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Robust Markers of Coffee Consumption Identified Among the Volatile Organic Compounds in Human Urine

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Abbreviations: CAR (carboxen); DVB (divinylbenzene); MSTUS (mass spectral total useful signal); PA (polyacrylate); PDMS (polydimethylsiloxane); PQN (probabilistic quotient normalization); QC (quality control); UT (untargeted/targeted); VOC (volatile organic compounds)

Keywords: coffee, dietary intake markers, nutrimetabolomcis, untargeted HS-SPME-GC×GC-MS, volatilomics

Abstract

Scope: The human volatilome has gained high interest for the discovery of potential biomarkers of diseases. However, knowledge about the diet as a crucial factor affecting the volatilome is scarce. Therefore, the search for disease biomarkers, as well as the potential use of volatiles as dietary markers is so far limited. The aim of this study was to investigate the influence of the diet on the urinary volatilome with the special task to find potential markers of coffee consumption in 24 h urine samples from the KarMeN study.

Methods and results: Acidified urine samples were analyzed using an untargeted HS-SPME-GC×GC-qMS approach. Overall, 138 reliably occurring volatiles were detected. To account for the unequally concentrated urine samples, results of six different commonly used normalization methods were compared. Statistical analysis evidenced six potential markers of coffee consumption, the most promising being 3,4-dimethyl-2,5-furandione. A correlation analysis between the 24 h dietary recall data and the urinary volatilome revealed further promising associations.

Conclusion: The human urinary volatilome is highly affected by the diet, enabling access to a high level of information about potential diet-related biomarkers. Therefore, it is a very promising source for further investigation about dietary intake markers.

1. Introduction

Volatile organic compounds (VOCs) are formed by several chemical and biochemical processes and are therefore omnipresent in the environment [1, 2]. VOCs play important roles in many different aspects of life such as aroma components of food, animal or plant hormones and pheromones, microbial products, combustion or industrial processes. These exogenous volatiles can easily enter the human body via diverse paths (e.g. inhalation, ingestion, dermal absorption, resorption in the gut), are distributed between different compartments of the body or can be easily excreted via diverse paths (e.g. exhalation, excretion, secretion). At the same time the human body itself can produce a variety of VOCs endogenously through normal or abnormal physiologic processes or even through residing commensal microorganisms [2-6]. The combination of these exogenous and endogenous VOCs emitted by the human body are defined as the human volatilome, which can be measured in various specimen such as breath, skin emanations, sweat, saliva, blood, urine or feces [3, 6, 7]. So far, the highly complex interplay between the various factors that influence the volatilome are not well understood [4, 5, 8-11]. Nonetheless, there is a growing interest to exploit the volatilome for early diagnosis of diseases [4, 5, 12] or metabolic disturbances like, among others, cancer [9, 13, 14] and diabetes [15, 16]. However, identifying distinguishable VOC profiles representing the healthy and diseased status is complicated by the huge inter-individual variability caused by many factors such as genetic background, life style, diet, age, sex, physiologic differences in metabolism, or residing commensal bacteria [4, 5, 8-11]. Therefore, it is necessary to understand the interplay between and the impact of these influencing factors on the human volatilome.

Every-day experience demonstrates that diet may have a major (and sometimes olfactorily well-perceivable) impact on urinary volatilome. So far, only a few systematic investigations of this phenomenon have been performed [17-21], but it is likely that VOCs could be valuable for nutritional research. For example, with respect to epidemiologic studies where under- and/or overreporting complicate interpretation of results along the diet-health/disease-trajectory [22], valid dietary markers would be extremely useful. While non-volatile metabolites in urine as potential dietary markers have

been in the focus of nutrition research for some years, so far little attention has been paid to volatile metabolites.

Breath and skin emanations are objects of several studies as they can be sampled easily and non-invasively [5, 7, 9]. Urine, as another readily accessible specimen, has gained popularity in the last years [23, 24]. It has the additional advantage of pre-concentration in the kidneys and of being more easily storable than, for example, breath or skin emanations. Even so, a huge disadvantage of urine is the differently diluted excretion, which necessitates (pre- or post-analytical) normalization. There are many normalization methods that can be applied, among others normalization on creatinine, osmolality, specific gravity, urine volume, MSTUS (mass spectral total useful signal) [25] or PQN (probabilistic quotient normalization) [26]. So far a scientific consensus on the evaluation and application of these methods is still missing [27-30].

Coffee, with more than 800 volatile constituents or flavorants [17] and more than 150 million (60 kg) bags consumed worldwide in 2016 [31], is an important and promising food to study the informative potential of its volatiles as dietary markers. Wagenstaller *et al.* [17] already described an increase of some VOCs in urine after the ingestion of coffee in an acute intervention study with a targeted method based on a limited population (n = 14). Otherwise markers for coffee consumption are manly from among non-volatile metabolites [32].

This study aims to identify potential markers of coffee consumption in 24 h urine samples from a selected subset of the observational KarMeN [33] study. Urinary VOCs were sampled by HS-SPME and analyzed by GC×GC-qMS. Different normalization methods on urinary VOC responses were tested to achieve a higher confidence level in results interpretation. Additionally, through the use of a 24 h dietary recall the influence of diet apart from coffee consumption on the urinary volatilome was investigated.

2. Materials and methods

2.1. Study design and subjects

A subset of 24 h urine samples from the cross-sectional study KarMeN, performed at the Max Rubner-Institut in Karlsruhe, Germany (2011-2013), was analyzed. The study design and examination procedures were described elsewhere [33]. The study was approved by the ethics committee of the State Medical Chamber of Baden-Württemberg (F-2011-051) and was in accordance with the 1964 Helsinki declaration and its later amendments. The study was listed at the German Clinical Study Register (DRKS00004890) and has the WHO universal trial number: U1111-1141-7051. The subset consisted of 97 healthy participants (18-80 years), of these 53 were male and 44 female. According to the conducted 24 h dietary recall, 48 participants drank coffee in the 24 hours of urine collection (coffee consumers) and 49 participants did not drink coffee during that period (non-consumers). The subset was chosen randomly among coffee consumers and non-consumers of the KarMeN population with the precondition, that about half of the coffee consumers and non-consumers respectively were male and the other half female. Additionally, for each consumers and non-consumers a uniform distribution of age was intended. For participant characteristics of the subset see Supporting Information Table S1.

Participants collected a 24 h urine sample in urine containers that were kept cooled throughout the collection period. The urine volume was recorded. A urine portion was centrifuged at 1850 × g at 20 °C and aliquoted. Aliquots were frozen at -20 °C for one day, and afterwards cryopreserved at -196 °C until analysis. A quality control (QC) sample was prepared by mixing fresh spot urine of 4 male and 4 female volunteers shortly before the measurement series, then it was aliquoted and frozen for a short period at -20 °C until analysis.

The food consumption (in g/d) of each participant during the 24 hours of urine collection was assessed in a personal interview using the 24 h dietary recall method and the software EPIC-Soft [34, 35], now renamed into GloboDiet.

2.2. Volatile profiling of urine samples using HS-SPME-GC×GC-qMS

2.2.1. Sample preparation and HS-SPME sampling conditions

A short screening with different fibres (polyacrylate (PA), polydimethylsiloxane (PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB) and divinylbenzene/carboxen/polydimethyl-siloxane (DVB/CAR/PDMS)) as well as comparing sample preparation either with β-glucuronidase (*Helix Pomatia*, ≥100000 units/mL, pH: 4.5-5; sigma-aldrich) or with addition of a pH modifier, i.e., HCl, was performed before setting the method sampling protocol (see section 1.2.1 of the Supporting Information).

The SPME fibre chosen for urinary VOCs sampling was the DVB/CAR/PDMS with d_f 50/30 µm and 2 cm length. A set of six fibres, all from Supelco (Bellefonte, PA, USA), was conditioned before use as recommended by the manufacturer and tested for response standardization.

For each 24 h urine sample, a headspace crimp top vial (22 mL) was filled with 5 mL of deionized water to enable an efficient heat transfer toward the inert glass insert of 6 mL volume used for urine sampling. The reduced headspace volume enabled achieving adequate sensitivity for effective VOCs monitoring. To the 6 mL insert 0.16 ± 0.001 g of NaCl were added together with 800 µL of urine sample. 200 µL of HCl (1 mol/L) were finally added to lower the pH, before capping the vial and mixing with a Vortex unit. QC sample aliquots and study samples were prepared in the same manner. QC samples were used for the correction of batch or drift effects. Each day, 11 study samples, 3 QC samples and 1 blank sample (consisting only of the internal standard solution) were prepared and analyzed. All study samples were measured twice.

For each run, 2 µL of internal standard solution (10 ppm tridecane in dibutylphthalate) was placed into a 22 mL glass vial for pre-loading onto the fibre for 30 min at 65 °C (standard-in-fibre procedure [36]), at the same time and temperature the urine sample was pre-equilibrated. Then, the fibre was exposed to the urine sample for 60 min at 65 °C. Afterwards the fibre was introduced into the injector of the GC×GC system at 250 °C and thermally desorbed for 5 min.

2.2.2. Instrumental setup for HS-SPME-GC×GC-qMS

HS-SPME-GC×GC-qMS analysis was performed on an Agilent 6890 unit coupled to an Agilent 5975C MS detector (Agilent, Little Falls, DE, USA), equipped with a MPS-2 multipurpose sampler (Gerstel, Mülheim a. d. Ruhr, Germany) and a two-stage KT 2004 loop-type thermal modulator (Zoex Corporation, Houston, TX) cooled with liquid nitrogen. Data were acquired by Agilent MSD ChemStation ver. E.02.01.00. In the Supporting Information Table S2, the different GC parameters including modulation parameters and the different MS parameters for the GC×GC-qMS measurement are shown.

2.2.3. Data processing

Processing of raw data was performed using GC Image™ GC×GC Software version 2.5 (GC Image, LLC Lincoln NE, USA). The sequential workflow described by Magagna *et al.* [37] was slightly modified and applied. After file import, rasterization, colorization, baseline correction, 2D-peak detection and integration, a template with known analytes (targeted 2D peaks) was built using GC Image™ standard workflow [37, 38]. Targeted peaks were identified by EI-MS fragmentation pattern (NIST MS Search algorithm, ver. 2.0, National Institute of Standards and Technology, Gaithersburg, MD, USA; with Direct Matching threshold 900 and Reverse Matching threshold 950) with those collected in commercial (NIST2014 and Wiley7n) and in-house databases. As a further parameter to support identification, linear retention indices based on *n*-alkanes were considered and experimental values were compared with tabulated NIST reference indices. The target template consisting of 63 known volatiles was matched to all 2D-chromatograms and results (positive matches) saved (GC Project™) to enable further processing.

Processed runs were then used to generate an untargeted/targeted (UT) template, following the UT fingerprinting principles [37, 38], including all reliable 2D peaks (known and unknown reliable peaks) that matched in all-but-one chromatograms of the set. The fully automated procedure is implemented in a dedicated software ambient named GC Investigator™. The algorithm performs a comprehensive pair-wise peak matching and alignment of all 2D chromatograms based on retention

times in both chromatographic dimensions and EI-MS fragmentation pattern (mode for preferred reliable peaks: most relaxed).

After application of the UT template to all samples (GC Project^{TM}), the resulting 2D chromatograms were supervised and checked for the correct alignment of all reliable peaks. A preliminary data matrix with 172 features was obtained for which an analyte-specific batch correction was performed based on the measured QC samples. Afterwards, analytes were evaluated with respect to their repeatability in QC samples (corrected inter-day CV > 35% were excluded) and the occurrence of 2D peaks in heavily crowded chromatogram regions leading to a final data matrix consisting of 138 peak features. Before statistical analysis, missing values were replaced analyte-wise by half of the minimal value. Afterwards, normalization for the differential concentration of urine samples was carried out. For comparative reasons we used not normalized data and data normalized using osmolality, creatinine, urine volume, MSTUS and PQN. While scaling factors for osmolality, creatinine and urine volume are based on physiologic properties, those derived from MSTUS and PQN are data driven with the objective to prevent the impact of sample concentration variability. MSTUS is similar to normalizing on total signal, but uses only a subset of ions/features (instead of including all measured ions (total ion count (TIC), which would also include noisy or specimen-specific ions/features) for a more reliable normalization [25]. PQN adjusts each sample of a metabolite profile according to the most probable dilution factor with regard to a reference sample to achieve a virtual overall concentration [26]. The reference sample can be either an elected biological study sample or a calculated median metabolite profile based on all measured biological samples, respectively. In recent publications [30, 39], when urine volume was used as the normalization reference, signal response was normalized by dividing by the urine volume and as this lead to an increased biological variance, it was concluded that urine volume normalization is not appropriate [30, 39]. However, compared to the direct proportionality between creatinine content or osmolality and the overall urine concentration, the urine volume is inversely correlated to the concentration of urine analytes (see Figure 1). Consequently, dividing by urine volume should lead to an increased variation contrary to the intention of

normalization. A physiologically more reasonable option based on the principles of human renal physiology may be to normalize signal intensities by multiplication with urine volume. For comparison, we applied both methods in this manuscript (urine volume div and urine volume mult). After assessment of data quality, some technical replicates were excluded. For the replicates the mean was calculated and further statistical analysis and data interpretation were carried out on the resulting seven data matrices, one for each different normalization methods, as well as on the raw data with no normalization.

2.3. Statistical analysis

Statistical analysis was carried out using JMP® (version 13, SAS Institute Inc., Cary, NC, 1989-2007). Data matrices were built with 97 study samples (48 coffee consumers and 49 non-consumers) and 138 UT peak features.

2.3.1. Comparison of normalization methods

In a first step, the overall impact of the different normalization methods on the data set was assessed using a correlation analysis. For this, we calculated Spearman correlation coefficients for the scaling factors used to normalize the data. In a second step, the coefficients of variation (CV) for all the 138 VOCs in all study samples and as well in the two groups of coffee consumers and non-consumers were calculated before and after application of all normalization methods.

2.3.2. Potential markers of coffee consumption

Firstly, an ANOVA based response screening analysis including correction for multiple testing [40] was performed to identify volatiles differentiating the two groups of coffee consumers and non-consumers. The workflow includes a first ANOVA with correction for multiple testing, a second ANOVA and post-hoc tests (each depending on the distribution and homogeneity of variance). The ANOVA based response screening was performed for the data without normalization, and the normalized data sets (creatinine, osmolality, urine volume div, urine volume mult, MSTUS and PQN). Only significant volatiles showing a median fold change > 1.5 for at least one of the normalization method were considered. Secondly, a correlation analysis using Spearman correlation

coefficients was performed to consider the potential dose-response relation between the coffee consumption and the volatiles. Therefore, Spearman correlation coefficients of the volatiles with the amount of consumed coffee were calculated before and after normalization. Significance of the correlation was tested and only those reported that remained significant after Bonferroni correction, which in this case corresponded to $\rho > 0.35$. In the Supporting Information Figure S1 a workflow for the raw data processing as well as the statistical analysis is depicted.

2.3.3. Relevance of age or sex for potential markers of coffee consumption

The potential influence of sex or age on the potential markers of coffee consumptions was evaluated. A Wilcoxon Test was performed to ascertain the influence of sex, while Spearman correlation coefficients were calculated for the age effect.

2.3.4. Other potential volatile markers related to food consumption

To get a further impression on the usability of volatiles as potential markers for food consumption in general and the influence of the diet on the urinary volatilome, the food consumption during the 24 hours of urine collection (overall 81 different food consumption and nutrient intake variables see [41]) was correlated with the detected volatiles in 24 h urine samples before and after normalization. Only Spearman correlation coefficients with $\rho > +0.35$ for at least three normalization methods and more than 25 participants ate certain food items were considered further. Occurring negative correlations of VOCs in urine with some food items were not considered at this stage because they could be related to endogenous metabolism and their relationship would be of a complex nature. Additionally, a Wilcoxon test was performed (lowest against highest tertile) for the normalization methods with the highest Spearman correlation coefficients.

3. Results

3.1. Analytical characteristics

As a first step different stationary phases of the fibre and sample treatment used for SPME were screened either with glucuronidase or HCl. Comprehensive approaches for selection of the fibre, sample treatment and extraction conditions can be found elsewhere [13, 14, 24, 42-45]. In Table 1, the number of detected 2D peaks is compared for the different fibres as well as the different sample preparations tested, including a blank sample with only glucuronidase and buffer. In the Supporting Information Figures S2 and S3 GC×GC-MS chromatograms for all fibres as well as the different sample preparations are depicted. The possibly gentler treatment with β-glucuronidase (*Helix pomatia*, ≥100000 units/mL, pH: 4.5-5; sigma-aldrich) was excluded as the measurement of the blank enzyme sample revealed high abundance of VOCs (see Table 1 and Supporting Information Figure S3). Enzyme preparations are usually isolated from living organisms (e.g. *Helix pomatia*) and can be complex mixtures. Therefore, it is highly recommendable to measure the blank enzyme before applying it to metabolomics studies.

Our short comparison gave the best result, i.e. the highest number of detected 2D peaks, for the DVB/CAR/PDMS fibre and the acidic treatment with HCl, which is in good agreement with literature evidences [14, 43, 45]. Therefore, these conditions were chosen for the analysis of the 24 h urine samples of the KarMeN study. Overall, 138 reliable 2D peaks were detected (at least in 70% of study samples) of which 63 were identified (targeted analytes). Repeatability of the measurement series was satisfactory with 66% of the volatiles having a CV < 25% in the batch corrected QC samples (see Supporting Information Table S3).

3.2. Comparison of normalization methods

In Figure 1 Panel A, the scatter plots and Spearman correlation coefficients are shown for the different normalization methods. The associations between the normalization methods are of different type and strength. While normalization based on creatinine and osmolality is highly correlated ($\rho = 0.901$) and thus almost equivalent in this specific dataset, the effects of other normalization methods are

obviously different and may lead to less comparable datasets and thus, at least in part, different results. Additionally, the inverse relationship of urine volume with the overall urine concentration and the other normalization methods can clearly be observed. In Figure 1 Panel B, the inter- and intra-group variation before and after the application of the different normalization methods is depicted in a violin plot. The densest region of the CV's was at similar values (CV ~ 50%) before normalization and after normalization using creatinine, osmolality, MSTUS and PQN. Interestingly, calculating total peak feature excretion per 24 hours (urine volume mult) leads to a similar densest region, whereas normalization by dividing by urine volume (urine volume div) showed a broader distribution of CV's and the highest density lay at higher CV's (CV ~ 125%).

3.3. Potential markers of coffee consumption

The results of the ANOVA based response screening (see Figure 2) and correlation analysis, as well as the potential influence of sex and age are summarized in Table 2. For the response screening, listed volatiles had a median fold change higher 1.5 or, in case of the correlation analysis, a Spearman correlation coefficient above 0.35. In case of the ANOVA based response screening further volatiles were found to distinguish between coffee consumers and non-consumers, but these volatiles were increased in non-consumers and additionally showed only a weak median fold change (see Supporting Information Table S4). Therefore, they are not discussed in this paper. The most interesting volatile marker candidate was 3,4-dimethyl-2,5-furandione, which was present in significantly higher levels in the samples of coffee consumers independent of the normalization method. This metabolite showed the highest fold changes (6.61-8.19) and correlations (ρ = 0.606-0.683). The other potential markers for coffee consumption were 2-methylfuran, guaiacol (i.e., 2-methoxyphenol), 2-/3-methylbutanoic acid and 2-vinylfuran. The compounds exhibited a significant difference only for some of the different normalization methods (see Table 2). In case of methylbutanoic acid both the 2-methyl- and 3-methylbutanoic acid isomer are coeluting, therefore we report them as a sum parameter. To elucidate whether only 2-methyl-, only 3-methylbutanoic acid or both are potential marker candidates, the raw data was reintegrated using a peak region over the coeluting isomers including two quantifier ions

(m/z 57 and 60). This enabled a distinction of these two isomers. After applying the different normalization methods and performing a Wilcoxon-test as well as a correlation with the coffee intake, it becomes clear that both isomers are equally increased after coffee intake (see Supporting Information Figure S4).

In the Supporting Information Figure S5 both boxplots from the ANOVA screening as well as scatterplots from the correlation analysis are shown. 2-Methylfuran and 2-vinylfuran were found to significantly differ between males and females at least in one of the normalization methods, but not all. Median fold changes ranged between 1.18-1.38, therefore we believe the influence of sex to be negligible. Guaiacol showed a rather weak correlation with age in case of two normalization methods.

3.4. Other potential volatile markers related to food consumption

With the aim to demonstrate the general capability of volatiles as potential markers of food consumption and to show the relevant influence of the diet on the urinary volatilome, we performed an additional Spearman correlation analysis with the food variables reported in the 24 h recall during the 24 hours of urine collection with the volatiles detected in these samples. Although the 97 participants were chosen with the specific aim to find markers of coffee consumption (see section 3.3), we were able to detect further associations between volatile compounds in urine and dietary intake (see Table 3).

4. Discussion

4.1. Analytical characteristics

Using the optimized HS-SPME-GC×GC-qMS method we detected 138 volatile compounds reliably and reproducibly in 97 (24 h) urine samples of the KarMeN study. The 63 identified VOC include different substance classes, among others alcohols, aldehydes, ketones, carboxylic acids, furan derivatives, terpenoids and phenols. It is noteworthy, that the 24 h urine samples were cryopreserved at -196 °C for 3-4 years and stored for a short period (3 months) at -80 °C before they were measured. Although investigations on the influence of storage conditions on the volatilome of urine advice not to store samples longer than 6-9 months at -80 °C [44, 46], we observed a complex profile of VOCs in 24 h urine samples measured. Nonetheless, it is to be expected that very volatile compounds will successively be lost even at cryopreservation temperatures (-196 °C). However, this will occur for all urine samples in a similar manner and therefore a relative comparison as performed in this study is still feasible. Semi- and less volatile compounds are even after 3-4 years of cryopreservation well detectable.

4.2. Normalization

In literature many comparisons about the advantages and disadvantages of normalization methods can be found [30, 39]. The most often applied criteria for comparing normalization methods is the number of distinguishing metabolites between groups. If considered exclusively, this criterion is highly questionable as a higher number of potential biomarkers does not necessarily prove the validity of these potential markers. Therefore, objective methods for the evaluation of normalization methods are needed. Some publications proposed application of multiple evaluation criteria (a.o. reduction of intra-group variation or consistency of identified markers) [27, 28]. Additionally, experimental design, the dataset and the underlying research question are important criteria for selecting a normalization method [28]. The proposed schemes [27, 28] are sophisticated approaches, but they necessitate a deep understanding of the implemented statistical and bioinformatical methods for their

application. Additionally, if the implementation of these statistical and bioinformatical methods is not performed by oneself, complete transparency can hardly be guaranteed.

We applied six normalization methods (creatinine, osmolality, urine volume div, urine volume mult, MSTUS and PQN), not with the aim of choosing one over another, but to objectively compare results with respect to their intrinsic similarity, their ability for reduction of inter- and intra-group variation and differences and similarities in the identified potential markers for coffee consumption (discussed in section 4.3). For all applied normalization methods correlations in different strength were observed (see Figure 1, Panel A). Therefore, statistical group comparison can be different depending on the applied normalization. All normalization methods as well as no applied normalization had comparable variation (see Figure 1, Panel B), with the exception of normalization on urine volume div, which caused a higher inter- and intra-group variation. This is in agreement with other reports in recent literature [30, 39] and confirms our assumption that normalizing on 24 h urine volume by division does not lead to an equalization of the differentially diluted urine samples, but to a higher variation due to its inversely proportional relationship with the overall urine concentration. Thus, the normalization on urine volume div cannot be recommended. Nevertheless, from the physiological point of view, urine volume can indeed be considered as a meaningful normalization reference for 24 h urine samples, because it allows the calculation of the total excretion of metabolites – which may, in case of an untargeted analysis and using the normalization on urine volume mult, be expressed as signal intensity per day which is proportional to the excreted amount per day. This allows investigating associations of 24 h dietary/substrate intake and corresponding urinary metabolite excretion in the same 24 hours. 24 h urine collections are not easily applicable in most studies and therefore, spot urine samples are taken instead, which necessitate normalization methods other than urine volume. On the other hand, as shown in Fig. 1 panel B, normalization using creatinine, osmolality, MSTUS or PQN was obviously equally appropriate at the global level. Further, any normalization based on urine volume is probably not appropriate if the urinary metabolite profiles of healthy people and those with impaired renal functions will be compared.

4.3. Potential markers of coffee consumption

Using the ANOVA based response screening and the correlation analysis, six potential markers for coffee consumption were found, 3,4-dimethyl-2,5-furandione, 2-methylfuran, guaiacol, 2- and 3-methylbutanoic acid and 2-vinylfuran (see Table 2). Only 3,4-dimethyl-2,5-furandione was found to be significant with all normalization methods, while the other potential markers were only significant for some of the normalization methods. For the normalization on urine volume div, which even introduced variation to the data, only 3,4-dimethyl-2,5-furandione was found to be significant. In case of the other potential marker candidates for some of the normalization methods the increased variation due to dividing by the urine volume masked the difference between coffee consumers and non-consumers. Applying normalization can reduce overall variation and therefore enable marker identification, but unsuitable normalization can as well introduce additional and artificial variation and is therefore a potential source of errors. However, biologically highly relevant markers, as 3,4-dimethyl-2,5-furandione (see Figure 2), will be found independent of the normalization method. Therefore, comparing the influence of different normalization methods on your dataset is a worthwhile and useful step.

3,4-Dimethyl-2,5-furandione is the most promising of the found potential markers for coffee consumption (see Figure 2). It had by far the highest median fold changes and correlation coefficients, additionally neither sex nor age showed any influence on the urinary levels of this VOCs. Most likely 3,4-dimethyl-2,5-furandione is a product of the roasting process of coffee beans. It was described in the headspace of roasted Robusta coffee beans [47] and brews prepared with these beans [48], but not in the headspace above green Robusta coffee beans [47]. Next to *Coffea canephora* (Robusta coffee), 3,4-dimethyl-2,5-furandione was as well detected in extracts from *Coffea arabica* varieties of different geographic origins [49]. Therefore, 3,4-dimethyl-2,5-furandione as potential marker of coffee consumption is plausible. However, roasting processes are applied to many foods and 3,4-dimethyl-2,5-furandione was as well found in roasted chicory [50], Ugandan vanilla beans (but not bourbon vanilla beans) [51], caramel [52] and in flue-cured tobacco leaves [53], affecting specificity

of this potential marker candidate. Most of the mentioned foods are consumed seldomly or in small amounts and the influence of smoking would have to be evaluated separately, because the KarMeN participants were all non-smokers. Nonetheless, with high median fold changes (6.61-8.19), no overlap of the boxes in the boxplots of coffee consumers vs. non-consumers (see Supporting Information Figure S4) and good correlations (ρ = 0.606-0.683) in a cross-sectional study with participants on an unrestricted diet, 3,4-dimethyl-2,5-furandione remains a highly interesting potential marker of coffee consumption. This is especially true because recording details of coffee consumption are not part of a 24 h recall, but coffee is drunk in many different ways. Not recorded details of coffee consumption are i) differentiated amounts of different coffee beverages (among others filtered coffee, instant coffee beverages or espresso), ii) brewing habit (e.g. amount and length), iii) degree of dilution with milk to some extent, iv) coffee variety (Arabica, Robusta etc.), and v) the roasting process, its intensity and the grounding. All these factors may be causal for the broad scattering of data points that can be observed in the scatter plots (see Supporting Information Figure S5) and for this reason correlation coefficients can only be indicators of associations. Therefore, a detailed quantitative assessment of coffee consumption is not easily possible, but the 3,4-dimethyl-2,5-furandione might allow a robust, qualitative assertion about general coffee consumption.

The other significantly different VOCs (2-methylfuran, guaiacol, 2-methyl- and 3-methylbutanoic acid and 2-vinylfuran) between coffee consumers and non-consumers had weaker effects (median fold changes < 1.9, $\rho < 0.56$). The influence of sex and age on these potential markers seems to be of negligible importance, as only in some of the normalization methods slight tendencies for differences in sex or age were observed (see Table 2). 2-Methylfuran, guaiacol, 3-methylbutanoic acid and 2-vinylfuran were detected in roasted coffee and are – like 3,4-dimethyl-2,5-furandion – a result of coffee roasting [47, 49, 54], therefore they can be viewed as plausible markers. Specificity is similarly questionable due to their formation during roasting processes. 2-Methyl-, 2-vinylfuran and 2-methyl- and 3-methylbutanoic acid are formed during heating of glucose [55], while guaiacol is formed from the thermal or microbial degradation of lignin or phenolic acids such as ferulic acid [56].

Wagenstaller et al. [17] described guaiacol and 3-methylbutanoic acid to increase after coffee consumption in a targeted analysis of selected VOCs of urine samples obtained from 14 volunteers. 3,4-dimethyl-2,5-furandion, our most relevant VOC, was not measured. Wagenstaller et al. [17] observed further VOCs to increase after coffee consumption, such as a.o. 4-vinylguaiacol and *β*-damascenone, these VOCs were identified in our analysis as well, while others might have remained unidentified or were not detected, but were all below the significance threshold for marker candidates of coffee consumption in our statistical analysis. Most likely due to the more controlled conditions in an intervention study, the small number of volunteers and the measurement of spot urine samples in comparison to the KarMeN cross-sectional study with 97 participants on an unrestricted diet and the usage of 24 h urine samples, Wagenstaller et al. [17] were able to detect a potential larger number of marker candidates of coffee consumption. However, our identified markers may be considered as more robust against all kinds of potentially interfering factors (e.g. other food consumption) and are based on a broader population.

4.4. Other potential volatile markers related to food consumption

The aim of the correlation analysis between the 24 h dietary intake data and the VOCs in 24 h urine was to explore the potential of using VOCs as dietary markers, not a comprehensive evaluation of further potential dietary markers. In the literature, VOCs have gained major interest for the diagnosis of diseases and although many authors assume that the volatilome is influenced by our diet [4, 5, 7, 9, 12], specific reports are still mostly missing. Our study shows that dietary intake has a noticeable influence on the urinary volatilome. Even though this study was specifically designed to investigate the consumption of coffee and therefore the strongest associations were observed with coffee, we were nonetheless able to observe further interesting correlations (see Table 3). Next to these positive correlations some negative correlations (ρ = -0.3514 to -0.4522) were observable, due to their more difficult interpretation with respect to food consumption and because most of the volatiles were not identified, they are not discussed here. Some of the positive correlations seem plausible, exemplary mentioned here: i) 2-pentylfuran is a known constituent of the volatile profile of bread [57]; ii)

octanoic acid is a known constituent of milk and other dairy products; iii) *p*-cymene and calamenene are typical constituents of different culinary herbs applied for taste and as antimicrobial agent in sausages [58-61] or iv) ethanol is part of alcoholic beverages. All these plausible dietary marker candidates highlight the future potential of investigations of the volatilome in this respect.

5. Concluding remarks

Acidified urine samples of the KarMeN study showed a rich VOC profile with 138 reliable volatiles (untargeted and targeted analytes) within samples collected from coffee consumers and non-consumers. Storage for 3-4 years at -196°C preserved the urinary volatilome information enabling effective and consistent fingerprinting by HS-SPME-GC×GC-qMS, even without any particular storage containers designed with respect to preservation of VOCs. The urinary volatilome is suitable for the identification of dietary markers, which could be illustrated by six identified potential markers of coffee consumption, the most promising being 3,4-dimethyl-2,5-furandione. These potential markers are plausible, but due to their likely formation via roasting processes their specificity is not ensured and necessitates future validation in further studies. Nonetheless, these markers were found in 24 h urine samples of 97 participants of an observational study on an unrestricted diet. The fact that this was possible, despite not recording details of coffee consumption in a 24 h dietary recall, underlines the robustness of the marker candidates. Applying five different normalization methods gave similar results. With the exception of normalization on urine volume by dividing by it (urine volume div), all other normalization methods showed similar variation in the data as no normalization method. Nevertheless, markers that are highly relevant and show huge biological differences, such as in case of 3,4-dimethyl-2,5-furandione, will be identified independent of normalization procedures. The additionally performed correlation analysis with the general food consumption during the 24 h of urine collection enabled us to illustrate the future potential of VOCs as dietary markers. Our data highlights the substantial impact of dietary intake on the urinary volatilome, therefore in future work it is necessary to investigate this influence, especially in relation to potential disease biomarkers.

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SK, CW, CC, CB: designed the research project; SK, AB, IH: developed the KarMeN concept and design; CM: conducted the analytical experiment; CC, EL: supervised the analytical measurement; CM, BE, CW, CC: analyzed data and performed statistical analysis; CM: wrote the initial draft of the manuscript; CW, SK, CC, EL: critically reviewed and contributed to the manuscript; CM, SK: had primary responsibility for final content. All authors read and approved the final version of the manuscript. None of the authors has a conflict of interest to declare.

7. References

[1] Zlatkis, A., Brazell, R. S., Poole, C. F., The role of organic volatile profiles in clinical diagnosis. *Clinical Chemistry* 1981, *27*, 789-797.

[2] Buettner, A., Wagenstaller, M., Beauchamp, J., in: Parker, J. K., Elmore, J. S., Methven, L. (Eds.), *Flavour Development, Analysis and Perception in Food and Beverages*, Woodhead Publishing 2015, pp. 387-407.

[3] Cumeras, R., Volatilome Metabolomics and Databases, Recent Advances and Needs. *Current Metabolomics* 2017, *5*, 79-89.

[4] Shirasu, M., Touhara, K., The scent of disease: volatile organic compounds of the human body related to disease and disorder. *The Journal of Biochemistry* 2011, *150*, 257-266.

[5] Broza, Y. Y., Mochalski, P., Ruzsanyi, V., Amann, A., Haick, H., Hybrid volatolomics and disease detection. *Angew Chem Int Ed Engl* 2015, *54*, 11036-11048.

[6] Amann, A., Costello Bde, L., Miekisch, W., Schubert, J., *et al.*, The human volatilome: volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva. *J Breath Res* 2014, *8*, 034001.

[7] Costello, B. d. L., Amann, A., Al-Kateb, H., Flynn, C., *et al.*, A review of the volatiles from the healthy human body. *Journal of Breath Research* 2014, *8*, 014001.

[8] Kimball, B. A., Volatile metabolome: problems and prospects. *Bioanalysis* 2016, *8*, 1987-1991.

[9] Schmidt, K., Podmore, I., Current Challenges in Volatile Organic Compounds Analysis as Potential Biomarkers of Cancer. *Journal of Biomarkers* 2015, *2015*, 16.

[10] Wagenstaller, M., Buettner, A., Characterization of odorants in human urine using a combined chemo-analytical and human-sensory approach: a potential diagnostic strategy. *Metabolomics* 2013, *9*, 9-20.

[11] Wagenstaller, M., Buettner, A., Quantitative Determination of Common Urinary Odorants and Their Glucuronide Conjugates in Human Urine. *Metabolites* 2013, *3*, 637.

[12] Angle, C., Waggoner, L. P., Ferrando, A., Haney, P., Passler, T., Canine Detection of the Volatilome: A Review of Implications for Pathogen and Disease Detection. *Frontiers in Veterinary Science* 2016, *3*, 47.

[13] Silva, C. L., Passos, M., Camara, J. S., Investigation of urinary volatile organic metabolites as potential cancer biomarkers by solid-phase microextraction in combination with gas chromatography-mass spectrometry. *Br J Cancer* 2011, *105*, 1894-1904.

[14] Monteiro, M., Carvalho, M., Henrique, R., Jerónimo, C., *et al.*, Analysis of volatile human urinary metabolome by solid-phase microextraction in combination with gas chromatography–mass spectrometry for biomarker discovery: Application in a pilot study to discriminate patients with renal cell carcinoma. *European Journal of Cancer* 2014, *50*, 1993-2002.

[15] Rhodes, G., Miller, M., McConnell, M. L., Novotny, M., Metabolic abnormalities associated with diabetes mellitus, as investigated by gas chromatography and pattern-recognition analysis of profiles of volatile metabolites. *Clinical Chemistry* 1981, *27*, 580-585.

[16] Liebich, H. M., Al-Babbili, O., Zlatkis, A., Kim, K., Gas-Chromatographic and Mass-Spectrometric Detection of Low-Molecular-Weight Aliphatic Alcohols in Urine of Normal Individuals and Patients with Diabetes Mellitus. *Clinical Chemistry* 1975, *21*, 1294-1296.

[17] Wagenstaller, M., Buettner, A., Coffee aroma constituents and odorant metabolites in human urine. *Metabolomics* 2013, *10*, 225-240.

[18] Roscher, R., Koch, H., Herderich, M., Schreier, P., Schwab, W., Identification of 2,5-dimethyl-4-hydroxy-3[2H]-furanone β-d-glucuronide as the major metabolite of a strawberry flavour constituent in humans. *Food and Chemical Toxicology* 1997, *35*, 777-782.

[19] Pelchat, M. L., Bykowski, C., Duke, F. F., Reed, D. R., Excretion and Perception of a Characteristic Odor in Urine after Asparagus Ingestion: a Psychophysical and Genetic Study. *Chemical Senses* 2011, *36*, 9-17.

[20] Zeller, A., Horst, K., Rychlik, M., Study of the Metabolism of Estragole in Humans Consuming Fennel Tea. *Chemical Research in Toxicology* 2009, *22*, 1929-1937.

[21] Zachariadis, G. A., Langioli, A. V., Headspace Solid Phase Microextraction for Terpenes and Volatile Compounds Determination in Mastic Gum Extracts, Mastic Oil and Human Urine by GC–MS. *Analytical Letters* 2012, *45*, 993-1003.
[22] Kuhnle, G. G. C., Nutritional biomarkers for objective dietary assessment. *Journal of the Science of Food and Agriculture* 2012, *92*, 1145-1149.
[23] Rocha, S. M., Caldeira, M., Carrola, J., Santos, M., *et al.*, Exploring the human urine metabolomic potentialities by comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry. *J Chromatogr A* 2012, *1252*, 155-163.
[24] Mills, G. A., Walker, V., Headspace solid-phase microextraction profiling of volatile compounds in urine: application to metabolic investigations. *Journal of Chromatography B: Biomedical Sciences and Applications* 2001, *753*, 259-268.
[25] Gagnebin, Y., Tonoli, D., Lescuyer, P., Ponte, B., *et al.*, Metabolomic analysis of urine samples by UHPLC-QTOF-MS: Impact of normalization strategies. *Analytica Chimica Acta* 2017, *955*, 27-35.
[26] Dieterle, F., Ross, A., Schlotterbeck, G., Senn, H., Probabilistic Quotient Normalization as Robust Method to Account for Dilution of Complex Biological Mixtures. Application in 1H NMR Metabonomics. *Analytical Chemistry* 2006, *78*, 4281-4290.
[27] Bo Li, J. T., Qingxia Yang, Shuang Li, Xuehiao Cui, Yinghong Li, Yuzong Chen, Weiwei Xue, Xiaofeng Li, Feng Zhu, NOREVA: normalization and evaluation of MS-based metabolomics data. *Nucleic Acids Res* 2017.
[28] De Livera, A. M., Olshansky, G., Simpson, J. A., Creek, D. J., NormalizeMets: assessing, selecting and implementing statistical methods for normalizing metabolomics data. *Metabolomics* 2018, *14*, 54.
[29] De Livera, A. M., Sysi-Aho, M., Jacob, L., Gagnon-Bartsch, J. A., *et al.*, Statistical methods for handling unwanted variation in metabolomics data. *Analytical chemistry* 2015, *87*, 3606-3615.
[30] Warrack, B. M., Hnatyshyn, S., Ott, K. H., Reily, M. D., *et al.*, Normalization strategies for metabonomic analysis of urine samples. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009, *877*, 547-552.
[31] D'Elia, L., La Fata, E., Galletti, F., Scalfi, L., Strazzullo, P. J. E. J. o. N., Coffee consumption and risk of hypertension: a dose–response meta-analysis of prospective studies. 2017.
[32] Rothwell, J. A., Madrid-Gambin, F., Garcia-Aloy, M., Andres-Lacueva, C., *et al.*, Biomarkers of intake for coffee, tea, and sweetened beverages. 2018, *13*, 15.
[33] Bub, A., Kriebel, A., Dörr, C., Bandt, S., *et al.*, The Karlsruhe Metabolomics and Nutrition (KarMeN) Study: Protocol and Methods of a Cross-Sectional Study to Characterize the Metabolome of Healthy Men and Women. *JMIR Research Protocols* 2016, *5*, e146.
[34] Slimani, N., Deharveng, G., Charrondiere, R. U., van Kappel, A. L., *et al.*, Structure of the standardized computerized 24-h diet recall interview used as reference method in the 22 centers participating in the EPIC project. European Prospective Investigation into Cancer and Nutrition. *Computer methods and programs in biomedicine* 1999, *58*, 251-266.
[35] Slimani, N., Ferrari, P., Ocke, M., Welch, A., *et al.*, Standardization of the 24-hour diet recall calibration method used in the european prospective investigation into cancer and nutrition (EPIC): general concepts and preliminary results. *Eur J Clin Nutr* 2000, *54*, 900-917.
[36] Wang, Y., O'Reilly, J., Chen, Y., Pawliszyn, J., Equilibrium in-fibre standardisation technique for solid-phase microextraction. *Journal of Chromatography A* 2005, *1072*, 13-17.
[37] Magagna, F., Valverde-Som, L., Ruiz-Samblas, C., Cuadros-Rodriguez, L., *et al.*, Combined untargeted and targeted fingerprinting with comprehensive two-dimensional chromatography for volatiles and ripening indicators in olive oil. *Anal Chim Acta* 2016, *936*, 245-258.
[38] Bressanello, D., Liberto, E., Collino, M., Chiazza, F., *et al.*, Combined untargeted and targeted fingerprinting by comprehensive two-dimensional gas chromatography: revealing fructose-induced changes in mice urinary metabolic signatures. 2018, *410*, 2723-2737.

[39] Edmands, W. M. B., Ferrari, P., Scalbert, A., Normalization to Specific Gravity Prior to Analysis Improves Information Recovery from High Resolution Mass Spectrometry Metabolomic Profiles of Human Urine. *Analytical Chemistry* 2014, *86*, 10925-10931.
[40] Weinert, C. H., Empl, M. T., Kruger, R., Frommherz, L., *et al.*, The influence of a chronic L-carnitine administration on the plasma metabolome of male Fischer 344 rats. *Mol Nutr Food Res* 2017, *61*.
[41] Mack, C. I., Weinert, C. H., Egert, B., Ferrario, P. G., *et al.*, The complex human urinary sugar profile: determinants revealed in the cross-sectional KarMeN study. *The American Journal of Clinical Nutrition* 2018, *108*, 502-516.
[42] Zhang, S., Liu, L., Steffen, D., Ye, T., Raftery, D., Metabolic profiling of gender: Headspace-SPME/GC–MS and 1H NMR analysis of urine. *Metabolomics* 2012, *8*, 323-334.
[43] Bojko, B., Reyes-Garcés, N., Bessonneau, V., Goryński, K., *et al.*, Solid-phase microextraction in metabolomics. *TrAC Trends in Analytical Chemistry* 2014, *61*, 168-180.
[44] Živković Semren, T., Brčić Karačonji, I., Safner, T., Brajenović, N., *et al.*, Gas chromatographic-mass spectrometric analysis of urinary volatile organic metabolites: Optimization of the HS-SPME procedure and sample storage conditions. *Talanta* 2018, *176*, 537-543.
[45] Cozzolino, R., De Magistris, L., Saggese, P., Stocchero, M., *et al.*, Use of solid-phase microextraction coupled to gas chromatography–mass spectrometry for determination of urinary volatile organic compounds in autistic children compared with healthy controls. *Analytical and Bioanalytical Chemistry* 2014, *406*, 4649-4662.
[46] Esfahani, S., Sagar, N. M., Kyrou, I., Mozdiak, E., *et al.*, Variation in Gas and Volatile Compound Emissions from Human Urine as It Ages, Measured by an Electronic Nose. *Biosensors (Basel)* 2016, *6*.
[47] Nebesny, E., Budryn, G., Kula, J., Majda, T., The effect of roasting method on headspace composition of robusta coffee bean aroma. *European Food Research and Technology* 2007, *225*, 9-19.
[48] Budryn, G., *HS-SPME/GC/MS Profiles of Convectively and Microwave Roasted Ivory Coast Robusta Coffee Brews*, 2011.
[49] Cheong, M. W., Tong, K. H., Ong, J. J. M., Liu, S. Q., *et al.*, Volatile composition and antioxidant capacity of Arabica coffee. *Food Research International* 2013, *51*, 388-396.
[50] BAEK, H. H., CADWALLADER, K. R., Roasted Chicory Aroma Evaluation by Gas Chromatography/Mass Spectrometry/Olfactometry. *Journal of Food Science* 1998, *63*, 234-237.
[51] Zhang, S., Mueller, C., Comparative Analysis of Volatiles in Traditionally Cured Bourbon and Ugandan Vanilla Bean (Vanilla planifolia) Extracts. *Journal of Agricultural and Food Chemistry* 2012, *60*, 10433-10444.
[52] Paravisini, L., Prot, A., Gouttefangeas, C., Moretton, C., *et al.*, Characterisation of the volatile fraction of aromatic caramel using heart-cutting multidimensional gas chromatography. *Food Chemistry* 2015, *167*, 281-289.
[53] Xiang, Z., Cai, K., Liang, G., Zhou, S., *et al.*, Analysis of volatile flavour components in flue-cured tobacco by headspace solid-phase microextraction combined with GC×GC-TOFMS. *Analytical Methods* 2014, *6*.
[54] Amaral, M. S. S., Marriott, P. J., Bizzo, H. R., Rezende, C. M., Ionic liquid capillary columns for analysis of multi-component volatiles by gas chromatography-mass spectrometry: performance, selectivity, activity and retention indices. *Analytical and Bioanalytical Chemistry* 2018, *410*, 4615-4632.
[55] S. Fagerson, I., *Thermal degradation of carbohydrates: A review*, 1969.
[56] Dorfner, R., Ferge, T., Kettrup, A., Zimmermann, R., Yeretzian, C., Real-Time Monitoring of 4-Vinylguaiacol, Guaiacol, and Phenol during Coffee Roasting by Resonant Laser Ionization Time-of-Flight Mass Spectrometry. *Journal of Agricultural and Food Chemistry* 2003, *51*, 5768-5773.
[57] Birch, A. N., Petersen, M. A., Hansen, Å. S., The aroma profile of wheat bread crumb influenced by yeast concentration and fermentation temperature. *LWT - Food Science and Technology* 2013, *50*, 480-488.

[58] Meynier, A., Novelli, E., Chizzolini, R., Zanardi, E., Gandemer, G., Volatile compounds of commercial Milano salami. *Meat Science* 1999, *51*, 175-183.
[59] BARBUT, S., JOSEPHSON, D. B., MAURER, A. J., Antioxidant Properties of Rosemary Oleoresin in Turkey Sausage. 1985, *50*, 1356-1359.
[60] Busatta, C., Vidal, R. S., Popiolski, A. S., Mossi, A. J., *et al.*, Application of Origanum majorana L. essential oil as an antimicrobial agent in sausage. *Food Microbiology* 2008, *25*, 207-211.
[61] Aminzare, M., Aliakbarlu, J., Tajik, H., The effect of Cinnamomum zeylanicum essential oil on chemical characteristics of Lyoner- type sausage during refrigerated storage. *Veterinary Research Forum* 2015, *6*, 31-39.

8. Tables

Table 1. Comparison of detected blobs for different fibres and different sample preparation.

	DVB/CAR/PDMS	PDMS/DVC	PDMS	PA
NaCl + HCl (1 M) + urine	656	628	411	429
NaCl + buffer + *β*-glucuronidase + urine	640			
NaCl + buffer + *β*-glucuronidase + water	440			

Table 2. Results for potential markers of coffee consumption. In the first part "ANOVA screening", the median fold changes for those volatiles that were tested as significantly differentiating between coffee consumers and non-consumers after the post hoc test are listed. The second part "Correlation analysis" lists the significant Spearman correlation coefficients of volatiles with the amount of consumed coffee after Bonferroni correction. The third part "Influence of sex" lists the significant p-values for a Wilcoxon test with males and females. The fourth part "Influence of age" lists the significant correlations of volatiles with age. If no value is included it was not tested as significant for this volatile or normalization method.

		3,4-Dimethyl-2,5-furandione	2-Methyl-furan	Guaiacol	2-/3-Methyl-butanoic acid[a)]	2-Vinylfuran
ANOVA screening (median fold change)	**No normalization**	7.81	1.83	1.55[b)]	1.69	1.68
	Creatinine	7.08	1.35	-	-	1.46
	Osmolality	6.61	1.42	-	1.39	1.33
	Urine volume div	8.19	-	-	-	-
	Urine volume mult	7.31	1.49	-	-	1.52
	MSTUS	7.36	1.76	1.51[b)]	1.48	1.58
	PQN	8.15	1.68	-	1.66	1.51
Correlation analysis (correlation coeff. ρ)	**No normalization**	0.683	0.541	0.486	-	0.432
	Creatinine	0.657	0.431	-	-	-
	Osmolality	0.673	0.493	0.360	-	0.359
	Urine volume div	0.606	0.404	0.384	-	0.364
	Urine volume mult	0.686	0.526	0.430	-	0.401
	MSTUS	0.682	0.559	0.515	-	0.406
	PQN	0.682	0.557	0.524	-	0.449
Influence of sex (p-values)	**No normalization**	-	-	-	-	-
	Creatinine	-	0.0242	-	-	-
	Osmolality	-	-	-	-	-
	Urine volume div	-	-	-	-	-
	Urine volume mult	-	0.026	-	-	-
	MSTUS	-	-	-	-	0.0362
	PQN	-	-	-	-	-
Influence of age (p-values)	**No normalization**	-	-	-[c)]	-	-
	Creatinine	-	-	0.352	-	-
	Osmolality	-	-	-[c)]	-	-
	Urine volume div	-	-	-	-	-
	Urine volume mult	-	-	-[c)]	-	-
	MSTUS	-	-	-[c)]	-	-[3]
	PQN	-	-	0.338	-	-

[a)] 2- and 3-methylbutanoic acid are coeluting compounds. [b)] Tukey HSD as post hoc test, all other volatiles and normalization methods used Steel-Dwass as post hoc test. [c)] correlations were tested as significant, but $\rho < \left| 0.3 \right|$.

Table 3. Further associations of volatile compounds with dietary intake. The number of participants having ingested a certain food variable (n_{ing}), the Spearman correlation coefficients for all normalization methods and the p-values of the additionally performed Wilcoxon test (lowest against highest tertile) using the in bold highlighted normalization method with the highest ρ are listed.

Food variable[1]	Volatile compound	n_{ing}	No normalization	Creatinine	Osmolality	Urine volume div	Urine volume mult	MSTUS	PQN	Wilcoxon
Bread sum	2-Pentylfuran	93	**0.452**	-	-	0.370	-	0.439	0.387	<0.0001
Cereal and cereal products	2-Pentylfuran	65	**0.420**	-	-	0.372	-	0.383	0.379	<0.0001
Polysaccharides	2-Pentylfuran	97	0.436	-	-	-	-	**0.459**	0.420	<0.0001
Baked goods	Octanoic acid	47	-	0.421	**0.422**	-	-	0.378	-	0.0005
Dairy products sum	Octanoic acid	85	0.394	0.363	-	-	-	0.408	**0.432**	0.0010
Milk sum	Octanoic acid	70	0.370	-	-	-	-	0.379	**0.397**	0.0002
Fat sum	2-Pentylfuran	80	-	-	0.357	-	-	**0.375**	0.373	0.0002
	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	80	0.356	-	-	-	-	**0.416**	0.404	0.0002
Sausage products	p-Cymene	50	0.437	0.353	-	-	-	**0.467**	0.447	<0.0001
	Calamenene	50	0.379	-	-	-	0.380	0.386	**0.391**	0.0039
Non-alcoholic beverages sum	Benzaldehyde	97	0.370	0.431	**0.455**	-	-	0.405	0.397	<0.0001
	4-Methylbenzaldehyde	97	-	0.391	**0.414**	-	-	0.362	-	<0.0001
	Butylbenzoate	97	-	0.446	**0.541**	-	-	0.446	0.371	<0.0001
	(14)	97	-	0.397	**0.437**	-	0.408	-	-	<0.0001
	(162)	97	-	0.368	**0.405**	-	0.384	-	-	0.0003
	Butyrolactone derivative	97	-	0.415	0.485	-	**0.489**	-	-	<0.0001
	(236)	97	-	0.383	0.415	-	**0.425**	-	-	0.0003
	1-Dodecanol	97	-	0.381	**0.467**	-	0.440	-	-	<0.0001
	Phthalic acid derivative	97	-	0.423	**0.528**	-	0.503	-	-	<0.0001
	Phthalic acid derivative	97	-	0.424	**0.501**	-	0.482	-	-	<0.0001

	Di-ter-butyl-phenol	97	-	0.418	0.456	-	**0.457**	-	-	<0.0001
	Dibutylmaleate	97	-	0.394	**0.505**	-	0.426	-	-	<0.0001
Alcoholic beverages	Ethanol	31	**0.569**	0.526	0.525	0.544	0.565	0.541	0.551	<0.0001
Carrot	(149)	29	0.379	0.459	**0.464**	-	0.412	0.393	0.407	0.0001
	(122)	29	-	**0.469**	0.458	-	0.375	0.369	0.378	0.0003
	(78)	29	0.354	**0.541**	0.527	-	0.389	0.417	0.436	<0.0001
	Methylsalicylate	29	-	0.420	**0.435**	-	-	0.355	0.367	0.0225
Fruit sum	4-Methylbenzaldehyde	78	0.369	0.390	**0.406**	-	0.380	-	0.359	0.0014
	(38)	78	-	0.4082	**0.4485**	-	0.4196	-	-	<.0001
	(40)	78	-	0.4179	**0.4565**	-	0.4087	-	-	<.0001
	(43)	78	-	0.3838	**0.4175**	-	0.3659	-	-	0.0002
	(68)	78	-	0.3889	**0.4048**	-	0.3634	-	-	0.0008
	Trimethyl-2-cyclohexenone	78	-	0.3703	**0.4052**	-	0.3539	-	-	0.0008
	(89)	78	-	**0.4147**	0.4073	-	0.3651	-	-	0.0001
	(98)	78	-	0.4378	**0.4773**	-	0.3981	-	-	<.0001
Apples raw	(24)	36	-	0.3953	**0.4161**	-	0.3799	-	-	<.0001
	(49)	36	-	0.3906	**0.4203**	-	0.3784	-	-	<.0001
	(58)	36	-	0.3636	**0.3964**	-	0.3874	-	-	<.0001
Nuts and seeds	(68)	25	-	**0.3557**	0.3537	-	0.3518	-	-	0.0008

9. Figures

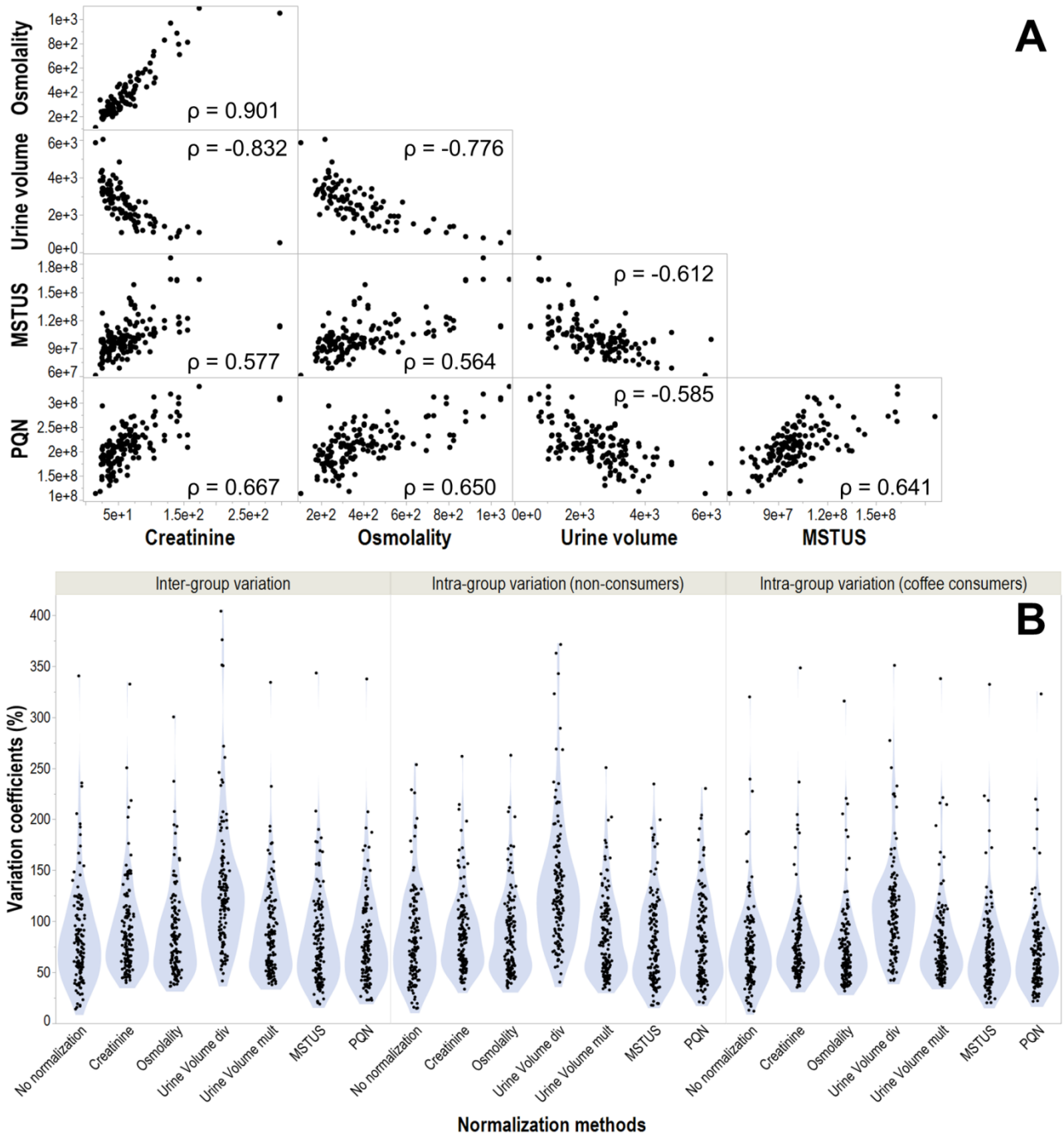

Figure 1. Normalization methods. Panel A: Scatter plots of the different normalization methods including Spearman correlation coefficient. Panel B: Violin plot for the CV's of all volatiles after the different normalization methods have been applied, grouped by inter- (CV over all study samples) and intra-group (CV within the two groups of coffee consumers and non-consumers) variation. The contour of the violins shows regions of data density, density is the highest where the violin is the broadest.

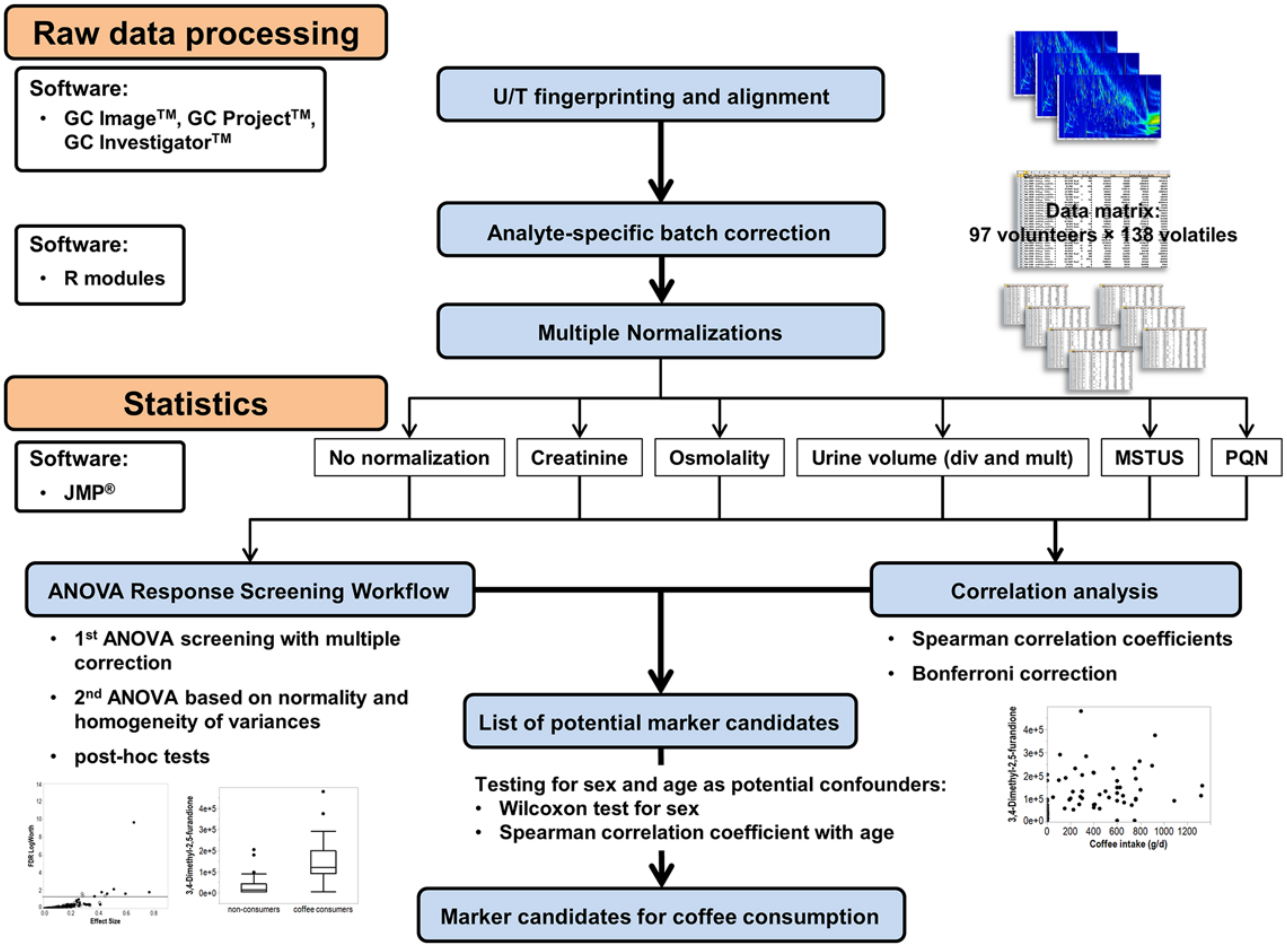

Figure 2. Workflow for the raw data processing and statistical analysis for potential coffee markers

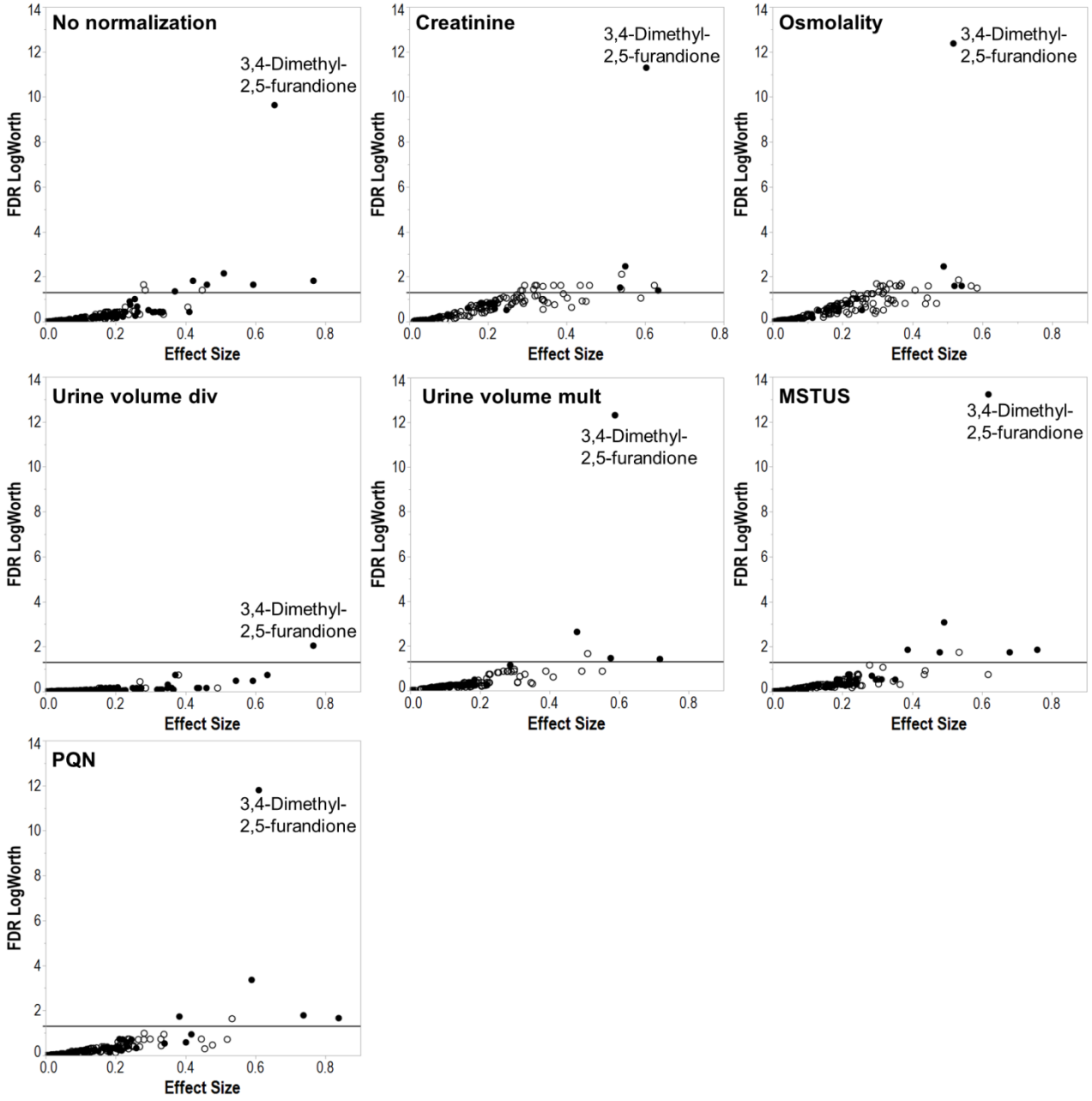

Figure 3. Results of the ANOVA based response screening for all normalization methods. FDRLogWorth = -log(p-value). Black line indicates p-value threshold of 0.05. ● higher in coffee consumers; ○ higher in non-consumers.