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Determination of synthetic phenolic antioxidants and their metabolites in water samples by downscaled solid-phase extraction, silylation and gas chromatography–mass spectrometry

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ABSTRACT

The development and performance evaluation of an analytical method dedicated to the comprehensive determination of the most relevant antioxidants and their metabolites in aqueous environmental samples is presented. This was achieved by a miniaturised solid-phase extraction (SPE) with 10 mg Oasis HLB cartridges, which allow to achieve a concentration factor of 200, reducing organic solvent wastes (1 mL of ethyl acetate suffices for complete elution) and SPE costs and eliminating the need for solvent evaporation that otherwise compromises the recoveries of butylated hydroxytoluene (BHT) and 2,6-di-*tert*-butylcyclohexa-2,5-diene-1,4-dione (BHT-Q). Analytes were then determined by gas chromatography–mass spectrometry (GC–MS) after derivatisation with N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) in a single run. BHT-d_7 and *n*-propyl-paraben-d_4 (PrP-d_4) were used as surrogate internal standards. These surrogates allowed obtaining relative recoveries in the 80–110% range for all analytes even with complex wastewater samples and LODs at the 2–44 ng L^{-1} level taking into account blank issues often associated to antioxidants analysis. The method was applied to sewage and river waters, showing that the seven analytes could be detected in raw wastewater. BHT and BHT-Q were the most concentrated species in that type of sample (in the 275–871 ng L^{-1} range). On the other hand two metabolites of BHT, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT-CHO) and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-COOH) appeared to be the most ubiquitous species, being found in all samples in the 10–150 ng L^{-1} concentration range.

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1. Introduction

Antioxidants are substances which prolong the shelflife of foodstuffs by protecting them against deterioration caused by oxidation, such as fat rancidity and colour changes. Since natural antioxidants are usually of poor stability, manufacturers prefer to use synthetic antioxidants. Many synthetic compounds are active as antioxidants, but only a few are used because of very strict safety regulations. The most frequently used are the synthetic phenolic antioxidants (SPA). FDA [1] and EU [2] have established the permitted food phenolic antioxidants and amounts of their allowable usage. SPAs currently permitted for use in food are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), propyl gallate, octyl gallate and dodecyl gallate, usually at concentrations up to 100–200 $\mu g\,g^{-1}$ of SPAs in oils or fats, either singly or in combination. The use of SPAs is not restricted to foodstuffs. Thus, they are permitted in many types of packaging materials, in adhesives that come in contact with food and also in cosmetics, personal care products and pharmaceuticals. Among the SPAs, BHA and BHT are the most used antioxidants.

The results of scientific studies about the consumption of these additives are controversial since several studies have shown a potential link between BHA, BHT and cancer [3,4], while other studies have shown no link [5,6], and even a protective effect [7]. Nevertheless, their degradation products should be evaluated since they may pose an environmental or human health risk [8].

Studies on the metabolism of BHT have revealed that there are two main metabolic processes [9]; that is, oxidation of the alkyl substituent and oxidation of the aromatic ring system. 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-COOH) is a major metabolite formed by oxidation of the alkyl substituent and may be generated via the corresponding alcohol (BHT-OH) and aldehyde (BHT-CHO). Moreover, oxidation of the π-system of BHT leads, amongst others,

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Table 1
Analyte abbreviations, structures and other relevant data.

Abbreviation	IUPAC name	CAS	Formula	Estructura	Monisotopic MW	Log K_{ow}[a]	pK_a[a]	P_v (Torr)[a]
BHT	2,6-di-*tert*-Butyl-4-methylphenol	128-37-0	$C_{15}H_{24}O$	OH	220.18	5.319 ± 0.235	12.75 ± 0.4	0.00624
BHA	2-*tert*-Butyl-4-methoxyphenol	25013-16-5	$C_{11}H_{16}O_2$	O, OH	180.12	2.998 ± 0.235	11.82 ± 0.18	4.46E−3
BHT-CHO	3,5-di-*tert*-Butyl-4-hydroxybenzaldehyde	1620-98-0	$C_{15}H_{22}O_2$	O, H, HO	234.16	4.769 ± 0.279	8.33 ± 0.40	1.28E−3
BHT-COOH	3,5-di-*tert*-butyl-4-Hydroxybenzoic acid	1421-49-4	$C_{15}H_{22}O_3$	O, OH, HO	250.17	4.796 ± 0.253	4.77 ± 0.10	3.28E−5
BHT-Q	2,6-di-*tert*-Butylcyclohexa-2,5-diene-1,4-dione	719-22-2	$C_{14}H_{20}O_2$	O, O	220.15	3.902 ± 0.381	–	2.81E−3
BHT-OH	2,6-di-*tert*-Butyl-4-(hydroxymethyl)phenol	88-26-6	$C_{15}H_{24}O_2$	OH, HO	236.18	3.675 ± 0.251	12.00 ± 0.40	3.37E−4
TBHQ	2-*tert*-Butylbenzene-1,4-diol	1948-33-0	$C_{10}H_{14}O_2$	OH, HO	166.10	2.333 ± 0.225	10.78 ± 0.18	1.12E−3

[a] Software calculated value, from SciFinder Scholar Database 2006: http://www.cas.org/products/sfacad/.

to 2,6-di-*tert*-butylcyclohexa-2,5-diene-1,4-dione (BHT-Q). On the other hand, the degradation of BHA produces TBHQ.

Most of the methods described in the literature for the quantitative analysis of antioxidants or antioxidant mixtures have been developed for the analysis of foodstuffs and food packaging [10]. In these cases, liquid chromatography with UV detection was the most common determination technique following the extraction by liquid–liquid extraction (LLE) or solid-phase extraction (SPE) of the sample. However, those methods are not applicable for trace analysis in environmental matrices because they do not offer the necessary selectivity and sensitivity. Hence, the establishment of sensitive and selective analytical methods to monitor the widespread in the environment of antioxidants and their degenerative products is a real need.

In aqueous environmental samples (i.e. wastewater samples and river water) most of the data of occurrence of these analytes have been obtained in multi-residue studies which evaluated the presence of one or two antioxidants together with a wide range of other organic compounds such as pharmaceuticals, phthalates, phenols, etc. In those methods, LLE [11], solid-phase microextraction (SPME) [12] and mainly SPE [13,14] have been used as pre-concentration techniques followed by GC–MS determination. However, studies dedicated to the development of analytical methods and subsequently the occurrence of SPAs and their metabolites in the aqueous environment are very scarce. The only exceptions are the works of Fries and Püttman, who studied BHT together with its metabolite BHT-CHO in river, ground and wastewater samples of Germany, where these pollutants were typically detected in the 10–2000 ng L^{-1} range, depending on the sample nature [15,16].

It is then necessary to develop analytical methods that allow the determination of antioxidants and a broader range of metabolites in the aqueous environment. Therefore, the goal of this work was the development and performance evaluation of a method that allows the determination of the three main synthetic phenolic antioxidant (i.e. BHT, BHA and TBHQ) together with their four most relevant metabolites (BHT-CHO, BHT-COOH, BHT-OH and BHT-Q; TBHQ is also a metabolite of BHA) in water by GC–MS combined to SPE for the enrichment of samples. Moreover, critical aspects associated

Table 2
GC–MS experimental data.

Abbreviation	Retention time (min)	M⁺	Quantification ion (*m/z*)	Qualifier ions (*m/z*)
BHT-Q	8.43	220	177	220, 205
BHT-d7	8.86	227	212	227
BHT	8.92	220	205	220, 177
BHA	12.08	294	237	294, 181
PrP-d4	13.16	298	241	298
BHT-OH	14.12	350	293	276, 219
TBHQ	14.81	394	394	281, 337
BHT-CHO	15.12	348	291	333, 348
BHT-COOH	15.62	364	307	263, 233

with the determination of these analytes at trace level have also been considered and discussed.

2. Materials and methods

2.1. Chemicals and stock solutions

Analytes' names, abbreviations and other relevant data are shown in Table 1. BHT, BHA, BHT-COOH, BHT-Q, BHT-OH and TBHQ were obtained from Sigma–Aldrich (Steinheim, Germany) and BHT-CHO from TCI Europe (Zwijndrecht, Belgium). Deuterated BHT (2,6-di-(*tert*-butyl-d_1)-4-methyl-d_3-phenol-3,5-d_2; BHT-d_7) and *n*-propyl paraben (*n*-propyl 4-hydroxybenzoate-2,3,5,6-d_4; PrP-d_4), used as surrogate internal standards (ISs) were obtained from CDN Isotopes (Quebec, Canada).

Methanol and ethyl acetate (all of chromatographic analysis grade) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Panreac (Castellar del Vallès, Spain). Pure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

The derivatisation reagents N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) were supplied by Aldrich.

Individual stock solutions were prepared in acetone at the 2 mg mL^{-1} level. Mix standard solutions were prepared at the 20 μg mL^{-1} in acetone and subsequently diluted as necessary. Calibration standards were prepared in ethyl acetate.

2.2. Samples

Surface water and WWTP influent and effluent samples were used along this study. The WWTP is located near Santiago de Compostela (Galicia, NW Spain) and receives urban and hospital wastewater from ca. 100,000 inhabitants. Surface water was collected from the river Sar in Galicia (NW Spain), 4 km downstream the WWTP effluent discharge. All samples were taken in amber glass bottles previously rinsed with Milli-Q water and methanol and stored in the dark at 4 °C for a maximum of 48 h prior to their analysis. Samples were filtered using cellulose acetate membranes (47 mm diameter, 0.45 μm pore size).

2.3. Sample extraction

SPE of samples was carried out with a Visiprep SPE manifold (Supelco, Bellefonte, PA, USA). Oasis HLB (10 and 60 mg) obtained from Waters (Mildford, MA, USA) were used.

In the optimised method, samples (200 mL) were adjusted to pH 2.5 and spiked with the deuterated ISs (200 ng L^{-1}). Oasis HLB (10 mg) cartridges were sequentially conditioned with 1 mL ethyl acetate, 1 mL MeOH and 1 mL pure water (pH 2.5). Cartridges were then loaded with the samples, washed with 3 mL Milli-Q water (pH 2.5) and dried with nitrogen for 30 min. Finally, elution of analytes was performed with ethyl acetate (1 mL).

tert-Butyldimethylsilyl derivatives were obtained by addition of 50 μL of MTBSTFA to an extract aliquot (50 μL). The derivatisation was performed at 80 °C during 90 min

2.4. GC–MS determination

GC–MS determination was performed on an HP 7890A system (Agilent Technologies, Palo Alto, CA, USA) equipped with a mass spectrometer detector MSD 5975C and a 7693 autosampler. The injector was set to 270 °C and injection volume was 2 μL. An HP-5MS capillary column (30 m × 250 μm i.d., 0.25 μm film thickness) was used with the following oven temperature program: 1 min at 90 °C, first ramp at 10 °C min^{-1} to 270 °C and second ramp at 25 °C min^{-1} to 290 °C (held for 10 min). Helium was used as carrier gas with a constant flow of 1 mL min^{-1}. Transfer line, MS quad

Table 3
Experimental domain and relative importance (with their sign) of the main effects associated to each factor and second order interactions in the Box–Behnken design.

Factors	Temperature (°C)	Time (min)	MTBSTFA/extract volume ratio	Interactions					
Low Level	40	15	0.1						
Central Level	60	52.5	0.55						
High Level	80	90	1						
Relative effects	A	B	C	AA	BB	CC	AB	AC	BC
BHA	++	++	++	+	+	+	+	++	++
BHT-OH	–	+	++	+	+	+	+	+	++
TBHQ	++	++	++	+	+	+	+	++	++
BHT-CHO	–	–	++	+	+	–	+	–	+
BHT-COOH	–	–	–	++	+	+	–	+	+
Selected conditions	80	90	1						

++ or – – indicate a statistically significant effect (95% confidence level), positive or negative respectively.
+ or – indicate that the effect was not statistically significant.

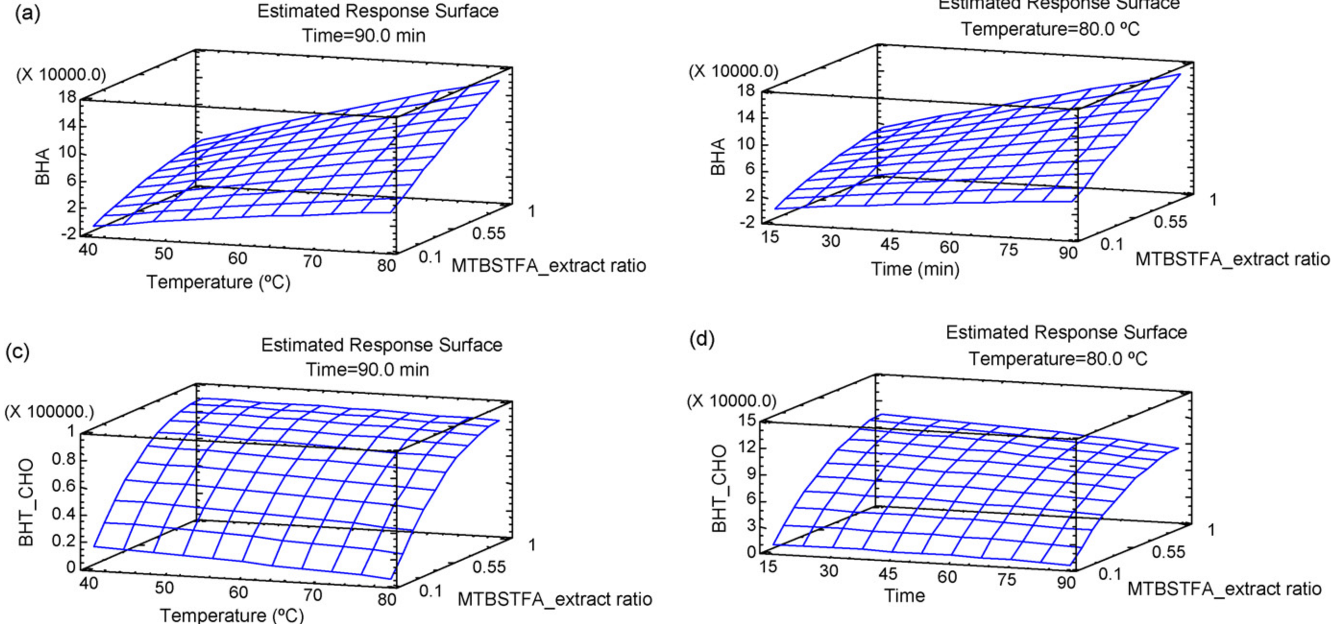

Fig. 1. Response surface plots for BHA (a and b) and BHT-CHO (c and d).

and MS source were maintained at 290, 150 and 230 °C, respectively. Detection was carried out by electron ionisation (70 eV) in single ion monitoring (SIM) mode, by considering two (in the case of ISs) or three (analytes) characteristic ions for each compound. The characteristic ions together with the substance-specific GC retention times for each studied compound are shown in Table 2.

2.5. Software

Experimental design creation and analysis was performed with the software package Statgraphics 5.1 (Statpoint Technologies, Warrenton, VA, USA).

3. Results and discussion

3.1. Derivatisation–GC–MS

In general, the studied compounds contain groups (phenols, alcohols and carboxylic acids) which can be derivatised to improve the chromatographic properties and separation on the GC-column [17]. The most common derivatisation procedure of compounds containing –OH and $–CO_2H$ groups is silylation [18,19]. Among the many possibilities of silylating agents, derivatisation experiments were performed by considering BSTFA, MSTFA and MTBSTFA. Derivatisation was accomplished by addition of 100 μL of the derivatisation reagent to a solution (900 μL) of the analytes in ethyl

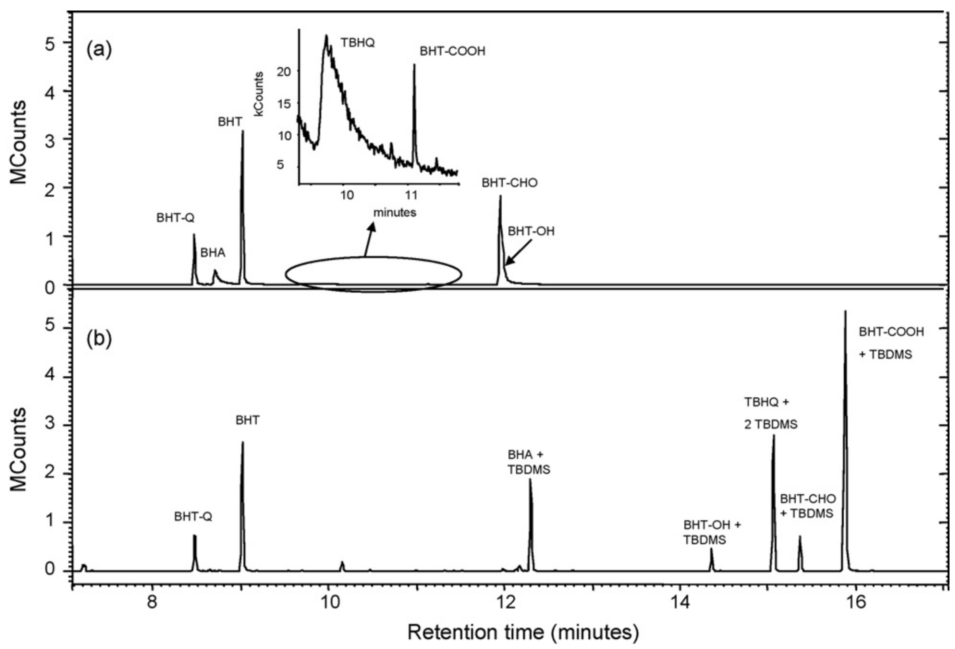

Fig. 2. Chromatograms of 1 μg mL^{-1} standard: (a) non-derivatised and (b) derivatised with MTBSTFA.

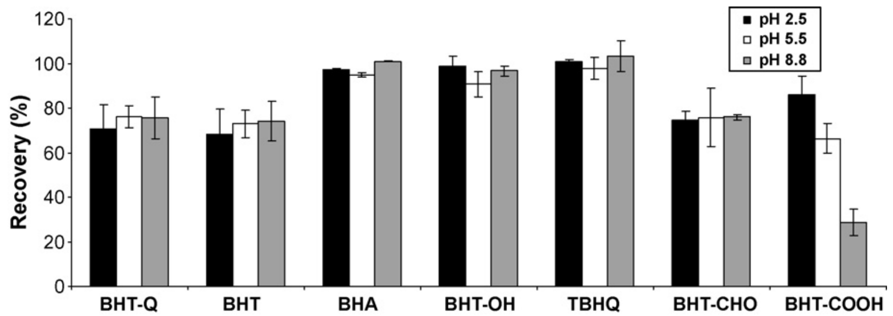

Fig. 3. Effect of sample pH on the SPE recoveries with 10 mg Oasis HLB cartridges. Sample volume: 100 mL; $n = 3$.

Table 4
Repeatability, linearity and detection and quantification limits of the GC–MS method.

	Internal standard	Repeatability (RSD, %) [a]	Linearity (R^2) [b]	LOD ($\mu g\,L^{-1}$) [c]	LOQ ($\mu g\,L^{-1}$) [c]
BHT-Q	BHT-d_7	2	0.999	0.6	2.1
BHT	BHT-d_7	4	0.997	0.2	0.6
BHA	PrP-d_4	3	0.994	0.8	2.7
BHT-OH	PrP-d_4	3	0.995	0.3	1.2
TBHQ	PrP-d_4	2	0.996	0.03	0.1
BHT-CHO	PrP-d_4	6	0.994	0.9	2.9
BHT-COOH	PrP-d_4	6	0.993	0.1	0.5

[a] 200 $\mu g\,L^{-1}$ standard ($n = 6$).
[b] 2–2000 $\mu g\,L^{-1}$; 10 levels in duplicate.
[c] Calculated for a signal-to-noise ratio of 3 (LODs) or 10 (LOQs).

acetate (1 $mg\,L^{-1}$). The mixtures were heated at 60 °C for 1 h prior to their injection in the chromatographic system. The proposed silylation reaction with MTBSTFA produced the successful derivatisation of all the studied compounds, except BHT, whose hydroxyl group is sterically hindered by two *tert*-butyls, and the quinone BHT-Q. Nevertheless both BHT and BHT-Q are easily determined by GC without the need of derivatisation. This derivatisation reaction leads to the formation of mono-*tert*-butyldimethylsilyl (TBDMS) derivates of BHA, BHT-OH, BHT-CHO and BHT-COOH while it produces the di-TBDMS derivate of TBHQ. On the other hand, the use of BSTFA and MSTFA leads to mixture of mono- and di-trimethylsilyl (TMS) derivates of BHT-CHO. Therefore, MTBSTFA was selected as the derivatisation agent because its ability to derivatise the studied compounds and the proved thermal and hydrolytic stability of the TBDMS derivates [20–22]. In addition, the resulting TBDMS derivatives produce very characteristics mass spectra by electron ionisation-mass spectrometry, with a molecular ion quite weak or even absent but dominated by the loss of the *tert*-butyl moiety $[M-57]^+$ (Table 2) [21–23].

The yield of derivatisation is affected by several variables such as derivatisation temperature and time and MTBSTFA/solvent volumes ratio. In order to optimise the derivatisation process, a Box–Behnken experimental design (with 3 central points; i.e. 15 experiments), was carried out. The use of Box–Behnken experimental design minimizes the number of experiments for 3 factors [24]. The factor levels were selected according to the literature [21–23], and bearing also in mind that: a higher MTBSTFA/solvent ratio would result in excessive dilution; temperature was limited to 80 °C to avoid overpressure in the vial and 90 min was considered the maximum reasonable derivatisation time. The experimental domain and the results of the analysis of the experimental design are shown in Table 3. Maximisation of peak area of the derivatised analytes was the target of the optimisation process. A pooled extract obtained from several treated wastewater samples spiked at 1 $mg\,L^{-1}$ for each compounds was used for the optimisation.

As shown in Table 3, the MTBSTFA/extract volume ratio had a statistically significant positive effect for most analytes (i.e. BHA, BHT-OH, TBHQ and BHT-CHO) except BHT-COOH. Temperature and time were only statistically significant for BHA and TBHQ and its effect was positive on the derivatisation yield. Finally, the MTBSTFA/extract volume ratio showed a positive statistically significant interaction with temperature and time for both BHA and TBHQ. Fig. 1 exemplarily shows the surface plots for BHA and BHT-CHO. Thus, the highest levels for all factors were selected as optimium for further experiments: 80 °C during 90 min with a ratio MTBSTFA/extract of 1. Fig. 2 presents the chromatograms of a standard

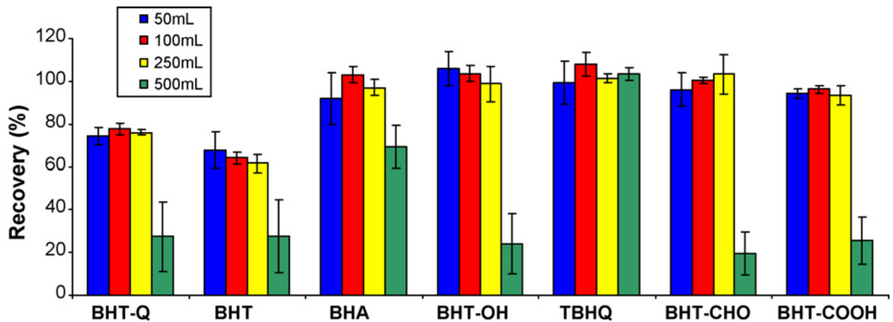

Fig. 4. Breakthrough volume study with Oasis HLB 10 mg cartridges. Sample pH adjusted to 2.5; $n = 3$.

Table 5
Relatives recoveries ($n = 4$) of analytes considering the surrogate ISs and detection limits of the whole analytical method.

	% Relative recovery (%RSD)				LODs[a] (ng L^{-1})	
	Milli-Q water (50 ng L^{-1})	Milli-Q water (500 ng L^{-1})	Treated wastewater (500 ng L^{-1})	Raw wastewater (1000 ng L^{-1})	Milli-Q, river and treated WW	Raw WW
BHT-Q	100 (7)	111 (7)	81 (8)	98 (18)	17[b]	39[b]
BHT	95 (10)	111 (6)	87 (4)	104 (8)	19[b]	44[b]
BHA	95 (4)	84 (5)	88 (3)	93 (12)	3	4
BHT-OH	100 (5)	80 (8)	83 (12)	98 (2)	8	8
TBHQ	81 (1)	84 (4)	80 (14)	83 (12)	2	3
BHT-CHO	86 (9)	110 (6)	98 (9)	110 (3)	10[b]	14[b]
BHT-COOH	98 (7)	95 (5)	108 (4)	108 (8)	2[b]	2[b]

[a] Calculated for a signal-to-noise ratio of 3 (LODs) from the second most intense ion.
[b] Calculated as three times the standard deviation of the blanks ($n = 6$).

either subjected or not to the final derivatisation procedure. Under these conditions, a complete derivatisation was observed, thus, no-derivatised analytes were not found in the chromatograms

Table 4 summarises the figures of merit of the derivatisation–GC–MS procedure. The developed GC–MS chromatographic method exhibits excellent linearity ($R^2 > 0.993$) in the 2–2000 μg L^{-1} range (duplicate injection, ten levels), and precision from the injection of a 200 μg L^{-1} standard (RSD < 6%), with detection limits (for S/N = 3) between 0.03 and 0.9 μg L^{-1}. BHT-d_7 and PrP-d_4 were selected as internal standards in order to correct possible variation in the whole process. BHT-d_7 was selected for the non-derivatised analytes (i.e. BHT-Q and BHT) while PrP-d_4 was selected for the derivatised ones.

3.2. Sample preparation

For the solid-phase extraction of the analytes, Oasis HLB cartridges were selected because of their ability to retain both non-polar and polar compounds providing good recoveries [25–27]. Thus, both parent compounds and their more polar metabolites can be recovered simultaneously with a single protocol and allow the possibility of decreasing SPE sorbent amount in order to decrease the organic solvent consumption and analytical costs. Hence, for a primary screening, different sorbent amounts (10 and 60 mg) were tested on their suitability for the SPE of the antioxidants into a single extraction method. Different parameters were evaluated for both phase amounts: elution volume of ethyl acetate, sample-pH and breakthrough volume.

First, the volume of ethyl acetate needed for a complete elution of the analytes was studied. Thus, 100 mL Milli-Q water samples spiked at the 20 μg L^{-1} level were percolated through the cartridges and eluted with 4 × 0.5 mL ethyl acetate (10 mg cartridges) or 4 × 1 mL ethyl acetate (60 mg cartridges), and the fractions were derivatised and analysed by GC–MS. The results of this study showed that a volume of 1 mL is enough for the complete elution of 10 mg cartridges. In the case of 60 mg cartridges, the elution is completed with 2 mL, except for TBHQ which needed 3 mL of ethyl acetate for the complete elution (data not shown).

Moreover, considering the different acidic character of the analytes, different sample-pH values were evaluated: 2.5, 5.5 and 8.8. Thus, 100 mL Milli-Q water samples adjusted to the different pH values indicated and spiked at the 20 μg L^{-1} level were percolated through the cartridge and eluted with 1 mL ethyl acetate (10 mg cartridges) or 3 mL ethyl acetate (60 mg cartridges). As expected (Fig. 3), the effect of the sample-pH is only significant for the most acidic compound (BHT-COOH); thus, the response decrease when sample-pH increases. Then, sample-pH should be adjusted to pH 2.5 prior to the extraction.

Finally, different sample volumes (50, 100, 250 and 500 mL) of river water spiked with the analytes (1 μg) were extracted with both cartridges in order to determine the breakthrough volume. Using 60 mg cartridges, breakthrough was never observed (data not shown), while it occurred for sample volumes higher than 250 mL for 10 mg cartridges (Fig. 4). Thus, in the case of 10 mg cartridges the extraction volume was limited to 200 mL.

In order to increase the enrichment factor, evaporation of ethyl acetate extracts (3 and 1 mL) containing the analytes to dryness and reconstitution in 100 μL ethyl acetate was performed. Possible losses of analytes during the extract evaporation step were studied, as this has been found as a critical step [28]. Absolute recoveries below 40% were obtained for all the analytes. In order to overcome this limitation, new experiments were performed evaporating the extract directly to 100 μL (avoiding dryness of the extract). Under these conditions absolute recoveries were slightly higher but still low (≈50%) and also showed a poor repeatability (RSD ≈ 40%). Since losses could not be overcome, no evaporation was performed in further experiments. Thus, in order to obtain the higher enrichment factor 10 mg cartridges were selected since 1 mL of ethyl acetate is enough for a complete elution and this critical step is no longer necessary. A further advantage of 10 mg cartridges is their lower price.

Under these conditions, absolute recoveries ($n = 4$) were between 70 and 115% in the evaluated matrices (Milli-Q water: 50 and 500 ng L^{-1}, treated wastewater: 500 ng L^{-1} and raw wastewater: 1000 ng L^{-1}) for all analytes but for BHT and BHT-Q (35–61%). Thus, possible losses of BHT and BHT-Q during different steps of

Table 6
Concentration (ng L^{-1}) ± standard deviation of antioxidants found in river and wastewater samples ($n = 3$ replicates of the same sample).

	February 2010			March 2010		
	River	Treated	Raw	River	Treated	Raw
BHT-Q	nd	nd	771 ± 100	nd	228 ± 22	871 ± 155
BHT	32 ± 5	nd	275 ± 21	112 ± 12	251 ± 30	801 ± 34
BHA	nd	nd	100 ± 4	nd	nd	135 ± 19
BHT-OH	nd	nd	55 ± 7	nd	nd	64 ± 6
TBHQ	nd	nd	9 ± 1	nd	nd	9 ± 2
BHT-CHO	26 ± 2	57 ± 3	144 ± 15	13 ± 1	24 ± 4	31 ± 6
BHT-COOH	13 ± 1	90 ± 9	67 ± 7	24 ± 2	61 ± 12	65 ± 8

nd: not detected (<LOD).

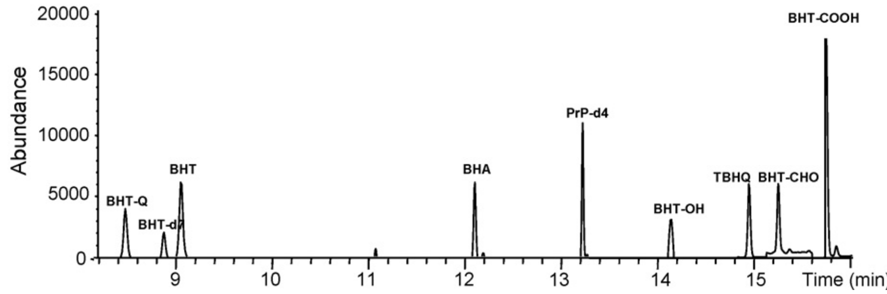

Fig. 5. Chromatogram of a real raw wastewater sample taken in March 2010. See Table 6 for concentration details.

the procedure were evaluated, viz. filtration, adsorption on SPE glassware and tubing, cartridges drying and degradation of the analytes. Filtration was evaluated by analysis of Milli-Q water spiked with the analytes ($2\,\mu g\,L^{-1}$) before and after the filtration and then submitted to SPE. No losses were observed during filtration (data not given). Obviously, this does not mean that some antioxidants/metabolites adsorbed to particulate matter will remain on the filter, so that only the dissolved fraction is measured. Possible adsorption of analytes on glassware and SPE tubing was studied by addition of different percentages of MeOH (0, 2.5, 5 and 10%) to a Milli-Q sample ($2\,\mu g\,L^{-1}$) prior its extraction by SPE. No improvement on SPE were observed at the different levels of methanol, only a slight decrease in the response at 20% methanol due to breakthrough of analytes at this high alcoholic level (data not shown). In order to estimate possible losses during cartridges drying, Oasis HLB cartridges were directly spiked with the analytes ($4\,\mu g$) and dried for different periods of time (0, 30 and 60 min) and then eluted and analysed. No losses were observed (data not shown). Finally, degradation of BHT and BHT-Q was evaluated by analysis of Milli-Q water spiked with a sole analyte, either BHT or BHT-Q at high concentration levels ($20\,\mu g\,L^{-1}$) and submitted to the sample preparation protocol. Although, dimerisation of BHT has been reported in the literature [15], formation of these dimers or any other degradation products was not observed.

Thus, the explanation for low recoveries of BHT and BHT-Q remains unclear. Fortunately, the labelled standard (BHT-d_7) is commercially available, permitting recovery correction and thus achieving good accuracy for these compounds (see Section 3.3).

3.3. Performance of the analytical method

Table 5 summarises the figures of merit of the whole analytical procedure, including SPE, derivatisation and final GC–MS determination. After the enrichment of 200 mL Milli-Q water samples spiked at two levels (50 and 500 $ng\,L^{-1}$) the obtained relative recoveries were satisfactory for all compounds (80–111%). Recoveries were also evaluated from raw and treated wastewater samples spiked at 500 and 1000 $ng\,L^{-1}$ level, respectively. Each sample was processed in quadruplicate. Non-spiked aliquots of each sample were also analysed and obtained peak areas subtracted from those corresponding to the spiked ones. The use of the surrogated IS (BHT-d_7) corrected satisfactorily the losses and the relative recoveries obtained for BHT and BHT-Q were between 81 and 104% with RSD values in the 4–18% range independently of the sample matrix. For the remaining analytes, PrP-d_4 was used as surrogate IS, as no other isotopically labelled analogous compounds were commercially available and PrP-d_4 is also derivatised with MTBSTFA. For those five analytes, relative recoveries (81–110%) and RSD (2–12%) values were also satisfactory (Table 5).

LODs were calculated by two different approaches: based on blank assays of Milli-Q water samples ($n = 6$) as blank signal plus 3 times the standard deviation of the blank; and defined for a signal-to-noise ratio of 3 and calculated on the basis of extracts of Milli-Q water samples spiked at $50\,ng\,L^{-1}$ level from the second most intense ion. LODs were established based on the highest value of these two approaches for each compound. The obtained LODs ranged from 2 to $19\,ng\,L^{-1}$. In the literature, BHT blank levels up to $2\,\mu g\,L^{-1}$ have been reported due to plastic cartridges usage that authors could only reduce to the $25\,ng\,L^{-1}$ by replacing plastic 200 mg Oasis HLB cartridges by glass ones [13]. In this work, with 10 mg Oasis HLB plastic cartridges, no such high blank levels were found, and blanks remained bellow the $5\,ng\,L^{-1}$ level. Thus, in spite of the measures taken with glassware, etc. blank contamination problems cannot be completely eliminated, but however the levels of BHT, BHT-Q, BHT-CHO and BHT-COOH could be maintained at a constant level in this work, which permitted to obtain acceptable LOD levels.

3.4. Application to samples

The SPE–GC–MS method was applied to the determination of antioxidants in 2 sets of (raw and treated) wastewater and 2 river water samples collected in February and March 2010. Blanks (from Milli-Q water) were processed with each SPE sample lot and subtracted for concentration calculations. Also, positives were confirmed from the ratio of the quantification and qualifiers ions, according to the European Union *Commission Decision 2002/657/EC*. A chromatogram of a raw wastewater sample is presented in Fig. 5.

As summarised in Table 6, BHT-Q and BHT were the two analytes detected in higher concentrations in raw wastewater samples (between 275 and $871\,ng\,L^{-1}$). Also, BHA was found at high concentration levels in raw wastewater (100 and $135\,ng\,L^{-1}$). However, these compounds seem to be partially removed during the wastewater treatment and lower concentrations were found in treated wastewater samples. These results are in agreement with the ones found in the literature for BHT and BHA, since these compounds were consistently detected in concentration at several hundred parts-per-trillion ($ng\,L^{-1}$) level in raw wastewater while the concentration in treated wastewater effluents were at from several tenths to hundred parts-per-trillion ($ng\,L^{1}$), suggesting that conventional wastewater treatment plants are capable of removing these chemicals with efficiencies varying between 65 and 99% [13].

It is noteworthy, that BHT-CHO and BHT-COOH were found in all the analysed samples, including in river water, and the concentration of BHT-COOH is constant and even increases during the treatment of wastewater. BHT-CHO was also previously detected in river water samples at higher concentrations levels (up to $233\,ng\,L^{-1}$) [15,16]. Obviously, these are grab samples that do not represent average concentrations and a deeper study is needed.

4. Conclusions

An SPE–derivatisation–GC–MS method has been developed for the determination of two synthetic phenolic antioxidants and their

five main metabolites in water. This is the first published method dedicated solely to the determination of this chemical class in water samples.

Extraction on 10 mg Oasis HLB cartridges provides a satisfactory enrichment factor for environmental samples avoiding the need of solvent evaporation and reducing SPE costs and organic solvent wastes. After extraction, polar metabolites are derivatised with MTBSTFA to produce stable, less polar analytes that are determined by GC–MS at low levels. The usage of two surrogate internal standards results in a method providing good accuracy, with relative recoveries between 80 and 110%, and limits of detection (2–44 ng L^{-1}).

The application of the method to wastewater and river samples showed BHT and BHT-Q as the compounds in higher concentrations in wastewater (up to 800 ng L^{-1}) and the metabolites BHT-CHO and BHT-COOH as the most resistant to water treatment, being at the 10–100 ng L^{-1} in sewage and river samples.

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