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Blank and sample handling troubleshooting in ultratrace analysis of alkylphenols and bisphenol A by liquid chromatography tandem mass spectrometry

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ABSTRACT

Blank contamination is a notorious problem in the ultratrace analysis of alkylphenols and bisphenol A. The achievement of low detection limits is complicated due to the high background signals. Furthermore, overestimations and underestimations in the analytical results can occur when blank levels are not stable. Thus, a review of sources of blank contamination in this type of analysis was carried out. Several sources of contamination were identified and useful guidelines are proposed for the determination of these compounds in water samples by liquid chromatography coupled with mass spectrometry. The system contamination was maintained below 0.09 ng (reagent blank) for all compounds and below 0.003 $\mu g\,L^{-1}$ (procedure blank). The main improvement was obtained by using LC-MS grade solvent in the mobile phase and PTFE syringe filters for the filtration of the sample extracts. Sample handling aspects such as filtration and storage of the water samples were also considered. The filtration of the samples should be avoided because both contamination and adsorption problems were observed when different kinds of filters were assayed. The refrigerated storage of water samples should be limited to 5 days (without addition of methanol) or 8 days (with 5% methanol).

1. Introduction

Alkylphenols (APs) and bisphenol A (BPA) are of increasing concern because of their endocrine disrupting properties [1]. APs are the degradation products of the non-ionic surfactants AP polyethoxylates (APEs), which are widely used worldwide in agricultural, industrial and domestic applications. Furthermore, these compounds are used as plasticizers in high density polyethylene (HDPE), polyethylene terephthalate (PET) and polyvinyl chloride (PVC) [2] and also in the manufacture of textiles, paper and agricultural chemical products.

Nonylphenol (NP), which is a technical mixture of 211 branched nonyl-chain isomers (~90% *para*-nonylphenols (4-NPs)), and 4-*tert*-octylphenol (4-*t*OP) are the most important alkylphenols due to their toxicological properties [3].

Bisphenol A (BPA) has been used as a material for the production of epoxy resins, phenol resins, polycarbonates, polyesters, lacquer coatings on food cans, and also in flame retardants, adhesives, and as a component of electronic circuits [4].

The behaviour of APs and BPA as xenoestrogens and their frequent presence in the environment mean that these compounds are highly regulated. The new European water legislation, Directive 2008/105/EC [5], establishes very strict environmental quality standards (EQS) for the presence of 4-NP ($2\,\mu g\,L^{-1}$ maximum level and $0.3\,\mu g\,L^{-1}$ annual average) and 4-*tert*-octylphenol ($0.1\,\mu g\,L^{-1}$ and $0.01\,\mu g\,L^{-1}$ annual average) in surface waters. Bisphenol A is included in Annex II of the Directive 2008/105/EC as a substance to be regulated in the future.

In order to confirm the required low levels of the aforementioned compounds, very selective and sensitive analytical methods are necessary. The chromatographic determination of these compounds is performed by either liquid chromatography (LC) [4,6–8] or gas chromatography (GC) [9–13] coupled with mass spectrometry detection. Due to the polarity and low volatility of alkylphenols, analysis by GC requires in most cases the derivatization of the compounds to obtain good chromatographic peaks and good precision [14]. For this reason, the technique most frequently used for the determination of APs and BPA is liquid chromatography coupled to mass spectrometry (LC-MS) [8,15,16].

'Blank' contamination problems, i.e., large peaks observed even without a sample (procedure blank), are often encountered during the analysis of alkylphenols (especially NP) and BPA. This blank

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contamination could result in the high detection limits. Blank subtraction does not always correct blank problems efficiently. When these blanks do not remain constant, the subtraction can cause quantitation errors. Consequently, it is important to maintain the blank contamination as low as possible, below a critical value.

There are numerous factors that could contribute to the presence of blank contamination in AP and BPA analysis. As pointed out before, AP and BPA are used in the manufacture of plastics and therefore, the use of plastic materials should be avoided in all steps of the analytical determination. AP polyethoxylates are also used in the fabrication of cleaning products, and for this reason it is also important to avoid the use of detergents in the cleaning of laboratory glassware in order to reduce blank contamination problems.

NP can also be present in laboratory air. For example, concentrations of 64 ng m^{-3} for 4-*t*OP and 103 ng m^{-3} for NP were found in the air of a typical laboratory [17] and it has also been reported that an LC-MS vial with fresh MeOH left in the autosampler of the instrument can absorb NP from the laboratory air within weeks [3]. Septa vials and solvents used as mobile phases can also cause blank contamination [17]. BPA contamination was observed in ultrapure water and this was caused by the water purification system [18,19]. Certain components of the LC instrument, such as mobile phase plastic tubes and connections, could be another important source of contamination [20,21].

Sampling, storage of samples, filtration and sample treatment are also important sources of blank contamination. APs and BPA in water samples are commonly extracted by LLE [11] or solid-phase extraction (SPE) [7,12,15,22,23], but this procedure can also cause contamination due to the plastic materials of the SPE cartridges [3], the multiple steps involved and the use of relatively high volumes of solvents. Nowadays SPE is being replaced by other techniques like solid-phase microextraction (SPME) [24,25], dispersive liquid liquid microextraction (DLLME) [26] and stir bar sorptive extraction (SBSE) [10,27,28]. These techniques minimize the waste of organic solvents and simplify the extraction process (fewer steps), in accordance with the principles of Green Analytical Chemistry [29]. As a result, the sources of contamination are also reduced.

In this work a study of blank contamination problems in all the steps involved in the ultratrace analysis of APs and BPA in water samples was carried out. A literature review was performed and, although some papers mentioned blank contamination problems in the analysis of these compounds, to the best of our knowledge this is the first study that considers in detail several sources of contamination and proposes different guidelines to minimize blank problems. In fact, a considerable improvement in the procedure blanks was achieved by following these guidelines (<0.003 μg L^{-1}). In this way, a smaller volume of water sample is needed to achieve the low limits required and green analytical methods could be applied. In this case, the extraction of aqueous samples was carried out by DLLME, thus reducing the sample manipulation and consequently the sources of contamination. Furthermore, sample handling aspects such as filtration and storage of the aqueous samples were considered. Although the paper is focused on the analysis of aqueous samples, some of the points studied – such as instrument blank contamination, cleaning of material and filtration of extracts – are also applicable in the analysis of AP and BPA in any kind of liquid or solid sample.

2. Experimental

2.1. Chemicals and reagents

Nonylphenol technical mixture (NP) (94% purity, ±1% tolerance) and 4-*n*-nonylphenol (4-*n*-NP) (99.9% purity, ±0.5% tolerance) were obtained from Riedel-de Haën (Seelze, Germany). Bisphenol A (BPA) (99% purity, ±0.5% tolerance), 4-*tert*-octylphenol (4-*t*OP) (97% purity, ±0.5% tolerance) and 4-octylphenol (4-OP) (99% purity, ±0.5% tolerance) were obtained from Sigma-Aldrich (Steinheim, Germany). All standard solutions (1000, 10 and 1 mg L^{-1}) containing the aforementioned compounds were prepared in methanol (SPS grade) from Romil Ltd. (Cambridge, United Kingdom) and stored at 4 °C. As surrogate internal standards, 4-*n*-nonylphenol-2,3,5,6-d_4 (NP-d_4) (99.3%) was obtained from CDN Isotopes (Pointe-Claire, Canada) and a solution of bisphenol A-d_{16} (BPA-d_{16}) (99.5%) in acetonitrile (100 mg L^{-1}) was supplied by Dr. Ehrenstorfer GMBH (Augsburg, Germany).

For the determination, HPLC grade methanol, LC-MS PAI methanol and ammonia (30%) for instrumental analysis were obtained from Panreac (Barcelona, Spain). Water was purified with a Direct 5 Milli Q system (Millipore, Bedford, MA, USA). For the extraction, Chromasolv® 1-octanol (HPLC grade, 99%) was obtained from Sigma-Aldrich Co. (Madrid, Spain). Seawater samples were used for the sample handling assays.

Glass vials with a white screw cap with bonded PTFE/silicone septa (Waters, Milford, MA, USA) were used for the injection. The syringe filters tested were as follows: Iso-Disc™ N-13-2, (nylon 0.2 μm) supplied by Supelco (Bellefonte, PA, USA), PTFE (polytetrafluoroethylene) 0.2 μm filter (Teknokroma, Barcelona, Spain), and PVDF (polyvinylidene fluoride) 0.45 μm Millex ®HV (Millipore, Carrigtwohill, Ireland).

Filtration assays of samples were carried out using 0.6 μm glass fibre filters MN GF-6 (Macherey Nagel, Düren, Germany), 0.45 μm cellulose nitrate-acetate filters and GNWP 0.2 μm nylon membrane filters (Millipore, Bedford, MA, USA).

2.2. Liquid chromatography-tandem mass spectrometry

LC separation was performed using an Agilent HP-1200 Series LC system fitted with an autosampler (injected volume 25 μL), a binary solvent pump and a thermostatic column oven. The chromatographic separation was carried out with a Hypersil Gold C_{18} column (150 × 2.1 mm, 3 μm, Thermo Fisher Scientific Inc., Waltham, MA, USA), using a mobile phase consisting of A (water) and B (methanol) both with 0.05% of ammonia as modifier. The initial equilibrium time was 7 min at 20% B. The gradient was then performed as follows: 20% B during 1 min; increased from 20% to 40% B from 1 min to 4 min; hold at 100% B from 4 to 12 min and return to initial conditions in 2 min. The total run time was 14 min. The flow rate was 0.250 mL min^{-1} and the oven temperature was set at 40 °C.

The MS/MS determination was performed in an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA, USA). The system was equipped with an APCI/ESI source from Agilent Technologies (Palo Alto, CA, USA). Optimisation of MS/MS settings was performed by an automatic function of the MS software (Analyst 1.4 Applied Biosystems, Carlsbad, CA, USA) using a direct infusion of individual standard solutions. APs and BPA were analysed by electrospray ionization in negative ion mode. The ion spray voltage was set at the maximum level (−4500 V). The relevant instrument settings for each compound are shown in Table 1 [30]. In the case of BPA-d16, the parent ion was 243 instead of 244 because this compound becomes BPA-d15 in water [31].

2.3. Extraction and quantitation

Water samples were analysed by dispersive liquid–liquid microextraction [30]. In brief, the sample (30 mL) was placed in a glass centrifugation tube and extracted with 100 μL of 1-octanol on a Vibrax-VXR agitation plate (IKA, Staufen, Germany) during

5 min at 1200 rpm. Separation of two phases occurred upon centrifugation (Eppendorf 5804, Madrid, Spain) at 3500 rpm for 3 min. The fine droplets of 1-octanol were collected and the volume was adjusted to 1 mL with methanol due to the immiscibility of the 1-octanol with the LC mobile phase. Finally, the extract was passed through a 0.45 μm PTFE syringe filter prior to injection into the HPLC system.

Quantitation was carried out using the deuterated compounds as internal standards in blank assays and as surrogate standards in the analysis of samples.

3. Results and discussion

3.1. Cleaning of laboratory glassware

The effective cleaning of laboratory glassware is mandatory in order to reduce blank problems. It is known that detergents should be avoided in the analysis of APs and BPA; however, there is a lack of agreement in the literature concerning the cleaning of glassware for routine analysis. The most frequent cleaning process involves rinsing with water and then with an organic solvent (typically acetone) followed by baking the glassware (except for volumetric glassware) for 2 h at 400 °C [32] or 4 h [23] or more than 8 h at 120 °C [7], 320 °C [33] or 450 °C [17]. Alternative approaches include cleaning with AP-13 Extran alkaline soap, rinsing with acetone and water and then baking at 110 °C overnight [34] or cleaning with chromic acid [35]. UNE EN 18857-1 Standard Method proposes the cleaning of glassware by rinsing with acetone or baking at 250 °C for 2 h [11].

In this work, three different glassware cleaning procedures were evaluated: (i) cleaning with alkaline soap (24 h) and then rinsing with milli-Q water, acetone and methanol; (ii) cleaning with solvents: acetone and methanol; or (iii) rinsing with acetone, baking at 350 °C overnight and rinsing with methanol in order to remove possible residues of calcination.

The glassware cleaned by each of the three procedures was then used for the analysis of blank water samples, using the procedure described above, and the obtained extracts ($n=2$) were injected into the LC-MS system.

As can be seen in Fig. 1, blank contamination is too high for 4-*t*OP, NP and BPA whereas it is minimal for 4-OP and 4-*n*-NP. All of the cleaning procedures gave similar results in the case of alkylphenols. However, in the case of BPA, higher blank contamination was observed when cleaning was carried out with soap. Rinsing with acetone and methanol was chosen as cleaning procedure because this is the fastest and simplest protocol that gives low blanks.

3.2. LC-MS system: instrument blanks

As mentioned in the introduction, the LC-MS system itself can be a source of contamination in the analysis of plasticizers.

Table 1
Parent and fragment ions, retention times and MS/MS parameters for each compound.

Analyte	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Declustering potential (V)	Collision energy (V)
BPA	9.40	227 $[M-H]^-$	212*	−40	−24
			133		−32
BPA-d^{16}	9.39	241 $[M-D]^-$	142*	−45	−44
			221		−46
4-*t*OP	10.62	205 $[M-H]^-$	133*	−45	−30
			116		−76
NP	10.89	219 $[M-H]^-$	133*	−40	−38
			116		−74
4-OP	10.99	205 $[M-H]^-$	106*	−50	−26
			–		–
4-*n*-NP	11.23	219 $[M-H]^-$	106*	−45	−28
			119		−44
NP-d^4	11.23	223 $[M-D]^-$	109*	−45	−28
			129		−46

* Quantitation ion.

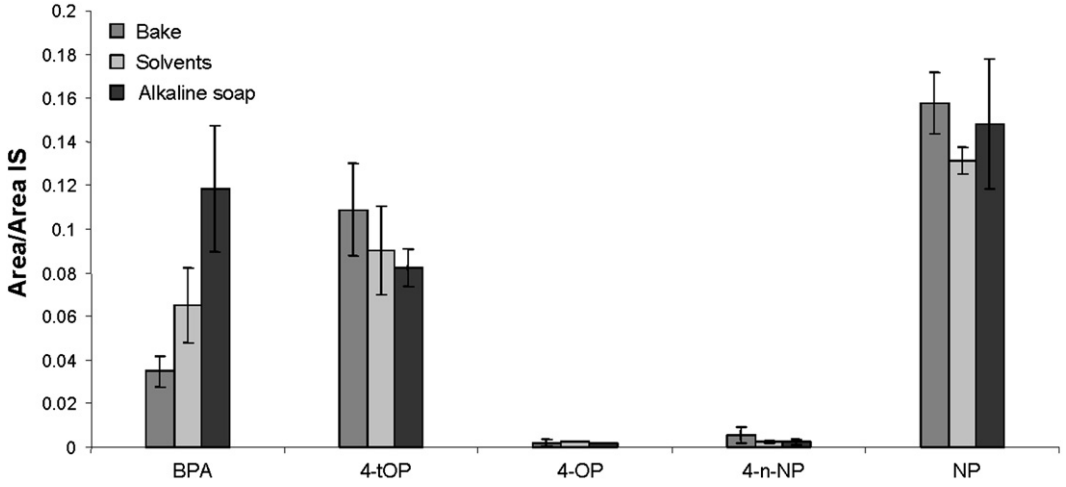

Fig. 1. Influence of the glassware cleaning method on the blank contamination.

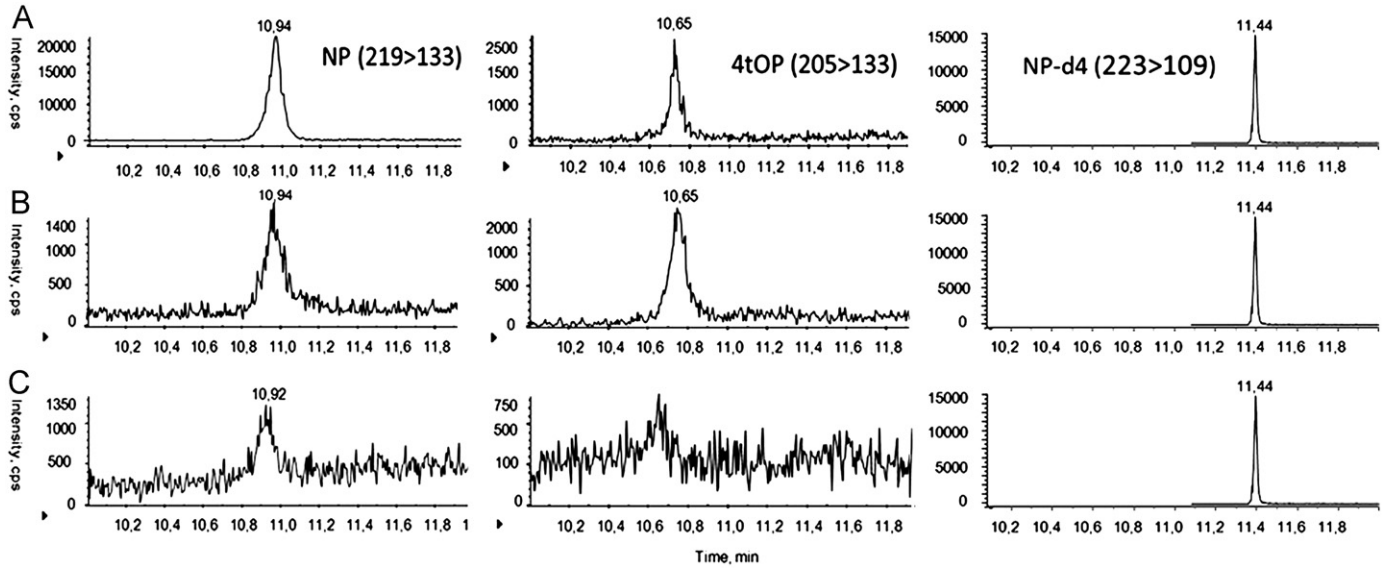

Fig. 2. Chromatograms for the analysis of 20 μL of methanol at initial conditions (A), when the water purification system was changed (mobile phase) (B), and using LC-MS grade methanol as the mobile phase (C).

According to the literature, septa vials, mobile phase plastic tubing and connectors [20,21] and solvents [3] could be sources of blank contamination in the analysis of these compounds. Recently, some authors employed an additional isolator column attached before the injection valve to separate background contaminants [36]. However, this proposal can produce loss of sensitivity and asymmetric peaks (with tails and/or fronts). Moreover, overpressures and clogging can occur; consequently, greater system maintenance is required. In order to minimize the blank signal and avoid these disadvantages, other cheaper and simpler alternatives were considered, taking into account all sources of contamination.

Substantial peaks for alkylphenols (especially NP) were initially observed when 20 μL of methanol (reagent blank) was directly injected into the HPLC-MS/MS system. Three replicates of reagent blank were carried out and an average concentration of 2.2 ng for 4-*t*OP and 26 ng for NP were obtained. The chromatogram of one of these injections was shown in Fig. 2A. BPA, 4-OP and 4-*n*-NP were not detected. In order to evaluate the main sources of contamination and reduce reagent blanks, the previously mentioned LC-MS sources were studied.

Septa vials were evaluated as a possible source of this contamination. Methanol samples from a vial with a PTFE/silicone septum and from a vial without a septum were injected and compared. Significant differences were not observed between the two experiments and therefore, septa vials were ruled out as a source of blank contamination.

Moreover, an instrument blank (run without injection) was performed in the same conditions and differences between this injection and the MeOH injection (reagent blank) were not detected; consequently, it can be concluded that contamination is originated in the LC system itself.

After that, mobile phase solvents were tested. HPLC grade methanol and water from a Milli-Q Gradient A10/Elix water-purification system were the mobile phases used in the experiments represented in Fig. 2A. The water purification system was replaced by a Milli-Q Direct 5 system, which minimizes the contact with plastic reservoirs. As can be seen from the chromatogram in Fig. 2B, the blank reduction was considerable, with peaks for 4-*tert*-octylphenol and nonylphenol that correspond to an average concentration ($n=3$) of 0.15 ng and 1.6 ng, respectively. LC-MS quality solvents were also tested as the mobile phase. A reduction in blanks was not observed when milli-Q water was replaced by the LC-MS grade water. Nevertheless, a significant decrease in the 4-*tert*-octylphenol peak area was obtained when LC-MS grade methanol was used, and the nonylphenol was reduced to 0.09 ng ($n=3$), as it was shown in Fig. 2C.

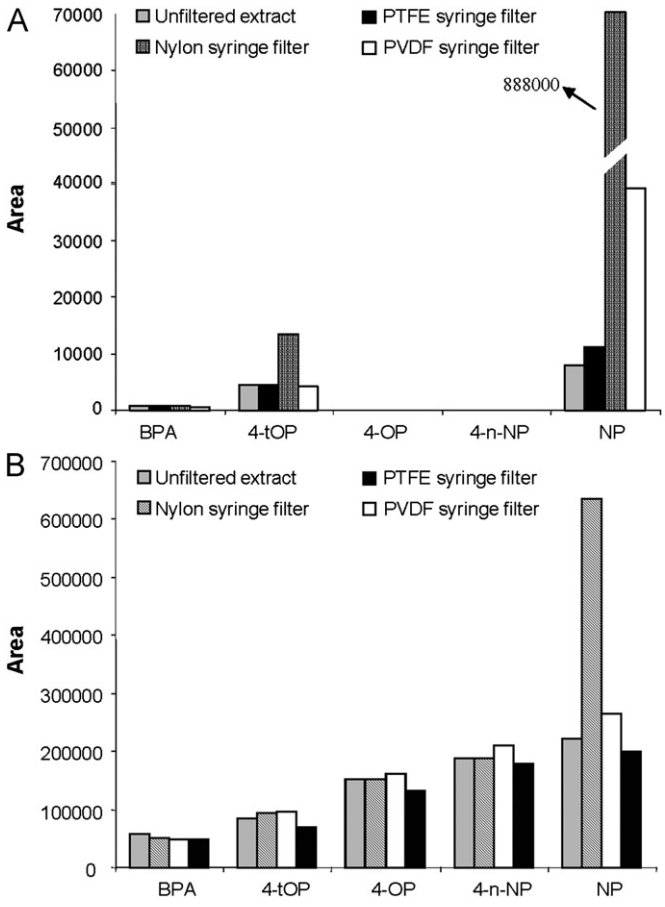

Fig. 3. Study of different syringe filters. Blanks obtained (A) and adsorption of APs and BPA on filters (B).

In the final part of this study, the LC mobile phase tubing and connectors were tested. In order to evaluate the possible contribution of the tube material, Teflon® tubes were replaced by PEEK tubes. This change did not lead to an effect on the NP blank signal.

3.3. Filtration prior to LC-MS injection

Even sample solutions that appear to be particulate-free can contain small amounts of solids that can clog the pores of the LC column inlet frit. In an effort to prevent this problem, solutions are commonly filtered prior to injection into the LC system. Syringe filters that have been used in the analysis of alkylphenols include hydrophilic polypropylene membrane filters (GHP Acrodisc, 0.45 μm) [37] and polyvinylidene fluoride filters (PVDF, 0.2 μm) [23].

A study of the blank contamination and losses caused by the syringe filters was carried out in order to identify the most appropriate filter. The syringe filters assayed in this work were 0.2 μm nylon, 0.2 μm PTFE and 0.45 μm PVDF (polyvinylidene fluoride) Fig. 3.

Blank water samples ($n=2$) were extracted according to the procedure outlined in the experimental section. One of the samples was unfiltered and the others were passed through the filters listed above. As can be observed in Fig. 3A, contaminated blanks of NP were obtained with nylon and PDVF filters.

The adsorption of compounds on the filters was evaluated. Blank samples ($n=2$) were extracted and the organic extracts (1 mL) were spiked with APs and BPA at a concentration of 1.7 μg L^{-1} and then filtered through each of the filters. The results of these assays were compared with the result obtained when a spiked water sample at the same concentration level was extracted but not filtered (Fig. 3B). Significant retention was not observed with any of the filters assayed. PTFE filters were finally selected because they gave similar results to the unfiltered samples and provided the lowest blanks without retention of the compounds.

3.4. Analysis of water samples: procedural blanks and other aspects

3.4.1. Storage of samples

According to the literature, samples are commonly stored in a refrigerator at 4 °C prior to analysis. The maximum reported storage time varied between studies, with times of 24 h [23,38–40], 48 h [14,33], a week [41] or unspecified [10,22,27,28,42,43]. In order to evaluate the degradation of the APs and BPA in seawater samples and to determine the maximum permissible storage time, a stability study was carried out.

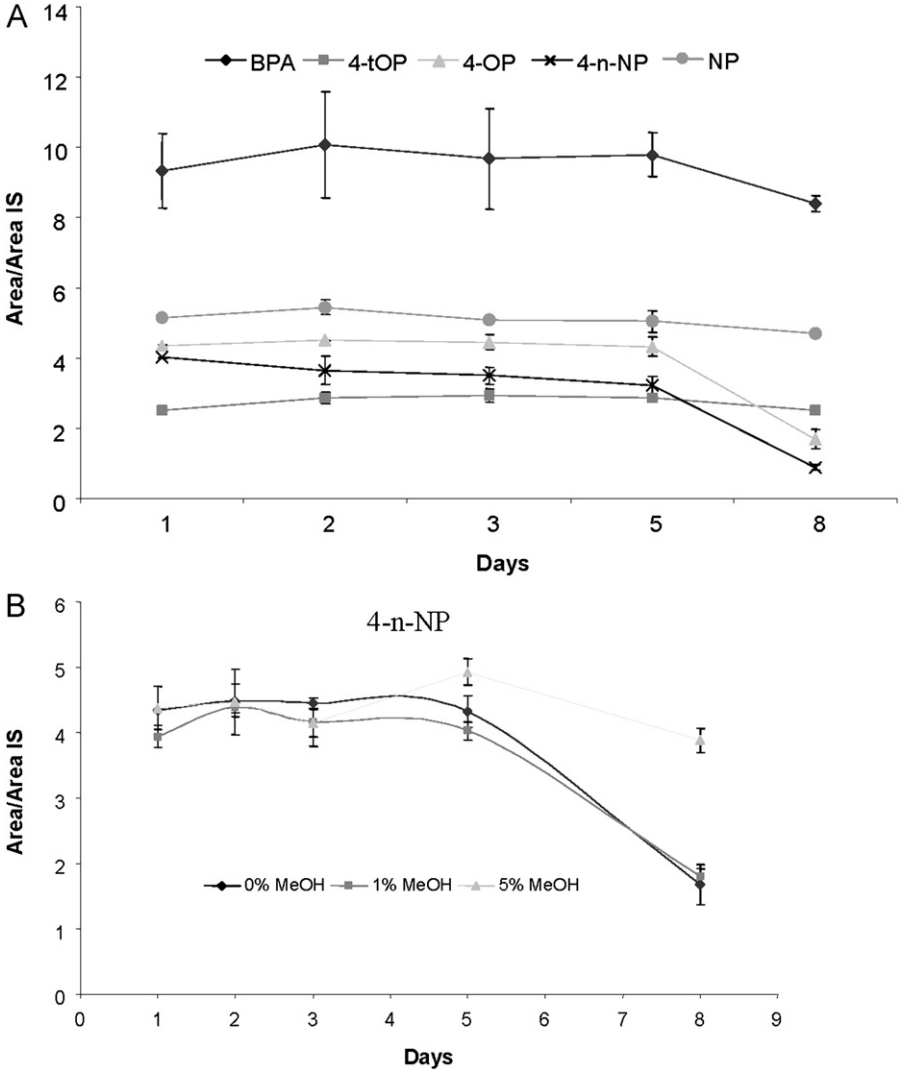

Fig. 4. Study of storage of samples ($n=2$) without additives (A) and with different percentages of methanol added for 4-*n*-NP (B).

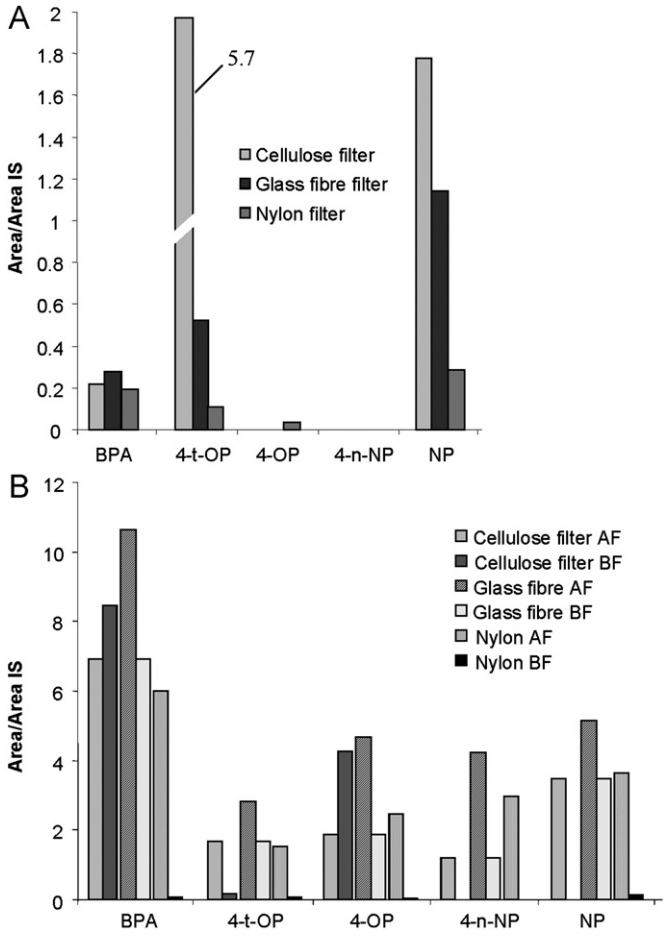

Fig. 5. Study of the filtration of samples: Blanks obtained (A) and adsorption of APs and BPA to different filters (B).

Ten aliquots of a seawater sample ($n=2$) were spiked with alkylphenols and bisphenol A at a level of 1.7 μg L^{-1} and then refrigerated at 4 °C. Two aliquots were analysed immediately (day 1), after 24 h (day 2), after 48 h (day 3), after 5 days and after a week (day 8). As can be seen in Fig. 4A, the response remains almost constant for all the alkylphenols until day 5. However, a sharp decrease (~30%) in the response was observed at day 8 for 4-OP and 4-*n*-NP. This decrease could be due to sorption problems or degradation of the compounds under investigation.

In order to avoid adsorption of the compounds onto the glassware, some authors add methanol to the water samples as a modifier or acidify the samples to pH 2.5–3. The percentage of methanol added varies between 0.1% and 10% depending on the author. Typical methanol percentages reported are 5% [22,44] and 0.1% [4], whereas some authors do not add any modifier at all [10,15,23,33,39,45].

Therefore, in order to assess the possible sorption of 4-OP and 4-*n*-NP onto containers, the experiment was repeated ($n=2$) with a small percentage (1% and 5%) of methanol added as a modifier. The results of this experiment are shown in Fig. 4B. The addition of 5% of methanol could reduce the adsorption. Nevertheless, the presence of methanol in the sample can also affect the DLLME extraction efficiency; methanol can modify the analyte-extraction solvent partition or the separation between the aqueous and organic phases. In this particular case, the addition of a modifier is not recommended and the maximum storage time should be less than 5 days in order to avoid analyte losses.

3.4.2. Filtration of samples

There is no general consensus in the literature concerning the prefiltration of water samples prior to analysis. The use of different kinds of filters has been reported and in some cases filtration is not carried out. Glass fibre filters (0.7 μm) are the most frequently used, along with glass microfibre prefilters (1 μm) [23] or with 0.45 μm membrane filters [22,46]. Other pore sizes used in glass fibre filters are 1 μm [16,32] and 1.2 μm [27,45]. Membrane filters (0.45 μm) [39–41], cellulose filters (0.45 μm) [42,47] and nylon filters (0.45 μm) [33] are other alternatives.

The contribution of the prefiltration step to the procedural blanks was evaluated along with possible losses of compounds due to adsorption on the filter. Three different filters were assayed: 0.45 μm cellulose filters, 0.2 μm nylon filters and 0.6 μm glass fibre filters. A volume of 100 mL of water sample ($n=2$) was filtered using each filter and then extracted according to the procedure described in Section 2.3. The relative signals of the blanks obtained with each kind of filter are shown in Fig. 5A. It can be seen that the highest blank was obtained with the cellulose filters, especially for 4-*tert*-octylphenol and nonylphenol. The lowest blank was obtained with the nylon filter.

The possible losses caused by the filtration were evaluated by comparison of the results obtained when a spiked water sample was filtered and then extracted with those obtained when a water sample was filtered and then spiked before the extraction ($n=2$). The results are shown in Fig. 5B. Nylon filters gave rise to a high level of retention of all the analytes (>95%), whereas cellulose filters retained 4-*n*-NP (72%) and 4-*t*OP (60%). The lowest retention was obtained with the glass fibre filters (~40%).

Bearing in mind the retention of the target compounds and the contaminated blanks obtained with all the filters assayed, the use of filters was discarded and prefiltration of samples is not recommended.

4. Concluding remarks

Nonylphenol, 4-*tert*-octylphenol and bisphenol A are compounds that are affected a great deal by blank contamination. In this work, the main contamination sources in DLLME-LC-MS/MS were considered and >90% of the contamination could be removed by following the guidelines described here.

The importance of avoiding the use of plastic material in any step of the analysis was demonstrated, even in the water purification system. The use of detergents should be also avoided. The cleaning of the glassware with milli-Q water, acetone and methanol before using is proposed as a cleaning procedure for routine analysis.

A significant reduction in 4-*tert*-octylphenol blanks was achieved using LC-MS grade methanol in the mobile phase, but an improvement was not observed using LC-MS quality water. The use of 0.2 μm PTFE syringe filters is recommended because these gave lower blanks. The reduction of reagent blanks observed on employing these conditions (<0.09 ng) provides increased sensitivity in alkylphenols and bisphenol A determination and allows low detection limits to be achieved.

The considerations outlined above could be applied to the analysis of these compounds in any liquid or solid samples. Moreover, the following recommendations should be considered in the analysis of water samples in order to reduce procedure blanks.

The addition of 5% MeOH to water samples is recommended in order to avoid adsorption of the compounds onto the glassware. In this way, samples can be stored in a refrigerator at 4 °C for at least a week. When the addition of methanol is not possible

because of the extraction procedure selected, the maximum storage time is decreased to 5 days in order to avoid losses of compounds.

Filtration of the water samples is not recommended because of the high blank signal caused by the filters assayed and the retention of compounds by the filter ($<40\%$ in the best case). When the prefiltration is mandatory (because of the extraction method selected or the presence of particulates), the use of glass fibre filters is recommended. In this case, the filter should also be extracted in order to recover all of the compounds and the blanks should also be taken into account.

Low blank contamination and low detection limits could be achieved in the analysis of water samples. The procedure blanks were minimized and were kept below $0.003\ \mu g\ L^{-1}$. Consequently, the low limits established for APs in the Directive 2008/105/EC were achieved.

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