Fast and Sensitive LC-MS Detection of Bisphenol A and Butylhydroxyanisole in WWTP Sewage Sludge

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In this study, a new LC-MS/MS method was developed and optimized in order to detect two omnipresent additives, Bisphenol A (BPA) and Butylhydroxyanisole (BHA), in WWTP sewage sludge. Both analytes are synthetic phenolic compounds known for their endocrine disruptive and toxic properties. BPA and BHA were isolated from sludge samples using ultrasonic assisted liquid-solid extraction followed by silicagel clean-up to remove interferences, and evaporation to dryness and extract re-dissolution with 1 mL methanol prior to LC-MS analysis. All LC-MS parameters were optimized in order to obtain high sensitivity and selectivity. MS detector response was linear in the range 1 ÷ 200 µg/L with correlation coefficient R² > 0.999 for both analytes. Intra-day and inter-day precision expressed as RSD values were 4.7% and 10.3% for BPA, whereas for BHA 7.8% and 13.8% RSD values were obtained. Recovery values after ultrasonic assisted extraction were 85.5% for BPA and 79.3% BHA with internal standard correction. Overall method LOQs were established at 1.97 ng/g (BPA) and 1.86 ng/g (BHA) on dry weight. Optimized chromatographic parameters allowed separation and detection of the two additives in less than 10 minutes. Both BPA and BHA were detected in all tested sludge samples with higher levels for BPA (34.6 - 132.6 ng/g), whereas BHA was found at lower levels in the range 3.16 - 5.64 ng/g.

Keywords: Additives, BPA, BHA, LC-MS, ultrasonic assisted liquid-solid extraction, WWTP sewage sludge

Additives of general use, such as synthetic phenolic compounds, have become widely used at industrial level over the past decades such that their impact on ecosystems and population has raised the scientific community interest due to their toxicological impact. These compounds pose a serious threat to the health of the environment, humans and wildlife. Among these synthetic phenolic compounds, some of the most commonly used are Bisphenol A (BPA) and Butylhydroxyanisole (BHA).

BPA is mainly used in polycarbonate and epoxy resin materials manufacture with a wide range of applications. These include composites and sealants in the dental industry [1, 2], reusable plastic bottles, baby bottles, microwave containers, inner wrapping for food and beverage containers, sunglasses, building materials and medical devices [3]. Incomplete polymerization process of some compounds can lead to leaching of these materials in the environment [4, 5]. Studies have shown that incomplete polymerization of BPA during production and repeated exposure to heat and / or acidic / basic conditions (even in the case of complete BPA polymerization) can promote BPA infiltration from plastics into the environment [6]. Human exposure to BPA is caused by the hydrolysis of polycarbonate plastics and epoxy resins, leading to the penetration of small concentrations of BPA into food and liquids [7]. The use of polycarbonate and epoxy resin products is on the rise, leading to widespread exposure to the population [8]. Butylhydroxyanisole (BHA) is widely used as an antioxidant in food, food packaging [9], cosmetics, and pharmaceuticals [10, 11]. The commercial mixture contains 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole isomers, the latter having better antioxidant properties, representing 90% of the BHA in mixture.

Both BPA and BHA are known for their adverse effects on the endocrine system, being among the most well-known endocrine disruptors used on industrial scale. In case of BPA, these adverse effects are attributed to its ability to act as an estrogen agonist promoting the endogenous effect of 17β-estradiol [7]. Increased levels of BPA have been correlated with various diseases. Health complications associated with increased BPA exposure levels have been recently reported. These include diabetes [12] because low levels of BPA inhibit the release of adiponectin from human adipose tissue [13], cardiovascular diseases [12, 14] and modification of liver enzymes levels [12]. Data from a recent study suggests that prenatal exposure to BPA (assessed by measurements of maternal BPA levels) may be associated with increased aggressivity and hyperactivity at 2 years age children, especially in females [15].

Regarding BHA, recent studies have shown that BHA has a weak estrogenic effect and also anti-androgenic properties [16], while an *in vivo* study has been shown to have anti-estrogenic properties [17]. At present, however, there is insufficient data available to reach a conclusion on the safety of BHA when referring to its endocrine disruptive character. In order to manufacture polycarbonate plastic materials, BPA can be used as such or in mixture with various additives such as antioxidants, plasticizers, clarifiers, colorants and/or extenders, one of the most commonly used antioxidants being BHA. Since polymerization of monomers is rarely complete and additives are not chemically bonded to the polymeric structure, they can easily leach in small concentrations from plastics and generate thus adverse effects in young mammals. This leakage of monomers and additives from a plastic material is often accelerated if the product is exposed to common usage demands such as UV radiation in the sunlight, microwave radiation and/or wet heating by boiling or washing vessels.

Due to their relatively high hydrophobicity (log Kow = 3.32 and 3.50 respectively), BPA and BHA strongly interact with organic molecules and as a result are predominantly found in sewage sludge produced in wastewater treatment plants [18]. Because of this, both Bisphenol A and

Butylhydroxyanisole are frequently detected in sewage sludge (0.1-3.2 × 10^{7} μg/kg BPA [19] and 5-10 ng/g BHA [20]). In recent years, an increasing number of studies were reported on the BPA levels in the environment and its possible biological effects [21], both BPA and BHA being considered omnipresent in the environmental compartments over the last decades [22, 23].

In the present work we developed a highly sensitive and selective LC-MS/MS method coupled to an ultrasonic assisted liquid-solid extraction procedure able to extract, separate and detect BPA and BHA from WWTP sewage sludge at trace level concentration. The method was applied to establish the presence of both phenolic compounds in sewage sludge samples collected from different waste water treatment plants located in Romania.

Experimental part
Reagents and chemicals
Individual high purity standards of BPA, BHA and isotopically labeled $^{13}C_{12}$-BPA which was used as internal standard were purchased from Sigma-Aldrich. Solvents used to prepare the mobile phase and for sewage sludge samples extraction were: acetonitrile (ACN) and methanol (MeOH) from Merck (Darmstadt, Germany), dichloromethane (DCM) and hexane from Sigma-Aldrich (Germany).

LC-MS instrumentation and conditions
Experiments were performed using an Agilent 1260 series LC system (Waldbronn, Germany) consisting of: degasser, binary pump, autosampler and thermostatted column compartment coupled with an Agilent 6410B triple-quadrupole mass spectrometer with electrospray ionization source (ESI). All chromatographic runs were carried out on a Luna C18 column (150 x 2.0 mm, 3.0 μm) from Phenomenex which was kept at 35°C. All experiments were performed in isocratic elution conditions at a flow-rate of 0.15 mL/min. Mobile phase consisted of a binary mixture of Aq. 0.01% Acetic acid / Acetonitrile = 40/60 (v/v) (v/v). Method injection volume was 10μL using MeOH as sample diluent. MS detection was achieved using Multiple Reaction Monitoring (MRM) acquisition mode. Full-Scan MS spectra was acquired in the range 60 - 300 Da to establish MRM transitions. Acquisition segments used, retention time, MRM transitions, fragmentor voltages, collision energies and other MS parameters are given in tables 1 and 2. ESI ionization source was operated in negative mode with 300°C as the drying gas temperature, 6 L/min drying gas flow, 40 psi nebulizer pressure and 6000 V capillary voltage.

Results and discussions
LC separation optimization
In order to obtain optimum LC separation, hydrophobicity index of BPA and BHA was taken into consideration (BPA log Kow = 3.32, whereas BHA log Kow = 3.50) when the chromatographic column and mobile phase were chosen [24, 25]. Because of these high values both analytes show increased retention on the chosen C18 phase. Therefore, a rich organic modifier mobile phase (60%) was chosen to allow both analytes and internal standard to elute over a reasonable period of time (about 10 minutes). Also, due to the difference in log Kow and to increased values of this parameter, separation of the two analytes was easily done in isocratic mode at 40% aqueous / 60% ACN mobile phase composition. Internal diameter of the chosen LC column (2.0 mm) allowed working at a reduced flow rate (0.15 mL/min) which enhances analyte ionization in ESI source leading to increased method sensitivity, whereas the small size of the particle size of the stationary phase (3 μm) resulted in high efficiency narrow peaks (also increased method sensitivity). Chromatographic column temperature was varied between 25 and 40°C (with 5°C steps). Highest BPA and BHA peak efficiencies were observed at 35°C and hence this temperature was chosen for the final optimized LC method.

Regarding the effect on MS detection, several aqueous component mobile phases containing 0.01%, 0.05%, 0.1% CH_3COOH and 0.1% HCOOH were prepared. Upon injection of analyte mixture, a significant increase in the analytical signal was observed for the 0.01% CH_3COOH aqueous component of the mobile phase when compared to the other choices. Thus, the former was chosen as aqueous mobile phase component. The LC method allowed very good separation between BPA and BHA peaks. This allowed detection of BPA and BHA in different time segments (acquisition windows) working thus with a minimum number of MRM transitions leading to increased method sensitivity. LC method run-time was less than 10 min. (fig. 1).

MS detection optimization
In order to obtain MS spectra corresponding to BPA and BHA, a mass range of 60 - 300 Da was used in MS scan mode. Quantitation was performed in the MRM (Multiple Reaction Monitoring) mode of the MS detector. In order to establish the most sensitive MRM transitions for the identification and quantitative determination of the studied analytes, after identification of molecular ions of the analytes from the MS spectra, the latter were isolated and fragmented in the collision cell in order to obtain specific product ions using Product Ion Scan mode. Positive and negative ion modes were simultaneously monitored for method optimization. Negative ionization mode was selected because two product ions were obtained for both analytes, one for quantitation and one for confirmation and also due to higher selectivity when compared to positive ionization mode. In order to quantitate extremely low levels of BPA and BHA from sewage sludge samples (ng/g as magnitude order), at which these compounds may be are present, acquisition mode of the Triple Quad detector was set to MRM mode with distinct time segments (acquisition

Table 1

MS PARAMETERS FOR THE DETECTION OF BPA, BHA AND $^{13}C_{12}$-BPA INTERNAL STANDARD (MRM TRANSITIONS, COLLISION ENERGY AND OTHERS)

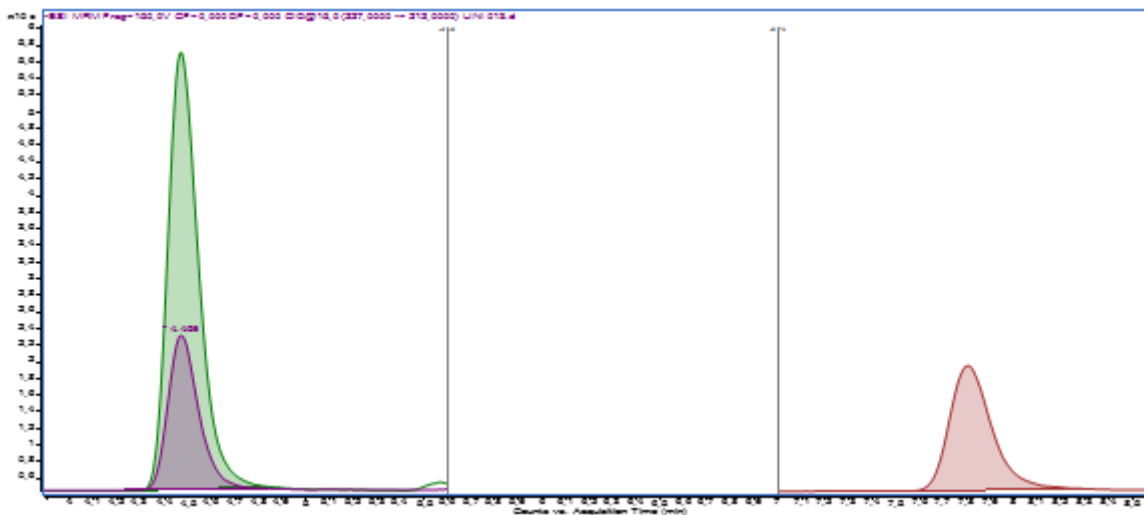| Analyte	Retention time (min)	MRM transition	Fragmentor voltage (V)	Collision energy (V)	Dwell time (msec)	Cell Accelerator Voltage	Time segment
BPA	4.475	227 → 212 (Q)	150	15	150	2	2
		227 → 133 (q)		22		6	
$^{13}C_{12}$-BPA	4.469	239 → 224 (Q)	150	15	150	1	2
BHA	7.807	179 → 163 (Q)	130	25	150	2	4
		179 → 149 (q)		10		8	

Fig. 1. MRM overlaid extracted ion chromatograms of a mixed 100 µg/L methanol solution of the BPA, BHA and the labeled $^{13}C_{12}$-BPA internal standard

Table 2

ACQUISITION WINDOWS SET FOR HIGH SENSITIVITY DETECTION OF BPA AND BHA

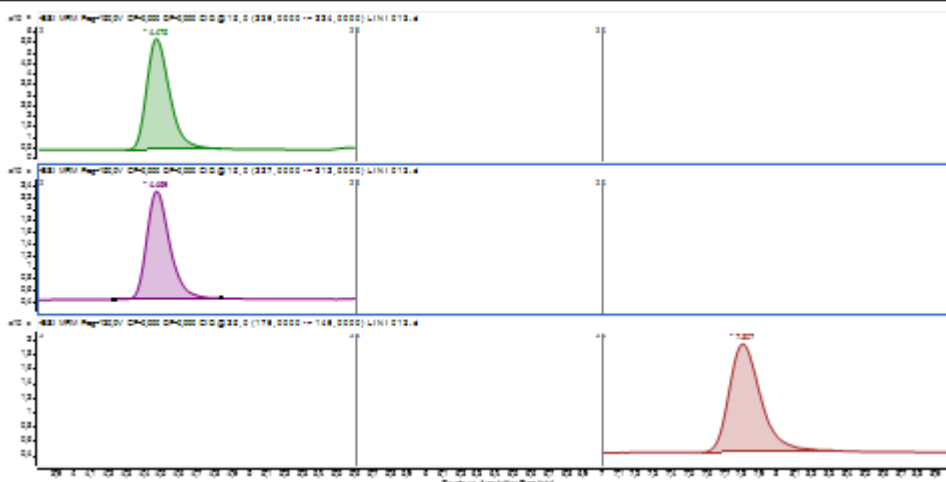| Time Segment	Start Time (min)	Scan Type	Ion Mode	Divert Valve	Store
1	0.0	MRM	ESI	To Waste	No
2	3.8	MRM	ESI	To MS	Yes
3	5.6	MRM	ESI	To Waste	No
4	7.0	MRM	ESI	To MS	Yes

Fig. 2. MRM overlaid extracted ion chromatograms of a mixed 100 µg/L methanol solution of the BPA, BHA and the labeled $^{13}C_{12}$-BPA internal standard on time segments

windows) one for each analyte (a single time segment was necessary for BPA and the internal standard) and one segment for BHA (tables 1-2). Thus, a small number of MRM transitions were monitored per time segment (3 MRM for BPA and $^{13}C_{12}$-BPA time segment and 2 MRMs for BHA time segment) which allowed setting the Dwell time parameter at high values (200 msec for BPA / $^{13}C_{12}$-BPA and 250 msec for BHA). This led to MRM chromatograms with reduced noise and hence high S/N ratio which means low detection limits (fig. 2).

Next, all detection parameters of the triple quadrupole MS detector (QQQ) were optimized in order to obtain the highest signal possible for both analytes. This was done after the detector tuning procedure also needed to obtain

the best settings of the intrinsic parameters of the detector. The optimized parameters were: Fragmentor voltage, Collision energy (CE), Cell accelerator voltage, MRM acquisition time (Dwell time), Capillary voltage and Drying gas flow. A 100 µg/L standard solution of BHA and BPA was used to optimize these parameters. With the modification of the mass spectrometric detection parameters, their effect on the peak area and the signal to noise ratio (S/N) was monitored. Collision energy (CE) applied in the collision cell (Q2) to the precursor ions to dissociate them and obtain product ions was varied also between 5 and 30 V with a 5 V increment. The maximum response for MRM transitions was obtained at 15 V for BPA and 25 V for BHA (table 3).

Table 3

COLLISION ENERGY OPTIMIZATION (PEAK AREA)

Collision energy Q2 (V)	10	15	18	20	22	25	30
BPA	2184	**2913**	2499	1938	1485	769	162
BHA	36	135	280	391	565	**757**	690

Table 4
FRAGMENTOR VOLTAGE OPTIMIZATION (PEAK AREA)

Fragmentor voltage (V)	130	135	140	145	150
BPA	1777	2025	1838	2344	2545
BHA	588	521	391	380	317

Table 5
CAPILLARY VOLTAGE OPTIMIZATION (PEAK AREA)

Capillary voltage (V)	3000	3500	4000	4500	5000	6000
BPA	1187	1683	2363	2816	3343	4131
BHA	753	1185	1647	2025	2717	3599

Table 6
CELL ACCELERATOR VOLTAGE (PEAK AREA)

Cell accelerated voltage (V)	2	4	6	8
BPA	3354	1838	2154	1361
BHA	347	391	412	459

Table 7
CORRELATION COEFFICIENTS (R^2), INTRA-DAY AND INTER-DAY PRECISION, ANALYTE RECOVERY, INSTRUMENTAL QUANTITATION LIMIT (IQL) AND METHOD QUANTITATION LIMIT (LOQ)

Analyte	R^2	Precision (%) Intra-day	Precision (%) Inter-day	Overall method recovery (% ± RSD%)	IQL (ng/mL)	LOQ (ng/g dw)
BPA	0.9995	4.7	10.3	85.8 ± 4.7	0.84	1.97
BHA	0.9997	7.8	13.8	79.3 ± 7.8	0.72	1.86

Using the optimal collision energy values, the fragmentor voltage representing the acceleration voltage applied between the skimmer and the first quadrupole to ions transfer from the ionization source to the latter one. The values selected for testing were 130, 135, 140, 145, 150V. The peak areas generated are given in table 4. The maximum signal was obtained by applying a 150 V for BPA and 130 V for BHA.

Based on previous results, sensitivity optimization was continued with other parameters of the MS detector, namely capillary voltage and cell accelerator voltage in collision cell. The capillary voltage was varied between 3000 and 6000 V, and the acceleration voltage between 2 and 8 V. The peak area data are given in tables 5 and table 6 respectively.

After optimization of all MS parameters, the instrumental quantitation limits (IQL) were determined to be between 0.84 μg/L for BPA and 0.72 μg/L for BHA respectively. IQL values were calculated as the concentration for which a S/N ratio of approximately 10 was obtained.

Sewage sludge sample extraction
For the optimization of extraction and clean-up procedure, real WWTP sewage sludge samples were used. In the first step, the samples were freeze dried and then the lyophilized material was grounded, homogenized, and stored in sealed containers at -20 °C until extraction. 0.5 g of dried sample was spiked with target analytes (50 ng/g) and IS. Extraction was carried out by sonication using 20 mL of DCM:hexane (1:1), in two portions. Samples were sonicated for 15 minutes and the collected organic layers were dried on sodium sulphate and then filtered. The crude extracts were purified with a silica gel column and eluted with 30 mL DCM. Under a gentle nitrogen stream, organic phase was evaporated to dryness and re-dissolved with 1.0 mL MeOH prior to LC-MS.

LC-MS/MS method validation
To account for its performance, the developed LC-MS/MS method was validated with respect to specificity, linearity, precision, accuracy and limit of quantitation. The calibration curves for the two compounds were set in the concentration range 1 - 200 μg/L, using internal standard method. MS detector response was linear for both compounds with correlation coefficients higher than 0.999. Intra-day and inter-day method precision was tested on 6 replicates by spiking 50 ng/g BPA and BHA mixture in lyophilized sewage sludge. RSD% values were 4.7% for BPA and 7.8% for BHA for intra-day precision and 10.3 and 13.8% respectively for inter-day precision. Method accuracy was tested also at 50 ng/g and the obtained analyte recovery were 85.5 and 79.3% with internal standard correction for BPA and BHA respectively, as can be observed in table 7. Overall method LOQs were 1.97 ng/g for BPA and 1.86 ng/g dry weight for BHA. All validation parameters are given in table 7.
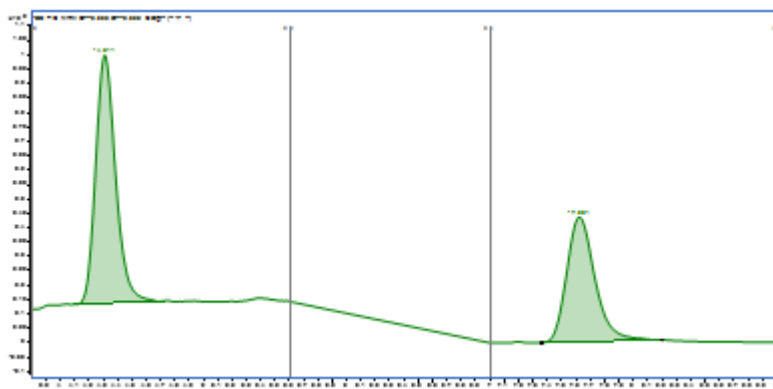

Fig. 3. Combined MRM chromatogram obtained for Sample S7 (Peak identification in elution order: 1. BPA and 2. BHA)

Occurrence of BPA and BHA in WWTP sewage sludge

The developed method was used to determine the presence of the target compounds in sewage sludge samples collected from waste water treatment plant in Romania. The samples were collected in brown glass containers and stored at 4°C during transport to the laboratory. All sewage sludge samples were lyophilized and the target analytes were isolated after the extraction and purification procedures. Both compounds were successfully isolated from the complex matrix of sludge from the treatment plant and were detected in all tested samples. Figure 3 shows the presence of BPA and BHA in one of the tested WWTP sewage sludge samples (S7).

Both analytes were detected in all tested WWTP sludge samples (table 8). In all analyzed samples, the BPA concentration values were situated between 34.6 and 132.6 ng/g dw. BHA concentration levels were significantly lower than BPA, which is normal considering the higher levels of the latter given its increased usage. In S1-S2 samples BHA was below LOQ, whereas in S3-S8 samples BHA was determined between 3.16 and 5.64 ng/g dw very similar to other reported studies.

Table 8

BPA AND BHA CONCENTRATION LEVELS FOUND IN WWTP SEWAGE SLUDGE OF DIFFERENT TREATMENT PLANTS IN ROMANIA

Samples	Concentration (ng/g, dw)	
	BPA	BHA
S1	77.11	<LOQ
S2	44.00	<LOQ
S3	60.17	4.08
S4	84.68	4.52
S5	61.15	3.91
S6	97.15	3.19
S7	34.60	5.64
S8	51.83	5.20
S9	93.39	5.27
S10	132.62	3.16

Conclusions

In this study, a robust, sensitive and fast LC-ESI(-)MS/MS method was developed for the determination of two synthetic phenolic compounds, BPA and BHA, in WWTP sewage sludge. Both compounds are known as omnipresent pollutants in the environment with endocrine disruptor properties. Chromatographic conditions and MS/MS parameters were optimized for separation and high sensitivity detection in order to be able to determine trace amounts of these contaminants in a very complex matrix like WWTP sewage sludge. Ultrasonic liquid-solid extraction was optimized as a suitable extraction method for the target analytes in sludge samples. DCM/Hexane mixture (1:1) showed higher extraction efficiency compared with other extraction solvents. In sewage sludge samples, recovery efficiencies for BPA and BHA were 85.7 and 75.8% respectively, with intra-day and inter-day precision better than 7.8 and 13.8%. The overall method quantitation limits (LOQ) were situated between 1.86 ng/g for BHA and 1.97 ng/g for BPA. Both target analytes were detected in all WWTP sludge samples with BPA being detected in the highest amounts (34.6 - 132.6 ng/g), whereas BHA was found at much lower levels between 3.16 and 5.64 ng/g.

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