MASARYK UNIVERSITY

FACULTY OF SCIENCE

RESEARCH CENTER FOR TOXIC COMPOUNDS IN THE ENVIRONMENT

DETERMINATION OF ALKYLPHENOLS IN ENVIRONMENTAL MATRICES

DISSERTATION THESIS

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DECLARATION/PROHLÁŠENÍ

I declare that I have produced this thesis independently using the cited literature and under the guidance of supervisor assoc. prof. RNDr. Zdeněk Šimek, CSc.

Prohlašuji, že jsem tuto práci vypracoval samostatně s použitím uvedené literatury a pod vedením vedoucího práce doc. RNDr. Zdeňka Šimka, CSc.

Brno 4. 3. 2020 …….

Mgr. Marek Pernica

ABSTRACT

The presented thesis deals with the determination of alkylphenols in environmental matrices, water and soil. Attention was devoted mainly to the industrially most widespread analytes such as 4-tert-octylphenol (4-t-OP), 4-octylphenol (4-OP), 4-n-nonylphenol (4-n-NP) isomers of 4-nonylphenol (iso-NP) called also technical nonylphenol, bisphenol A (BPA) and bisphenol F (BPF).

The aim of the thesis was to optimize, validate and use analytical methods for the determination of the above-mentioned alkylphenols and bisphenols in environmental matrices (water and soil) applying 5- (dimethylamino) naphthalene-1-sulfonyl chloride (dansyl chloride, DNSC) as a derivatizing agent. For the separation and quantification of the dansyl derivatives, the high-performance liquid chromatography method with a tandem mass spectrometer detector was used. The ionization was performed using the positive mode of electrospray (HPLC-ESI$^+$/MS/MS) **(Article I)**. Soxhlet extraction, ultrasound extraction (USE), accelerated solvent extraction (ASE) and fast, easy, cheap, efficient, robust and safe extraction (QuEChERS) were tested for soil as a more complex matrix. The QuEChERS technique has several modifications such as original, citrate and acetate, which have also been tested for selected soils based on total organic carbon (TOC) **(Article II)**.

The analytical methods were applied to real samples of drinking water from the water pipeline **(Article III)** as well as freshwater ponds and reservoirs affected by water blooms where estrogenic activity and estrogenic concentration were determined **(Article IV)**. Other real samples were bottled drinking water in PET bottles, sewage water, artificial soil, and real soils selected from the Soil Bank from the RECETOX Research Center.

Additionally, experiments were conducted on the analysis of the blanks as a notorious problem in ultra-trace analysis of alkylphenols **(Article V)**.

The published papers which are an integral part of the presented thesis **(Articles I–V)** give a detailed description of the issue.

ABSTRAKT

Předkládaná disertační práce se zabývá stanovením alkylfenolů v environmentálních matricích jako je voda a půda. Pozornost byla věnována především průmyslově nejrozšířenějším analytům, jako jsou: 4-terc-oktylfenol (4-t-OP), 4-oktylfenol (4-OP), 4-n-nonylfenol (4-n-NP), isomery 4-nonylfenolu (iso-NP) nazývány také jako technický nonylfenol, bisfenol A (BPA) a bisfenol F (BPF).

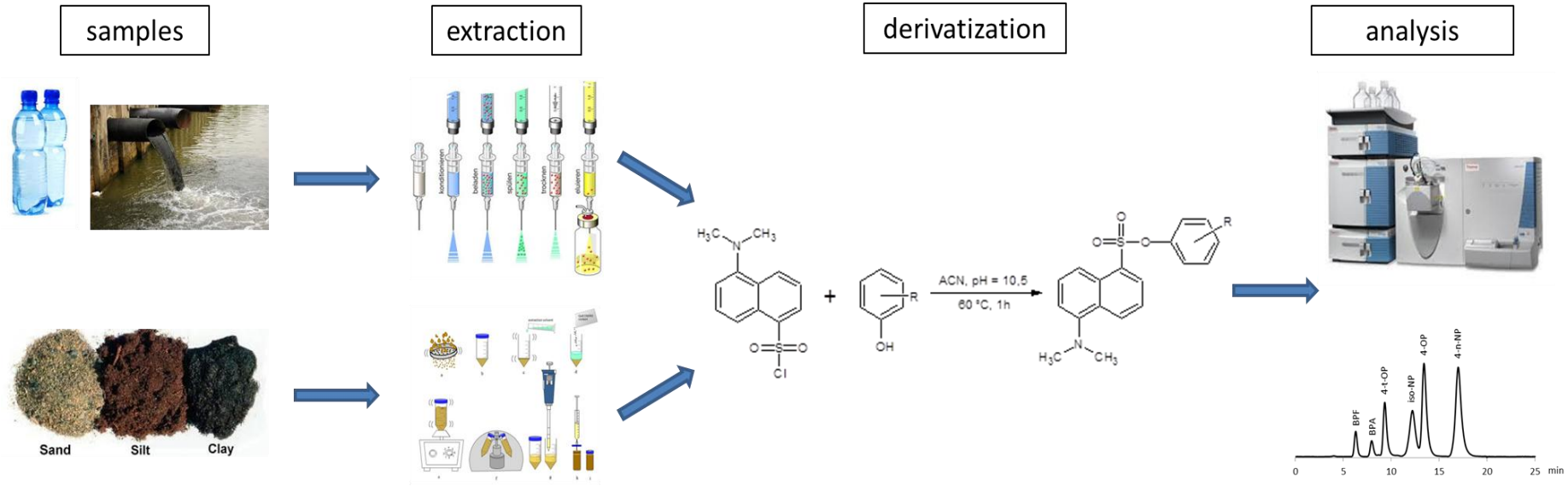
Náplní práce bylo optimalizovat a validovat analytickou metodu pro stanovení výše uvedených alkylfenolů a bisfenolů v environmentálních matricích (voda, půda) s využitím 5-(dimethylamino) naftalen-1-sulfonyl chloridu (dansyl chlorid, DNSC) jako derivatizačního činidla. Pro separaci a kvantifikaci dansyl derivátů alkylfenolů byla použita metoda vysokoúčinné kapalinové chromatografie s tandemovým hmotnostně spektrometrickým detektorem, přičemž ionizace probíhala s využitím elektrospreje v pozitivním módu (HPLC-ESI$^+$/MS/MS) **(Článek I)**. Pro půdu jako komplexnější matrici byly testovány čtyři extrakční techniky jakými byly Soxhletova extrakce, extrakce pomocí ultrazvuku (USE), urychlená extrakce rozpouštědlem (ASE) a rychlá, snadná, levná, efektivní, robustní a bezpečná extrakce nazývaná QuEChERS. QuEChERS technika má mnoho modifikací jako je originální, citrátová a acetátová, které byly také testovány pro vybrané půdy v závislosti na celkovém organickém uhlíku (TOC) **(Článek II)**.

Analytické metody byly aplikovány na reálné vzorky pitné vody z vodovodního potrubí **(Článek III)**, sladkovodní rybníky a nádrže ovlivněné vodním květem, u kterých byla měřena estrogenní aktivita a koncentrace estrogenních látek **(Článek IV)**. Dalšími reálnými vzorky byla balená pitná voda v PET lahvích, voda z čističky odpadních vod, artificiální půdy a sada půd vybraná z banky půd Centra pro výzkum toxických látek v prostředí (RECETOX).

Dále byly realizovány experimenty týkající se analýzy blanku, jako notoricky známý problém v ultrastopové analýze těchto analytů **(Článek V)**.

Publikované práce, které jsou součástí předkládané disertační práce **(Články I–V)** obsahují detailní popis dané problematiky.

GRAPHICAL ABSTRACT



CONTENTS

MAIN OBJECTIVES AND OUTLINE OF THE THESIS

The presented dissertation thesis deals with the determination of alkylphenols and bisphenols in environmental matrices such as water and soil. Attention is paid mainly to a selected group of analytes and their derivates with 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl chloride, DNSC) agent, respectively.

The main objectives of the dissertation thesis were as follows:

- To optimize and validate an analytical method for LC/MS determination of selected alkylphenols in water and soil **(Articles I, II)**
- To apply a new analytical method for determination of alkylphenols in environmental samples such as water from a treatment plant **(Article I)**, residential water pipes **(Article III)**, freshwater ponds **(Article IV)** and various soil types **(Article II)**
- To evaluate and assess water and soil blanks **(Article V)**

INTRODUCTION

1) Alkylphenols and Bisphenols

Alkylphenols (APs) and bisphenols (BPs) have caused extensive scientific, environmental, and political concerns in recent years because of their worldwide pollution and serious potential effects on humans and wildlife (Li et al. 2013). Alkylphenols and bisphenols are classified as endocrine-disrupting compounds (EDCs) which have a negative effect on the hormonal system of many organisms including humans (Balabanič et al. 2013; Lauretta et al. 2019). Research into the presence of APs and BPs in the environment has dramatically increased over the last two decades.

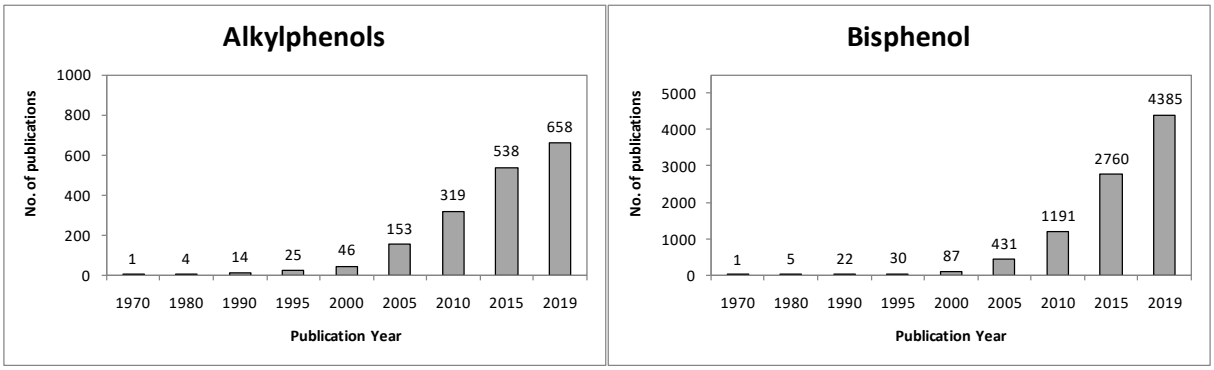


Figure 1. *Number of publications with keywords "Alkylphenols" and "Bisphenol" in the last decades.* (CAB Direct as of 6. 9. 2019)

Alkylphenols are the final products in the biodegradation of alkylphenol polyethoxylates, which are non-ionic surfactants widely used in detergents, paints, pesticides, cosmetics and other formulated products (Balabanič, et al. 2013). Due to extensive long-term application and the particularly difficult degradation of alkylphenols, these compounds are present at low concentrations in most environmental and biological matrices. In context to *log* K_{ow}, these compounds indicate lipophilicity and a tendency to enter the solid phase in the environment (Sabik et al. 2003; Soares, et al. 2008). Alkylphenols are the great family of organic compounds formed by a substituted phenolic ring and an alkyl chain. The alkyl chain can be attached at various locations around the phenolic ring and therefore, *meta*-, *ortho*- and *para*-alkylphenols (also called 2-, 3- and 4-alkylphenols, respectively) can be distinguished. From all alkylphenols, 4-octylphenol (4-OP) and 4-nonylphenol (4-NP) are the most important because of their higher level of use in industrial and household applications as well as their higher disruption capabilities. Branched and linear alkyl of alkylphenols isomers can be identified depending on the structure of the octyl- and nonyl alkyl group. While

branched 4-OP (4-*tert*-OP) is a unique compound, more than 211 isomers are part of branched 4-NP (also called technical mixture). Although linear isomers (4-*n*-OP and 4-*n*-NP) are scarcely used for industrial purposes, they can be present in aquatic systems and can bioaccumulate in organisms as has been demonstrated in various environmental and ecotoxicological studies (Salgueiro-González et al. 2017). The relative estrogenic potencies of alkylphenolic compounds are OP > NP carboxylic acid > NP > NP ethoxylate, with potencies approximately 1×10^5 less than that of E2 (Balabanič et al. 2013).

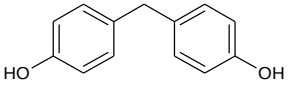
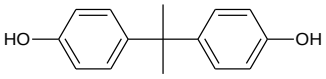
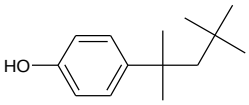
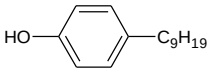
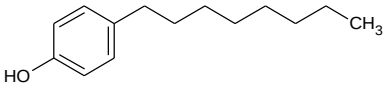
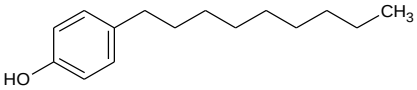
Bisphenols are a group of chemical compounds with two hydroxyphenyl functionalities. Most of them are based on diphenylmethane. The most popular representative of this group is bisphenol A (BPA), often simply called "bisphenol", although there are many structural analogues (Fiege et al. 2000; Regueiro et al. 2015). Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is one of the highest-volume chemicals in the world. The main market for BPA is the production of polycarbonate, epoxy resins, polyesters and flame retardants. Thus, it is present in electronic components, construction materials, beverage and food containers and medical devices (Ballesteros-Gómez et al. 2009; Salgueiro-González et al. 2017). Bisphenol A is a lipophilic compound like alkylphenols and can easily contaminate foods of animal origin (e.g. milk, meat, cheese), which are thought to represent the most important sources of human exposure (Shao et al. 2007). BPA demonstrates estrogenic activity and is considered to be an important organic pollutant (Amiridou and Voutsa, 2011). The estrogenic potency is approximately 1×10^4 less than that of E2 (Balabanič, et al. 2013). The estrogenic activity of BPA was first reported in 1993. Because of the high volume, wide dispersive use, and endocrine-disrupting and toxic properties of BPA, it is a clear candidate to be included in the list of substances subject to authorization in the new policy on chemicals approved by the EU, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals, Annex XIV) along with alkyphenols (Baugros et al. 2008; Ballesteros-Gómez et al. 2009).

Of concern is the fact that EDCs can adversely affect human health. Alkylphenols and bisphenols are endocrine disruptors like polychlorinated compounds (dioxins, furans and biphenols), polybrominated diphenyl ethers, phthalates and steroid sex hormones. These various chemical substances may have synergistic or antagonistic effects on each other. In most documented studies, animals were exposed to a few chemical substances for a certain period of time. However, humans are exposed to a great number of chemical substances simultaneously and throughout their lifetime, usually several decades. The largest source of EDCs is industry. Measures need to be taken to minimise the release of these substances

into the environment, thus reducing the impact of these pollutants on human health (Balabanič et al. 2013; Petrović et al. 2001).

Table 1. *Physiochemical properties and structure of the studied compounds*.

(https://chemaxon.com/products/chemicalize)

Compound	Acronym	CAS-number	Molecular weight	log P	Structure
Bisphenol F	BPF	620-92-8	200,23	3,46	
Bisphenol A	BPA	80-05-7	228,29	4,04	
4-*terc*- octylphenol	4-t-OP	140-66-9	206,32	4,69	
Nonylphenol (isomer mixture)	iso-NP	84852-15-3	220,35	~5,74	
4-octylphenol	4-OP	1806-26-4	206,32	5,30	
4-nonylphenol	4-NP	104-40-5	220,35	5,74	

BPA, OP and NP have been placed in the contaminant candidate list for compounds that may require regulations under the Safe Drinking Water Act. The total allowable concentration (TAC) for BPA is 100 $\mu g \cdot L^{-1}$ in drinking water. The Integrated Risk Information System (IRIS) of the US EPA has proposed reference dose values for chronic oral exposure (RfD) based on chronic health hazard assessment for non-carcinogenic effects for BPA at 5×10^{-2} mg/kg bw/day (Willhite et al. 2008; EPA 2009).

The European Commission has elaborated a draft "Working Document on Sludge" that proposes maximum levels for some micro-contaminants, including alkyl ethoxylates (AEOs) and alkylphenol ethoxylates (APEOs). The maximum value for their concentration in sludges for agricultural soils is 50 $mg \cdot kg^{-1}$ of dry matter. This value includes the nonylphenol and nonylphenol ethoxylates with one or two ethoxy groups (Andreu et al. 2007).

The hygienic limits for these substances are not determined in air. In 2011, the use of bisphenol A in baby bottles was banned in all EU countries, and some countries have further extended this ban to food packaging and thermopaper in cash receipts. In 2013, BPA was added to the list of carcinogenic and hormone disrupting agents (Arnika; Björnsdotter et al. 2017).

2) Sample preparation and cleanup

Most environmental samples are not directly compatible with the chromatographic system to be analyzed in the final step. The reason for this is the high complexity of the environmental sample with a high proportion of ballasts that affect the analyte, often present in trace concentrations relative to other matrix components. The sample preparation step is focused on isolation, clean-up and/or preconcentration of analytes of interest from the complex matrix (Nováková 2013; Salgueiro-González et al. 2017).

Sample preanalysis affects all subsequent sample work steps and is a critical and time-consuming sample-handling step. The choice of sample preparation technique is thus crucial for the accuracy and precision of the obtained data, as the analytes are often present at very low concentrations (Nováková 2013).

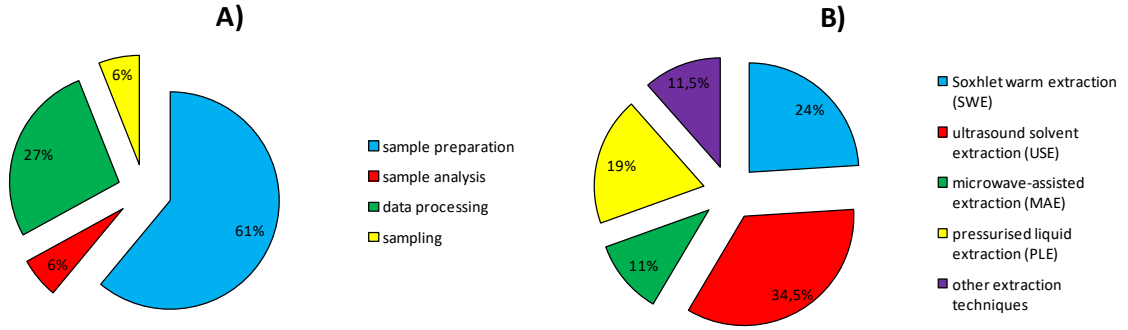


Figure 2A) *Representation of the time required for each step of the chromatographic method. The time-consuming step of sample treatment may be higher or lower than shown. This factor strongly depends on the nature of the sample* (Nováková & Douša 2013). **2B)** *Sample treatment techniques for the extraction of alkylphenols and bisphenol A* **(**Salgueiro-González et al. 2018).

The sample-preparation techniques available for environmental samples can be divided into two main groups based on the history and frequency of their use. The first group, conventional sample preparation methods, is represented by well-established techniques such as Soxhlet warm extraction (SWE), ultrasound solvent extraction (USE), supercritical fluid extraction (SFE), pressurised liquid extraction (PLE) also known as accelerated solvent extraction (ASE), microwave-assisted extraction (MAE), liquid-liquid extraction (LLE) and solid-phase extraction (SPE). On the other hand, these techniques have several drawbacks: they use large quantities of toxic organic solvents, are not environmentally friendly, and they are labour-intensive, difficult to automate and time-consuming. The second group includes

more recently developed techniques belonging to modern approaches in the field of sample preparation. The main requirements for modern sample treatment techniques are reducing the time required, the consumption of solvent, the number of steps, and the costs of analysis and also enabling automation (Nováková 2013; Salgueiro-González et al. 2018; Atapattu & Rosenfeld, 2020). Extraction techniques suitable for the determination of alkylphenols and bisphenols and yet in accordance with Green Chemistry principles are e.g. solid phase microextraction (SPME), stir bar sorptive extraction (SBSE), dispersive liquid-liquid microextraction (DLLME), immunoaffinity solid-phase extraction (IA-SPE), molecularly imprinted polymers (MIP) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe extraction) (Ferguson et al. 2001; Alexiadou et al. 2008; Li et al. 2008; Salgueiro-González et al. 2017; Salgueiro-González et al. 2018; Pernica et al. 2019).

A combined extractive/clean-up extraction technique is the QuEChERS method initially developed by (Anastassiades et al. 2003) for extraction of pesticides with a wide polarity range from fruits and vegetables. This method consists of an extraction with acetonitrile in the presence of salts. There are three dominant varieties of the QuEChERS method for extraction and cleanup of analyte: the original QuEChERS method (unbuffered QuEChERS method), citrate buffered QuEChERS method (EU vision) and acetate buffered QuEChERS method (AOAC vision) (Lehotay et al. 2010; Andraščíková & Hrouzková 2013). Apart from these three QuEChERS types, there are a number of modifications and QuEChERS versions for the extraction and cleanup of analytes such as mycotoxins, pharmaceuticals, polycyclic aromatic hydrocarbons and other in a wide variety of complex matrices (González-Curbelo et al. 2015). The characteristic features of the original QuEChERS method are as follows: extraction with acetonitrile in a disposable tube, followed by the salting out and removal of water from acetonitrile using sodium chloride (NaCl) and anhydrous magnesium sulphate ($MgSO_4$), and purification with dispersive solid phase extraction (d-SPE), in which the extract is processed by shaking with either primary-secondary amine (PSA), silica gel alone, or PSA and C18 or graphite carbon black (GCB). The biggest advantage of using this method is that the time required to perform the assay is reduced since there are only two steps involved (Lehotay et al. 2010).

3) Derivatization

Derivatization is a commonly-used technique for augmenting chromatography analysis. It requires efficient and effective reagents that can modify the behaviour of complex compounds and allow their detection in chromatographic analysis. Derivatization is used in many areas of chemistry such as medical, forensic, food science, and environmental disciplines. In general, analytical derivatization is employed for two reasons (Knapp 1979; Sigma-Aldrich: derivatization):

- to permit analysis of compounds with inadequate volatility or stability,
- to improve chromatographic behaviour or detectability.

Derivatization is a specific chemical reaction. A reactive functional group in the target compound and the corresponding reaction group(s) of the derivatization reagent are the prerequisites for derivatization (Figure 3). The selection of a derivatization reagent mainly depends on the reactive functional group of the target compounds and the purpose of the research.

Derivatization aims to modify the structure of the target compounds and, as a consequence, the chemical and physical properties. Upon derivatization, the chemical and physical properties of the compound will charge, thus affecting compound stability, polarity, solubility, retention, and ionization efficiency. For effective derivatization analysis, the derivatization reaction should be fast, efficient, and specific, and form stable products.

Sensitive and selective methods for the determination of trace-level compounds in complex matrices are essential in many research fields. Due to its inherent sensitivity and selectivity, liquid chromatography-mass spectrometry (LC/MS) has become one of the most prominent analytical techniques. However, many compounds cannot be analysed well by LC/MS, especially if they are difficult to ionize or fragment, as that makes detection sensitivity extremely low. Chemical derivatization has proven to be a powerful strategy for improving the detection characteristics of compounds, and not only in LC/MS.

Recently, a considerable number of derivatization reagents have been synthesized and derivatization methods have been established. Many type of derivatization still exist, including formation of byproducts, non-quantitative reaction, requirement for harsh reaction conditions, long reaction time, and product degradation.

Derivatization approaches can be divided, according to the place where the derivatization reaction takes place, into the categories pre-column derivatization, post-column derivatization and on-column derivatization (Nováková, L., & Douša, M. 2013; Qi et al. 2014).

Recently, the term "green chemistry" has been come into vogue and it has been translated into "green derivatization" in analytical chemistry (Anastas & Warner 1998; Lavillaet et al. 2014). In general, an analytical method is not considered green derivatization if it uses a persistent, bioaccumulative and toxic (PBT) reagent, hazardous reagent, or if the pH is less than 2 or greater than 12 and the waste generated is greater than 50 g (Lavillaet et al. 2014).

An overview of selected derivatizing agents for the determination of alkylphenols and bisphenols is summarized in Table 2.

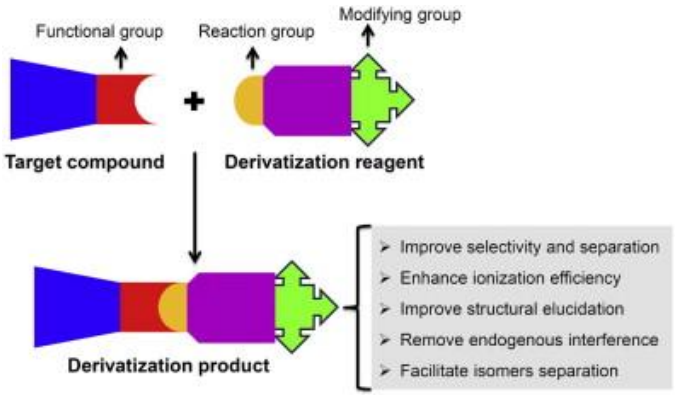


Figure 3. *General reaction mechanism between target compound and derivatization reagent and advantages of derivatization-based LC-MS methods*. (Qi et al. 2014)

Table 2. *Selected derivatizing agents for the determination of alkylphenols and bisphenols.*

Matrix	Analytes	Derivatization agents	Detection	Reference
water	APs, BPA	dansylchloride [Dns-Cl; DNSC; 5-(dimethylamino)naphthalene-1-sulfonyl chloride]	HPLC-ESI/MS/MS	(Pernica et al. 2015)
water	APs, BPA	BSTFA [bis(trimethylsilyl)trifluoroacetamide] + TMCS [trimethylchlorosilane]	GC-MS	(Li et al. 2001)
	Bisphenols	pyridine3-sulfonyl chloride	LC/MS	(Regueiro et al. 2015)
water	APs, BPA	BSTFA [bis(trimethylsilyl)trifluoroacetamide]	GC-MS	(Basheer & Lee, 2004).
sediment	Bisphenols	AA [acetic anhydride]	GC-MS	(Wang et al. 2017)
water	APs	PFPdy [pentafluoropyridine]	GC-MS	(Kojima et al. 2003)
rat serum	APs, BPA	2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole	HPLC-FLR	(Katayama et al. 2001)
Aquaculture pond water	APs, BPA	BCEC-Cl [2-(11H-benzo[a]carbazol-11-yl) ethyl carbonochloridate]	HPLC-FLR	(Wu et al. 2016)
urine	APs, BPA	PFBBr [pentafluorobenzyl bromide]	GC-MS	(Kuklenyik et al. 2003)
water	APs	MTBSTFA [N-tert-butyl-dimethylsilyl-Nmethyltrifluoroacetamide]	GC-MS	(Pan & Tsai, 2008)
water	APs	MSTFA [N-methyl-N-(trimethylsilyl)-trifluoroacetamide]	GC-MS	(Voutsa et al. 2006)
water	APs, BPA	EASC [10-ethyl-acridone-2-sulfonyl chloride]	HPLC-FLR	(Zhang et al. 2012 A)
sediment	APs, BPA	CEOC [2-(9H-carbazol-9-yl)ethyl carbonochloridate]	HPLC-FLR	(Zhang et al. 2012 B)
sewage sludge	APs, BPA	dansylchloride [Dns-Cl; DNSC; 5-(dimethylamino)naphthalene-1-sulfonyl chloride]	HPLC-FLR	(Naassner et al. 2002)
urine	NP, BPA	p-nitrobenzoyl chloride	HPLC-FLR	(Mao et al. 2004)
plastic samples	NP, BPA	DIB-Cl [4-(4,5-diphenyl-1H-imidazole-2-yl)benzoyl chloride]	HPLC-FLR	(Sun et al. 2001)

4) Residue of alkylphenols and bisphenols

Blank contamination is a generally known problem in the ultra-trace analysis of alkylphenols and bisphenol A. Achieving low detection limits is complicated due to the high background signals (Salgueiro-González et al. 2012). The primary purpose of analysis of blanks is to trace sources of artificially introduced contamination. The source of contamination introduced in the field or laboratory can be deduced by comparing blank results. The equipment blank could potentially be contaminated in the field, during transport to the lab, or in the lab. Using all the blanks described in this fact sheet will facilitate the identification of contamination sources (EPA, 2015).

Secondary contamination of analysed samples can come from laboratory air, septa vials, solvents and other chemicals used. Plastic tubes and connections could be another important source of sample contamination. This can be a problem especially in the determination of alkylphenols and bisphenol A at low environmentally relevant concentrations (Salgueiro-González et al. 2012).

Terms such as ghost peaks, artifact peaks, artefact peaks, system peaks, pseudo peaks, vacancy peaks, eigenpeaks, induced peaks and spurious peaks can be found in published papers. These terms are called undesirable peaks in the blank systems (Williams, 2004).

It is important to fully characterise the analytical performance in order to understand capability and limitations, and to ensure that they are “fit for purpose”. Terms such as LOB (limit of blank), LOD (limit of detection), and LOQ (limit of quantification) describe the lowest concentration of an analyte that can be reliably determined by an analytical procedure. LOB and LOD are important parameters for tests used to distinguish between the presence or absence of an analyte and LOQ to reliably determine low levels of analyte and should be incorporated as part of any method evaluation (Armbruster & Pry 2008).

• Blank equipment (equipment blank) includes the total field and laboratory contamination sources, which are determined by sputtering the blank over the decontaminated field-sampling device before sampling the environment. The objective of the assessment is to assess the total contamination of the sampling.

• Field blank includes overall ambient conditions during sampling and laboratory pollution sources. Field blank determination is done as follows: a blank sample is transferred to the sampling container and then sent to a laboratory with a field sample. The aim is to assess contamination from field conditions during collection.

• Trip blank includes shipping and laboratory contamination sources only for volatile substances. The transport blind sample is a sample which is transported from the laboratory to the point of collection and transported back to the laboratory without being subjected to a sampling procedure.

• The blank method (method blank) only shows laboratory sources of contamination. Blank methods are prepared and analyzed exactly in the same way as a field sample. The aim is to assess the contamination during sample preparation.

• The instrumental blank (instrument blank) only shows instrumental sources of contamination. The blank itself is parsed with the field sample and the goal is to assess the presence or absence of device contamination (EPA 2015).

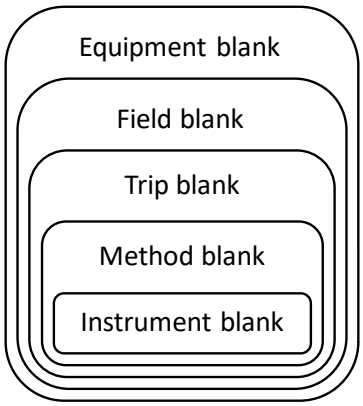


Figure 4. *Comparison of different blank.*

Nonylphenols can also be present in laboratory air. For example, concentrations about 100 ng/m^3 were found in the air of a typical laboratory and it has also been reported that an LC-MS vial with methanol left in the autosampler of the instrument can absorb NP from the laboratory air within weeks. Septa vials and solvents used as mobile phases can also cause blank contamination. BPA contamination caused by the water purification system was observed in ultrapure water. Sampling, storage of samples, filtration, and sample treatment are also important sources of blank contamination. APs and BPA from water samples are commonly extracted by liquid-liquid extraction (LLE) or solid-phase extraction (SPE). However, this procedure can also cause contamination due to the plastic materials of the SPE cartridges, the multiple steps involved and the use of relatively high volumes of solvent.

A quality assurance/quality control (QA/QC) protocol in the analysis of APs and BPs is necessary because of possible contamination during the various steps from initial sampling to final analysis (Xie et al. 2006; Salgueiro-González et al. 2012; Salgueiro-González et al. 2018).

5) Determination of alkylphenols and bisphenols

In order to assess the risk of contamination of the environment by alkylphenols and bisphenols it is necessary to have sensitive and selective methods for qualitatively and quantitatively analyzing them. Current requirements for analytical methods include high sensitivity, high throughput, ease of use, precision, accuracy, robustness and automation. Although instrumentation meets some of these requirements, sample preparation and cleanup that take place prior to instrument analysis is still the subject of active research and development (Atapattu & Rosenfeld, 2013).

The analysis of APs and BPs can be carried out by immunochemical methods (Estevez-Alberola & Marco, 2004), micellar liquid chromatography (Romero-Cano, et al. 2015), gas chromatography or liquid chromatography with different detectors. Gas chromatography with flame ionization detection (GC-FID), highperformance liquid chromatography coupled to a fluorescence detector (HPLC-FLD) and ultraviolet-visible detector (HPLC-UV-Vis) are not widely used. Overlapped and tailed peaks are observed because of the complexity of the matrices and the lower selectivity of the detectors. Therefore, an exhaustive cleanup step is normally required (Salgueiro-González et al. 2018).

The most widely employed techniques for the separation and detection of these compounds are gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to mass spectrometry (LC-MS) for their sensitivity, specificity, and fast response. The use of liquid chromatography followed by (tandem) mass spectrometry has grown steeply in the last decade. Soft ionization techniques like electrosprey (ESI) and atmospheric pressure chemical ionization (APCI) are the most widely employed (Atapattu & Rosenfeld, 2013; Salgueiro-González et al. 2018).

The (ESI)MS/MS transitions commonly using a triple quadrupole as analyser for the qualitative and quantitative analyses of alkylphenols and bisphenols are as follows: 227 > 212 for BPA, 205 > 133 and 205 > 106 for 4-t-OP and 4-n-OP respectively, 219 > 133 and 219 > 106 for NP and 4-n-NP, respectively. Methanol, acetonitrile and water with some modifiers (acetic acid, ammonium salts, ammonia) added to improve the chromatographic separation and its sensitivity are commonly used as mobile phases (Seifertová & Simek 2012; Salgueiro-González et al. 2018).

The alkylphenols and bisphenols can be analyzed directly or after derivatization with various agents as shown in Table 2.

Dansyl chloride (DNSC) has been widely used as a derivatizing reagent for fluorescence detection and for facilitating the MS detection of phenols and amines (Tang & Guengerich, 2010). The reaction occurs in middle alkaline acetone-aqueous medium at multiple excess of derivatization reagent. Both degrees of derivatization and hydrolysis of derivatives increase with increasing pH of the reaction medium. Therefore, the recommended pH of reaction medium has to be middle alkaline. Derivatization is carried out exclusively before LC-MS analysis as a pre-column procedure. The disadvantage of dansyl derivation may be its long reaction time and high reaction temperature (Wiedmeier et al. 1982; Seifertová & Simek 2012). The dansylation of phenolic hydroxyl groups introduces ionizable basic nitrogen that enhances the mass spectrometric response of the target analytes. The MS/MS analysis of such a derivative utilizes the formation of product ion with m/z 171 corresponding to the protonated
5-(dimethylamino) naphthalene. LC-ESI-MS/MS analysis based on this product ion is highly selective and sensitive and is used for MS/MS detection of dansyl derivatives of phenolic compounds. The efficiency of electrospray ionization (ESI) of alkylphenols and bisphenols without derivatization is much lower than those of derivatized APs and BPs, and not only in environmental samples (Seifertová & Simek 2012).

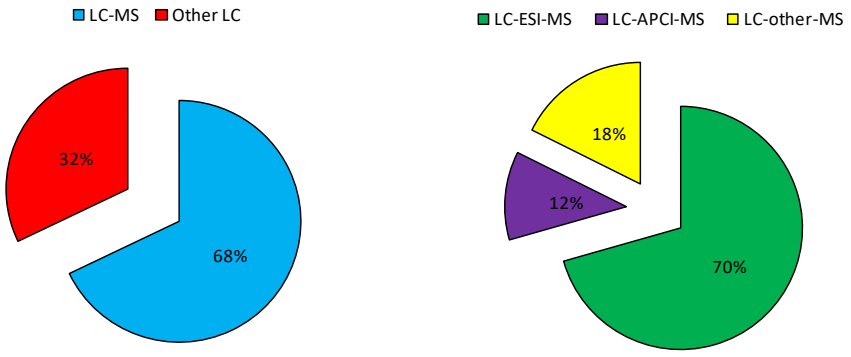


Figure 5. *Percentage of number of publications with the keywords "Bisphenol A" and "Instrumental techniques" in the last decades.* (CAB Direct as of 5. 11. 2019).

CONCLUSION

Alkylphenols and bisphenols are ubiquitous compounds that can affect the endocrine systems of animals and human at low concentrations. Because of their physicochemical properties, these compounds can be detected in all environmental matrices (water, soil, sediment and air). In this context, analytical methods that allow the determination of these compounds at residue concentrations have been developed in recent years, according to trends in environmental analytical techniques as well as the marked goal in each application.

Sampling and sample preparation are critical and time-consuming points in the analysis of alkylphenols and bisphenols in environmental samples. Conventional extraction techniques have been largely replaced by alternative techniques for extraction of these compounds. A new trend in analytical chemistry (QuEChERS, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe extraction) merits greater attention and can be applied for the analysis of these pollutants. The alkylphenols and bisphenols can be analyzed directly and/or after derivatization with various reagents as shown in Table 2. Analytical derivatization of target analytes can substantially improve chromatographic separation as well as the sensitivity and selectivity of detection.

The determination of APs and BPs can be carried out by high-performance liquid chromatography and gas chromatography coupled to various detectors. However, the complexity of environmental samples can hinder the identification and quantification of target compounds using a detector such as a flame ionization detector (FID), fluorescence detector (FLD) or ultraviolet-visible detector (UV-Vis). The limitations of these detectors can be avoided using mass spectrometry. About 70% of the recently published work for the determination of alkylphenols and bisphenols is based on the use of LC-MS/MS. The most commonly employed ionization technique is electrospray (ESI), followed by atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI).

Special attention should be paid to blank contamination problems and matrix effects. Blank contamination is a common problem in the ultra-trace analysis of alkylphenols and bisphenols. A quality assurance/quality control (QA/QC) protocol in the analysis of APs and BPs is necessary because of possible contamination during the various steps from initial sampling to final analysis.

Finally, further research is needed to develop an effective multiresidue methodology for determination of APs (including individual isomers) and BPs in environmental samples.

REFERENCES

Alexiadou, D. K., Maragou, N. C., Thomaidis, N. S., Theodoridis, G. A., & Koupparis, M. A. (2008). Molecularly imprinted polymers for bisphenol A for HPLC and SPE from water and milk. *Journal of Separation Science, 31*(12), 2272-2282.

Amiridou, D., & Voutsa, D. (2011). Alkylphenols and phthalates in bottled waters. *Journal of Hazardous Materials, 185*(1), 281-286.

Anastas, P. T., & Warner, J. C. (1998). Green chemistry. *Frontiers, 640*.

Anastassiades, M., Lehotay, S. J., Štajnbaher, D., & Schenck, F. J. (2003). Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *Journal of AOAC International, 86*(2), 412-431.

Andraščíková, M., & Hrouzková, S. (2013). A comparative study of three modifications of the QuEChERS method for determination of endocrine disrupting pesticide residues in lemon matrices by fast GC-MS. *Analytical Methods, 5*(6), 1374-1384.

Andreu, V., Ferrer, E., Rubio, J. L., Font, G., & Picó, Y. (2007). Quantitative determination of octylphenol, nonylphenol, alkylphenol ethoxylates and alcohol ethoxylates by pressurized liquid extraction and liquid chromatography–mass spectrometry in soils treated with sewage sludges. *Science of the Total Environment, 378*(1-2), 124-129.

Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *The Clinical Biochemist. Reviews, 29 Suppl 1*(Suppl 1), S49–S52.

Atapattu, S. N., & Rosenfeld, J. M. (2013). Solid phase analytical derivatization as a sample preparation method. *Journal of Chromatography A, 1296*, 204-213.

Atapattu, S. N., & Rosenfeld, J. M. (2020). Solid-phase analytical derivatizations. In *Solid-Phase Extraction* (pp. 551-571). Elsevier.

Balabanič, D., Rupnik, M., & Klemenčič, A. K. (2011). Negative impact of endocrine-disrupting compounds on human reproductive health. *Reproduction, Fertility and Development, 23*(3), 403-416.

Ballesteros-Gómez, A., Rubio, S., & Pérez-Bendito, D. (2009). Analytical methods for the determination of bisphenol A in food. *Journal of Chromatography A, 1216*(3), 449-469.

Basheer, C., & Lee, H. K. (2004). Analysis of endocrine disrupting alkylphenols, chlorophenols and bisphenol-A using hollow fiber-protected liquid-phase microextraction coupled with injection port-derivatization gas chromatography–mass spectrometry. *Journal of Chromatography A, 1057*(1-2), 163-169.

Baugros, J. B., Giroud, B., Dessalces, G., Grenier-Loustalot, M. F., & Cren-Olivé, C. (2008). Multiresidue analytical methods for the ultra-trace quantification of 33 priority substances present in the list of REACH in real water samples. *Analytica Chimica Acta, 607*(2), 191-203.

Björnsdotter, M. K., de Boer, J., & Ballesteros-Gómez, A. (2017). Bisphenol A and replacements in thermal paper: A review. *Chemosphere, 182*, 691-706.

EPA (2009), Integrated Risk Information System, U.S. Environmental Protection Agency.

Estevez-Alberola, M. C., & Marco, M. P. (2004). Immunochemical determination of xenobiotics with endocrine disrupting effects. *Analytical and Bioanalytical Chemistry*, *378*(3), 563-575.

Ferguson, P. L., Iden, C. R., McElroy, A. E., & Brownawell, B. J. (2001). Determination of steroid estrogens in wastewater by immunoaffinity extraction coupled with HPLC− electrospray-MS. *Analytical Chemistry*, *73*(16), 3890-3895.

Fiege, H., Voges, H. W., Hamamoto, T., Umemura, S., Iwata, T., Miki, H., Fujita, Y., Buysch, H. J., Garbe, D.& Paulus, W. (2000). Phenol derivatives. *Ullmann's Encyclopedia of Industrial Chemistry*.

González-Curbelo, M. Á., Socas-Rodríguez, B., Herrera-Herrera, A. V., González-Sálamo, J., Hernández-Borges, J., & Rodríguez-Delgado, M. Á. (2015). Evolution and applications of the QuEChERS method. *TrAC Trends in Analytical Chemistry*, *71*, 169-185.

https://arnika.org/

https://chemaxon.com/products/chemicalize

https://www.epa.gov/sites/production/files/2015-06/documents/blanks.pdf

https://www.sigmaaldrich.com › sigma-aldrich › Derivatization

Katayama, M., Sasaki, T., Matsuda, Y., Kaneko, S., Iwamoto, T., & Tanaka, M. (2001). Sensitive determination of bisphenol A and alkylphenols by high performance liquid chromatography with pre-column derivatization with 2-(4-carboxyphenyl)-5, 6-dimethylbenzimidazole. *Biomedical Chromatography*, *15*(6), 403-407.

Knapp, D. R. (1979). *Handbook of Analytical Derivatization Reactions*. John Wiley & Sons.

Kojima, M., Tsunoi, S., & Tanaka, M. (2003). Determination of 4-alkylphenols by novel derivatization and gas chromatography–mass spectrometry. *Journal of Chromatography A*, *984*(2), 237-243.

Kuklenyik, Z., Ekong, J., Cutchins, C. D., Needham, L. L., & Calafat, A. M. (2003). Simultaneous measurement of urinary bisphenol A and alkylphenols by automated solid-phase extractive derivatization gas chromatography/mass spectrometry. *Analytical Chemistry*, *75*(24), 6820-6825.

Lauretta, R., Sansone, A., Sansone, M., Romanelli, F., & Appetecchia, M. (2019). Endocrine Disrupting Chemicals: Effects on Endocrine Glands. *Frontiers in Endocrinology*, *10*, 178.

Lavilla, I., Romero, V., Costas, I., & Bendicho, C. (2014). Greener derivatization in analytical chemistry. *TrAC Trends in Analytical Chemistry*, *61*, 1-10.

Lehotay, S. J., Son, K. A., Kwon, H., Koesukwiwat, U., Fu, W., Mastovska, K.,Hon, E., & Leepipatpiboon, N. (2010). Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. *Journal of Chromatography A*, *1217*(16), 2548-2560.

Li, D., Park, J., & Oh, J. R. (2001). Silyl derivatization of alkylphenols, chlorophenols, and bisphenol A for simultaneous GC/MS determination. *Analytical Chemistry*, *73*(13), 3089-3095.

Li, L., Wang, J., Zhou, S., & Zhao, M. (2008). Development and characterization of an immunoaffinity monolith for selective on-line extraction of bisphenol A from environmental water samples. *Analytica Chimica Acta*, *620*(1-2), 1-7.

Li, Y., Zhang, S., Song, C., & You, J. (2013). Determination of bisphenol A and alkylphenols in soft drinks by high-performance liquid chromatography with fluorescence detection. *Food Analytical Methods*, *6*(5), 1284-1290.

Mao, L., Sun, C., Zhang, H., Li, Y., & Wu, D. (2004). Determination of environmental estrogens in human urine by high performance liquid chromatography after fluorescent derivatization with p-nitrobenzoyl chloride. *Analytica Chimica Acta*, *522*(2), 241-246.

Naassner, M., Mergler, M., Wolf, K., & Schuphan, I. (2002). Determination of the xenoestrogens 4-nonylphenol and bisphenol A by high-performance liquid chromatography and fluorescence detection after derivatisation with dansyl chloride. *Journal of Chromatography A*, *945*(1-2), 133-138.

Nováková, L. (2013). Challenges in the development of bioanalytical liquid chromatography–mass spectrometry method with emphasis on fast analysis. *Journal of Chromatography A*, *1292*, 25-37.

Nováková, L., & Douša, M. (2013). *Moderní HPLC separace v teorii a praxi*. Lucie Nováková.

Pan, Y. P., & Tsai, S. W. (2008). Solid phase microextraction procedure for the determination of alkylphenols in water by on-fiber derivatization with N-tert-butyl-dimethylsilyl-N-methyltrifluoroacetamide. *Analytica Chimica Acta*, *624*(2), 247-252.

Pernica, M., Poloucká, P., Seifertová, M., & Šimek, Z. (2015). Determination of alkylphenols in water samples using liquid chromatography–tandem mass spectrometry after pre-column derivatization with dansyl chloride. *Journal of Chromatography A*, *1417*, 49-56.

Pernica, M., Lesniaková, R., Doležalová D., & Šimek, Z., (2019). Analysis of alkylphenols and bisphenols in soils using liquid chromatography-tandem mass spectrometry, *International Journal of Environmental Analytical Chemistry*, DOI: 10.1080/03067319.2019.1679804

Petrović, M., Eljarrat, E., de Alda, M. J. L., & Barceló, D. (2001). Analysis and environmental levels of endocrine-disrupting compounds in freshwater sediments. *TrAC Trends in Analytical Chemistry*, *20*(11), 637-648.

Qi, B. L., Liu, P., Wang, Q. Y., Cai, W. J., Yuan, B. F., & Feng, Y. Q. (2014). Derivatization for liquid chromatography-mass spectrometry. *TrAC Trends in Analytical Chemistry*, *59*, 121-132.

Regueiro, J., Breidbach, A., & Wenzl, T. (2015). Derivatization of bisphenol A and its analogues with pyridine-3-sulfonyl chloride: multivariate optimization and fragmentation patterns by liquid chromatography/Orbitrap mass spectrometry. *Rapid Communications in Mass Spectrometry*, *29*(16), 1473-1484.

Romero-Cano, R., Kassuha, D., Peris-Vicente, J., Roca-Genovés, P., Carda-Broch, S., & Esteve-Romero, J. (2015). Analysis of thiabendazole, 4-tert-octylphenol and chlorpyrifos in waste and sewage water by direct injection–micellar liquid chromatography. *Analyst*, *140*(5), 1739-1746.

Sabik, H., Gagné, F., Blaise, C., Marcogliese, D. J., & Jeannot, R. (2003). Occurrence of alkylphenol polyethoxylates in the St. Lawrence River and their bioconcentration by mussels (Elliptio complanata). *Chemosphere, 51*(5), 349-356.

Salgueiro-González, N., Concha-Graña, E., Turnes-Carou, I., Muniategui-Lorenzo, S., López-Mahía, P., & Prada-Rodríguez, D. (2012). Blank and sample handling troubleshooting in ultratrace analysis of alkylphenols and bisphenol A by liquid chromatography tandem mass spectrometry. *Talanta, 101*, 413-419.

Salgueiro-González, N., Muniategui-Lorenzo, S., López-Mahía, P., & Prada-Rodríguez, D. (2017). Trends in analytical methodologies for the determination of alkylphenols and bisphenol A in water samples. *Analytica Chimica Acta, 962*, 1-14.

Salgueiro-González, N., Castiglioni, S., Zuccato, E., Turnes-Carou, I., López-Mahía, P., & Muniategui-Lorenzo, S. (2018). Recent advances in analytical methods for the determination of 4-alkylphenols and bisphenol A in solid environmental matrices: a critical review. *Analytica Chimica Acta, 1024*, 39-51.

Seifertova, M., & Simek, Z. (2012). Determination of alkylphenol and bisphenol a in environmental matrices using HPLC-MS/MS methods. *CHEMICKE LISTY, 106*, S109-S111.

Shao, B., Han, H., Li, D., Ma, Y., Tu, X., & Wu, Y. (2007). Analysis of alkylphenol and bisphenol A in meat by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry. *Food Chemistry, 105*(3), 1236-1241.

Soares, A., Guieysse, B., Jefferson, B., Cartmell, E., & Lester, J. N. (2008). Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environment International, 34*(7), 1033-1049.

Sun, Y., Wada, M., Kuroda, N., Hirayama, K., Nakazawa, H., & Nakashima, K. (2001). Simultaneous determination of phenolic xenoestrogens by solid-phase extraction and high-performance liquid chromatography with fluorescence detection. *Analytical Sciences, 17*(6), 697-702.

Tang, Z., & Guengerich, F. P. (2010). Dansylation of unactivated alcohols for improved mass spectral sensitivity and application to analysis of cytochrome P450 oxidation products in tissue extracts. *Analytical Chemistry, 82*(18), 7706-7712.

Voutsa, D., Hartmann, P., Schaffner, C., & Giger, W. (2006). Benzotriazoles, alkylphenols and bisphenol A in municipal wastewaters and in the Glatt River, Switzerland. *Environmental Science and Pollution Research, 13*(5), 333-341.

Wang, Q., Zhu, L., Chen, M., Ma, X., Wang, X., & Xia, J. (2017). Simultaneously determination of bisphenol A and its alternatives in sediment by ultrasound-assisted and solid phase extractions followed by derivatization using GC-MS. *Chemosphere, 169*, 709-715.

Wiedmeier, V. T., Porterfield, S. P., & Hendrich, C. E. (1982). Quantitation of Dns-amino acids from body tissues and fluids using high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications, 231*(2), 410-417.

Willhite, C. C., Ball, G. L., & McLellan, C. J. (2008). Derivation of a bisphenol A oral reference dose (RfD) and drinking-water equivalent concentration. *Journal of Toxicology and Environmental Health, Part B, 11*(2), 69-146.

Williams, S. (2004). Ghost peaks in reversed-phase gradient HPLC: a review and update. *Journal of Chromatography A, 1052*(1-2), 1-11.

Wu, H., Li, G., Liu, S., Hu, N., Geng, D., Chen, G., Sun, Z., Zhao, X., Xia, L., & You, J. (2016). Monitoring the contents of six steroidal and phenolic endocrine disrupting chemicals in chicken, fish and aquaculture pond water samples using pre-column derivatization and dispersive liquid–liquid microextraction with the aid of experimental design methodology. *Food Chemistry, 192*, 98-106.

Xie, Z., Selzer, J., Ebinghaus, R., Caba, A., Ruck, W. (2006). Development and validation of a method for the determination of trace alkylphenols and phthalates in the atmosphere. Analytica chimica acta, 565(2): 198–207.

Zhang, S., You, J., Sun, Z., Song, C., Ning, S., Zhao, C., & Suo, Y. (2012). A sensitive method for extraction and determination of endocrine-disrupting compounds from wastewater using 10-ethyl-acridone-2-sulfonyl chloride as pre-column labeling reagent by high-performance liquid chromatography with fluorescence detection. *Microchemical Journal*, *103*, 90-96. A

Zhang, S., You, J., Song, C., Chen, G., & Suo, Y. (2012). Purification and determination of bisphenol A and alkylphenol in river sediments by high performance liquid chromatography with fluorescence detection. *Analytical Methods*, *4*(12), 4030-4036. B

RESULTS AND DISCUSSION

The dissertation thesis is conceived as a set of five peer-reviewed articles, three of which are first-authored and the other two are co-authored. The main result of the dissertation thesis is the optimization of the conditions of the derivatization reaction of alkylphenols with dansyl chloride, hitherto unpublished. The optimised derivatization enables a significant drop in the limits of quantification (from 0.08 to 0.83 pg/injection) for alkylphenols on the levels often required for endocrine-disrupting compounds in the environment. The effectiveness of the use of the optimised method was verified in the subsequent environmental and ecotoxicological studies.

The first article entitled **"Determination of alkylphenols in water samples using liquid chromatography-tandem mass spectrometry after pre-column derivatization with dansyl chloride"** describes the optimization and validation of the derivatization reaction for the determination of alkylphenols and bisphenol A in drinking water and water from a waste water treatment plant (WWTP).

The second article, entitled **"Analysis of alkylphenols and bisphenols in soils using liquid chromatography-tandem mass spectrometry"**, aims to optimize extraction and purification techniques for the determination of alkylphenols and bisphenols in soil based on total organic carbon (TOC).

Co-authored articles entitled **"Drinking water contaminants from epoxy resin-coated pipes: A field study"** and **"Estrogenic activity and contributing compounds in stagnant water bodies with massive occurrence of phytoplankton"** describe the use of the optimised analytical method for the determination of alkylphenols and bisphenols in residential water pipes and freshwater ponds.

The last work **"An analysis of residual alkylphenols and bisphenol A using liquid chromatography-tandem mass spectrometry"** focuses on the contamination of the blank as a notorious problem in residual analysis. Part of this study describes the comparison of plastic and glass SPE columns, and recommendations for working with alkylphenols and bisphenols on level ultra-trace analyses.

List of publications

Pernica, M., Poloucká, P., Seifertová, M., & Šimek, Z. (2015). Determination of alkylphenols in water samples using liquid chromatography-tandem mass spectrometry after pre-column derivatization with dansyl chloride. *Journal of Chromatography A*, 1417, 49-56. **(Q1, IF = 3.93)**

Pernica, M., Lesniaková, R., Doležalová D., & Šimek, Z., (2019). Analysis of alkylphenols and bisphenols in soils using liquid chromatography-tandem mass spectrometry, *International Journal of Environmental Analytical Chemistry*, 1-15. **(Q2, IF = 1.267)**

Rajasärkkä, J., **Pernica, M**., Kuta, J., Lašňák, J., Šimek, Z., & Bláha, L. (2016). Drinking water contaminants from epoxy resin-coated pipes: A field study. *Water Research*, 103, 133-140. **(Q1, IF = 5.99)**

Prochazkova T., Sychrova E., Vecerkova J., Javurkova B., Otoupalikova A., **Pernica M.**, Simek Z., Lepsova-Skacelova O., Hilscherova K. (2018) Estrogenic activity and contributing compounds in stagnant water bodies with massive occurrence of phytoplankton.*Water Research*, 136, 12-21 **(Q1, IF = 6.94)**

Pernica, M., & Simek, Z. (2019). An analysis of residue alkylphenols and bisphenol A using liquid chromatography-tandem mass spectrometry. *MendelNet*, 26, 601-605.

Article I

Pernica, M., Poloucká, P., Seifertová, M., & Šimek, Z. (2015). Determination of alkylphenols in water samples using liquid chromatography-tandem mass spectrometry after pre-column derivatization with dansyl chloride. *Journal of Chromatography A*, 1417, 49-56. **(Q1, IF = 3.93)**

Marek Pernica designed and conducted the research and wrote the manuscript.

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Determination of alkylphenols in water samples using liquid chromatography–tandem mass spectrometry after pre-column derivatization with dansyl chloride

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Chemical derivatization of alkylphenols

ABSTRACT

The present study describes an effect of reaction condition of pre-column derivatization of alkylphenols (APs): bisphenol A (BPA), 4-tert-octylphenol (4-t-OP), 4-octylphenol (4-OP), 4-n-nonylphenol (4-n-NP), and isomers of 4-nonylphenol (iso-NP) with 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl chloride, DNSC) on their LC–ESI–MS/MS determination in water samples. Chemical derivatization improves the sensitivity and selectivity of LC–MS/MS analysis. In principle, alkylphenols can be analyzed by LC–MS/MS without derivatization. However, pre-column derivatization of APs increases the sensitivity up to 1000 times in comparison with the analysis of underivatized alkylphenols. Reaction conditions affecting formation of the DNSC-derivatives, such as various solvent, reaction temperature, reaction time, DNSC concentration and pH values were tested. The most suitable conditions, in terms of achieving a high sensitivity, resulting from this study are: acetonitrile as reaction solvent, 60 min as reaction time, 60 °C as reaction temperature, pH values 10.5, 0.5 mg mL^{-1} as DNSC concentration. Calibration curves are linear at least in the range of 1–1000 ng mL^{-1}, limits of detection (LOD) and limits of quantification (LOQ) ranging from 0.02 to 0.25 pg/injection and from 0.08 to 0.83 pg/injection, respectively. The improved procedure was successfully applied for the analysis of APs and BPA in real water samples. The median concentration of BPA and iso-NP obtained in bottled waters was 4.7 ng L^{-1} and 33.5 ng L^{-1}, respectively. The median concentration of 4-t-OP was 1.3 ng L^{-1}.

1. Introduction

Alkylphenols (APs) are toxic, xenobiotic substances classified as endocrine disruptors, which have a negative effect on the hormonal system of many organisms including humans. APs are among the ubiquitous environmental contaminants and are frequent contaminants in wastewater [1,2]. These compounds are also discharged into the environment as metabolites of alkylphenol ethoxylates (APE) mainly by biodegradation in sewage treatment plants. Bisphenol A (BPA) is used as a monomer in the preparation of epoxide resins, polycarbonate plastics and as an antioxidant or stabilizer in polyvinylchloride. BPA can be released into the environment from packaging materials, food and feed as a result of (i) diffuse environmental pollution and direct uptake by animals via food or air and potential bioaccumulation and transfer through the food web, (ii) food processing by contact with plastics, resins, lacquers, surfactants, containers, and (iii) migration from packaging and bottling material, and printer ink [3]. The presence of alkylphenols residues in bottled water is attributed to (i) water contamination in the bottling plant, (ii) migration of plasticizers from the bottle material to the water since quality may vary depending on the raw material as well as the technology used in bottle production [4]. Alkylphenols including linear octylphenol (OP) and nonylphenol (NP) are widely used as intermediates in production of surfactant (anionic and non-ion surfactants) and as a stabilizers of ethylcellulose resin, oil-soluble, phenols and esters. Octylphenol (log K_{OW} 4.12), nonylphenol (log K_{OW} 4.48) and bisphenol A (log K_{OW} 3.32) are lipophilic compounds. Therefore, they can easily contaminate foods of animal origin (e.g., meat, milk, cheese), which are thought to represent the most important source of human exposure to many organic pollutants [3,5].

BPA, OP and NP are nominated in the contaminant candidate list for compounds that may require regulations under the Safe Drinking Water Act [6]. Total allowable concentration (TAC) for BPA is 100 μg L^{-1} in drinking water [7]. The Integrated Risk Information System (IRIS) of US EPA proposed values of

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Table 1
Comparison of instrumental methods used for analyses of APs and BPA with and without derivatization reagents.

Analytes	Derivatization agents	Detection	LOD	Reference
OP, NP	–	HPLC-UV	NP 0.2 μg L^{-1} OP 0.1 μg L^{-1}	[22]
APs, BPA	–	HPLC-UV	2–6 μg L^{-1}	[23]
APs, BPA	BSTFA + 10% TMCS	GC–MS	0.001–0.02 μg L^{-1}	
NP	–	GC–MS	27 ng L^{-1}	[16]
BPA	–	GC–MS	<0.3 μg L^{-1}	[24]
NP, OP	–	HPLC-DAD	NP 0.06 ng μL^{-1} OP 0.04 ng μL^{-1}	[25]
APs, BPA	BSTFA	GC–MS	BPA 0.15 ng L^{-1} NP 0.60 ng L^{-1} OP 1.50 ng L^{-1}	[19]
APs	Pentafluororyridine	GC–MS	NP 85.2 ng L^{-1} OP 12.5 ng L^{-1}	[15]
BPA, NP, OP	MTBSTFA	GC–MS	4–6 ng L^{-1}	[26]
APs, BPA	–	HPLC–ESI/MS/MS	*12–240 ng mL^{-1}	[18]
APs, BPA	DNSC	HPLC–ESI/MS/MS	*4–197 pg mL^{-1}	[27]

BSTFA, N,O-bis (trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; MTBSTFA, N-methyl-N-(tert-butyl-dimethylsilyl)-trifluoroacetamide; DNSC, 5-(dimethylamino) naphthalene-1-sulfonyl chloride.
* IDL, instrument detection limit.

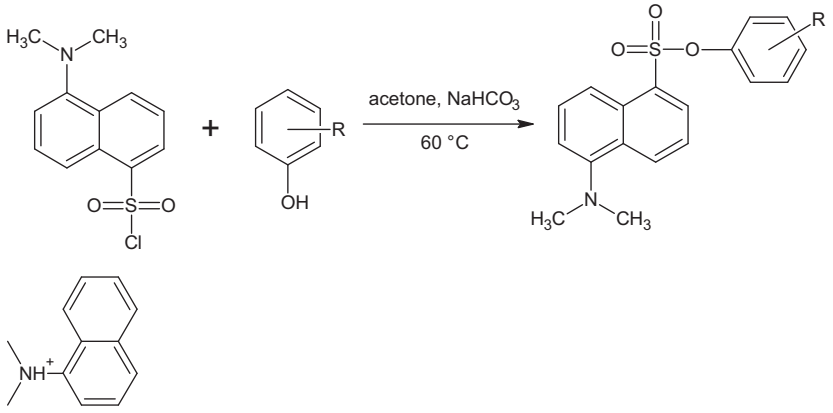

Fig. 1. Reaction scheme of dansylation of alkylphenols. Ion used for MS/MS quantification [5].

reference dose for chronic oral exposure (RfD) based on chronical health hazard assessment for non-carcinogenic effects for BPA 5×10^{-2} mg/kg bw/day [8,9].

High concentrations of BPA, OPs and NPs can be found in wastewater at the outlet of the wastewater treatment plant (WWTP). Acute toxicity levels for BPA, defined as the concentration at which half of the organisms survive (LC_{50}), have been measured in a variety of aquatic organisms, including freshwater and saltwater algae, invertebrates (daphnia and mysid shrimp) and fish. LC_{50} values range from 1 to 20 mg L^{-1} [10]. Similar study describes the values for nonylphenol toxicity to aquatic organisms; LC_{50} values for nonylphenol are in range from 20 to 1590 μg L^{-1}. For instance, NPs at concentrations below 350 μg L^{-1} did not have any toxic effect (EC_{50}) on *Caenorhabditis elegans* (a sediment-dwelling nematode) but enhanced its growth and reproduction [11].

In order to assess the risk of contamination of environment by alkylphenols it is necessary to have sensitive and selective methods for their quantitative analyses. Current requirements for analytical methods include the high sensitivity, high throughput, ease of use, precision, accuracy and automation. Although instrumentation meets some of these requirements, sample preparation that takes place prior to instrumental analysis is still under active research and development [12]. Chromatographic methods are used for determination of alkylphenols, for example micellar liquid chromatography (MLC) [13], gas chromatography [14–16] and liquid chromatography [17–19]. The alkylphenols and bisphenol A can be analyzed directly and/or after pre-column derivatization with various agents as shown in Table 1. Analytical derivatization of target analytes can substantially improve chromatographic separation as well as the sensitivity and selectivity of detection [12]. Dansyl chloride has been widely used as a derivatizing reagent for fluorescence detection and for facilitating the MS detection of phenols and amines, but not for general alcohols [20]. The general derivatization reaction of alkylphenols with DNSC is described in Fig. 1. The reaction occurs in mildly alkaline solution acetone-aqueous medium at multiple excess of reagent. Both degree of derivatization and hydrolysis of derivatives increase with increasing pH of the reaction medium. The recommended pH of reaction medium is pH 9.5–10. Derivatization is carried out exclusively before LC–MS analysis as a pre-column procedure. The disadvantage of dansylderivation may be long reaction time (2–60 min) and high reaction temperature (60–100 °C) [18,21].

The dansylation of phenolic hydroxyl groups introduces ionizable basic nitrogen that enhances the mass spectrometric response of the target analytes. The MS/MS analysis of such a derivative utilizes the formation of product ion with *m/z* 171 corresponding to the protonated 5-(dimethylamino) naphthalene (Fig. 1). LC–ESI–MS/MS analysis based on this product ion is highly selective and sensitive and is commonly used for MS/MS detection of dansylderivatives of phenolic compounds [28,29]. The efficiency of electrospray ionization (ESI–MS/MS) of APs without pre-column derivatization is much lower than those of derivatized APs. The difference in LOQ reaches up to 3 orders [18]. Due to different conditions of derivatization used and presented in various studies, the

Table 2
Precursor and product ions and collision energies of MRM transitions used for BPA and APs analyses.

Analyte	BPA	4-t-OP	iso-NP	4-OP	4-n-NP
Transitions monitored	695.3 → 171 695.3 → 461	440.3 → 171 440.3 → 156	454.3 → 171 454.3 → 156	440.3 → 171 440.3 → 156	454.3 → 171 454.3 → 156
Collision energy (eV)	44	38	34	34	44

verification of derivatization procedure of alkylfenols has to be carried out in order to achieve the highest possible yield of the reaction. The main aim of our study was to test the effect of reaction conditions of dansylderivatisation on the sensitivity of LC–ESI–MS/MS analysis of the most frequently monitored alkylphenols. The reason was because of limited information on the effect of individual parameters on efficiency of derivatization reaction found in published papers. Bisphenol A, 4-*tert*-octylphenol, 4-octylphenol, mixture of nonylphenol isomers called also as technical nonylphenol and 4-n-nonylphenol were selected for these purposes. The usability of improved derivatization reaction is demonstrated on LC–ESI–MS/MS analysis of alkylphenols and bisphenol A in drinking water and wastewater samples.

2. Experimental

2.1. Reagents and chemicals

Analytical standards (Sigma–Aldrich, Germany): 4-n-nonylphenol (99.9%), bisphenol A (>99%), 4-octylphenol (99%), 4-tert-octylphenol (97%), nonylphenol – mixture of ring and chain isomers (iso-NP, analytical grade), solvents: acetone (99.8%), dichloromethane (DCM, 99.8%), toluene (99.8%) (LAB-SCAN, Poland), acetonitrile (ACN, 99.9%), methanol (MeOH, for pesticide residue analysis), water for LC/MS (impurities <0.0001%, Fluka, Sigma–Aldrich, Germany). Derivatizing agent dansyl chloride (Fluka, Sigma–Aldrich, Germany), pH adjusting agents and additives for HPLC-MS: sodium bicarbonate, sodium hydroxide, formic acid (Fluka, Sigma–Aldrich, Germany). Methanol for mobile phase preparation (LC-MS, Biosolve, Netherlands).

2.2. Derivatization conditions

The efficiency of dansyl derivatization depends on experimental conditions. The conditions of dansylderivatization of APs were tested using various solvents for preparation of standard solutions, reaction time, reaction temperature, pH value of reaction mixture and concentration of DNSC. Optimal parameter was always used in a test of the next parameter. Derivatization reaction was carried out in a 2 mL amber glass sample vial with 200 μL standard solution of alkylphenols (1 ng mL^{-1} each AP) in selected reaction solvent. Fifty microliters of 100 mM $NaHCO_3$ in water were added and the content was mixed (vortex mixer) for 1 min. Then 200 μL of DNSC in acetone (0.5 mg mL^{-1}) were added and mixed again. Subsequently the mixture was incubated for selected time at selected temperature. The reaction mixtures were then cooled to room temperature and evaporated to dryness under gentle stream of nitrogen. The residue was redissolved in 1 mL of methanol and mixed. The solution was injected into the LC–ESI–MS/MS. The derivatization parameters were tested in the order: effect of reaction solvents (acetone, ACN, MeOH, water, toluene, DCM), reaction time (1, 3, 10, 30, 60, and 120 min), reaction temperature (5, 20, 40, 60, 80, and 100 °C), pH value in the range from 8.25 to 11.9, and concentration of DNSC in the range from 0.0005 to 5 mg mL^{-1}. The changes of LC–ESI–MS/MS peak area were used for assessment of effect of different derivatization conditions.

Table 3
Characteristics of samples of bottled drinking water.

	A	B	C	D	E	F
Bottle type	PET	PET	PET	PET	PET	PET
Colour	Clear	Clear	Clear	Dark blue	Light green	Clear
Volume (L)	1.5	1.5	1.5	1.5	1.5	1.5
Water type[a]	BW	MSW	NMW	NMW	NMW	MSW
Σ Cations (mg L^{-1})	79.5	104.7	36.6	213.6	179.4	110.7
Σ Anions (mg L^{-1})	259.0	386.1	114.9	981.1	580.5	361.1

[a] BW, baby water; MSW, mountain spring water; NMW, natural mineral water; PET, polyethylene.
Σ Cations: Na^+; K^+; Mg^{2+}; Ca^{2+}.
Σ Anions: SO_4^{2-}; HCO_3^-; Cl^-; NO_3^-.

2.3. LC–ESI–MS/MS analysis

Chromatographic separations were performed using an Agilent 1200 Infinity Series (Agilent Technologies, Santa Clara, CA, USA) with chromatographic column ACE 5 C18, 150 mm × 4.6 mm i.d., 5 μm particle size (ACE, Scotland, UK). The column temperature was maintained at 40 °C and the injection volume was 10 μl. Methanol (A) and water containing 7 mM formic acid (B) were used as a mobile phase. The isocratic elution of 90% (A) + 10% (B) was used at flow rate 0.5 mL min^{-1}. The Agilent 6410 Triple Quadrupole (Agilent Technologies, Santa Clara, CA, USA) was used for MS/MS analysis. The instrument was operated in the ESI-positive MRM mode. Two MS/MS transitions were used for MS analyses. Fragment ion *m*/*z* 171 was chosen for the final quantification, fragment ion *m*/*z* 156 was used for confirmation, except of BPA (Table 2). The structure of quantification ion is described in the Fig. 1. The optimized instrument conditions were as follows: drying gas temperature 350 °C; capillary voltage 2 kV; nebulizer gas pressure 50 psi. LC–MS/MS parameters are summarized in Table 2 [5].

2.4. SPE procedure

The following procedure was used for treatment of water samples for alkylphenols and bisphenol A analyses. The SPE columns (octadecyl C 18, 500 mg) were conditioned with (2 mL of methanol and 2 mL of water (purity for LC–MS). The water samples (100 mL) were passed through the SPE columns using vacuum manifold flowrate 5 mL min^{-1}. After extraction, the columns were dried with air for 5 min. Elution was performed with ACN (5 mL). The eluate was evaporated and converted into mini-vial derivatized with optimized dansyl chloride procedure and analyzed by LC/ESI/MS/MS [27].

2.5. Water samples

Six samples of drinking water packed in 1.5 L PET bottle were purchased in the wholesale network in December 2014 for determination of alkylphenols and bisphenol A. Water samples were lettered A–F, to avoid the legal dispute (Table 3). At the same time a composite water sample was taken at effluent of the wastewater treatment plant in Brno – Modřice for direct comparison of the

content of alkylphenols and BPA in bottled drinking water and water leaving the treatment plant. The samples were stored at 4 °C in cool box until analyzed.

2.6. QA/QC

Because the BPA and/or APs are present in various laboratory equipment, the use of any plastic and rubber material was avoided to minimize possible contamination of the samples during sample collection, storage, transport, treatment, analyte extraction, derivatization procedure, calibration and LC/MS/MS analyses and also volume measurements and weighing. Only glass and/or stainless steel materials were used. No detergents were used for glassware cleaning. All glassware was rinsed with water, acetone, methanol and acetonitrile purity for LC/MS. The composite samples were collected into amber glass bottle treated by the same procedure as for other glassware.

Solvent blanks and standard solutions were analyzed in sequence with each set of analyzed samples for control of background contamination. Blanks were processed in the same way as the samples. All of the standard solutions used for the calibration curves were injected before and after each analysis of a set of samples to check for instrumental sensitivity and reproducibility. To assess the efficiency of the SPE method used for extraction of BPA and APs from water samples, preliminary experiments were performed. BPA and APs were spiked in to same volume of water for LC/MS as the volume of analyzed real water samples. Only glass SPE columns were used for extraction of analytes from water samples. The recovery calculated from analysis of six parallel spiked water samples was used for correction of data obtained from LC/MS/MS analyses of real samples [27].

In order to obtain high selectivity and sensitivity of LC/MS analyses, multiple-reaction-monitoring (MRM) was chosen as a data acquisition mode. Three identification ions (one precursor ion and two products ions) were used for each analyte. The relative response between the two MRM transitions together with retention times was used as a criterion for the identification of the compounds. Agilent Mass Hunter quantitation software was used for these purposes. The uncertainty of the ratio of the quantification ion to the qualifier ion of individual analytes was set at 20% of the expected values according MassHunter standard evaluation procedure of MassHunter software.

Quantitation of analytes was performed using a seven-point calibration curve in the concentration range of 1–1000 ng L^{-1}. Internal standard 4-n-NP–d8 was added to all samples and calibration point s. Instrumental limits of quantification was determined from the injected amount of standard solutions that provide signal-to-noise ratio of S/N = 10. The limits of detection was determined from the amount of analytes that provides signal-to-noise ratio of S/N = 3. Repeated analysis (n = 6) of standard solutions was carried out for these purposes.

Accuracy and precision of the method used for the LC/MS/MS determination of alkylphenols were calculated for six parallel samples and three levels of concentrations (50 ng L^{-1}, 250 ng L^{-1}, 1000 ng L^{-1}). The concentration levels were chosen based on the content of alkylphenols commonly present at the outlet of the water treatment plants.

Single factor analysis of variance ANOVA was used to compare the differences of experimental data obtained from the study of the effect of investigated parameter of derivatization reaction on LC/MS/MS signal (peak area) for each of compound. The null hypothesis was that investigated parameter has no effect on LC/MS/MS signal; it means that the yield of derivatization reaction is not dependent on parameter in tested region. If the probability (p-value) <0.05 then the null hypothesis was rejected.

3. Results and discussion

3.1. Effect of reaction conditions on the yield of derivatization reaction of alkylphenols

3.1.1. Effect of a sample solvent

The first factor, which can affect the derivatization reaction, is a solvent in which sample and/or standard are dissolved. Six different solvents (acetone, ACN, MeOH, water, DCM and toluene) were used for these purposes. Different solvents were used to prepare a standard solution of analytes. The ANOVA analysis was used for testing the differences in peak areas obtained for individual analytes when different solvents were used (see ANOVA result in Section 3.3). As shown in the Fig. 2A, regarding the sum of the detector responses (peak area) of individual analytes, acetonitrile is the most suitable solvent for the mixture of alkylphenols. Water could be preferable as the reaction solvent if only iso-NP have to be analyzed. Completely unsuitable sample solvent is methanol. Selection of the appropriate sample solvent may depend on the method used for extraction and/or purification of extracts of alkylphenols from various types of matrices. From the results of the test of suitability of selected solvents follows that methanolic SPE eluates from purification processes have to be converted into another solvent after evaporation, preferably into acetonitrile [27]. The similar effect was described in the case of study focussed on dansylderivatisation of fluorotelomers. The decrease of sensitivity of derivatization reaction in methanol was assigned to the reaction of DNSC with methanol. Due to reactivity of DNSC preferably with primary amino, hydroxyl and carboxylic groups the differences in effect of other tested solvents cannot be associated with reaction of solvents with DNSC [30].

3.1.2. Effect of reaction time

Reaction time is one of the most important factors in derivatization procedures. In our study reaction time was tested in the range of 1–120 min; the results you can see in Fig. 2B. Changes of reaction time from 30 to 120 min do not lead to significant changes in peak areas (see ANOVA result in Section 3.3). Based on these results, 60 min was selected for further experiments. The shorter time is recommended in several studies [18,27]. However shorter reaction time especially in the range of 1–10 min may cause poor repeatability due to possible variability of time delays between the individual steps of sample derivatization.

3.1.3. Effect of reaction temperature

To evaluate which value of recommended temperature is optimal in terms of yield of derivatization reaction and the time of sample preparation for analysis, the effect of a wide range of temperatures on the yield of derivatization was tested. The starting point was below the laboratory temperature (5 °C); the last point (100 °C) was slightly above boiling point of acetonitrile chosen as reaction solvent (82 °C), in order to test the whole temperature range recommended in the literature. Sixty minutes were used as the reaction time. As is apparent from Fig. 2E, the suitable reaction temperature is 60 °C for the whole mixture of alkylphenols. No significant differences were found in the region of 60–100 °C, except for iso-NP (see ANOVA result in Section 3.3). The different shape of iso-NP dependence curve could be caused by different physico-chemical properties of individual isomers in the mixture. The rigorous explanation could be carried out only if standards of individual isomers will be available.

3.1.4. Effect of pH

The pH of the reaction mixture affects the efficiency of derivatization due to the degree of dissociation of phenolic hydroxyl (pK_a of alkylphenols ranged from 9.9 to 10.9 pK_a of BPA is lower – approx.

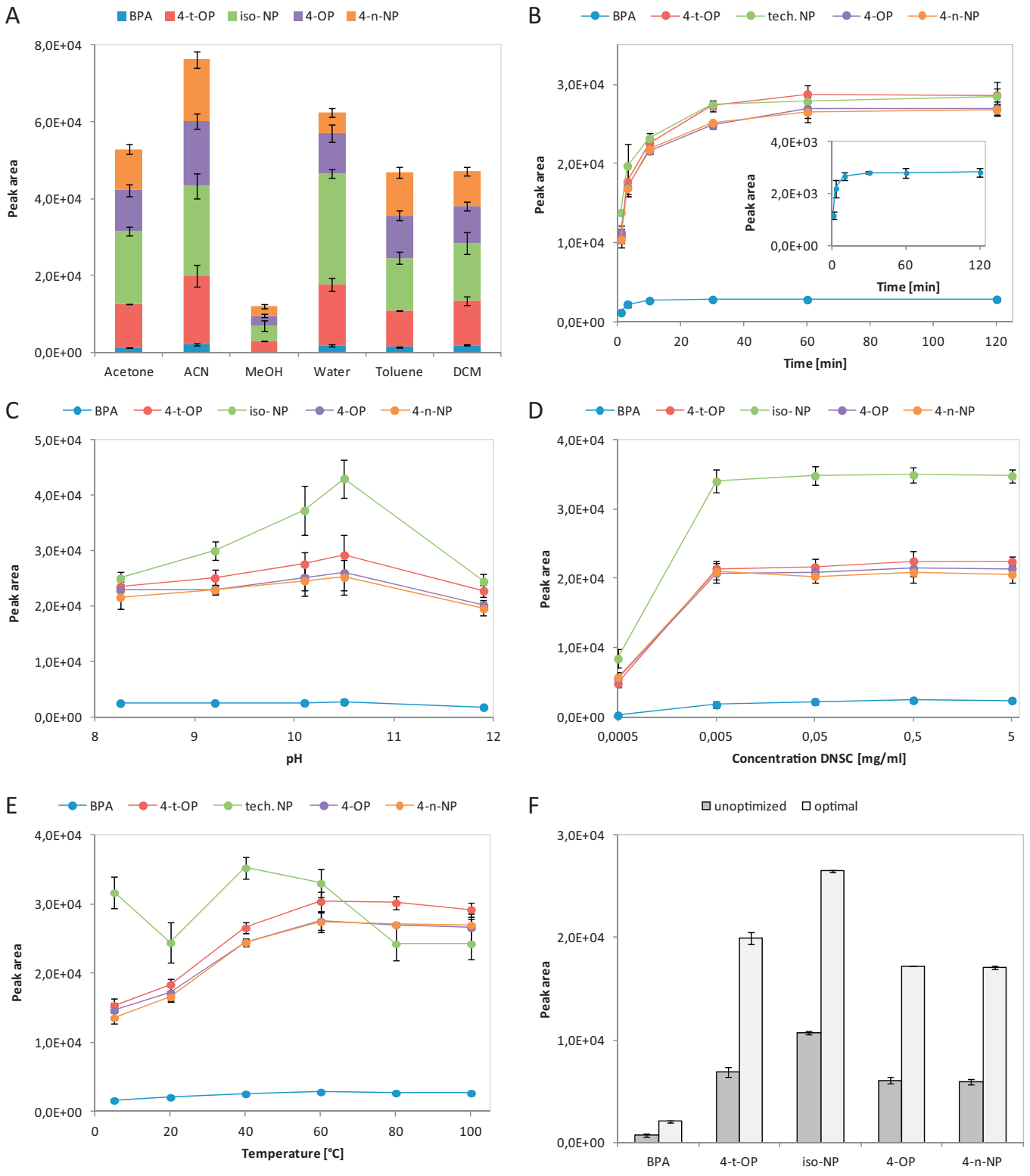

Fig. 2. Effect of reaction properties on the yield of derivatization of APs and BPA expressed as chromatographic peak areas of LC/MS/MS analysis. Effect of: (A) solvent, (B) reaction time, (C) pH values, (D) DNSC concentration and (E) reaction temperature. (F) compares the yield of optimized derivatization reaction with unoptimized.

8.5–9.2 depending on environment) and the speed of hydrolysis of dansyl-derivatives [21]. Series of experiments were carried out to determine the effect of pH in the following manner. The sodium bicarbonate at a concentration of 100 mM was adjusted using 3 M NaOH to a pH in the range of 8.25–11.9 usually recommended in the literature. The significant effect of pH on the peak area was confirmed by ANOVA for all investigated analytes (Section 3.3). The highest effect was obtained for the mixture of iso-NPs that can have different pK_a values depending on chain structure. The maximum peak area was obtained at pH 10.5, as you can also see in Fig. 2C.

Based on these facts, the pH of the sample was adjusted to pH 10.5 in the subsequent experiments.

3.1.5. Effect of concentration of dansyl chloride

The effect of the concentration of dansyl chloride on the yield of derivatization procedure was investigated in the range of concentrations 0.0005 and 5 mg mL^{-1} which are recommended for determination of alkylphenols on the level of ng mL^{-1} and lower. Concentration of each alkylphenols used for this study was 1 ng mL^{-1}, typical for environmental samples, such as wastewater, soil, sediments. As is shown in Fig. 2D the DNSC concentration of 0.005 mg mL^{-1} (i.e. 5000 times greater for each alkylphenols) provides maximum yield of derivatization reaction, that did not change with additional surplus. No significant differences were found in the region of 0.005–5 mg mL^{-1} (see ANOVA result in Section 3.3). As to concentration of alkylphenols expected in environmental samples and as to the content of other compounds which can be present in the matrix the DNSC concentration of 0.5 mg mL^{-1} was chosen as sufficient excess.

If above optimized derivatization conditions are used for determination of alkylphenols under investigation, two times higher responses can be obtained comparing to conditions of most often recommended for derivatization of phenolic compounds (Fig. 2F). As can be seen especially in the case of effect of temperature and reaction time, the use of appropriate values in the plateau of the curves (Fig. 2B and E) allow decreasing the experimental errors caused by eventual fluctuation of these reaction conditions outside of plateau. The following "unoptimized" conditions were used for comparison in our study: 100 mM solution of $NaHCO_3$ in water, acetone as reaction solvent, 0.5 mg mL^{-1} dansyl chloride solution in acetone, reaction time 3 min, reaction temperature 60 °C [18].

3.2. Chromatographic analysis of derivatized alkylphenols

The chromatographic analysis of derivatized alkylphenols obtained under above specified conditions selected on the basis of previous experiments is shown in Fig. 3. As you can see poor separation was obtained for the mixture of nonylphenol isomers (iso-NP). A focus on a better resolution of mixture of iso-NP is not necessary because, as is common, iso-NP quantification is based on the sum of signals of isomers in the mixture [14,31]. The mixture of ring and chain nonylphenol isomers (iso-NP) present in Sigma–Aldrich standard mixture was used for quantification of nonylphenols except the linear nonylphenol (n-NP). The length of chromatogram can be modified depending on the aim of chromatographic analyses and properties of sample matrix. Especially an increase of column temperature can be used for effective time shortening up to one half.

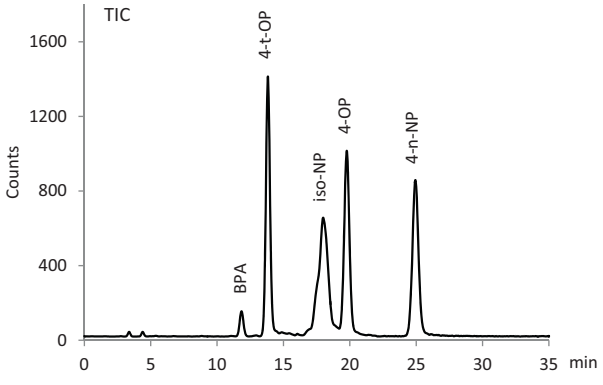

Fig. 3. Chromatogram of the dansylderivatives of the alkylphenolic compounds (c = 1 ng mL^{-1}).

Table 4
Validation parameters of selected alkylphenols and bisphenol A.

Compounds	Regression equation	R^2	[pg/inj]		t_R [min]
			LOD	LOQ	
BPA	$y = 8270x$	0.9933	0.25	0.83	11.8
4-t-OP	$y = 39788x$	0.9988	0.02	0.08	13.8
iso-NP	$y = 48426x$	0.9989	0.06	0.20	17.9
4-OP	$y = 72487x$	0.9995	0.06	0.19	19.7
4-n-NP	$y = 79749x$	0.9996	0.04	0.14	24.9

Table 5
p-values of ANOVA of effect of parameters of derivatization reaction on peak area of LC/MS/MS analyses of alkylphenols.

	pH	Temperature	Time	DNCS concentration	Solvents
BPA	0.044	0.663	0.973	0.235	1.08E−03
4-t-OP	0.021	0.472	0.338	0.513	6.68E−05
iso-NP	2.72E−05	4.38E−03	0.261	0.821	1.34E−05
4-OP	0.031	0.719	0.085	0.765	9.19E−04
4-n-NP	4.28E−03	0.843	0.131	0.931	1.78E−05

3.3. Method validation

Validation parameters for selected alkylphenols and bisphenol A investigated under optimized conditions are listed in Table 4. Good linearity of calibration curves was obtained in the whole concentration range. Instrumental limits of quantification and limits of detection determined from the injected amount of individual analytes ranged from of 0.08 to 0.83 pg/injection and 0.02–0.25 pg/injection, respectively.

Accuracy and precision of the method used for the LC/MS/MS determination of alkylphenols in water samples involving SPE procedure (Section 2.4) and derivatization of analytes in the preconcentrated sample, ranged from 71 to 99% and from 16 to 3%, respectively.

Uncertainties of the results of the study of effect of individual reaction conditions and results of analysis of real samples expressed as RSD ranged for selected analytes as follows: BPA 0.6–34.9%, 4-t-OP 0.3–16.1%, iso-NP 0.9–31.8%, 4-OP 0.9–21.7%, and 0.6–23.7% for 4-n-NP. The highest values were found only for experiments with methanol as reaction medium, which is not suitable for derivatization of alkyphenols and also for lowest concentration of DNSC which represents insufficient surplus of DNSC. Uncertainties of LOQ expressed as RSD were in the range of 4.5–14.8%.

Table 5 lists p-values of ANOVA of effect of reaction parameters for individual compounds. The significant difference was indicated for effect of pH of reaction mixture and reaction solvents because p-values are <0.05. The p-values of effect of reaction time, DNSC concentration and reaction temperature were higher than 0.05 for values in the plateau of the experimental curves (regions of 30–120 min, 60–100 °C and 0.005–5 mg mL^{-1}, respectively) therefore the effect of that parameters is non-significant in mentioned region. The p-value of effect of temperature for iso-NP was an exception (for explanation see Section 3.1.3).

3.4. Determination of alkylphenols in water samples

Improved method was used in our study for analysis of the content of alkylphenols and bisphenol A in various commercially available drinking waters. The method was used also for analyses of bisphenol A, nonylphenols and octylphenols in wastewater collected as 24 composite samples at the output from wastewater treatment plant (WWTP). Three parallel samples were analyzed in each case (Fig. 4).

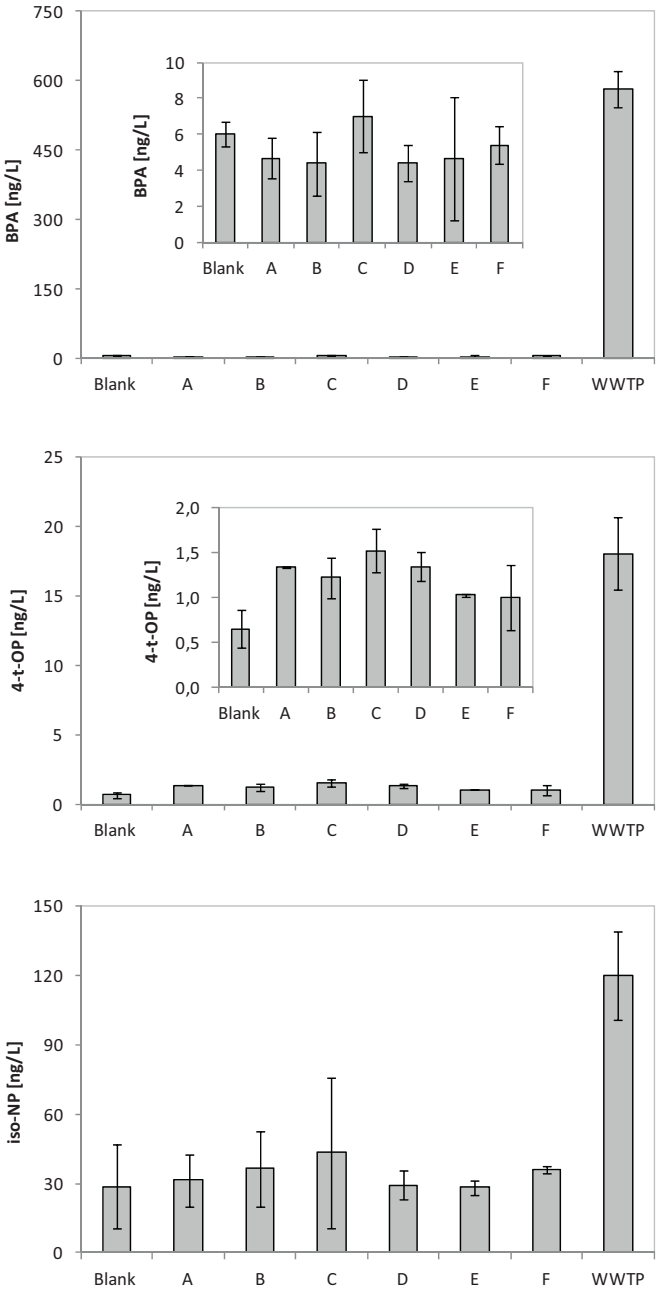

Fig. 4. Content of APs and BPA in drinking and wastewater ($n = 3$).

The median concentration of BPA and iso-NP obtained for bottled waters was 4.7 ng L^{-1} and 33.5 ng L^{-1}, respectively. These values are not distinguishable from the blanks in case of all samples. The median concentration of 4-t-OP was 1.3 ng L^{-1} approximately two times higher than blank.

A relatively high level of alkylphenols and bisphenol A in blank samples comparing to water samples is caused by common contamination of samples coming from laboratory air, septa vials, and solvents and chemicals used. This can be a problem especially in determination of alkylphenols and bisphenol A at low concentrations, as in mineral water.

Concentration of BPA and alkylphenols in determined in bottled drinking waters was well below the guideline values for drinking waters. Daily intake of these compounds from drinking bottled water will be low and does not exceed the acceptable value of income relative to the reference doses.

The daily intake of these compounds via consumption of drinking water is low, contributing to respective tolerable intakes with compared to reference doses. Equally, there is no special hazard arising from the presence of alkyphenols and BPA in the effluent of water treatment plants relative to the reference values. Equally, no risk of water presents in the effluent of a wastewater treatment plant in comparison with the reference values. The linear alkylphenols such as 4-OP and 4-n-NP in real samples did not appear in any of the cases.

4. Conclusions

The aim of our study was testing the conditions of derivatization reaction of selected analytes (BPA, 4-t-OP, iso.-NP, 4-OP and 4-n-NP) with dansyl chloride.

The study of effect of conditions of derivatization reactions of alkylphenols using dansyl chloride reagent was carried out, because limited information on the effect of individual parameters on efficiency of derivatization reaction can be found in published papers. Improved conditions enable to obtain more reproducible results comparing to results obtained by previously recommended method. Especially the use of appropriate value of temperature and reaction time is critical, due to need to reduce errors caused by changes during derivatization procedures of samples. Experimental results have shown, that the developed method enables to improve limits of detection and limits of quantification comparing to recommended method.

The improved method was used for the determination of analytes in real samples. The concentration of BPA and APs in bottled drinking waters and wastewater analyzed in our study was below the maximum safe doses for human and other organism exposure. The relatively high levels of alkylphenols and bisphenol A in blank samples are a serious problem in the analysis. Especially for analyses of trace levels of alkylfenols and BPA occurring in environmental samples, further research focussed on sample treatment and final analytical procedures is necessary. Elimination or at least decrease of sources of contamination of treated sample by APs and BPA in laboratory materials and chemicals will be the aim of following research.

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References

[1] N. Salgueiro-González, E. Concha-Graña, I. Turnes-Carou, S. Muniategui-Lorenzo, P. López-Mahía, D. Prada-Rodríguez, Blank and sample handling troubleshooting in ultratrace analysis of alkylphenols and bisphenol A by liquid chromatography tandem mass spectrometry, Talanta 101 (2012) 413–419.

[2] L. Xu, D.C. Spink, 1,2-Dimethylimidazole-4-sulfonyl chloride, a novel derivatization reagent for the analysis of phenolic compounds by liquid chromatography electrospray tandem mass spectrometry: application to 1-hydroxypyrene in human urine, J. Chromatogr. B 855 (2007) 159–165.

[3] B. Shao, H. Han, D. Li, Y. Ma, X. Tu, Y. Wu, Analysis of alkylphenol and bisphenol A in meat by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry, Food Chem. 105 (2007) 1236–1241.

[4] D. Amiridou, D. Voutsa, Alkylphenols and phthalates in bottled waters, J. Hazard. Mater. 185 (2011) 281–286.

[5] M. Seifertová, Determination of Alkylphenols in Indoor Air (Rigorous thesis), Masaryk University, Brno, 2013.

[6] EPA, Summary of Nominations for the Third Contaminant Candidate List, EPA 815-R-09-011, 2009, August.

[7] C.C. Willhite, G.L. Ball, C.J. McLellan, Derivation of a bisphenol A oral reference dose (RfD) and drinking-water equivalent concentration, J. Toxicol. Environ. Health B 11 (2008) 69–146.

[8] EPA, Integrated Risk Information System, U.S. Environmental Protection Agency, 2009.
[9] EFSA-Q-2005-100, EFSA J. 428 (2006) 1–75.
[10] C.A. Staples, K. Woodburn, N. Caspers, A.T. Hall, G.M. Klecka, A weight of evidence approach to the aquatic hazard assessment of bisphenol A, Hum. Ecol. Risk Assess. (2002) 1083–1105.
[11] A. Soares, B. Guieysse, B. Jefferson, E. Cartmell, J.N. Lester, Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters, Environ. Int. 34 (2008) 1033–1049.
[12] S.N. Atapattu, J.M. Rosenfeld, Solid phase analytical derivatization as a sample preparation method, J. Chromatogr. A 1296 (2013) 204–213.
[13] R. Romero-Cano, D. Kassuha, J. Peris-Vicente, P. Roca-Genoves, S. Carda-Broch, J. Esteve-Romero, Analysis of thiabendazole 4-tert-octylphenol and chlorpyrifos in waste and sewage water by direct injection – micellar liquid chromatography, Analyst 140 (2015) 1739–1746.
[14] A.R. Fischer, N.T.P. Lan, C. Wiedemann, P. Heide, P. Werner, A.W. Schmidt, G. Theumer, H.J. Knőlker, Determination of 4-nonylphenol in water samples using 4-(2,6-dimethylhept-3-yl)phenol as new internal standard, J. Chromatogr. A 1217 (2010) 2950–2955.
[15] M. Kojima, S. Tsunoi, M. Tanaka, Determination of 4-alkylphenols by novel derivatization and gas chromatography–mass spectrometry, J. Chromatogr. A 984 (2003) 237–243.
[16] D. Jahr, Determination of alkyl, chloro and mononitrophenols in water by sample-acetylation and automatic on-line solid phase extraction gas chromatography mass spectrometry, Chromatographia 47 (1998) 49–56.
[17] M. Cantero, S. Rubio, D. Pérez-Bendito, Determination of alkylphenols and alkylphenol carboxylates in wastewater and river samples by hemimicelle-based extraction and liquid chromatography–ion trap mass spectrometry, J. Chromatogr. A 1120 (2006) 260–267.
[18] M. Seifertová, Z. Šimek, Determination of alkylphenol and bisphenol A in environmental matrices using HPLC MS/MS methods, Chem. Listy 106 (2012) 109–111.
[19] R. Jeannot, H. Sabik, E. Sauvard, T. Dagnac, K. Dohrendorf, Determination of endocrine-disrupting compounds in environmental samples using gas and liquid chromatography with mass spectrometry, J. Chromatogr. A 974 (2002) 143–159.
[20] Z. Tang, F.P. Guengerich, Dansylation of unactivated alcohols for improved mass spectral sensitivity and application to analysis of cytochrome P450 oxidation products in tissue extracts, Anal. Chem. 82 (2010) 7706–7712.
[21] V.T. Wiedermeier, S.P. Porterfield, C.E. Hendrich, Quantitation of DNS-amino acids from body tissues and fluids using high-performance liquid chromatography, J. Chromatogr. 231 (1982) 410.
[22] A.M. Kvistad, E. Lundanes, T. Greibrokk, Determination of alkylphenols in water samples by solid-phase extraction on to poly(styrene-divinylbenzene) and quantification by liquid chromatography with UV-detection, Chromatographia 48 (1998) 707.
[23] R.A. Rudel, S.J. Melly, P.W. Geno, G. Sun, J.G. Brody, Identification of alkylphenols and other estrogenic phenolic compounds in wastewater, septage, and groundwater on Cape Cod, Massachusetts, Environ. Sci. Technol. 32 (1998) 861.
[24] D.A. Markham, D.A. McNett, J.H. Birk, G.M. Klecka, M.J. Bartels, C.A. Staples, Quantitative determination of bisphenol-A in river water by cool on-column injection-gas chromatography–mass spectrometry, Int. J. Environ. Anal. Chem. 69 (1998) 83–98.
[25] R. Gadzała-Kopciuch, A. Filipiak, B. Buszewski, Isolation, purification and determination of 4-n-nonylphenol and 4-tert-octylphenol in aqueous and biological samples, Talanta 74 (2008) 655–660.
[26] H.G.J. Mol, S. Sunarto, O.M. Steijger, Determination of endocrine disruptors in water after derivatization with N-methyl-N-(tert-butyldimethylsilyl-trifluoroacetamide) using gas chromatography with mass spectrometric detection, J. Chromatogr. A 879 (2000) 97–112.
[27] P. Poloucká, The Study of Alkylphenols in Water (Diploma thesis), Masaryk University, Brno, 2014.
[28] X. Zhuang, Y. Zhong, M. Yuan, H. Li, Pre-column derivatization combined with UHPLC–MS/MS for rapid and sensitive quantification of bakuchiol in rat plasma, J. Pharm. Biomed. Anal. 75 (2013) 18–24.
[29] F. Zhang, M.J. Bartels, D.R. Geter, M.S. Carr, L.E. McClymount, T.A. Marino, G.M. Klecka, Simultaneous quantitation of testosterone, estradiol, ethinyl estradiol, and 11-ketotestosterone in fathead minnow fish plasma by liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 23 (2009) 3637–3646.
[30] H. Peng, K. Hu, F. Zhao, J. Hu, Derivatization method for sensitive determination of fluorotelomer alcohols in sediment by liquid chromatography–electrospray tandem mass spectrometry, J. Chromatogr. A 1288 (2013) 48–53.
[31] M. Naassner, M. Margler, K. Wolf, I. Schuphan, Determination of the xenoestrogens 4-nonylphenol and bisphenol A by high-performance liquid chromatography and fluorescence detection after derivatisation with dansyl chloride, J. Chromatogr. A 945 (2002) 133–138.

Article II

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Analysis of alkylphenols and bisphenols in soils using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Methods useful for the extraction of alkylphenols from soil samples were assessed. A comparison is made of the four extraction techniques that are most commonly used to extract phenolic compounds from soil, such as bisphenol A, bisphenol F and alkylphenols (4-t-OP, iso-NP, 4-OP and 4-n-NP). Soxhlet extraction, ultrasonic-assisted extraction (USE), accelerated solvent extraction (ASE) and the so-called Quick, Easy, Cheap, Effective, Rugged and Safe extraction (QuEChERS) were included. Modifications of the QuEChERS technique such as original, citrate and acetate were investigated. The effects of basic purification sorbents used for QuEChERS extracts such as a primary and secondary amine (PSA), C18 endcapped sorbents and graphitised carbon black (GCB) were tested. A derivatisation step prior to the final analyses by liquid chromatography coupled to a triple quadrupole analyser operating in tandem mass spectrometry (LC–MS/MS) was performed by using dansyl chloride (DNSC). The best extraction of the monitored compounds from spiked soil was obtained using QuEChERS extraction. Recoveries of phenolic compounds using the QuEChERS technique were in the range from 67% to 114%. The lowest extraction recoveries (16–73%) were obtained using ultrasonic extraction. The QuEChERS method was successfully applied for the analysis of alkylphenols in soil samples. Instrumental limits of quantification and limits of detection ranged from 0.08 to 0.83 pg per injection and 0.02 to 0.25 pg per injection, respectively.

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1. Introduction

Phenolic compounds such as bisphenol A (BPA), octylphenol (OP) and nonylphenol (NP) are widespread all over the globe. They are highly toxic, xenobiotic and relatively stable in the environment. Alkylphenols are also known as *endocrine disrupting compounds* (EDCs) because they possess an ability to disrupt endocrine functions by binding to the hormone receptors 17β-oestradiol (E2) inducing synthesis of vitellogenin (Vtg) and zona radiate protein (Zrp) [1–5]. The presence of alkylphenols (APs) in the environment originates principally from the degradation of alkylphenol ethoxylates (APEs) which are widely used as surfactants and as stabilisers of ethylcellulose resin. BPA is used as a monomer in the process of preparing epoxide resins, polycarbonate plastics and as an antioxidant or stabiliser of polyvinyl chloride

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[6]. The occurrence of APs and BPA in the environment is clearly correlated with anthropogenic activities such as wastewater treatment, landfilling and sewage sludge recycling [7,8]. Selected phenolic compounds are found often in matrices such as sewage sludge, effluents from sewage treatment works, river water and sediments, soil and groundwater [9]. Although it has been shown that concentration of phenolic compounds in the environment is decreasing, they are still found at a concentration in the $\mu g.L^{-1}$ in bodies of water and in the mg/kg in sediments and soils [10–12]. The occurrence of BPA, OP and NP at relatively high concentrations and their toxic effect on many living organisms including humans are reasons for monitoring on all levels [13]. It is crucial to have a proper analytical detection method and extraction technique for extracting and isolating these compounds from solid environmental matrices such as soil. A variety of extraction techniques have been reported in the literature for the analysis of phenolic compounds in soil matrices, though most of these techniques have only been used for the simultaneous analysis of one or several alkylphenols belonging to the same family. For instance, accelerated solvent extraction (ASE) has been applied for the analysis of 4-*tert*-octylphenol (4-*t*-OP) and nonylphenol isomers (iso-NP), also called technical mixture [11], ultrasonic-assisted extraction (USE) for the analysis of 4-*t*-OP [14] and Soxhlet extraction for bisphenol A, 4-*t*-OP and iso-NP [15,16]. In the last few years, a new procedure known as the QuEChERS method (an acronym derived from Quick, Easy, Cheap, Effective, Rugged and Safe) has been applied for the extraction of pesticides with a wide polarity range from fruits and vegetables [17]. This method is based on the salting-out of analytes into acetonitrile. Three different modifications of the QuEChERS method for extraction of analytes and extract clean-up can be used depending on the sample origin: the original QuEChERS method, the citrate buffered QuEChERS method (EU version) and the acetate buffered QuEChERS method (AOAC version) [18]. Final extracts can be analysed directly and/or after pre-column derivatisation using various reagents. Different approaches have been reported for the analysis of phenolic compounds, for example, micellar liquid chromatography (MLC) [19], although gas chromatography (GC) and liquid chromatography (LC) have been mainly used. Currently, the use of GC and LC coupled with mass spectrometers (MS) [20–22] is widespread in the analysis of environment because they provide high selectivity and sensitivity, especially when operating in the selected reaction monitoring (SRM). Therefore, a derivatisation step is mandatory to improve the chromatographic performance and sensitivity, offering better results than direct LC-MS/MS, without a derivatisation step [5]. Analytical derivatisation can substantially improve chromatographic separation as well as the sensitivity and selectivity of detection [13]. The most often-used derivatisation reagents for phenolic compounds are bis(trimethylsilyl) trifluoroacetamide (BSTFA), pentafluoropropionic acid anhydride (PFPA), *N*-methyl-*N*(*tert*.-butyldimethyltrifluoroacetamide) (MTBSTFA) [23], pentafluoropyridine [24], 1,2-dimethylimidazole-4-sulphonyl chloride (DMISC) and 5-(dimethylamino) naphthalene-1-sulphonyl chloride (DNSC). Dansyl chloride (DNSC) contains a sulphonyl group that can readily react with phenols through the dansylation reaction mechanism. The dimethylamino group of DNSC can serve as an ionisation moiety and the dansyl derivatives exhibit excellent MS ionisable potential. When SRM mode is used, the product ion at *m/z* 171 generated from the DNS-derivative can greatly enhance the detection sensitivity [25].

The European Commission has elaborated a draft 'Working Document on Sludge' that proposes maximum levels for some micro-contaminants, including alkyl ethoxylates (AEOs) and alkylphenol ethoxylates (APEOs). The maximum value for their concentration

in sludges for agricultural soils is at 50 mg·kg^{-1} of dry matter, and this includes the nonylphenol and nonylphenol ethoxylates with one or two ethoxy groups [26].

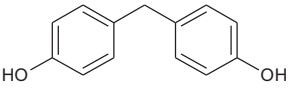
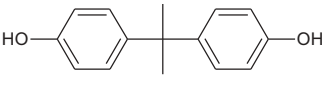
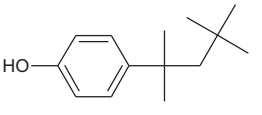
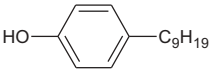
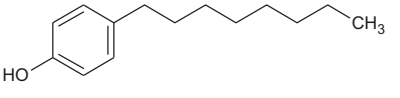
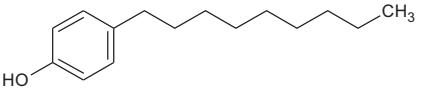
The objective of our study was to compare the extraction efficiencies of four extraction methods optimised and recommended for the extraction of alkylphenols from soil using samples with different content of total organic carbon (TOC). The aim was also to compare these methods with three modifications of the QuEChERS method (original, citrate buffered and acetate buffered) and compare suitable clean-up techniques for determination of alkylphenols and bisphenols in soils. The improved procedure was successfully applied for the analysis of APs, BPA and BPF in soil samples.

2. Experimental

2.1. Reagents and chemicals

The analytical standards were bisphenol F (>99%), bisphenol A (>99%), 4-n-nonylphenol (99.9% purity), 4-octylphenol (99%), 4-tert-octylphenol (97%) and nonylphenol- the mixture of ring and chain isomers (Sigma-Aldrich, Germany) (see Table 1). Each compound was

Table 1. Physicochemical properties and structure of the phenolic compounds used [27].

Compound	Acronym	CAS-number	Molecular weight	log P	Structure
Bisphenol F	BPF	620-92-8	200,23	3,46	
Bisphenol A	BPA	80-05-7	228,29	4,04	
4-*terc*-octylphenol	4-t-OP	140-66-9	206,32	4,69	
Nonylphenol (isomers mixture)	iso-NP	84,852-15-3	220,35	~5,74	
4-octylphenol	4-OP	1806-26-4	206,32	5,30	
4-nonylphenol	4-NP	104-40-5	220,35	5,74	

dissolved in methanol to form a stock solution at a concentration of 0.1 mg.mL^{-1}. The stock solution was stored in the dark at 4°C. The stock solution was diluted with acetonitrile to prepare working solutions at the required concentrations. A standard solution (1 ng.µL^{-1} in acetone) of isotopically labelled 4-n-nonylphenol D8 (98%) obtained from Dr Erhenstofer (Augsburg, Germany) was used as an internal standard (IS). The solvents were acetone (99.8%), dichloromethane (DCM, 99.8%, LAB-SCAN, Poland), acetonitrile (ACN, 99.9%), methanol (MeOH, for pesticide residue analysis) and water for LC/MS (impurities <0.0001%), all from Sigma-Aldrich (Fluka, Sigma-Aldrich Germany). A granular substance with the trade name Hydromatrix^{TM} (Dionex, Sunnyvale, CA, USA) was used for the ASE method. Magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), acetic acid (HOAc), sodium acetate (NaOAc), sodium citrate dihydrate ($Na_3Cit{\cdot}2H_2O$), disodium hydrogen citrate sesquihydrate ($Na_2HCit{\cdot}1.5H_2O$) all from Sigma-Aldrich (Fluka, Sigma-Aldrich, Germany) were used for the QuEChERS technique. Primary and secondary amine (PSA), C18 endcapped sorbents and graphitised carbon black (GCB) for clean-up processes (all from Supelco, Sigma-Aldrich, Germany) were used. Derivatising agent- dansylchloride (Fluka, Sigma-Aldrich, Germany), pH adjusting agents-sodium bicarbonate and sodium hydroxide, (Fluka, Sigma-Aldrich, Germany) were used for derivatisation reaction. Methanol (LC-MS – Biosolve, Netherlands) and formic acid (Fluka, Sigma-Aldrich, Germany) as additive were used for the mobile phase preparation.

2.2. Sample collection

Seven soil samples with different properties and a large scale of TOC values were selected from RECETOX bank of soil samples for the development of an analytical method for analyses of alkylphenols in soil. The samples were collected from the RECETOX reference sites in the Czech Republic. The soil samples with extreme TOC values (G01-0.44 and G13- 6.31) were chosen to optimise the extraction procedures. The characteristics of the test soils are presented in Table 2. Soils were stored in the dark at laboratory temperature. Ten grams of each homogenised sample of soil were weighed into 20 mL glass vials. Each extraction technique was performed in five replicates. Control samples (blank) were performed in three replicates. Soil samples were spiked with a mixture of alkylphenol standards. The final concentration of substances in the soil was 10 ng per gram of soil. An artificial soil sample consisting of 10% dried peat (horticultural grade 1, topsoil, pH 3.5–5.0, Agro CS, homogenised over a 2 mm screen), 20% kaolin clay with a minimum kaolin content of 30% (Sigma – Aldrich, Cat. No. 18,672-25KG), 70% quartz sand with a minimum of 50% grains of 0.05–0.2 mm (Sand 0.2–0.5, Filtering Sands, Chlum, Hornbach) and calcium carbonate for pH adjustment, was prepared for comparison. Soil samples with varying TOC values (see Table 2) were used for analysing the content of alkylphenols and bisphenols.

2.3. Extraction techniques

Ten grams of spiked soils were weighed and extracted using four different extraction methods. Soxhlet Warm Extraction (SWE), Accelerated Solvent Extraction (ASE,) Ultrasonic Extraction (USE) and QuEChERS extraction were used for these purposes. The extracts in

Table 2. Characteristics of soils selected for the determination of alkylphenols and bisphenols.

Soil code		G 01	G 04	G 07	G 08	G 10	G 11	G 13
Locality character			Arable			Grassland		
Soil type		cambisol	luvisol	phaeozem	chernozem	luvisol	Cambisol	podzol
Land cover		Arable	Arable	Arable	Pastures	Pastures	Orchard	Moors
Horizon		A	A	A	A	A	A	A
TOC		0.44	1.22	2.49	0.93	1.73	3.86	6.31
pH (H2O)		5.95	5.85	6.69	7.78	4.55	7.70	4.57
HA		0.12	0,22	0.60	0.16	0.30	0.44	1.07
FA		0.11	0.21	0.36	0.11	0.46	0.48	1.03
Particle size distribution	≤2 µm	10.3	25.4	33.0	21.4	9.9	10.8	4.3
	<10 µm	18.3	33.9	39.7	29.0	30.1	11.9	10.4
	10–50 µm	15.8	34.9	41.7	53.4	45.0	6.3	21.0
	50–100 µm	20.1	18.8	14.8	14.2	11.0	8.9	17.4
	100–2000µm	45.8	12.4	3.9	3.4	13.9	72.9	51.3

* HA – Humic acid, FA – Fluvic acid

different extraction solvents were evaporated under a nitrogen stream to dryness, and redisolved in 10 mL of acetonitrile before purification. In order to avoid a questionable interpretation of the results, identical procedures were used to purify the extracts obtained by all extraction methods. Blank samples were extracted simultaneously using each of the extraction techniques.

A B-811 automatic extractor (Büchi, Flawil, Switzerland) was used for Soxhlet Warm Extraction. Target analytes were extracted for 60 min (40 min extraction, 20 min dropping) using 100 mL of DCM. The acquired extract (approx. 70 mL, the rest of the solvent was evaporated during the extraction) was concentrated to 10 mL and transferred quantitatively into a 20 mL glass vial. Concentrated extracts were stored in a cold and dark place before purification [16].

Pressurised liquid extraction (PLE) was performed using an ASE 150 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA). The soil sample was mixed with Hydromatrix^{TM} and transferred into a 10 mL stainless steel extraction cell. A filter paper was placed at each end of the cell. One static extraction cycle lasting 5 min was used and hexane/acetone (in the ratio 1:1 v/v) was chosen as the extraction solvent. The extraction temperature was 60°C and the extraction pressure was 112 bar. Ten millilitres of extract were collected in 40 mL vials with Teflon septa and stored in a refrigerator before purification [26].

Ultrasonic-assisted extraction (USE) was carried out in a SONOREX RK106 ultrasonic bath (Bandelin, Berlin, Germany). The spiked soil sample was transferred into a 50 mL extraction tube and 10 mL of methanol as the extraction solvent was added. Extraction in the ultrasonic bath lasted 30 min. Supernatant was transfer into a micro vial and stored in the refrigerator [14].

Three modifications of the QuEChERS method were used:

- *unbuffered QuEChERS method (original QuEChERS method)*. The spiked soils were transferred into a 50 mL extraction tube and extracted with 10 mL of ACN followed by liquid-liquid partitioning with 1 g NaCl and 4 g $MgSO_4$ and manual shaking of the mixture for 1 min. Subsequently, the mixture was centrifuged (Centrifuge Z 36 HK, HERMLE, Germany) at 4000 rpm for 5 min. The portion of the upper layer (1 mL) was transferred into a 2 mL centrifuge tube for clean-up processes [17].
- *buffered QuEChERS method (AOAC 2007.01)*. Ten grams of a soil sample, 10 mL of 1% HOAc in CAN, 4 g $MgSO_4$ and 1 g NaOAc was added to a 50 mL centrifuge tube and shaken by hand for 1 min. Subsequently, the mixture was centrifuged at 4000 rpm for 5 min. The portion (1 mL) of the upper layer was transferred into a 2 mL centrifuge tube for clean-up processes [28].
- *citrate buffered QuEChERS method (EN 15,662)*. Ten grams of a spiked sample, 10 mL of ACN and a mixture of 4 g $MgSO_4$, 1 g NaCl, 1 g $Na_3Citrate{\cdot}2H_2O$ and 0.5 g $Na_2Hcitrate{\cdot}1.5H_2O$ were added into 50 mL extraction and shaken by hand for 1 min. Subsequently, the mixture was centrifuged at 4000 rpm for 5 min. The portion (1 mL) of the upper layer was transferred into a 2 mL centrifuge tube for clean-up processes [29].

2.4. Clean-up of extract

Three different sorbents were tested for clean-up of extract: primary and secondary amine (PSA), C18 endcapped sorbents and graphitised carbon black (GCB). One millilitre of the extract was transferred into a 2 mL centrifuge tube. To the tube were added 150 mg $MgSO_4$ and 50 mg of cleaning sorbent and the tube was shaken by hand for 1 min. Finally, the mixture was centrifuged at 5000 rpm for 5 min. Two hundred microlitres of the upper layer were transferred to a mini vial and internal standard was then added. The extracted and purified samples were derivatised with DNSC and analysed using LC-MS/MS.

2.5. Conditions of derivatisation of alkylphenols

The derivatisation reaction (see Figure 1) was carried out in a 2 mL amber glass sample vial with 200 µL of purified extracts. Fifty microlitres of 100 mmol.L^{-1} $NaHCO_3$ (pH = 10.5) were added and the mixture was mixed (Vortex) for 1 min. Then, 200 µl of DNSC in acetone (0.5 mg·mL^{-1}) were added and the mixture was mixed again. Subsequently, the mixture was incubated for 60 min at 60°C. The reaction mixtures were then cooled to room temperature and evaporated to dryness under N_2. The residue was re-dissolved in 1 mL of methanol and mixed. The solution was injected into the LC-MS/MS. Optimisation of the alkylphenol derivatisation method was carried out in a previous study [13].

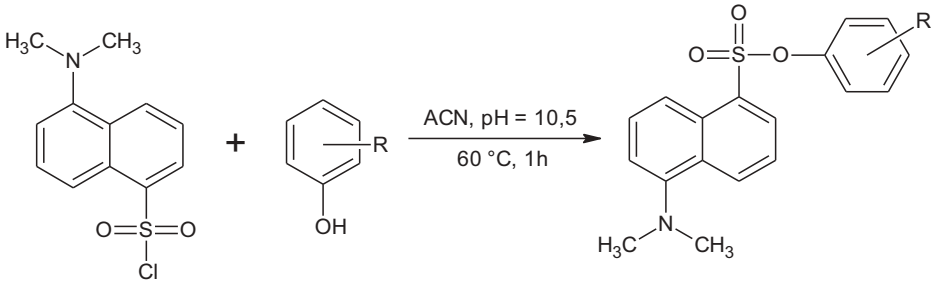


Figure 1. *Reaction scheme of dansylation of alkylphenols* [13].

2.6. LC-MS/MS analysis and detection

Chromatographic separations were performed using an Agilent 1200 Infinity Series (Agilent Technologies, Santa Clara, CA) with chromatographic column ACE 5 C18, 100 × 4.6 mm i.d., 5 µm particle size (ACE, Scotland, UK). The column temperature was maintained at 40°C. The injection volume was 10 µl. Methanol (A) and demineralised water containing 7 mmol L^{-1} formic acid (B) were used as a mobile phase. The isocratic elution of 90% (A) + 10% (B) was used at a flow rate of 0.5 mL min^{-1}. The Agilent 6410 Triple Quadrupole (Agilent Technologies, Santa Clara, CA) was used for MS/MS analysis and this instrument was operated in the ESI-positive SRM mode. Two MS/MS transitions were used for MS analyses. The optimised instrument conditions were as follows: drying gas temperature 350°C, capillary voltage 2 kV, nebuliser gas pressure 50 psi. MS/MS parameters are summarised in Table 3. The most abundant fragment ion m/z 171 of the precursor of the derivatised analyte was chosen for

the analyte quantification. The second most abundant fragment ion of each precursor was used to confirm the presence of the analyte.

A method of internal standard calibration in the concentration range of 1.0 – 100 ng mL^{-1} was used for final determination of the analysed compounds in the respective extract. Calibration curves were constructed using an internal standard of 4-n-NP D8 at a concentration of 20 ng mL^{-1}. Instrumental limits of quantification and limits of detection determined from the injected amount of individual analytes ranged from 0.08 to 0.83 pg per injection and 0.02 to 0.25 pg per injection, respectively, [13]. The calibration curves were linear over the concentration range used, with $R^2 \geq 0.99$.

3. Results and discussion

3.1. Extraction methods

As stated before, sample preparation is critical for the determination of phenolic compounds in soils. Four extraction methods were evaluated in order to obtain conditions for the simultaneous extraction of BSA and alkylphenols from soils. Solid-liquid extraction techniques (SLE) were evaluated with soil samples fortified by target analytes on the level of 10 µg kg^{-1}. SWE, USE, ASE and the original QuEChERS extraction methods were tested for their efficiency at extracting the selected alkylphenols (BPF, BPA, 4-t-OP, iso-NP, 4-OP and 4-n-NP) depending on the content of total organic carbon of the soil sample. Two soils with the lowest (TOC – 0.44) and highest (TOC – 6.31) content of total organic carbon were selected for this purpose (see Figure 2).

In the case of soil with low-TOC content, the least effective extraction technique was USE with the recovery in the range from 15% to 73% depending on the analytes. The recovery of extraction using SWE was 26% to 95%. The recovery of extraction using ASE was 33% to 103%. The best extraction technique for all monitored compounds was the QuEChERS method with recoveries of phenolic compounds in the range from 67% to 114%. Recoveries using the QuEChERS method were significantly higher for BPF and BPA (80% and 67%) compared to other extraction methods. Values are significantly different from the values obtained by other methods (evaluated by ANOVA, $p \leq 0.05$).

In the case of soil with a high content of TOC, the result is different compared to the result obtained for low-TOC soil. It is evident that the yield is compound-dependent. Generally, the best extraction efficiency was achieved by ASE extraction, in the range from 32% to 82%. The recovery obtained by USE ranged from 13% to 70%. The recovery of QuEChERS ranged from 34% to 77%. The extraction recovery obtained by SWE ranged from 20% to 148%. However, the variance of recovery values in the QuEChERS method is the best. In the case of iso-NP the recovery is very high (148%).

Moreover, the determination of a mixture of nonylphenol isomers (iso-NP) is complicated. It should be noted that technical nonylphenol is a mixture of isomers with regard to the branching of the alkyl chain [30]. Technical nonylphenol used in industry is classified as a mixture of C3 – C10 alkylphenols, where 4-NP is the main component. Technical mixture contains from 153 to 204 alkylphenols. Thus, when analysing the total concentration of 4-NP isomers, the content is identified and quantified as a mixture of 4-NP isomers [31].

The QuEChERS extraction technique was chosen as the most suitable extraction technique for further experiments. The QuEChERS method not only provides the highest

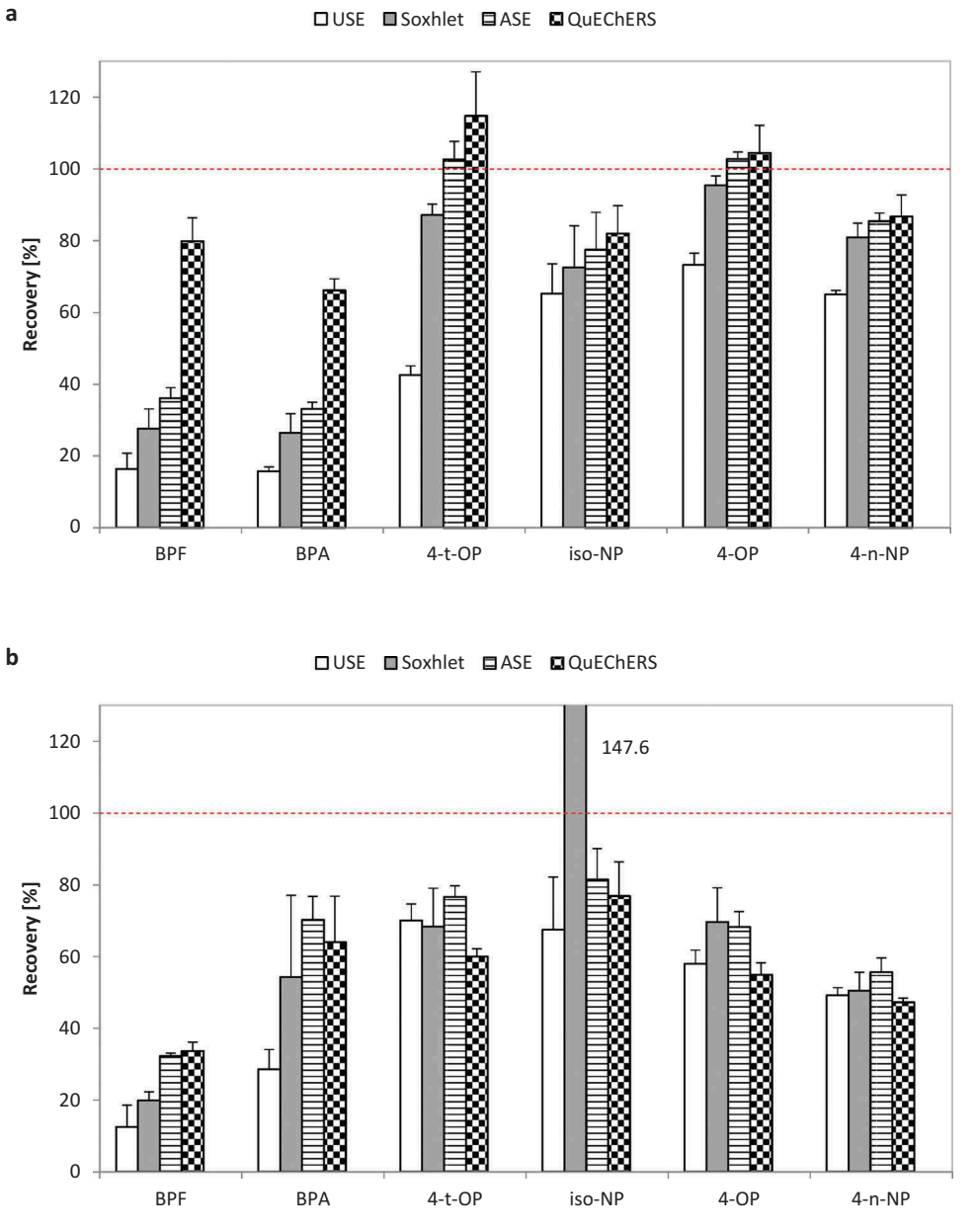


Figure 2. Recovery values of the extraction of alkylphenols and bisphenols from soils (a) with lowest TOC and (b) with the highest TOC obtained using different extraction technique.

extraction yield and the lowest scattering of recovery values, it is also the least instrumentally demanding and consumes the least solvent.

In order to obtain the best results with the QuEChERS technique, different experimental conditions of the method were tested. This procedure has been widely used for the simultaneous extraction of compounds with a wide range of polarity. Therefore, three different experimental conditions were examined: unbuffered QuEChERS method

(original QuEChERS method), buffered QuEChERS method (AOAC 2007.01), citrate buffered QuEChERS method (EN 15,662).

The results for the unbuffered and citrate buffered QuEChERS method were very similar both for the soil with a low and high content of total organic carbon (see Figure 3).

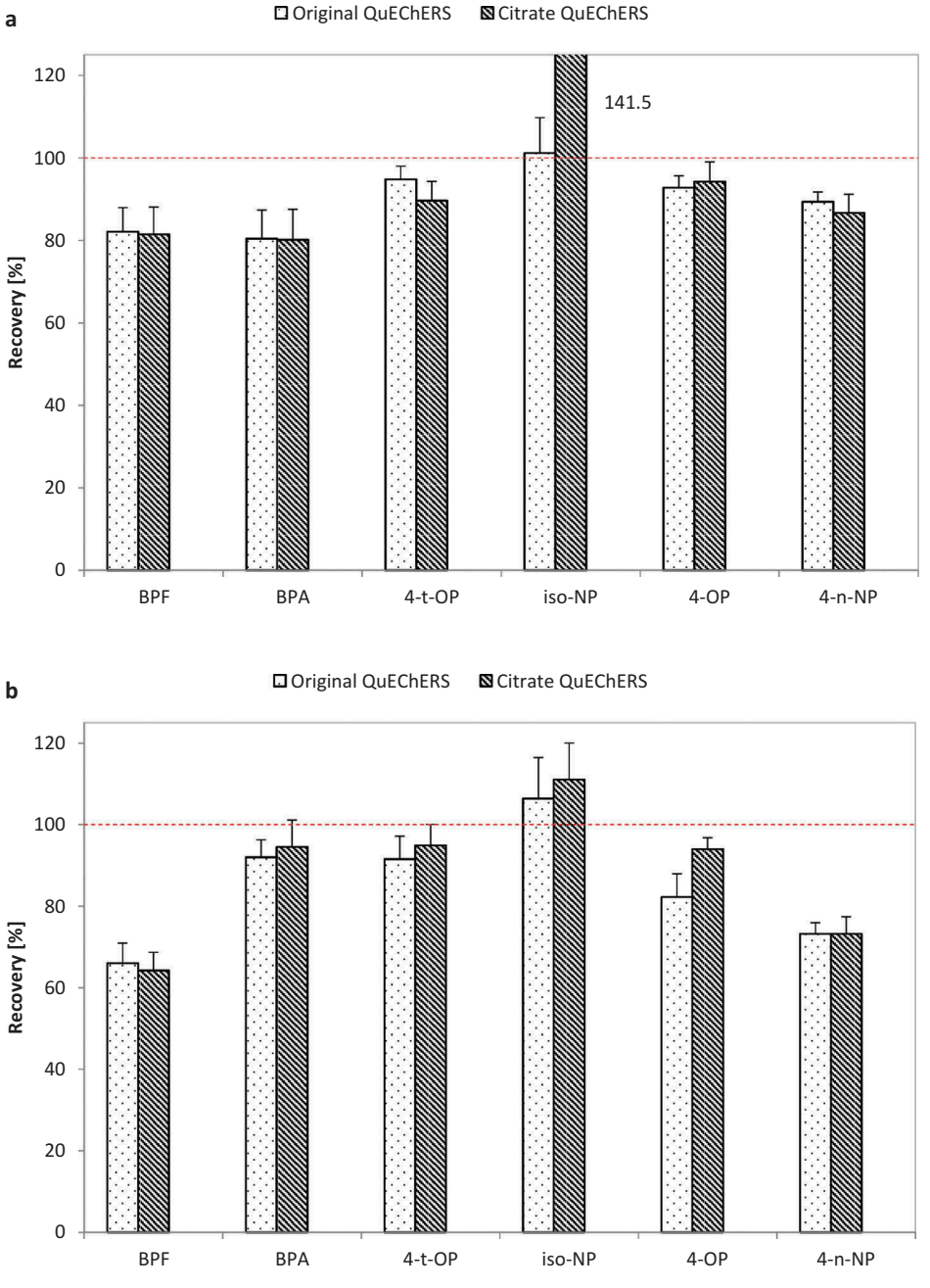


Figure 3. Recovery values of the extraction of alkylphenols and bisphenols from soils (a) with lowest TOC and (b) with the highest TOC obtained using two different QuEChERS procedures.

Table 3. SRM transitions used for LC-MS/MS determination of dansyl derivatives of phenolic compounds.

Analyte	BPF	BPA	4-t-OP	iso-NP	4-OP	4-n-NP
Transitions monitored	667.2 → 171	695.3 → 171	440.2 → 171	454.2 → 171	440.2 → 171	454.2 → 171
	667.2 → 433	695.3 → 461	440.2 → 156	454.2 → 156	440.2 → 156	454.2 → 156
Fragmentor voltage (V)	150	150	150	150	150	150
Collision energy (eV)	40	44	38	34	34	44

Recovery was within the range of 80.1–141.5% and 64.3–111.0% for soils G01 and G13, respectively. The acetate buffered QuEChERS method was disadvantageous for the determination of all monitored phenolic compounds. The response of the mass spectrometry detector was in all cases under signal/noise = 3 and therefore under the limit of detection. The explanation is that 1% HOAc in ACN is inappropriate for derivatisation of alkylphenols and bisphenols, which was demonstrated after drying the extract under a stream of nitrogen and converting only to acetonitrile. Subsequently, the redissolved extract was derivatised and recovery increased to a value comparable to unbuffered and citrate buffered QuEChERS methods. A statistical test found that the difference between the unbuffered and citrate buffered QuEChERS methods is not statistically significant. This was the reason why we used the unbuffered QuEChERS method in our study.

The matrix effect (ME%) was calculated according to the formula (1):

$$ME\% = \left(\frac{A_{matrix} - A_{blank}}{A_{standard}} - 1\right) \times 100 \quad (1)$$

where A_{matrix} is the LC/ESI/MS peak area of known concentration of alkyphenols added to the soil extract, A_{blank} corresponds to the area of the alkylphenol peak in the non-spiked extract, and $A_{standard}$ is the area of the alkylphenol peak in a standard solution with the same concentration as alkylphenol added into the extracted matrix. If ME% >0, the matrix enhances the signal of the analyte; if ME% <0, the matrix decreases the signal of the analyte.

The results show that the effect of the matrix extracted from soil by the unbuffered QuEChERS method on the determination of alkylphenols by LC- ESI-MS/MS is higher in the soil with higher TOC value. The ME was in the range from −17.5% to −19.2% for soil G01 and in the range from −20.4% to – 23.8% for soil G13.

The precision determined as the standard deviation for repeating analyses of spiked soil samples was in the range from 7% to 15%.

3.2. Clean-up of the extract

The cleaning step was used for reducing the possible matrix effect, which can have a significant impact on LC-MS/MS analysis. The disperse-SPE (d-SPE) technique was used for clean-up of the extract of alkylphenols from soils. Clean-up of extracts of alkylphenols from soils spiked by 20 ng of analyte per gram was performed in a 2 mL centrifuge tube. The mixture of 150 mg of $MgSO_4$ and various amounts of sorbent (PSA, Si-C18 or GCB) was added to 1 mL of extract. PSA as a weak anion exchanger based on silica gel chemically modified with primary and secondary amine functional groups allows removing a wide variety of polar organic acids, polar pigments, certain carbohydrates and

fatty acids. GCB as a graphitised carbon removes sterols and pigments such as chlorophyll. Si-C18 as a silica gel modified by hydrophobic hydrocarbon chains is used for removing non-polar interfering substances, such as lipids [32]. PSA was used in the range 0–150 mg, while GCB and Si-C18 were used in the range 0–75 mg.

Different results were achieved with the tested sorbents, depending on the soil type and monitored compound (Table 4).

In the case of soils with a low content of TOC, use of the PSA sorbent is not necessary for extract purification. The maximum amount of other sorbents is 50 mg for all analytes, because in the case of higher quantities, the yield of BPF and iso-NP is rapidly reduced. The opposite situation occurs in soils with high TOC. Here, the purification step is recommended in a minimum quantity of at least 25 mg of PSA, and preferably 50 mg PSA. This is probably due to an increased interaction of analytes with the soil organic matrix. Thus, in the case of soils with low content of TOC, analytes are retained less and are better extractable.

Quite similar results were provided by sorbent GCB for soils with a low content of TOC, which can be used directly for analyses of raw extract. In this case, the cleaning step may be omitted altogether, or only a very small quantity of sorbent may be used up to a maximum of 10 mg of GCB for all the compounds under investigation. For soils with a high content of TOC, 10–20 mg of GCB sorbent is recommended to clean up the extract. If BPA will be the target analyte, the amount of sorbent does not depend on the TOC content. Unfortunately, this finding cannot be used for other analytes in our study.

Table 4. Results of ANOVA analysis of the effect of the amount of sorbent used for purification of the extract from the soil with lowest (left) and highest (right) TOC level (samples were spiked to reach 20 µg of each analytes per kg of soil.

PSA [mg]							PSA [mg]						
BPF	0	25	50	75	100	150	BPF	0	25	50	75	100	150
BPA	0	25	50	75	100	150	BPA	0	25	50	75	100	150
4-t-OP	0	25	50	75	100	150	4-t-OP	0	25	50	75	100	150
iso-NP	0	25	50	75	100	150	iso-NP	0	25	50	75	100	150
4-OP	0	25	50	75	100	150	4-OP	0	25	50	75	100	150
GCB [mg]							**GCB [mg]**						
BPF	0	5	10	20	50	75	BPF	0	5	10	20	50	75
BPA	0	5	10	20	50	75	BPA	0	5	10	20	50	75
4-t-OP	0	5	10	20	50	75	4-t-OP	0	5	10	20	50	75
iso-NP	0	5	10	20	50	75	iso-NP	0	5	10	20	50	75
4-OP	0	5	10	20	50	75	4-OP	0	5	10	20	50	75
Si-C18 [mg]							**Si-C18 [mg]**						
BPF	0	5	10	20	50	75	BPF	0	5	10	20	50	75
BPA	0	5	10	20	50	75	BPA	0	5	10	20	50	75
4-t-OP	0	5	10	20	50	75	4-t-OP	0	5	10	20	50	75
iso-NP	0	5	10	20	50	75	iso-NP	0	5	10	20	50	75
4-OP	0	5	10	20	50	75	4-OP	0	5	10	20	50	75

For soil with low TOC, the sorbent Si-C18 can be used in the range from 5 mg to 20 mg for all analytes, the higher content of sorbent Si-C18 may be statistically significant (see Table 5). For more polar substances such as BPF and BPA, the sorbent Si-C18 can be used in the whole range from 0 mg to 75 mg, if soil with a high content of TOC was used. In principle, for non-polar substances such as (4-t-OP and 4-OP) a maximum of 10 mg of sorbent Si-C18 can be used.

Table 5. Statistic evaluation of R_E – ANOVA, statistical significance $p < 0.05$ highlighted in bold.

TOC[%]	Clean up	BPF	BPA	4-t-OP	iso-NP	4-OP
0,44	PSA	0.1762	**0.0345**	0.2707	0.1961	0.3450
	GCB	0.3237	0.2087	0.0800	0.4015	**0.0031**
	Si-C18	0.0977	0.9295	0.3516	0.6498	0.0925
6,31	PSA	0.4557	0.3224	0.0865	0.5715	0.0623
	GCB	0.1539	0.4220	0.2433	0.5797	0.0756
	Si-C18	0.3236	**0.0040**	0.6265	**<0.0001**	0.8777

3.3. Determination of alkylphenols in soil samples

The above-mentioned improved method was used for analysing the content of alkylphenols and bisphenols in various soil samples. Ten grams of investigated soil (G01, G04, G07, G08, G10, G11 and G13) were extracted using the unbuffered (original) QuEChERS method. One millilitre of supernatant was purified using a mixture of 150 mg $MgSO_4$ and 50 mg PSA. Subsequently, the derivatisation of analytes was performed (see Section *2.5. Conditions of derivatisation)*. The median concentration of BPA in samples G7 and G13 was 0.41 $\mu g \cdot kg^{-1}$ and 0.47 $\mu g \cdot kg^{-1}$, respectively. In other soil samples, the content of BSA was below the limit of quantification. The median of BPF concentrations in soil samples G11 and G13 were 0.20 $\mu g \cdot kg^{-1}$ and 0.14 $\mu g\ kg^{-1}$, respectively. The linear alkylphenols such as 4-OP and 4-n-NP were not detected in analysed soil samples. Also, the artificial soil sample did not contain any of the target analytes. The median concentrations of 4-t-OP in soil samples G1, G7, G8 and G13 were 0.12 $\mu g\ kg^{-1}$, 0.16 $\mu g\ kg^{-1}$, 0.06 $\mu g\ kg^{-1}$ and 0.44 $\mu g\ kg^{-1}$, respectively. Relatively high concentrations of iso-NP were found in soils G7 and G13, the median concentrations of iso-NP were 10.89 $\mu g\ kg^{-1}$, and 9.62 $\mu g\ kg^{-1}$, respectively. The concentrations of alkylphenols in the examined soils do not pose any health risk to humans. The reference dose (RfD) for oral exposure is 0.01 mg/kg/day for BPA and 0.10 mg/kg/day for nonylphenols [33].

4. Conclusions

The aim of this study was to investigate the occurrence of alkylphenols and bisphenols (BPF, BPA, 4-t-OP, iso-NP, 4-OP and 4-n-NP) in soils using optimised extraction, clean-up of extract and derivatisation of selected analytes. The recoveries of extraction of alkylphenols and bisphenols from soils obtained using three conventional extraction techniques and the promising QuEChERS method were compared. Two soils with the lowest (TOC – 0.44%) and highest (TOC – 6.31%) content of total organic carbon were tested. USE was the least effective extraction technique for soil with a low TOC content. SWE and ASE provided low extraction recoveries or unsatisfactory repeatability in the case of BPF and BPA. These two extraction techniques are also more time-consuming compared to USE and the QuEChERS method. The comparison of the results of the extraction methods was carried out using three modifications of the QuEChERS method (original, citrate buffered and acetate buffered) and an appropriate cleaning technique. The best extraction procedure for all monitored compounds was the unbuffered QuEChERS method. Recoveries obtained by unbuffered QuEChERS are significantly higher for BPF and BPA in comparison with other extraction methods. For soil with a high content of TOC, the result is ambiguous. It is obvious that it depends on the monitored compounds.

The optimised method was successfully used for the determination of target alkylphenols in soil samples. The concentrations of alkylphenols and bisphenols in soils ranged from 0.06 μg kg^{-1} and 10.89 μg kg^{-1}. These values do not pose any risk to human health.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] R. Gadzała-Kopciuch, A. Filipiak and B. Buszewski, Talanta **74**, 655 (2008). doi:10.1016/j.talanta.2007.06.028.
[2] Y. Kawagoshi, Y. Tsukagoshi and I. Fukunaga, J. Environ. Monitor **4**, 1040 (2002). doi:10.1039/b206161a.
[3] A.L. Capriotti, C. Cavaliere, V. Colapicchioni, S. Piovesana, R. Samperi and A. Lagana, J. Chromatogr. A **1313**, 62 (2013). doi:10.1016/j.chroma.2013.06.054.
[4] J. Wang, H. Pan, Z. Liu and F. Ge, J. Chromatogr. A **1216**, 2499 (2009). doi:10.1016/j.chroma.2008.12.003.
[5] J.A. Padilla-Sánchez, P. Plaza-Bolaños, R. Romero-González, A. Garrido-Frenich and J.L.M. Vidal, J. Chromatogr. A **1217**, 5724 (2010). doi:10.1016/j.chroma.2010.07.004.
[6] S. Almeida, A. Raposo, M. Almeida-Gonzalez and C. Carrascosa, Compr. Rev. Food Sci. F. **17**, 1503 (2018). doi:10.1111/1541-4337.12388.
[7] M. Zhen, X.F. Zheng, Y.Q. Zhang, X.X. Tao, Y. Yan Li and W. Wang, Int. J. Mol. Sci **13**, 491 (2012). doi:10.3390/ijms13010491.
[8] F.M. Ghazali and W.L.W. Johari, J. Biochem. Microbiol. Biotechnol **3**, 30 (2015).
[9] S. Lee, C. Liao, G.J. Song, K. Ra, K. Kannan and H.B. Moon, Chemosphere **119**, 1000 (2015). doi:10.1016/j.chemosphere.2014.09.011.
[10] A. Soares, B. Guieysse, B. Jefferson, E. Cartmell and J.N. Lester, Environ. Int **34**, 1033 (2008). doi:10.1016/j.envint.2008.01.004.
[11] J. Gong, L. Xu, Y. Yang, D.Y. Chen and Y. Ran, J. Hazard. Mater **192**, 643 (2011). doi:10.1016/j.jhazmat.2011.05.071.
[12] M. Petrovic, S. Tavazzi and D. Barcelo, J. Chromatogr. A **971**, 37 (2002). doi:10.1016/S0021-9673(02)01026-9.
[13] M. Pernica, P. Poloucká, M. Seifertová and Z. Šimek, J. Chromatogr. A **1417**, 49 (2015). doi:10.1016/j.chroma.2015.09.030.
[14] J. Llorca-Pórcel, M. Martínez-Parreño, E. Martínez-Soriano and I. Valor, J. Chromatogr. A **1216**, 5955 (2009). doi:10.1016/j.chroma.2008.12.003.
[15] R. Gibson, M.J. Wang, E. Padgett and A.J. Beck, Chemosphere **61**, 1336 (2005). doi:10.1016/j.chemosphere.2005.03.072.

[16] M.I. Helaleh, K. Tanaka, S.I. Fujii and T. Korenaga, Anal. Sci **17**, 1225 (2001). doi:10.2116/analsci.17.1225.
[17] M. Anastassiades, S.J. Lehotay, D. Štajnbaher and F.J. Schenck, J. AOAC Int **86**, 412 (2003).
[18] M. Andraščíková and S. Hrouzková, Anal. Methods-UK **5**, 1374 (2013). doi:10.1039/c3ay26434c.
[19] R. Romero-Cano, D. Kassuha, J. Peris-Vicente, P. Roca-Genovés, S. Carda-Broch and J. Esteve-Romero, Analyst **140**, 1739 (2015). doi:10.1039/C4AN01782J.
[20] O. Ros, A. Vallejo, L. Blanco-Zubiaguirre, M. Olivares, A. Delgado, N. Etxebarria and A. Prieto, Talanta **134**, 247 (2015). doi:10.1016/j.talanta.2014.11.015.
[21] Y.Y. Gu, X.J. Yu, J.F. Peng, S.B. Chen, Y.Y. Zhong, D.Q. Yin and X.L. Hu, J.Chromatogr. B **965**, 164 (2014). doi:10.1016/j.jchromb.2014.06.024.
[22] A.M. Gómez-Caravaca, V. Verardo and M.F. Caboni, J. Chromatogr. A **1217**, 7411 (2010). doi:10.1016/j.chroma.2010.09.054.
[23] H.G. Mol, S. Sunarto and O.M. Steijger, J. Chromatogr. A **879**, 97 (2000). doi:10.1016/S0021-9673(00)00124-2.
[24] M. Kojima, S. Tsunoi and M. Tanaka, J. Chromatogr. A **984**, 237 (2003). doi:10.1016/S0021-9673(02)01836-8.
[25] B.L. Qi, P. Liu, Q.Y. Wang, W.J. Cai, B.F. Yuan and Y.Q. Feng, TrAC **59**, 121 (2014).
[26] V. Andreu, E. Ferrer, J.L. Rubio, G. Font and Y. Picó, Sci. Total Environ **378**, 124 (2007). doi:10.1016/j.scitotenv.2007.01.024.
[27] www.chemicalize.org by ChemAxon.
[28] S.J. Lehotay, J. Tully, A.V. Garca, M. Contreras, H. Mol, V. Heinke and M.E. Poulsen, J. AOAC Int **90**, 485 (2007).
[29] S.J. Lehotay, K.A. Son, H. Kwon, U. Koesukwiwat, W. Fu, K. Mastovska and N. Leepipatpiboon, J. Chromatogr. A **1217**, 2548 (2010). doi:10.1016/j.chroma.2010.01.044.
[30] M. Naassner, M. Mergler, K. Wolf and I. Schuphan, J. Chromatogr. A **945**, 133 (2002). doi:10.1016/S0021-9673(01)01522-9.
[31] R.P. Eganhouse, J. Pontolillo, R.B. Gaines, G.S. Frysinger, F.L. Gabriel, H.P.E. Kohler and L. B. Barber, Environ. Sci. Technol **43**, 9306 (2009). doi:10.1021/es901110m.
[32] A. Wilkowska and M. Biziuk, Food Chem **125**, 803 (2011). doi:10.1016/j.foodchem.2010.09.094.
[33] A. Careghini, A.F. Mastorgio, S. Saponaro and E. Sezenna, Environ. Sci. Pollut. R **22**, 5711 (2015). doi:10.1007/s11356-014-3974-5.

Article III

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Marek Pernica conducted the research of alkylphenols and bisphenols

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Drinking water contaminants from epoxy resin-coated pipes: A field study

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ABSTRACT

Rehabilitation of aged drinking water pipes is an extensive renovation and increasingly topical in many European cities. Spray-on-lining of drinking water pipes is an alternative cost-effective rehabilitation technology in which the insides of pipes are relined with organic polymer. A commonly used polymer is epoxy resin consisting of monomer bisphenol A (BPA). Leaching of BPA from epoxy lining to drinking water has been a concern among public and authorities. Currently epoxy lining is not recommended in some countries. BPA leaching has been demonstrated in laboratory studies but the behavior and ageing process of epoxy lining in situ is not well known. In this study 6 locations with different age epoxy linings of drinking water pipes done using two distinct technologies were studied. While bisphenol F, 4-n-nonylphenol, and 4-t-octylphenol were rarely found and in trace concentrations, BPA was detected in majority of samples. Pipes lined with the older technology (LSE) leached more BPA than those with more recent technology (DonPro): maxima in cold water were 0.25 μg/L and 10 ng/L, respectively. Incubation of water in pipes 8–10 h prior to sampling increased BPA concentration in cold water 1.1–43-fold. Hot water temperature caused even more BPA leaching - at maximum 23.5 μg/L. The influence of ageing of epoxy lining on BPA leaching on could be shown in case of LSE technology: locations with 8–9 years old lining leached 4–20-fold more BPA compared to a location with 2-year-old lining. Analysis of metals showed that epoxy lining can reduce especially iron concentration in water. No significant burden to water could be shown by the analyzed 72 volatile organic compounds, including epichlorhydrin, precursor used in epoxy resin. Estrogenicity was detected in water samples with the highest BPA loads. Comparable responses of two yeast bioreporters (estrogen receptor α and BPA-targeted) indicated that bisphenol-like compounds were the main cause of estrogenicity. Compared to the estimated average daily BPA exposure, additional BPA load via cold drinking water in the studied locations was low, maximum 8.7%. However, hot water should also be considered as exposure source due to higher BPA concentrations. Epoxy lined locations should be monitored in future in order to evaluate ageing process and control increasing leaching of potentially harmful chemicals.

1. Introduction

Access to pure drinking water is one of the most important issues for human population. Drinking water distribution system plays a key role in delivering this resource to consumers. In addition to pathogenic microbes which are the major threat to drinking water quality in the world, chemical safety is another emerging challenge (WHO, 2011). Chemical contaminants can enter to drinking water in several stages: they can originate from source water, purification process, and distribution system components, or via backflow, breakdowns, leakage, or vandalism.

Drinking water is exposed to many different pipe materials in distribution system. Water can reside in the pipes for several days during which corrosion and leaching of compounds from the pipes can affect water quality (Kekki et al., 2007). Small pipes in households, however, can have potentially greater impact on water compared to large main pipes in the distribution system where the quality of water remains more stable due to larger pipes and more

Abbreviations: BPA, Bisphenol A; BPF, Bisphenol F; CPDW, Construction products in contact with drinking water; DWTP, Drinking water treatment plant.

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constant flow (Kekki et al., 2007).

Rehabilitation of old drinking water pipes is an increasingly topical issue in many European countries. Majority of apartment houses have been built in the decades after the Second World War (Meijer et al., 2009), and their water pipes are coming to the end of their life time. Old drinking water pipes suffer from corrosions, blockages, biofilm formation, and they have been shown to leach metals such as iron, zinc, copper, and lead to the drinking water (Kekki et al., 2007).

Pipe rehabilitation is an extensive renovation, and consequently expensive and time-consuming. Usually the apartments are uninhabitable during the time of pipe replacements which may take up to several months. For these reasons alternative pipe rehabilitation methods are attractive. Relining of the inside surface of pipes has been used as such a method. The so-called spray-on-lining is the oldest used technology to restore the functioning of the pipe and stop internal corrosion (Ellison et al., 2010). Organic polymers such as epoxy resins and polyurethane are most commonly used lining materials. Epoxy resins have good thermal stability, tight attachment to metal and ceramic surfaces, and they can span over small holes in the pipe (Ellison et al., 2010; Kekki et al., 2007). Spray-on epoxy lining is suitable also for narrow pipes because the used thickness is only about 0.3–4 mm (Ellison et al., 2010; Pekkinen, 2011).

The epoxy-based spray-on-lining procedure typically includes several steps such as drying the pipes, cleaning with pressure and sand blasting, application of the epoxy mass with pressure, and drying. After the lining procedure the pipes are ready to be flushed and used within 12–24 h (Ellison et al., 2010; Pekkinen, 2011). Due to the non-invasive nature of this technology and no need for renovation of other surfaces, the costs are generally about half of the traditional pipe replacement renovation (Pekkinen, 2011). For this reason there is still growing interest towards this technology, was commonly used in 1980's and 1990's in Europe and in the USA (Ellison et al., 2010).

Spray-on-lining has several challenges. The most important issues include proper cleaning of the pipe inner surface, efficient spreading of the lining material, correct formula and sufficient curing time of the epoxy resin (Ellison et al., 2010; Pelto-Huikko and Kaunisto, 2011). All of these factors affect the attachment of the resin to the pipe and consequently leaching of contaminant into drinking water. Detected errors in lining include drainage of the resin in branches and turns, weak cleaning and resin attachment, and breakage of the lining due to errors in subsequent couplings (Ellison et al., 2010).

Epoxy resins used for pipe lining commonly comprise of two main components: bisphenol A (BPA) and epichlorhydrin. These two react to different polymer length, consisting of pre-polymer unit bisphenol A diglycigyl ether (BADGE). As BPA is a well-characterized endocrine-disrupting compound (e.g. reviewed by Flint et al., 2012, and Rubin, 2011), the safety of epoxy resin-based lining technology has caused concern among consumers and authorities. It has been shown that epoxy coating can leach BPA, BADGE, and other related organic compounds (Bruchet et al., 2014; Kosaka et al., 2012; Umweltbundesamt, 2010). Currently, epoxy-based lining is not allowed in Germany, and not recommended in Sweden (Pelto-Huikko and Kaunisto, 2011). The information on the use of epoxy lining in other regions is limited and it follows highly variable country-specific regulations and procedures. In addition, insurance companies have varying policies, and many do not insure renovations based on spray-on-lining (Pelto-Huikko and Kaunisto, 2011).

In this study the impact of epoxy lining on drinking water quality was studied using the field research in 8 houses with different age rehabilitations in Helsinki, Finland. The effects of age, used lining technology, temperature, and incubation time on the occurrence and burden of chemical compounds (i.e. metals, volatile compounds and organic chemicals - including BPA) were evaluated. In addition, total estogenic potential of water extracts were measured using bioassays and compared to concentrations of BPA in order to evaluate the proportion of estrogenicity caused by BPA. Finally, risk assessment based on highest detected BPA concentrations was done.

2. Methods and materials

2.1. Samples

Samples were collected in 6 apartment houses with epoxy lined drinking water pipes in Helsinki capital area, Finland, during July–April in 2015 (Table 1). Additional samples were taken from two other locations. Location TR (for Traditional Renovation) had been renovated using traditional pipe-replacement technology. This location was in a close distance from location D, and it was both built and its pipes rehabilitated at the same time as in location D. Location NR (for Non-Renovated) was built in 1960's and had original non-renovated drinking water pipes. TR and NR locations were sampled in December 2015. Finally, a drinking water treatment plant (DWTP) in Helsinki was sampled for treated drinking water in both summer 2015 and December 2015 to assess original chemical burden of water before entering the distribution system.

The epoxy lining of drinking water pipes at locations A-F were done during years 2006–2013 using two spray-on lining technologies LSE and DonPro (Table 1). LSE technology was developed by a Swiss engineering LSE-SYSTEM AG in 1987. Since then it has been often used in Europe, and adopted in Finland in 2005, being the first used spray-on-lining technology for drinking water pipes (Markelin-Rantala and Rautiainen, 2007). DonPro technology was further developed from LSE by Donauer & Probst GmbH & Co in 1994. The main difference between the two technologies is pipe cleaning where LSE method uses sandblasting with over-pressure while DonPro uses a more pipe-friendly under-pressure cleaning (personal communication with contractor II). No specific formula of the epoxy resin materials is available. LSE epoxy material is marketed under the name LSE-001 NA (tested according to ANSI/NSF standard 61), while in DonPro the Tubeprotect-coating is used (Table 1). Service life expectancy of the coating work is generally 30–50 years with guarantee of 2–5 years.

All locations were sampled twice on different days in order to have an idea of daily variation. In all sampling places the residents were asked not to use water in the whole apartment for about 8–10 h before sampling. This was done in order to assess the leaching of chemicals from pipes during incubation of water in pipes. Location D was an exception because the apartment was uninhabited, and incubation times for the two samplings were about 1 month and 1 week, respectively. Kitchen tap was sampled for pipe-incubated water from both cold and hot water lines, and water from both lines after 2 min flowing on full pressure. One more sample was taken from a water tap as close to the plot pipe as possible (usually ground floor/basement tap) to represent water coming to the house from communal distribution system. This sample was collected after 2 min flowing from the cold water line. In total 5 samples were studied at each location - (i) incoming water, (ii) cold water incubated, (iii) cold water flowed, (iv) hot water incubated, and (v) hot water flowed.

2.2. Sample handling

All samples were collected in amber glass bottles which were cleaned by rinsing with acetone and distilled water, and baked in

Table 1
Epoxy coated sampling locations, and epoxy lining technology characteristics.

Sampling location	Building time	Pipe renovation characteristics			
		Year	Contractor	Coating technology	Epoxy coating material
A	1960's	2013	II	DonPro (Donauer & Probst GmbH & Co)	Tubeprotect
B	1950's	2013	I	LSE (LSE-SYSTEM AG)	LSE-001 NA
C	1950's	2012	II	DonPro (Donauer & Probst GmbH & Co)	Tubeprotect
D	1950's	2007	I	LSE (LSE-SYSTEM AG)	LSE-001 NA
E	1910's	2006	I	LSE (LSE-SYSTEM AG)	LSE-001 NA
F	1930's	2013	II	DonPro (Donauer & Probst GmbH & Co)	Tubeprotect
NR	1960's	–	Non-renovated location		
TR	1950's	2007	Renovated by traditional pipe replacement		

50 °C until dry. Water was carefully drained along the side of bottle to avoid excess loss of volatile compounds. Sample volume was 0.93–1.29 L, except for drinking water treatment plant, where 2 L sample was taken. Based on pipe charts of one of the locations, 1 L volume was calculated to be roughly the volume of water pipes of a particular apartment, and thus a good sampling volume for pipe-incubated water. One extra 1 L sample was taken at each sampling location and spiked with 1 μg of BPA and 0.5 μg of bisphenol F (BPF), 4-n-nonylphenol (4-n-NP), and 4-t-octylphenol (4-t-OP)) to test primarily for stability and yield of chemicals after sample handling and processing. This sample was co-extracted and analyzed with other samples. The measured concentrations were compared to a non-extracted reference sample to calculate yield, and then used to correct the measured concentrations of other samples accordingly.

Samples were stored for maximum 20 h in 4 °C before extraction.

Sub-samples for analyses of volatile compounds were taken immediately after sampling to two 40 mL vials (or to one 500 mL bottle during March 2016 additional sampling for dedicated analyses of epichlorhydrin). The vessels were filled head-space-free and stored in 4 °C until analysis.

For metal analysis, 25 mL aliquot was taken to acid-cleaned LDPE bottles and acidified 1% v/v with nitric acid. Samples were stored in 4 °C until analyzed.

Rest of the water sample was extracted by solid phase extraction (SPE) using Oasis HLB 200 mg glass cartridges (Waters Corporation, USA), and slightly modified method published by Baugros et al. (2008). Shortly, cartridges were conditioned with 10 mL MeOH, 10 mL 50% MeOH, and 10 mL LC-MS Ultra Chromasolv water (Sigma-Aldrich, Germany), then loaded with sample water at flow rate of approximately 10 mL/min. Analytes were eluted from columns with 10 mL acetone and 10 mL ethyl acetate. The elute was evaporated to smaller volume, divided in two parts, then evaporated to dryness and each aliquot was reconstituted to 100 μL acetonitrile for alkylphenol analysis or DMSO for bioassays.

2.3. Alkyl phenol analysis and volatile organic compounds

Alkyl phenols were analyzed from 50 μL volume of the acetonitrile extracts using dansyl chloride derivatization and LC–ESI–MS/MS described by Pernica et al. (2015).Extracts were analyzed for BPA, BPF, 4-t-OP, and mixture of nonyl phenols (iso-NP), 4-n-NP. Injection volume was 10 μL and instrumental detection limit was 0.83 pg/injection for all analytes.

Volatile organic compounds were analyzed in contracted accredited laboratory ALS Global in Brno, Czech Republic. The analytes were in total 71 and are listed in Certificate of Accreditation No. 819/2015 of 30/11/2015 (http://alsglobal.cz/website/var/assets/media-general/certificates-2015/819_2015_eng_whole_cai_pdf.pdf), and they were analyzed using US EPA 8260 (GC-MS) method. In addition, epichlorhydrin was analyzed in extra collected samples in March 2016 by ALS Global using a GC-MS method with detection limit of 100 μg/L.

2.4. Metal analysis

Inductively Coupled Plasma – Mass Spectrometry (Agilent 7700x ICP-MS, Agilent Technologies, Japan) was used for determination of metals (iron, copper, zinc and lead) in the acidified water samples. Elements were measured in He collision mode using Octopole Reaction System for reduction of polyatomic interferences coming from sample matrix. 200 ppb solution of internal standards (Ge and Bi) was used for correction of matrix effects and drift.

2.5. Bioreporter assays

The DMSO extracts were tested for total estrogenicity and bisphenol A-equivalent activity using yeast bioreporters with human estrogen receptor α (Leskinen et al., 2005) and bisphenol A-targeted receptor (Rajasärkkä and Virta, 2013). The BPA-targeted receptor (BPA-R) is insensitive to estradiols at least up to 190 μg/L, and has about 4-fold greater potency towards BPA than ERα (Rajasärkkä and Virta, 2013; Rajasärkkä et al., 2011). The BPA-R has also at least 15 times higher potency towards BPA than bisphenols F or S, whereas ERα has similar potency towards BPA and BPF (Rajasärkkä et al., 2014).

The yeast assays were done as described by Leskinen et al. (2005).Briefly, yeast cells were grown overnight in 5 mL of minimal SD medium (containing Yeast Nitrogen Base Without Amino Acids (Sigma-Aldrich, Germany), 2% glucose and supplemented with adenine, histidine and leucine) at 30 °C with 250 rpm shaking, and diluted in the morning in fresh SD medium to OD600 of 0.4. The culture was further grown for approximately 2 h until OD600 reached 0.6–0.7. Assays were performed in white 96-well plates (Nunc, ThermoFischer Scientific) by first pipetting 2 μL of sample extracts (diluted 1:1 in DMSO), standard chemical solutions, or blank solvent (100% DMSO) in the wells. Then 98 μL of yeast culture was added, and the plates were incubated for 2.5 h at 30 °C after which 100 μL 1 mM D-luciferin stock solution in 0.2 M sodium citrate buffer (pH 5) was added to the wells and immediately measured for luminescence using BioTek Synergy 4 microplate reader. Data was analyzed as described in Rajasärkkä and Virta (2013). Fold luminescence signals of samples were compared to the BPA standard dose-response curve, and BPA equivalent concentrations (BPAeq) were calculated.

3. Results and discussion

3.1. Metals

The European Drinking Water Regulations 2014 (European

Union, 2014) set standards for 48 microbiological, chemical and indicator parameters which should be monitored regularrly to assure good water quality. The parameters also include maximum limits for metals, such as copper (2 mg/L), iron (200 μg/L) and lead (10 μg/L).

Galvanized steel was the most common material installed in household cold water lines between 1900 and 1970 in Finland (Kekki et al., 2007), and it was expected that iron and zinc would be good indicators of pipe ageing in the studied houses.

Indeed, iron concentrations in the non-renovated location NR were high in cold water samples (Fig. 1). Even in flowed cold water iron concentration exceeded the maximum limit during one of the two repeated samplings. Also zinc concentrations were high at site NR (>280 μg/L) in incubated and incoming water samples (Table S1 in supplemetrary material).

In majority of epoxy lined locations (A-F) iron and zinc concentrations in cold water were clearly lower compared to location NR (Fig. 1 and Table S1) which indicates that epoxy lining can lower the levels of iron and zinc in drinking water, but metal concetrations should have been measured before renovations for conclusive confirmation. Locations B and C, however, still had relatively high concentrations of iron (Fig. 1). In location D the incubated samples had high iron concentrations most likely due to very long incubation times (1 month and 1 week, respectively) but for all other samples collected at the location the concentrations were below 50 μg/L, i.e. at about the same level with the close-by location TR which had traditional pipe rehabilitation (Fig. 1 and Table S1).

Pipes made of copper have been commonly used in household hot water pipes, but since mid 1970's they have been applied to cold lines as well (Kekki et al., 2007). In the present study copper concentrations were generally higher in hot water samples with an average of 157 μg/L, whereas cold water had only on average 31 μg/L. None of the samples exceeded the EU quality limit 2 mg/L, and epoxy lining seems to have no effect on copper concentration in water (Table S1).

Lead has not been used extensively in water pipes in Finland (Kekki et al., 2007), and lead concentrations in all studied samples were below the recommended quality level of 10 μg/L (Fig. 2). Similar to profiles of iron and zinc, incubated samples in location D and the traditional renovated location (TR) had relatively high concentrations of lead. Possible sources can be connectors or sealings.

Metal concentrations measured at the DWTP in Helsinki in this study were in good agreement with the results obtained by the DWTP in August 2015: 20.4/21.6 μg/L vs. 31 μg/L for iron, and 5.2/4.8 μg/L vs. 5 μg/L for copper. Measured concentrations for zinc and lead by the DWTP were below LOD, while 0.7 and 0.03 μg/L were measured in this study (Table S1).

The incoming water samples had great varation in metal concentrations between different locations, although they were generally lower than those in corresponding cold flowed water samples (Fig. 1). Communal distribution system can be the source, however, in Finland it comprises on average 61% of plastic pipe materials, and only 36% steel or iron pipes, much less than in Europe and the USA (Gonzalez et al., 2013; Kekki et al., 2007). Possibly plot pipes, i.e. pipes branching from communal system to houses, can be made of old steel pipes contributing to observed variability.

3.2. Volatile organic chemicals

Of the 71 volatile compounds analyzed the chlorination by-product chloroform was detected in all samples at levels 0.1–0.99 μg/L with an average of 0.21 μg/L (Table S2 supplementary data). Another chlorination by-product bromo-dichloromethane was occasionally detected in DWTP and few other samples at concentrations 0.10–0.16 μg/L (Table S2). In the incubated samples at location D methyl-tertbutylether (MTBE) and tert-butylalcohol (TBA) were detected in concentrations 1.45–11.7 μg/L and 47–535 μg/L, respectively. MTBE and TBA have been associated with polyethylene water pipes as cross-linking reagent by-product (Whelton and Nguyen, 2013). Detectable concentrations in incubated samples from location D can likely be associated with very long incubation times, possibly originating from plastic couplings or sealings.

No epichlorhydrin was detected in any of the samples (detection limit 100 ng/L) upon resampling campaign in March 2016. Epichlorhydrin is a potential carcinogen according to the US EPA (Group B2), and WHO classifies it as “probably carcinogenic to humans (IARC Group 2A). A maximal limit of 100 ng/L has been set in The European Drinking Water Regulations (2014) (European Union, 2014). The compound is very reactive and probably not detectable even if small amounts would be leaching from the resin.

3.3. Alkylphenols

For alkylphenol analysis the water samples were extracted by solid phase extraction. Based on yield controls, the yields for studied compounds were on average 83% for BPF, 82% for BPA, 120% for 4-t-octylphenol, and 58% for 4-n-nonylphenol. Yield-corrected detection limits (LOD) for concentration in water samples were 0.43 ng/L for BPF and BPA, 0.04 ng/L for 4-t-octylphenol, and

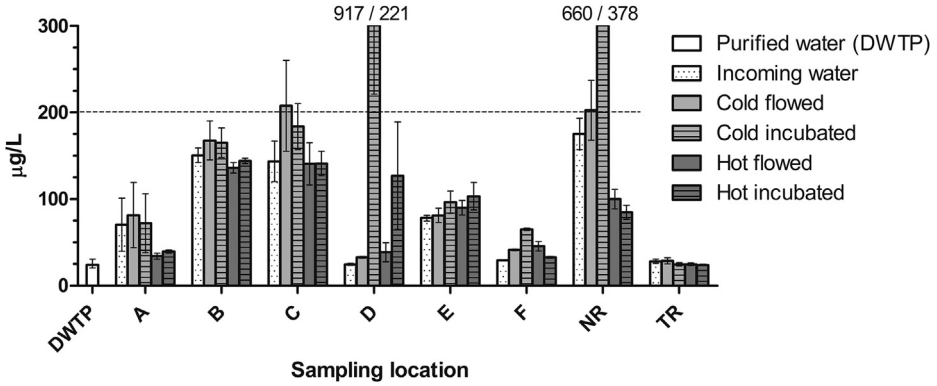

Fig. 1. Iron concentrations in tap water samples. Average with range of the two sampling time points are shown. The two actually measured concentrations in two repeated samplings are also shown for bars exceeding the scale of Y-axis. DWTP = drinking water treatment plant, NR = non-renovated location, TR = traditional renovated location (pipe replacement). Horizontal dashed line: recommended maximal limit for iron (200 μg/L).

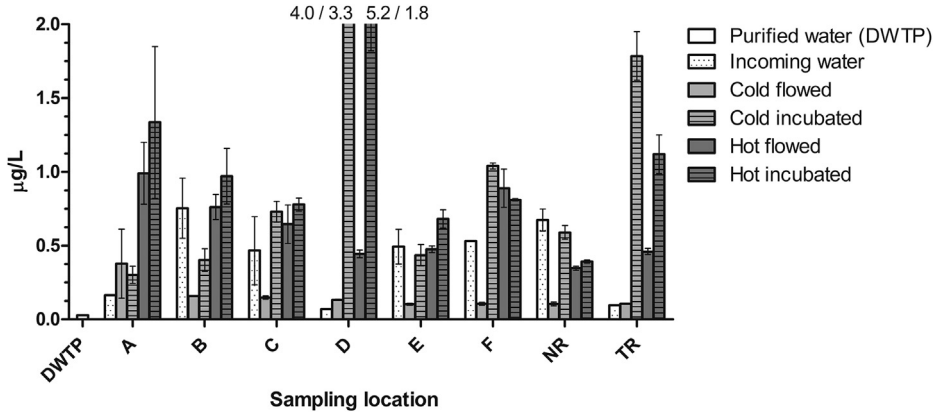

Fig. 2. Lead concentrations in tap water samples. Average with range of the two sampling time points are shown. The two actually measured concentrations in two repeated samplings are also shown for bars exceeding the scale of Y-axis. DWTP = drinking water treatment plant, NR = non-renovated location, TR = traditional renovated location (pipe replacement).

0.16 ng/L for 4-n-nonylphenol.

BPF was detected only in the long-incubated water samples at location D, and the concentrations were generally low, 8–24 ng/L (Table S3, supplementary material) indicating that the resin may contain residues of BPF. Interestingly, in another study with epoxy lined pipes BPF concentrations were clearly higher, 25–1050 ng/L even without long incubation time (Bruchet et al., 2014). Occurrence of BPF in water thus probably depends on the resin formulation.

Minimal occurrence and low concentrations were observed for 4-n-nonylphenol (two samples 1.6 ng/L and 13 ng/L) while concentrations of iso-NP mixture exceeded those of blanks (max 32.5 ng/L) on only two occasions in hot incubated water in location E (69 ng/L) and in hot flowed water in location F (66 ng/L) (Table S3). 4-t-octylphenol was detected in low concentrations in 8 samples of the epoxy lined locations (0.7–22 ng/L), in all three DWTP samples (0.5–1.2 ng/L) and in relatively high frequency at locations NR (non-renovated) and TR (traditional renovation), in 14 samples of total 20 samples (0.9–33.5 ng/L, see Table S3). Concentrations of 4-t-octylphenol were highest in location TR in the incubated samples, probably due to new plastic pipe or coupling materials.

BPA and BPF were not detected in DWTP samples nor at location NR (Table S3). At location TR BPF and BPA was detected only once in incubated cold water sample at low concentrations of 4 and 5 ng/L, respectively.

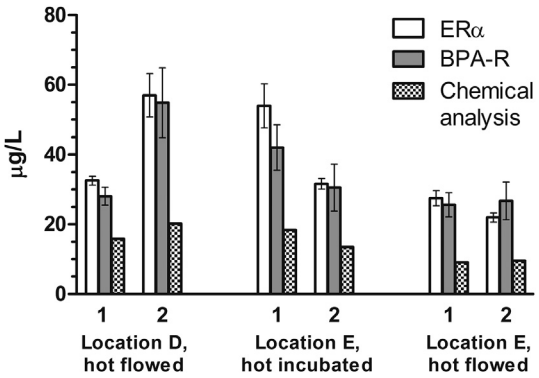

Fig. 3. Bisphenol A equivalent concentrations in the hot water samples from locations D and E measured by yeast ERα and BPA-R bioreporters compared to BPA concentrations measured by chemical analysis. The bioassay results are given as average ± standard deviation of two independent analyses, each with 3 parallels.

BPA was detected in majority of the samples from locations with epoxy resin lining. The frequency of detection was lowest in the incoming water samples (Fig. 3 and Table 2), of which BPA was detected only in locations B, C, and D. Probably in these locations there was a stretch of epoxy lined pipe upstream of the sampled tap. It is also possible that the sampled taps (sauna at the basement, laundry room in ground floor, and water/heat supply room for B, C, and D, respectively) were less frequently used, and remainders of contaminants were still detectable even after 2 min of water flowing.

In cold flowed water BPA concentrations were low, maxima 10–11 ng/L at locations A-C and F, while locations D and E maxima were 148–252 ng/L (Table 2). Flowing water for 2 min before its use is not, however, a common practice for residents. To simulate water standing in the pipes e.g. overnight or during working day, the residents were asked not to use water in the apartment for 8–10 h before sampling. Incubation did increase the concentration of BPA in all locations 1.1–43-fold, up to maximum of 505 ng/L at location E (Table 2), indicating that incubation increases the BPA-burden of water.

Similar BPA concentrations in cold water have been observed in other studies. In laboratory scale experiments pipes lined with epoxy resin were shown to leach BPA in concentrations of few nanograms to 250 ng/L (Bruchet et al., 2014; Kosaka et al., 2012). Examination of houses with epoxy lined pipes in France showed BPA levels of 18–421 ng/L (Bruchet et al., 2014). According to German Umweltbundesamt (Umweltbundesamt, 2010), BPA concentrations in studied renovated locations in Germany were below 1 µg/L.

In this study hot water samples were also studied and clearly higher BPA concentrations were compared to cold water were observed with maximum of about 23 µg/L in locations D and E (Fig. 3, Table 2.). Hot temperature has been shown previously to increase BPA leaching from epoxy resin in laboratory experiments (Bae et al., 2002). Umweltbundesamt (Umweltbundesamt, 2010) has also reported that hot water samples from houses with epoxy lined pipes had BPA concentrations above 30 µg/L of BPA, and even up to 280 µg/L in one location with poorly assembled lining. In the present study incubation of water increased BPA concentration in hot water lines by maximum 2-fold in location E, whereas in other location it had no or low effect (Fig. 3 and Table 2). It is possible that in warm temperature BPA reacts with other compounds or may undergo degradation. In location E the pipes were not insulated and hot pipes are probably cooled down faster.

Table 2
Bisphenol A concentrations in sampling locations A-F, NR (non-renovated), TR (traditional renovation), and DWTP (drinking water treatment plant). Concentrations of both sampling time points are shown.

Sampling location	Bisphenol A concentration in water (ng/L)									
	Incoming		Cold flowed		Cold incubated		Hot incubated		Hot flowed	
A	<LOQ		10	<LOQ	410	<LOQ[a]	593	422[a]	713	349
B	52	<LOQ	11	<LOQ	40	20	1264	2183	2536	1567
C	200	82	10	<LOQ	51	<LOQ	660	347	640	383
D	10	9	148	175	416	290	2134	397	18 460	23 504
E	<LOQ		252	236	505	269	23 174	17 058	11 512	12 102
F	nd	<LOQ	<LOQ		34	29	716	613	517	424
NR	<LOQ									
TR	<LOQ		<LOQ		<LOQ	5	<LOQ		< LOQ	
DWTP	<LOQ									

nd: not determined. <LOQ: below the limit of quantification.
[a] Sample was not incubated as requested.

In addition to temperature, pipe lining technology had a great influence on BPA concentration in water. The highest BPA concentrations were observed in locations B, D, and E rehabilitated using the LSE technology, while locations A, C, and F (DonPro technology) had low concentrations (Fig. 3, Table 2). This was especially clear in hot water samples in which DonPro-renovated locations had at maximum 0.72 μg/L of BPA, while LSE locations had 1.57–23.5 μg/L. In cold water, LSE-locations D and E had 15–25 fold higher BPA concentrations than locations with DonPro lining. Although LSE lining in location B was of similar age with DonPro lining locations A, C, and F, BPA concentrations were clearly higher in hot water (Tables 1 and 2, Fig. 3).

Ageing of LSE lining was also reflected in water BPA-burden. Compared to older LSE locations D and E, location B still had about 4–20-fold lower concentrations of BPA in water (Fig. 3, Table 2), and it may be expected that BPA concentrations in this location will increase during the following years. Increasing concentrations of BPA in water can thus be a sign of accelerating degradation of the resin, and BPA concentration in water should be monitored regularly also in hot water in which degradation of the resin is potentially faster.

Attachment of the resin to the inner surface of water pipe is an important factor for the expected life time for the lining. For example, in location D the resin was visibly crumbling from the pipes and blocking filters in taps after only about 8 years (personal communication with local service). Without structural inspection of pipe samples it is difficult to estimate life time of the lining work. Such inspection was done by Swiss authority EMPA (EMPA, 2004) by studying a sample of a 16-old LSE epoxy lined pipe. The pipe was reported to have slightly impaired attachment of the lining but it still fulfilled its purpose as corrosion inhibition. EMPA estimated that the lining would fulfill its purpose for another 5–10 years, resulting in total life time of 21–26 years.

The results of this study indicate that LSE technology is less favorable spray-on-lining technology than DonPro in respect of BPA leaching. This is possibly due to better attachment of the resin to the pipes (following more efficient pipe cleaning used in DonPro) or different resin composition. It should be pointed, however, that all studied DonPro locations were of about same age, and no conclusions about changes during time in these locations can be done.

3.4. Yeast bioreporter assays

In addition to analyses of individual chemicals, integrative effect-based monitoring approaches are currently highlighted as a viable water quality assessment tool for detection of biological effects of unidentified compounds and mixtures (Escher et al., 2014; Wernersson et al., 2015), of which estrogenicity is of main concern (Jarosova et al., 2014).

As yeast LODs for BPA were relatively high, 601 ± 122 μg/L for ERα bioreporter and 145 ± 38 μg/L for BPA-R bioreporter (average ± standard deviation, given as total concentration in mixture with yeast cells), only those water sample extracts with highest BPA concentrations induced the yeast bioreporters. These were the flowed hot water samples from location D, and both flowed and incubated hot water samples from location E, while none of the other samples from other locations elicited significant potency above bioassay LOD. The bioassay-based BPAeq concentrations in positive samples were similar with both bioreporters in all samples, indicating that bisphenol-like compounds were the main cause of estrogenic activity in the studied samples (Fig. 3).

The yeast bioreporter results were in line with chemical analysis results: BPAeq concentrations were highest in hot flowed sample no. 2 from location D and hot incubated sample no. 1 from location E, which also had the highest measured BPA concentrations in LC-MS analysis (Fig. 3). However, all bioassay-derived BPAeq concentrations were about 2–3-fold higher than concentrations measured by HPLC, and it is thus possible that samples could contain other bisphenol-like and estrogenic compounds that have potency in the yeast assay were not instrumentally analyzed.

For example, chlorinated bisphenols can be formed in pipes when chlorine used for disinfection reacts with epoxy resin components. It has been shown that when chlorinated water was exposed to epoxy-coated pipes, chlorinated bisphenol A derivatives and degradation product 2,4,6-trichlorophenol (a probable human carcinogen) could be detected at concentrations 8–96 ng/L and 50–16 000 ng/L, respectively (Bruchet et al., 2014; Kosaka et al., 2012). It was also noted in both studies that the epoxy resin is able to accumulate the compounds on its surface. Interestingly, chlorinated bisphenol A derivatives have been shown to have greater estrogenic potential than non-chlorinated BPA (Hu et al., 2002), and thus presence of chlorinated bisphenol A derivatives could be responsible for higher detected estrogenicity than would be expected by BPA alone.

3.5. Exposure and risk assessment

Analyses of VOCs, metals as well as majority fo alkylphenols in the present study indicate negligible health risks of the investigated epoxy linings. Only rare occurrences and very low concentrations of VOCs (no epichlorhydrin) as well as alkylphenols (BPF, 4-n-NP and 4-t-OP) were observed. Metal concentrations were - with few exceptions - generally below the limits set by the European Drinking Water Regulations 2014 (European Union, 2014) being 2 mg/L for copper, 200 μg/L for iron, and 10 μg/L for lead.

With regard to BPA, there is no generally accepted regulatory

limit in drinking water. A few countries have set their limit values. For example, in France a reference value of 100 ng/L for BPA is recommended (Bruchet et al., 2014), whereas in Germany the limit value is 30 μg/L (Umweltbundesamt, 2010) and in Japan 100 μg/L (Kosaka et al., 2012). In addition, the US ANSI/NSF-61 standard suggests total allowable concentration of 200 μg/L of BPA permitted in drinking water. Compared to these limits, maximum detected BPA concentrations in cold flowed drinking water samples in the present study were well below the French limit of 100 ng/L - except for locations D and E where up to 252 ng/L were detected.

BPA exposure of residents was assessed according to the estimated 2 L/day consumption of drinking water for adults by U. S. EPA (U.S. EPA, 2000). Assuming a 70 kg person uses 2 L of water per day for drinking and cooking, then BPA exposure with the above-mentioned national limits would be 0.003 μg/kg, 0.86 μg/kg, and 2.86 μg/kg per day for France, Germany, and Japan, respectively. None exceeds the current tolerable daily intake of 4 μg/kg for BPA set by the European Food Safety Authority (EFSA, 2015).

However, EFSA has estimated that average daily dietary BPA intake is 0.132 μg/kg for adult women, 0.126 μg/kg for men, and 0.375 μg/kg for children. Using water with BPA concentration at the German limit would cause over 6-fold increase in average adult BPA intake, while with the French limit the increase would be about 2%. Thus, the French limit should be more applicable for regulatory purposes, especially if target is to ensure only minor increase in overall BPA exposure.

Intake rates of BPA for adults via cold water in the studied locations with epoxy lining was neligible: 0.27–7.2 ng/kg/day for adults and 0.58–15.3 ng/kg/day for small children, estimated with the average intake rates by the US EPA (Table 3). These correspond to a 0.2–5.7% increase in the average daily BPA intake via food (EFSA, 2015).

Hot water, which is not generally considered to be drinking water, contained from 45 to over 200-fold more BPA than cold water in the studied locations. For this reason unintentional exposure via hot water can potentially cause increase in daily intake of BPA. Direct consumption of hot water could happen due to improper flowing in water, as well as accidental ingestion or water aerosol aspiration in shower or bath. Unintended ingestion of water during swimming has been evaluated by the U. S. EPA (U.S. EPA, 2011). According to the estimated ingestion rate during swimming (21 mL/h for adults and 49 mL/h for children) and average showering and bathing time of 17.1 min/day, BPA exposure was found low, 0.04–2 ng/kg/day for adults, and 0.66–21.9 ng/kg/day for children (Table 3).

Summarized alltogether, the highest BPA exposures were 8.2 ng/kg/day and 32.5 ng/kg/day for adults and children, respectively, corresponding to 6.5% and 8.7% increase in average intake of BPA.

Table 3
Calculated BPA exposure levels via cold drinking water and ingestion via hot water in shower or bath for adults (>18 years, 70 kg) and children (toddlers 2–3 years, 15 kg). Highest measured concentration in each location was used.

Location	Adult (ng/kg/day)			Child (ng/kg/day)		
	Cold water[a]	Hot water[b]	Sum	Cold water[a]	Hot water[b]	Sum
A	0.27	0.06	0.33	0.58	0.66	1.24
B	0.32	0.22	0.53	0.67	2.36	3.03
C	0.28	0.05	0.33	0.59	0.60	1.19
D	4.99	2.01	7.00	10.62	21.88	32.50
E	7.20	1.03	8.23	15.31	11.27	26.58
F	–	0.04	0.04	–	0.48	0.48

[a] Daily consumption for adults 2 L and for child (2–3 years) 0.912 L (U. S. EPA, 2000 and 2011).
[b] Intake rate 21 mL/h for adults and 49 mL/h for children, time 17.1 min/event (U.S. EPA, 2011).

Another exposure route can be dermal absorption in shower and bathing, however, currently there are no recommended methods for dermal exposure via this route.

4. Conclusions

BPA concentrations were higher in locations with epoxy lining done using LSE lining technology than those using DonPro technology. Higher leaching of BPA can be due to less efficient cleaning of pipes and consequently less tight attachment of the resin to the pipes. Also differences in resin compositions can effect leaching.

Locations D and E with 8–9 years old LSE coating leached more BPA compared to 2 years old location B. Thus ageing of the resin was seen as increased BPA leaching to water. No conclusions about ageing effect concerning locations with DonPro technology could be done due to the same age of these locations.

In all samples hot water contained more BPA than cold water. Life expectancy of epoxy lining can be shorter in hot water lines than in cold water lines, and should be taken into account when estimating working life for the lining work. More studies concerning ageing should be done, especially with hot water lines.

Intake of BPA via water in the studied locations is low and correspond maximum of 8.7% increase in average BPA intake via other sources.

Formation of chlorinated bisphenols is possible in epoxy coated water pipes with chlorinated water. This should be further studied and taken into account in risk assessment due to higher biological potency of chlorinated BPAs.

Drinking water pipe lining can be a reasonable option for pipe renovation if.

- an efficient pipe cleaning and thus good resin attachment can be ensured,
- harmful amounts of metals, especially iron and lead, occur in drinking water
- a non-invasive renovation is strongly preferred due to, for example, recent renovation of other surfaces,
- it is acceptable that rehabilitation by lining might not have as long life time as conventional rehabilitation by pipe replacement.

Author contributions

J.R. and L.B. designed research; J.R., M.P, J.K., J.L., and Z.S. performed research and analyzed data; J.R. wrote the paper; J.R., Z.S. and L.B. contributed to the financial support of the research. All the authors contributed to the interpretation of the data and paper finalization.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.07.027.

References

Bae, B., Jeong, J.H., Lee, S.J., 2002. The quantification and characterization of endocrine disruptor bisphenol-A leaching from epoxy resin. Water Sci. Technol. 46, 381–387.

Baugros, J.B., Giroud, B., Dessalces, G., Grenier-Loustalot, M.F., Cren-Olivé, C., 2008. Multiresidue analytical methods for the ultra-trace quantification of 33 priority substances present in the list of REACH in real water samples. Anal. Chim. Acta 607, 191–203. http://dx.doi.org/10.1016/j.aca.2007.11.036.

Bruchet, A., Elyasmino, N., Decottignies, V., Noyon, N., 2014. Leaching of bisphenol A and F from new and old epoxy coatings: laboratory and field studies. Water Sci. Technol. Water Supply 14, 383–389. http://dx.doi.org/10.2166/ws.2013.209.

Eidgenössische Materialprüfungs- und Forschungsanstalt (EMPA), 2004. Beurteilung der LSE-SYSTEM-Rohr-Innenwand- Beschichtung einer Kaltwasserleitung, Prüfbericht Nr. 432984/1.

Ellison, D., Sever, F., Oram, P., Lovins, W., Romer, A., Duranceau, S.J., Bell, G., 2010. Global Review of Spray-On Structural Lining Technologies, ISBN 9781605730943. Water Research Foundation and U.S. EPA.

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J.Y.M., Burg, B., Van De Linden, S.C., Van Der Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X., Leusch, F.D.L., 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. Environ. Sci. Technol. 48, 1940–1956.

European Food Safety Authority, 2015. Draft Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: part I - exposure assessment. EFSA J. 13 http://dx.doi.org/10.2903/j.efsa.2015.3978.

European Union, 2014. European Union Drinking Water Regulations. S.I. No. 122 of 2014.

Flint, S., Markle, T., Thompson, S., Wallace, E., 2012. Bisphenol A exposure, effects, and policy: a wildlife perspective. J. Environ. Manage 104, 19–34. http://dx.doi.org/10.1016/j.jenvman.2012.03.021.

Gonzalez, S., Lopez-Roldan, R., Cortina, J.-L., 2013. Presence of metals in drinking water distribution networks due to pipe material leaching: a review. Toxicol. Environ. Chem. 95, 870–889. http://dx.doi.org/10.1080/02772248.2013.840372.

Hu, J.-Y., Xie, G.-H., Aizawa, T., 2002. Products of aqueous chlorination of 4-nonylphenol and their estrogenic activity. Environ. Toxicol. Chem. 21, 2034–2039.

Jarosova, B., Ersekova, A., Hilscherova, K., Loos, R., Gawlik, B.M., Giesy, J.P., Blaha, L., 2014. Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. Environ. Sci. Pollut. Res. 21, 10970–10982. http://dx.doi.org/10.1007/s11356-014-3056-8.

Kekki, T.K., Keinänen-Toivola, M.M., Kaunisto, T., Luntamo, M., 2007. Talousveden Kanssa Kosketuksissa Olevat Verkostomateriaalit Suomessa, ISBN 978-952-99840-1-5. WANDER Nordic Water and Materials Institute.

Kosaka, K., Hayashida, T., Terasaki, M., Asami, M., Yamada, T., Itoh, M., Akiba, M., 2012. Elution of bisphenol A and its chlorination by-products from lined pipes in water supply process. Water Sci. Technol. Water Supply 12, 791–798. http://dx.doi.org/10.2166/ws.2012.055.

Leskinen, P., Michelini, E., Picard, D., Karp, M., Virta, M., 2005. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61, 259–266. http://dx.doi.org/10.1016/j.chemosphere.2005.01.080.

Markelin-Rantala, L., Rautiainen, L., 2007. Asuinrakennusten Viemäri- Ja Käyttövesiputkistojen Pinnoitusmenetelmät - Esiselvitys, Raportti Nro VTT-s-05086–08.

Meijer, F., Itard, L., Sunikka-Blank, M., 2009. Comparing European residential building stocks: performance, renovation and policy opportunities. Build. Res. Inf. 37, 533–551. http://dx.doi.org/10.1080/09613210903189376.

Pekkinen, J., 2011. Putkistojen Pinnoitus Vaihtoehtona Perinteiselle Saneeraukselle. Metropolia Ammattikorkeakoulu. Building Services Engineering. (thesis).

Pelto-Huikko, A., Kaunisto, T., 2011. Vesijohtojen Saneerauspinnoitus, ISBN 978-952-67166-7-1. WANDER Nordic Water and Materials Institute.

Pernica, M., Poloucka, P., Seifertova, M., Simek, Z., 2015. Determination of alkylphenols in water samples using liquid chromatography-tandem mass spectrometry after pre-column derivatization with dansyl chloride. J. Chromatogr. A 1417, 49–56. http://dx.doi.org/10.1016/j.chroma.2015.09.030.

Rajasärkkä, J., Hakkila, K., Virta, M., 2011. Developing a compound-specific receptor for bisphenol A by directed evolution of human estrogen receptor α. Biotechnol. Bioeng. 108, 2526–2534. http://dx.doi.org/10.1002/bit.23214.

Rajasärkkä, J., Koponen, J., Airaksinen, R., Kiviranta, H., Virta, M., 2014. Monitoring bisphenol A and estrogenic chemicals in thermal paper with yeast-based bioreporter assay. Anal. Bioanal. Chem. 406, 5695–5702. http://dx.doi.org/10.1007/s00216-014-7812-x.

Rajasärkkä, J., Virta, M., 2013. Characterization of a bisphenol a specific yeast bioreporter utilizing the bisphenol a-targeted receptor. Anal. Chem. 85, 10067–10074. http://dx.doi.org/10.1021/ac401614c.

Rubin, B.S., 2011. Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. J. Steroid Biochem. Mol. Biol. 127, 27–34. http://dx.doi.org/10.1016/j.jsbmb.2011.05.002.

U. S. Environmental Protection Agency (U.S. EPA), 2000. Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health. EPA/822/B-00/004.

U.S. Environmental Protection Agencey (EPA), 2011. Chapter 3-Ingestion of Water and Other Select Liquids, in: Exposure Factors Handbook. EPA/600/R-09/052F.

Umweltbundesamt, 2010. Bisphenol a, an Industrial Chemical with Adverse Effects. http://dx.doi.org/10.4126/38M-005166122.

Wernersson, A.-S., Carere, M., Maggi, C., Tusil, P., Soldan, P., James, A., Sanchez, W., Dulio, V., Broeg, K., Reifferscheid, G., Buchinger, S., Maas, H., Van Der Grinten, E., O'Toole, S., Ausili, A., Manfra, L., Marziali, L., Polesello, S., Lacchetti, I., Mancini, L., Lilja, K., Linderoth, M., Lundeberg, T., Fjällborg, B., Porsbring, T., Larsson, D.J., Bengtsson-Palme, J., Förlin, L., Kienle, C., Kunz, P., Vermeirssen, E., Werner, I., Robinson, C.D., Lyons, B., Katsiadaki, I., Whalley, C., den Haan, K., Messiaen, M., Clayton, H., Lettieri, T., Carvalho, R.N., Gawlik, B.M., Hollert, H., Di Paolo, C., Brack, W., Kammann, U., Kase, R., 2015. The European technical report on aquatic effect-based monitoring tools under the water framework directive. Environ. Sci. Eur. 27, 7. http://dx.doi.org/10.1186/s12302-015-0039-4.

Whelton, A.J., Nguyen, T., 2013. Contaminant migration from polymeric pipes used in buried potable water distribution systems: a review. Crit. Rev. Environ. Sci. Technol. 43, 679–751. http://dx.doi.org/10.1080/10643389.2011.627005.

WHO (World Health Organization), 2011. Guidelines for Drinking-water Quality, fourth ed. ISBN 978 92 4 154815 1.

Article IV

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Estrogenic activity and contributing compounds in stagnant water bodies with massive occurrence of phytoplankton

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ABSTRACT

Stagnant water bodies have generally received little attention regarding the presence of endocrine disruptive compounds, although they can integrate diverse pollutants from multiple different sources. Many compounds of anthropogenic as well as natural origin can contribute to the overall estrogenicity of surface waters and some of them can exhibit adverse effects on aquatic biota even in very low concentrations. This study focused on freshwater ponds and reservoirs affected by water blooms and determined the estrogenic activity of water by *in vitro* bioassay as well as concentrations of several important groups of estrogenic compounds (estrogenic hormones, alkylphenols, and phytoestrogens) by LC-MS/MS analyses. Estrogenic hormones were found at concentrations up to 7.1 ng.L^{-1}, similarly to flavonoids, whose concentrations did not exceed 12.5 ng.L^{-1}. Among alkylphenols, only bisphenol A and 4-*tert*-octylphenol were detected in levels reaching 100 ng.L^{-1} at maximum. Estrogenic activity of water samples varied from below the quantification limit to 1.95 ng.L^{-1}. There does not seem to be any general causal link of the massive phytoplankton occurrence with the estrogenicity of water or concentration of phytoestrogens, since they showed no direct relationship with the phytoplankton abundance or composition across sites. The contribution of the analysed compounds to the estrogenic activity was calculated in three scenarios. In minimum scenario, just the compounds above quantification limit (LOQ) were taken into account and for most samples, only minor part (<6%) of the biological activity could be explained. In the mean and maximum scenarios, we included also compounds below LOQ into the calculations at the level of LOQ/2 and LOQ, respectively. In these cases, a considerable part of the estrogenic activity could be attributed to the possible presence of steroid estrogens below LOQ. However, for the samples with estrogenic activity greater than 1 ng.L^{-1}, more than 50% of the estrogenic activity remained unexplained even in the maximum scenario. Probably other compounds or possible interactions between individual substances cause the estrogenic activity in these types of water bodies and in this case, the results of LC-MS/MS analyses cannot sufficiently predict the biological effects. A complex approach including bioassays is needed when assessing the estrogenicity of these types of surface waters.

1. Introduction

Endocrine disruptive compounds that can cause a disturbance in hormone signalling and adverse effects in biota even at very low concentrations received much attention in recent years. In surface waters, namely potential presence of estrogenic compounds has been investigated as it has been associated with the occurrence of intersex in fish in numerous studies (reviewed e.g. in Söffker and Tyler, 2012).

Main attention was paid to rivers and wastewater treatment plant effluents as important sources of estrogens to the environment. Steroid estrogenic hormones were found to be the main contributors to the estrogenicity in these waters (Sumpter and Jobling, 2013). Therefore, three estrogens (estrone, 17β-estradiol and 17α-ethynylestradiol) have been included in the European Water Framework Directive (WFD) watch list (COM, 2011-876). For

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17β-estradiol and 17α-ethynylestradiol, the annual average environmental quality standards were proposed to be 400 pg.L^{-1} and 35 pg.L^{-1}, respectively.

With chemical analytical techniques, it is often very difficult to establish methods with detection limits low enough to enable reliable monitoring in these concentration levels in complex environmental samples. On the other hand, biodetection systems have the advantage of detecting these extremely low concentrations of estrogens. They also integrate the effects of all compounds in the sample and possible interactions in the mixture (Charles et al., 2002; Frische et al., 2009). This is especially important as many more compounds, than just estrogenic hormones, were found to interfere with estrogen signalling. Industrial chemicals (like alkylphenols, DDT, PCBs, parabens or phthalates) and phytochemicals (flavonoids, lignans) can also induce the estrogenic response (Giesy et al., 2002). Some compounds bind to the estrogen receptor but do not activate it and so they exert antiestrogenic effects. These include for example polycyclic musks, organic ultraviolet absorbents or textile dyes (Ihara et al., 2014). All these compounds can occur together in a complex mixture and contribute to the overall activity. However, they are rarely measured simultaneously and for flavonoids, data on a broader spectrum of compounds are missing.

Endocrine disruptive potential has been recently associated also with phytoplankton biomass and water blooms, which could possibly contribute to elevated levels of phytoestrogens. Gong et al. (2014) observed a correlation between the estrogenicity of sea water and phytoplankton concentration and they also reported the estrogenic activity of two isolated phytoplankton species. Stepankova et al. (2011) described the estrogenic activity of environmental cyanobacterial water bloom extracts in the *in vitro* bioassay. The induction of vitellogenin, a protein whose synthesis is under the control of estrogen receptor, was also described after the exposure to cyanobacterial biomass in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) (Marie et al., 2012; Rogers et al., 2011). These studies did not identify the responsible compounds. However, cyanobacteria and algae were reported to contain estrogenic flavonoids (Goiris et al., 2014; Klejdus et al., 2010).

In stagnant waters, massive development of phytoplankton can occur under suitable conditions, potentially influencing the estrogenic activity of these waters. However, studies focusing on estrogenicity of stagnant waters and contributing compounds, including flavonoids, are missing. Therefore, we aimed to investigate the estrogenic activity of freshwater ponds and lakes affected by massive occurrence of phytoplankton and estimate the contribution of various estrogenic compounds, including flavonoids, which could be potentially connected to the phytoplankton occurrence. We have also examined the hypothesis that higher phytoplankton abundance can be associated with greater estrogenic activity and higher levels of flavonoids. The specific goals of this study were to a) evaluate the concentrations of a broad spectrum of estrogenic compounds from different structural groups and of different origin in the freshwater reservoirs and ponds affected by water blooms of cyanobacteria or massive development of other phytoplankton groups, b) determine the estrogenic activity of water samples from these localities in the *in vitro* bioassay and c) calculate the potential contribution of individual compounds to the overall biological activity.

2. Materials and methods

2.1. Sampling

Water samples from ponds and reservoirs with water blooms were collected from 19 localities across the Czech Republic and Hungary during summer 2013 and 2014 (Table 1). Coordinates of sampling points and sampling dates are presented in Supplementary material S1. Samples were collected in 2.5L amber-glass bottles, transported on ice to the laboratory, and stored before extraction in dark at 4 °C for up to 24 h. Species determination and cell counting were done using microscope Olympus BX 51 and Bürker chamber. Cell counts were converted to biovolumes using databases of cell dimensions of individual species (Skácelová and Lepš, 2014) on the basis of the commonly used method described by Hillebrand et al. (1999). The composition of phytoplankton is expressed as a share of biovolume in particular sample, concentration of phytoplankton in cell numbers per millilitre.

2.2. Sample preparation

From each locality, 4 L of water were centrifuged (3910 g, room temperature, centrifuge HanilCombi 514R, HanilBioMed Inc., Gwangju, Korea) and vacuum filtered through 0.6 μm paper filters (Macherey-Nagel, Dueren, Germany). Water samples were then subjected to solid phase extraction (SPE) using Oasis HLB sorbent (1g, Waters, Milford, USA) in two setups as previously described in Procházková et al. (2017). The first setup (V1) is commonly used for the assessment of estrogenicity (e.g. Alvarez et al., 2013; Jugan et al., 2009) and the second setup (V2) was designed to increase the extraction efficiency of less polar compounds. Briefly, sorbent was conditioned with 15 mL of methanol and 15 mL of distilled water and eluted with 15 mL of methanol in the V1 setup. Setup V2 consisted of sorbent conditioning with 6 mL of ethylacetate, 6 mL of methanol and 12 mL of 20% 2-propanol in water. 2-propanol was also added to water samples before the extraction to achieve its 20% content. Elution was performed with 15 mL of ethylacetate. The eluates were evaporated to near dryness under a gentle stream of nitrogen (LabEva Visible, Labicom, Czech Republic) and reconstituted in methanol to obtain extracts with a concentration factor of 4000x compared to the original water sample. In the end, we obtained two individual samples from each locality. Blank samples from the deionized water were also prepared for each SPE setup to be analysed together with the environmental samples.

2.3. Chemical analyses

Concentrations of selected flavonoids, alkylphenols and estrogenic hormones were assessed in all samples by LC-MS/MS methods. Limits of quantification (LOQs) were derived as a concentration with a signal to noise ratio 10:1.

2.3.1. Analysis of flavonoids

Eight flavonoids (biochanin A, coumestrol, daidzein, equol, formononetin, genistein, naringenin and apigenin) were analysed using the LC-MS/MS method described in Procházková et al. (2017).

2.3.2. Analysis of estrogenic hormones

Concentrations of four compounds were determined: natural estrogens estrone (E1), 17β-estradiol (E2), and estriol (E3) and synthetic hormone 17α-ethynylestradiol (EE2). 17α-ethynylestradiol-2, 4, 16, 16-d4, 17β-estradiol-2, 4, 16, 16-d4 and 16α-hydroxy-17β-estradiol-2, 4-d2 were used as instrumental internal standards.

The SPE water extracts were evaporated to dryness under a nitrogen atmosphere. Twenty microliters of acetone were added and mixed. After 1 min, 50 μL of 0.1 mM $NaHCO_3$ were added (the pH of $NaHCO_3$ solution was adjusted to 10.5 with NaOH solution). The mixtures were vortexed and then allowed to stand for 1 min. Fifty microliters of dansyl chloride solution (1 mg mL^{-1} in acetone) were added, mixed and incubated for 3 min at 60 °C. Samples were then evaporated to dryness and redissolved in 1 mL of a methanol:water (4:6) mixture.

Table 1
Characteristics of the sampling locations – area of the waterbody, species composition of phytoplankton (the percentage expresses a share of biovolume in particular sample) and the cell density of the phytoplankton.

	locality	area (km^2)	phytoplankton composition[a]			cell density (10^6 cells.mL^{-1})
			species	group		
1	Šutrák gravel pit	0.01	*Anabaenopsis elenkinii*	CYA[b]	91.0%	12.58
2	Kamenický Šutrák gravel pit	0.01	*Plantothrix agardhii*	CYA	80.0%	5.56
3	Dolní Lužický lake	0.13	*Coelastrum microporum*	CHL[c]	24.0%	38.87
			Planctonema lauterbornii	CHL	12.0%	
			Planktothrix agardhii	CYA	11.0%	
			Oocystis marssonii	CHL	11.0%	
4	Vranov reservoir	7.63	*Microcystis aeruginosa*	CYA	37.0%	90.85
			Microcystis viridis	CYA	28.0%	
			Coelastrum microporum	CHL	25.0%	
			Woronichinia naegeliana	CYA	11.0%	
5	Landštejn reservoir	0.38	*Dolichospermum macrosporum*	CYA	88.0%	0.04
6	Öreg-tó lake	2	*Microcystis wesenbergii*	CYA	49.0%	0.28
			Microcystis aeruginosa	CYA	20.0%	
7	Várpalota lake	0.08	*Komvophoron constrictum*	CYA	37.0%	0.52
			Planktolyngbya spp. + *Limnothrix redekei*	CYA	45.0%	
8	Szelidi-tó lake	0.46	*Cylindrospermopsis raciborskii*	CYA	47.0%	0.68
			Tetraedron minimum + *Oocystis* sp.	CHL	22.0%	
9	Staňkov lake	2.41	*Dolichospermum mucosum*	CYA	27.0%	0.20
			Woronichinia naegeliana	CYA	22.7%	
			Microcystis wesenbergii	CYA	15.7%	
			Dolichospermum curvum	CYA	10.6%	
10	Jamský lake	0.43	*Pediastrum boryanum*	CHL	28.9%	0.67
			Phacus longicauda + *P. pleuronectes*	EUG[d]	18.9%	
11	Opatovický lake	1.66	*Dolichospermum viguieri*	CYA	42.0%	1.28
			Cuspidothrix issatchenkoi	CYA	16.0%	
			Woloszynskia sp.	DIN[e]	10.0%	
12	Záhřebský pond	0.05	*Dolichospermum crassum*	CYA	60.9%	1.93
13	Libořezy village water tank	0.001	*Microcystis aeruginosa*	CYA	65.7%	0.79
14	Příbraz pond	0.003	*Desmodesmus communis*	CHL	93.7%	0.61
15	Žíteč village water tank	0.002	*Desmodesmus communis*	CHL	29.6%	0.50
			Centrales indet.	DIA[f]	23.4%	
			Desmodesmus opoliensis	CHL	10.5%	
16	Hejtman lake	0.8	*Dolichospermum planctonicum*	CYA	46.3%	2.33
			Dolichospermum smithii	CYA	22.2%	
17	sand mine near Veselí nad Lužnicí	0.11	*Pediastrum boryanum*	CHL	25.6%	0.03
			Trachelomonas hispida	EUG	20.3%	
			Dinobryon cylindricum var. *palustre*	CHR[g]	11.7%	
18	Velký Horusický lake	4.4	*Pediastrum boryanum*	CHL	25.4%	0.12
			Ceratium hirundinella	DIN	23.7%	
19	Kukla malá pond	0.03	*Desmodesmus subspicatus*	CHL	23.4%	0.12
			Desmodesmus magnus	CHL	21.0%	
			Pediastrum boryanum	CHL	13.9%	

[a] Dominants forming more than 10% of total biomass.
[b] Cyanobacteria.
[c] Chlorophyta.
[d] Euglenozoa.
[e] Dinoflagellate.
[f] Diatoms.
[g] Chrysophytes.

An Agilent 1200 series liquid chromatograph equipped with an ACE3 C18 column (150 mm × 2.1 mm, 3 μm, Advanced Chromatography Technologies Ltd.) coupled to an Agilent 6410 triple-quadrupole mass spectrometer was used for the analysis. The mobile phase consisted of water with 7 mM formic acid (A) and acetonitrile (B). The initial composition of the mobile phase used for gradient elution was 60% B held for 3.5 min, then increased to 92% B in 13.5 min and finally decreased back to 60% B in 1 min. The mobile phase flow rate was set at 0.3 mL/min. The column temperature was set to 25 °C; 10 μL were used for sample injection.

The ionization of analytes was performed using an ESI source set in positive mode. The drying gas temperature was 320 °C, the drying gas flow was 8 L/min, and the nebulizer pressure was 344.7 kPa; the capillary and fragmentor voltages were 4500 V and 250 V, respectively. Collision energies were in the range of 38–42 V. MRM transitions $[M+H]^+ \rightarrow 171$ and $[M+H]^+ \rightarrow 156$ were used for the quantification and confirmation of each analyte, respectively.

2.3.3. Analysis of phenolic compounds

Concentrations of bisphenol A, bisphenol F, 4-*tert*-octylphenol, 4-octylphenol and 4-n-nonylphenol were measured. Deuterated n-nonylphenol was used as internal standard.

Samples were derivatized with dansyl chloride using the procedure optimized by Pernica et al. (2015). Concentrated SPE extract in a 2 mL amber glass sample vial was evaporated to dryness and redissolved in 200 μL acetonitrile. Fifty microliters of 100 mM $NaHCO_3$ in water were added and the content was mixed (vortex mixer) for 1 min. Then 200 μL of dansyl chloride in acetone (0.5 mg mL^{-1}) were added and mixed again. Subsequently, the mixture was incubated for 60 min at 60 °C. The reaction mixtures were then cooled to room temperature and evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 1 mL of methanol and mixed. The solution was injected into the LC-ESI-MS/MS. Chromatographic separations and MS detection were performed as described in Pernica et al. (2015). Product ions used

for the detection of bisphenol F were 433 and 171.

2.4. Assessment of estrogenic activity

The *in vitro* biotest with stably transfected hERα-HeLa-9903 cell line was used to determine total estrogenicity of the samples as well as relative potencies (RPs) of individual steroid estrogens (E1, E2, E3 and EE2) and alkylphenols (bisphenol A, bisphenol F, 4-*tert*-octylphenol, 4-octylphenol and 4-n-nonylphenol). The procedure described in Procházková et al. (2017) was used. Briefly, the cells were seeded in 96-well microplates and exposed to the dilution series of the tested compounds, environmental samples and E2 (as a reference estrogen) and blank and solvent controls. Exposure was conducted in three replicates for each sample concentration. After the 24 h exposure, the intensity of luminescence was measured. Detected induction of luminescence was related to the maximal response of E2 (E2max) and converted into percentages of E2max, while solvent control and blank provided a baseline. Nonlinear logarithmic regression of concentration-effect curve of steroid estrogens, alkylphenols as well as environmental samples and calibration standard was calculated in GraphPad Prism (GraphPad Software, San Diego, USA) for determination of ECx (Effective Concentration of x% of maximal response). Environmental samples were tested in the range 2.5-20x of concentration factor compared to the original water sample. The estrogenic equivalent (EEQ) was derived as the ratio of EC50 of E2 and EC50 of the sample. For samples, whose response did not reach 50%, the EC20 values were used for the calculations. The dilution series of standard compounds were tested up to 3.3×10^{-7} M for EE2, 5×10^{-7} M for E1 and E3 and up to 5×10^{-5} M for alkylphenols. RPs were then determined as the ratio of molar EC50 of the E2 and EC50 of the compound except for 4-octylphenol and 4-n-nonylphenol, whose response did not reach 50% and therefore the EC20 was used for the calculation of their RPs. RPs of flavonoids were assessed previously (Procházková et al., 2017) and the reported values were used for calculations of their contribution to the estrogenic activity also in this study.

Estrogenic equivalents derived from chemical analyses (cEEQs) were calculated from molar concentrations determined by LC-MS/MS analyses and relative potencies obtained from the *in vitro* assay using following equation: $cEEQ = \sum c_i * RP_i$. Three scenarios were considered to evaluate the potential impact of the quantification limits on the explicability of the effects by the studied compounds. They were designed to show the whole range of estrogenicity the analysed compounds could account for. In the minimum scenario, the compounds that were below LOQ were not included in the calculations of cEEQ. This scenario shows the minimum contribution of the analysed compounds as it considers only positively detected and quantified compounds. The mean scenario includes the compounds below LOQ to the contribution calculations at the theoretical level of LOQ/2 to indicate the average estimate of the estrogenicity that can be explained by the analysed compounds. In the maximum scenario, the concentration of compounds below LOQ was set as LOQ for the calculation of cEEQ. This scenario demonstrates the maximum theoretical contribution of the analysed compounds and the impact of LOQs on the contribution calculations.

3. Results and discussion

Generally, relatively little attention is paid to pollution of stagnant waters, although they can integrate pollutants from different sources (e.g. domestic wastewaters, animal waste from farms and field fertilising, field runoff, decaying plant material or metabolites produced by water bloom forming organisms). In this study, we focused on three groups of estrogenic compounds – flavonoids (a group of phytoestrogens), alkylphenols (anthropogenic contaminants) and estrogenic hormones (including natural E1, E2, E3 and synthetic EE2) – and we assessed their concentrations and potential contribution to the estrogenicity of stagnant waters with massive occurrence of phytoplankton. Sampled localities are listed in Table 1 along with the area of sampled water bodies, cell densities and species composition of phytoplankton. This study covers different types of stagnant water bodies – from small ponds to large reservoirs. Various phytoplankton species dominated at individual sites – from well-known toxic cyanobacteria (like *Microcystis aeruginosa* at locality 13) to green microalgae (like *Desmodesmus communis* at locality 14).

3.1. Occurrence of flavonoids in stagnant waters

Flavonoids were detected at all localities (Fig. 1A, for detailed concentrations, see Supplementary material S2). Different concentrations of compounds in V1 and V2 samples could be anticipated from different recoveries of the two extraction methods reported in Procházková et al. (2017). Detected concentrations of flavonoids were generally in the order of $ng.L^{-1}$ with a maximum of 18 $ng.L^{-1}$ for the sum of all detected compounds at reservoir Vranov (locality 4). These levels are similar to the previously reported content of flavonoids in freshwater reservoirs as well as rivers (Jarošová et al., 2015; Procházková et al., 2017). Of particular interest are the concentrations of equol at Jamský lake and Záhřebský pond (localities 10 and 12), where it exceeded 5.8 $ng.L^{-1}$. These levels were recently shown to induce intersex in medaka fish (*Oryzias latipes*) after exposure from hatching for 100 days (Wang et al., 2016).

3.2. Occurrence of alkylphenols in stagnant waters

Among the studied alkylphenols, 4-octylphenol, 4-n-nonylphenol and bisphenol F were below LOQ (0.07, 0.03 and 0.04 $ng.L^{-1}$ respectively) in all samples. 4-tert-octylphenol was detected at 5 localities, but its concentration reached only 1.15 $ng.L^{-1}$ at maximum. The most abundant was bisphenol A, which was detected at 18 (out of 19) localities (Fig. 1B, for detailed concentrations, see Supplementary material S2). Its concentrations ranged from 3 to 100 $ng.L^{-1}$. Similar concentration range (0.5–410 $ng.L^{-1}$) of bisphenol A was reported also by Fromme et al. (2002) from German surface waters with no difference observed between rivers and lakes. These levels seem to be common in surface waters around the world as reviewed by Careghini et al. (2015). Bisphenol A is mainly used in the production of polymers, which have wide applications e.g. in water pipes, bottles, medical equipment or electronic devices. It is also used in thermal paper production or tin coatings (Michałowicz, 2014). All these products can be a source of bisphenol A as it can leach from the materials. Industrial as well as household wastewaters were reported to contain concentrations of bisphenol A in the order of $\mu g.L^{-1}$ (Fürhacker et al., 2000). Nonylphenol and octylphenol were previously found in surface waters in concentrations similar to bisphenol A (Careghini et al., 2015; Jin et al., 2004). However, these reports may cover all isomers of these compounds. Nevertheless, most data are available for rivers and there is very limited knowledge related to stagnant waters.

3.3. Occurrence of estrogenic hormones in stagnant waters

Steroid estrogens were found at 11 localities (Fig. 1C, for detailed concentrations, see Supplementary material S2). Their concentration range was similar to flavonoids with the highest amount of

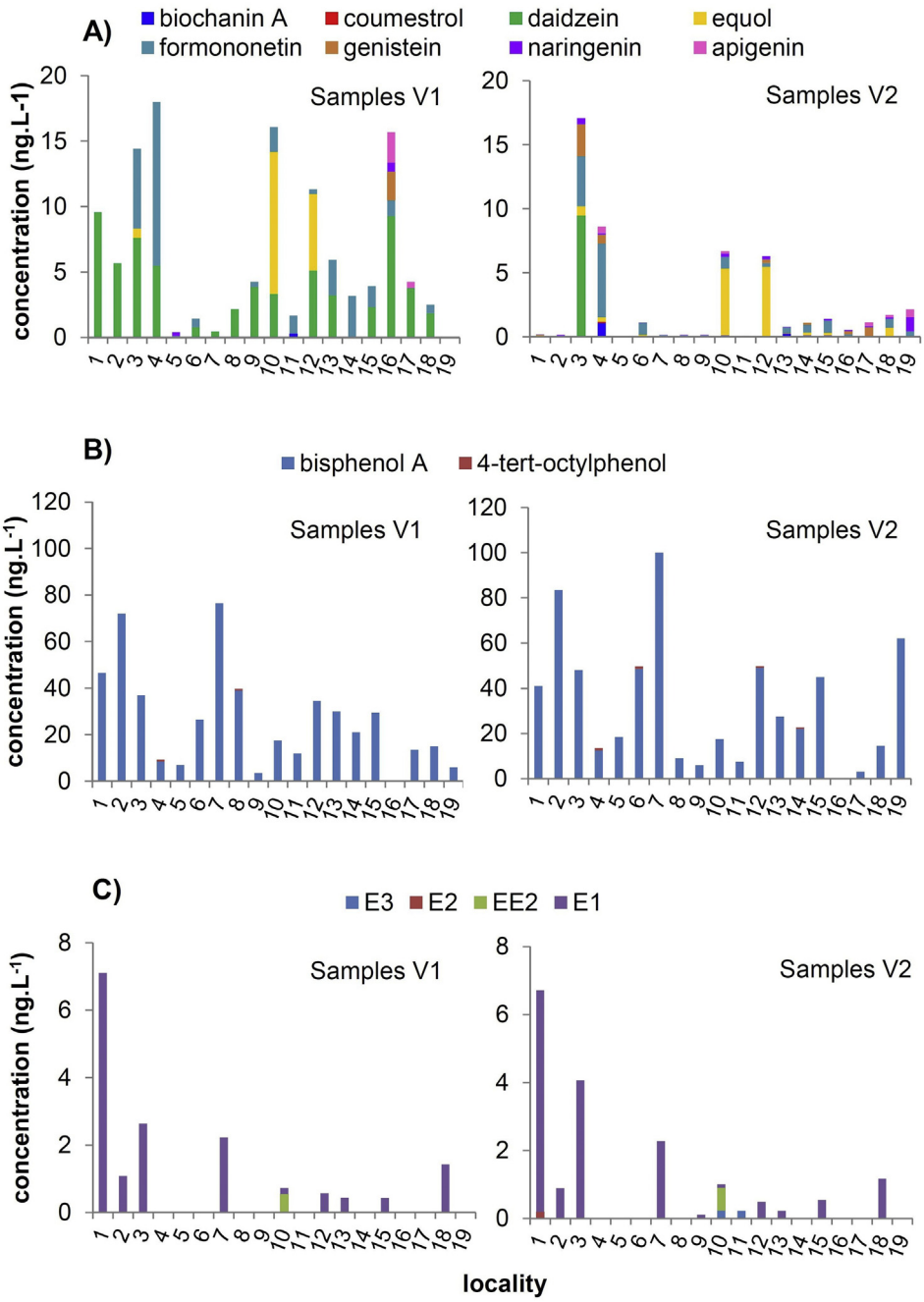

Fig. 1. Detected concentrations of A) flavonoids, B) alkylphenols and C) estrogenic hormones in water samples.

7.1 ng.L^{-1} of estrogens at gravel pit Šutrák (locality 1). These levels are in agreement with previously published studies on the presence of steroid estrogens in surface waters, although they focused on rivers and provided no data for stagnant waters (Esteban et al., 2014; Laganà et al., 2004; Miege et al., 2009; Patrolecco et al., 2015). The most common estrogen in our samples was E1, which was detected at 10 localities. E1 is also the most commonly reported estrogen in rivers. This is attributed to the fact that it can also be formed as a transformation product of E2 (Ternes et al., 1999). As for the other three estrogens (E2, E3 and EE2), they were present only rarely in our samples – at 1–2 localities and at lower concentrations than E1 ($\leq$0.7 ng.L^{-1}), close to their LOQs (0.2 ng.L^{-1}). These LOQs correspond to the sensitivity of other recently published methods (Conley et al., 2017; Čelić et al., 2017; Česen and Heath, 2017). When compared with the proposed environmental quality standards from the European WFD watch list (COM, 2011-876), concentrations of E2 were always below this threshold of 0.4 ng.L^{-1}. For EE2 it is not possible to assess the exceedance since the LOQ of our method (0.2 ng.L^{-1}) was higher than the proposed environmental quality standard (35 pg.L^{-1}). However, the concentrations of EE2 on locality 10 (Jamský lake) were in both samples higher than our LOQ and also more than 10 times higher than the proposed environmental quality standard.

3.4. Relative potencies of analysed compounds

Relative estrogenic potencies (RPs) of individual compounds can vary greatly between different *in vitro* bioassays. For example, RPs

of estriol reported in literature ranged from undetectable estrogenic response in YES assay (Alvarez et al., 2013) to 0.4 in bioassay with HGELN cell line (Gutendorf and Westendorf, 2001). Similarly, RP of 4-octylphenol derived in MVLN cells was 8.3×10^{-5}, whereas it was 8.0×10^{-4} in HGELN cells (Gutendorf and Westendorf, 2001). Therefore, in order to calculate the contribution of individual compounds to the overall estrogenic activity of water samples, we determined RPs of all analysed estrogenic hormones and alkylphenols using the same bioassay as for the assessment of estrogenicity of field water samples. RPs of flavonoids were established previously (Procházková et al., 2017) and these values were used also in this study.

Fig. 2 shows representative concentration-response curves obtained in the *in vitro* hERα-HeLa-9903 bioassay for all analysed alkylphenols and estrogenic hormones. Estrogenic hormones were the most potent among the studied compounds with RPs 0.02–1.36. EE2 with RP 1.36 induced the estrogenic response in similar concentrations to E2. E1 and E3 were less active in our bioassay and their RPs were 0.02 and 0.06, respectively. Alkylphenols had generally lower estrogenic activity (similar to flavonoids) with RPs in the range of $6.1 \times 10^{-7} - 5.5 \times 10^{-5}$. RPs of 4-*tert*-octylphenol and bisphenols A and F were all in a close range, being 4.2×10^{-5}, 5.5×10^{-5} and 3.3×10^{-5}, respectively. 4-octylphenol and 4-n-nonylphenol had considerably lower RPs of 8.4×10^{-7} and 6.1×10^{-7}, respectively. All RPs are listed also in Supplementary material S2. Takeyoshi (2006) reported EC50s of a few compounds analysed also in our study using the same cell line. Although they used a different approach to calculate these EC50s as they did not normalize the response to E2max, RPs that could be derived from those values were in good agreement with our results. Namely, there was a very good correspondence in case of EE2, E1, 4-*tert*-octylphenol and bisphenol A, while some difference occurred for E3 and 4-nonylphenol.

3.5. Estrogenic activity of water samples and relative contribution of analysed compounds

Estrogenicity of stagnant waters is largely unexplored, even though they can receive polluting estrogenic compounds of different origin and from various sources. In our case, estrogenic activity was commonly detected in studied stagnant water samples – 32 out of 38 samples induced estrogenic response in *in vitro* assay with the hERα-HeLa-9903 cell line. EEQs of water samples (shown in Table 2) ranged from below 0.02 $ng.L^{-1}$ to 1.95 $ng.L^{-1}$. The differences between EEQs of samples V1 and V2 from one locality were up to 0.55 $ng.L^{-1}$. However, none of the two SPE setups showed systematically higher estrogenic activities in the samples. Setup V1 is generally used to extract various polar compounds,

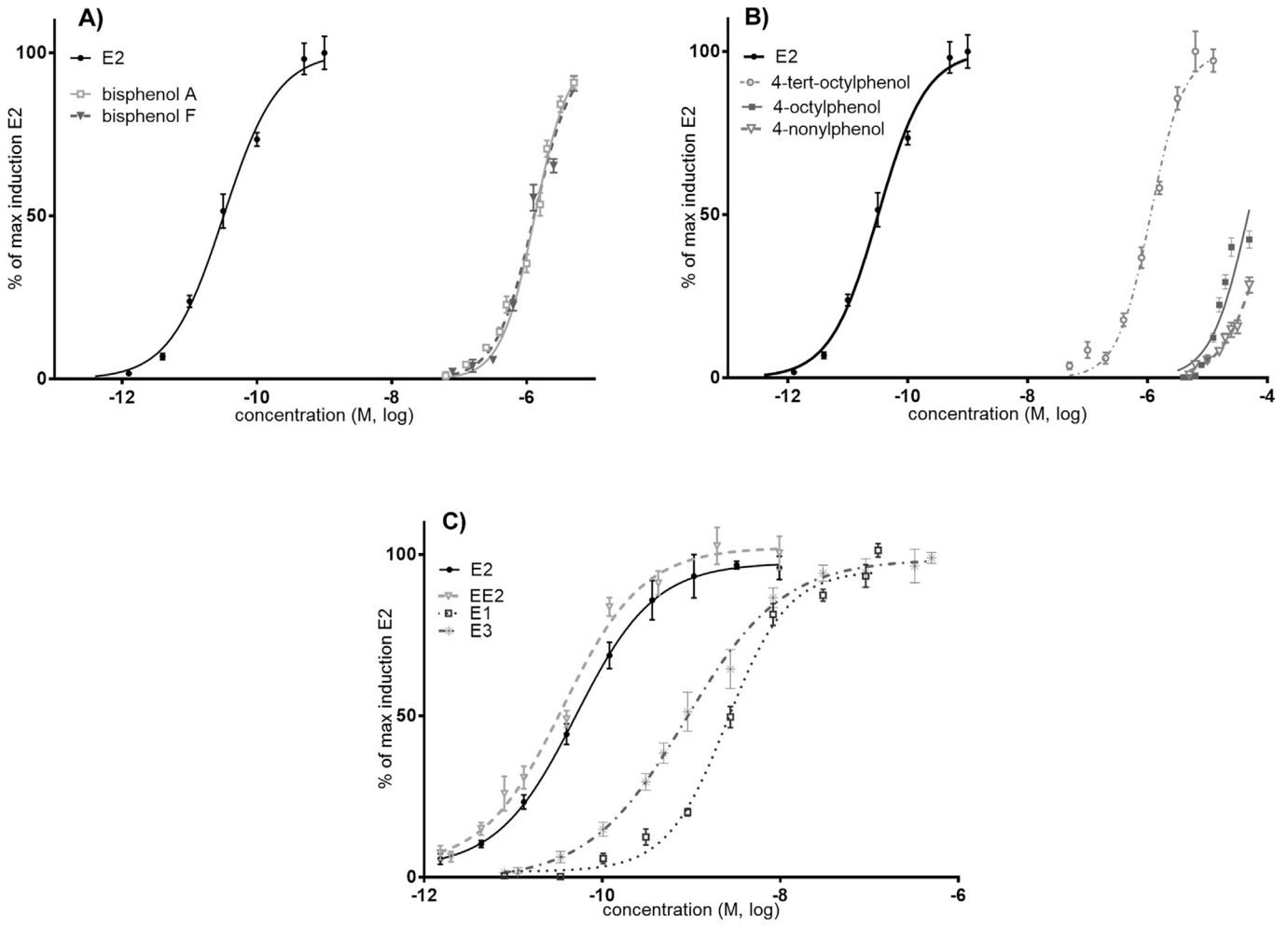

Fig. 2. Concentration–response curves for the estrogenic activity of A) alkylphenols bisphenol A and bisphenol F, B) alkylphenols 4-*tert*-octylphenol, 4-octylphenol and 4-nonylphenol and C) estrogenic hormones (E1, E2, E3 and EE2) in hERα-HeLa-9903 cell line after 24 h exposure. Data show representative results expressed as % of maximal estrogen receptor-mediated induction caused by standard estrogen 17β-estradiol (E2max), values represent the mean ± standard error (n = 3).

Table 2
Estrogenic equivalents (EEQ, pg.L^{-1}) of water samples determined in the *in vitro* bioassay with hERα-HeLa-9903 cell line, chemicals-based estrogenic equivalents (cEEQ, pg.L^{-1}) calculated from analysed groups of estrogenic compounds in minimum scenario (compounds below LOQ not considered) and the contribution of each group (expressed in % of the EEQ) to the estrogenicity of samples.

		EEQ	cEEQ of estrogenic hormones	contribution to the EEQ	cEEQ of flavonoids	contribution to the EEQ	cEEQ of alkylphenols	contribution to the EEQ
1	V1	560[a]	140	25	1.3	0.24	2.1	0.38
	V2	850[b]	330	38	0.046	0.0054	1.9	0.22
2	V1	600[a]	22	3.6	0.79	0.13	3.3	0.55
	V2	600[b]	18	3.0	0.017	0.0029	3.8	0.64
3	V1	1950[a]	53	2.7	2.7	0.14	1.7	0.087
	V2	1530[a]	82	5.3	3.6	0.23	2.2	0.14
4	V1	≤20	–	–	3.7	–	0.42	–
	V2	≤20	–	–	2.1	–	0.61	–
5	V1	≤20	–	–	0.030	–	0.32	–
	V2	≤20	–	–	0.0045	–	0.85	–
6	V1	330[a]	–	–	0.26	0.080	1.2	0.37
	V2	≤20	–	–	0.27	–	2.3	–
7	V1	260[a]	45	17	0.064	0.024	3.5	1.3
	V2	180[b]	46	25	0.033	0.019	4.6	2.5
8	V1	90[b]	–	–	0.30	0.34	1.8	2.0
	V2	≤20	–	–	0.030	–	0.41	–
9	V1	120[b]	–	–	0.63	0.53	0.16	0.13
	V2	460[a]	2.1	0.46	0.023	0.0050	0.27	0.060
10	V1	730[a]	810	111	4.3	0.60	0.80	0.11
	V2	970[a]	990	102	1.9	0.20	0.80	0.082
11	V1	880[a]	–	–	0.35	0.039	0.55	0.062
	V2	1210[a]	13	1.1	0.015	0.0012	0.34	0.028
12	V1	1120[a]	12	1.0	2.6	0.24	1.6	0.14
	V2	570[a]	9.6	1.7	2.0	0.34	2.3	0.40
13	V1	750[a]	8.7	1.2	1.1	0.15	1.4	0.18
	V2	370[b]	4.4	1.2	0.17	0.045	1.3	0.34
14	V1	140[b]	–	–	0.74	0.53	0.96	0.69
	V2	170[b]	–	–	0.29	0.17	1.0	0.60
15	V1	870[a]	8.6	0.99	0.70	0.081	1.3	0.15
	V2	410[b]	11	2.6	0.30	0.073	2.1	0.50
16	V1	90[b]	–	–	2.8	3.1	–	–
	V2	100[b]	–	–	0.18	0.18	–	–
17	V1	80[b]	–	–	0.58	0.72	0.62	0.77
	V2	130[b]	–	–	0.35	0.27	0.14	0.11
18	V1	930[a]	28	3.1	0.41	0.044	0.69	0.074
	V2	620[a]	23	3.8	0.40	0.064	0.66	0.11
19	V1	1250[a]	–	–	–	–	0.27	0.022
	V2	920[a]	–	–	0.19	0.021	2.8	0.31

[a] EEQ calculated from EC50.
[b] EEQ calculated from EC20.

including estrogenic hormones, with good recoveries (Labadie and Budzinski, 2005). Setup V2 was, on the other hand, designed to give better recoveries of less polar compounds - e.g. sterols, as reported in our previous study (Procházková et al., 2017). The results indicate at some sites contribution to estrogenic activity also from less polar compounds that can only be extracted by more complex extraction approaches covering compounds with wider polarity range.

To our knowledge, the only study on estrogenic activity in ponds and wetlands was conducted in the USA and 13 out of 20 selected sites showed EEQ (E-screen assay) in the range of 0.03–0.27 ng.L^{-1} (Shappell, 2006). Most information on the estrogenic activity of surface waters comes from rivers affected by wastewaters. Their EEQs were reported to reach up to 78.8 ng.L^{-1} in Liao River (Wang et al., 2011) and 9.4 ng.L^{-1} in Yellow River in China (Wang et al., 2012). Jugan et al. (2009) determined that EEQs of river Seine were 2.5 ng.L^{-1} at maximum. In Swiss rivers, observed estrogenicity was up to 7 ng.L^{-1} (Vermeirssen et al., 2005) and in American streams up to 4.6 ng.L^{-1} (Alvarez et al., 2013). Leusch et al. (2010) found the estrogenic activity of Australian river water below 1 ng.L^{-1} of EEQ at all studied sites. Comparing to our results, estrogenic activity at some sites in waterbodies with high phytoplankton biomass including cyanobacterial blooms can reach levels in the same order of magnitude as estrogenicity of river water under wastewater treatment plants.

It is difficult to generalize the potential sources of estrogenicity in our samples as estrogenic activity was detected in small village water tanks and gravel pits that can receive run-offs from the surrounding surfaces, households or septic tanks (e.g. localities 13, 14 or 15) and also in lakes in the vicinity of animal farms (e.g. localities 1 or 2) or agricultural fields (e.g. localities 10 or 18). Interestingly, the localities with the highest EEQs (localities 3, 11, 12 and 19) are managed as breeding ponds/lakes, where next to adjacent agricultural activities, also some management interventions could influence the observed pollution.

The predicted no effect concentration (PNEC) of E2 was derived by Caldwell et al. (2012) to be 2 ng.L^{-1}. For the assessment of PNEC, they considered effects on fish reproduction (e.g. feminization, egg production, fertilization or hatching) as the most sensitive endpoints. In our study, only one sample reached similar level of EEQ (1.95 ng.L^{-1} – sample V1 from Dolní Lužický lake). However, Lahnsteiner et al. (2006) described significant decrease of semen fertility of rainbow trout (*Oncorhynchus mykiss*) also after exposure to concentration 1 ng.L^{-1} of E2. This level of estrogenic activity was observed in 4 lakes/ponds from our study in at least one sample (Table 2). When we compare the EEQs with the proposed environmental quality standard for E2 from the European WFD watch list (COM, 2011-876), the level 0.4 ng.L^{-1} was exceeded on 11 (out of 19) localities. Ten sites also exceeded the recently suggested effect-

based trigger value for *in vitro* estrogenicity assessment of 0.5 ng.L^{-1} EEQ (van der Oost et al., 2017). Unlike the above-mentioned levels, which were derived specifically for E2 and therefore considered only the studies focusing on this compound, the effect based trigger value of van der Oost et al. (2017) was estimated for the effect observed in the bioassay. So in the process of derivation of this value, EC50 values of different estrogenic compounds were converted to equivalent concentrations of the reference compound (E2) and included in the assessment. This trigger value was derived as a concentration that will adversely affect 5% of the species and was meant to prioritize the localities where detailed chemical analyses for characterisation of the responsible compounds are required.

From concentrations of individual compounds and their RPs, we calculated the cEEQs considering 3 scenarios. Minimum scenario takes into account only the concentrations above LOQ and simulates the lowest potential estrogenicity contributed by the analysed estrogenic compounds. However, also the compounds below LOQ could be present in the samples in the concentration range 0 – LOQ. To demonstrate the potential contribution of the compounds below LOQ and relative importance of their limits of quantification, we have calculated also the hypothetical mean and maximum scenarios. Mean scenario shows the average estimate of the contribution of all compounds analysed in this study as we set the concentration of compounds below LOQ to the level of LOQ/2. In the maximum scenario, we set the concentration of analytes below LOQ to the level of LOQ to demonstrate the highest potential contribution of the compounds to the estrogenicity of water. It is not probable that all the unquantified compounds would be present at LOQ levels, but this maximum scenario demonstrates the impact of LC-MS/MS method LOQs on the explicability of biological effects. It also helps to estimate the minimal part of bioactivity that cannot be explained by the presence of analysed compounds in a simple additive model. The results of minimum scenario cEEQs are shown in Table 2, the comparison of all three scenarios is shown in Supplementary material S2. Contributions of individual compounds to cEEQs are presented in Supplementary materials S3 – S5.

The mass-balance calculations are frequently used to assess the potential contribution of analysed compounds to detected bioactivities (e.g. Chou et al., 2015; Grund et al., 2011; Miege et al., 2009). In these calculations, the compounds below the LOQ are frequently not considered. Thus, only the minimum scenario is presented and discussed. However, as is demonstrated by our results, even the values below LOQs could play a significant role, especially in case of highly potent compounds (e.g. natural and synthetic steroid estrogens) or too high LOQs of less potent compounds.

We observed large differences between the minimum, mean and maximum scenario cEEQs of estrogenic hormones and flavonoids. The largest differences were in the cEEQs of estrogenic hormones, especially due to E2 and EE2. These two compounds are the most potent (RPs 1 and 1.36, respectively), but were rarely detected above their LOQ of 200 pg.L^{-1}. Despite the applied sensitive analytical methods with relatively low LOQs of E2 and EE2, which are comparable or better than for other recently published methods (Conley et al., 2017; Čelić et al., 2017; Česen and Heath, 2017), even the levels below LOQ could be significant. Together, they add 248 pg.L^{-1} to the mean scenario cEEQ and 496 pg.L^{-1} to the maximum scenario cEEQ of samples where they were found below the LOQ. In case of flavonoids, cEEQs were sometimes up to 56 and 100 times higher for the mean and maximum scenario, respectively, when compared with the minimum scenario. Despite this, the difference between the minimum and maximum scenarios was less than 1 pg.L^{-1} in all cases. cEEQs of alkylphenols were almost the same in all scenarios as the RPs of these compounds ($\leq 5.45 \times 10^{-5}$) and also their LOQs ($\leq$70 pg.L^{-1}) are very low. Therefore, their influence on maximum scenario cEEQ is negligible.

In the next step, EEQs of water samples determined in the biotest were compared with cEEQs calculated for the studied groups of estrogenic compounds (expressed as a contribution to the EEQ in Table 2 and Supplementary material S2 and S5). Using the minimum scenario, EEQs estimated from *in vitro* assay were higher or comparable to cEEQs (cEEQs corresponded to 0.022–112% of EEQs). In this scenario, the contribution of all analysed compounds was mostly very low – for 26 samples from 14 localities, more than 94% of the total estrogenic activity remained unexplained. Only in Jamský lake (locality 10), concentrations of analysed compounds completely explained the observed activity in V1 and V2 sample, and in both cases, the estrogenic hormones were entirely responsible for the estrogenic effects (Table 2). Alkylphenols and flavonoids generally accounted for less than 1% of the estrogenic activity of water samples with the maximum contribution of 3% from both these groups of compounds.

In the mean scenario, compounds below LOQ could add up to 260 pg.L^{-1} to the cEEQ. This of course significantly increased the part of bioactivity attributable to our analytes. The estrogenicity observed in bioassay could be entirely explained in 12 samples and the lowest contribution of cEEQ to the EEQ was 16%.

Using the maximum scenario, on the other hand, all analysed compounds at their LOQ level would together account for 510 pg.L^{-1}, which would entirely explain the activity of 14 out of the 32 samples that showed detectable estrogenic activity. For the samples with the EEQ below 1 ng.L^{-1}, the maximum scenario cEEQ shows that with the contribution of the compounds at their LOQ levels, the estrogenic activity would be explained by more than 50%. However, for the samples with EEQ > 1 ng.L^{-1}, the higher the EEQ determined from the biotest, the lower was the contribution of the analysed compounds. For the sample with the highest estrogenic activity (locality 3, sample V1, EEQ 1950 pg.L^{-1}), the maximum scenario cEEQ accounted for only 29% of the EEQ. This indicates that other compounds or possible interactions between individual estrogenic substances play an important role in estrogenic activity of stagnant surface waters.

Other studies also report estrogenic hormones as the main contributors to the estrogenic activity of surface waters, but all these studies were conducted on rivers. For example, in the study of Miege et al. (2009), concentrations of estrogenic hormones determined by LC-MS/MS method explained at least about 50% of EEQs observed in the bioassay for river water samples. Chou et al. (2015) explored the contribution of estrogenic hormones and alkylphenols to the estrogenicity of Taiwanese rivers and attributed from 28% to more than 100% of the estrogenic activity to these compounds, with E1 and E2 being the most significant. Chemical (LC-MS/MS) and biological (YES assay) analyses of Danube river sediments also revealed E1 as the main contributor to the estrogenic activity from the selected set of compounds (E1, E2, EE2, bisphenol A and nonylphenol) (Grund et al., 2011). Still, in comparison with the biologically determined estrogenicity of sediment extracts, the LC-MS/MS analyses explained about 55% of the activity on one locality and less than 6% on the other four. These studies (Chou et al., 2015; Grund et al., 2011; Miege et al., 2009) therefore report considerable part of the biological activity of some samples (45–98%) as unexplained. However, they also did not consider compounds below quantification limits, which could increase the part of estrogenicity attributed to the analysed estrogens.

Although estrogenic hormones are often reported as the main contributing compounds, Oh et al. (2009) associated also alkylphenols with a considerable portion of estrogenic activity of river water. They found alkylphenols at concentrations up to 2 μg.L^{-1} in samples that exhibited estrogenic activity and these levels

explained 34% of the total estrogenicity. Phytoestrogens in surface waters usually do not reach concentrations that could cause an observable estrogenic effect. However in specific cases, like drainage water from fields of plants rich in phytoestrogens, their concentrations can be up to several $\mu g.L^{-1}$ and in such high levels, they can be also relevant and dominant drivers of estrogenicity (Hoerger et al., 2011). Nevertheless, there were relatively low levels of both phytoestrogens and alkylphenols with no significant contribution to estrogenicity across all different stagnant waters in our study.

The analysed compounds did not fully explain the observed *in vitro* estrogenic activity at some sites, even when the maximum uncertainty connected with the (in)ability of chemical analytical methods to detect the low concentrations of estrogenic compounds was taken into account. This indicates that other unidentified compounds are probably contributing to the observed estrogenicity and to identify them, e.g. the effect directed analysis to isolate these compounds would be needed. Compounds that could contribute to the total estrogenicity and were not analysed in this study include for example estrogens produced by fungi. Mycoestrogen zearalenone and its metabolites have RPs in the range of 5×10^{-4} – 6.8×10^{-1} and their concentrations can reach up to tens or hundreds of $ng.L^{-1}$ in surface waters (Jarošová et al., 2015).

Cyanobacteria and algae could be another potential source of estrogenic substances. It was reported that their biomass can contain low concentrations of phytoestrogens (Goiris et al., 2014; Klejdus et al., 2010). Their metabolites can also induce *in vivo* estrogenic activity (Marie et al., 2012; Rogers et al., 2011). However, in this study, there does not seem to be any direct relationship between the estrogenicity of water or occurrence of phytoestrogens and phytoplankton concentration or composition. The site with the highest and the second least density of phytoplankton (locality 4, 90.8×10^6 cells.mL^{-1} and locality 5, 0.04×10^6 cells.mL^{-1}, respectively) had both EEQ below LOQ (<20 $pg.L^{-1}$). On the other hand, the site with the second highest density (locality 3, 38.9×10^6 cells.mL^{-1}) had the highest EEQs (1950 and 1530 $pg.L^{-1}$ in sample V1 and V2, respectively). The estrogenic activity does not seem to be connected with a specific group of phytoplankton either. For example, cyanobacteria dominate at localities 12 and 16 at similar densities around 2×10^6 cells.mL^{-1}, yet the EEQs at locality 12 (1120 and 570 $pg.L^{-1}$) are at least 5 times higher than at locality 16 (90 and 100 $pg.L^{-1}$). Similarly, phytoplankton on localities 14 and 19 is dominated by green microalgae and the EEQs at site 14 (140 and 170 $pg.L^{-1}$) are 5 times lower than at site 19 (1250 and 920 $pg.L^{-1}$). Phytoestrogens generally occurred at low levels across all studied water bodies. The two sites with greatest biomass densities (locality 3, 4) belong to those with the greatest phytoestrogen concentrations, but comparable levels were found at sites 10 and 16 with much lower cell densities and very different species composition.

4. Conclusions

- Stagnant freshwaters are under multiple pressures especially in urbanized and agricultural areas, which leads to co-occurrence of various anthropogenic and natural estrogenic compounds
- Since estrogenic activities in these ecosystems can reach levels comparable to WWTP-affected rivers, greater attention needs to be paid to the endocrine disruptive potency of present compound mixtures, the major contributors and their sources need to be identified.
- Mass balance calculations of the contribution of analyzed compounds to detected bioactivities need to take into consideration their limits of quantification. Especially in the case of highly potent compounds, the contribution to the detected bioactivities might be significant even below these limits.
- A complex approach including bioassays is needed when assessing the estrogenicity of these types of surface waters as it could be underestimated if predicted from the concentrations of analysed estrogenic hormones, alkylphenols or phytoestrogens.
- Organisms in stagnant waters affected by cyanobacterial water blooms can be next to the different groups of estrogenic compounds co-exposed to other bioactive and toxic compounds from cyanobacteria as well as to other stressors associated with massive blooms, such as decreased oxygen levels or reduced light penetration. This can increase the vulnerability of exposed organisms and lead to adverse effects even below effective levels of the individual stressors.
- Mitigation actions should be implemented on the stagnant water bodies with longer term pollution and water bloom problems, including reduction of input of both nutrients and endocrine disruptive compounds.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.02.040.

References

Alvarez, D.A., Shappell, N.W., Billey, L.O., Bermudez, D.S., Wilson, V.S., Kolpin, D.W., Perkins, S.D., Evans, N., Foreman, W.T., Gray, J.L., Shipitalo, M.J., Meyer, M.T., 2013. Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations. Water Res. 47 (10), 3347–3363.

Caldwell, D.J., Mastrocco, F., Anderson, P.D., Länge, R., Sumpter, J.P., 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol. Environ. Toxicol. Chem. 31 (6), 1396–1406.

Careghini, A., Mastorgio, A.F., Saponaro, S., Sezenna, E., 2015. Bisphenol A, nonylphenols, benzophenones, and benzotriazoles in soils, groundwater, surface water, sediments, and food: a review. Environ. Sci. Pollut. Res. 22 (8), 5711–5741.

Čelić, M., Insa, S., Petrović, M., Škrbić, B., 2017. Development of a sensitive and robust online dual column liquid chromatography-tandem mass spectrometry method for the analysis of natural and synthetic estrogens and their conjugates in river water and wastewater. Anal. Bioanal. Chem. 409 (23), 5427–5440.

Česen, M., Heath, E., 2017. Disk-based solid phase extraction for the determination of diclofenac and steroidal estrogens E1, E2 and EE2 listed in the WFD watch list by GC–MS. Sci. Total Environ. 590–591, 832–837.

Charles, G.D., Gennings, C., Zacharewski, T.R., Gollapudi, B.B., Carney, E.W., 2002. Assessment of interactions of diverse ternary mixtures in an estrogen receptor-alpha reporter assay. Toxicol. Appl. Pharmacol. 180 (1), 11–21.

Chou, P.-H., Lin, Y.-L., Liu, T.-C., Chen, K.-Y., 2015. Exploring potential contributors to endocrine disrupting activities in Taiwan's surface waters using yeast assays and chemical analysis. Chemosphere 138, 814–820.

COM 2011/876-PrE of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.

Conley, J.M., Evans, N., Mash, H., Rosenblum, L., Schenck, K., Glassmeyer, S., Furlong, E.T., Kolpin, D.W., Wilson, V.S., 2017. Comparison of in vitro estrogenic activity and estrogen concentrations in source and treated waters from 25 U.S. drinking water treatment plants. Sci. Total Environ. 579, 1610–1617.

Esteban, S., Gorga, M., Petrovic, M., González-Alonso, S., Barceló, D., Valcárcel, Y., 2014. Analysis and occurrence of endocrine-disrupting compounds and estrogenic activity in the surface waters of Central Spain. Sci. Total Environ. 466–467, 939–951.

Frische, T., Faust, M., Meyer, W., Backhaus, T., 2009. Toxic masking and synergistic modulation of the estrogenic activity of chemical mixtures in a yeast estrogen screen (YES). Environ. Sci. Pollut. Res. 16 (5), 593–603.

Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J., Wenzel, A., 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water Res. 36 (6), 1429–1438.
Fürhacker, M., Scharf, S., Weber, H., 2000. Bisphenol A: emissions from point sources. Chemosphere 41 (5), 751–756.
Giesy, J.P., Hilscherova, K., Jones, P.D., Kannan, K., Machala, M., 2002. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Mar. Pollut. Bull. 45 (1–12), 3–16.
Goiris, K., Muylaert, K., Voorspoels, S., Noten, B., De Paepe, D., Baart, G.J.E., De Cooman, L., 2014. Detection of flavonoids in microalgae from different evolutionary lineages. J. Phycol. 50 (3), 483–492.
Gong, Y., Wang, X., Indran, I.R., Zhang, S.-J., Lv, Z., Li, J., Holmes, M., Tang, Y.Z., Yong, E.L., 2014. Phytoplankton blooms: an overlooked marine source of natural endocrine disrupting chemicals. Ecotoxicol. Environ. Saf. 107, 126–132.
Grund, S., Higley, E., Schonenberger, R., Suter, M.J.F., Giesy, J.P., Braunbeck, T., Hecker, M., Hollert, H., 2011. The endocrine disrupting potential of sediments from the Upper Danube River (Germany) as revealed by in vitro bioassays and chemical analysis. Environ. Sci. Pollut. Res. 18 (3), 446–460.
Gutendorf, B., Westendorf, J., 2001. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166 (1–2), 79–89.
Hillebrand, H., Dürselen, C.-D., Kirchtel, D., Pollingher, U., Zohary, T., 1999. Biovolume calculation for pelagic and benthic microalgae. J. Phycol. 35 (2), 403–424.
Hoerger, C.C., Wettstein, F.E., Bachmann, H.J., Hungerbuhler, K., Bucheli, T.D., 2011. Occurrence and mass balance of isoflavones on an experimental grassland field. Environ. Sci. Technol. 45 (16), 6752–6760.
Ihara, M., Ihara, M.O., Kumar, V., Narumiya, M., Hanamoto, S., Nakada, N., Yamashita, N., Miyagawa, S., Iguchi, T., Tanaka, H., 2014. Co-occurrence of estrogenic and antiestrogenic activities in wastewater: quantitative evaluation of balance by in vitro ERα reporter gene assay and chemical analysis. Environ. Sci. Technol. 48 (11), 6366–6373.
Jarošová, B., Javůrek, J., Adamovský, O., Hilscherová, K., 2015. Phytoestrogens and mycoestrogens in surface waters — their sources, occurrence, and potential contribution to estrogenic activity. Environ. Int. 81, 26–44.
Jin, X., Jiang, G., Huang, G., Liu, J., Zhou, Q., 2004. Determination of 4-tert-octylphenol, 4-nonylphenol and bisphenol A in surface waters from the Haihe River in Tianjin by gas chromatography–mass spectrometry with selected ion monitoring. Chemosphere 56, 1113–1119.
Jugan, M.L., Oziol, L., Bimbot, M., Huteau, V., Tamisier-Karolak, S., Blondeau, J.P., Lévi, Y., 2009. In vitro assessment of thyroid and estrogenic endocrine disruptors in wastewater treatment plants, rivers and drinking water supplies in the greater Paris area (France). Sci. Total Environ. 407 (11), 3579–3587.
Klejdus, B., Lojkova, L., Plaza, M., Snoblova, M., Sterbova, D., 2010. Hyphenated technique for the extraction and determination of isoflavones in algae: ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry. J. Chromatogr. A 1217 (51), 7956–7965.
Labadie, P., Budzinski, H., 2005. Development of an analytical procedure for determination of selected estrogens and progestagens in water samples. Anal. Bioanal. Chem. 381 (6), 1199–1205.
Laganà, A., Bacaloni, A., De Leva, I., Faberi, A., Fago, G., Marino, A., 2004. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. Anal. Chim. Acta 501 (1), 79–88.
Lahnsteiner, F., Berger, B., Kletzl, M., Weismann, T., 2006. Effect of 17β-estradiol on gamete quality and maturation in two salmonid species. Aquat. Toxicol. 79 (2), 124–131.
Leusch, F.D.L., De Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., Tremblay, L.A., Wilson, V.S., Chapman, H.F., 2010. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ. Sci. Technol. 44 (10), 3853–3860.
Marie, B., Huet, H., Marie, A., Djediat, C., Puiseux-Dao, S., Catherine, A., Trinchet, I., Edery, M., 2012. Effects of a toxic cyanobacterial bloom (Planktothrix agardhii) on fish: insights from histopathological and quantitative proteomic assessments following the oral exposure of medaka fish (Oryzias latipes). Aquat. Toxicol. 114–115, 39–48.
Michałowicz, J., 2014. Bisphenol A – sources, toxicity and biotransformation. Environ. Toxicol. Pharmacol. 37 (2), 738–758.
Miege, C., Karolak, S., Gabet, V., Jugan, M.L., Oziol, L., Chevreuil, M., Levi, Y., Coquery, M., 2009. Evaluation of estrogenic disrupting potency in aquatic environments and urban wastewaters by combining chemical and biological analysis. Trac. Trends Anal. Chem. 28 (2), 186–195.
Oh, S.M., Kim, H.R., Park, H.K., Choi, K., Ryu, J., Shin, H.S., Park, J.-S., Lee, J.S., Chung, K.H., 2009. Identification of estrogen-like effects and biologically active compounds in river water using bioassays and chemical analysis. Sci. Total Environ. 407 (21), 5787–5794.
Patrolecco, L., Capri, S., Ademollo, N., 2015. Occurrence of selected pharmaceuticals in the principal sewage treatment plants in Rome (Italy) and in the receiving surface waters. Environ. Sci. Pollut. Res. 22 (8), 5864–5876.
Pernica, M., Poloucká, P., Seifertová, M., Šimek, Z., 2015. Determination of alkylphenols in water samples using liquid chromatography–tandem mass spectrometry after pre-column derivatization with dansyl chloride. J. Chromatogr. A 1417, 49–56.
Procházková, T., Sychrová, E., Javůrková, B., Večerková, J., Kohoutek, J., Lepšová-Skácelová, O., Bláha, L., Hilscherová, K., 2017. Phytoestrogens and sterols in waters with cyanobacterial blooms - analytical methods and estrogenic potencies. Chemosphere 170, 104–112.
Rogers, E.D., Henry, T.B., Twiner, M.J., Gouffon, J.S., McPherson, J.T., Boyer, G.L., Sayler, G.S., Wilhelm, S.W., 2011. Global gene expression profiling in larval zebrafish exposed to microcystin-LR and Microcystis reveals endocrine disrupting effects of cyanobacteria. Environ. Sci. Technol. 45 (5), 1962–1969.
Shappell, N.W., 2006. Estrogenic activity in the environment: municipal wastewater effluent, river, ponds, and wetlands. J. Environ. Qual. 35 (1), 122–132.
Skácelová, O., Lepš, J., 2014. The relationship of diversity and biomass in phytoplankton communities weakens when accounting for species proportions. Hydrobiologia 724 (1), 67–77.
Stepankova, T., Ambrozova, L., Blaha, L., Giesy, J.P., Hilscherova, K., 2011. In vitro modulation of intracellular receptor signaling and cytotoxicity induced by extracts of cyanobacteria, complex water blooms and their fractions. Aquat. Toxicol. 105 (3–4), 497–507.
Sumpter, J.P., Jobling, S., 2013. The occurrence, causes, and consequences of estrogens in the aquatic environment. Environ. Toxicol. Chem. 32 (2), 249–251.
Söffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish – a critical review on effects and possible consequences. Crit. Rev. Toxicol. 42 (8), 653–668.
Takeyoshi, M., 2006. Draft Report of Pre-validation and Inter-laboratory Validation for Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity. The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-He-La-9903 Cell Line OECD document der EDTA.
Ternes, T.A., Kreckel, P., Mueller, J., 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants — II. Aerobic batch experiments with activated sludge. Sci. Total Environ. 225 (1–2), 91–99.
van der Oost, R., Sileno, G., Suárez-Muñoz, M., Nguyen, M.T., Besselink, H., Brouwer, A., 2017. SIMONI (Smart Integrated Monitoring) as a novel bioanalytical strategy for water quality assessment: Part I – model design and effect-based trigger values. Environ. Toxicol. Chem. 36 (9), 2385–2399.
Vermeirssen, E.L.M., Burki, R., Joris, C., Peter, A., Segner, H., Suter, M.J.F., Burkhardt-Holm, P., 2005. Characterization of the estrogenicity of Swiss midland rivers using a recombinant yeast bioassay and plasma vitellogenin concentrations in feral male brown trout. Environ. Toxicol. Chem./SETAC 24 (9), 2226–2233.
Wang, C., Zhang, S., Zhou, Y., Huang, C., Mu, D., Giesy, J.P., Hu, J., 2016. Equol induces gonadal intersex in Japanese medaka (Oryzias latipes) at environmentally relevant concentrations: comparison with 17β-estradiol. Environ. Sci. Technol. 50 (14), 7852–7860.
Wang, L., Ying, G.-G., Zhao, J.-L., Liu, S., Yang, B., Zhou, L.-J., Tao, R., Su, H.-C., 2011. Assessing estrogenic activity in surface water and sediment of the Liao River system in northeast China using combined chemical and biological tools. Environ. Pollut. 159 (1), 148–156.
Wang, L., Ying, G.G., Chen, F., Zhang, L.J., Zhao, J.L., Lai, H.J., Chen, Z.F., Tao, R., 2012. Monitoring of selected estrogenic compounds and estrogenic activity in surface water and sediment of the Yellow River in China using combined chemical and biological tools. Environ. Pollut. 165, 241–249.

Article V

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Marek Pernica designed and conducted the research and wrote the manuscript.

An analysis of residue alkylphenols and bisphenol A using liquid chromatography-tandem mass spectrometry

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Abstract: Bisphenol A (BPA) and alkylphenols (APs) such as 4-*tert*-octylphenol (4-t-OP) and mixture of nonylphenol isomers (iso-NP) are frequent contaminants of various environmental analyses. Analyse of various blanks is a serious problem in the residue analysis. Secondary contamination of analysed samples can come from laboratory air, septa vials, solvents and other chemicals used. Plastic tubes and connections could be another important source of sample contamination. This can be a problem especially in determination of alkylphenols and bisphenol A at low environmentally relevant concentrations. The analytical method for determination of residues of pollutants in water samples using solid phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is presented here. In principle, alkylphenols can be analysed without derivatization. However, pre-column derivatization with 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansylchloride, DNSC) improves the sensitivity and selectivity of LC-MS/MS analysis. The instrumental blank for all compounds is under the limit of detection (LOD). The system contamination was maintained about 0.5 ng (reagent blank), and below 0.05 µg/L for the glass SPE columns and below 0.1 µg/L for the plastic columns (procedure blank).

Key Words: blank, blank contamination, LC-MS, alkylphenols, bisphenol A

INTRODUCTION

Alkylphenols and bisphenol A as well known endocrine disrupting compounds (EDCs) are able to mimic or antagonize the female estrogens 17 β-estradiol (Salgueiro-González et al. 2012b). Nonylphenols, which form a mixture of 211 branched nonyl-chain isomers, and 4-tert-octylphenol are the most important alkylphenols due to their toxicological properties. Bisphenol A 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 (Salgueiro-González et al. 2012a). The concentrations of BPA, APs and their parent compounds have been measured worldwide in all compartments of the environment and even in food products for human consumption (Xie et al. 2006).

The primary purpose of analysis of blanks is to trace sources of artificially and or randomly introduced contamination of real samples. The diagram (see Figure 1) shows how comparison of different blank sample results can be used to identify and isolate the source of contamination introduced in the field or the laboratory. The source of contamination introduced in the field or laboratory can be deduced by comparing blank results. An equipment blank could potentially be contaminated in the field, during transport to the lab or in the lab. The method blank, on the other hand, could only be contaminated in the lab. Using all the blanks described in this fact sheet will facilitate the identification of contamination sources (EPA 2009).

Nonylphenols can also be present in laboratory air. For example, concentrations around 100 ng/m^3 were found in the air of a typical laboratory and it has also been reported that an LC-MS vial with methanol left in the autosampler of the instrument can absorb NP from the laboratory air within weeks. Septa vials and solvent used as mobile phases can also cause blank contamination.

BPA contamination caused by the water purification system was observed in ultrapure water. Sampling, storage of samples, filtration and sample treatment are also important sources of blank contamination. APs and BPA from water samples are commonly extracted by liquid-liquid extraction (LLE) or solid-phase extraction (SPE). However, this procedure can also cause contamination due to the plastic materials of the SPE cartridges, the multiple steps involved and the use of relatively high volumes of solvent (Xie et al. 2006, Salgueiro-González et al. 2012a).

The terms such as ghost peaks, artifact (and artefact) peaks, system peaks, pseudo peaks, vacancy peaks, eigenpeaks, induced peaks and spurious peaks can be found in published papers. These terms are called peaks that are undesirable in the blank systems (Williams 2004).

It is important to fully characterise the analytical performance in order to understand their capability and limitations, and to ensure that they are "fit for purpose". The terms such as LOB (limit of blank), LOD (limit of detection), and LOQ (limit of quantification) describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. LOB and LOD are important for tests used to distinguish between the presence or absence of an analyte and LOQ to reliably measure low levels of analyte and should be incorporated as part of any method evaluation (Armbruster and Pry 2008).

• Blank equipment (equipment blank) includes the total field and laboratory contamination sources, which are determined by sputtering the blank over the decontaminated field sampling device before sampling the environment. The objective of the assessment is to assess the total contamination of the sampling.

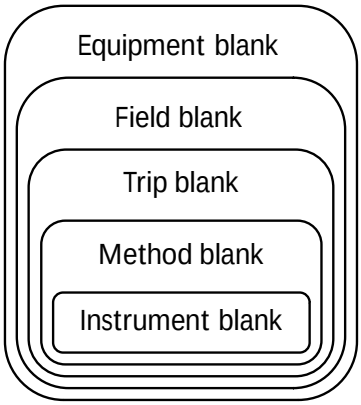
• Field blank includes overall ambient conditions during sampling and laboratory pollution sources. Blank determination is done as follows, a blank sample is transferred to the sampling container and then sent to a laboratory with a field sample. The aim is to assess contamination from field conditions during collection.

• Trip blank includes shipping and laboratory contamination sources, only for volatile substances. The transport blind sample is a sample which is transported from the laboratory to the point of collection and transported back to the laboratory without being subjected to a sampling procedure.

• The blank method (method blank) only shows laboratory sources of contamination. Blank methods are prepared and analyzed exactly in the same way as a field sample. The aim is to assess the contamination during sample preparation.

• The instrumental blank (instrument blank) only shows instrumental source of contamination. The blank itself is parsed with the field sample and the goal is to assess the presence or absence of device contamination (EPA 2009).

Figure 1 Comparison of different blank (EPA 2009)



The main purpose of our study was to compere individual types of blanks such as instrumental blank, reagent blank, method blank and also evaluated to effect use of glass and plastic SPE columns in residual analysis of alkylphenols and bisphenol A. Bisphenol A, 4-tert-octylphenol, 4-octylphenol, isomers of nonylphenol and 4-n-nonylphenol were selected for these purpose. The LC-ESI-MS/MS was used for intended study using pre-column derivatisation with dansyl chloride.

MATERIAL AND METHODS

Instruments and chemicals

Chromatographic separations were performed using an Agilent 1200 Infinity Series (Agilent Technologies, Santa Clara, CA, USA) with chromatographic column ACE 5 C18, 150 mm × 4.6 mm i.d., 5µm particle size (ACE, Scotland, UK). The Agilent 6410 Triple Quadrupole (Agilent Technologies, Santa Clara, CA, USA) was used for MS/MS analysis. The glass and plastic SPE columns octadecyl C18, 500 mg (Supelco) was used for analysis. The analytical standards, derivatizing agent DNSC and water (ultrapure water for HPLC) were purchased from Sigma-Aldrich (Germany).

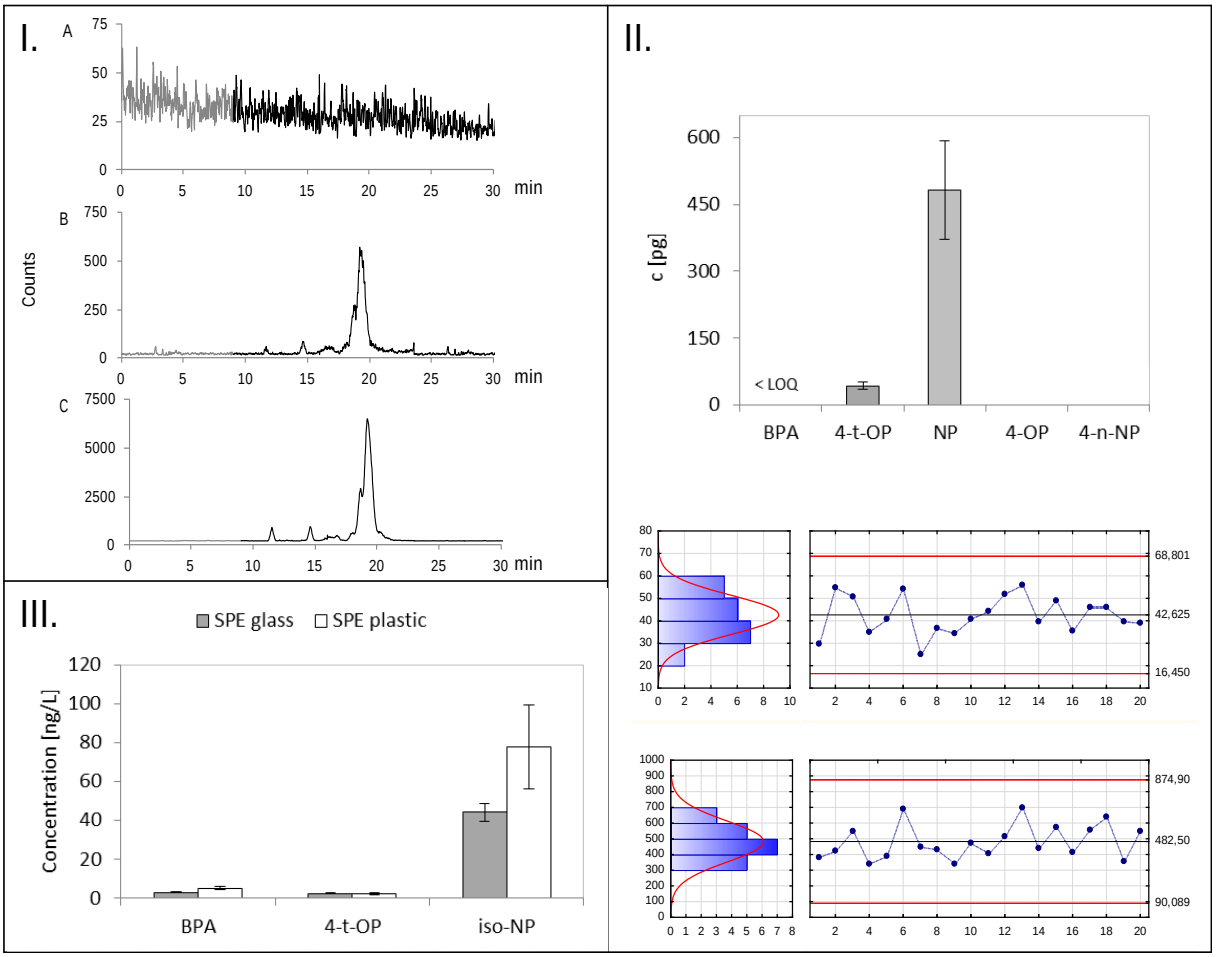
Alkylphenols measurements

Alkylphenols and bisphenol A were measured in 3 levels blank samples such as instrumental blank, reagent blank and method blank. Sample of ultrapure water for HPLC was used for determination of instrumental source contamination (the instrumental blank) during analyses by LC-MS/MS method, as described by Pernica et al. (2015). Analyse of *the reagent blank* was used in this study to assess relationship between the instrumental blank and the method blank. *The reagent blank* was prepared in ultrapure water for HPLC with all of chemicals used for derivatization of alkylphenols by dansyl chloride (DNSC). The derivatization reaction was carried out in a 2 mL amber glass sample vial with 200 µL ultrapure water for HPLC. Fifty microliters of 100 mmol/L $NaHCO_3$ (pH = 10.5) were added and the mixture was stirred (vortex) for 1 minute. Next 200 µL of DNSC in acetone (0.5 mg/mL) were added and stirred again. Subsequently the mixture was incubated for 60 minutes and 60 °C. The reaction mixtures were then cooled to the room temperature and evaporated to dryness under nitrogen atmosphere. The residue was redissolved in 1 mL of methanol and stirred (Pernica et al. 2015). The following procedure was used as the method blank for residue analyses of alkylphenols and bisphenols A. The glass and plastic SPE columns were conditioned with 2 mL of methanol and 2 mL of water (purity for LC/MS). 100 mL of ultrapure water for HPLC was passed through the SPE column using vacuum manifold flow rate 5 mL/min. After extraction, the columns were dried with air for 5 minutes. Elution was performed with 5 mL acetonitrile. The eluate was evaporated and converted into a mini-vial, derivatized with dansyl chloride and analysed by LC/ESI/MS/MS as described by Pernica et al. (2015). The method blank was measured 3 times for glass and plastic SPE columns.

RESULTS AND DISCUSSION

The instrumental blank was under the limit of detection (LOD) for selected compounds, such as bisphenol A (BPA), 4-tert-octylphenol (4-t-OP), mixture of nonylphenol isomers (iso-NP, technical mixture), 4-octylphenol (4-OP), and 4-n-nonylphenol (4-n-NP). Only BPA, 4-t-OP and iso-NP were detected and only 4-t-OP and iso-NP were quantified in the reagent blank. Three analytes were quantified in the method blank, which is about 10-times higher than the counts of chromatogram in the natural reagent blank. Repeatability of the reagent blank was measured once per day for a period of 20 days, when BPA was under of quantification (LOQ). The average concentration of 4-t-OP and iso-NP measured in the reagent blank was 42.6 pg/mL and 482.5 pg/mL, respectively. QC histograms for significant evaluation were used, the critical upper limit and lower limit was not exceeded in any way. Finally, a SPE glass column and SPE plastic column were compared in the method blank. The median concentration of BPA for the glass SPE column was 3.1 ng/L and 5.3 ng/L for the plastic SPE column. No significant differences were found in SPE columns for 4-t-OP. The median concentration of 4-t-OP was 2.45 ng/L for the glass SPE column and 2.42 ng/L for the plastic SPE column. The result of iso-NP was statistically significant with a concentration of 44.3 ng/L for the glass SPE column and 77.8 ng/L for the plastic SPE column (see Figure 2). The plastic SPE columns provided a higher method blank than the SPE glass columns. However, the SPE glass column had better repeatability than plastic materials. The SPE plastic columns provided relatively higher levels of alkylphenols and bisphenol A caused by common contamination of the prepared samples coming from laboratory air, septa vials, and solvents and chemicals used. These sources of contamination cannot be affected. The linear alkylphenols such as 4-OP and 4-n-NP were not present in any blank.

Figure 2 I. Chromatograms of blanks (A) instrumental blank (B) reagent blank (C) method blank. II. Intermittent repeatability (n = 20) of the reagent blank for selected alkylphenols and bisphenol A, include QC histograms for 4-t-OP and iso-NP. III. Comparison of SPE columns made of glass and plastic (n = 3).



CONCLUSIONS

Significant blank contamination is a notorious problem in ultratrace analyses of alkylphenols and bisphenol A, especially when we perform an analysis at concentrations close to the LOQ. A blank contamination may result in a significant increase in the detection limit. A systematic study and analysis of blanks together with analyses of a set of treated and analysed samples is necessary. In order to keep blank contamination as low as possible, the minimization of the number of potential contamination sources is required, such as plastic laboratory containers and plastic aids, water prepared in plastic devices etc., the work in a laboratory devoted solely to analyzes of alkylphenol is the strong requirement.

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REFERENCES

Armbruster, D. A., Pry, T. 2008. Limit of blank, limit of detection and limit of quantitation. The Clinical Biochemist Reviews, 29(Suppl 1): S49.

EPA Government ©2009. Quality Control Tools: Blanks. [Online]. Available at: https://www.epa.gov/sites/production/files/2015-06/documents/blanks.pdf. [2019-09-19].

Pernica, M., Poloucká, P., Seifertová, M., Šimek, Z. 2015. Determination of alkylphenols in water samples using liquid chromatography–tandem mass spectrometry after pre–column derivatization with dansyl chloride. Journal of Chromatography A, 1417: 49–56.

Salgueiro-González, N., Concha-Graña, E., Turnes-Carou, I., Muniategui-Lorenzo, S., López-Mahía, P., Prada-Rodríguez, D. 2012a. Blank and sample handling troubleshooting in ultratrace analysis of alkylphenols and bisphenol A by liquid chromatography tandem mass spectrometry. Talanta, 101:413–419.

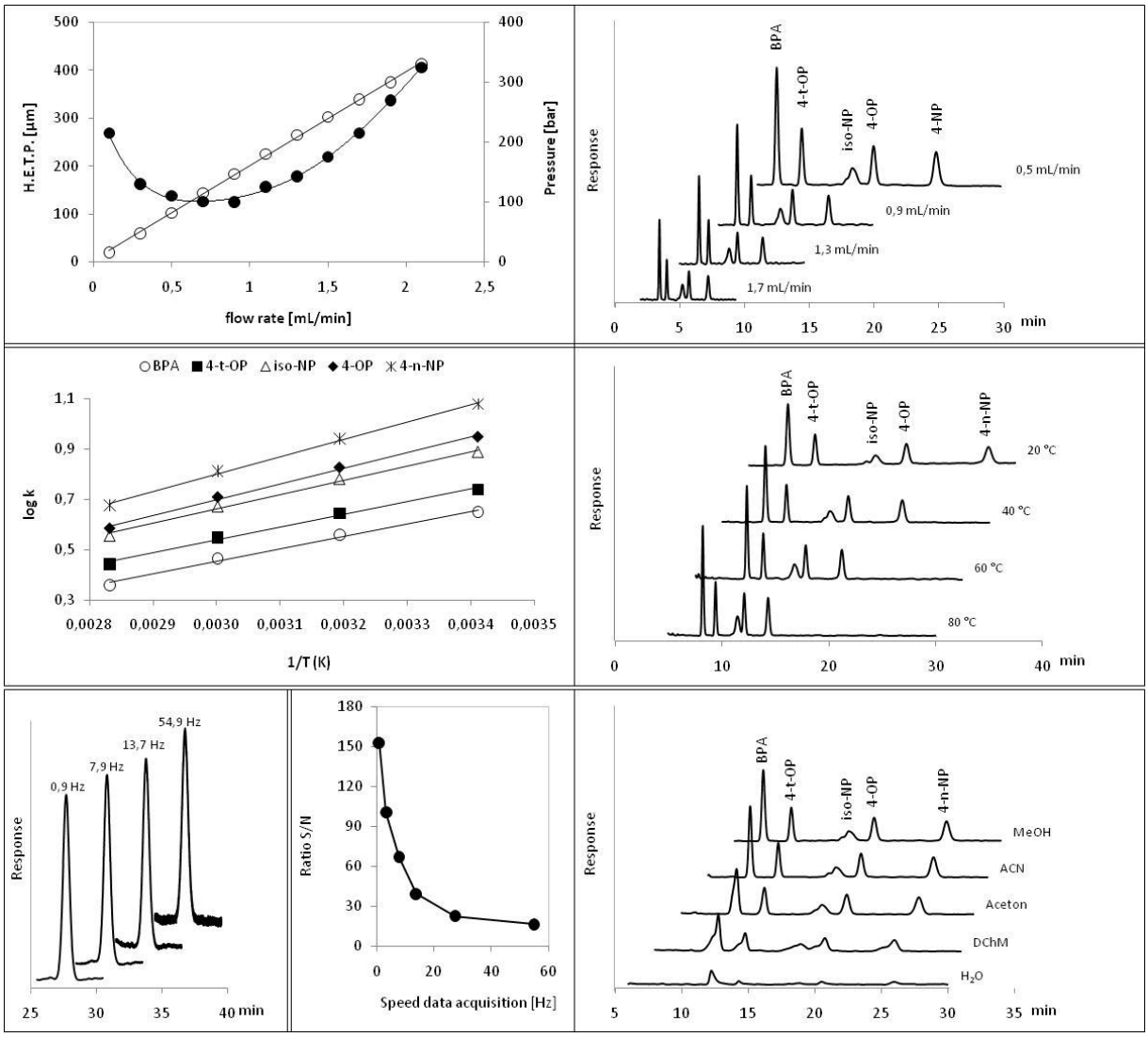
Salgueiro-González, N., Turnes-Carou, I., Muniategui-Lorenzo, S., López-Mahía, P., Prada-Rodríguez, D. 2012b. Fast and selective pressurized liquid extraction with simultaneous in cell clean up for the analysis of alkylphenols and bisphenol A in bivalve molluscs. Journal of Chromatography A, 1270: 80–87.

Williams, S. 2004. Ghost peaks in reversed-phase gradient HPLC: a review and update. Journal of Chromatography A, 1052(1–2): 1–11.

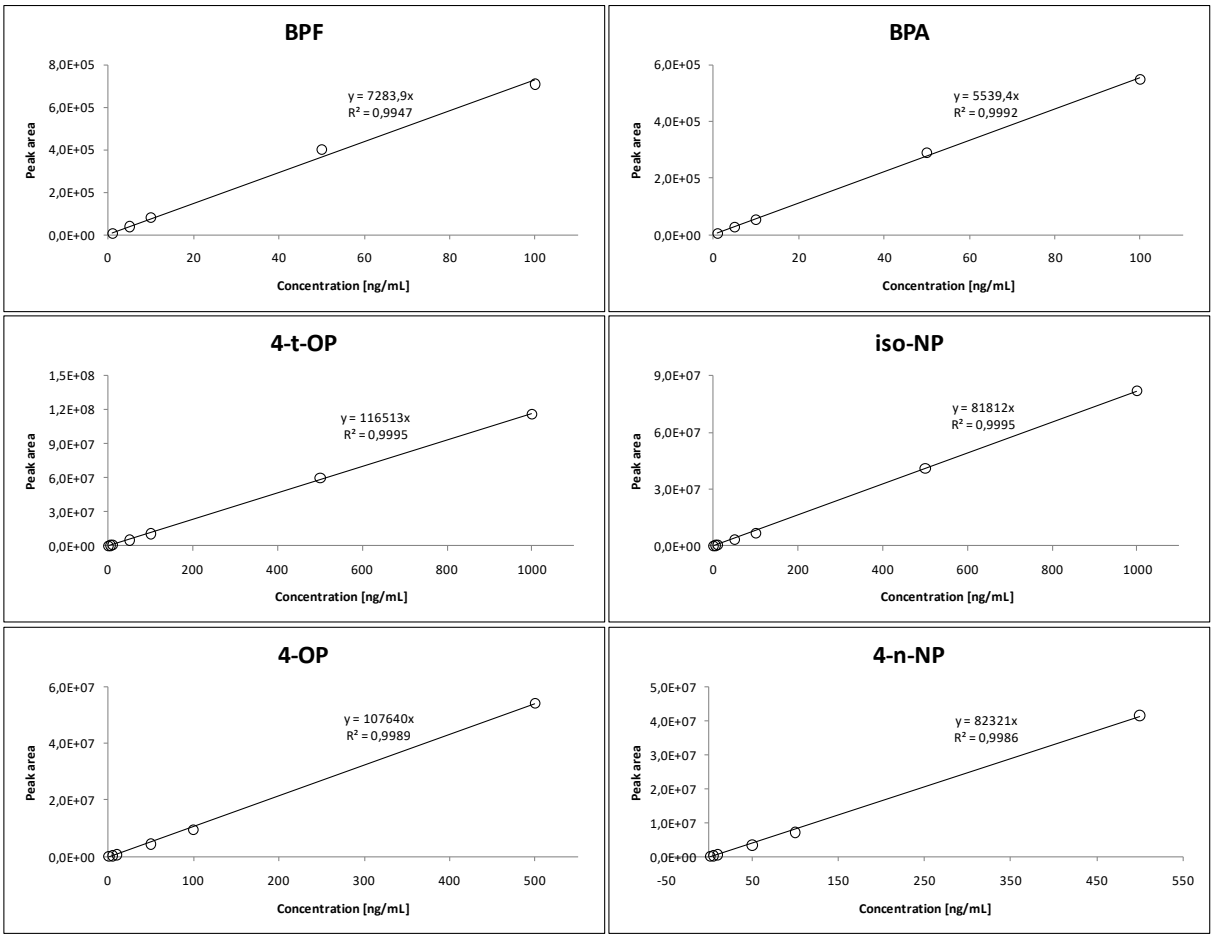
Xie, Z., Selzer, J., Ebinghaus, R., Caba, A., Ruck, W. 2006. Development and validation of a method for the determination of trace alkylphenols and phthalates in the atmosphere. Analytica Chimica Acta, 565(2): 198–207.

APPENDICES

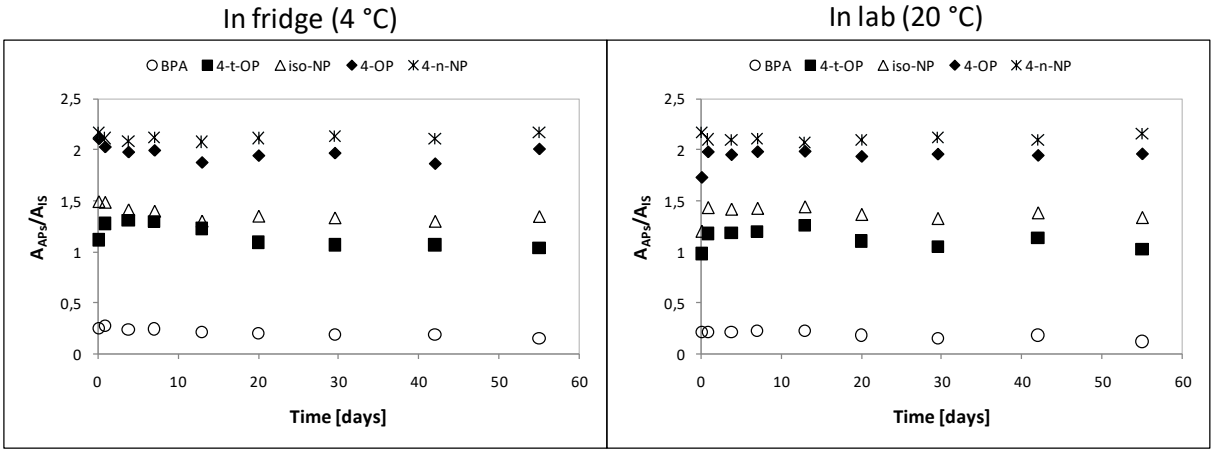
Appendix I: Influence of mobile phase flow, temperature, sample solvent and data collection rate on chromatographic parameters of derivatized alkylphenols.



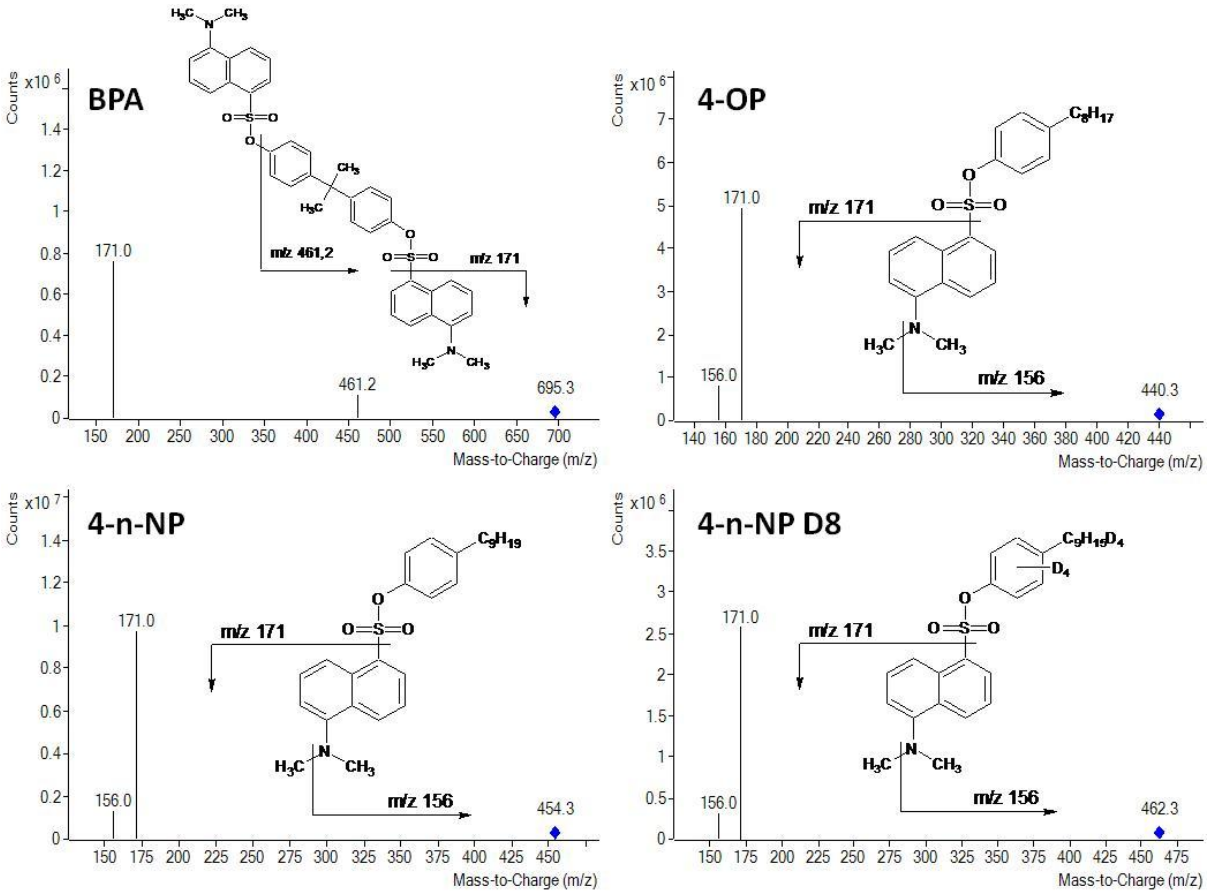
Appendix II: Calibration curves of dansyl-derivatives of alkylphenols and bisphenols.



Appendix III: The stability of the derivatized alkylphenols and bisphenol A in MeOH was studied at room temperature (20 °C) and at refrigerated temperature (4 °C). Two standards of derivatized alkylphenols and bisphenol A at 100 ng/mL were prepared and stored in a refrigerator and in a lab and analyzed at a time interval of 0–60 days.



Appendix IV: Mass spectra of octylphenol (4-OP), nonylphenol (4-n-NP), bisphenol A (BPA) and internal standard (4-n-NP D8).



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Publications

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Rajasärkkä, J., **Pernica, M.**, Kuta, J., Lašňák, J., Šimek, Z., & Bláha, L. (2016). Drinking water contaminants from epoxy resin-coated pipes: A field study. *Water Research, 103*, 133-140.

Prochazkova T., Sychrova E., Vecerkova J., Javurkova B., Otoupalikova A., **Pernica M.**, Simek Z., Lepsova-Skacelova O., Hilscherova K. (2018) Estrogenic activity and contributing compounds in stagnant water bodies with massive occurrence of phytoplankton. *Water Research,* 136, 12–21

Pernica, M., Piacentini, K. C., Benešová, K., Čáslavský, J., & Běláková, S. (2019). Analytical techniques for determination of mycotoxins in barley, malt and beer: A review. *KVASNY PRUMYSL*, *65*(2), 46-57.

Piacentini, K. C., Běláková, S., Benešová, K., **Pernica, M.**, Savi, G. D., Rocha, L. O., & Corrêa, B. (2019). Fusarium mycotoxins stability during the malting and brewing processes. *Toxins*, *11*(5), 257.

Pernica, M., Lesniaková, R., Doležalová, D., & Šimek, Z. (2019). Analysis of alkylphenols and bisphenols in soils using liquid chromatography-tandem mass spectrometry. *International Journal of Environmental Analytical Chemistry*, 1-15.

Pernica, M., Piacentini, K.C., Bosko, R., & Belakova, S. (2019) The determination of deoxynivalenol and zearalenone in barley from Brazil and malted barley. *MendelNet,* 26, 596-600.

Pernica, M., & Simek, Z. (2019) An analysis of residue alkylphenols and bisphenols A using liquid chromatogramy-tandem mass spectrometry. *MendelNet,* 26, 601-605.

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