Analysis of bisphenols and bisphenol A diglycidyl ethers by stable isotope dilution assay liquid chromatography-tandem mass spectrometry

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Abstract

Bisphenol A (BPA) and bisphenol A digycidyl ether (BADGE) are widely applied chemicals, frequently used in food packaging and thermal paper production. From packaging materials they show a high potential to migrate into foodstuffs. Their precise analysis is crucial to maintain product safety and consumer protection. For bisphenol analysis, BPA-d_{16} and $^{13}C_{12}$-BPA are commonly applied. Isotope standards for further bisphenols such as BPF and BPS, as well as for bisphenol A diglycidyl ethers (BADGEs) were hardly available.

Within the first project, the isotope standards BPA-d_4, BPF-d_4, and BPS-d_4 were synthesized and applied for quantification of BPA, BPF, and BPS from thermal paper in form of cash register receipts. 13 out of 14 receipts contained BPA, while the 14th one contained BPS in comparable amounts. Thus, the conclusion was drawn that in Germany no general substitution of BPA in this application field occurred until 2014.

Within the second project, BADGE-d_4, BADGE·H_2O-d_4, and BADGE·$2H_2O$-d_4 were synthesized, to complement the set of standards for the aimed application: Determination of nine bisphenols and BADGEs, including BPA, BPF, and BPS as well as BADGE with its two hydrates and three chlorohydrins, from canned beers. A sensitive method for the simultaneous LC-MS/MS analysis of bisphenols and BADGEs in one run could be established. A combined analysis of both substance groups applying mass spectrometric detection was presented for the first time. All 14 beers examined were tested positive for BPA and BADGE·$2H_2O$, respectively, which is consistent with other studies. Several findings of BPF in small concentrations were surprising. Other substances were found in marginal concentrations only, complying with given specifications from European law.

Within the third project, the aforementioned isotope standards were applied to realize a more technical approach: Development of a LC-MS/MS rapid test for quantification of the lead analytes BPA and BADGE·$2H_2O$, along with BPF and BPS from beverages. By this, previously observed sources of contamination were avoided by combining a minimalistic in-vial sample workup with an automated dilute-and-shoot technique using the autosampler. Workup consisted of sample transfer and degassing, followed by aspiration, spiking, and dilution of the sample in the needle of the LC-MS/MS system. For beer and mixed beer samples examined, results were achieved in accordance with project two, verifying, in addition to recovery experiments, the suitability of the rapid test. Alcopops on cola basis examined, showed increased BPA and BADGE·$2H_2O$ concentrations, mainly assumed to be a consequence of higher alcohol content.

In conclusion, the six synthesized isotope standards applied in the developed LC-MS/MS methods enable a fast, sensitive, precise and robust bisphenol determination. Quantification and proof of absence of bisphenolic compounds from different matrices are essential for food monitoring and assurance of consumer safety.

Zusammenfassung

Bisphenol A (BPA) und Bisphenol-A-diglycidylether (BADGE) sind weltweit eingesetzte Chemikalien, die oft zur Fertigung von Lebensmittelverpackungen oder Thermopapier verwendet werden. Aus Lebensmittelkontaktmaterialien zeigen sie ein hohes Migrationspotential, was sodann zur Kontamination des Lebensmittels führt. Eine präzise Bisphenolanalytik ist essentiell, um Lebensmittelsicherheit und Verbraucherschutz zu gewährleisten. Im Rahmen der Analytik werden BPA-d_{16} und $^{13}C_{12}$-BPA weitverbreitet eingesetzt. Isotopenmarkierte Standards für weitere Bisphenole, wie BPF und BPS, sowie für BADGEs sind nur eingeschränkt kommerziell verfügbar.

Im Rahmen von Projekt 1 wurden die Isotopenstandards BPA-d_4, BPF-d_4 und BPS-d_4 synthetisiert und zum Nachweis von BPA, BPF und BPS aus Thermopapier, insbesondere aus Kassenzetteln eingesetzt. 13 von 14 Kassenzetteln wurden dabei positiv auf BPA getestet, der verbleibende Kassenzettel enthielt BPS in vergleichbarer Menge. Dieser Befund ermöglichte die Schlussfolgerung, dass BPA auf Thermopapier in Deutschland bis 2014 nicht nennenswert ersetzt wurde.

Im Rahmen von Projekt 2 wurden die vorherigen Isotopenstandards um die Substanzen BADGE-d_4, BADGE·H_2O-d_4 und BADGE·$2H_2O$-d_4 ergänzt, um die geplante Anwendung zu realisieren: Der Nachweis von neun Bisphenolen und BADGEs aus Dosenbier. Namentlich wurden die Substanzen BPA, BPF und BPS, sowie BADGE, die beiden BADGE-Hydrate und die drei BADGE-Chlorhydrine untersucht. Eine empfindliche Methode für den gemeinsamen Nachweis der beiden Substanzgruppen wurde etabliert; unter Anwendung massenspektrometrischer Detektion, gibt es keine vergleichbaren Veröffentlichungen. Alle 14 untersuchten Biere enthielten BPA und BADGE·$2H_2O$. Diese Befunde werden durch andere Studien gestützt. Der mehrheitliche Nachweis von BPF-Spuren war überraschend. Weitere Substanzen wurden in Spuren nachgewiesen, wobei dabei keine Probe als auffällig im Sinne der EU-Gesetzgebung zu bewerten war.

Im Rahmen von Projekt 3 wurden die vorab synthetisierten Isotopenstandards zur Umsetzung eines eher technischen Unterfangens eingesetzt: Der Entwicklung eines LC-MS/MS-Schnelltests zum Nachweis der Leitsubstanzen BPA und BADGE·$2H_2O$, zusammen mit BPF und BPS aus Getränken. Kontaminationen konnte in diesem Projekt während der Probenaufbereitung erfolgreich entgegengewirkt werden. Dies wurde durch eine minimalistische In-Vial-Probenaufbereitung mit anschließender automatischer Weiterverarbeitung (Verdünnen, Zusatz von Isotopenstandards, Injizieren) durch den Autosampler realisiert. Die Untersuchungsergebnisse für Bier und Mixed-Bier, spiegelten die Ergebnisse aus Projekt 2 wider, was zusammen mit den durchgeführten Wiederfindungsversuchen die Eignung der Methode für die Getränkeuntersuchung belegt. Alkopops auf Cola-Basis zeigten erhöhte Ergebnisse für BPA und BADGE·$2H_2O$. Dies wird primär auf den erhöhten Alkoholgehalt zurückgeführt.

Abschließend kann gesagt werden, dass die synthetisierten Isotopenstandards zusammen mit den zugehörigen LC-MS/MS-Methoden eine schnelle, präzise und robuste Bisphenolanalytik ermöglichen. Quantifizierung, aber auch Ausschluss, von Bisphenolen und BADGEs aus komplexen Matrices sind zwei wichtige Anwendungsfelder in Lebensmittelüberwachung und Verbraucherschutz, die hiermit bedient werden können.

1 Introduction

Bisphenol A (BPA) has attracted attention from scientists, politicians and the general public for decades. The number of scientific publications related to bisphenol A (BPA) stagnated at an average of 17,000 a year from 2010 to 2014, while from 2000 to 2004 numbers of publications constantly increased from 4,380 to 7,630[1]. An overview on relevant data will be given in the following sections.

1.1 Application fields of bisphenol A and bisphenol A diglycidyl ether

There is a vast number of bisphenol applications. The main use of BPA is as a monomeric starting substance in the synthesis of plastic materials. Two relevant fields to be examined in detail are thermoplastic materials and epoxy resins on bisphenol basis. Thermoplastic materials made from BPA are for example polycarbonate and polysulfone (Figure 1). Both are synthesized by polycondensation of BPA with chlorine containing reaction partners. Application for those plastics are manifold: bottles, including infant feeding bottles, mouth guards, (swimming) glasses, dishes, CDs, DVDs, and Blu-ray disks are only some distinctive examples of BPA containing plastic ware.

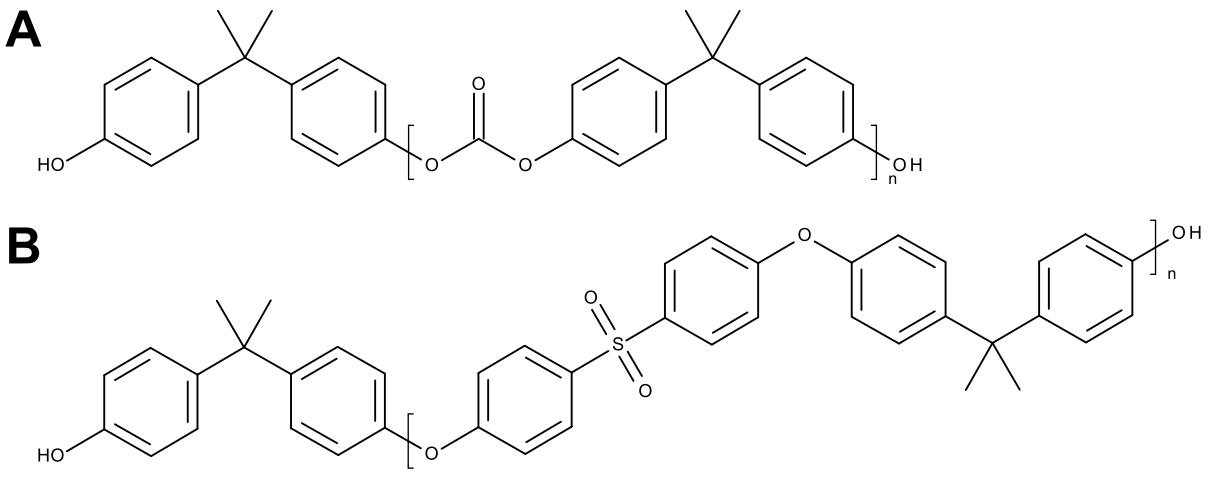

Figure 1 Structures of polycarbonate (**A**) and polysufone (**B**), both synthesized using BPA as a starter monomer

For common epoxy resins, BPA also serves as a starter monomer. In a first step BPA is reacted with epichlorohydrin to form bisphenol A diglycidyl ether, commonly abbreviated as BADGE. Two equivalents of BADGE can co-polymerize with one equivalent of BPA,

[1] Determined by use of google scholar, search terminus “bisphenol a”.

respectively, to form longer-chained epoxy resins (Figure 2). Those resins are commonly applied as basis for linings in food packaging but also in tanks and water pipes. A major application of epoxy resins is their use in beverage cans, which will be discussed in detail within this work.

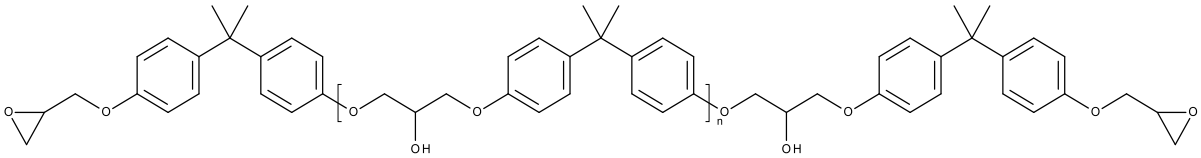

Figure 2 Structure of an epoxy resin produced by co-polymerization of one equivalent of BPA with two equivalents of BADGE (for n = 1)

Another key application of BPA is its use in thermal paper production. Here, in contrast to aforementioned applications, BPA is applied in its monomeric form and acts as developer. This means, that BPA provides a proton, which is needed to initiate the transformation of the leuco dyestuff from its white/invisible form to its colored/visible structure during heating process [1, 2].

1.2 Metabolic pathways

The various applications of BADGE and BPA pose a risk of migration/leaching from materials. In plastics, such as polycarbonate and expoxy resins, a certain amount of unreacted BADGE and BPA remains after polymerization. Those monomers were shown to migrate into foods and beverages. Hence, the main route of exposure for BPA and BADGE uptake is ingestion from food and beverages. Other routes of exposure as trans-dermal-absorption, e.g. from thermal paper [3, 4], inhalation, e.g. from lacquers [5] or dust [6] are considered to contribute a rather small amount to the overall exposition.

Since BPA uptake and metabolism is discussed extensively and species differences seem to be relevant, in the presented work focus is set on studies regarding the metabolic pathway in humans, rather than in rodents or other species.

1.2.1 The fate of BPA in the human organism

After ingestion and absorption by the gastrointestinal tract BPA is metabolized almost completely by human liver microsomes. The BPA-monoglucuronide was proven to be the main metabolite of BPA [7–9]; additionally sulfate conjugates, and sulfate/glucuronide

diconjugates have been identified [8]. From the liver, the conjugates are then transported to plasma which enables renal clearance and excretion through the urine [7, 10]. While BPA-glucuronide is recognized as being biological inactive, unbound or so-called “free” BPA is claimed to cause several adverse health effects. Plasma concentrations of total BPA are reported to be in ng/ml range [11]. The detection rate of BPA accounted for 90 % in adipose fat samples [12].

Urinary concentrations of free and total BPA have been determined from different proband groups [5, 13–16], including children [17, 18], pregnant women [16, 18, 19] and neonates [9]. Sometimes standardization to creatinine was performed, but this is not consistent between the studies. This is a great handicap in comparison and interpretation of the published data. Summarizing, it can be said, that usually the level of free BPA in urine lays below limit of quantification in pg/ml range, while the level for total BPA (after deconjugation) is in the one-digit ng/ml range. Detection rate for total BPA accounts for approximately 90 to 100 %, while quantifiable amounts of free BPA were reported as below 15 % [20].

1.2.2 The fate of BADGE in the human organism

For BADGE the initial position is a bit more complicated. Since BADGE possesses two highly reactive epoxy groups, it already shows a series of reactions before ingestion. Hence, more than one metabolic pathway including multiple degradation/transformation products and their metabolism must be considered. So far there is no exhaustive information on the metabolic pathways of BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, and BADGE·HCl, as well as further BADGEs – mainly peptide and protein bound ones were reported to be significant and of high number [21, 22]. In the following the pathway of BADGE is outlined.

In aqueous media BADGE is easily hydrolyzed in a two-step reaction: Initially BADGE is converted into BADGE·H_2O. Since BADGE H_2O is a rather unstable and reactive intermediate it is further hydrolyzed into BADGE·2H_2O [23, 24].

BADGE hydrolysis described above is in accordance to data from plasma [12] and urine [25–27]. In both liquids BADGE·2H_2O was found in high concentrations compared to the amounts of BADGE and further BADGE derivatives (in focus of this work). A median concentration of

7.2 ng/ml BADGE·2H_2O was quantified in plasma, in relation to 2.3 ng/ml BADGE·H_2O and below LOQ for BADGE, BADGE·H_2O·HCl, BADGE·2HCl, and BADGE·HCl [12]. In urine 0.6 ng/ml BADGE·2H_2O were determined, in comparison to concentrations of 0.1 ng/ml BADGE, BADGE·H_2O, and BADGE·H_2O·HCl (the chlorohydrins of BADGE where not examined in this study) [26]. But it is even more complicated: Applying enzymatic deconjugation through application of β-glucuronidase, it could be proven that for all examined analytes additionally conjugated precursors were present. The molecular weight of BADGE·2H_2O-monoglucuronide accounts for 552.6 Da. This is around the molecular size cutoff for urinary excretion in the human organism (470 Da [28] to 550 Da [29]). Hence, next to renal excretion, there is a possibility for biliary excretion of BADGE·2H_2O-glucuronide. Additionally, enterohepatic circulation has to be considered [26] and – from rodents – was included in risk assessment of BADGE [30]. Summarizing, it must be underlined, that within aqueous food and beverage systems BADGE is hydrated at an early stage to BADGE·2H_2O. Thus, concentrations of BADGE·2H_2O and BADGE·2H_2O-glucuronide in urine and plasma probably derive from ingestion of BADGE·2H_2O rather than from ingestion of native BADGE. Detection rates in urine of 90 % [27] to 100 % [26] for total BADGE·2H_2O, lay within the detection rates determined for BPA and BPA-glucuronide. For plasma detection rates of 70 % were shown for free BADGE·2H_2O [12].

Detection rates and partition in adipose fat [12] differed from those determined in plasma and urine samples. Here, next to BADGE·2H_2O (detection rate of 60 %), all other BADGEs could be detected (detection rates from 5 to 25 % for BADGE·HCl = BADGE·2HCl < BADGE·H_2O < BADGE·H_2O·HCl < BADGE). These findings prove the accumulation of unpolar derivatives in adipose tissue. To draw a conclusion regarding persistence of the substances, further studies need to be accomplished.

1.3 Adverse health effects and risk assessment of bisphenolic compounds

For most of the bisphenolic compounds examined within this study, a comprehensive risk assessment has been accomplished. Within those assessments, the first step is a hazard evaluation based on scientific data relating to toxicity in animal and human studies. The second step consists of risk characterization which is focused on clarification of the impact of the observed hazard on human health. Current consumption data and intake through

exposure routes such as ingestion, inhalation, or skin contact are major building blocks of hazard evaluation.

1.3.1 The endocrine system and endocrine disrupting chemicals (EDCs)

Numerous functions within the human and wildlife organism are regulated by second messengers also called hormones. Hormones are produced by different glands – organs or tissues – and mostly transported by blood stream to their target organs. Here, they regulate processes on biochemical and physiological level [31]. Within the endocrine system hormones affect processes as growth, metabolism and sexual development. One of the most significant sexual hormones is 17ß-estradiol, which serves as reference in various test systems concerning hormone activity. Next to biosynthesized hormones from within the organism, also external substances can have an impact on the endocrine system. If they cause adverse health effects, they are assigned to the inhomogeneous group of endocrine disrupting chemicals (EDCs). Widely present man-made EDCs in daily life are for example phthalates and polychlorinated biphenyls (PCBs). BPA is also recognized as EDC [32, 33]. Next to anthropogenic substances also natural substances (phytoestrogens) can mimic hormone-like action. Isoflavones for example are contained in plants as flaxseeds, soybeans, and hops [34, 35] and can enter the food-chain despite processing. Most EDCs and phytoestrogens show structural similarities to 17β-estradiol.

1.3.2 Assessment of bisphenols

BPA has been shown to cause estrogenic, anti-estrogenic, androgenic, and anti-androgenic effects by different test systems *in vivo* and *in vitro* [36–39]. Compared to 17β-estradiol, hormone activity of BPA is described as rather low. Nevertheless, in 2011 a law was introduced to ban the usage of BPA in polycarbonate infant drinking bottles [40]. In 2015, a further and complete risk assessment has been concluded by the European Food Safety Authority (EFSA) [41]. Reported, and accepted by EFSA, adverse health effects of BPA are the following: toxicological effects on liver and kidneys, effects on the mammary glands of rodents [42], and disturbance of fertility and development. But, those effects are underlined to occur only if exposition is inflated a factor 10,000 in relation to the temporary tolerable daily intake (tTDI) of 4 µg/kg body weight/day. Some scientists on the other side report, that for BPA a so-called low dose effect has been observed [33], which stands in conflict to the current European risk assessment, which is based on a conservative dose-response approach

– “the dose makes the poison”. Recent cross-sectional-studies related increased urinary levels of BPA to e.g. heart disease in adults [33] and obesity in children and adolescence [34]. Concerning consumption, an effect between urinary level and high consumption of canned fish could be shown [18], which corresponds with proven migration rates for BPA from tuna cans using aqueous food simulants [43]. Also soda, school lunches, and meals prepared outside the home were proven to be related to extended BPA uptake [44]. From Spain inflated BPA urinary concentrations were proven for “pregnant women who were younger, less-educated, smoked, and who were exposed to second-hand tobacco smoke […] BPA concentrations were also higher in children exposed to second-hand tobacco smoke” [18]. As a matter of fact, this study indicates that adverse health effects might be wrong-correlated to BPA only. Additionally, the lifestyle summarized for this group of test persons, might e.g. include a high consumption of plastic packed or canned fast food, which certainly contains raised levels of BPA. Thus, it is very well possible, that a lot more factors, e.g. from nutrition, play a crucial role, but are not included all-encompassing in cross-sectional studies.

Compared to BPA, data on BPF and BPS is scarce, thus, as potential substitutes of BPA, question was if they are less hazardous alternatives? Hormone activity of BPS and BPF was confirmed to be comparable to hormone activity of BPA, resulting equally in estrogenic, antiestrogenic, androgenic, and antiandrogenic effects *in vitro* and *in vivo* [36, 45–47]. Thus, it can be concluded that substitution of BPA using BPS or BPF, seems only limited reasonable. Recently a complete risk assessment for BPF has been completed, due to findings in the ones mg/kg level of BPF in white and yellow mustard [48]. In this report it was concluded, that from regular consumption of BPF contained in mustard, considering the actual scientific data, only a marginal risk is expected for human health. The knowledge on the existence of BPF from biosynthesis is new and might represent a milestone in the ongoing discussion of bisphenols.

1.3.3 Assessment of BADGEs

Concerning toxicology, for BADGE the absence of reproductive, developmental, endocrine and carcinogenic effects was described [49]. Based on the evaluated data, a clear dependence was seen between BADGE and its hydrates. Thus, for the sum of BADGE and its two hydrates a TDI of 150 µg/kg body weight and day was defined [30, 50]. Additionally,

specific migration levels (SML) were established accounting to 9 mg/kg food or food simulant for BADGE and BADGE hydrates and for 1 mg/kg for BADGE chlorohydrins [50].

1.4 Analytics: Determination of bisphenols and BADGEs

Measurement of bisphenolic compounds is challenging, since application fields for BPA and BADGE are wide spread and relevant matrices are usually complex. Matrices include urine, plasma and tissues from the biomonitoring sector; foodstuffs and beverages in regard to food safety and consumer protection; as well as polymers or plastic products, e.g. within quality control. Hence, BADGE and BPA migrates are often determined from the same samples and their origin, mostly epoxy resins, is known to be identical. Nevertheless, co-determination of the two substances (in combination with the other BADGEs) is rare. Bisphenols can be determined well by gas chromatography, mostly after derivatization procedure [13, 51, 52, 52, 53] or liquid chromatography, applying mass spectrometric [11, 15, 27] or fluorescence detection [54–59] [59]. BADGEs on the other side possess a rather low volatility (for GC measurement) and thus, are more likely measured by LC-MS/MS [11, 60]. Determination of bisphenols and BADGEs in one run is unusual and was only performed by LC fluorescence from food samples [54, 55, 57, 59]. One method states the determination of BPA and BADGE·$2H_2O$ from plasma by LC-MS/MS [11].

Frequently, analytes are concentrated by sample work-up using solid phase extraction [13, 53, 53, 54] or (dispersive) liquid-liquid extraction [58, 61–63] to make them accessible for instrumental analysis. At best, such techniques concentrate the target analyte, while separating it from disturbing matrix compounds at the same time. But they also bear the risk, that target analyte is lost during sample processing. Here, application of isotopically labeled standards is advantageous. Stable isotope dilution assay presents a powerful tool for quantification of bisphenolic compounds from foods, beverages, and biomatrices as urine, plasma, and fatty tissue. Talking of biomatrices, isotopically labeled standards also open up possibilities for better elucidation of metabolic pathways in vivo [7]. BPA-d_{16} and $^{13}C_{12}$-BPA are the two commonly used isotope standards for bisphenol analysis [12, 13, 15, 20, 64]. Both have been commercially available for some time; additionally, synthesis of BPA-d_{16} was published [52]. Standards of further bisphenols and BADGEs (as e.g. BADGE-d_6 [12]) have been lacking in former times; some entered the market recently. Prior to that, matrix

matched calibration (including standard addition) was applied for analysis [54, 62, 65] or isotope standards were self-synthesized [22, 51, 52].

2 Scopes and objectives

Project 1:

Replacement of BPA is mainly caused by law changes and suspiciousness of the general public. In various applications, BPF and BPS are potential substitutes. Consequently, for reasons of food safety and consumer protection, analytic systems have to be adjusted or need to be developed for control of these substances. Thus, the aim of the first project was to synthesize d_4-isotope standards for the analytes BPA, BPF, and BPS and apply them for determination from thermal paper.

Project 2:

Literature reflected an urgent need for a simultaneous analysis of the two analyte groups of bisphenols and BADGEs. While combined determination from canned food samples, which contain comparable high amounts of the target analytes, is possible by HPLC fluorescence, measurements in the field of beverages or biomonitoring are more demanding. Here, both analytes groups are analyzed separately by LC-MS/MS to achieve the needed sensitivity. Hence, the aim of the second project was first to complement the bisphenol isotope standards through BADGE-d_4, BADGE-H_2O·d_4, and BADGE·$2H_2O$-d_4 and second to develop a LC-MS/MS method for sensitive and simultaneous determination of bisphenols and BADGEs (also including the BADGE chlorohydrins).

Project 3:

The idea of this project derived from certain pitfalls observed within the former projects as well as the literature: Here, frequently leakages of BPA and BADGE, from laboratory equipment, solvents, and other sources were reported. A relation between elaborate sample workup and contamination risk is obvious. Therefore, the aim of the third project was to establish a sample workup of minimalistic design in regard to glassware and solvents used. Further sample processing was intended to be performed automatically. Since not all examined analytes from project two were of urgent relevance in beverages, decision was made to reduce the target analytes for this project to the lead analytes for aqueous media, BPA and BADGE·$2H_2O$, as well as the potential BPA substitutes BPF and BPS.

3 Project I: Stable isotope dilution assay of bisphenol A, bisphenol F, and bisphenol S from thermal paper using high-performance liquid chromatography-tandem mass spectrometry

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Summary

A stable isotope dilution assay (SIDA) was developed to quantify bisphenol A (BPA), bisphenol F (BPF), and bisphenol S (BPS) from thermal paper by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The SIDA was verified for BPA, BPF, and BPS resulting in average recoveries from 106 to 115%. d_4-Isotope standards were applied to determine bisphenols contained on supermarket receipts (*n* = 14) from Berlin, Germany. BPA values of 0.07 to 20.1 mg/g paper weight were determined. One receipt contained 6.43 mg/g BPS; BPF was not detected. Synthesis of 2,6-dideuterio-4-[1-(3,5-dideuterio-4-hydroxy-phenyl)-1-methyl-ethyl]phenol (BPA-d_4), 2,6-dideuterio-4-[(3,5-dideuterio-4-hydroxy-phenyl)methyl]phenol (BPF-d_4), and 2,6-dideuterio-4-(3,5-dideuterio-4-hydroxy-phenyl)sulfonyl-phenol (BPS-d_4) is described. BPA and BPF were labeled directly via acid induced hydrogen-deuterium exchange reaction in deuterium oxide, while BPS was deuterated by labeling 4-(4-hydroxyphenyl)sulfanylphenol followed by oxidation of the intermediate.

Zusammenfassung

Eine Stabilisotopenverdünnungsanalyse (SIDA) zum Nachweis von Bisphenol A (BPA), Bisphenol F (BPF) und Bisphenol S (BPS) aus Thermopapier mittels Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS) wurde entwickelt. Die SIDA wurde mit Wiederfindungen zwischen 106 und 115% als geeignet zur Quantifizierung von BPA, BPF und BPS aus Papier nachgewiesen. Die entwickelte Methode wurde zur Bestimmung von Bisphenolgehalten in Kassenzetteln (*n* = 14) aus Berlin, Deutschland angewandt. Für BPA

[2] Author contributions: J.Z. and L.-A.G. designed research; J.Z. performed synthesis; A.M. performed NMR measurements; C.C., K.N., and L.-A.G. supported in the field of synthesis; J.Z. and A.-K.S. performed analytics; J.Z. analyzed data; J.Z. wrote the paper

wurden dabei 0,07 bis 20,1 mg/g Papier quantifiziert. Ein Kassenzettel enthielt 6,43 mg/g BPS; BPF wurde nicht nachgewiesen. Die Synthesen von 2,6-Dideuterio-4-[1-(3,5-dideuterio-4-hydroxy-phenyl)-1-methyl-ethyl]phenol (BPA-d_4), 2,6-Dideuterio-4-[(3,5-dideuterio-4-hydroxy-phenyl)methyl]phenol (BPF-d_4) und 2,6-Dideuterio-4-(3,5-dideuterio-4-hydroxy-phenyl)sulfonyl-phenol (BPS-d_4) werden beschrieben. BPA und BPF wurden mittels säurekatalysierter Wasserstoff-Deuterium-Austauschreaktion in Deuteriumoxid markiert. BPS-d_4 wurde durch Deuterierung von 4-(4-Hydroxyphenyl)sulfanylphenol und anschließender Oxidation des Zwischenprodukts dargestellt.

3.1 Introduction

Bisphenol A (BPA; 4-[1-(4-hydroxyphenyl)-1-methyl-ethyl]phenol) is topic of scientific, political, and public concern. In the European Union a temporary tolerable daily intake (t-TDI) of 4 µg/kg body weight per day was defined (January 2015) [41]. Changing laws [40, 66] and the increasing suspiciousness in BPA in public enforce the search for substitutes [67] and lead to replacement of BPA[3]. Bisphenol F (BPF; 4-[(4-hydroxyphenyl)methyl]phenol) and Bisphenol S (BPS; 4-(4-hydroxyphenyl)sulfonylphenol) as structural related compounds might be adequate [2, 67]. An increasing demand on BPA analytics in food contact materials is seen and might rise in 2015 (law changes in France)[1].

Earlier exclusively bisphenol absorption via oral intake was examined, mainly focusing on BPA. Nowadays additional sources like aspiration [6, 13, 41, 68, 69] and contact exposure [3, 4, 41] are studied. Sources for aspiration can be uptake from the air or from household dust. Trans-dermal absorption is mainly caused from handling thermal paper, especially from supermarket receipts. BPA is used as developer in thermal paper. In the color active layer of the paper the weak acidic bisphenol serves as proton donor causing the leuco dyestuffs, mostly spiro lactones, to undergo a structural change from the white/invisible to the visible molecule upon heating [1, 2]. Reported findings of BPA from thermal paper receipts vary around the milligram per gram range [3, 70, 71]. About 1 µg of BPA is transferred to the fingers while handling a receipt [3]. Biotransformation of BPA during trans-dermal passage accounted for 27% of the applied dose in human [4]. These facts evidence that exposition through contact is high and that trans-dermal absorption of BPA adds significantly to the

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total bisphenol exposition of humans. Free and therefore reactive BPA acts as endocrine disrupting chemical in the human organism [32, 72].

For analysis of bisphenols liquid or gas chromatography coupled with mass selective detectors (LC-MS; GC-MS) are common. To obtain precise and accurate results the use of isotope standards is advantageous. Synthesis and application of BPA-d_{14} is described [52], commercial standards from d_6 to d_{16} are available. In chromatography highly deuterated standards often show isotope effects resulting in separation of analyte and standard. This might result in different interferences for analyte and standard. For quantification of bisphenolic compounds next to BPA matrix matched calibration [53] or BPA isotope standards [6, 73] as internal standard are used; implying that there is a demand for isotope standards. The aim of the study presented was to develop d_4-labled standards for BPA, BPF, and BPS. Isotope standards and analytes are supposed to co-elute within application experiments. The standards should be applicable for determination of bisphenolic compounds using gas or liquid chromatography with mass selective detection.

Bisphenol content on German supermarket receipts was not published (as at October 2014). Thus for application of the isotope standards BPA, BPF, and BPS were quantified by SIDA-LC-MS/MS from receipts randomly collected in stores in Berlin, Germany.

3.2 Experimental

3.2.1 Chemicals and reagents

Synthesis: Bisphenol A, bisphenol S, 4-(4-hydroxyphenyl)sulfanylphenol, and deuterium oxide were purchased from Sigma-Aldrich (Steinheim, Germany). Bisphenol F was obtained from Alfa Aesar (Karlsruhe, Germany). Diethyl ether was purchased from Merck (Darmstadt, Germany), distilled prior use and stored over potassium hydroxide. Further solvents and salts were obtained from different suppliers and used without purification. NMR: Dimethylsulfoxide-d_6 and acetone-d_6 were purchased from ABCR (Karlsruhe, Germany), trichloromethane-d_1 was purchased from Roth (Karlsruhe, Germany). GC-MS: BSTFA was purchased from Sigma-Aldrich (Steinheim, Germany), Helium 5.0, (99.9990% purity) was purchased from Air Liquide GmbH (Berlin, Germany). LC-MS/MS: Acetonitrile (LC-MS grade) was purchased from VWR International GmbH (Darmstadt, Germany). Methanol (HPLC grade) and syringe filters (nylon, 0.2 µm) were purchased from Roth (Karlsruhe, Germany).

Milli-Q water was provided through a Synergy® UV ultrapure water system from Millipore Corporation (Molsheim, France) after a rinsing period of 15 min.

3.2.2 Synthesis of 2,6-dideuterio-4-[1-(3,5-dideuterio-4-hydroxy-phenyl)-1-methyl-ethyl]phenol (BPA-d_4)

1 g BPA (4.4 mmol), 10 g deuterium oxide, and a droplet of H_2SO_4 (96%) were stirred and heated in a closed steel vessel in a high-pressure laboratory autoclave (Roth, Karlsruhe, Germany) for 2 h at 160 °C. After cooling to room temperature for 1 h the raw product was poured into 50 ml of aqueous $NaHCO_3$-solution (saturated) and extracted with diethyl ether (2 x 25 ml). The combined organic phases were dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The dried residue was resuspended and purified by column chromatography (20 g silica gel 60, petroleum ether/ethyl acetate 9/1, v/v). Elution was accomplished by gravity. BPA-d_4 was obtained after evaporation to dryness under reduced pressure. ^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C): δ (ppm) = 7.09 (s, 4H, H_{Ar}), 4.66 (br s, 2H, OH), 1.62 (s, 6H, CH_3). ^{13}C-NMR (75 MHz, $CDCl_3$, 25 °C): δ (ppm) = 153.3 (C_q), 143.5 (C_q), 128.0 (CH), 114.6 (t, $J_{C\text{-}D}$ = 23.4 Hz, C^2H), 41.8 (C_q), 31.2 (CH_3).

3.2.3 Synthesis of 2,6-dideuterio-4-[(3,5-dideuterio-4-hydroxy-phenyl)methyl]phenol (BPF-d_4)

BPF-d_4 was synthesized analog to BPA-d_4 using 1 g BPF (5.0 mmol), 40 g deuterium oxide, and a heating period of 8 h. ^{1}H-NMR (400 MHz, acetone-d_6, 25 °C): δ (ppm) = 8.05 (br s, 2H, OH), 7.01 (s, 4H, H_{Ar}), 3.77 (s, 2H, CH_2). ^{13}C-NMR (100 MHz, acetone-d_6, 25 °C): δ (ppm) = 156.2 (C_q), 133.7 (C_q), 130.4 (CH), 115.6 (t, $J_{C\text{-}D}$ = 24.2 Hz, C^2H), 40.8 (CH_2).

3.2.4 Synthesis of 2,6-dideuterio-4-(3,5-dideuterio-4-hydroxy-phenyl)sulfonyl-phenol (BPS-d_4)

BPS-d_4 was synthesized applying two steps. First 1 g 4-(4-hydroxyphenyl)sulfanylphenol (4.6 mmol) was deuterated as described for BPA-d_4 with a heating period of 24 h. Second the purified intermediate was oxidized: 4-(4-hydroxyphenyl)sulfanylphenol-d_4 was suspended in as few glacial acetic acid as possible. 4 ml of H_2O_2 (30%) were added dropwise and the solution was refluxed for 30 min [74]. After standing overnight 20 ml ice-cold water were added and extraction was performed with diethyl ether (2 x 25 ml). Remaining acetic acid was cleared by washing with $NaHCO_3$-solution (saturated) until neutral pH was achieved. The

diethyl ether phase was dried over Na_2SO_4 and evaporated to dryness under reduced pressure. ^{1}H-NMR (300 MHz, DMSO-d_6, 25 °C): δ (ppm) = 10.53 (br s, 2H, OH), 7.70 (s, 4H, H_{Ar}). ^{13}C-NMR (75 MHz, DMSO-d_6, 25 °C): δ (ppm) = 161.5 (C_q), 132.1 (C_q), 129.3 (CH), 115.6 (t, $J_{C\text{-}D}$ = 19.8 Hz, C^2H).

3.2.5 ^{1}H- and ^{13}C-Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were recorded on a Bruker Avance II 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz), Bruker Avance III 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz), and Bruker Avance 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer at room temperature. The chemical shifts, *δ,* were referenced versus residual solvent shifts in parts per million (ppm). The coupling constants, *J*, are reported in Hertz (Hz). Multiplicities are indicated as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br).

3.2.6 Gas chromatography-mass spectrometry (GC-MS)

Purity of isotope standards as well as isotopologue patterns were confirmed by GC-MS with and without silylation using *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (1/1; v/v, 80 °C, 15 min). GC-MS analysis was performed using a GCMS-QP2010plus system (Shimadzu, Duisburg, Germany). Chromatographic separation was achieved on a DB-5ms UI capillary column (30 m x 0.25 mm; 0.25 µm; Agilent J&W Scientific, Böblingen, Germany). Helium was used as the carrier gas at a constant flow of 1.1 ml/min. The oven temperature was raised from an initial temperature of 150 °C to 300 °C at 15 °C/min; 300 °C were maintained for 10 min. Sample injections were performed using a split of 1:10. The injector was set to 240 °C. Electron impact ionization-mass spectrometry (70 eV) was performed with ion source and interface temperatures of 200 and 250 °C, respectively. The scan range measured from m/z 70 to 500.

3.2.7 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed using a modular HPLC system including degasser, binary pump (LC-20AD), autosampler, and column oven (Shimadzu, Duisburg, Germany) coupled to a QTRAP® 5500 mass spectrometer (AB Sciex, Darmstadt, Germany). Analysis of mass spectrometric data was performed with Analyst 1.5.1 software.

Chromatographic separation was achieved using an OTU TriKala C_{18} column (125 x 3 mm; 5 µm; Application & Chromatography, Oranienburg, Germany) connected to a C_{18} guard

column (4 x 3 mm; Phenomenex, Aschaffenburg, Germany). Mobile phase A consisted of water, mobile phase B of acetonitrile. The mobile phase flow rate was 500 µL/min. Gradient elution was achieved by ramping from 25% B to 58% B within 11 min, followed by a cleaning step at 90% B for 3 min and re-equilibration (16 min). An aliquot of each calibration/sample solution was filtrated using a 0.2 µm nylon filter prior to injection into the LC-MS/MS system. The injection volume was 5 µL.

For MS/MS detection negative electrospray ionization (ESI(-)) multiple-reaction monitoring (MRM) mode was used. The MS/MS parameters were optimized by direct infusion via syringe pump and flow injection analysis via LC-system of the target analytes and isotope standards into the mass spectrometer. Nitrogen was used as both a curtain and a collision gas. Curtain gas was maintained at 40 psi. Optimized ion spray voltage was -4500 V, the source temperature was 500 °C. The final MRM transitions and optimized analyte specific parameters are shown in Table 1.

3.2.8 Stable isotope dilution assay of bisphenols from thermal paper

Prior to use all required glass ware was washed with methanol and heated to 200 °C. The use of plastic ware was prevented.

Calibration

Appropriate aliquots of analytes and isotope standards were weighed in separately and diluted with acetonitrile to a concentration of 1 mg/ml. The stock solutions were stored at -25 °C until use. BPA-d_4, BPF-d_4 and BPS-d_4 stock solutions were mixed and diluted with methanol/water (50/50; v/v) to result in an isotope standard working solution of 5 µg/ml. BPA, BPF and BPS stock solutions were mixed and diluted with methanol/water (50/50; v/v) to result in an analyte working solution of 500 ng/ml. Working solutions were stored for no more than 4 weeks at 4 °C.

Calibration was conducted for BPA:BPA-d_4 and BPF:BPF-d_4 in concentration ratios from 1:10 to 20:1, for BPS:BPS-d_4 from 1:10 to 2:1. Isotope standard concentration was kept constant at 50 ng/ml. Calibration curves were constructed by plotting the peak area ratios (analyte/isotope standard) over the corresponding concentration ratios. The resulting equations were y = 1.54x + 0.0111 (BPA/BPA-d_4, R^2 = 0.9991), y = 1.07x - 0.0566 (BPF/BPF-d_4, R^2 = 0.9976), and y = 1.84x + 0.0499 (BPS/BPS-d_4, R^2 = 0.9983).

Sample preparation

Liquid-solid extraction was developed based on literature [19, 11]. In short: Three 1 by 1 cm squares were cut out of the middle of each tested receipt. Each square was divided into small stripes, weighed, and transferred to a glass centrifuge tube. 0.5 to 5 ml of isotope standard solution was added, corresponding to 2.5 to 25 µg of each isotope standard. 6 ml of methanol/water (50/50; v/v) were added and the samples were shaken (200 min^{-1} Certomat®, B. Braun, Melsungen, Germany) for 30 min. Samples were then centrifuged for 5 min at 3000 x g (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) and the supernatant was transferred to a 50 ml graduated flask. The extraction was repeated once and the supernatants were combined and filled up with acetonitrile/water (25/75; v/v). Prior LC-MS/MS analysis samples were diluted to achieve an isotope standard concentration of 50 ng/ml. With every extraction series a blank was carried along.

Recovery experiments, limit of detection, and limit of quantification

The accuracy of the developed SIDA was proven by recovery experiments. Photocopying paper was used as a blank matrix and spiked with analytes at concentrations of 1 and 10 mg/g. Sample preparation and measurement were conducted as described before. Recovery experiments were performed in triplicates. From resulting signal-to-noise ratios of the 1 mg/g spiking level limit of detection (LOD) and limit of quantification (LOQ) were estimated.

3.3 Results and discussion

3.3.1 Synthesis and characterization of deuterated bisphenols

BPA-d_4 and BPF-d_4 could be synthesized successfully from unlabeled BPA and BPF, respectively. Hydrogen-deuterium exchange in acidulated deuterium oxide, liquid-liquid extraction, and column chromatography yielded d_4-isotope standards. Synthesis of BPA-d_{14} is described taking 12 days [52]. In comparison the presented BPA-d_4 can be synthesized within one workday! For BPA, an extended heating period yields higher deuterated products (data not shown). BPF-d_4 takes longer due to the prolonged heating period of eight hours. Concerning BPS direct deuteration as described for BPA and BPF resulted in poor deuteration efficiency, mainly expressing d_0 to d_2 isotopologues (data not shown). Synthesis of BPS-d_4 demanded a “detour”: changing the educt for deuteration from BPS to 4-(4-

hydroxyphenyl)sulfanylphenol and subsequent oxidation of the intermediate. All three isotope standards have been characterized by NMR, GC-MS, and LC-MS/MS.

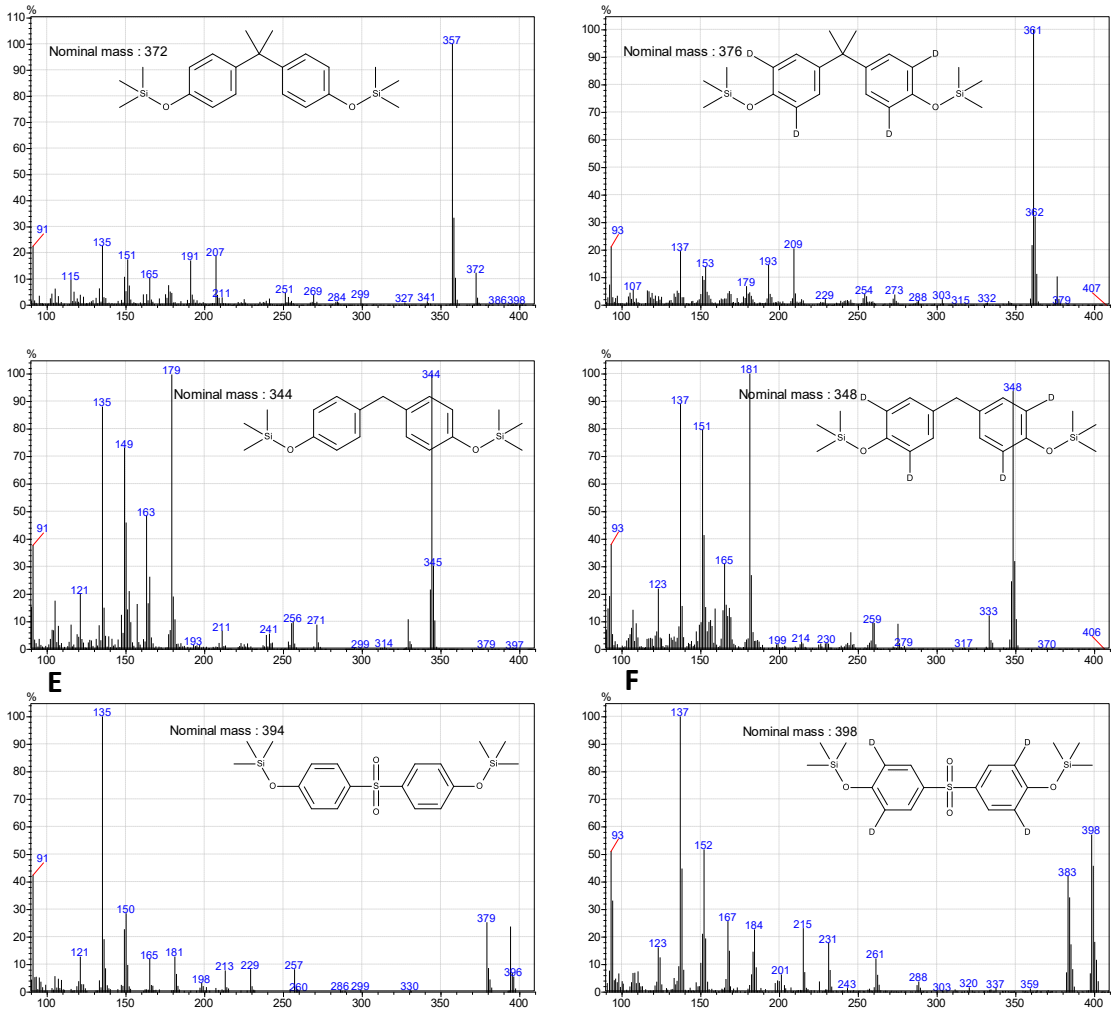

Figure 3 Structures, nominal masses and electron impact mass spectra of the O-bis(trimethylsilyl) derivatives of BPA (**A**), BPA-d_4 (**B**), BPF (**C**), BPF-d_4 (**D**), BPS (**E**) and BPS-d_4(**F**)

Electron impact ionization GC-MS analysis of deuterated bisphenols is known to be challenging, since deuterium/hydrogen exchange might occur [75] when no hydroxyl group derivatization is performed. Additionally BPS does not pass the column when applied in its native state. When trimethylsilyl-derivatization is used the original isotopologue distribution of the standards is adulterated slightly due to the natural isotope pattern of silicon [52]. However, combining NMR data with GC-MS and LC-MS/MS measurements it could be

proven that no d_0-isotopologue was contained in any of the isotope standards as well as that the targeted d_4-isotopologue was the prevalent one. GC-MS spectra of silylated bisphenols are shown in Figure 3. NMR data is placed in sections 3.2.2 to 3.2.4 beneath synthesis descriptions. Figure 4 shows a comparison of LC-MS/MS product ion spectra belonging to analytes and their corresponding isotope standards. The d_4-deuteration of the bisphenols (d_2 on each phenol ring, respectively) ensures that d_4- and d_2-fragments are suitable for LC-MS/MS quantification.

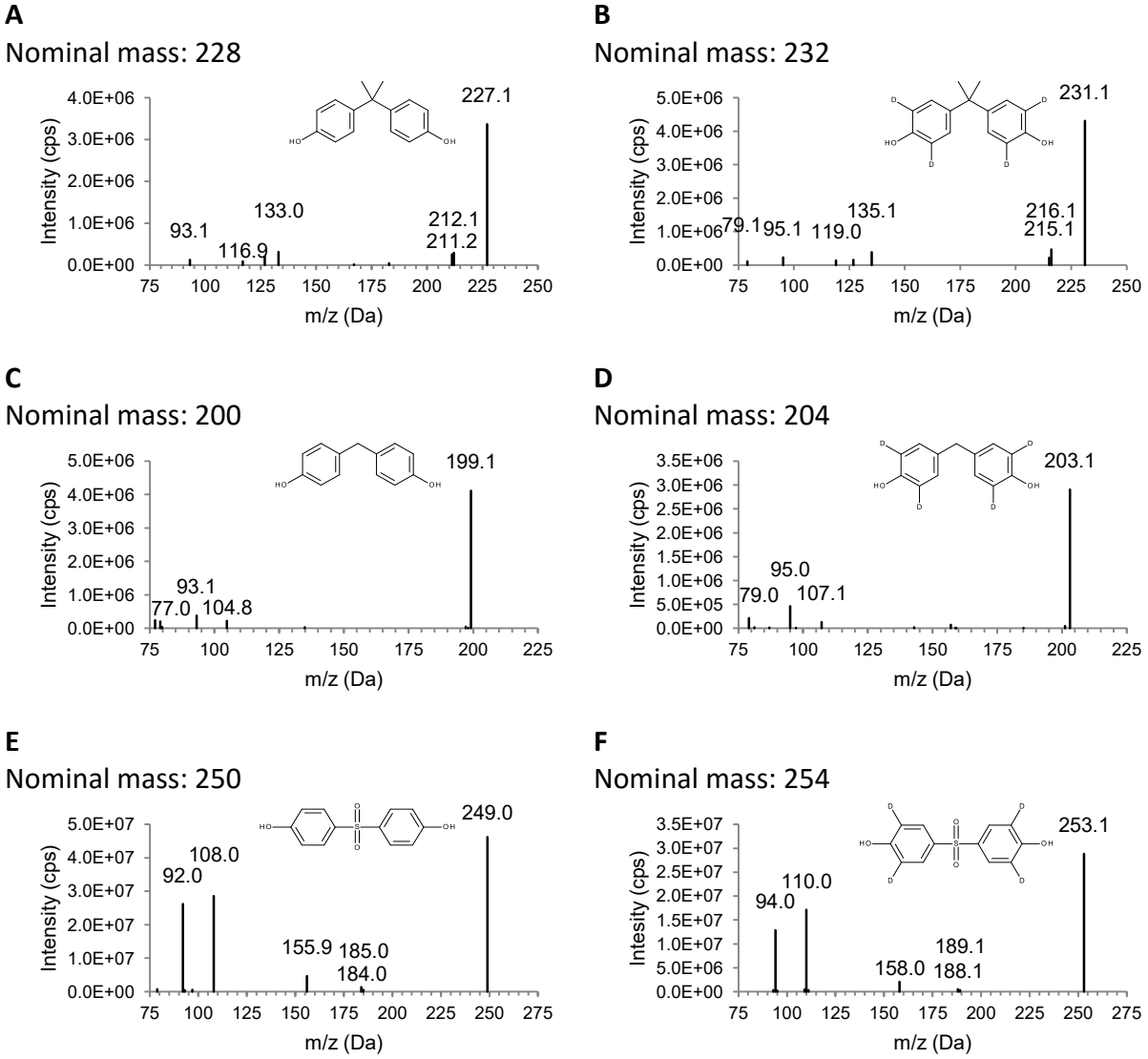

Figure 4 Structures, nominal masses, and ESI(-)-MS/MS spectra of BPA (**A**), BPA-d_4 (**B**), BPF (**C**), BPF-d_4 (**D**), BPS (**E**), and BPS-d_4 (**F**)

3.3.2 Stable isotope dilution assay (SIDA)

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The target bisphenols were separated on a C_{18} column in a time of 11 min. One run including rinsing step and re-equilibration lasted 30 min. Gradient elution using water and acetonitrile was applied. LC-MS/MS chromatograms (Figure 5) show that using the established method co-elution of isotope standards and target analytes was achieved to a great extent. Isotope standards elute only 0.04 to 0.07 min before the analytes ensuring a great overlap of the corresponding peak areas used for quantification.

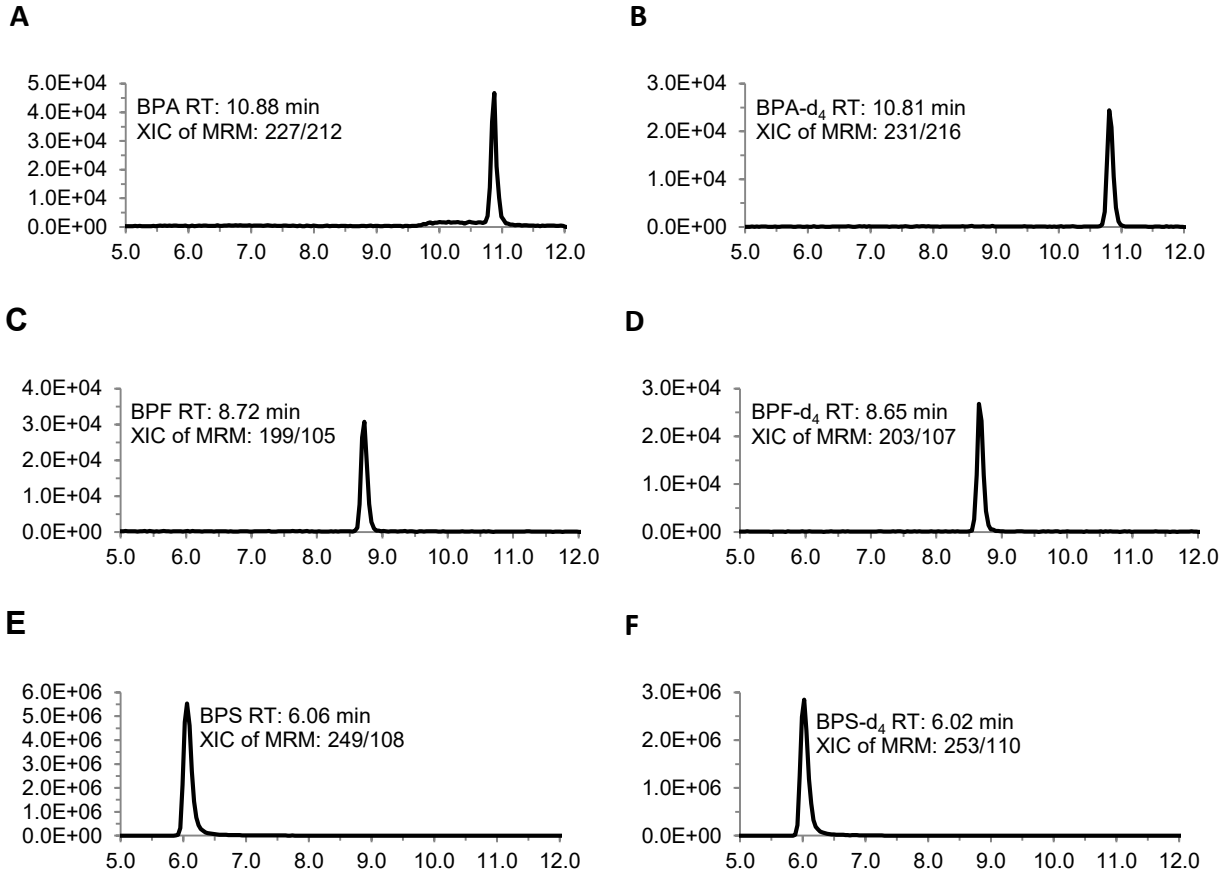

Figure 5 Extracted LC-ESI(-)-MS/MS chromatograms of quantifier ion transitions showing retention times of BPA (**A**), BPA-d_4 (**B**), BPF (**C**), BPF-d_4 (**D**), BPS (**E**), and BPS-d_4 (**F**)

MS/MS parameters were optimized according to common procedures (direct infusion and flow injection analysis). For the examined bisphenols and their isotope standards precursor ions corresponded to the deprotonated molecules $[M-H]^-$ in ESI(-). Fragmentation patterns from isotope standards correlated highly with those of the non-labeled compounds (Figure 4), so corresponding ion transitions were chosen for quantification. Extracted ion chromatograms of quantifier ion transitions are shown in figure 5. Quantifier and qualifier

ion transitions are listed in table 1. For BPA and BPS the most intense ion transitions were selected as quantifiers. For BPF the 199/93 ion transition (most intense after optimization) showed a slight ion suppression within recovery experiments. Thus the 199/105 ion transition was used as quantifier. Ionization of BPS is about 10 times more sensitive compared to BPA and BPF, which show almost similar intensities. Signals for BPS concentrations above 100 ng/ml resulted in signal saturation. Thus the working range had to be reduced compared to BPA and BPF.

Table 1 ESI(-)-MS/MS parameters: Ion transitions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP)

	Ion transitions[a]					
Compound	**Precursor ion $[M-H]^-$ (m/z)**	**Product ion (m/z)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
BPA	227.0	212*	-55.0	10	-26.0	-15.7
		133	-55.0	10	-33.3	-9.70
		93.0	-55.0	10	-58.7	-7.70
BPA-d_4	231.0	216*	-53.3	10	-26.0	-9.00
		135	-53.3	10	-37.3	-11.7
		94.9	-53.3	10	-60.0	-10.3
BPF	199.0	92.9	-96.7	10	-29.3	-7.60
		105*	-96.7	10	-29.3	-6.30
		77.0	-96.7	10	-32.7	-6.30
BPF-d_4	203.0	95.0	-55.0	10	-30.0	-9.70
		107*	-55.0	10	-30.0	-9.00
		79.0	-55.0	10	-32.7	-11.0
BPS	248.9	108*	-16.7	10	-36,0	-9.70
		91.9	-16.7	10	-47.3	-9.00
		156	-16.7	10	-30.0	-17.3
BPS-d_4	252.9	110*	-41.7	10	-37.3	-9.70
		93.8	-41.7	10	-48.0	-9.00
		158	-41.7	10	-30.0	-9.00

[a] Quantifier ion transitions are marked with an asterisk

Calibration curves obtained by plotting peak area ratios of analyte and isotope standard over concentration ratios revealed a linear relationship over the described ranges. Applying linear regression correlation coefficients $R^2 > 0.997$ were achieved for all bisphenols examined.

3.3.3 Sample preparation

Liquid-solid extraction could be optimized with regard to reducing methanol usage and time in comparison to published methods [3, 73]. Both methods use methanol as an extraction agent, but for the presented SIDA methanol/water (50/50, v/v) was tested to be sufficient. Liao et al. (2012) conducted three 30 min extraction steps [73], while Biedermann et al. (2010) extracted overnight applying 60 °C [3]. In the presented work two extraction steps of 30 min each at room temperature were sufficient. Extraction of the paper samples was performed after addition of the d_4-isotope standards. Due to severe differences in bisphenol content in thermal paper, for some samples more internal standard had to be added in a second workup to provide a sample measurement within calibration working ranges.

3.3.4 Recovery experiments, limit of detection, and limit of quantification

Recoveries were determined for concentration levels of 1 mg/g and 10 mg/g using photocopying paper as a blank matrix. SIDA was conducted in triplicates resulting in recoveries from 106% to 115% (Table 2). Relative standard deviations (RSD) ranged from 2.8% to 9.5% which meet the demands of modern analytic procedures. For the 10 mg/g samples the BPS peak areas where out of the linear range after dilution (IS 50 ng/ml). Since BPS was found only once within application experiment and the amount correlated rater with the 1 mg/g sample, the recovery experiment was not repeated with a greater volume of isotope standard. Limit of detection (LOD) and limit of quantification (LOQ) were estimated based on the signal-to-noise ratios of the 1 mg/g spiking samples. Signal-to-noise ratio of 3 gave the following LODs: 0.73 µg/g for BPA, 0.66 µg/g for BPF, and 0.08 µg/g for BPS. LOQs were determined as the concentrations ensuing in a signal-to-noise ratio of 10, resulting in 2.44 µg/g for BPA, 2.21 µg/g for BPF, and 0.27 µg/g for BPS.

3.3.5 SIDA-LC-MS/MS of bisphenols from thermal paper

The developed method was applied to quantify BPA, BPF, and BPS from 14 receipts as one of the most discussed thermal paper samples. Results show that 13 out of 14 receipts contained BPA. BPS was found in one sample, while BPF was not detected in any sample. Detailed results are shown in table 3. RSD from application experiment (conducted in triplicates) show a very high precision ranging from 2.0% to 6.2%. Sample 4 shows an abnormal low amount of BPA (0.07 mg/g) in comparison to the other samples (0.5% of the BPA sample average of 14.6 mg/g). Since the blank of the corresponding series was negative

and analysis was conducted in triplicates with a resulting RSD of only 3.6% a cross-contamination was disproved. A potential source of BPA in this receipt could be the usage of recycling paper even though this sample does not have a different appearance as the rest of the set. There is probably a different developer applied on the paper, assuming that the detected amount of BPA is not sufficient for coloring reaction.

Table 2 Recoveries (%) of SIDA-LC-MS/MS in photocopying paper (n = 3)

Photocopying paper	**Analyte**	**Spiked conc. (mg/g)**	**Measured conc. mean ± SD (mg/g)**	**RSD (%)**	**Recovery (%)**
1-3	BPA	0.97	1.11 ± 0.09	8.2	114
	BPF	0.98	1.06 ± 0.10	9.5	108
	BPS	0.98	1.06 ± 0.10	9.5	108
4-6	BPA	9.54	11.0 ± 0.64	5.9	115
	BPF	9.54	10.1 ± 0.29	2.8	106
	BPS	9.53	out of linear range		

3.4 Conclusions

The isotope standards BPA-d_4, BPF-d_4, and BPS-d_4 have been synthesized cost- and time-saving applying a few synthesis steps only. The deuteration of BPF and BPS is shown for the first time. The presented BPA-d_4-synthesis taking 8 h illustrates an improved concept compared to Varelis & Balafas (2000) BPA-d_{14}-synthesis [52] taking 12 days.

For application a SIDA-LC-MS/MS method was developed for the quantification of BPA, BPF, and BPS from thermal paper receipts. In the examined set of samples from Berlin, Germany (in 2014) BPA is the most common developer. A shift towards the usage of other bisphenols was not seen yet. The BPA results (arithmetic mean: 14.6 mg/g, minimum 0.07 mg/g, maximum 20.1 mg/g) correspond with published results from other countries. The finding of 6.43 mg/g BPS in one receipt allows the assumption that BPS can be used in corresponding concentrations as BPA in thermal paper. Findings of BPS are reported in thermal paper receipts in one former study [73], but interpretation is difficult since ranges are very large

and the sets of samples do not show Gaussian distribution. In short, there is not enough comparison data existent yet.

Table 3 Concentration ± standard deviation (mg/g) of bisphenols in super market receipts from Berlin, Germany (n = 3)

Receipt	**BPA (mg/g)**	**RSD (%)**	**BPF (mg/g)**	**RSD (%)**	**BPS (mg/g)**	**RSD (%)**
1	19.5 ± 0.95	4.9	ND		ND	
2	20.1 ± 0.51	2.5	ND		ND	
3	1.58 ± 0.73	4.6	ND		ND	
4	0.07 ± 0.00	3.6	ND		ND	
5	10.1 ± 0.28	2.8	ND		ND	
6	18.0 ± 0.71	3.9	ND		ND	
7	10.5 ± 0.21	2.0	ND		ND	
8	ND		ND		6.43 ± 0.20	3.0
9	20.0 ± 0.20	6.2	ND		ND	
10	19.7 ± 0.69	3.5	ND		ND	
11	17.9 ± 0.36	2.0	ND		ND	
12	10.9 ± 0.28	2.6	ND		ND	
13	16.8 ± 0.84	5.0	ND		ND	
14	10.6 ± 0.26	2.5	ND		ND	

The application of the developed isotope standards within further projects in regard to articles of daily use as well as food, and food contact materials is planned. Their suitability for further clean up and extraction procedures will be examined.

3.5 Acknowledgements

Special thanks go to Dr. P. Schmieder (Leibniz-Institut für Molekulare Pharmakologie) and Dr. S. Kemper (Department of Chemistry, Technische Universität Berlin) for providing the machines and software for NMR measurements as well as Dr. A. Mattulat for the insides in tendencies within service analytics of bisphenol A.

4 Project II: Determination of bisphenols, bisphenol A diglycidyl ether (BADGE), BADGE chlorohydrins and hydrates from canned beer by high-performance liquid chromatography-tandem mass spectrometry

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Descriptors

Bisphenol A, BADGE, can coatings, migration, Stable isotope dilution assay (SIDA), LC-MS/MS

Abstract

A set of 9 bisphenolic compounds has been analyzed from canned beer after liquid-liquid extraction (LLE). Namely bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), bisphenol A diglycidyl ether (BADGE), BADGE·2H_2O, BADGE·H_2O, BADGE·HCl·H_2O, BADGE·2HCl, and BADGE·HCl have been examined. Stable isotope dilution assay (SIDA) using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) was applied. Spiking and extraction experiments from Pilsner, wheat, and black beer resulted in recoveries from 75 to 118 %. For application experiment, 14 canned beer samples have been examined for bisphenolic content. Lead substances BPA and BADGE·2H_2O were tested positive in 14 out of 14 samples. Concentration ranges varied from 0.10 to 2.54 µg/l and 0.64 to 14.3 µg/l, respectively. Concentrations of BADGE·2H_2O were herein in accordance with the specific migration limit set by the European Union. For BPA and BADGE·2H_2O dietary intakes were calculated using the highest concentrations determined. The resulting daily intakes amounted to below 1 % of the current tolerable daily intakes. It can thus be concluded, that the examined set of canned beers can be considered to be safe for the consumer.

4.1 Introduction

Can linings are functional barriers to prevent canned beverages from disadvantageous influences from metal cans such as off flavor, color change and oxidation. One common

[4] Author contributions: J.Z. designed research; N.R. and L.-A.G. reviewed the designed research; J.Z. performed synthesis; A.M. performed NMR measurements; J.Z. and S.M. performed analytics; J.Z. analyzed data; J.Z. wrote the paper

epoxy resin consists of polymerized bisphenol A (BPA) and bisphenol A diglycidyl ether (BADGE). Both monomers can migrate into foodstuff. Additionally monomeric BADGE tends to react with ingredients due to its two reactive epoxy groups. Reported products of these reactions are substances as BADGE hydrates and BADGE chlorohydrins [22, 24, 76, 77]. In the following BADGE and BADGE reaction products will be referred to as “BADGEs”.

Due to the enduring scientific, political and public debate about BPA it is likely that substitutes as bisphenol F (BPF) and bisphenol S (BPS) are established within can lining production as can be seen in other industry branches, e.g. thermal paper [2, 67, 73] or infant feeding bottles [78]. Scientific examination and discussion about the question if BPS and BPF are safe substitutes already started [45].

Within the European Union a regulatory framework is set for the examined BPA based monomeric substances. An overview of the substances examined within the presented work and information as specific migration limit (SML) and tolerable daily intake (TDI) can be seen in table 4. For BADGE and BADGE hydrates sum parameters are defined [50] due to their close dependence as well as for the BADGE chlorohydrins.

For trace analysis of bisphenols mainly chromatography coupled to mass selective detectors is used as LC-MS/MS and GC-MS. Table 5 gives an overview on literature data which presents examinations of bisphenolic compounds in beer. The application of isotope standards within quantification is beneficial [79], especially to compensate for potential analyte losses during extraction procedure. For BPA, BPS, and BADGE labeled standards are commercially available (as at 2015), from which especially the labeled BPA standards are used often [51, 80–82]. Synthesis of deuterated BPA, BPF, BPS, and BADGE is described [22, 51, 52, 83]. Isotope standards of BADGE hydrates and chlorohydrins are not available, even though especially BADGE·2H_2O is of great interest because of its frequent occurrence within beverage samples [76, 80]. Findings of bisphenols and BADGEs in canned beer are also stated in table 5.

The attempt of the developed method was to accelerate the determination of bisphenols and BADGEs in particular by combining both sets of analytes within one LC-MS/MS analysis. To facilitate quantification, d_4-labeled standards for the BADGE hydrates should be applied

in addition to the former existent isotope standards. The effectiveness of the SIDA was verified by recovery experiments. A set of 14 canned beer samples was examined.

Table 4 Analytes examined, with CAS numbers, IUPAC names and regulatory framework

Substance **IUPAC**	**CAS**	**TDI (mg/kg body weight/day)**	**SML (mg/kg)**	**Source**
Bisphenol A 2,2-bis(4-hydroxyphenyl)propane	80-05-7	0.004 temporary	0.6*	[41, 84]
BADGE 2,2-bis(4-hydroxyphenyl)propanebis(2,3-epoxypropyl)ether	1675-54-3	0.15 sum parameter	9 sum parameter	[50]
BADGE·H_2O 3-(4-{2-[4-(2-Oxiranylmethoxy)phenyl]-2-propanyl}phenoxy)-1,2-propanediol	76002-91-0			
BADGE·2H_2O 3-(4-{2-[4-(2-Oxiranylmethoxy)phenyl]-2-propanyl}phenoxy)-1,2-propanediol	5581-32-8			
BADGE·HCl 1-Chloro-3-(4-{2-[4-(2-oxiranylmethoxy)phenyl]-2-propanyl}phenoxy)-2-propanol	13836-48-1	none	1 sum parameter	[50]
BADGE·HCl·H_2O 3-(4-{2-[4-(3-Chloro-2-hydroxypropoxy)phenyl]-2-propanyl}phenoxy)-1,2-propanediol	227947-06-0	none		
BADGE·2HCl 1,1'-[2,2-Propanediylbis(4,1-phenyleneoxy)]bis(3-chloro-2-propanol)	4809-35-2	none		
Bisphenol S 4,4'-Sulfonyldiphenol	80-09-1	none	0.05	[84]
Bisphenol F 4,4'-Methylenediphenol	620-92-8	none	none	

* SMLs for BPA and BPS are not valid for surface coatings as used in beverage cans.

Table 5 Overview on findings (µg/l) of bisphenolic compounds in canned beer

Substances examined	**Findings in canned beer**	**Range**	**Technique**	**Source**
BPA (BPB)	BPA	0.29 to 4.70 µg/l	GC-MS	[80]
BADGE·2H_2O BADGE·H_2O BADGE·HCl·2H_2O BADGE·HCl BADGE·2HCl	BADGE·2H_2O	5.1 and 4.3 µg/kg	LC-MS/MS	[76]
BPA BADGE	BPA BADGE	1.26 to 3.79 µg/l 0.11 to 0.74 µg/l	HPLC fluorescence	[85]
BPA	BPA	0.081 to 0.54 µg/l	GC-MS	[86]
BPA	BPA	1.9 to 6.6 µg/l	GC-MS	[51]
BPA	BPA	1.5 µg/l	HPLC fluorescence	[56]

4.2 Materials and Methods

4.2.1 Synthesis of isotope standards

The isotope standards BADGE-d_4, BADGE·2H_2O-d_4, and BADGE·H_2O-d_4 were prepared as described by Rauter et al. (1999) for the non-labeled substances [24] with only slight modifications within sample clean-up for BADGE. Detailed information on synthesis and characterization of isotope standards can be seen in appendix.

4.2.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed using a modular HPLC system (Shimadzu, Duisburg, Germany) composed of degasser, pump (LC-20AD), autosampler, and column oven coupled to a QTRAP 5500 mass spectrometer (AB Sciex, Darmstadt, Germany). Analyst 1.5.1 software was used for data analysis. Bisphenols and BADGEs were separated using an OTU TriKala C_{18} column (250 x 3 mm; 5 µm; Application & Chromatography, Oranienburg, Germany) connected to a C_{18} guard column (4 x 3 mm; Phenomenex, Aschaffenburg, Germany). A gradient consisting of water, acetonitrile, and methanol (all LC-MS grade, purchased from

VWR International GmbH, Darmstadt, Germany) was applied at a flow rate of 500 µl/min and 25 °C (table 6). The injection volume was 25 µl.

Table 6 HPLC gradient for separation of bisphenols and BADGEs

Time (min)	**Water (%)**	**Acetonitrile (%)**	**Methanol (%)**
0	75	25	0
13	49	51	0
16	43	0	57
27	5	0	95
29	5	0	95
29.9	5	0	95
30	75	25	0
35	75	25	0

MS/MS detection was performed in periods of positive and negative electrospray ionization (ESI). Multiple-reaction monitoring (MRM) was applied. The MS/MS parameters of the target analytes and isotope standards were optimized via direct infusion and flow injection analysis into the mass spectrometer. Nitrogen served as curtain and as collision gas and was varied between periods. The source was heated to 350 °C. The final MRM transitions and collision energies are shown in table 7.

4.2.3 Calibration

Separate stock solutions of 10 µg/ml bisphenolic compounds (all purchased from Sigma-Aldrich, Steinheim, Germany) and isotope standards were prepared in acetonitrile. The stock solutions were stored at -25 °C until use. An isotope standard mix was prepared in acetonitrile containing 100 µg/l of BPA-d_4, BPF-d_4, BADGE·$2H_2O$-d_4, and BADGE·H_2O-d_4 and 10 µg/l of BADGE-d_4 and BPS-d_4. Calibration was conducted for bisphenols and BADGEs corresponding to their working ranges, which can be seen in table 8.

4.2.4 Sample preparation

Beer samples were degassed in an ultrasonic bath. Aliquots of 200 µl were taken and spiked with 5 µl of isotope standard mix. 1 ml of ethyl acetate:hexane (1:1; v:v; both HPLC grade, VWR) was added rapidly. The samples were shaken for 10 sec by vortex mixer. After

centrifugation for 1 min at 20,000 x g the supernatant was separated and the residual sample was extracted one more time. Combined supernatants were dried under nitrogen and vacuum. Residues were dissolved in 100 µl of acetonitrile:water (25:75; v:v) and injected into LC-MS/MS system.

Table 7 List of target analytes with MS/MS parameters

Period	**Analyte**	**Precursor (m/z) [type]^{charge}**	**Fragment (m/z)**	**Isotope standard**	**Collision Energy (V)**
1	BPS	249 $[M-H]^-$	108*	BPS-d_4 (253/110)	-36
			92	BPS-d_4 (253/94)	-47
2	BADGE·$2H_2O$	394 $[M+NH_4]^+$	209*	BADGE·$2H_2O$-d_4 (398/211)	21
			135	BADGE·$2H_2O$-d_4 (398/137)	45
3	BPF	199 $[M-H]^-$	105*	BPF-d_4 (203/107)	-29
			77	BPF-d_4 (203/79)	-33
	BPA	227 $[M-H]^-$	212*	BPA-d_4 (231/216)	-26
			133	BPA-d_4 (231/135)	-33
4	BADGE·HCl·H_2O	412 $[M+NH_4]^+$	227*	BADGE·H_2O-d_4 (380/211)	23
			135	BADGE·H_2O-d_4 (380/211)	47
	BADGE·H_2O	376 $[M+NH_4]^+$	209*	BADGE·H_2O-d_4 (380/211)	19
			191	BADGE·H_2O-d_4 (380/193)	27
5	BADGE	358 $[M+NH_4]^+$	191*	BADGE-d_4 (362/193)	19
			135	BADGE-d_4 (362/137)	47
	BADGE·HCl	394 $[M+NH_4]^+$	227*	BADGE-d_4 (362/193)	19
			135	BADGE-d_4 (362/193)	43
	BADGE·2HCl	430 $[M+NH_4]^+$	135*	BADGE-d_4 (362/193)	47
			107	BADGE-d_4 (362/193)	71

* Quantifier ions are marked with an asterisk.

4.2.5 Recovery experiment and definition of working ranges

For calculation of recoveries, spiking experiments were conducted at two concentration levels using three different beer matrices. Pilsner was chosen because it is a rather light beer with low haze; besides, it holds the greatest market share in Germany. Wheat and black beer were chosen since color and turbidity might lead to interferences within measurements. Bottled beers served as blank matrices. Spiking levels were 0.5, and 1.0 µg/l for all analytes besides BPS and BADGE which were examined at 0.05 and 0.1 µg/l. For analytes with working ranges starting at 0.1 µg/l this level was examined additionally. SIDA was performed in triplicates.

4.2.6 Application

Bisphenolic content was quantified from 14 canned beer samples from randomly picked suppliers applying the described SIDA-LC-MS/MS method. In line with recovery experiments the beers were grouped in the categories Pilsner, wheat beer, black beer, and others. Quantification was performed in triplicates.

4.3 Results and discussion

4.3.1 Synthesis and characterization of deuterated bisphenols

BPA-d_4, BPF-d_4, and BPS-d_4 "remained" from a previous work [83] and were used after GC-MS control of deuteration (data not shown). BADGE-d_4, BADGE·H_2O-d_4, and BADGE·$2H_2O$-d_4 were synthesized using BPA-d_4 as educt. Applying three basic synthesis steps BPA-d_4 could be converted over BADGE-d_4 to BADGE·H_2O-d_4 and BADGE·$2H_2O$-d_4. Details of synthesis procedure and ^1H- and ^{13}C-NMR data of labeled hydrated BADGEs is provided in appendix. All three isotope standards showed the d_4-isotopologue as the prevalent one, which makes them suitable for LC-MS/MS application. ESI-MS/MS product ion spectra of the ammonium adducts (as used in analysis) of the labeled and unlabeled BADGEs are shown in figure 6. The d_4-labeling of BADGEs (d_2 on each phenol ring, respectively) results in d_4- and d_2-labeled product ions after fragmentation.

4.3.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Separation of the targeted bisphenolic compounds was achieved within 30 min. One sample measurement including rinsing step and re-equilibration of the column took 39 min. A gradient consisting firstly of water and acetonitrile and later of water and methanol was

developed to not only separate bisphenols and BADGEs, but also to provide essential solvent composition to support electrospray ionization (ESI) for the two groups.

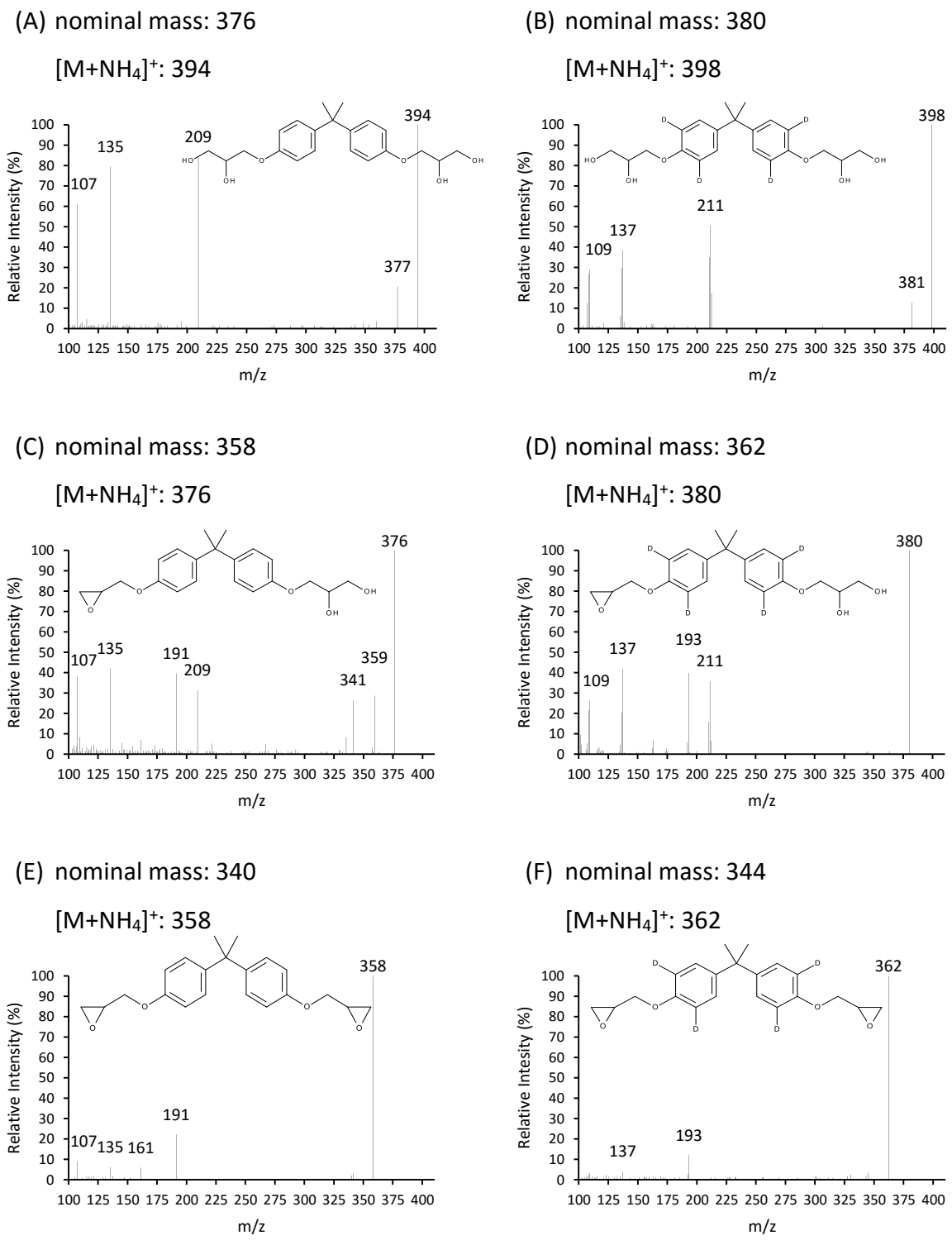


Figure 6 Structures and nominal masses of BADGE·2H_2O (A), BADGE·2H_2O-d_4 (B), BADGE·H_2O (C), BADGE·H_2O-d_4 (D), BADGE (E), and BADGE-d_4 (F) and ESI(+)-MS/MS spectra of corresponding ammonium adducts $[M+NH_4]^+$

In general, bisphenols ionize very sensitive in acetonitrile and water mixtures, while BADGEs ionize most sensitive in methanol and water systems. Small amounts of methanol (1 to 5 %) suppress ionization of BPA and BPF significantly (data not shown). Since methanol supported the ionization of BADGEs strongly, acetonitrile was substituted completely within the gradient.

MS/MS parameters were optimized according to common procedures (direct infusion and flow injection analysis). For the examined bisphenols and their isotope standards, precursor ions corresponded to the deprotonated molecules $[M-H]^-$ in ESI(-). Fragmentation patterns from isotope standards correlated highly with those of the non-labeled compounds, so corresponding ion transitions were chosen for quantification. For BADGEs, protonated molecules $[M+H]^+$ and ammonium adducts $[M+NH_4]^+$ were examined as precursors, from which the adducts (figure 6) showed better sensitivity after optimization. Since BPS and BADGE ionized about a factor 10 more sensitive than the other target compounds their concentration ranges for calibration and recovery experiments have been adjusted.

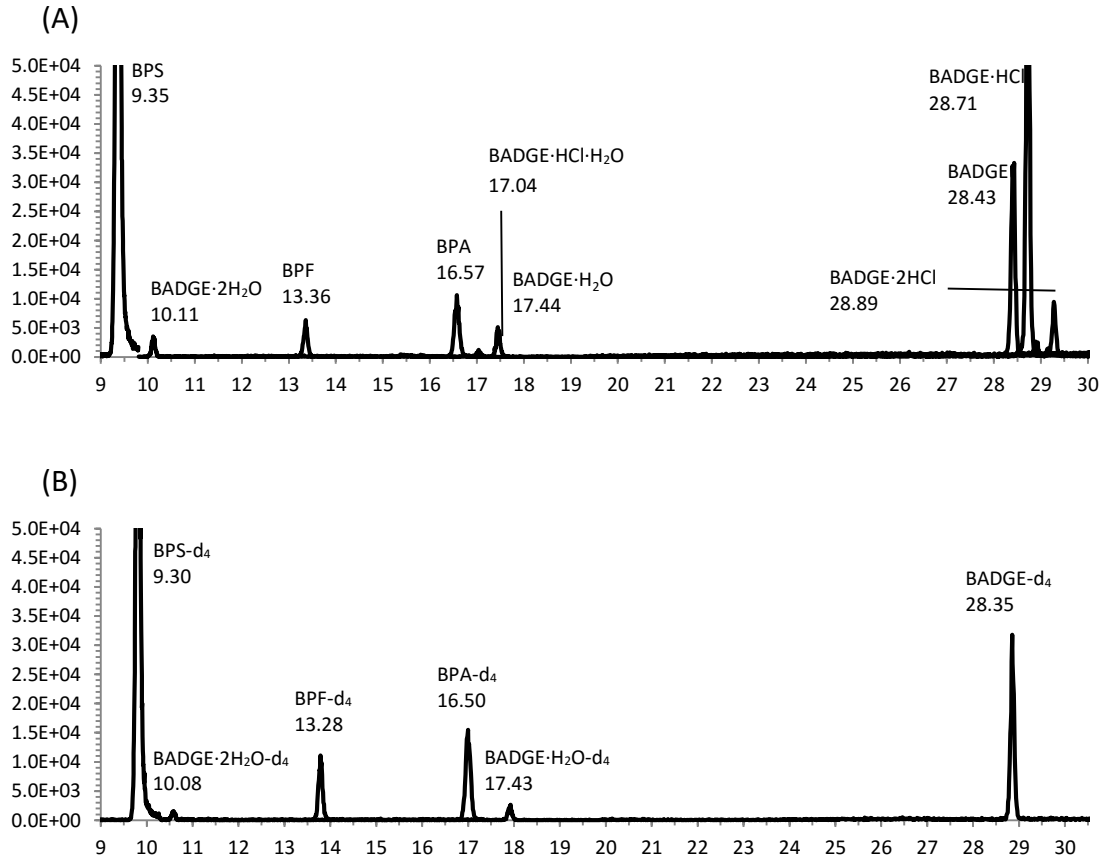


Figure 7 Extracted LC-ESI-MS/MS chromatograms of quantifier ion transitions (A) and isotope standards (B) in solvent at a concentration of 1.0 µg/l + 2.5 µg/l isotope standard

Figure 7 shows an extracted ion chromatogram of a solvent run presenting all quantifier ion transitions as well as corresponding isotope standard ion transitions. Quantifier and qualifier ion transitions are listed in table 7, an overview on the used periods (column 1) is given.

Calibration curves were generated by plotting ratios of peak areas over ratios of concentrations (analyte divided by isotope standard, respectively). Evaluation of the linear equations revealed a linear relationship over the described working ranges (table 8). Correlation coefficients greater 0.998 were achieved for all bisphenols and BADGEs examined. BPS was excluded from quantification, because from recovery experiment on contamination of BPS in chemical blank was observed.

Table 8 Performance parameters of developed SIDA

Analyte	**Working range (µg/l)**	**Isotope standard conc. (µg/l)**	**linear equation y = mx + n**	**R^2**
BPS	0.01 - 0.55	0.25	y = 2.65x - 0.0119	1.0000
BADGE·2H_2O	0.5 - 10	2.5	y = 5.51x - 0.0131	1.0000
BPF	0.1 - 5	2.5	y = 1.23x + 0.0035	0.9999
BPA	0.1 - 5	2.5	y = 1.46x + 0.0244	0.9999
BADGE·HCl·H_2O	0.5 - 10	2.5	y = 1.25x - 0.0000	1.0000
BADGE·H_2O	0.1 - 5	2.5	y = 4.26x + 0.0680	0.9996
BADGE	0.05 - 1	0.25	y = 2.5x + 0.2180	0.9989
BADGE·HCl*	0.1 - 10	0.25	y = 0.444x + 0.0279	1.0000
BADGE·2HCl	0.5 - 10	0.25	y = 0.0175x - 0.0040	0.9998

* The commercial standard of BADGE·HCl (purity of > 90 %) contained BADGE in significant amounts. Thus BADGE·HCl was calibrated separately from the other analytes.

4.3.3 Method characteristics

Within recovery experiments results from 75 % to 118 % were achieved. Detailed data can be seen in table 9. Relative standard deviation accounted, with exception of one spiking experiment, for less than 25 %, which is appropriate for trace level analysis. A third series of recovery experiments has been performed for the 0.1 µg/l level (data not shown), which enabled to expand the working ranges down to 0.1 µg/l for BPA, BPF, BADGE·H_2O and BADGE·HCl. BADGE and BPS can be measured down to 0.05 µg/l.

4.3.4 SIDA-LC-MS/MS of bisphenols from canned beers

Bisphenol content of 14 canned beers has been examined. Results are shown in table 10. Results show that BPA-based epoxy resins dominate the market within can lining production. 14 out of 14 samples were tested positive for BPA. The 13 samples within the working range contained rather low concentrations from 0.10 to 2.54 µg/l (arithmetic mean: 0.81 µg/l, median 0.31 µg/l). The second compound which was tested positive in all beers examined was BADGE·2H_2O, which could be quantified in 11 cases. With results ranging from 0.64 µg/l to 14.3 µg/l (arithmetic mean: 3.09 µg/l, median 0.96 µg/l) BADGE·2H_2O content is about three times higher than BPA content. Results for BPA and BADGE·2H_2O are comparable with other publications as described in table 5. They also correspond to findings in other canned beverages [51, 53, 80, 82]. Noticeable is the finding of traces of BPF in 11 out of 14 beer samples, which is usually not monitored in beverages. Comparable data is missing for canned beers in detail and beverages in general; only one study stated findings of BPF below LOQ of 0.05 µg/kg in the sum category beverages [82]. There, the four beers examined were bottled samples and did not contain any bisphenols[5].

Table 9 Recoveries (%) from spiked Pilsner, wheat, and black beer samples, relative standard deviation (%) is given in parentheses

Analyte	**Pilsner**		**Wheat beer**		**Black beer**	
	0.5 µg/l	1.0 µg/l	0.5 µg/l	1.0 µg/l	0.5 µg/l	1.0 µg/l
BPS*	80 (13)	88 (11)	117 (6)	94 (10)	84 (13)	85 (0)
BADGE·2H_2O	89 (5)	103 (10)	119 (14)	108 (5)	100 (22)	99 (7)
BPF	96 (2)	88 (4)	97 (9)	90 (5)	95 (7)	95 (1)
BPA	89 (9)	100 (7)	85 (9)	92 (7)	64 (24)	81 (12)
BADGE·HCl·H_2O	89 (34)	79 (8)	100 (11)	76 (7)	112 (15)	103 (12)
BADGE·H_2O	105 (4)	110 (3)	102 (3)	95 (3)	101 (3)	118 (5)
BADGE*	101 (2)	93 (3)	124 (18)	108 (9)	78 (5)	88 (2)
BADGE·HCl	97 (4)	90 (7)	94 (12)	87 (6)	88 (5)	83 (7)
BADGE·2HCl	89 (15)	75 (9)	119 (4)	100 (13)	111 (6)	115 (6)

* Recoveries of BADGE and BPS have been determined at 0.05 and 0.1 µg/l

[5] Personal contact: Dr. C. Liao

4.3.5 Assessment of consumer risks from the intake of bisphenolic compounds from canned beer

Concentrations of the lead substances BPA and BADGE·2H_2O in canned beers were low especially in comparison to canned food samples. If an adult of 60 kg body weight consumes 350 ml beer per day [87], the resulting dietary intakes amount to 0.015 µg/kg of body weight/day for BPA and 0.083 µg/kg of body weight/day for BADGE·2H_2O. Both estimations are based on the highest value for beer consumption (350 g/day, males from 51-64 years) from the German National Consumption Survey II [87], assuming that exclusively canned beer was consumed with the highest concentrations determined within the presented work (compare table 10). For BPA, the estimated amount corresponds to 0.38 % of the current temporary TDI of 4 µg/kg body weight/day established by the European Union; for BADGE·2H_2O, to 0.06 % of the TDI of 150 µg/kg body weight/day. While the SML for BPA only applies for plastic materials, the SML for BADGE·2H_2O (summed with BADGE and BADGE·H_2O) applies also to surface coatings as used in beverage can production. The migration of BADGE·2H_2O determined corresponds to 0.16 % of the SML, set at 9 mg/kg food or food simulant. Thus, the determined migration for BADGE·2H_2O and the estimated intakes for both lead analytes are in accordance with the limits set by the European Union and can be considered to be safe for the consumer.

For BPF, which was found in traces, no TDI is available. For evaluation of the results, the margin of exposure (MOE) approach can be used. The MOE describes the ratio between the lowest observed adverse effect level (LOAEL) and the dietary intake of a substance from e.g. a beverage. On basis of toxicological data, a MOE can be defined at or above which the intake of an undesired substance can be assumed to be safe for consumer's health. Recently a complete risk assessment for BPF (in mustard) was published [48]. There, it was stated that the safe ratio between LOAEL of 20 mg/kg body weight/day and dietary intake should amount to 1,800 or above. If applied to the results of the presented work, again using the highest concentration of BPF (0.30 µg/l, dietary intake of 0.002 µg/kg body weight/day) determined, a MOE of 1.9 million was calculated. Thus, the determined BPF content in canned beer can be evaluated as a minor risk to consumer's health basing on the current scientific and toxicological knowledge.

Questionable is the origin of the BPF findings. Epoxy resins made from diglycidyl ether of BPF (BFDGE) could be source of a BPF leakage. Generally, application of BFDGE is allowed for epoxy resin coating of large tanks, but tanks used in brewing industry are usually not coated.

Table 10 Quantitation results (µg/l) of bisphenols and BADGEs in canned beer (n = 14), relative standard deviation (%) is given in parentheses

Category	**No.**	**BPF**	**BPA**	**BADGE**	**BADGE·2H$_2$O**	**BADGE·H$_2$O**	**BADGE·HCl·H$_2$O**	**BADGE·2HCl**	**BADGE·HCl**
Pilsner	1	n.d.[a]	1.11 (6)	< 0.05	3.94 (5)	n.d.	< 0.5	n.d.	< 0.1
	2	n.d.	1.56 (20)	< 0.05	4.86 (9)	n.d.	< 0.5	n.d.	< 0.1
	3	0.21 (2)	1.96 (1)	n.d.	0.65 (3)	n.d.	< 0.5	n.d.	n.d.
	4	0.14 (8)	0.66 (11)	n.d.	7.98 (9)	n.d.	< 0.5	n.d.	n.d.
Wheat beer	1	< 0.1	0.24 (17)	n.d.	< 0.5	n.d.	n.d.	n.d.	n.d.
	2	< 0.1	1.80 (7)	n.d.	14.3 (8)	n.d.	< 0.5	n.d.	n.d.
	3	< 0.1	0.16 (29)	n.d.	6.16 (10)	n.d.	< 0.5	n.d.	n.d.
Black beer	1	0.10 (8)	0.31 (6)	n.d.	< 0.5	n.d.	n.d.	n.d.	n.d.
	2	< 0.1	0.10 (33)	n.d.	0.64 (6)	n.d.	n.d.	n.d.	n.d.
Others	1	< 0.1	0.26 (20)	n.d.	0.96 (7)	n.d.	< 0.5	< 0.5	n.d.
	2	0.30 (3)	2.54 (7)	n.d.	1.2 (9)	n.d.	< 0.5	n.d.	n.d.
	3	n.d.	0.31 (16)	n.d.	0.97 (5)	n.d.	0.65 (8)	0.55 (12)	n.d.
	4	< 0.1	0.22 (6)	n.d.	0.85 (9)	n.d.	n.d.	n.d.	n.d.
	5	0.11 (10)	< 0.1	n.d.	< 0.5	n.d.	n.d.	n.d.	n.d.

[a] n.d. – not detected

4.3.6 Pitfalls in analytic procedure

While content of bisphenolic substances in food contact material is mostly regulated, it is not in analytical equipment as plastic tubings, pipette tips, filter materials, needles etc.

Within the presented work, background contamination/leakage prevented quantification of BPS from beer samples. The source of suddenly appearing BPS contamination of samples, blanks and standards was a bottle of LC-MS water used for dilution of samples and standards that was contaminated in the µg/l range. To avoid false positive and false quantitated results, carrying along a chemical blank with each extraction is mandatory.

4.4 Conclusion/Summary

A sensitive stable isotope dilution assay for simultaneous extraction and quantification of BPA, BPF, BPS, BADGE, BADGE chlorohydrins, and BADGE hydrates from different beers was developed. Common isotope standards as BPA-d_{16} or BPA-d_4 have been complemented through synthesis of BADGE-d_4, BADGE·$2H_2O$-d_4, and BADGE·H_2O-d_4. The method was verified by recovery experiments using bottled Pilsner, wheat, and black beer as blank matrices. Recoveries from 75 to 118 % were achieved. Examination of 14 canned beers showed an omnipresence of BPA and BADGE·$2H_2O$. 11 beers contained traces of BPF. Migrations levels of the three substances have been evaluated in accordance to European regulatory framework and proven to be safe for consumers' health. The developed method is an adequate tool for trace analysis of bisphenols and BADGEs in beer. An application for monitoring purposes is possible as well as control of samples whose containers are declared "BPA-free".

4.5 Appendix

4.5.1 Synthesis

Unless otherwise mentioned used chemicals were of synthesis and p.a. grade from different suppliers.

Synthesis of 2-[[2,6-dideuterio-4-[1-[3,5-dideuterio-4-(oxiran-2-ylmethoxy)phenyl]-1-methyl-ethyl]phenoxy]methyl]oxirane (BADGE-d_4)

1.78 g BPA-d_4 (7.7 mmol) were solved with 0.87 g of KOH (15.6 mmol) in 8 ml of ethanol at 60 °C. Ethanol was removed by rotary evaporator and the dried residue was reacted with 2.34 ml of freshly distilled epichlorohydrin (29.8 mmol) at 80 °C for 20 min. After cooling to room temperature the reaction product was solved in acetone and drawn on silica gel by rotary evaporation. The dried BADGE-d_4 raw product-silica gel mixture was applied on the

column and column chromatography was performed using petrol ether/ethyl acetate (9:1; v:v).

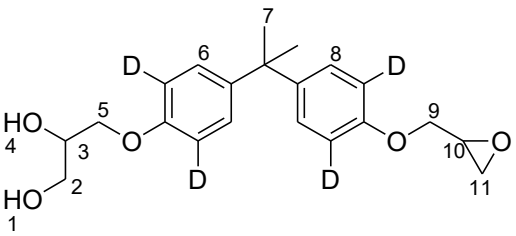
Synthesis of 3-[2,6-dideuterio-4-[1-[3,5-dideuterio-4-(2,3-dihydroxypropoxy)phenyl]-1-methyl-ethyl]phenoxy]propane-1,2-diol (BADGE·2H_2O-d_4) and 3-[2,6-dideuterio-4-[1-[3,5-dideuterio-4-(oxiran-2-ylmethoxy)phenyl]-1-methyl-ethyl]phenoxy]propane-1,2-diol (BADGE·H_2O-d_4)

286 mg BADGE-d_4 were solved in 115 ml of water:acetone (3:2; v:v) and refluxed at 105 °C. Reaction progress was monitored by HPLC-UV. After 45 h reaction was stopped and the substances BADGE-d_4, BADGE·2H_2O-d_4, and BADGE·H_2O-d_4 were separated by column chromatography on silica gel using ethyl acetate as solvent.

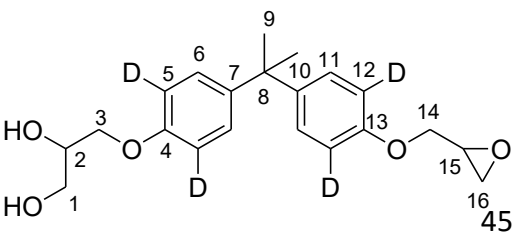
4.5.2 Characterization

NMR spectra were recorded in methanol-d_1 (Aldrich, Steinheim, Germany) and DMSO-d_6 (ABCR, Karlsruhe, Germany) on an Avance II 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) and Avance III 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer (Bruker, Rheinstetten, Germany) at room temperature. The chemical shifts, *δ,* were referenced versus residual solvent shifts in parts per million (ppm). The coupling constants, *J*, are reported in Hertz (Hz). Multiplicities are indicated as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m) and broad (br).

BADGE·H_2O-d_4



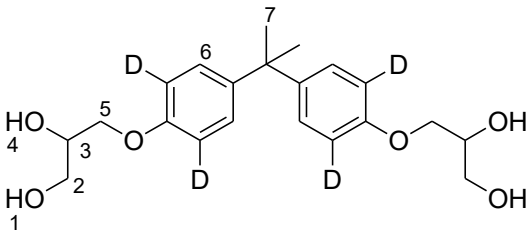
^{1}H-NMR (400 MHz, DMSO-d_6, 25 °C): δ (ppm) = 7.09, 7.10 (2 x br s, 4H, H-6 and H-8), 4.95 (d, 1H, *J* = 4.5 Hz, H-4), 4.68 (t, 1H, *J* = 4.9 Hz, H-1), 4.26 (dd, 1H, *J* = 11.3, 2.7 Hz, H-9), 3.94 (dd, 1H, *J* = 9.4, 4.0 Hz, H-5), 3.83-3.73 (m, 3H, H-5', H-9' and H-3), 3.43 (dd appears as t, 2H, *J* = 4.9 Hz, *J* = 4.9 Hz, H-2), 3.32-3.27 (m, 1H, H-10), 2.82 (dd appears as t, 1H, *J* = 4.9 Hz, *J* = 4.9 Hz, H-11), 2.69 (dd, 1H, *J* = 5.1, 2.7 Hz, H-11'), 1.57 (s, 3.9H, H-7).



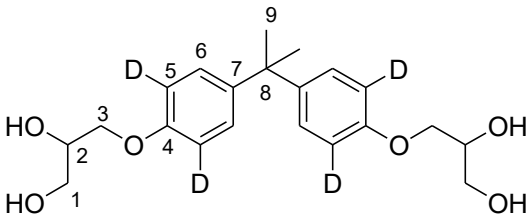
^{13}C-NMR (100 MHz, MeOH-d_1, 25 °C): δ (ppm) = 157.2 (C_q, C-4), 156.9 (C_q, C-13), 144.0, 143.7 (2 x C_q, C-7 and C-10), 2 x 127.8 (2 x CH, C-6 and C-11)

113.9, 113.8 (2 x C_q, 2 x t, J_{C-D} = 23.8, 24.0 Hz, C-5), 71.0 (CH, C-2), 69.4 (CH_2, C-3), 69.3 (CH_2, C-14), 63.4 (CH_2, C-1), 50.5 (CH, C-15), 44.1 (CH_2, C-16), 41.8 (C_q, C-8), 30.7 (CH_3, C-9).

BADGE·2H_2O-d_4



^{1}H-NMR (400 MHz, DMSO-d_6, 25 °C): δ (ppm) = 7.09 (br s, 3.4H, H-6), 4.92 (d, 2H, *J* = 3.9 Hz, H-4), 4.65 (br s, 2H, H-1), 3.94 (dd, 2H, *J* = 9.5, 4.1 Hz, H-5), 3.83-3.75 (m, 4H, H-5' and H-3), 3.43-3.42 (m, 4H, H-2), 1.57 (s, 3.4H, H-7).



^{13}C-NMR (100 MHz, DMSO-d_6, 25 °C): δ (ppm) = 156.4 (C_q, C-4), 142.5 (C_q, C-7), 127.3 (CH, C-6), 113.6 (C_q, t, J_{C-D} = 23.1 Hz, C-5), 70.0 (CH, C-2), 69.5 (CH_2, C-3), 62.8 (CH_2, C-1), 41.2 (C_q, C-8), 30.8 (CH_3, C-9).

5 Project III: LC-MS/MS rapid test system for quantification of bisphenol A, bisphenol F, bisphenol S, and bisphenol A bis(2,3-dihydroxypropyl) ether from canned beverages

in preparation

Authors[6]: Zech, J.; Malchow, S.; Rettberg, N.; Garbe, L.-A.

Abstract

A procedure for fast and reliable bisphenol determination from beverages was developed. Degassing of samples was directly performed in the HPLC vial. For dilution, addition of isotope standard mix, and injection into the LC-MS/MS a procedure related to sandwich injection was applied. Bisphenol A (BPA) and bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE·2H_2O), as the lead components migrating from epoxy can linings, as well as bisphenols F (BPF) and S (BPS) as potential substitutes have been examined in different beverage samples. Recovery experiments verified the suitability of the method to quantify bisphenol content from beer, mixed beer, and cola. Recoveries for all analytes ranged from 87 to 122 % in relevant concentration levels. BADGE·2H_2O was the analyte with the lowest sensitivity with a working range starting from 0.5 µg/l. BPA and BPF can be quantified from 0.1 µg/l on, while BPS can be determined at a level of 0.01 µg/l. In this study 24 beverages have been examined for their bisphenol content.

5.1 Introduction

Today, beverages are frequently packed in plastic or plastic coated containers, cans, and boxes. The use of these packagings can lead to migrations from packaging material into foodstuff. For migration from epoxy can linings, bisphenol A (BPA) and bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE·2H_2O) can be defined as lead substances. BPA migration during pasteurization of canned products has been proven [39, 43, 88]. Storage of cans can, but must not have, an effect on BPA migration in foods and beverages. Here migration highly depends on the coating used and the physicochemical properties of the canned foodstuff [43]. In contrast to BPA, BADGE·2H_2O is not applied as a starting material for

[6] Author contributions: : J.Z. designed research; N.R. and L.-A.G. reviewed the designed research; J.Z. and S.M. performed analytics; J.Z. analyzed data; J.Z. wrote the paper

coating production, but is formed through hydrolysis of bisphenol A diglycidyl ether (BADGE). Factors such as heat and acidity promote this conversion [23]. Additionally, Bisphenol S (BPS) and bisphenol F (BPF) are monitored within this work. Both are used as substitutes for BPA, which is in the focus of scientific, political, and public debates due to its endocrine activity. The ubiquity of BPA represents a challenge within analytical approaches. Trace levels of other bisphenols have also been observed [82]. In order to perform accurate and reasonable analysis of bisphenols, work-intensive countermeasures are needed to prevent falsely positive or increased results. Those problems might occur in case sample pretreatment is not checked thoroughly for contamination sources [89]. Leakage of BPA, BADGE·2H_2O, and further BADGE derivatives from metal needles is described [76, 90]. In former projects [91] contamination of solvents was observed.

Taking this into account, the idea of the work presented herein, was to reduce glass ware and (different) solvents to a minimum and to use the pipetting function of the autosampler (rather than applying disposable tips or needles) to process sample treatment and isotope standard addition within a tightly controlled environment.

5.2 Materials and Methods

5.2.1 Reagents and calibration

Acetonitrile and water were both of LC-MS grade (VWR International GmbH, Darmstadt, Germany). Stock solutions of 1 mg/ml concentration of BPA, BPF, BPS, and BADGE·2H_2O (Sigma-Aldrich, Steinheim, Germany) and corresponding d_4-deuterated isotope standards [83, 91] were prepared separately in acetonitrile. Until use, the stock solutions were stored at -25 °C. Calibration solutions were prepared in acetonitrile/water (25/75, v/v) ranging from 0.1 to 10 µg/l for BADGE·2H_2O, BPF, and BPA; from 0.01 to 1 µg/l for BPS. Isotope standard mix was prepared daily in acetonitrile/water (25/75, v/v) containing 40 µg/l BADGE·2H_2O-d_4, 10 µg/l BPF-d_4 and BPA-d_4, and 1 µg/l BPS-d_4 and installed in the autosampler of the LC-MS/MS. Addition of isotope standard mix to calibration solutions or samples was performed automatically by the autosampler before injection.

5.2.2 Application

A set of 24 samples was collected from local stores and analyzed for their bisphenol content within a time frame of four weeks. The samples can be divided into three general categories:

beers, mixed beers (with sparkling lemonade), and alcopops (on cola basis). Beer samples were taken into account since German males consume an average of 299 g beer per day [87]. Mixed beers - there is a great regional variety of those beverages in Germany - and alcopops were chosen because their market shares are still growing. Cola was chosen, because it is common, contains a high sugar content, is colored, and caffeine was determined to have an effect on BPA migration [88]. Additionally, cola is often basis of mixed beverages as mixed beer and alcopops. Alcopops are of special interest due to their increased sugar and alcohol content. And, all these beverages are filled in cans frequently.

5.2.3 Sample preparation

Canned beverages were mixed by gentle shaking, prior to opening. Samples that contained sediments (as wheat beer based beverages) were shaken upside down for 20 s, allowed to sit for 10 min and opened afterwards. By Pasteur pipette 0.5 to 0.75 ml beverage were transferred into an HPLC vial, which was closed with a screw cap. After treatment in ultrasonic bath for 20 s, the samples were allowed to sit for a moment, before short vortex mixing. To prevent overpressure caused by degassing, aeration was achieved by loosening and retightening the screw caps. Samples could now be inserted into the autosampler of the LC-MS/MS system. Each sample was prepared in triplicates.

5.2.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed using a modular HPLC system (Shimadzu, Duisburg, Germany) coupled to a QTRAP 5500 mass spectrometer (AB Sciex, Darmstadt, Germany). The HPLC was composed of degasser, pump (LC-20AD), autosampler (SIL20AC) including solvent station, and column oven. Analyst 1.5.1 software was used for data analysis. Bisphenols and BADGE·$2H_2O$ were separated using an OTU TriKala C_{18} column (250 x 3 mm; 5 µm; Application & Chromatography, Oranienburg, Germany) connected to a C_{18} guard column (4 x 3 mm; Phenomenex, Aschaffenburg, Germany). Mobile phase A consisted of water, mobile phase B of acetonitrile. Mobile phase was pumped at a flow rate of 500 µl/min at a column temperature of 25 °C. The HPLC gradient is shown in Table 11. The HPLC autosampler was programmed to first aspirate 10 µl of isotope standard mix and second 10 µl of pure sample (based on sandwich technique from polymer measurements and gas chromatography). Both zones were then injected together into the LC-MS/MS system, resulting in a one-to-two dilution for the sample. Alternatively, for a one-to-four

dilution 10 µl of isotope standard mix were injected together with 5 µl of sample and 5 µl of 25 % acetonitrile.

Table 11 HPLC gradient for separation of BPS, BADGE·2H_2O, BPF, and BPA and their corresponding d_4-isotope standards

Time (min)	**A (%)**	**B (%)**
0	75	25
13	49	51
16	10	90
17	75	25
24.75	75	25

MS/MS detection was performed switching from negative to positive electrospray ionization (ESI) mode and back. Multiple-reaction monitoring (MRM) was applied. Nitrogen served as curtain and as collision gas and was varied between periods to achieve the highest sensitivity for each analyte. The source was heated to 400 °C. Periods of ESI positive and ESI negative, MRM transitions, and further MS/MS relevant parameters are shown in Table 12.

5.2.5 Recovery experiment and definition of working ranges

To verify the suitability of the developed method for different beverages, three matrices were chosen: Pilsner beer (from glass bottle), mixed beer (from glass bottle), and cola (from PET bottle). Spiking experiments were performed using three spiking levels per analyte: 0.1, 0.5, and 1.0 µg/l for BADGE·2H_2O, BPF, and BPA and 0.01, 0.05, and 0.1 µg/l for BPS.

5.3 Results and discussion

5.3.1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

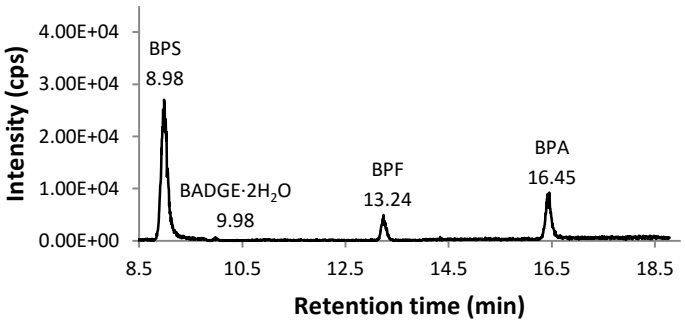
Based on a former project which described a liquid-liquid extraction for extraction, concentration, and quantification of bisphenols and BADGE derivatives from beer samples by stable isotope dilution assay [91], the presented LC-MS/MS rapid test was developed. Since BPS, BPF, and BPA require measurment in negative ionization modus as $[M-H]^-$ ions, while BADGE·2H_2O ionizes as ammonium adduct $[M+NH_4]^+$ in positive mode, each run was devided into four periods. This enables the measurement of each analyte with optimized parameters. A chromatogram of the quantifier ions is shown in figure 8.

Table 12 List of target analytes with MS/MS parameters

Period	Analyte	Precursor (m/z) [type]^{charge}	Curtain gas (psi)	Fragment (m/z)	Isotope standard (ion transition)	Collision energy (V)
1	BPS	249 $[M-H]^-$	36.6	108*	BPS-d_4 (253/110)	-36.0
				92	BPS-d_4 (253/94)	-47.3
				156	BPS-d_4 (253/158)	-30.0
2	BADGE·$2H_2O$	394 $[M+NH_4]^+$	33.3	209*	BADGE·$2H_2O$-d_4 (398/211)	21.0
				135	BADGE·$2H_2O$-d_4 (398/137)	45.0
				107	BADGE·$2H_2O$-d_4 (398/109)	67.0
3	BPF	199 $[M-H]^-$	30.0	105*	BPF-d_4 (203/107)	-29.3
				77	BPF-d_4 (203/79)	-32.7
				93	BPF-d_4 (203/95)	-29.3
4	BPA	227 $[M-H]^-$	32.5	227*	BPA-d_4 (231/216)	-26.0
				135	BPA-d_4 (231/135)	-33.3
				93	BPA-d_4 (231/95)	-58.7

* Quantifier ions are marked with an asterisk.

(A)



(B)

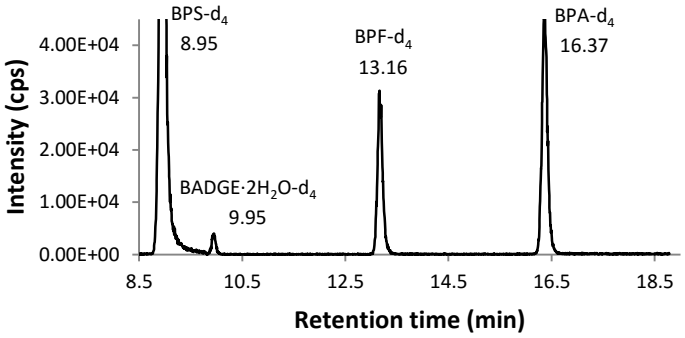


Figure 8 Extracted ion chromatograms of 0.55 µg/l standard (BPS 0.055 µg/l) for separation of BPS, BADGE·2H_2O, BPF, and BPA (**A**) and their corresponding d_4-isotope standards (**B**)

Due to application of SIDA, analyte quantification was possible without the need of several matrix calibrations. Performance parameters of the developed method are shown in table 13. Applying external (solvent) calibration would not have been possible, since ion suppressions have been observed for all analytes examined.

Table 13 Performance parameters of the developed SIDA

Analyte	**Working range (µg/l)**	**linear equation y = mx + n**	**R^2**
BPS	0.01 – 1	y = 2.38x + 0.0015	0.9999
BADGE·2H_2O	0.5 – 10	y = 5.07x - 0.0083	0.9996
BPF	0.1 – 10	y = 1.19x - 0.0050	0.9999
BPA	0.1 – 10	y = 1.50x - 0.0146	0.9998

5.3.2 Development of degassing and automated injection procedure

Final sample processing consisted of sample transfer into the vial by hand and degassing by ultrasonic bath, followed by automated dilution, spiking with isotope standards, and

injection performed by autosampler. Anyway, for thought-provoking impulse some words are lost from development process.

How much sample volume must be transferred to the vial to enable sufficient degassing (for carbonated beverages) while providing the needed 500 µl fill volume needed for injection at the same time? A series of degassing experiments was performed to examine these points in detail. Beer and sparkling mineral water were used as test matrices. Sample was filled in the vial half quantitative, marks on the vial were used as orientation. Control of success was performed by testing if pipetting of the treated sample was possible or not. Table 14 gives an overview on degassing experiments performed and results achieved. To enable a batch processing of samples, from all successful varieties, treatment of 500 to 750 µl of sample for 20 s in ultrasonic bath was chosen as the most practical approach. After degassing, all samples were vortex mixed for approximately 5-10 s to clean foam residues from glass wall and screw cap.

Table 14 Degassing of carbonated beverages in HPLC vials, an overview on different approaches with control of success

Technique	**Volume (ml)**	**Time (s)**	**Septum**	**Beer**		**Mineral water**	
				Comment	**Success**	**Comment**	**Success**
ultrasonic bath	0.5	20	intact	foam formation	yes		yes
ultrasonic bath	0.5	20	perforated	foam formation	yes		yes
ultrasonic bath	1.0	20	intact	foam formation	no		no
ultrasonic bath	1.0	20	perforated	foam formation	no		no
vortex mixer	0.5	20	intact		yes		yes
vortex mixer	0.5	20	perforated		yes		yes
vortex mixer	1.0	60	intact	foam formation	no		no
oven 80 °C	0.5	60	intact		no		yes
oven 80 °C	0.5	60	perforated		no	spilled	yes
water bath 50 °C	0.5	60	intact		no		yes
water bath 50 °C	0.5	60	perforated		no		yes
microwave (800 W)	0.5	60	intact	septum inflated	yes		yes
microwave (800 W)	0.5	60	perforated	septum inflated	yes	spilled	yes

Development of sample treatment in the autosampler started out with the idea to insert samples on the one side and empty vials on the other. Using the solvent station of the autosampler, the samples of interest should be pipetted and diluted. This technique quickly revealed a set of issues. The pipetting of greater volumes, e.g. 100 µl, takes more time than pipetting the corresponding volume by hand. Mixing is performed by aspiration and dispensing of the sample from and into the vial, which is also a time consuming step. Depending on vial size and usage of inlays more or less mixing steps are needed for homogenization. Each sample needs two places in the autosampler, which reduced sample throughput overnight and the weekend; only 70 spots were available in the autosampler used.

By using sandwich technique a higher sample throughput could be achieved. Here, only one position is needed for internal standard mix and, if further dilution is used, one for solvent. The remaining 68 positions could be used for sample vials. The advantages of this injection procedure are a faster sample processing and less volume errors occurring during pipetting, dispensing, and volume distortion (droplets of inhomogeneous solutions at the glass wall or the septum of the vial). The established procedure was verified by a comparing reference measurement of standard samples pipetted by hand and by autosampler. The peak intensities achieved of both methods matched with standard deviations of below 10 %. This clearly indicates the functionality of the automated method. The programming of the autosampler is provided in appendix.

5.3.3 Method characteristics

Sample selection included beer, mixed beer beverages and alcopops. Since this is a very inhomogeneous set of samples a variety of recovery experiments was performed. Table 15 shows the results for the matrices examined. All experiments were performed in triplicates.

Recoveries ranging from 87 to 122 % for all analytes over the observed concentration ranges fulfill the demand of modern analytics. Relative standard deviations equal or below 11 % are presentable for automated dilute and shoot experiments in trace level analytics.

Table 15 Recoveries (%) from spiked Pilsner, mixed beer, and cola samples, relative standard deviation (%) is given in parentheses

Analyte	Pilsner			Mixed beer			Cola		
	0.1 µg/l	**0.5 µg/l**	**1.0 µg/l**	**0.1 µg/l**	**0.5 µg/l**	**1.0 µg/l**	**0.1 µg/l**	**0.5 µg/l**	**1.0 µg/l**
BPS*	87 (2)	88 (6)	92 (5)	101 (4)	106 (2)	112 (3)	99 (11)	116 (3)	118 (2)
BADGE·2H$_2$O	-$^+$	103 (11)	114 (11)	-	122 (2)	113 (1)	-	120 (10)	116 (7)
BPF	107 (16)	102 (1)	101 (5)	103 (3)	96 (4)	88 (5)	120 (4)	107 (4)	101 (1)
BPA	104 (3)	106 (1)	104 (2)	106 (8)	98 (4)	96 (8)	106 (5)	111 (2)	111 (6)

* Recoveries of BPS were determined at 0.01, 0.05 and 0.1 µg/l spiking concentration.

$^+$ Signal-to-noise of BADGE·2H$_2$O laid below 10 for 0.1 µg/l spiking concentration.

5.3.4 LC-MS/MS rapid test of bisphenols from canned beverages

Beverages from the categories beer, mixed beer and alcopops have been examined for bisphenols and BADGE·2H$_2$O content using the developed LC-MS/MS rapid test system. Table 16 gives a summary on the results grouped according to analyte and sample type. Detailed results are available in appendix. On average, findings of BPA, BPF, and BPS, if existent in quantifiable amounts, are in ng/l concentrations. Average findings of BADGE·2H$_2$O for the whole set of samples lay with 8.30 µg/l (arithmetic mean) and 4.32 µg/l (median) about 10-fold higher. If the beverage categories are compared for BADGE·2H$_2$O, a difference between beer and mixed beer cannot be observed in the (small) set of samples examined; beer possesses with 3.17 µg/l the higher median, while mixed beer presents with 5.83 µg/l the higher arithmetic mean. While mean and median for the beer samples almost correspond to each other after examination of 12 samples, the set of 7 mixed beers doesn't show Gaussian distribution, which aggravates interpretation of the results. Raised amounts of BADGE·2H$_2$O have been observed in alcopops. Different reasons for this are reported: the alcopops contained with 10 % alcohol more alcohol than the samples from the other categories, which promotes migration in general and transformation of BADGE into BADGE·2H$_2$O in particular [23]. Regarding beer, products with high sugar contents are treated more intensely regarding to pasteurization units [92–94] than products with fewer carbohydrates; this might also be the case for alcopops. No information was found regarding the pasteurization of alcopops, which combine raised sugar content with raised alcohol content. Also caffeine content might have an impact, which was shown for BPA before [88]. Regarding caffeine, one mixed beer contained cola instead of lemonade and achieved a BPA

result of 0.58 µg/l, which was the highest within this category. Additionally a BADGE·2H_2O content of 9.8 µg/l was determined, ranking second within the category of mixed beers and fifth if compared to all 24 samples examined.

Table 16 Summarized results from sample measurements (µg/l) performed in triplicates

Analyte	**Beer (n = 12)**			**Mixed beer (n = 7)**			**Alcopops (n = 5)**		
	Range	**Ar. mean**	**Median**	**Range**	**Ar. mean**	**Median**	**Range**	**Ar. mean**	**Median**
BPS	0.03 – 0.13	0.08	0.08	n.d.*	-	-	n.d.	-	-
BADGE·2H_2O	0.63 – 10.5	4.37	3.17	0.55 – 17.3	5.83	2.49	2.39 – 39.8	15.9	6.64
BPF	0.11 – 0.26	0.16	0.14	0.24	-	-	0.20 and 0.22	-	-
BPA	0.10 – 0.28	0.18	0.15	0.11 – 0.58	0.32	0.26	0.18 - 1.67	0.61	0.37

* n.d. – not detected

Evaluation of consumer safety is complex in the field of bisphenolic compounds. For BADGE·2H_2O, BADGE·H_2O, and BADGE a summed specific migration limit (SML) is set at 9 mg/kg food or food simulant [50]. Further examinations showed that in aqueous samples BADGE·H_2O and BADGE contributed only in negligible amounts to the sum of the three substances, e.g. in beer [91], thus they were not included in the developed rapid test system. In the current study the highest BADGE·2H_2O amount measured 39.8 µg/l in an alcopop sample. This amount lays around a factor 225 under the summed SML. It can thus be expected that all samples examined can be evaluated as safe for consumption and no further tests must be performed to safeguard the results concerning BADGE·H_2O and BADGE concentrations. For BPA and BPS evaluation is performed via the tolerable daily intakes (TDI). For BPA the temporary TDI (tTDI) amounts to 4 µg/kg body weight/day [41], for BPS to 50 µg/kg body weight/day [84]. Since for alcopops and mixed beers no consumption data is present, the possible daily intake to reach the TDIs was calculated rather than an estimation of the realistic daily intake performed. Thus, it could be determined that an adult of 60 kg body weight would need to drink 144 l of alcopop to reach the tTDI of BPA or 23,077 l of

beer to reach the TDI of BPS. Both calculations were based on the highest determined amounts and prove very descriptive that a normal consumption (compare e.g. National German Consumption Study II [87]) of the examined beverages lays within a safe range. For BPF no regulation is set. Nevertheless, there are two ways to evaluate the achieved results. Both ways were carried out to evaluate findings of BPF from mustard in 2015. One way is to perform a risk assessment via the margin of exposure (MOE) [48]. The other way is to evaluate the findings via the TDI of a structural related compound, here BPA [95]. Using the before described calculation of the possible daily intake, the result is that an adult can drink 923 l of canned beer until reaching the tTDI of BPA. Thus, it can be concluded that none of the samples examined will pose a risk to consumers' health.

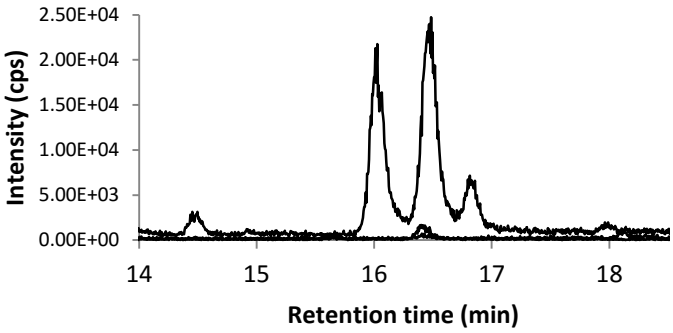
From all samples and calibration points examined, only one measurement showed an abnormal high amount of BPA, which could not be clarified. Procedural blanks showed only traces of analytes below limit of detection (s/n = 3). Thus, a correction of data was not necessary/possible. The run was excluded from the set. One alcopop sample showed an interference for the quantifier ion transition (m/z: 227/212) of BPA. Control of the ion ratios between the ion transitions one, two and three belayed, that the result from the quantifier was inflated. Since ratios between transitions 2 and 3 were in a reasonable range, qualifier 2 was used for quantification in this respective sample. Figure 9 shows a comparison of chromatograms belonging to the peculiar sample and a standard run.

5.4 Conclusion/Summary

In the current study, the successful development and validation of a LC-MS/MS based rapid test system for quantification of BPA, BPF, BPS, and BADGE·2H_2O from canned beverages is described. This method presents a powerful tool for high throughput bisphenol analysis of complex liquid samples such as beer, mixed beer, and cola-based alcopops. Due to the minimal sample processing, analyte loss during extraction, resulting in loss of sensitivity at the same time, is prevented. As a consequence very low concentration levels as 0.01 µg/l for BPS, 0.1µg/l for BPF and BPA or 0.5 µg/l for BADGE·2H_2O can be quantified. Matrix-matched calibration technique, which is needed in external calibration procedures, could be omitted by application of internal d_4-labled isotope standards in combination with sensitive LC-MS/MS analysis. Features as solvent stations enable such automated approaches today. If automation is utilized at full capacity, the time consuming step of sample pretreatment is

almost dispensable. This does not only facilitate and accelerate workflow, but most importantly minimizes the contamination risks within bisphenol analytics.

(A)



(B)

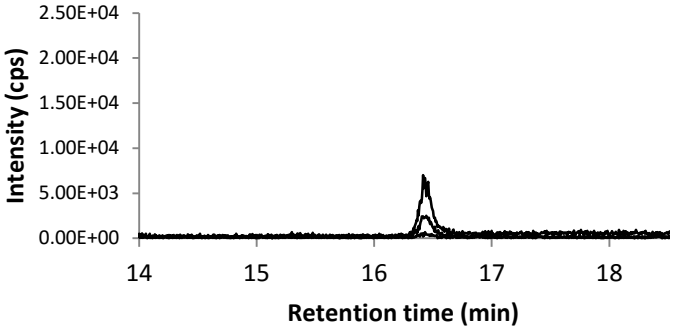


Figure 9 BPA ion transitions for peculiar alcopop sample (**A**) and 0.1 µg/l standard (**B**)

5.5 Appendix

Table 17 Programming of the autosampler SIL-20AC (Shimadzu, Duisburg, Germany) for sandwich injection procedure

Line	Command	Parameter	Remarks
0	A0=	4	Rinses the needle at the rinsing port using rinse liquid 2 (RS2 – acetonitrile).
1	IRINSE	250,35	Replaces the interior of the needle and sample loop using 250 µl of acetonitrile at a flow rate of 35 µl/s.
2	INJ.P		Moves the needle to the injection port.
3	V.INJ		Switches the high-pressure valve to inject position (sample loop is introduced to column).
4	WAIT	2.4	Column is equilibrated and sample loop is filled with mobile phase.
5	V.LOAD		Switches high pressure valve to load position (sample loop is taken out of the flow path).
6	VIAL	70	Moves needle to vial 70, which contains the isotope standard mix.
7	N.STRK	52	Lowers the needle 52 mm into the vial.
8	ASPIR	10,5	Aspirates 10 µl of isotope standard mix at a speed of 5 µl/s.
9	VIAL	SN	Moves the needle to the sample injection vial programmed in batch file.
10	N.STRK	52	Lowers the needle 52 mm into the vial.
11	ASPIR	10,15	Aspirates 10 µl of sample at a speed of 15 µl/s.
12	INJ.P		Moves the needle to the injection port.
13	S.INJ		Switches the high pressure valve to inject position and starts analysis (data acquisition)
14	END		

Table 18 Quantitation results (µg/l) of bisphenols and BADGE2H_2O in canned beverages, relative standard deviation (%) is given in parentheses

Category	No.	Alc. (vol%)	BPS	BADGE·2H_2O	BPF	BPA
Alcopop	1	10.0	< 0.01	2.39 (13)	0.20 (2)	0.37 (5)
	2	10.0	< 0.01	39.8 (3)	< 0.1	0.19 (24)
	3	10.0	n.d.[a]	6.64 (2)	< 0.1	0.62 (7)
	4	10.0	< 0.01	3.74 (3)	0.22 (5)	1.67 (3)
	5	10.0	< 0.01	16.8 (4)	< 0.1	0.18 (11)
Beer	1	4.9	< 0.01	8.43 (9)	< 0.1	0.20 (9)
	2	4.9	n.d.	1.45 (13)	0.14 (7)	0.22 (7)
	3	5.0	n.d.	4.89 (14)	< 0.1	0.15 (33)
	4	4.9	n.d.	< 0.5	n.d.	< 0.1
	5	4.9	n.d.	0.85 (13)	< 0.1	0.14 (14)
	6	4.8	n.d.	7.75 (2)	< 0.1	0.23 (1)
	7	4.8	n.d.	7.39 (10)	0.11 (13)	0.14 (13)
	8	5.2	0.13 (9)	0.97 (17)	0.26 (5)	< 0.1
	9	4.9	n.d.	0.83 (5)	< 0.1	< 0.1
	10	7.5	n.d.	10.5 (11)	0.14 (9)	0.28 (8)
	11	< 0,5	n.d.	0.63 (8)	< 0.1	0.10 (2)
	12	5.2	0.03 (28)	< 0.5	< 0.1	0.14 (10)
Mixed beer	1	2.5	n.d.	17.3 (2)	0.24 (5)	0.55 (7)
	2	2.5	n.d.	2.49 (6)	< 0.1	< 0.1
	3	5.0	n.d.	1.15 (15)	< 0.1	0.26 (14)
	4	-	< 0.01	2.63 (5)	< 0.1	0.11 (6)
	5	2.5	n.d.	0.55 (3)	< 0.1	0.12 (4)
	6	2.5	n.d.	0.68 (4)	< 0.1	< 0.1
	7	3.1		9.80 (5)	< 0.1	0.58 (1)

[a] n.d. – not detected

6 Closing discussion

The presented work is composed of three chapters. All are concerned with BPA and related bisphenolic compounds. The work was initiated by the continuing controversial discussion on BPA and its endocrine effects, as well as a lack on isotopically labeled bisphenolic standards further than BPA-d_{16} and $^{13}C_{12}$-BPA.

One important field of BPA application is the production of thermal paper, here the substitution of BPA is recommended by authorities [67]. As a consequence of this recommendation BPS is more and more frequently traced in paper products and currency bills from different countries. Those finding prove, that occasionally substitution of BPA through BPS takes place [73]. In order to maintain the functionality of e.g. thermal paper products, similar amounts of BPS as otherwise of BPA are applied. As a consequence the entrance of BPS in the paper recycling cycle was observed [73]. Additionally, BPS and BPF were found in indoor dust from the United States and three Asian countries. In respect to the concentration, BPS ranked second and BPF third after BPA [6]. Those findings suggest that BPS and BPF might be the next substances with ubiquitary presence in environmental samples, and thus, will surely be subject in the ongoing debate of consumer safety and human health.

At the beginning of this work only isotope standards for BPA, namely BPA-d_{16} and $^{13}C_{12}$-BPA were commercially available. This fact was surprising, since stable isotope labeled standards, respectively their use as internal standards in mass spectrometry based methods, are regarded as being the superior technique for accurate and reliable quantification of trace contaminants. Our workgroup achieved good results with application of BPA-d_4 [51]. A reproducible protocol for synthesis and clean-up of this isotope standard was successfully developed [83]. In this protocol, deuterium labels were introduced by a cost effective and straightforward hydrogen-deuterium exchange reaction using unlabeled BPA as starting substance. Applying the same conditions on BPS no hydrogen-deuterium-exchange could be observed. This is due to strong deactivation of the aromatic system caused by the negative mesomeric effect (-M effect) of the sulfonyl group in BPS. Changing the educt for deuteration from BPS to 4,4’-thiodiphenole (+M effect), introduction of the four deuterium atoms could be achieved. BPS-d_4 was finally obtained by oxidation of the intermediate using hydrogen peroxide. In order to deuterate BPF, reaction conditions as described for BPA were

applied. The exchange reaction could be successfully completed, but a prolonged heating period compared to BPA-d_4 synthesis was necessary.

For Germany no data concerning application and determination of BPA, and the potential substitutes BPF and BPS, from thermal paper was available. Thus, a set of 14 cash register receipts was examined using aforementioned deuterium labeled standards within SIDA. Solid liquid extraction of spiked samples resulted in high recoveries for the analytes examined, proving the suitability of the developed SIDA. One receipt out of 14 contained BPS (6.4 mg/g), while all further receipts contained BPA (at average 14.6 mg/g). These results were in accordance to published data from other countries [3, 70, 71] – especially countries with no legal framework concerning BPA in paper products. Out looking on future projects, it might be worthy to examine paper products labeled as being “BPA free”. Since further substitutes than BPF and BPS can be expected, a non-target approach concerning substitution seems appropriate.

From an analytical perspective, thermal paper can be regarded as a matrix with only limited complexity, also bisphenols – applied in their native monomeric form – are contained in high amounts. In the next phase of the project, it was aimed to work with more complex matrices that contain fewer concentrations (to be expected) of the target analytes. Canned beer, as fermented beverage, seemed to be an appropriate sample type. Interferences have been shown in GC measurements [86]. Concerning leaching from epoxy can linings, next to BPA also BADGE and its derivatives were considered as analytes of interest. Thus, decision was taken to synthesize three more isotope standards based on BPA-d_4 and develop a LC-MS/MS method for simultaneous determination of bisphenols and BADGEs. BADGE chlorohydrins were excluded from isotope labeling, since only small amounts of them were expected in beverages [43, 53]. BADGE-d_4 served mainly as an intermediate from which BADGE·H_2O-d_4, and BADGE·$2H_2O$-d_4 could be yielded by hydrolysis. The latter compounds are not commercially available, yet. Great efforts in respect to experiments and time were needed for the development of a LC-MS/MS method, which not only separated the target analytes, but also provided adequate sensitive ionization at the same time. Bisphenols ionize very sensitive in negative electrospray ionization, whereas the solvent system of preference consists of acetonitrile and water. At the same time BADGEs ionize in positive electrospray ionization, showing a variety of adducts next to the single hydrogenated molecule $[M+H]^+$,

e.g. plus sodium, plus ammonium. The preferred system consists of methanol and water; in some cases modifiers like ammonium- or sodium acetate are used. Bisphenols are rather hydrophilic, eluting early from C_{18} stationary phases. BADGEs are rather hydrophobic compounds, thus, eluting towards the second half of the HPLC run. An experiment was performed to switch from one solvent system to the other within the run, obtaining promising results in preliminary tests. After optimization of the length and the gradient of the solvent substitution step and introduction of four periods, between which polarity was reversed for detection (in order: negative, positive, negative, and positive), the method could be applied for verification measurements and sample analysis. Summarized, a sensitive and robust LC-MS/MS method was developed and proven suitable by recovery experiments for trace determination of BPA, BPF, and BPS, as well as BADGE, its hydrates, and chlorohydrins [91]. To the best of our knowledge no such method was published before. In literature merely two HPLC fluorescence methods were found [54, 55], which were applied for analysis of BPA and BADGEs from food samples with working ranges about a factor ten above the ones needed for beverages. As has been stated before [81], a high percentage of beverage samples contain BPA concentrations below 1 µg/l. Concentrations of BADGE derivatives, BADGE·$2H_2O$ excluded, are usually present in even smaller concentrations. Thus, a lot of common methods are not sensitive enough for quantification of those substances from beverages. For application, bisphenols and BADGEs were determined from different beer types. Sample types examined were Pilsner, wheat beer, black beer, and others. Summarized, BPA and BADGE·$2H_2O$ were tested positive in 14 out of 14 samples. Except of three samples for BADGE·$2H_2O$ (below 0.5 µg/l) and one sample for BPA (below 0.1 µg/l) all samples contained the two analytes in quantifiable concentrations. Concentration of BPA at an average of 0.81 µg/l (median of 0.31 µg/l) corresponds at level with literature data [56, 86]; BPA concentrations for canned beers in the third project part likewise. BADGE·$2H_2O$ concentrations in canned beer ranged from 0.64 µg/l to 14.3 µg/l (arithmetic mean: 3.09 µg/l, median 0.96 µg/l) in the second project part. Comparison data on BADGE·$2H_2O$ for beer is scarce with only two single concentrations of 5.1 and 4.3 µg/l reported [76], which lay inside the determined range. Data from other canned beverages (containing less alcohol than beer) lay at the same level [76]. In accordance is also the (almost) absence of further BADGE derivatives. One single sample from the “others”-category contained amounts of BADGE·HCl·H_2O and BADGE·2HCl of 0.65 and 0.55 µg/l,

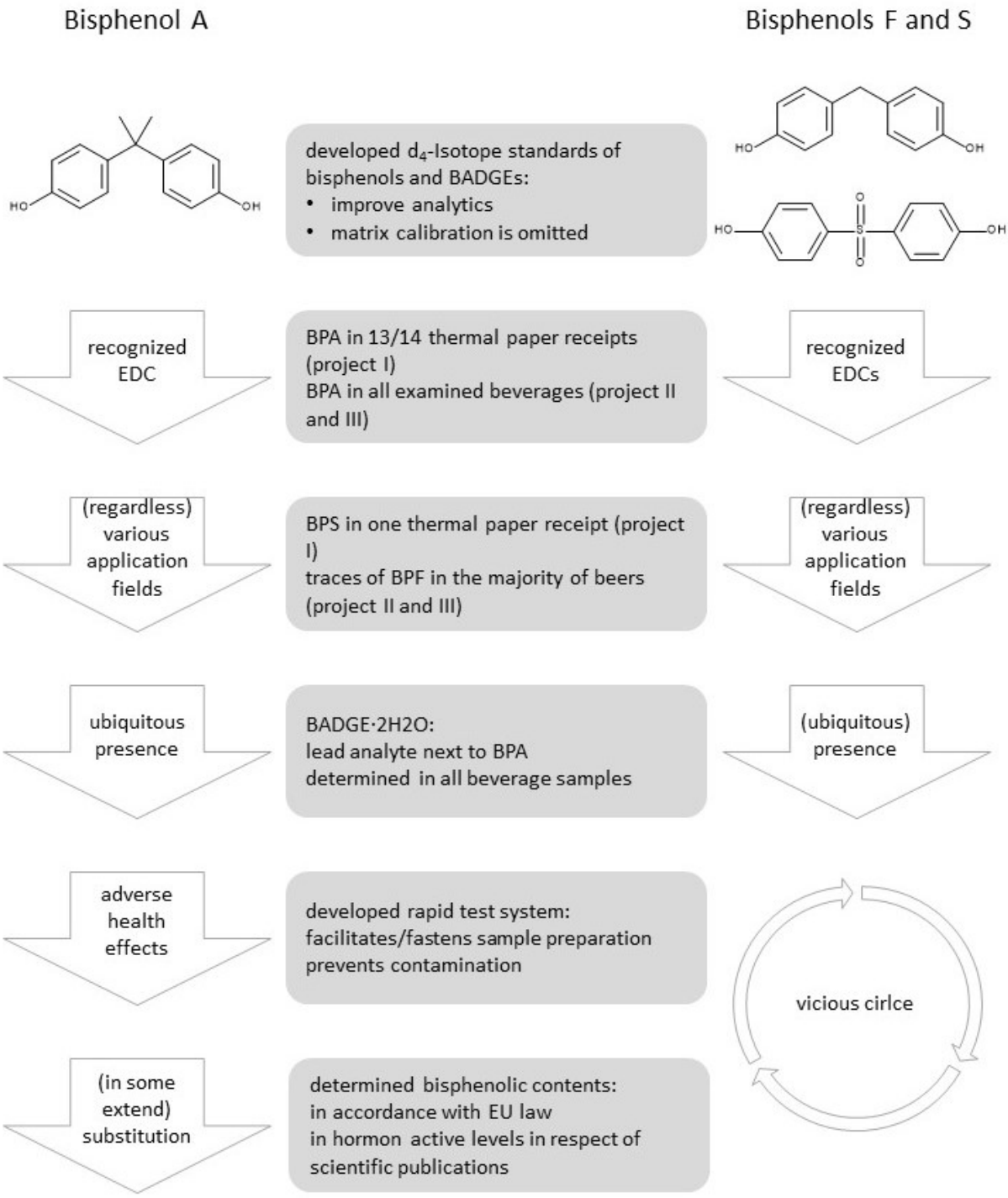
respectively. Debatable are the numerous findings of small amounts of BPF in beer samples in second and third project. To the best of our knowledge, BPF from canned beer was not examined before, but in soft drinks corresponding concentrations of 0.2 and 0.1 µg/l were stated for orange and lemon soda [53]. Recent findings proved the presence of BPF in white and yellow mustard assumed to be biosynthesized from glucosinalbin via 4-hydroxybenzyl isothiocyanate and 4-hydroxybenzyl alcohol [48]. 4-hydroxybenzyl alcohol was also determined at 2.3 mg/l in one high hopped beer sample before [96]. It must be underlined that this was only a single finding of two beer results published. Thus, it might be of value to examine if the presence of 4-hydroxybenzyl alcohol in high hopped beers can be reproduced. If present, further beer types should be tested for 4-hydroxybenzyl alcohol. Additionally the beers examined can be screened for BPF content to clarify, if there is a possibility of BPF in beers originating from biosynthesis rather than from migration. Naturally, those experiments should be performed using bottled beers rather than canned samples.

Some studies state peculiar high amounts of BPA in beer at an average of 2.9 µg/l [85] or 4.2 µg/l [51]. Even higher are published contents from beverages packaged in paper boxes (juice and milk above 40 µg/kg) [97]. Scrutinizing the results presented, it is obvious, that in two studies no recovery experiment is presented [51, 97]. In the other study the lowest spiking level for BPA is about a factor ten above the average concentration of 2.9 µg/l [85]. For BADGE, which also was determined, spiking level accounts for a factor 53 of the 0.38 µg/l average stated. Performing spiking experiments in unrealistic high concentrations, poses the risk to oversee contamination during sample treatment. Thinkable is that contamination of samples during pretreatment caused increased results detected. Carrying along of a procedural blank can help identify possible contaminations, but is not reported in either of the studies. For BPA and BADGE contamination from different sources has been stated [53, 89, 90]. Thus, the results of the three articles are questionable. In the here presented second project, false positive results for BPS were recognized right away. Since procedural blank showed peculiar increased results, sample treatment, glassware and solvents were critically observed for BPS leakage or contamination. Result was the finding of a contaminated bottle of water used during sample dilution. LC-MS water is filtered before filling. Filter membranes are often manufactured from polysulfone (also called polyethersulfone, PES). Here a carry-over of BPS from filter to water is thinkable. Since BPS ionizes with increased sensitivity

compared to the other bisphenols, already small contaminations through leakage can show severe signals in blank measurements and significantly adulterate results.

Building on the knowledge gained so far, it seemed reasonable to establish a rapid test system for determination of selected analytes from canned aqueous samples, respectively beverages. Bisphenol A and BADGE·2H_2O were defined as lead substances for determination. Additionally BPF and BPS were included in examinations. Goal was to combine the potencies of the sensitive measurement technique with the advantages of stable isotope dilution assay; additionally preventing potential contamination of sample material as far as possible. Thus, a fast in-vial sample workup was developed for carbonated beverages. Further sample treatment was substituted by automated dilute-and-shoot procedure using the autosampler of the LC-MS/MS system. On the basis of sandwich injection technique, sample was aspirated, diluted, and spiked with isotope standard inside of the injection needle. Working ranges reflect the sensitivity of the method, starting at 0.5 µg/l for BADGE·2H_2O, 0.1 µg/l for BPA and BPF, and 0.01 µg/l for BPS. The rapid test was utilized to investigate differences in bisphenol migrations from beverage cans to beers, mixed beer beverages, and alcopops on cola basis. Results for beers and mixed beers were in correspondence with the former measured and discussed data from project 2. For BPA, a slight increase was observed for arithmetic means and medians in the following order: beer < mixed beer < alcopops. For BADGE·2H_2O, differences between beer and mixed beer were not distinct. Concentrations of alcopops in contrast were obviously increased with an arithmetic mean of 15.9 µg/l (median: 6.6 µg/l) accounting for two to three times of the amounts determined from beer and mixed beer. Raised concentrations of BPA and BADGE·2H_2O in alcopops are probably caused by increased migration due to higher alcohol content. Additionally, but this could not be verified, pasteurization process might differ from beer and mixed beer (longer time, higher temperature), due to raised sugar content, which then also would have an impact. Summarized, the developed rapid test impresses by fast batch processing of samples. Applying the minimal manual workup followed by the automated workup developed, not only a reduction and savior of solvents and glassware was achieved, but also a solid prevention from contamination.

Figure 10 presents an overview on the achieved milestones of the three projects in relation to the former known background on BPA.



EDCs: endocrine disrupting chemicals

Figure 10 Background on bisphenols **(arrows)** in context with the performed work **(boxes)**

7 Summary

The presented work contains three individual projects, all concerned with the stable isotope dilution analysis of bisphenols. Within the first project, the bisphenolic isotope standards, BPA-d_4, BPF-d_4, and BPS-d_4 were successfully synthesized and applied for quantification of BPA, BPF, and BPS from thermal paper. Cash register receipts from Berlin, Germany were determined to contain BPA in the order of tens mg/g in 13 out of 14 receipts examined. These amounts were in accordance to data of comparable studies from other countries. The 14th sample contained BPS instead of BPA. Based on those findings it was concluded, that for Germany no extensive substitution of BPA in this application field occurred until 2014.

From an analytical perspective thermal paper can be regarded as a matrix with limited complexity, also bisphenols – applied in their native monomeric form – are contained in high amounts. Consequently, the second projects' aim was to quantify bisphenolic compounds from more complex matrices and in smaller concentrations as well as to broaden the range of analytes. For that reason, the existent isotope standards were complemented through synthesis of BADGE-d_4, BADGE·H_2O-d_4 and BADGE·$2H_2O$-d_4. In the following, a sensitive method for the combined analysis of bisphenols and BADGEs within one run could be established. A comparable LC-MS/MS method was not published before; this is possibly due to different analytical challenges concerning the two analyte groups (bisphenols and BADGEs). Especially severe needs in case of solvents and ionization modes aggravated the method development in the beginning. After optimization, nine analytes, including BPA, BPF, and BPS as well as BADGE with its two hydrates and three chlorohydrins, were examined from canned beers of different type. All 14 beers examined were tested positive for BPA and BADGE·$2H_2O$, mostly ranging in order of one hundreds ng/l and ones µg/l, respectively. These results were consistent with literature data. Several findings of BPF in small concentrations were debatable. Recently published data suggested a biosynthesis of BPF over the intermediate 4-hydroxbenzyl alcohol (occurring in mustard production). Since 4-hydroxybenzyl alcohol was once proven from high hopped beer, there might be a relation with the BPF findings presented, but this has to be verified. In general all findings were in accordance with European law as far as regulated. In contrast, it should be remarked that levels determined were at the same time in concentrations that have been shown to exhibit endocrine activity in several studies.

During the extensive work with bisphenols in the course of the works of the previous chapters, contamination of samples by lab ware, chemicals, as well as analytical instruments was identified as one of the major challenges in bisphenol analysis. As a consequence within the third project, a rapid test system for determination of BPA, BPF, BPS, and BADGE·$2H_2O$ from canned beverages was established. By establishment of an in-vial sample preparation and automated dilute-and-shoot technique using the LC-MS/MS autosampler, suitable results in recovery and sample measurements were achieved. It must be underlined, that due to the minimalistic approach concerning the usage of glassware and solvents, contamination of samples during sample workup could be effectively minimized.

In conclusion it can be said, that the synthesized isotope standards can contribute a part to sensitive, precise and robust quantification of bisphenolic compounds from different matrices. Independently from approach – solid liquid extraction, liquid-liquid extraction, and dilute-and-shoot were applied – a robust and reliable detection and quantification of the target analytes was achieved. Especially, applying the half automated rapid test system, time and expenses could be omitted due to advantages of stable isotope dilution assay. Availability of a rapid, reliable, and cost effective bisphenol analysis is an essential building block of ongoing research in this field of science. It is a fundamental tool to assure food and consumer safety as well as to support studies in the field of endocrinology.

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9 Appendix

List of publications related to the presented work

- Garbe, L.-A.; Cao, X.-L.; **Zech, J.**: New Data on bisphenol A (BPA) concentrations in canned beer, World Brewing Congress 2012 (poster presentation)
- **Zech, J.**; Garbe, L.-A.: Bestimmung von Bisphenol A aus Dosenbier mittels GC-MS, Lebensmittelchemikertag 2012 (poster presentation)
- **Zech, J.**; Manowski, A.; Schulze, A.-K.; Canitz, C.; Neumann, K.; Garbe, L.-A.: Stable Isotope Dilution Assay of Bisphenol A, Bisphenol F and Bisphenol S from Thermal Paper Using High-Performance Liquid Chromatography-Tandem Mass Spectrometry, Deutsche Lebensmittel-Rundschau, **111** (2015), pp. 129-136
- **Zech, J.**; Malchow, S.; Rettberg, N.; Garbe, L.-A.: Determination of bisphenols, bisphenol A diglycidylether (BADGE), BADGE chlorohydrines and hydrates from canned beer by LC-MS/MS, Tag der Biotechnologie 2015 (poster presentation)
- **Zech, J.**; Malchow, S.; Rettberg, N.; Garbe, L.-A.: Aktueller Stand zum Thema Bisphenole in Dosenbieren und -getränken, VLB-Oktobertagung 2015 – Meeting VLB Technical Scientific Committee (oral presentation)
- **Zech, J.**; Manowski, A.; Malchow, S.; Rettberg, N.; Garbe, L.-A.: Determination of Bisphenols, Bisphenol A Diglycidyl Ether (BADGE), BADGE Chlorohydrins and Hydrates from Canned Beer by High-Performance Liquid Chromatography-tandem Mass Spectrometry, BrewingScience - Monatsschrift für Brauwissenschaft, **68** (2015), pp. 102-109

List of further publications

- **Zech, J.**; Lorenz, E.; Nickel, D.; Garbe, L.-A.: Bestimmung von Glutathion, Cystein und deren Disulfiden aus Fermentationsproben mittels LC-MS/MS, Lebensmittelchemikertag 2013 (poster presentation)
- Malchow, S.; **Zech, J.**; Back, D.-A.; Garbe, L.-A.: Stabilitätsuntersuchungen von Cefoxitin in isotoner NaCl-Lösung mittels HPLC, Lebensmittelchemikertag 2013 (poster presentation)
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