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Determination of Bisphenols, Bisphenol A Diglycidyl Ether (BADGE), BADGE Chlorohydrins and Hydrates from Canned Beer by High-Performance Liquid Chromatography-tandem Mass Spectrometry

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.

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

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 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 [1–4]. 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 [5–7] or infant feeding bottles [8]. Scientific examination and discussion about the question if BPS and BPF are safe substitutes already started [9].

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 1. For BADGE and BADGE hydrates sum parameters are defined [10] 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 2 gives an overview on literature data which presents examinations of bisphenolic compounds in beer. The application of isotope standards within quantification is beneficial [13], 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 [14–17]. Synthesis of deuterated BPA, BPF, BPS, and BADGE is described [4, 15, 18, 19]. 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 [1, 14]. Findings of bisphenols and BADGEs in canned beer are also stated in table 2.

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

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Table 1 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*	[11, 12]
BADGE 2,2-bis(4-hydroxyphenyl)propanebis(2,3-epoxypropyl)ether	1675-54-3	0.15 sum parameter	9 sum parameter	[10]
BADGE·H$_2$O 3-(4-{2-[4-(2-Oxiranylmethoxy)phenyl]-2-propanyl}phenoxy)-1,2-propanediol	76002-91-0			
BADGE·2H$_2$O 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	[10]
BADGE·HCl·H$_2$O 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*	[11]
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

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.

2 Materials and Methods

2.1 Synthesis of isotope standards

The isotope standards BADGE-d$_4$, BADGE·2H$_2$O-d$_4$, and BADGE·H$_2$O-d$_4$ were prepared as described by Rauter et al. (1999) for the non-labeled substances [3] with only slight modifications within sample clean-up for BADGE. Detailed information on synthesis and characterization of isotope standards can be seen in section 5.

Table 2 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	[14]
BADGE·2H$_2$O BADGE·H$_2$O BADGE·HCl·2H$_2$O BADGE·HCl BADGE·2HCl	BADGE·2H$_2$O	5.1 and 4.3 µg/kg	LC-MS/MS	[1]
BPA BADGE	BPA BADGE	1.26 to 3.79 µg/l 0.11 to 0.74 µg/l	HPLC fluorescence	[20]
BPA	BPA	0.081 to 0.54 µg/l	GC-MS	[21]
BPA	BPA	1.9 to 6.6 µg/l	GC-MS	[15]
BPA	BPA	1.5 µg/l	HPLC fluorescence	[22]

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 3). The injection volume was 25 µl.

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 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

Table 3 **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

standard mix was prepared in acetonitrile containing 100 µg/l of BPA-d$_4$, BPF-d$_4$, BADGE·2H$_2$O-d$_4$, and BADGE·H$_2$O-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 5.

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 the LC-MS/MS system.

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.

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.

3 Results and discussion

3.1 Synthesis and characterization of deuterated bisphenols

BPA-d$_4$, BPF-d$_4$, and BPS-d$_4$ “remained” from a previous work [18] and were used after GC-MS control of deuteration (data not shown). BADGE-d$_4$, BADGE·H$_2$O-d$_4$, and BADGE·2H$_2$O-d$_4$ were

Table 4 **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	108*	BPS-d$_4$ (253/110)	-36
		$[M-H]^-$	92	BPS-d$_4$ (253/94)	-47
2	BADGE·2H$_2$O	394	209*	BADGE·2H$_2$O-d$_4$ (398/211)	21
		$[M+NH4]^+$	135	BADGE·2H$_2$O-d$_4$ (398/137)	45
3	BPF	199	105*	BPF-d$_4$ (203/107)	-29
		$[M-H]^-$	77	BPF-d$_4$ (203/79)	-33
	BPA	227	212*	BPA-d$_4$ (231/216)	-26
		$[M-H]^-$	133	BPA-d$_4$ (231/135)	-33
4	BADGE·HCl·H$_2$O	412	227*	BADGE·H$_2$O-d$_4$ (380/211)	23
		$[M+NH4]^+$	135	BADGE·H$_2$O-d$_4$ (380/211)	47
	BADGE·H$_2$O	376	209*	BADGE·H$_2$O-d$_4$ (380/211)	19
		$[M+NH4]^+$	191	BADGE·H$_2$O-d$_4$ (380/193)	27
5	BADGE	358	191*	BADGE-d$_4$ (362/193)	19
		$[M+NH4]^+$	135	BADGE-d$_4$ (362/137)	47
	BADGE·HCl	394	227*	BADGE-d$_4$ (362/193)	19
		$[M+NH4]^+$	135	BADGE-d$_4$ (362/193)	43
	BADGE·2HCl	430	135*	BADGE-d$_4$ (362/193)	47
		$[M+NH4]^+$	107	BADGE-d$_4$ (362/193)	71

* Quantifier ions are marked with an asterisk

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 ^{1}H- and ^{13}C-NMR data of labeled BADGE hydrates is provided in section 5. 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 1. 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.

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. 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 $[M+NH4]^+$ were examined as precursors, from which the adducts 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 2 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 4, 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 5). 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.

3.3 Method characteristics

Within recovery experiments, results from 75 % to 118 % were achieved. Detailed data can be seen in table 6. Relative standard deviation accounted, with exception of one spiking experiment, for less than 25 %, which is appropriate for trace level analysis.

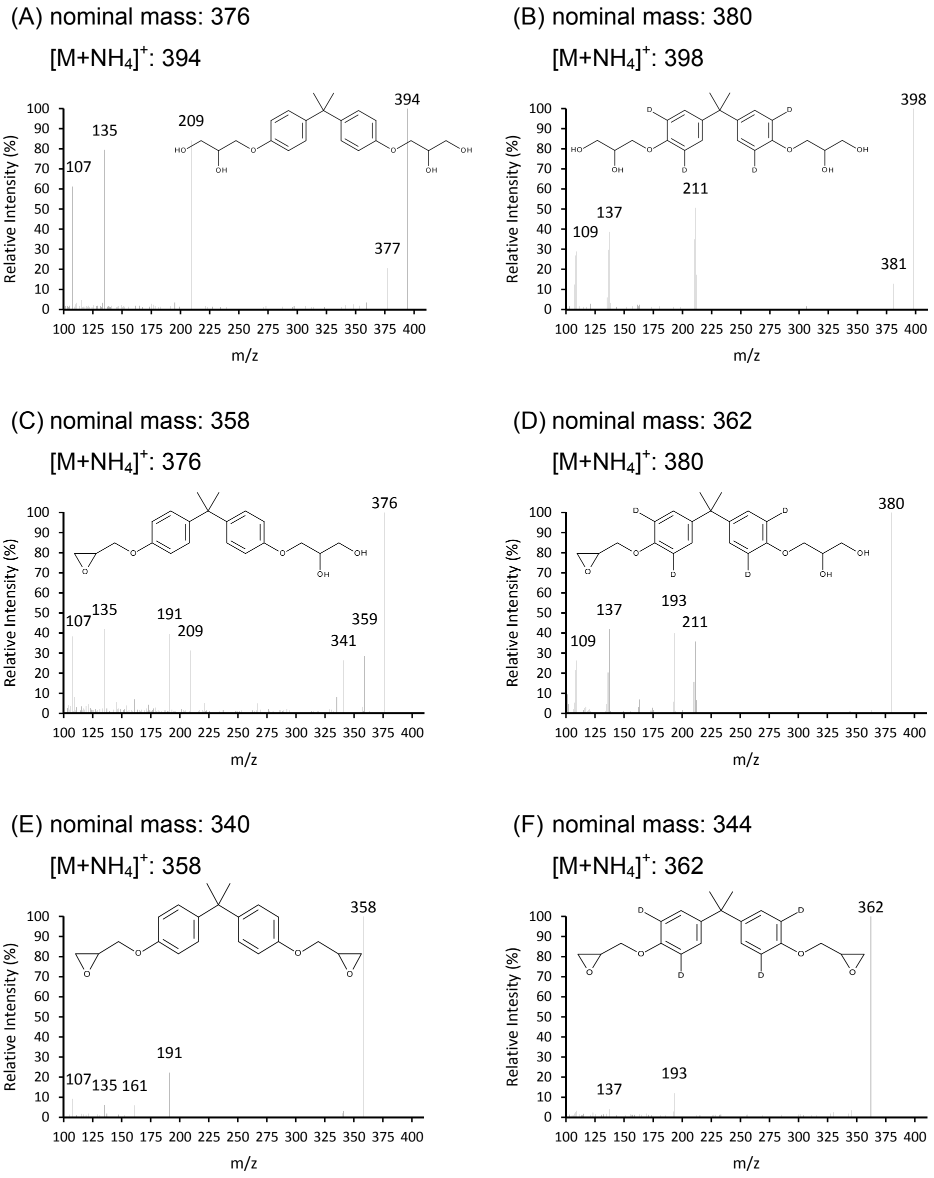


Fig. 1 Structures and nominal masses of BADGE·$2H_2O$ (A), BADGE·$2H_2O$-d_4 (B), BADGE·H_2O (C), BADGE·H_2O-d_4 (D), BADGE (E), and BADGE-d_4 (F) and ESI(+)-MS/MS spectra of corresponding ammonium adducts $[M+NH_4]^+$

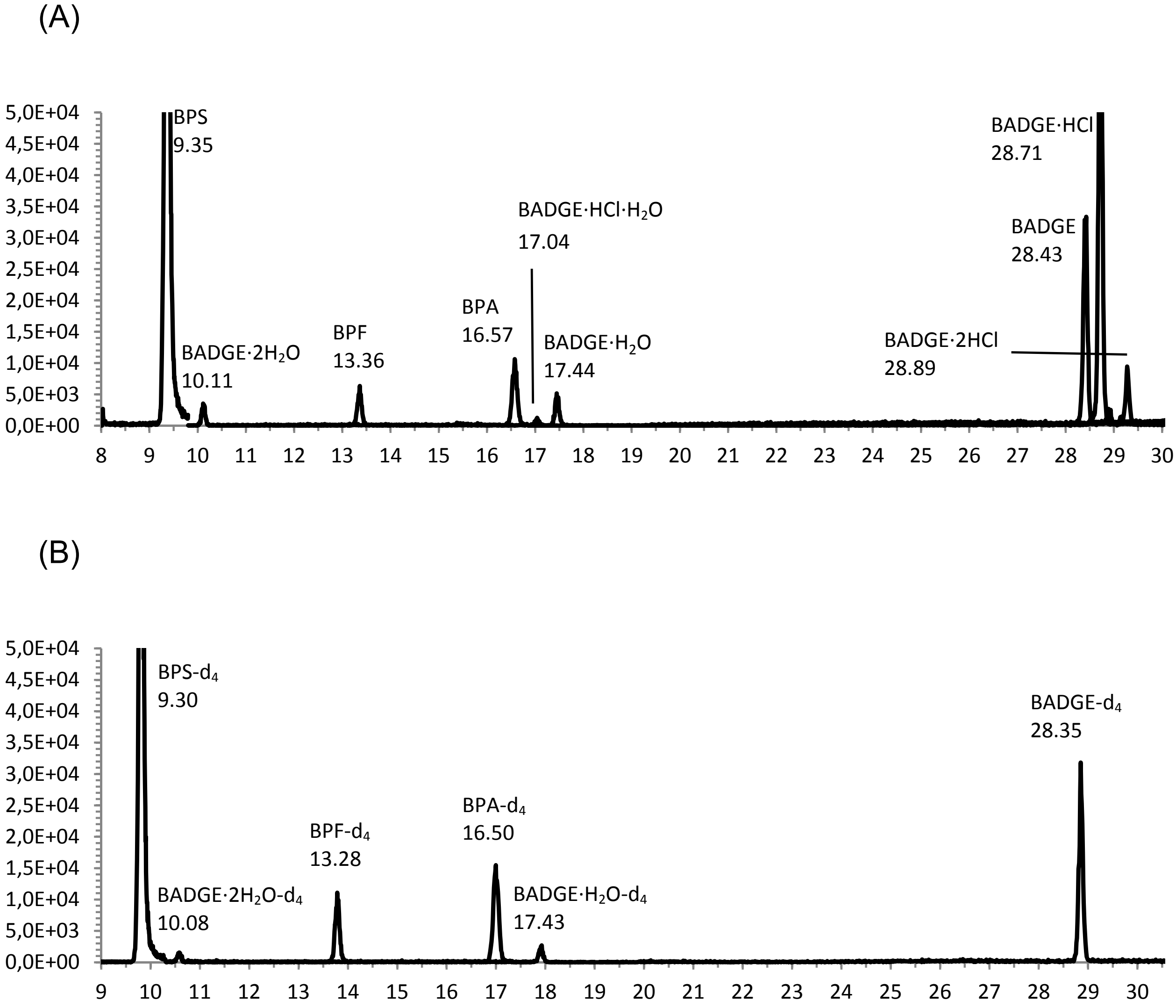


Fig. 2 Extracted LC-ESI-MS/MS chromatograms of quantifier ion transitions (A) and isotope standards (B) in solvent at a concentration of 1.0 µg/l + 2.5 µg/l isotope standard

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.

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 7. 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 2. They also correspond to findings in other canned beverages [1, 14, 15, 17, 23]. 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 [17]. There, the

Table 5 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

Table 6 **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

four beers examined were bottled samples and did not contain any bisphenols (personal contact: Dr. C. Liao).

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 [23], the resulting dietary intakes amount to 0.015 µg/kg body weight/day for BPA and 0.083 µg/kg 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 [23], assuming that exclusively canned beer was consumed with the highest concentrations determined within the presented work (compare Table 7). 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 consumers' health. Recently a complete risk assessment for BPF (in mustard) was published [24]. 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 consumers' 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.

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 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".

5 Appendix

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

Table 7 **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

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$_2$O-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$_2$O-d$_4$)

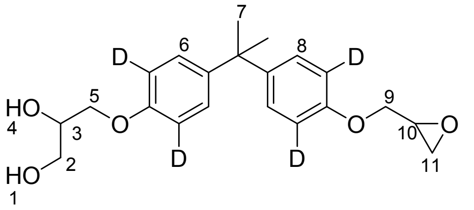
286 mg BADGE-d$_4$ were solved in 115 ml of water:acetone (3:2; v:v) and refluxed at 105 °C. Reaction progress was monitored by HPLC-UV. After 45 h, reaction was stopped and the substances BADGE-d$_4$, BADGE·2H$_2$O-d$_4$, and BADGE·H$_2$O-d$_4$ were separated by column chromatography on silica gel using ethyl acetate as solvent.

5.2 Characterization

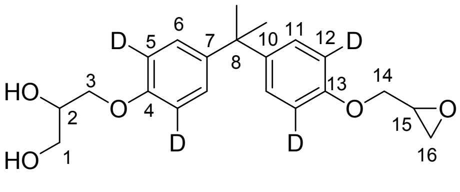
NMR spectra were recorded in methanol-d$_1$ (Aldrich, Steinheim, Germany) and DMSO-d$_6$ (ABCR, Karlsruhe, Germany) on an Avance II 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) and Avance III 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer (Bruker, Rheinstetten, Germany) at room temperature. The chemical 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$_2$O-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).

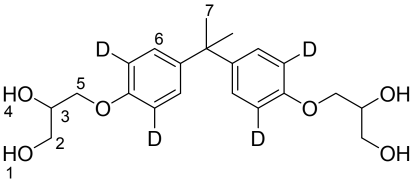


^{13}C-NMR (100 MHz, MeOH-d$_1$, 25 °C): δ (ppm) = 157.2 (C$_q$, C-4), 156.9 (C$_q$, C-13), 144.0, 143.7 (2 x C$_q$, C-7 and C-10), 2 x 127.8 (2 x CH, C-6 and C11) 113.9, 113.8 (2 x C$_q$, 2 x t, J_{CD} = 23.8, 24.0 Hz, C-5), 71.0 (CH, C-2), 69.4 (CH$_2$, C-3), 69.3 (CH$_2$, C-14), 63.4 (CH$_2$, C1), 50.5 (CH, C-15), 44.1 (CH$_2$, C-16), 41.8 (C$_q$, C-8), 30.7 (CH$_3$, C-9).

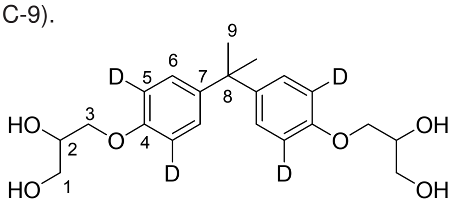


BADGE·2H$_2$O-d$_4$

^{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, H4), 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, H7).



^{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_{CD} = 23.1 Hz, C-5), 70.0 (CH, C-2), 69.5 (CH_2, C-3), 62.8 (CH_2, C1), 41.2 (C_q, C-8), 30.8 (CH_3, C-9).



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