrying out several rinses and allowed excellent recovery to be achieved with a good CV.

Please insert Figure 5 near here

The influence of the concentration in PBDE on the recovery was studied as follows. The cleanup was applied to ten samples of 500 mg of extracted cream; five were spiked with 5 ng and five were spiked with 50 ng of each PBDE. Considering each PBDE, the difference in mean recovery yields (comparable to those obtained in the experience described in the previous paragraph) between both concentrations did not exceed 9 % and showed no evidence of a correlation between the recovery yield of the cleanup step and the initial PBDE concentration in the sample.

Figure 6 shows the mean recovery yields for the method overall, i.e. taking into consideration ASE extraction and SPE cleanup. They were determined by spiking ten samples of cream, five at 10 ng/g and five at 100 ng/g in each PBDE. Mean recovery yields range from 65 to 101 % for samples at 10 ng/g and from 69 to 106 % for samples at 100 ng/g. There is no evident concentration effect since recovery yields do not significantly differ comparing both concentrations for a given PBDE. The associated coefficients of correlation range from 1.1 to 5.5 % for the samples spiked at 10 ng/g and from 3.4 to 9.3 % for the samples spiked at 100 ng/g, showing the good repeatability of the sample preparation procedure. Gel permeation chromatography (GPC) (Kazda et al. 2004; Dodder et al. 2002; Allae et al 2001) and adsorption chromatography (Päpke et al. 2004; Kazda et al. 2004; De Boer et al. 2000, 2001, 2002) have been widely used for the cleanup of PBDEs extracted from biological compounds. The former technique is not available in our laboratories. Nowadays, "classical" adsorption chromatography with silica and/or alumina columns tends to be replaced by SPE which reduces solvent consumption and allows total automation.

Please insert Figure 6 near here

Method validation

In preliminary tests, traces of PBDEs (especially BDE-47) were systematically detected in creams from different regions and contamination by plastic tubes has been suspected. The whole sample preparation method has been carried out two times on two cream samples. In the first extraction, Eppendorf plastic tubes were used to store samples; in the second one, plastic tubes were replaced by glass tubes. The comparison of results obtained by GC-MS analysis clearly showed a PBDE contamination of the sample by the plastic tubes. Even

though the amounts of PBDEs detected were very low (below the quantitation threshold of the method), plastic tubes were discarded of the sample extraction procedure in further work.

Cream samples were spiked at six concentrations ranging from 0.1 to 10 ng/g of fat for BDEs-28, -47, -99 and -100, and from 0.2 to 20.0 ng/g of fat for BDEs-153 and -154 for which preliminary tests showed higher detection limits. To estimate possible matrix effects, standard solutions of PBDEs were prepared at the same concentrations. In each case the internal standard (dibromobiphenyl) was added at 0.10 ng/g. The chromatographic response was proportional to the PBDE concentration in cream on all the studied concentration range with $r^2 > 0,995$ for each congener. The comparison of calibration lines with and without matrix interferences shows a matrix effect so slight that it can be neglected. Accuracy and repeatability were estimated on four consecutive dosages at two concentrations in PBDEs: 0.6 and 6.0 ng/g of fat for BDEs-28, -47, -99 and -100, 1.2 and 12.0 ng/g for BDEs-153 and -154. In terms of repeatability, the method provides satisfying CV ranging from 6.3 to 14.5 % at the lowest concentration and from 3.5 to 4.6 % at the highest one. In terms of accuracy, the determined amounts are systematically lower than expected: between 78 and 93 % of the expected amount at the lowest concentration and between 78 and 87 % of the expected amount at the highest one. The method systematically under evaluates the PBDE concentration in cream by about 10 to 20 %. Given the good recovery ratios (> 90 %) of the cleanup process, this under-evaluation suggests that all PBDEs are not in the fat and that some of them remain in the aqueous phase during the extraction step.

Application to real samples

Milk cream

Figure 7 shows the chromatograms obtained from batches of cream from four different suppliers. In all cases, traces amounts of BDE-47 were detected below the limit of quantitation of the method. The mean value of BDE-47 in these cream extracts could very empirically estimated at 0.02 ng/g of fat. Other PBDEs among the six congeners were not detected.

Please insert Figure 7 near here

Cream cheese

The applicability of the method was also tested on cream cheese. Four samples of 22 g of cream cheese underwent ASE extraction with poor recovery yields of fat ranging from 45 to 47 %. After SPE cleanup, the four extracts were analyzed by GC-MS. Quantification was not possible because of the difficulty to get good fat recovery from cream cheese, but there were

nevertheless indications of the presence of BDEs-47, -99 and -100 at low levels when samples were analysed.

Conclusion

A method has been developed for routine analysis of six polybromodiphenyl ethers (PBDE) in milk cream. Special attention has been focused on PBDEs extraction from cream. Sample preparation involves two steps. The first one consists in extracting fat from cream by ASE. The second step, referred as "cleanup", uses SPE to separate PBDEs from fat. Recovery yields of the whole sample preparation process were estimated between 65 and 101 % at 10ng/g, with CV ranging from 3.5 % to 14.5 % for n=6, according to the PBDE considered. PBDEs were analyzed by GC-NCI-MS. The GC-MS method suffers a lack of selectivity because the PBDEs which were analyzed provided only Br⁻ and HBr₂⁻ ions under electron attachment conditions, in agreement with previous work; nevertheless, it is very sensitive and permitted to reach detection thresholds of 0.1 ng/g of fat for tri-, tetra- and penta-BDEs and 0.2 ng/g of fat for hexa-BDEs. In terms of accuracy, the method systematically under estimates the PBDE concentration in cream by about 10 to 20 %, suggesting that some PBDEs remain in the aqueous phase during the extraction step. Evidence has been found of sample contamination by the PBDEs contained in plastic parts of the apparatus, which must be removed from the sample preparation equipment and replaced by glass parts. The analysis of real samples of cream and cream cheese showed the reliability of the method. Some traces of BDE-47 were detected in different milk cream batches. BDEs-47, -99 and -100 were found in cream cheese samples. In all cases, PBDEs were not detected at concentrations above the limit of quantification of the method (0.1 ng/g).

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Captions for figures

Figure 1. Experimental protocol used to compare the methods of extraction and to optimize the C18-SPE purification step

Figure 2. Mean recovery yields of fat from cream and corresponding CV (n=10) for each protocol of extraction tested. Numbers on the abscissa axis refer to the method used; the corresponding methods are described the text.

Figure 3. Recovery yields of fat and coefficients of variation associated to the SPE purification step as a function of the amount on fat deposited onto the 2g-C18 cartridge

Figure 4. Comparison of chromatograms recorded from extracts of cream. The upper chromatogram results from an extract purified on a SPE cartridge containing 2 g of C18 phase. The lower chromatogram results from the same extract purified on a SPE cartridge containing 5 g of C18 phase.

Figure 5. Cumulated recovery yields, for each PBDE, as a function of the number of 5 mL-eluted fractions collected

Figure 6. Mean recovery yields for the complete sample preparation process determined at two concentrations of PBDEs in cream: 10 ng/g and 100 ng/g

Figure 7. GC-NCI-MS chromatogram of a milk cream extract

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Figure 6. Mean recovery yields for the complete sample preparation process determined at two concentrations of PBDEs in cream: 10 ng/g and 100 ng/g

Analyte	LOD ^a (pg/mL)	LOQ ^b (pg/mL)	Linearity range	r ²	CV (%)	
					at 2.5 ng/mL	at 50.0 ng/mL
BDE-28	3	10	10 pg/mL 50 ng/mL	0.999	4.5	0.5
BDE-47	3	10	10 pg/mL 50 ng/mL	0.998	6.2	0.7
BDE-99	8	20	20 pg/mL 50 ng/mL	0.998	5.8	1.1
BDE-100	8	20	20 pg/mL 50 ng/mL	0.997	5.2	2.2
BDE-153	25	80	80 pg/mL 50 ng/mL	0.997	17.7	3.6
BDE-154	25	80	80 pg/mL 50 ng/mL	0.998	10.1	3.3

Table 1. Main characteristics of the GC-NCI- MS method on standard solutions

^a Limit of detection

^b Limit of quantitation