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Comparison of gas chromatography–mass spectrometry and high-performance liquid chromatography with coulometric electrode array detection for determination of alkylresorcinol metabolites in human urine

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ABSTRACT

Alkylresorcinols (AR) are amphiphilic compounds present at high concentrations in the outer parts of wheat and rye kernels. Due to their specificity to whole grain and bran products of these cereals, AR and their metabolites have been proposed as biomarkers for intake of such foods. Two alkylresorcinol metabolites, 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA), have previously been quantified in human urine using two different methodologies: high-performance liquid chromatography coupled to a coulometric electrode array detector (HPLC–CEAD) and gas chromatography in combination with mass spectrometry (GC–MS). In this study, these two methodologies were compared by analysing 114 urine samples from free-living Swedish subjects consuming their habitual diet. Data were evaluated by graphical investigation of difference-plots and statistical inference of agreement was assessed by weighted Deming regression analysis. The median DHBA concentrations were 11 μM (GC–MS) and 13 μM (HPLC–CEAD), respectively. Both difference-plot and regression analysis showed a small but statistically significant additive bias, with HPLC–CEAD resulting in a slightly higher DHBA concentration than GC–MS. The median concentration of DHPPA was 18 μM for both methods. Examination of the difference-plot of DHPPA did not indicate any systematic difference between the methods, but regression analysis showed small but statistically significant constant and proportional biases. The conclusion was that the two methodologies are equally suitable for analysis of alkylresorcinol metabolites in human urine and that any small systematic differences observed are most likely of limited practical importance.

1. Introduction

Alkylresorcinols (AR) are phenolic lipids present in high amounts in the outer parts of wheat and rye kernels (outer testa/inner pericarp) [1]. They are therefore present in whole grain products of these cereals, but absent or found in very low amounts in refined cereal products [2,3]. Although barley also contains AR in lower concentrations, the contribution from barley in AR consumption is marginal due to the limited role of barley in the human diet [4]. Both AR and their metabolites have been suggested as biomarkers for whole grain rye and wheat intake [5,6]. As such, they could aid understanding of the associations between whole grain intake and risk of chronic diseases observed in numerous epidemiological studies [7–10].

Once ingested, cereal AR are absorbed in the small intestine and suggested to be transported to the systemic circulation via the lymphatic system [5,11]. Due to their amphiphilic nature, AR are easily incorporated into biological membranes (e.g. in erythrocytes [12]) and are highly associated to lipoproteins in plasma [13]. Absorbed alkylresorcinols are eliminated rather quickly and hepatic metabolism of AR has been suggested to be similar to that of tocopherols, where the alkyl side-chain is oxidised by ω-oxidation initiated by CYP450 enzymes, followed by several cycles of β-oxidation [6]. This, in combination with conjugation of polar groups, increases the hydrophilicity and thus facilitates urinary excretion. Two major metabolites of AR have been identified in both urine [6] and plasma [14]: 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA).

Two methods for quantification of AR metabolites in urine, utilising different chromatographic and detecting techniques, have been reported previously [15,16]. The first published method has

Abbreviations: AR, alkylresorcinols; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; CEAD, coulometric electrode array detector; DHBA, 3,5-dihydroxybenzoic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-1-propanoic acid; HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; TMCS, trimethylchlorosilane.

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been used in several studies with different degrees of sample preparation [17,18], and is based on high-performance liquid chromatography coupled to a coulometric electrode array detector (HPLC–CEAD) [15]. The second method was published recently and uses gas chromatography and mass spectrometry (GC–MS) for separation and detection [16]. The same technique is used for quantification of intact alkylresorcinols in plasma [19,20], erythrocytes [12], and adipose tissue [21].

The aim of this study was to compare HPLC–CEAD and GC–MS for quantification of AR metabolites in deconjugated and extracted human urine. By excluding variation originating from pre-analysis sample treatment, the study focused on differences due to chromatography and detectors.

2. Materials and methods

2.1. Materials

Syringic acid and all enzymes were obtained from Sigma Chemicals (St. Louis, MO, USA). DHBA and formic acid were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and DHPPA from Isosep AB (Tullinge, Sweden). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Thermo Scientific (Rockford, IL, USA), while Oasis® MAX 60 mg solid-phase extraction cartridges were purchased from Waters (Milford, MA, USA). Ethyl acetate, hydrochloric acid, methanol, phosphoric acid, sodium acetate and sodium hydroxide were obtained from Merck KGaA (Darmstadt, Germany) and acetonitrile was purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). All solvents were of HPLC grade or equivalent.

2.2. Samples

Samples were 24-h urine collections obtained from 59 Swedish volunteers (43 female and 16 male), consuming their habitual diet. The urine was collected during two periods 2–3 months apart and volumes were recorded to enable calculation of daily excretion of AR metabolites. To each urine collection, 9 ml 20% HCl were added to prevent microbial growth and samples were stored at −80 °C until analysis. The study was approved by the local ethics committee for the Uppsala region (Sweden) and all participants gave their written consent.

2.3. Deconjugation, extraction and gas chromatographic–mass spectrometric analysis

Samples (n = 114) were hydrolysed and extracted as described elsewhere [16]. In brief, 15 nmol syringic acid (in 10 μl methanol) were added as internal standard to 50 μl urine, followed by addition of 740 μl hydrolysis solution (0.1 sodium acetate buffer [pH 5.0] containing 50 mU β-glucuronidase and >320 mU sulfatase from *H. pomatia*) and samples were incubated overnight at 37 °C. Analytes were protonated by addition of 15 μl concentrated HCl. The samples were then extracted twice with ethyl acetate (2 ml) and the combined organic phases were evaporated to dryness and reconstituted in methanol. The samples were applied to Oasis® MAX columns, conditioned with 0.1 M sodium hydroxide/methanol (30/70, v/v) and washed with methanol, and finally eluted with 3 ml 2% formic acid in methanol. Following solid phase extraction, 1 ml was removed from each sample and kept at −20 °C until analysis with HPLC–CEAD. The remaining 2 ml were evaporated, silylated and analysed by GC–MS as described previously [16]. In brief, 100 μl BSTFA with 1% TMCS were added to the evaporated extracts and the samples were silylated for 60 min at 60 °C before transfer to GC vials and analysis by GC–MS in single ion recording mode. Molecular ions (DHBA, m/z 370; DHPPA, m/z 398; syringic acid, m/z 342) were used for quantification of the analytes.

2.4. High pressure liquid chromatographic analysis

The procedure for analysis with HPLC–CEAD followed the described method [15] with some modifications. The eluents were delivered by two pumps, LC-10AD from Shimadzu Corporation (Kyoto, Japan), coupled to a degasser, SDU 2006, from Prolab GmbH (Reinach, Switzerland). A Midas 830 autosampler from Spark Holland BV (Emmen, The Netherlands) was used to introduce the samples to the system. The column, mobile phase and detector were similar to those described elsewhere [15]. The gradient used for analysis was as follows: 0–5 min, 10–20% mobile phase B; 5–20 min, 20–30%; 20–25 min, 30–50%; 25–30 min, 50–100%; 30–60 min, 100%; 60–65 min, 100–10%; 65–90 min, 10%. Syringic acid, DHBA and DHPPA were quantified at the channels where the highest response was obtained (470, 670, 600 mV, respectively). In some urine samples DHBA was quantified at 600 mV due to co-elution of interfering compounds.

The solid phase extract that was kept for HPLC–CEAD was evaporated to dryness under a stream of nitrogen, reconstituted in 50 μl methanol, 100 μl mobile phase (20% phase B/80% phase A [15]) was added, and the sample was filtered through a 0.45 μm syringe filter from Pall Corporation (Port Washington, NY, USA) before analysis by HPLC–CEAD.

Ten of the urine samples analysed with GC–MS were chosen to cover the concentration interval of the urine collections described above and were additionally analysed by HPLC–CEAD directly after deconjugation, with no further sample clean-up, according to a recently published protocol [18]. In brief, 2.1 nmol syringic acid (in 10 μl methanol) were added to 100 μl urine. After addition of 100 μl 0.1 M sodium acetate buffer (pH 5.0), containing 20 mU β-glucuronidase (from bovine liver) and 200 mU sulfatase (from *P. vulgata*), the samples were incubated overnight at 37 °C. After incubation, 50 μl were removed and diluted with 50 μl methanol and 650 μl mobile phase (20% phase B/80% phase A [15]). Before analysis by HPLC–CEAD, the samples were filtered as described above.

2.5. Method comparison

Agreement between the HPLC–CEAD and GC–MS methods was evaluated by graphical investigation of difference-plots [22] and by weighted Deming regression analysis [23]. For difference-plots, samples with differences between the two methods exceeding two standard deviations were rejected as outliers and were excluded from the calculation of the mean difference. Analyse-it version 2.22 from Analyse-it Software Ltd. (Leeds, UK) and Microsoft Office Excel 2007 from Microsoft Corporation (Redmond, WA, USA) were used for the difference-plots and weighted Deming regression analysis. In the weighted Deming regression, coefficients of variation for both methods and both analytes were assumed to be 10%, based on previously reported coefficients of variation for both methods [14,16]. Outliers were identified as previously described [24] and were excluded from the regression model. P-values <0.05 were considered statistically significant.

3. Results

3.1. GC–MS quantification

When measured by GC–MS, concentrations of DHBA in the samples were in the range 1.3–76 μM (median 11 μM) (Fig. 1). With the quantified concentrations the daily excretion was calculated and was found to range from 1.6 to 88 μmol/d, with a

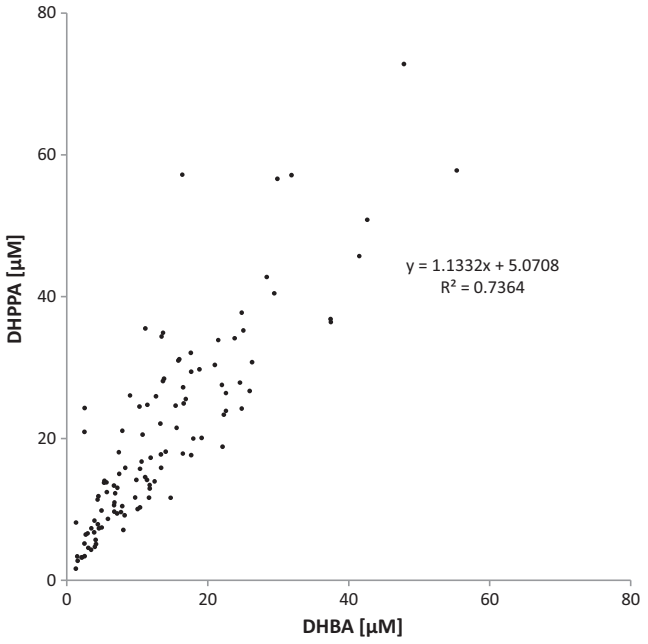

Fig. 1. Concentrations of DHBA and DHPPA in human urine quantified by GC–MS (n = 114). Equation of the linear regression and the coefficient of determination are indicated in the figure. One sample is not shown in the figure due to its very high concentration (DHBA: 76 µM, DHPPA: 220 µM) and was also excluded from the linear regression.

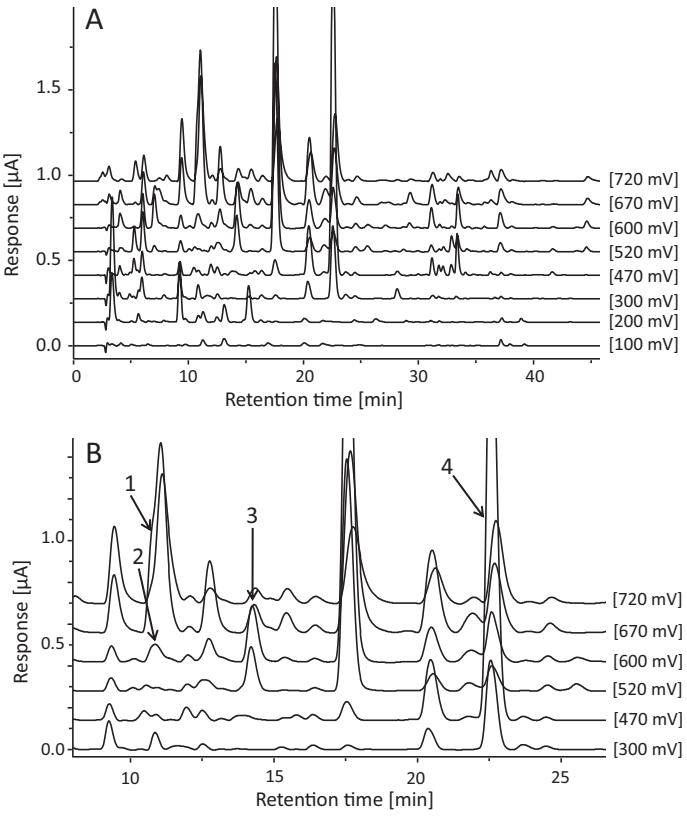

Fig. 2. HPLC–CEAD chromatogram of a deconjugated and extracted human urine sample with poor separation. (A) Whole chromatogram with all eight channels, (B) detailed part of A with six channels covering the elution of DHBA, DHPPA and syringic acid. Due to co-eluting compounds, detection of DHBA on the dominant channel (670 mV) (1) was impaired and therefore quantification was performed using the peak area on the channel immediately before the dominant channel (600 mV) (2). No apparent interference due to co-elution was observed for DHPPA (3) and syringic acid (4).

median value of 15 µmol/d. The concentrations of DHPPA determined by the same analysis were in the range 1.7–220 µM (median 18 µM), corresponding to daily DHPPA excretions in the range 4.5–260 µmol/d (median 24 µmol/d). The DHPPA/DHBA ratios in the samples ranged from 0.79 to 6.3 (median = 1.5, IQR = 0.80, SD = 1.2).

3.2. HPLC–CEAD quantification

Quantification of DHBA in the ten samples analysed directly after deconjugation was hindered due to coeluting compounds displaying oxidation pattern similar to DHBA. Coeluting compounds were also present to some extent when analysing urine samples subjected to subsequent purification, but here quantification of DHBA could be performed at channel 600 mV instead of the dominant channel (670 mV) (Fig. 2). The quantification of DHPPA with HPLC–CEAD did not suffer from co-eluting compounds to the same extent as DHBA.

The concentrations of DHBA quantified by HPLC–CEAD were in the range 0.91–110 µM (median 13) and, based on these concentrations, daily excretion was in the range 1.6–120 µmol/d (median 19 µmol/d). The DHPPA concentrations of the samples analysed by HPLC–CEAD were in the range 0.38–220 µM (median 19 µM), corresponding to daily excretion of 1.1–250 µmol/d (median 26 µmol/d). The DHPPA/DHBA ratio ranged from 0.14 to 14 (median = 1.3, IQR = 0.90, SD = 0.87).

3.3. Method comparison

One sample was excluded before evaluation of the agreement between the two methods due to its very high concentrations of DHBA (76 µM [GC–MS] or 110 µM [HPLC–CEAD]) and DHPPA (220 µM [both methods]).

The mean difference between the two methods was 1.6 µM, i.e. quantification by HPLC–CEAD resulted in average in concentrations 1.6 µM higher than quantification by GC–MS (Fig. 3A). The limits of agreement, corresponding to ±2 SD of observed differences, ranged from −7.2 µM to 10.4 µM. No obvious systematic trend in the difference could be observed in the plot, i.e. the differences between the two methods appeared to be random. Application of weighted Deming regression analysis resulted in a slope (0.92; 95% CI: 0.82, 1.02) not significantly different from unity (P = 0.12). However, the intercept (−0.9; 95% CI: −1.64, −0.16) differed significantly from 0 (P < 0.02), suggesting a small but statistically significant additive difference between the two methods (Fig. 3C). An additive difference is the indication of a constant bias between the methods, whereas a proportional difference is the result of a non-constant bias throughout the sample range.

For DHPPA, the mean difference between the two methods, determined by the difference-plot, was 0.27 µM (n = 110) (Fig. 3B). The limits of agreement for DHPPA were in the range −8.4 µM to 8.9 µM. The slope between the two methods was estimated to be 0.90 (95% CI: 0.83, 0.97) and the intercept to be 1.64 (95% CI: 0.94, 2.34) by weighted Deming regression (Fig. 3D). Both slope and intercept differed significantly in identity (P < 0.01), suggesting that agreement between the two methods was somewhat obscured by both a proportional and an additive difference.

4. Discussion

The AR metabolites DHBA and DHPPA have been suggested as biomarkers for intake of whole grain wheat and rye [6], as well as for cereal fibre [25]. Two methods for quantification of urinary alkylresorcinol metabolites [14,16], using liquid chromatography coupled with electrochemical detection and gas chromatography

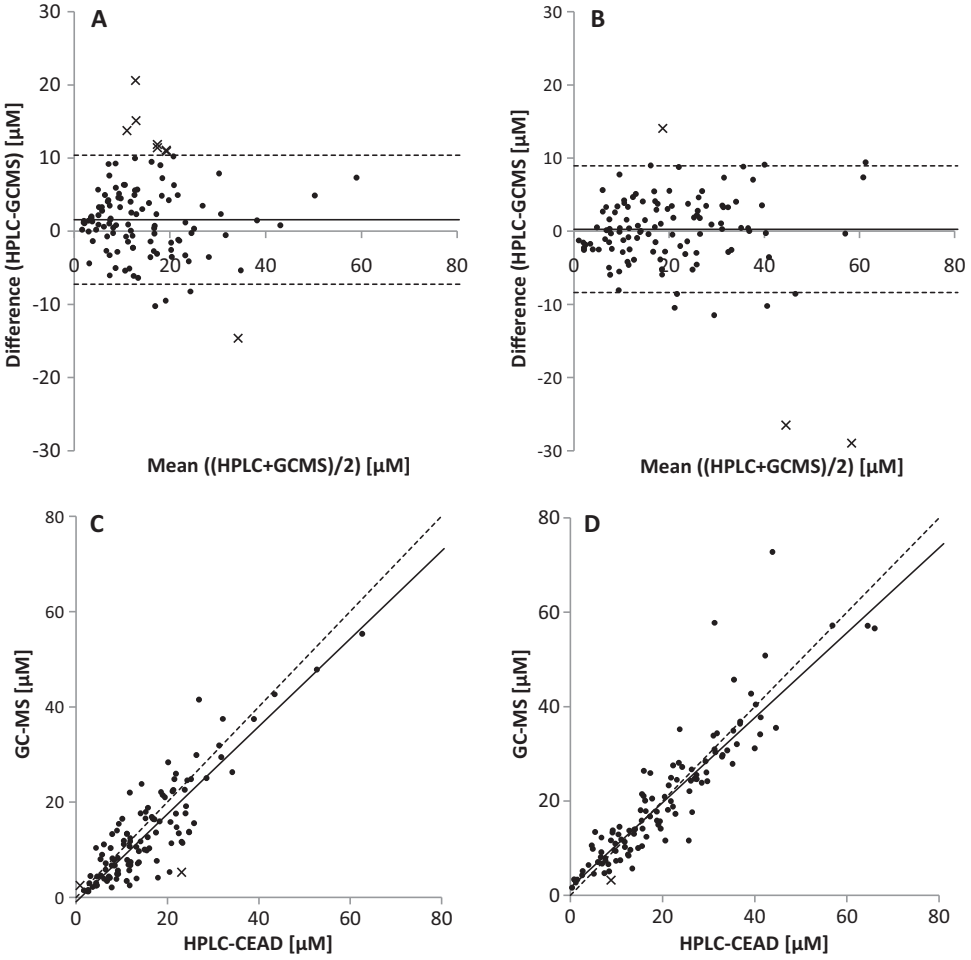

Fig. 3. Difference-plots and weighted Deming regressions of alkylresorcinol metabolites in human urine. In the difference-plots of DHBA (A) and DHPPA (B), the differences between the two methods ($C_{HPLC} - C_{GC-MS}$) are plotted against the mean concentrations by the two methods (($C_{HPLC} + C_{GC-MS}$)/2). The mean differences are shown (solid lines), as are the limits of agreement (broken lines), corresponding to ±2 SD. Concentrations quantified by GC–MS and HPLC–CEAD are plotted against each other (dots) for DHBA (C) and DHPPA (D) and the weighted Deming regressions (DHBA: $y = -0.93 + 0.92x$ and DHPPA: $y = 1.64 + 0.90x$) are indicated (unbroken lines), as are the identity lines (broken lines). Outliers excluded from the models are shown as crosses.

in combination with mass spectrometry were compared here for the first time.

Difficulties in detecting DHBA by HPLC–CEAD due to co-eluting compounds have been reported previously [17]. In this study, quantification of DHBA with HPLC–CEAD was impaired in some samples by co-eluting compounds displaying similar oxidation potentials to the analyte (Fig. 2). As expected, samples analysed after deconjugation, without liquid–liquid extraction and sample clean-up by solid phase extraction, resulted in erratic chromatograms and although additional liquid–liquid extraction and subsequent clean-up with solid phase extraction decreased the number of interfering peaks, the DHBA peak in some samples still suffered from co-eluting peaks. It is possible that analysis of deconjugated urine (without subsequent purification) could be sufficient for reliable quantification of DHPPA, but the few samples of deconjugated urine analysed in this study are deficient to provide reliable information.

The median DHPPA/DHBA ratio in the samples analysed is comparable to that reported in a study on urinary excretion of DHBA and DHPPA from free-living Finnish women (vegetarians and omnivores) [18] and in a previously published intervention study using Swedish subjects [26]. The wide range of ratios in the samples analysed here can most likely be attributed to the non-restricted diet of the contributing volunteers. However, samples with extreme ratio values may indicate alternative sources of DHPPA and/or DHBA, or differences in alkylresorcinol elimination between subjects.

Both difference-plots and regression analysis were used in the present study to assess method agreement. Difference-plots are simple and informative tools to display differences between analytical methods [27,28], but do not provide a suitable tool for statistical inference of systematic biases between methods [28]. Several possible regression analysis methods (e.g. ordinary least-squares and the Passing–Bablok rank method [29]) have been suggested for statistical evaluation of method comparisons. In this study, the weighted Deming regression analysis was selected over other regression analyses due to its suitability for comparison of methods where the measurement error is proportional to the analysed concentration throughout the measurement range (i.e. constant coefficients of variation). Moreover, Deming regressions account for measurement errors in both methods [24]. Because the method comparison was performed by analysing single samples by each method, the ratio of the coefficients of variation from each method (λ) was approximated to 1, based on previously published analytical coefficients of variation from HPLC–CEAD and GC–MS analyses [15,16]. The large range ratios (highest concentration/lowest concentration) for both analytes in the samples (>30) allowed some violation of the assumption of $\lambda = 1$ without affecting the model considerably [30].

For DHBA, the difference-plot showed that quantification by HPLC–CEAD, on average, yielded 1.6 µM higher values than the GC–MS method, irrespective of sample concentration. This was

confirmed by the weighted Deming regression, which showed a statistically significant intercept. The practical implication of this difference is small in most cases, as the typical sample concentration is high compared with the difference.

For DHPPA, the difference-plot did not reveal any obvious differences between the two methods, but the weighted Deming regression analysis showed that agreement was obscured to a small extent by a difference which was both additive and proportional, as indicated by a significant intercept and significant slope. However, in practice, these biases are critical only in lower concentrations, as the regression function deviates more than 10% from the identity line only at concentrations less than 8 μM, which only applied for 18% of the samples analysed in this study.

It has previously been reported that quantification of AR metabolites by HPLC–CEAD can be performed on deconjugated urine samples with and without subsequent extraction steps, with satisfying correlation [15]. However, other studies report obstacles in quantifying DHBA due to co-elution of interfering compounds [17]. In the present study, quantification was interfered by co-eluting compounds and the impact of interference was smaller when sample purification was applied compared to direct injection of sample extracts. Even in the purified extracts, the interference could not be completely avoided in all urine samples. These findings show that sample extraction and clean-up is probably necessary in order to ensure accurate results, particularly for DHBA. Although sample extraction and clean-up require several analytical steps, which is a time-consuming disadvantage compared with direct analysis of deconjugated urine, both time and labour can be reduced by the use of good laboratory infrastructure (e.g. automated solid phase extraction system). Analysis by GC–MS (in single ion recording mode) of purified urinary extract results in a chromatogram with no apparent interference of co-eluting peaks and the time between two consecutive samples is less than 30 min. This can be compared with the analytical run-time of HPLC–CEAD used in this study, which extended to 90 min between injections.

5. Conclusions

Detection and quantification of alkylresorcinol metabolites (DHBA and DHPPA) in human urine can be performed by both GC–MS and HPLC–CEAD. Quantification of DHBA with HPLC–CEAD suffered from interference from co-eluting compounds, suggesting that sample purification is necessary to ensure accurate quantification. Difference-plots and regression analysis revealed some degree of small systematic differences between the two methods, mostly affecting samples with low concentrations. The practical implications of these differences are probably negligible in most cases.

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