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Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by HLPC-MS/MS

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Abbreviations: BPA, bisphenol A; BPA-monoglucuronide, BPA-gluc; CAD, collision activated disotiation; DP, declustering potential; EPI, enhanced product ion; ER, enhanced resolution; IDA, information dependent acquisition; LIT, linear ion trap; LOD, limit of detection; LOQ, limit of quantitation; MRM, multiple reaction monitoring; S/N, signal to noise ratio

ABSTRACT

Bisphenol A (BPA) is a weak estrogen. Pharmacokinetic studies of BPA have demonstrated a rapid and extensive metabolism of BPA to the non-estrogenic BPA-monoglucuronide (BPA-gluc). Some investigators have reported that BPA was found at parts per billion concentrations in the tissues or urine of humans without known exposure to BPA. This work developed a rapid and sensitive method for the determination of BPA and BPA-gluc in plasma and urine based on LC-MS/MS. The LC-ESI-MS/MS method for quantitation of BPA and BPA-gluc uses stable isotope labelled internal standards. A linear ion trap mass spectrometer permits identification and quantitation of BPA-gluc and BPA without sample workup. Development of separation conditions reduced the BPA-background in solvent samples to below 2.5 pmol/mL for BPA. Limit of quantitation (LOQ) for BPA in control urine was 15 pmol/mL, LOQ for BPA-gluc was 65 pmol/mL. Application of the method to urine samples from human subjects (n = 6) after administration of 25 µg BPA/person (estimated maximum human daily intake) permitted the determination of excretion kinetics for BPA-gluc; BPA was below the LOD in all except two of the samples. In urine or blood samples of human subjects (n = 19) without intentional exposure to BPA, BPA-concentrations were always below the limit of detection (≈ 2.5 pmol/mL) with or without prior glucuronidase treatment.

The results show that care is required for analysis of BPA and its major metabolite BPA-gluc. The LOD obtained and the absence of detectable levels of BPA in samples from individuals suggests that general exposure of humans to BPA is much lower than worst-case exposure scenario developed.

Bisphenol-A (BPA) is a widely used industrial chemical with many applications. BPA has been reported to be weakly estrogenic and, due to possible low-dose effects of unknown toxicological relevance, is the subject of many investigations assessing its potential toxicity and human exposures for a more conclusive risk asssement (vom Saal et al., 1998; Ashby et al., 1999; Cagen et al., 1999; Takahashi and Oishi, 2000; Ema et al., 2001; Ramos et al., 2001). Human exposure to BPA is estimated to be mainly by food consumption and according to worst case scenarios may reach 40 µg/kg b.w. (EU-Report, 2003). Methods for the analytical determination of BPA in various biological matrices from rodents and humans using GC-MS, LC-MS/MS and HPLC with electrochemical or fluorescence detection and antibodies have been reported. Concentrations of BPA in human urine samples without intentional exposures to BPA were reported from up to 1.2 µg/L (mean) to up to 1 200 µg/L and blood serum concentration in unintentionally exposed individuals were reported to range from below 1 µg BPA/L up to 19 µg BPA/L (Yoo et al., 2000; Inoue et al., 2001; Long et al., 2001; Ikezuki et al., 2002; Inoue et al., 2002; Schönfelder et al., 2002; Takeuchi and Tsutsumi, 2002; Yamada et al., 2002; Kim et al., 2003; Kuklenyik et al., 2003; Matsumoto et al., 2003; Arakawa et al., 2004; Mao et al., 2004; Calafat et al., 2005). Most of these assays determined the parent compound BPA, both with and without pretreatment with glucuronidase, and different sample workup procedures in blood or urine. A major issue with these methods is that BPA-gluc, is not separately determined, since BPA-gluc is not estrogenic (Matthews et al., 2001). BPA-gluc is the exclusive metabolite of BPA detected in humans after oral BPA exposure (Fig. 1) (Völkel et al., 2002). Furthermore, BPA is subject to an efficient first-pass metabolism in the liver after oral administration and rapidly converted to BPA-gluc in humans and rats (Pottenger et al., 2000; Snyder et al., 2000; Inoue et al., 2001; Völkel et al.,

2002; Yamada et al., 2002). Moreover, methods using single chromatographic traces for BPA-detection may not be sufficiently specific compared to LC-MS/MS. Analytical procedures may also be complicated by complex sample preparations required and/or contamination due to release of BPA from reagents and materials used. The presence of background of BPA of unknown origin has forced the use of d_{16}-BPA to assess BPA toxicokinetics in humans after low doses (Völkel et al., 2002). Therefore, sensitive and selective methods to determine BPA in biological matrix are needed with reduced sample workup to reduce possible contamination of samples with BPA from unknown sources and to quantitate both BPA and BPA-gluc based on stable isotope labelled standards. The method development also considered the issue of background contamination with BPA present in solvents or released from material used for sample preparation by organic solvents. The combination with a linear ion trap mass spectrometer permits multiple reaction monitoring for quantitation and simultaneous recording of a product ion spectrum for conclusive identification.

MATERIAL AND METHODS

Chemicals. d_{16}-BPA (98 atom % D, fully labelled) and BPA were obtained from Sigma-Aldrich (Deisenhofen, Germany). Water and acetonitrile used as HPLC solvents were purchased in HPLC gradient grade quality from Carl Roth GmbH&Co (Karlsruhe, Germany). The glucuronides of d_{16}-BPA and BPA were generated and characterized as described previously (Völkel et al., 2002). All other reagents and solvents were reagent grade or better and obtained from several commercial

suppliers. Glucuronidase/sulfatase from helix pomatia (EC. 3.2.1.31) was obtained from Sigma-Aldrich.

Exposure of Human Subjects to BPA. BPA (25 µg/person) was orally administered in 50 mL water to 3 healthy female and 3 healthy male human subjects (Table 1). All subjects enlisted in the study had to refrain from alcoholic beverages and medicinal drugs 2 days before and throughout the experiment. Subjects did not abuse alcohol and were non-smokers. Subjects were healthy as judged by medical examination and clinical blood chemistry. The study was carried out according to the Declaration of Helsinki, after approval by the Regional Ethical Commitee of the University of Würzburg, Germany, and after written informed consent by the subjects. Urine samples from the subjects were collected in glass vessels at 0, 1, 3, 5, and 7 h after giving BPA. After urine volume was determined, two aliquots (1 mL and 50 mL) were stored at -20°C. Urine and plasma samples from human subjects without known exposure to BPA (age 26 – 50 years) were also collected in glass vessels (urine) or heparinized syringes at random times in the morning or in the afternoon. After urine volume was determined, two aliquots (1 mL and 50 mL) were stored at -20°C. For analysis, part of the plasma samples (100 µL) were diluted with an equal volume of methanol, subjected to centrifugation and 100 µL of acetonitrile was added followed by a second centrifugation. From the obtained supernatant, 20 µL were analyzed by LC-MS/MS. Quantitation of creatinine was performed in the 1 mL urine samples separately frozen for creatinine determination as described elsewhere (Amberg et al., 1999). Glucuronidase treatment of urine and plasma samples was performed as previously described (Völkel et al., 2002).

Identification of BPA-gluc by information dependent acquisition (IDA). To identify BPA-gluc in urine samples from humans by LC-MS/MS, an information

dependent acquisition (IDA) method consisting of multiple reaction monitoring (MRM), enhanced resolution (ER), and enhanced product ion (EPI) scan mode was developed. Urine samples (100 µL) were diluted with 100 µL of acetonitrile containing internal standard (d_{14}-BPA-gluc, 42 nM). Samples (10 µL) were separated by a Reprosil-Pur ODS-3 column (5 µm, 150 x 4.6 mm, Maisch, Ammerbuch, Germany) using an Agilent 1100 autosampler and an Agilent 1100 HPLC-pump. Gradient elution with water (solvent A) and acetonitrile (solvent B) with the following conditions was applied: 0% B for 2 min, followed by a linear gradient linear to 80% B within 20 min. Solvent composition was then kept at 80% B for 2 min. Flow rate was 300 µL/min. IDA experiments were performed on a linear ion trap (LIT) mass spectrometer (QTrap™, Applied Biosystems, Darmstadt, Germany) equipped with a TurboIon®Spray source connected to the HPLC-system. To record spectral data, a vaporizer temperature of 450°C and a TurboIonSpray voltage of -4.5 kV in the negative ionization mode were applied. Declustering potential (DP) was set to -40 V and N_2 was used as collision gas. The MRM mode used transitions from m/z 403 to 227 and from m/z 417 to 241 with a collision energy of -30 V and a collision gas setting of CAD = 4. The ER was performed with a scan rate of 250 amu/s and a fill time of 50 ms. For EPI scans, a scan rate of 4000 amu/s, a collision energy of -30 V and a fill time of LIT of 400 ms was used. In all experiments DP was set to -40 V. All IDA experiments were performed in the range of m/z 50 to m/z 420 with an entrance potential of 8 V and enabled Q_0-trapping.

Quantitation of BPA-gluc. All quantitations were performed using a triple quadrupole instrument (API 3000, Applied Biosystems, Darmstadt, Germany) equipped with a TurboIon®Spray source. The HPLC-parameters used were identical

as described above for identification. The HPLC system was directly coupled to the mass spectrometer. Analytes were detected in the negative-ion mode at a vaporizer temperature of 400°C and a TurboIon®Spray voltage of -4.0 kV. Spectral data were recorded with N_2 as collision gas (CAD = 4) in the multiple reaction monitoring (MRM) mode with a dwell time of 500 ms for each transition monitoring the MS/MS ion-transitions shown in table 1. Quantitation of BPA-gluc was based on calibration curves obtained after addition of known amounts of BPA-gluc (0 nM, 20 nM, 39 nM, 78 nM, 157 nM, 314 nM) to urine samples from the subjects collected before the exposure. Calibration curves were constructed from six data points using Analyst 1.3.1 (Applied Biosystems) with $R^2 > 0.99$. Intra assay variability was below 15%. Acquisition conditions for mass spectra are detailed in table 2. Under these conditions, calibration curves for BPA-gluc were linear up to concentrations of 1 900 nM with $R^2 = 0.998$ and an accuracy between 88% and 115% (Table 3).

Quantitation of BPA. Samples both before or after glucuronidase treatment were analyzed by LC-MS/MS using the API 3000. Separations were performed with water (A) and acetonitrile (B) as solvents: 60% B for 2 min, followed by a linear gradient to 80% B within 20 min. The solvent composition was then kept at 80% B for 2 min; flow rate was 300 µL/min. All other parameters were identical to those described for the analysis of BPA-gluc. Quantitation of BPA was also performed relative to the stable isotope labelled internal standard d_{16}-BPA (Table 2). With this method, calibration curves for BPA were linear up to concentrations of 772 nM with R^2=0.998 and an accuracy between 95% and 108% (Table 3).

RESULTS

Method development. Optimized mass spectrometric conditions for the quantitation of BPA and BPA-gluc were developed. In addition, the problem of interference of BPA from other sources eg. contamination of samples, reagents, and equipment was addressed. Due to the presence of BPA in plastics, contamination of biological samples with BPA from other sources may interfere with sensitive analytical methods and may indicate higher BPA-concentrations than actually present.

The developed methods apply stable isotope labelled standards as a basis for quantitation of both BPA and BPA-gluc and LC-MS/MS with negative ionization and were optimized for both analytes for optimal sensitivity (Tables 2 and 3). Under these conditions, both BPA and BPA-gluc are deprotonated to give $[M-H]^-$ (227.0 amu) for BPA, resp. $[M-H]^-$ (403.2 amu) for BPA-gluc. Addition of buffer salts or acid to improve separation was not possible since the addition of buffer salts or acids to the HPLC solvents dramatically reduced sensitivity. The use of stable isotope labelled internal standards permitted a clear identification of the signals for both BPA-gluc and BPA despite the small shifts in retention time observed in the absence of buffers (Table 3). Moreover, collision induced fragmentation results in characteristic product ions from the precursor ion 403.2 amu of BPA-gluc (Table 2). These fragmentations patterns were included into the aquisition method and quantitations of BPA and BPA-gluc are reported relative to the internal standards.

Two different HPLC separation methods were developed. The first method provided a separation of BPA and BPA-gluc and a separation of BPA-gluc from the solvent front. This method was initially intended for the simultaneous quantitation of both BPA and BPA-gluc in one separation. A clear separation of BPA-gluc from the salts and other components of urine or plasma, which elute close to the solvent front, is necessary since these components may suppress ionization of BPA-gluc and thus

reduce sensitivity. However, using this system, peaks indicative of the presence of BPA at the retention of authentic BPA (Kuch and Ballschmiter, 2001; Völkel et al., 2002) were observed when injecting only solvents (water/acetonitrile, 1:1, v/v), or even when the gradient was started without sample injection (Fig. 2A and B). Integration of peak areas observed suggested the presence of concentrations up to 25 nmol BPA/mL of urine or plasma. The peaks representing BPA increased over time after repeated injection of purified solvent. These observations suggest a contamination of the HPLC-solvents with BPA, a leaching of BPA from plastic equipment used and/or adsorption of BPA to the column material. Change or cleanup of solvents by distillation, use of different HPLC columns, or minor modifications of the gradient did not completely solve the problem of BPA background. Sample workup exclusively in purified glass vessels without contact to plastics somewhat reduced the suggested concentrations of BPA, but was also not able to reduce the BPA-contents in blanks to below the LOD.

Only a major change in gradient conditions reduced the background of BPA in blanks or injected solvents to below the LOD and permitted the analysis of samples with sufficent confidence (Fig. 2, C and D). Interferences with components of urine samples were not an issue using the modified conditions since no coeluting components with the mass transitions for BPA were present in urine samples. Unfortunately, the application of this gradient did not permit the sensitive quantitation of BPA-gluc in the same separation, since BPA-gluc eluted with the solvent front.

Therefore, separate methods were developed to quantify BPA-gluc and BPA. The method for BPA-gluc used the gradient starting with a high water content of the mobile phase and permitted the quantitation of BPA-gluc with a limit of detection of

0.9 pmol/mL when the standard was analyzed after dilution in purified water. However, despite the use of a specific analytical method including HPLC-separation, stable isotope internal standard, and presumably selective MS/MS transition, several peaks were observed in the region where BPA-gluc elutes from the column when analyzing urine samples from humans without intentional exposure to BPA (Fig. 3A and B). Although different HPLC-columns (Synergi Hydro 150 x 2 mm; 80 A, 4 µm, Nucleosil C18 150 x 2 mm; 100 A, 5 µm, ReproSil-Pur C18-AQ 150 x 4.6 mm; 120 A, 3 µm) were used, HPLC-solvents were acidified and gradient parameters were changed, the best resolution of these components and spiked BPA-gluc obtained is shown in Fig. 3 (panel B). The presence of these interfering compounds reduced the LOQ for BPA-gluc to 65 pmol/mL (S/N 8) in urine, due to a 50-fold reduced signal to noise ratio (S/N). Addition of BPA-gluc (final concentration: 500 pmol BPA-gluc/mL) to a urine sample resulted in a defined peak with an S/N of 31 presented in Fig. 3 (panel C). The peaks at retention times 8.85, 9.09 and 9.18 min presumably represent isobaric glucuronides which show identical fragmentation.

In urine samples from humans administered BPA, a peak with the mass spectral information supposed to be characteristic for BPA-gluc eluted at the expected retention time (Fig. 3, panel D). However, due to the closely eluting peaks of other compounds, confirmation of the identity of BPA-gluc was required and the presence of presumably present isobaric glucuronides indicate a need for a more conclusive identification of BPA-gluc to avoid false positive results. Therefore, urine samples from individuals given BPA were also analyzed by a LC-MS/MS QTrap^{TM} instrument using the IDA scan method to confirm the presence of BPA-gluc. The linear ion trap mass spectrometer combined with the software tool IDA allows mass scans including a MRM for both BPA-gluc and the internal standard d_{14}-BPA-gluc (no clear

separation of BPA-gluc and d_{14}-BPA-gluc was achieved) to select the analytes, an enhanced resolution to record higher resolved $[M-H]^-$ spectra and an enhanced product ion scan to record a mass spectrum of each analytes recognised by the MRM-mode. These mass spectral data are obtained in a single separation. In Fig. 4A, the characteristic EPI mass spectrum of BPA-gluc is presented and Fig. 4B shows the spectrum of BPA-gluc obtained by IDA analysis in an urine sample collected 3 h after application of 25 µg BPA to a human subject. If all EPI spectra of m/z 403 (BPA-gluc) and m/z 417 (d_{14}-BPA-gluc) recorded within the peak width of BPA-gluc are summed up, the resulting EPI mass spectrum gives a typical fragment (m/z 241) of d_{14}-BPA-gluc due to coelution and identical collision energy for both glucuronides (Fig 4B). In addition, the signal m/z 113 formed from both precursors (m/z 403 and m/z 417) represents the sum of both precursors and is therefore higher compared to the spectrum of the precursor m/z 403 alone (Fig 4A). In urine and calibration samples in water and acetonitrile (50:50, v:v) without addition of the internal standard d_{14}-BPA-gluc, the fragment of m/z 241 was not observed and the presumed isobaric glucuronides showed more or less changed mass spectra (data not shown). The combination of both LC-MS/MS methods permitted a conclusive identification of BPA-gluc even in low concentrations. These results are more reliable as compared to methods that only use single chromatographic traces and retention time.

Analysis of urine samples for BPA-gluc and BPA after oral administration of BPA to human subjects. The two procedures were applied to analyze samples from an application of BPA to human subjects in a single oral dose in the range of the worst-case assuption for daily exposure. All samples were analyzed independently

with both HPLC-systems and with and without glucuronidase treatment. This combination of analytical methods gives information on the presence of both BPA and BPA-gluc and on the total content of BPA in a sample. The excretion of BPA and BPA-gluc was quantitated over time in urine samples, but BPA-gluc was only quantified in samples where its presence could be confirmed by EPI-mass spectrometry (time points 1, 3, and 5 h). In male subjects, 93 ± 19 nmol (85% of the applied BPA-dose) and in female subjects, 83 ± 16 nmol (75% of the applied dose) were recovered as BPA-gluc (Fig. 5) within 5 hours after application. An elimination half-life of approximately four hours was calculated from the excretion rates. The half-life and the recovery is similar to that reported in a previous study (Völkel et al., 2002) which used higher doses of d_{16}-BPA applied to human subjects. Highest concentrations of BPA-gluc from 221 to 611 pmol/mg creatinine and 117 to 345 pmol/mg creatinine were observed in urine samples collected 1 h respectively 3 h after administration of 25 µg BPA/subject (Fig. 5). In plasma samples from this exposure, BPA-gluc could also be detected due to absence of interfering peaks (data not shown).

Application of the HPLC separation conditions to determine free BPA without background in the samples from the controlled exposure indicated BPA-concentrations above the LOD in only two of the urine samples (w2-5h and m4-1h). The BPA concentrations in these two samples were approximately 10 pmol BPA/mg creatinine corresponding to 1 - 2 nmol (< 1 µg/L) BPA in the total volume of urine released or less than 2% of BPA dose received. BPA-concentrations in all other samples were below the LOD (Fig. 6, A and B).

To determine the content of BPA representing both BPA-gluc and BPA, BPA-gluc was hydrolyzed by glucuronidase and the samples were then analyzed with the

method for determination of BPA. BPA-content determined in these samples was identical to the concentrations of BPA-gluc as determined by the method to quantitate BPA-gluc further supporting the presence of very low levels of free BPA even after a controlled exposure (Fig. 5). After glucuronidase treatment, 106 ± 16 nmol (97% of the applied dose) BPA and 92 ± 16 nmol (84% of the applied dose) BPA were recovered in male, resp. female subjects (Fig. 5). The higher recovery of BPA as compared to BPA-gluc may be explained by the lower sensitivity of the QTRAP system since only samples showing a characteristic EPI spectrum were used for quantitation of BPA-gluc.

BPA and BPA-gluc in urine and plasma samples from human subjects without intentional exposure to BPA. Urine and plasma samples from human subjects (7 males and 12 females) were randomly collected between 10 am and 7 pm on different days and analyzed for BPA-gluc and BPA. In most of the urine samples, small peaks with the characteristic MS/MS-transitions for BPA-gluc were detected at retention times very close to those of authentic BPA-gluc, but the concentrations were lower than 65 pmol/mL (LOQ) corresponding to < 65 nmol BPA-gluc in an average urine volume of 1 L. Due to the low concentrations and the presence of other analytes with identical mass spectral characteristics, an uneqivocal identification of BPA-gluc with IDA-MRM-ER-EPI was not possible. After glucuronidase cleavage, the BPA-concentrations determined in urine were always below the LOD of 1.14 µg/L (5 pmol/mL) suggesting the presence of very low concentrations of BPA, at best. However, addition of even small concentrations of BPA or BPA-gluc to the samples gave definite responses (Figs. 6 C and 7). None of the plasma samples analyzed (both with and without glucuronidase treatment)

contained detectable concentrations of BPA or BPA-gluc also indicating plasma concentrations of BPA below 0.5 µg/L.

DISCUSSION

The aim of the work presented here was to develop an optimized method for exposure analysis of BPA in humans using sensitive and selective methods such as LC-MS/MS. The original intention was to quantify both free BPA and BPA-gluc in one separation without detailed sample workup to avoid degradation of BPA-gluc and/or sample contamination. Due to the sensitivity and selectivity of LC-MS/MS, this was considered feasible with sufficient sensitivity. However, since glucuronides are urinary metabolites of many endogenous and exogenous compounds (Tephly and Burchell, 1990), signals with the presumed specific transitions for BPA-gluc were present at similar HPLC-retention times in urine samples from human subjects not intentionally exposed to BPA. For example, some isoflavones widely present in human diet such as resveratrol have an identical molecular weight as BPA and are also excreted as glucuronides (Bayer et al., 2001; Wang et al., 2004). Therefore, a more conclusive identification of BPA-gluc was necessary to avoid false positive results.

BPA-gluc was analyzed after fragmentation of the $[M-H]^-$ anion formed in the negative ionization mode of the electrospray source (Inoue et al., 2002; Völkel et al., 2002). With this technique, BPA-gluc may be identified by monitoring specific mass transitions. However, since the method included a loss specific for glucuronides, presumed isobaric glucuronides of unknown structures eluted closely to BPA-gluc.

The fragmentation of these compounds gave identical spectral information (fragment with m/z 113 and $[M-H]^-$ of the glucuronide moiety).

A linear ion trap mass spectrometer was therefore additionally used to confirm the presence of BPA-gluc in low concentrations. With this instrument, an EPI spectrum can be recorded simultaneously to the MS/MS transitions specific for BPA-gluc (Fig. 4). The detection of the M-glucuronide fragment of d_{14}-BPA-gluc in these spectra – not seen in samples without d_{14}-BPA-gluc – serves as additional evidence for the presence of BPA-gluc. Application of this combination of mass spectrometry procedures clearly confirmed that, after controlled exposure to low doses of BPA, BPA-gluc is excreted as a metabolite of BPA.

However, the method using the triple quadrupol was not sufficiently selective to be applied to samples from human subjects without intentional exposure to BPA. Therefore, the strategy was changed to determine free BPA in urine or blood samples both with and without glucuronidase cleavage. BPA may be quantified with high sensitivity by LC-MS/MS; however, issues with background contamination needed to be solved before the method could be applied. Likely, both water and/or acetonitrile used as HPLC solvents contain traces of BPA (Kuch and Ballschmiter, 2001; Völkel et al., 2002). Another source for contamination may be the release of BPA from plastics by organic solvents (Krishnan et al., 1993; Hutson, 1998; Fung et al., 2000). Indeed, sample workup with glasware gave a trend to lower BPA background levels. However, only a major change in HPLC-separation conditions gave BPA-concentrations below the limit of detection when solvent samples were injected. This could be explained by a trapping effect of the HPLC column for BPA at lower concentrations of organic solvents. Small amounts of BPA presumably contained in the HPLC-grade water may be trapped by the column, after reaching

higher concentrations of acetonitrile in the eluent, the trapped BPA elutes from the column (Meyer, 1999).

Based on these observations, the optimized analytical procedure for exposure monitoring of BPA was considered to be the analysis of free BPA, both with and without prior hydrolysis of BPA-gluc, to determine free BPA and total BPA. The content of free BPA is relevant in the context of possible effect concentrations of BPA, since some effects of low-dose BPA-administration (> 20 µg/kg bw per day) have been described (vom Saal et al., 1997; Takahashi and Oishi, 2000) and the blood levels of free BPA are relevant for assessment of endocrine activity since BPA-gluc is devoid of estrogenicity (Matthews et al., 2001).

When applying this procedure to a number of randomly collected blood and urine samples from human subjects without intentional exposure to BPA, the content of BPA in all samples was below the LOD of 5 pmol/mL (≈ 1.14 µg/L) suggesting that the human exposure to BPA from diffuse sources in the environment is low. The levels observed in European subjects are well below those reported previously for populations in Korea and Japan, where mean values of 2.8 µg/L, resp. 9.5 µg/L were found in urine (Ouchi and Watanabe, 2002; Kim et al., 2003; Yang et al., 2003). In recent studies in the United States, mean urinary concentrations of BPA were 1.33 µg/L determined by GC-MS after glucuronidase treatment (Calafat et al., 2005). In Japan, daily excretion of BPA with urine was reported as 1.2 µg/day (mean) using GC-MS/MS (Arakawa et al., 2004). The data presented here suggest that BPA concentrations in human urine in Europe are, at best, in a similar range.

Due to the rapid and complete elimination of BPA in humans as BPA-gluc in urine (Völkel et al., 2002), the determined concentration of BPA in urine suggest that the human exposure to BPA is less then 2.3 µg/person/day (based on a volume of 2 L of

urine excreted). This value corresponds to a BPA dose of 38 ng/kg bw per day for a 60 kg adult and thus is well below the daily doses of BPA which have sometimes been reported to cause responses of unknown toxicological relevance in highly sensitive animal systems (20 µg/kg/day) (Timms et al., 2005).

The lack of detection of BPA in plasma with a LOD of 0.5 ng/mL also suggests that the plasma levels of BPA resp. BPA-gluc in Europeans are below the BPA plasma concentrations reported in non-obese Japanese women (0.71 ± 0.09 ng/mL as analyzed by ELISA) (Takeuchi et al., 2004) and lower than the previously reported median of 3 ng BPA/mL plasma in pregnant females in Europe (Schönfelder et al., 2002).

The capacity of the procedure to detect low levels of BPA and BPA-gluc in samples from human subjects administered a low dose of BPA, which represents the estimated maximum human exposure (EU-Report, 2003), showed the capability of the procedure for exposure monitoring and also confirms previous observations on the rapid and almost complete excretion of BPA as BPA-gluc within a few hours after oral exposure. The rapid elimination of BPA as seen in this and a previous study of human excretion kinetics of BPA also indicated a need for a very frequent monitoring of BPA in blood and urine since BPA taken up from food will be rapidly eliminated and will only be present in detectable concentrations for a relatively short period.

In summary, the results show that exposure monitoring to potentially ubiquitous chemicals requires a very detailed approach to account for possible interference by other chemicals present in the media and also requires specific analytical methods such as MS/MS. However, even when using these specific methods, structure confirmation is required. Therefore, methods to quantify BPA or BPA-gluc by HPLC-UV, HPLC-FLD, or HPLC with electrochemical detection or after fluorophore

derivatisation in urine of non-intentionally exposed humans may have only limited applicability (Ouchi and Watanabe, 2002; Takeuchi and Tsutsumi, 2002; Matsumoto et al., 2003; Mao et al., 2004; Kawaguchi et al., 2005) since the use of internal standards with these methods is difficult and many possibly interfering compounds with fluorophores or electrochemical potential are present in biological samples. Quantitation of parent BPA with GC-MS has a sufficient sensitivity and a higher power of separation as compared to HPLC (Schönfelder et al., 2002), but the procedure requires solvent extraction and/or further sample preparation as a possible source of contamination with BPA and needs to be well evaluated to avoid production of false positive results. GC-MS analysis for BPA in human blood or urine samples also needs to account for the fact that, due to the rapid conjugation of BPA to BPA-gluc, most of the BPA is present as BPA-gluc. Therefore, a separation of BPA-gluc and free BPA must be achieved before extraction and derivatization since the derivatization at higher temperature may hydrolyse BPA-gluc and overestimate level of free BPA. Therefore, a LC-MS/MS method is more reliable and permits the quantitation of both free and conjugated BPA, which is relevant for assessment of postulated human health risks due to low dose BPA-exposures.

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Figure legends

Fig. 1: Biotransformation of BPA in humans

Fig. 2: Presence and absence of background contamination of samples with BPA by analysis with different gradient conditions by LC-MS/MS (m/z 227 to m/z 212). A, Separation of a sample of H_2O (10 µL injected) starting at 100% of solvent A (H_2O) suggesting presence of 51 pmol BPA /mL; B, an urine sample (10 µL injected) from a human subject without intentional exposure to BPA under same separation conditions (suggesting presence of 50 pmol BPA /mL); C, Separation of a sample of H_2O/MeOH (50/50 v/v, 10 µL injected) starting at 40% of solvent A (H_2O), D, an urine sample from a human subject (10 µL injected) without intentional exposure to BPA under same separation conditions as in panel C.

Fig. 3: Chromatographic trace (m/z 403 to m/z 113) obtained after separation of a solvent sample (A), an urine sample from human subject not intentionally exposed to BPA (B, suggested BPA-gluc concentration of 14 pmol/mL). Panel C, represents the separation of an urine sample from a human subject (10 µL injected) without intentional exposure to BPA under same separation conditions, spicked with BPA-gluc to give a concentration of 500 pmol/mL and Panel D an urine sample collected 1 h after oral administration of 25 µg BPA to a male subject (BPA-gluc concentration of 540 pmol/mL). Retention time of BPA-gluc is 8.95 min under the separation conditions used.

Fig. 4: EPI mass spectrum of the standard BPA-gluc in absence (A), and IDA-EPI mass spectrum of BPA-gluc in presence of d_{14}-BPA-gluc (B) in an urine sample collected 3 h after administration of 25 µg BPA.

Fig. 5: Kinetics of excretion with urine of BPA-gluc (■) or total BPA after glucuronidase treatment (◆) in three male and three female human subjects after oral administration of 25 µg BPA/person.

Fig. 6: Chromatographic traces (m/z 227 to m/z 212) indicative for BPA in human urine samples obtained without glucuronidase treatment: A, urine sample from not intentionally exposed human subject; B, urine sample (collected one hour after administration) from human subject receiving 25 µg BPA; C, urine sample from not intentionally exposed human subject spicked with BPA to give a concentration of 40 pmol BPA/mL.

Fig. 7: Chromatographic traces (m/z 227 to m/z 212) indicative for BPA in human urine samples obtained after glucuronidase treatment: A, urine sample from not intentionally exposed human subject; B, urine samples (collected one hour after administration) from human subject receiving 25 µg BPA after glucuronidase treatment (BPA concentration of 130 pmol/mL); C, urine sample from not intentionally exposed human subject spicked with BPA-gluc to give a concentration of 70 pmol BPA/mL after glucuronidase treatment.

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Table 1: Characteristics of human volunteers participating in the study.

Volunteer	Gender	Age	Height [cm]	Body weight [kg]
A	male	37	175	90
B	male	43	178	70
C	male	49	188	85
D	female	29	168	68
E	female	25	183	68
F	female	32	163	57

Table 2: MS/MS transitions and instrument parameters used to quantify BPA and BPA-gluc in biological fluids

Transition	Compound	DP* [V]	CE* [V]	Loss of fragment
227.0 – 212.0	BPA (quantifier)	- 40	- 28	- CH_3
227.0 – 133.0	BPA (qualifier)	- 40	- 38	-H, -C_6H_5O
241.1 – 223.3	d_{16}-BPA (quantifier, internal standard)	- 40	- 28	- CD_3
241.1 – 142.1	d_{16}-BPA (qualifier, internal standard)	- 40	- 38	-H, -C_6D_5O
403.2 – 113.1	BPA-gluc. (quantifier)	- 36	- 24	- H, - H_2O, - CO_2, - $C_{15}H_{15}O_2$ (BPA moiety)
403.2 – 227.0	BPA-gluc. (qualifier)	- 36	- 30	- $C_6H_8O_6$ (glucuronic acid moiety)
417.2 – 113.0	d_{16}-BPA-gluc. (quantifier, internal standard)	- 36	- 24	- H, - H_2O, - CO_2, - $C_{15}HD_{14}O_2$ (BPA moiety)
417.2 – 241.1	d_{16}-BPA-gluc. (qualifier, internal standard)	- 36	- 30	- $C_6H_8O_6$ (glucuronic acid moiety)

* DP = declustering potential; CE = Collision energy

Table 3: Method validation in human urine samples.

	LOD [pmol/mL] (µg/L)	LOQ [pmol/mL] (µg/L)	Accuracy [%]	mean retention time ±SD [min]
BPA	5 (1.14)	15 (3.42)	92 – 121	6.77 ± 0.06 (n = 55)
BPA-gluc	25* (10.1)	65 (26.26)	90 – 120	8.98 ± 0.05 (n = 33)

*LOD in water 0.9 pmol/mL

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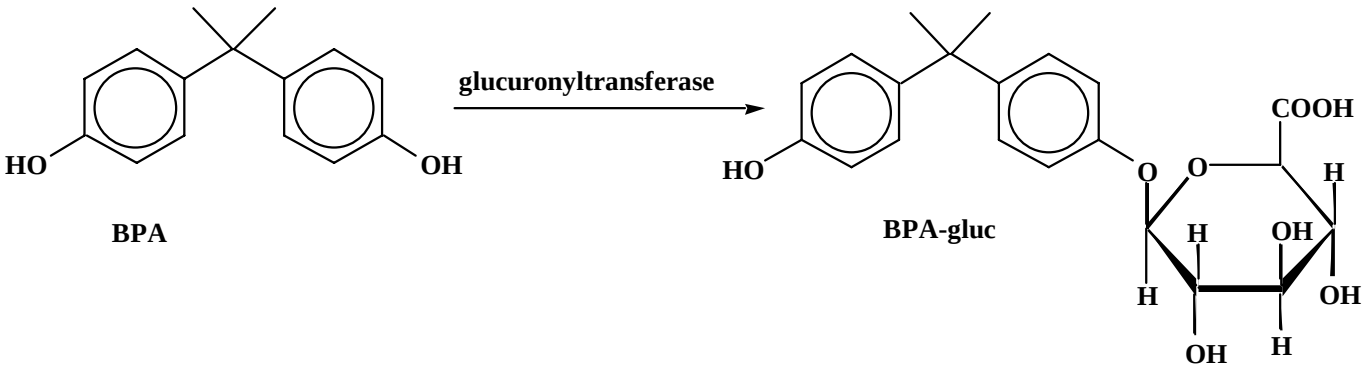
Fig. 1



BPA **BPA-gluc**

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Fig. 2

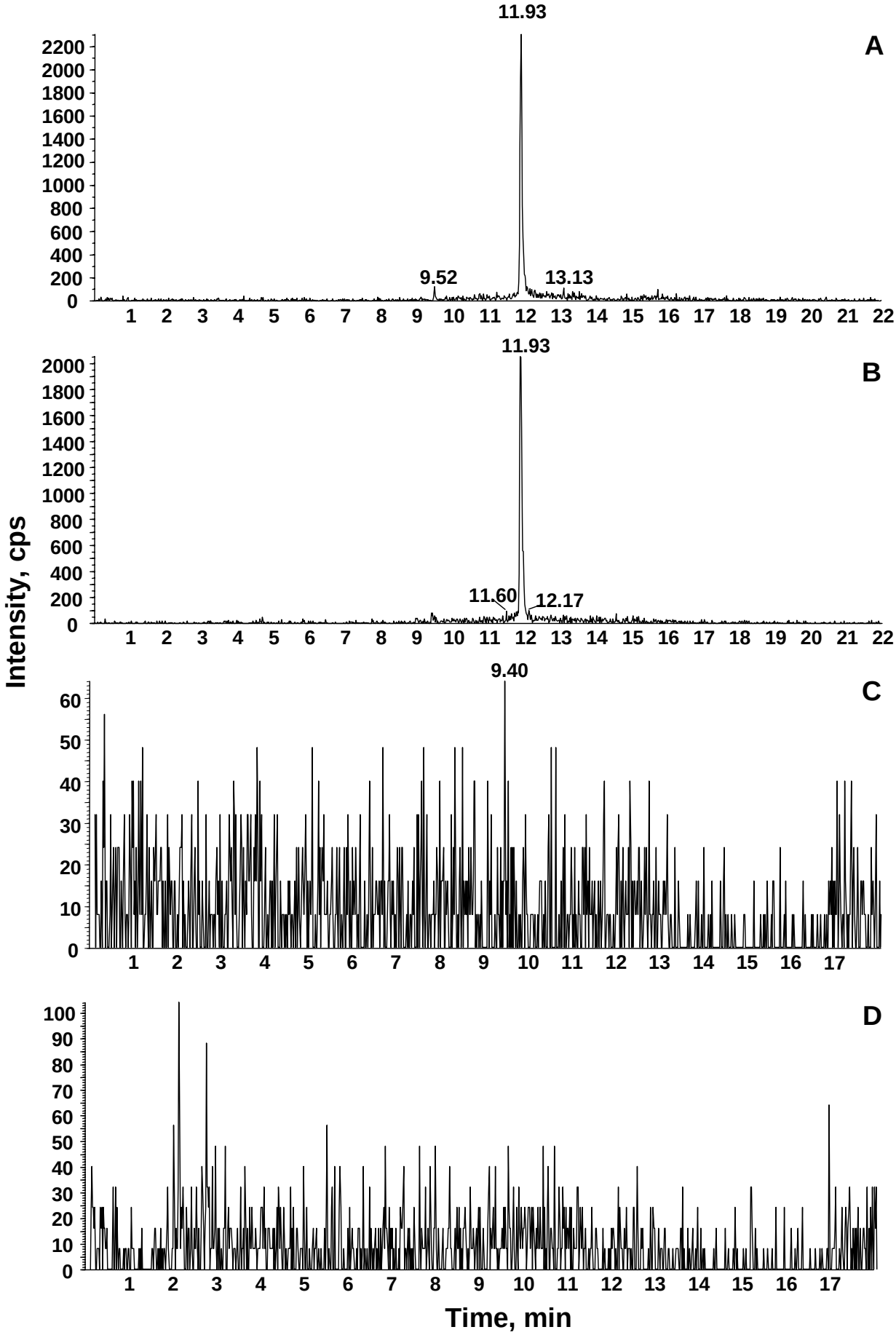

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Fig. 3

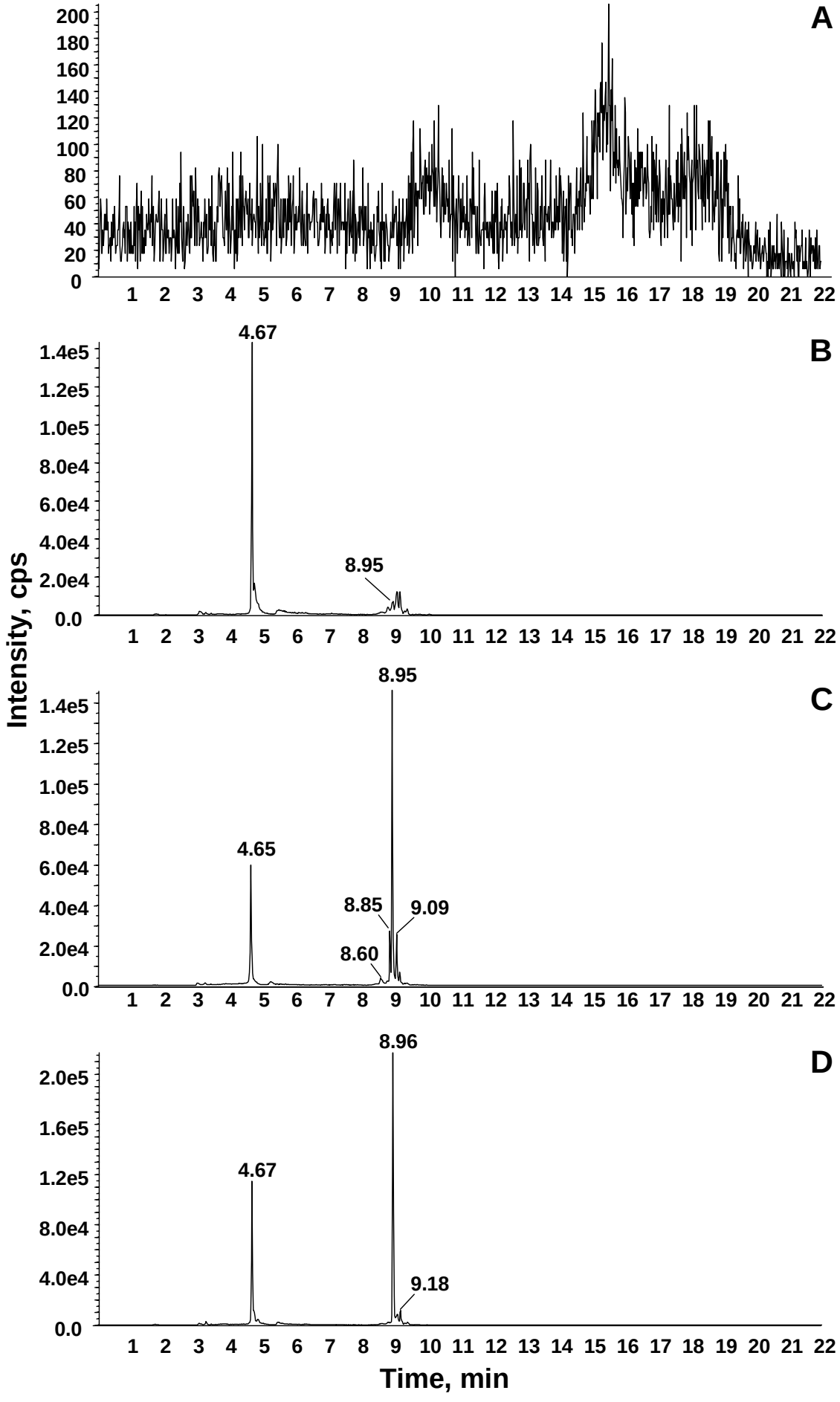

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Fig. 4

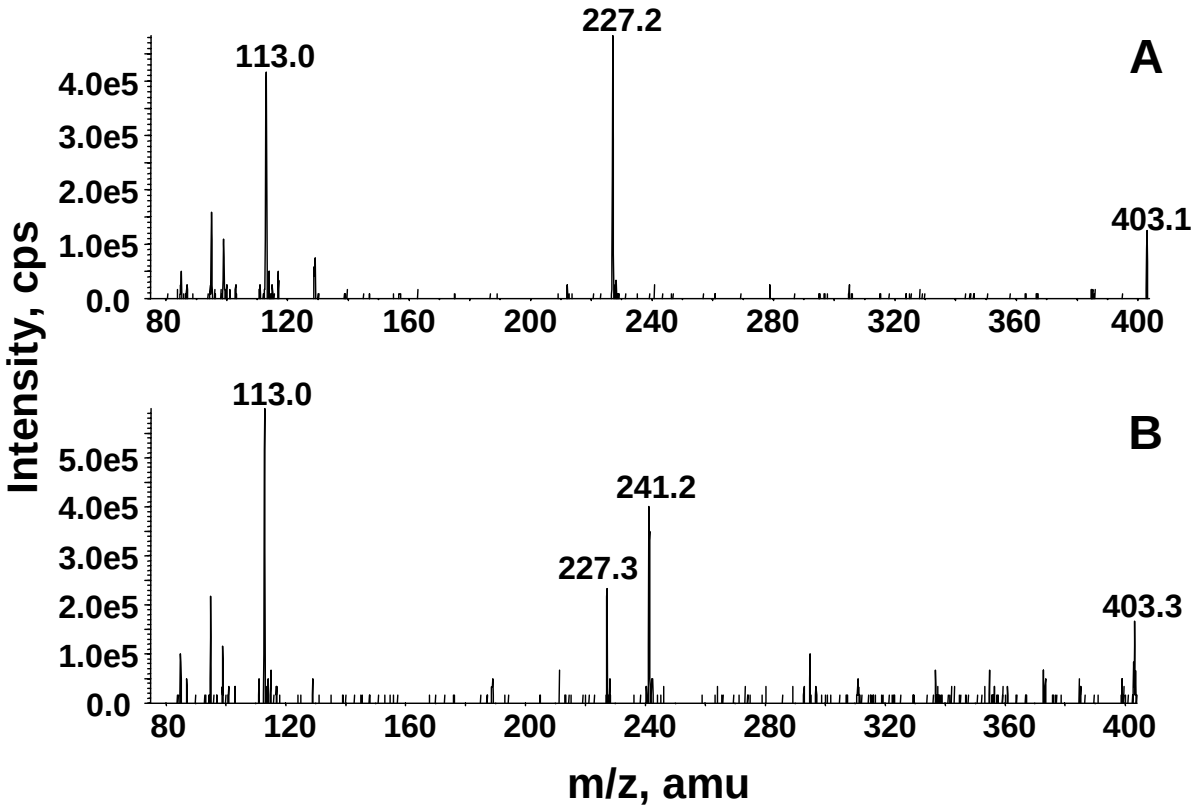

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Fig. 5

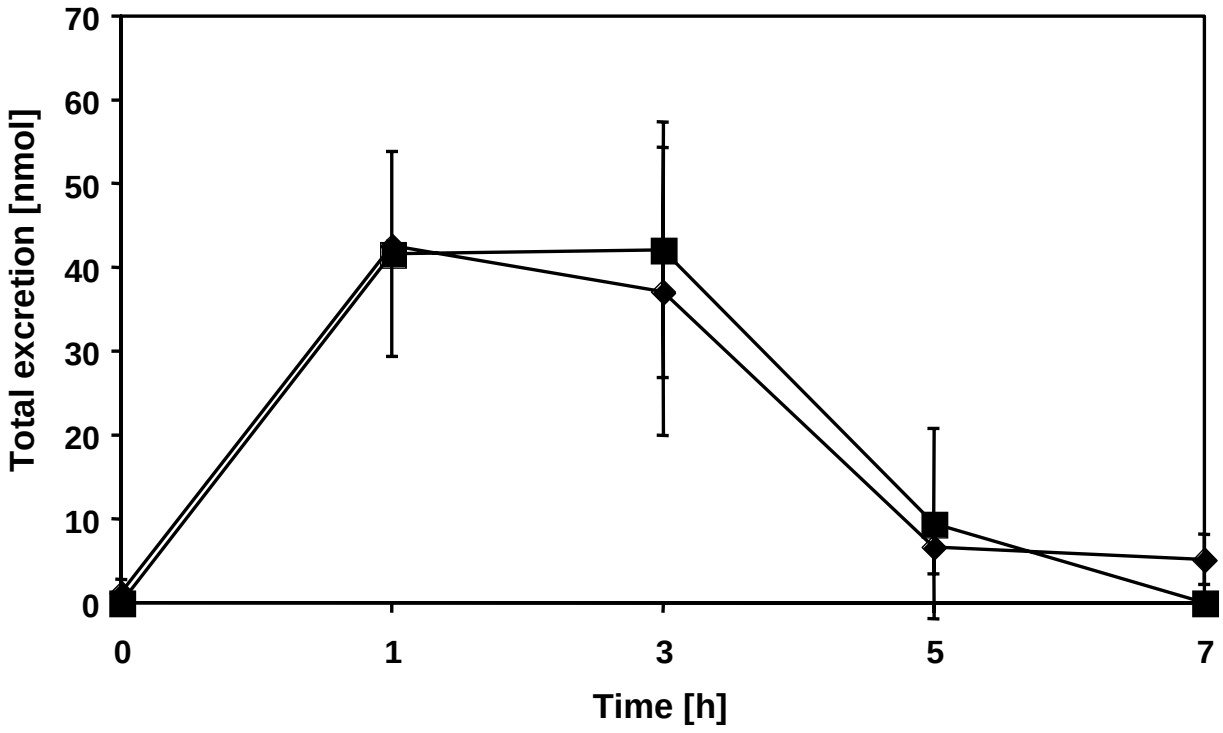

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Fig. 6

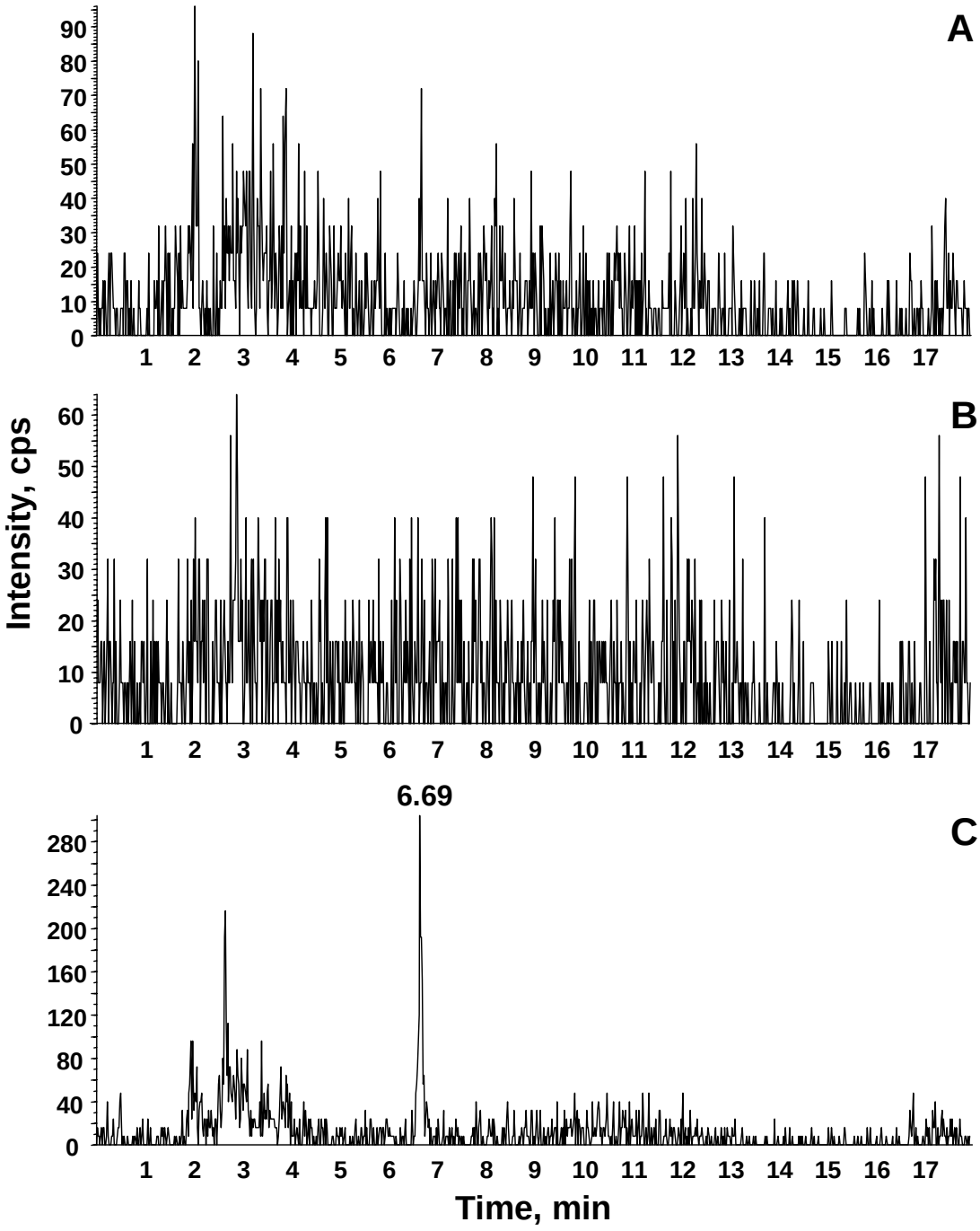

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Fig. 7