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Detecting beer intake by unique metabolite patterns

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Abbreviations

AA : Alpha acids

ABV : Alcohol By Volume

ASCA : ANOVA simultaneous component analysis

C : Control beverage (soft drink)

IAA : Iso-α-acids

IBU : International Bitterness Units

LB : Light beer

MSt1 : Meal Study 1

MSt2 : Meal Study 2

NMR : Nuclear magnetic resonance spectroscopy

NMT : N-methyl tyramine

pGlu-Pro : Pyro-glutamyl proline

PLSDA: Partial least squares discriminant analysis

RB : Regular beer

SCA : Simultaneous component analysis

SS : Sum of squares

UPLC-MS : Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry

Abstract

Evaluation of health related effects of beer intake is hampered by the lack of accurate tools for assessing intakes (biomarkers). Therefore, we identified plasma and urine metabolites associated with recent beer intake by untargeted metabolomics and established a characteristic metabolite pattern representing raw materials and beer production as a qualitative biomarker of beer intake. In a randomized, crossover, single-blinded meal study (MSt1) 18 participants were given one at a time four different test beverages: strong, regular and non-alcoholic beers and a soft drink. Four participants were assigned to have two additional beers (MSt2). In addition to plasma and urine samples, test beverages, wort and hops extract were analyzed by UPLC-QTOF. A unique metabolite pattern reflecting beer metabolome, including metabolites derived from beer raw material (i.e. N-methyl tyramine sulfate and the sum of iso-α-acids and tricyclohumols) and production process (i.e. pyro-glutamyl proline and 2-ethyl malate) were selected to establish a compliance biomarker model for detection of beer intake based on MSt1. The model predicted the MSt2 samples collected before and up to 12 h after beer intake correctly (AUC = 1). A biomarker model including four metabolites representing both beer raw materials and production steps provided a specific and accurate tool for measurement of beer consumption.

Keywords: Beer, Barley, Hops, Biomarker model, Hops, Metabolomics, Plasma, Urine, UPLC-QTOF

Introduction

Light to moderate alcohol consumption has been associated with decreased total mortality and with lower risk of metabolic diseases such as diabetes and cardiovascular disease[1, 2], but also with adverse effects on certain cancers[3]. The health related effects of consuming alcoholic beverages are mostly attributed to their alcohol content rather than specific components of different kinds of beverages, although there are some contradictory findings[4, 5]. One of the major issues in alcohol research is the assessment in observational studies of the consumption of specific alcoholic beverages usually obtained using self-reported questionnaires. These tools may provide only moderately reliable information, since reporting may be influenced by cultural and personal views on alcohol. Biomarkers of food intake are powerful tools for assessment of dietary exposure in clinical and observational studies. Some recent studies suggested resveratrol and isoxanthohumol as intake biomarkers for wine and beer consumption, respectively[6, 7]. However, resveratrol is also found in grapes and berries and isoxanthohumol has a highly variable rate of biotransformation into 8-prenylnaringenin (0-100% conversion) in the human gut making it a less than ideal biomarker[8, 9].

Beer is one of the most widely consumed beverages and is a very complex food with a metabolome originating from raw materials (e.g. barley and hops) and their transformation products formed during malting, boiling and fermentation. Consumption of beer may lead to direct absorption of its components but quite often their degradation or biotransformation products have also to be taken into consideration. Hops may be the most characteristic ingredient in beer and therefore the best candidate for single biomarker search strategies[10]. Iso-α-acids (IAAs) from hops, are specific to beer[11, 12] yet one major issue with IAAs as biomarkers is their high susceptibility to degradation, leading to multiple products; therefore their quantity varies after storage[13, 14]. In terms of metabolism of IAAs, only limited

number of studies is available[15, 16]. Therefore investigating the fate of not only IAAs but also their oxidation products in the human body would be a prerequisite for finding a suitable hops-derived biomarker.

Basic steps and raw materials in beer production are mostly universal, yet production processes by different producers vary, thereby further complicating the search for a global beer biomarker. Furthermore, human individuals differ with respect to many biotransformation pathways, making it even more challenging to find any single biomarker that is universal for beer intake. Finding markers representing each common raw material and step in beer production and mapping their biotransformation may therefore be a strategy to design more global aggregated biomarkers, representing the complete process. Beer as well as alcohol may affect metabolism by altering the glucose and insulin responses[17,18] and these parameters are therefore of potential importance as modifiers of biomarker responses.

Metabolomics resulting from the development of highly sensitive modern analytical instruments is an attractive tool for identification of novel intake biomarkers for specific diets, foods, food constituents and/or for further assessment of their health impacts[19-22]. This technology together with chemometrics also allows the identification of metabolite patterns as markers of intake[19]. Therefore, utilizing an LC-MS based metabolomics and chemometrics approach we aimed here to identify the plasma and urinary metabolites associated with recent beer intake and establish a novel and validated aggregated biomarker produced by combination of identified metabolites that represent each of the raw materials, processing steps, thereby ascertaining uniqueness to beer intake.

Materials and Methods

Subjects

37 healthy subjects within an age range of 18-60 years were recruited from the greater Copenhagen area by bulletins at educational institutions and on public websites for recruiting volunteers for clinical studies. Exclusion criteria were the following: systemic infections, psychiatric conditions and metabolic disease; alcohol intolerance; alcohol intake above the Danish Health and Medicines Authority's recommended limits of 7 and 14 units per week for females and males, respectively; donation of blood (>24 ml) less than 3 months before the study; breast feeding or pregnancy.

The subjects were given both verbal and written information about the study before providing their written consent. The study was carried out at the Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark. The research protocol was approved by the Ethical Committee of the municipalities of Copenhagen and Frederiksberg (H-1-2013-029) and the study has been registered at clinicaltrials.gov (NCT02449577).

Study Designs and Diets

Meal study 1(MSt1): 19 subjects were included in the main part of the study. A formal power calculation is not possible for explorative studies but several previous meal studies have indicated that 10-20 volunteers suffice. The study was performed as a randomized, crossover, single-blinded intervention with four study periods of three days and a wash-out period of >1 week between periods. The four periods consisted of a test day where subjects were served with one of the three different test beers or a control beverage in randomized order. The three test beers were a strong lager (SB) (Carlsberg Elephant, 8 % ABV, ~35 IBU), a regular lager (RB) (Carlsberg Pilsner, 4.6 % ABV, ~22

IBU), and a light/alcohol-free beer (LB) (Tuborg Super Light 0.1 % ABV, ~19 IBU). The control soft drink beverage (C) (Carlsberg Sports drink) was alcohol free but with the same energy content (carbohydrate and protein) as the average of the test beers.

The evening before the test day, subjects consumed a standardized dinner (spaghetti Bolognese) with water *ad libitum*. On the test day, after having a standardized breakfast (bread, butter, ham, cheese, orange juice) *ad libitum* between 7-8 am, subjects were instructed to fast for 3-4 hours (except for ~½ liter of water). Subjects were served a standardized lunch of Parisian toast/grilled cheese sandwiches (Tulip Food Company) at 11.35 am together with one of the test beers or the C (330 ml), all to be consumed within 25 min. The amount of toast was adjusted according to the type of drink served, so that the content of macronutrients was the same in each meal. Until 2.35 pm (last blood sampling) subjects did not consume any food or drink, but had water *ad libitum*. Thereafter, subjects were allowed to consume their habitual diet but were instructed to abstain from intake of alcohol in any form for 24 hours after the start of each test day (i.e. until 8 am in the following morning). They were otherwise instructed to retain their usual pattern of alcohol intake during the remaining 4 weekdays between each of the four test periods.

Meal study 2(MSt2): Four subjects were included in a second (validation) study; one of them also participated in the MSt1. The study was performed as a randomized, crossover intervention with all the same details as for MSt1, except that only two study periods of three days were conducted. The two periods consisted of a test day where subjects were served with two servings (660ml) of either of the two test beers in randomized order. Two test beers were used; one being a high hops moderate alcohol beer (HHB) (Lawn Mower, Carlsberg breweries, Sweden, 4.8 % ABV, ~31 IBU) and the other a low hops high alcohol beer (LHB) (T-beer, Tuborg breweries, Romania7-8 % ABV, ~8 IBU). These beers

were selected as extremes with regard to the two main raw materials in beer to serve as a test for the validity of the biomarker development concept.

On the test day volunteers in the MSt2 validation study had a standardized breakfast and lunch (multigrain bread containing also barley, cheese, ham, a red pepper fruit, radishes, apple, banana, orange juice and egg) according to calculated energy needs, and consumed the same standardized dinner together with 660 ml of either of the test beers. The following day, they consumed the same standardized meals but not the test beers.

In both studies volunteers were allowed otherwise to remain on their habitual diets, but were required to abstain from drinking all forms of alcohol and avoid heavy physical activity from 48 and 24 hours before the test days, respectively.

A simple randomization was used in both studies. The brewery delivered the four drinks in dark bottles, marked A, B, C, D for MSt1 and E and F for MSt2. A sequence table was generated for all volunteers so that they received the drinks in random order within each study. In principle participants, care providers and assessors were all blinded, however the participants could easily taste the nature of the soft drink. They were instructed not to tell the care providers.

Blood and Urine Sampling

Blood samples were drawn only in MSt1 on each test day 20 min before (baseline) and 0.45, 2 and 3 h after the subjects consumed the test beverages together with a standardized lunch. The blood samples were transferred into 4 mL vials containing heparin as an anticoagulant. The blood was centrifuged at 3,000 g, 4°C for 10 min. The plasma fraction was aliquoted into 2 mL cryotubes and stored at −80°C until further processing.

Urine samples were collected on each test day within the following time periods: from their morning void until 20 min before having their test drink (baseline); from baseline until 1.5 h; from 1.5 h to 3 h; and from 3 h to 21 h. In addition, 24 h pooled samples were created as a mixture of the urine samples collected in the four different time intervals in relative proportion to their volumes.

For the MSt2, only urine samples were collected: from lunch to dinner before having the test beer (baseline), baseline to 2 h, 2h to 4 h, 4h to 12 h, 12 h to 24h and 24 h to 40 h.

During sampling, urine was stored below 5 °C in cooling bags and after completion, sample aliquots were kept at -80 °C until further analysis.

Blood glucose and serum insulin

The blood samples were collected in dry tubes for the measurement of insulin and in fluoride citrate tubes for the measurement of glucose. Plasma glucose was measured with an enzymatic colorimetric method (ABX Pentra Glucose HK CP; Horiba, Montpellier, France) and analyzed with an ABX Pentra 400 (Horiba). Serum insulin was measured by a chemiluminescent immunometric assay with an Immulite 1000 Immunoassay System (Siemens Healthcare Diagnostics, West Sacramento, CA). All samples were analysed in one batch. Intra-assay and interassay coefficients of variation (CVs) for glucose and insulin were below 2.8%.

UPLC-QTOF Analysis

Plasma protein precipitation, urine centrifugation and dilution procedures were performed as described previously [23, 24]. The 288 plasma samples and 320 urine samples were placed in 96-well plates, such that all samples (urine or plasma) from the same individual were in the same plate (analytical batch)

while the individuals were randomized between batches (five per batch) and the order of samples was randomized within each batch. An ultra-performance liquid chromatography (UPLC) system coupled to a quadrupole time-of-flight mass spectrometer (Premier QTOF, Waters Corporation, Manchester, UK) was used for sample analysis. The mobile phase was 0.1% formic acid in water (solvent A) and 0.1% formic acid in 70% acetonitrile and 30% methanol (solvent B). Five µL of each sample were injected into a HSS T3 C18 column (2.1 x 100 mm, 1.8µm) coupled with a VanGuard HSS T3 C18 column (2.1 x 5mm, 1.8µm) and the gradient was operated for 7.0 min. The eluate was analyzed by electrospray ionization (ESI) in positive and negative mode, applying a capillary voltage of 3.2 kV and 2.8 kV, respectively and cone voltage of 20 kV. Ion source and desolvation gas (nitrogen) temperatures were set to 120 and 400°C, respectively. More detail on UPLC-QTOF analysis conditions can be found elsewhere[25]. Blanks (5% of acetonitrile:methanol 70:30 v/v in water) and external metabolomics standard mixtures were injected after every 30 samples, throughout each analytical batch[25].

In addition to plasma and urine samples, the test drinks, a beer wort extract, and hops extracts were analyzed with UPLC-QTOF using identical conditions. Sample preparation was comprised of dilution in solvent A for test drinks and wort whereas a mixture of solvent A and B (50-50 %) was used for dilution of the hops extract.

MS/MS fragmentation experiments were conducted for structural characterization of beer intake markers. The collision-induced dissociation (CID) was set to 10, 20 and 30 eV in separate runs and all other parameters were kept as for the MS full scan experiments as described above.

Data Preprocessing

Initially, raw data were converted to intermediate MZdata using an R function (convert.waters.Rd, https://github.com) operating on R software (ver. 3.1.0.). MZdata files were imported into MZmine 2.10 for data preprocessing including the following steps: mass detection, chromatogram builder, chromatogram deconvolution (local minimum search), isotopic peaks grouper, peak alignment (join aligner), duplicate peak filter, peak list row filter and gap filling (peak finder). The term 'feature' is used to refer to an ion with a specific retention time and mass over charge ratio (m/z) throughout this paper.

MZmine-preprocessed data were imported into MATLAB R2013a (ver. 8.1.0.604). Feature filtering was applied based on three criteria. First, the features detected earlier than 0.3 min and later than 6 min were removed. Second, if a feature has a significant peak area (>60, approx. 6 times the maximal background noise) in the first blank sample in at least one of the analytical batches, the feature was removed from the entire set as background noise. Third, if a feature had a peak area lower than 10 (considered as noise level or gap filling errors), in more than 60% of the samples within every sample group (specified with one test drink type and one time point), the feature was excluded (percent rule).

Urine samples were normalized to unit-length normalization to correct for urine concentration variations. Later, to remove inter-batch variation, each feature detected in urine/plasma samples within each batch was divided with the overall mean of its recordings included in the entire batch. This approach is justified by randomization of individuals between batches and of all samples from each individual within the same batch prior to analysis. Application of these pre-processing and normalization procedures separately on negative and positive mode data was followed by concatenation of the datasets from the two modes. In order to ease identification, features within the retention time

range of 0.02 were grouped and assigned as one feature group if they had correlation coefficients

higher than 0.7. Then, each feature group is considered as representation of one unique compound.

Data Analysis

ASCA

Data analysis was performed in MATLAB R2013a (ver. 8.1.0.604). For the plasma samples the

baseline level of each feature was subtracted from its subsequent recordings at each time point (0.45, 2

and 3 h) in order to reduce the influence of intra-individual factors. For urine, this procedure did not

affect the outcome and was therefore omitted (data not presented).

Unit scaled data were subjected to, ASCA (ANOVA Simultaneous Component Analysis)[26], to isolate

and further investigate the variation related to test drinks and to identify the contributions of time

course and test drink $\times$ time interactions.

In order to evaluate the significance of ASCA decomposed effect matrices, a permutation test was

employed [27]. The permutation test is based on comparison of sum of squares (SS) of each effect matrix

with SS distribution of matrices obtained with repeated random permutation of class labels. In this

case, the number of permutations was 100,000. The significance of each effect matrix was determined

as the fraction of data permuted matrices that had SS higher than the SS of the original effect matrix.

Later, simultaneous component analysis (SCA) was applied on individual effect matrices to interpret

the associated variation. SCA provides information only on the level averages, and to analyze the

variation between samples within each level, the residuals were projected on the principal component

subspace of effect matrices, as described previously[28].

An approach similar to ANOVA-PCA was carried out, where the residual matrix was added to test drink effect matrix [20, 28]. This provided the basis data matrix for the subsequent PLSDA.

ASCA was performed with in-house developed MATLAB functions.

PLSDA

PLS_Toolbox (version 6.5, Eigenvector Research, Inc., MA, US) was used for partial least squares discriminant analysis, PLSDA. PLSDA was applied to differentiate the plasma and urine metabolic patterns associated with beer intake (i.e. SB+PB+LB *vs.* C) within the consumed beers. In order to validate PLSDA models, ten different test sets each comprised of data from all samples (time points and treatments) from all possible combinations of two subjects were set aside. Variable selection was performed repeatedly by removing variables with selectivity ratio and variable importance in projection (VIP) values lower than 1 until no further increase in the cross-validation (eight-fold) classification errors could be observed. Final models with selected variables were evaluated using test set misclassification[29]. The variables that were selected in at least seven of the models were assigned as markers of beer intake.

Calibration and validation models

The calibration set was created from MSt1 including only the measurements of the selected metabolites for urine collected at 0-1.5 h, 1.5-3 h and 3-21 h. PLSDA was applied on the calibration set to discriminate the three beers (SB+RB+LB) from the soft drink, C. PLSDA models were built with 1 to 5 LV and optimum model complexity (i.e. number of components) was decided by evaluating misclassifications. This PLSDA model is used as the calibration model. MSt2 with the measurements

of selected metabolites for four subjects was used as the test set to validate the calibration model. The evaluation was based on how well the calibration model performs in classifying MSt2 samples collected after beer intake within the beer periods (i.e. SB+RB+LB in MSt1), as well as classifying MSt2 samples collected before beer intake within C (MSt1). Models were assessed by diagnostic statistics, area under receiver operating characteristic curve (AUC) and misclassification rate.

Standards and Identification

Four levels of chemical identification according to the published guidelines for chemical analysis were used [30]. Amino acids (i.e., tyrosine, leucine/isoleucine, proline) and the dipeptide pyroglutamyl proline (pGlu-Pro) were identified using an in-house metabolite database containing retention time information and MS spectra of reference substances. Reference standards for ICS-I3 (containing 62.3 % w/w of trans-IAA) and ICE-3 (containing 44.64 % w/w of AA) were obtained from Labor Veritas (Zurich, Switzerland). Isoxanthohumol and naringenin were purchased from Sigma (Schnelldorff, Germany). For unknown compounds with a signal area higher than 600, MS/MS spectra (10, 20 and 30 eV) were recorded in order to obtain fragmentation information to compare with data in available databases, such as Metlin (https://metlin.scripps.edu), hmdb (http://www.hmdb.ca), Chembank (http://chembank.broadinstitute.org/welcome.htm), Chemspider (http://www.chemspider.com), and similar sites. All six test drinks and beer wort and hop extract were kindly provided by Carlsberg A/S.

NMR analysis

The presence and identity of the dipeptide pGlu-Pro in beer was verified by 2D homo- and heteronuclear high-resolution NMR spectroscopy using beer fractionated by preparative UPLC. To this end, lyophilized UPLC fractions containing compound with a mass consistent with possible pGlu-Pro

were redissolved in 500 µL D_2O (Cambridge Isotope Laboratories, Andover, MA, USA). NMR

spectra including ^{1}H-^{13}C HSQC and ^{1}H-^{1}H TOCSY were recorded on a Bruker Avance II 800 MHz

spectrometer equipped with a TCI Z-gradient CryoProbe and an 18.7 T magnet (Oxford Magnet

Technology, Oxford, U.K.). All NMR spectra were recorded, processed and analysed with Bruker

Topspin 2.1 (Fällanden, Switzerland).

Selection of the aggregated biomarker for beer intake

Initially, the origin of each compound associated with beer intake was grouped based on their origin.

This is accomplished by comparing m/z and retention time of each beer marker with m/z and retention

time of the compounds detected in test beers, hops extract and wort reference samples. Accordingly,

beer intake markers were divided into four groups, originating from (1) hops, present only in hops

extract and test beers, (2) wort, present only in beer wort and test beers, (3) fermentation related

markers, present only in beer but not in hops or wort, (4) human metabolism of beer components,

present only in urine and plasma but not in beers, wort or hops. Since beer is a processed and complex

beverage, none of the markers originating from beer raw materials or their processing individually may

suffice as sufficiently specific and sensitive beer intake biomarkers. An aggregated biomarker

combining each raw material and process was therefore constructed based on the principle that each

included biomarker must be identified chemically and present as a signalling exposure marker clearly

increasing after intake of each kind of beer while having much smaller or undetectable levels in the

control period.

The calibration set for this aggregated biomarker was built from MSt1 including only the

measurements of the four selected metabolite groups in the 24 h pooled urines from each period and

from each of the 18 subjects. A PLSDA model with one component was established as the calibration

model. The performance of the calibration model was evaluated using metabolome profiles from MSt2

(validation set), which had not been used in model development and optimization. The validation set

included the measurements of the four selected metabolite groups in urine samples collected before (as

control) vs. 0-2 h, 2-3 h and 3-12 h after beer intake (representing beer intake) for all four individuals.

Results

Recruitment started April 2013 and the trials ended November 2013. A total of 18 completers in MSt1

were included in the analyses. One subject dropped out after the first out of four periods, because she

did not have enough time to participate anymore. All four completers in MSt2 were included. Data

acquisition ended in August 2014. The trials ended as scheduled. We report here only the primary and

some secondary aims according to the protocol. Baseline characteristics of the subjects are recorded in

Table 1. No adverse events for the trial were recorded.

Table 1. Participant characteristics of MSt1 and MSt2 at the time of enrollment

	MSt1 (n = 19)	MSt2 (n = 4)
Female [n (%)]	9 (47)	2(50)
Age Range	24-50	28-60
BMI	23.3±5.1	22.4±3.4
Blood Pressure (mm Hg)		
Systolic blood pressure	110.2±6.5	113.7±8.1
Diastolic blood pressure	62.0±8.0	75.7±11.4
Habitual Alcohol Consumption[1] [n]		
Moderate (1-2 drink/day)	11	2
Low (or abstained) (<1 drink/day)	7	2

[1]The levels of alcohol consumption is adapted from on NIH

Fasting whole-blood glucose and serum insulin levels were not different between treatments. There

were no significant differences between treatments when glucose and insulin response were expressed

in terms of total or incremental AUC.

Selection of metabolites associated with beer intake

The number of samples for plasma was 216 (i.e. 18 subjects × three time points × four test drinks) and 360 for urine samples (i.e. 18 subjects × (four time points + pool) × four test drinks). A total of 1503 and 8935 features remained after feature filtering for plasma and urine samples, respectively.

ASCA partitioned the total variation into contributions of corresponding experimental factors (i.e. test drink and time) and their interaction. A permutation test was applied in order to validate the ASCA factor models. Comparing the SS of 100,000 random class assignments with SS calculated from the original classes for each effect matrix, revealed that only the two main effects, time and test drink, were significant ($P<0.0001$), but not the interaction between time and test drink ($P>0.7$) (Figure S-1 and S-2). The percentage of explained variation of each factor was calculated as the SS of decomposed factor matrices (Table S-1).

SCA scores plot (Figure 1) displays the separation of the samples at the test drink level both for urine and plasma. For urine, the separation of beer samples was mainly explained by PC1, with lower PC1 score values for beer samples compared to C. On the other hand, for plasma the dominating factor was alcohol, explained by PC1, and the combination of PC1 and PC2 provides the separation of beer intake.

SCA scores plots for individual factors and their interaction are also given in Supplemental Material (Figures S-3 and 4).
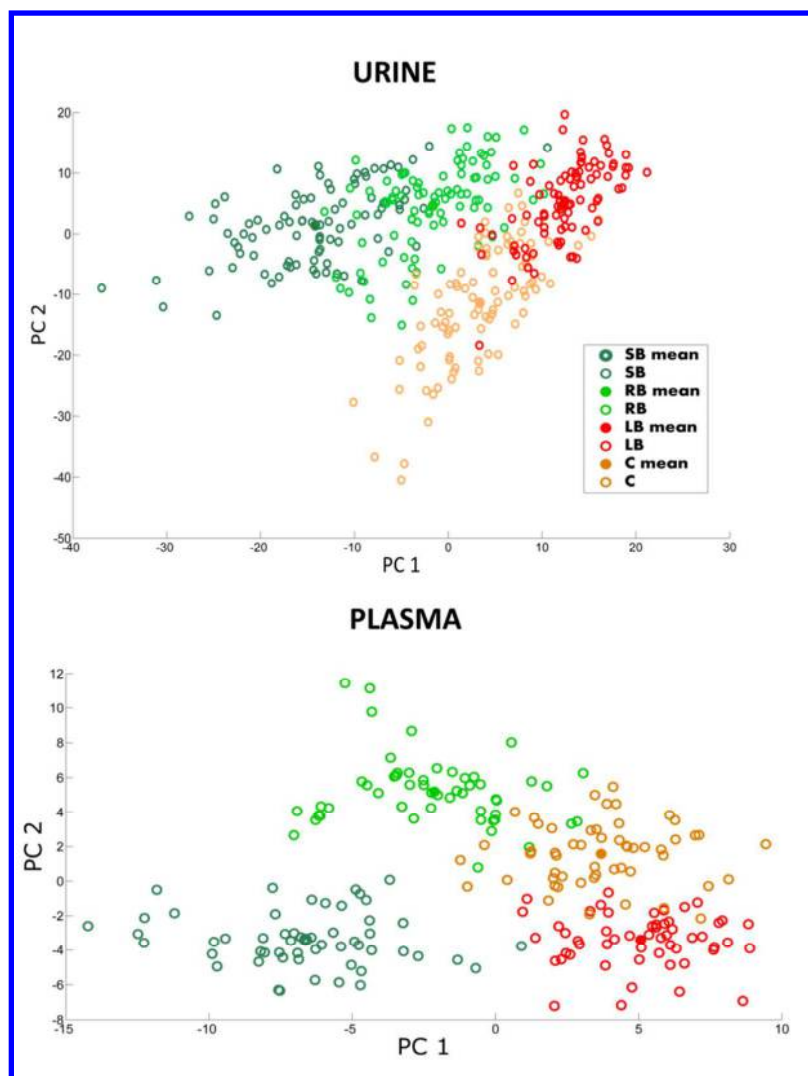
Figure 1. SCA score plot of the test drink matrix for both the urine (top) and plasma (bottom) metabolic profiles. An SCA model was applied to the effect matrix obtained from the ANOVA decomposition.

PLSDA was applied on the matrix where residuals were added to the decomposed test drink factor matrix. Three beers are assigned as one group providing a binary classification problem (i.e. SB+PB+LB *vs.* C). The mean of misclassification errors of selected test sets for plasma and urine were significantly higher than classifications errors of 1000 random class assignments (P-values were 0.04

for plasma and 0.004 for urine). The potential beer intake markers were selected by evaluating VIP values and selectivity ratio.

Assessment of the origin of putative beer intake biomarkers

Sixty nine compounds associated with beer intake were grouped based on their presence in test beers, wort and hops extracts as described in the Materials and Methods section. This comparison revealed 15 compounds originating directly from the hops extract (two in both plasma and urine, 13 only in urine), 19 directly from wort (one in both plasma and urine, three only in plasma, 14 only in urine), 2 from fermentation (only observed in urine) and 20 from human biotransformation (one only in plasma and 19 only in urine). The identified compounds are given in Table 2 and the list of unknown compounds and their source is given in Table S-2.

Table 2. Identified plasma and urine metabolites originating from beer raw materials hops and wort, fermentation process and human and/or microbial biotransformation of beer components.

No	m/z	RT	MS/MS	Annotation	Suggested Compound*	Biofluid	Specificity to beer intake/ Source**
1	363.18	4.08 4.17 4.2 4.24	too low signal	[M-H]⁻	Hydroxy alloiso-cohumulone and/or cohumulinone[3]	Urine	S/hops
2	365.19	4.15 4.22 4.27	347.1,269.1 251.1,237.1 209.1,193.1 181,141	[M-H]⁻	Tetra-cyclocohumol and/or tri-cyclocohumol[3]	Urine	S/hops
3	377.182 379.192	4.27 4.34 4.2	too low signal	[M-H]⁻ [M+H]⁺	Hydroxy alloiso-ad/humulones and/or ad/humulinone[3]	Urine	S/hops
4	379.21	4.29	361.2,310.1 283.1,265.1 223.1,207.1	[M-H]⁻	Tetra-cycload/humol[2]	Urine	S/hops

			195.1,155.1				
5	379.21	4.38 4.51	too low signal	[M-H]-	Tri-cycload/humol and/or double hydroxylated iso-cohumulone[3]	Urine	S/hops
6	347.19	4.53	329.2,285.2 259.1,253.2 235.2,217.1 183.1,165.1	[M-H]-	Iso-tricyclocohumene[2]	Urine	S/hops
7	347.19	4.61	329.2,279.1 251.1,233.1 209.1,181.1, 182.1	[M-H]-	Iso-cohumulone[1]	Urine Plasma	S/hops
8	361.20	4.59	343.2,299.2 259.1,223.1 197.1,179.1	[M-H]-	Iso-tricycload/humene[2]	Urine	S/hops
9	361.20	4.66	343.2,265.2 247.1,223.1 196.1,195.1	[M-H]-	Iso-n/ad-humulone[1]	Urine	S/hops
10	393.19	3.78 3.83	375.2,281.1 263.1,223.1 196.1,195.1	[M-H]-	Dihydroxylated iso-ad/humulone[2]	Urine	S/hops
11	393.182	3.82	too low signal	[M-H]-	Dihydroxylated iso-ad/humulone II[3]	Urine	S/hops
12	377.162	4.15	too low signal	[M-H]-	NO or CH_2O adduct of iso-cohumulone[3] M7 + 30.0	Urine	S/metabolised hops
13	391.176	4.19 4.28 4.42	347.2,329.2 303.2,292.1 265.1,247.1 196.1,195.1 163.1,125	[M-H]-	NO or CH_2O adduct of iso-ad/humulone[3] M8 + 30.0	Urine	S/metabolised hops
14	393.149	3.62 3.58	331.1,316.1 314.2,267.1 249.1,219.1 195.1,182.1 149.1,111.1	[M-H]-	NO_2 or CH_2O_2 adduct of iso-cohumulone[3] M7 + 46.0	Urine	S/metabolised hops
15	407.174	3.78 3.66 3.72	389.2,345.2 315.2,295.1 277.1,245.1 233.1,223.1 195.1,175 149.1,99.1	[M-H]-	NO_2 or CH_2O_2 conjugate adduct of iso-ad/humulone[3] M8 + 46.0	Urine	S/metabolised hops
16	514.176	3.41	too low signal	[M-H]-	Cysteine conjugate of NO_2 or CH_2O_2 adducted iso-cohumulone[3] M7 + 45.98 +	Urine	S/metabolised hops

					121.017		
17	528.191	3.47	407.1,363.2 351.1,345.2 281.1,245.1	[M-H]$^-$	Cysteine conjugate of NO_2 or CH_2O_2 adducted iso-ad/humulone[3] M8 + 45.98 + 121.017	Urine	S/metabolised hops
18	335.044	2.26		[M -H]$^-$	Unknown I Glucuronide I[4]	Urine	NS/metabolised beer component
			301.1	[M -H$_2$S]$^-$			
			289	[M -COOH$_2$]$^-$			
			243				
			193	[Glucuronide+H$_2$O]$^-$			
			175	[Glucuronide-H]$^-$			
			159	[M-Glucuronide-H]$^-$ ($C_6H_7O_3S$)			
			131				
			125	[M-Glucuronide-H$_2$S]$^-$			
			113,103,73				
19	335.044	2.47		[M-H]$^-$	Unknown I Glucuronide II[4]	Urine	NS/metabolised beer component
			317	[M-H$_2$0]$^-$			
			301	[M-H$_2$S]$^-$			
			247				
			193	[Glucuronide+H$_2$O]$^-$			
			175	[Glucuronide-H]$^-$			
			159	[M-Glucuronide-H]$^-$ ($C_6H_7O_3S$)			
			131				
			125	[M-Glucuronide-H$_2$S]$^-$			
			113,87,73				
20	238.969	1.95		[M-H]$^-$	Unknown I Sulfate[4]	Urine	NS/metabolised beer component
			175	[Sulfate-H]$^-$			
			159	[M-Sulfate-H]$^-$ ($C_6H_7O_3S$)			
			141				
			131	[M-Sulfate-H$_2$O]$^-$			
			125				
			115.3,103	[M-Sulfate-H$_2$S]$^-$			
			97,87.1, 80.9				
21	435.139	2.11		[M -H]$^-$	Unknown II Glucuronide[4]	Urine	NS/metabolised beer component
			259.1	[M-Glucuronide-H]$^-$			
			175.02	[Glucuronide-H]$^-$			
			153.1,129				
			117,113				
			103,99				
			95,85				
22	303.071	1.97		[M -H]$^-$	Unknown III Glucuronide[4]	Urine	NS/metabolised beer component
			261.1	[M-C$_2$OH$_2$]$^-$			
			175	[Glucuronide-H]$^-$			

			127	[M-Glucuronide-H]-			
			117,113,99				
			85,71,59				
23	290.072	2.62	too low signal	[M+H]+	Mercapturic acid conjugate of Unknown IV[4]	Urine	NS/metabolised beer component
	312.053			[M+Na]+			
	164.042			[M+H-126.03]+			
	162.022			[M-H-126.03]-			
	127.042			[M+H-163.03]+			
	122.029			[Cysteine + H]+			
24	365.105	0.88		[M+Na]+	Maltose[2]	Urine	NS/ Malting or Mashing
	127.037			[M-$C_6H_{15}O_8$]+			
	325.116						
	332.118			[M-OH]+			
	97.0292						
	85.0301						
25	225.089	1.93		[M-H]-	Pyroglutamyl proline[1]	Urine Plasma	NS/Malting
	227.104			[M+H]+			
			ESI+:209.1	[M-H_2O]+			
			181.1,	[M-COOH]+			
			116.1	[Proline+H]+			
			70.1				
			ESI- :				
			181.1,173				
26	166.12	1.13		[M+H]+	Hordenine[1]	Urine	NS/Barley
27	230.053	1.01		[M-H]-	N-methyl tyramine sulfate[2]		
	232.066			[M+H]+			
			ESI-:150.1,	[M-Sulfate]-			
			106.1,79.9				
28	182.082	0.78		[M+H]+	Tyrosine[1]	Plasma	NS/Barley
			ESI+: 119.1,				
			136.1,123,				
			165.1				
29	132.102	0.91		[M+H]+	Iso/Leucine[1]	Plasma	NS/Barley
			ESI+: 86.1				
30	161.044	1.79		[M-H]-	2-ethyl malate[2]	Urine	NS/ Fermentation
			ESI-:				
			143,101				
			71.1				

*identification level, ** source of unconjugated compound, S: Specific to beer intake; NS: Not specific to beer intake, UI: Unidentified

Identification of metabolites associated with beer intake.

Metabolites originating from hops: The identified metabolites originating from hops were IAAs and their oxidation and degradation products (Table 2, Metabolites 1 to 11). Detailed description of identification based on MS/MS is provided in the Supplementary material (Text S-2, Figure S-5 and S-6). IAAs, iso-cohumulone and the mixture of iso-humulone and iso-adhumulone were identified as markers of beer intake both in urine and plasma with very similar excretion profiles. Figure 2 illustrates the excretion profiles of iso-cohumulone after consumption of 330 mL of test drinks. In accordance to the measured IAA concentrations in test beers (Figure 2C), IAAs in urine and plasma were the highest following consumption of SB. In plasma they dropped rapidly within 3 h, on the other hand, IAAs peaked later in urine (pooled urine within 1.5-3 h) and were still present in urine collected from 3 h up to 21 h.

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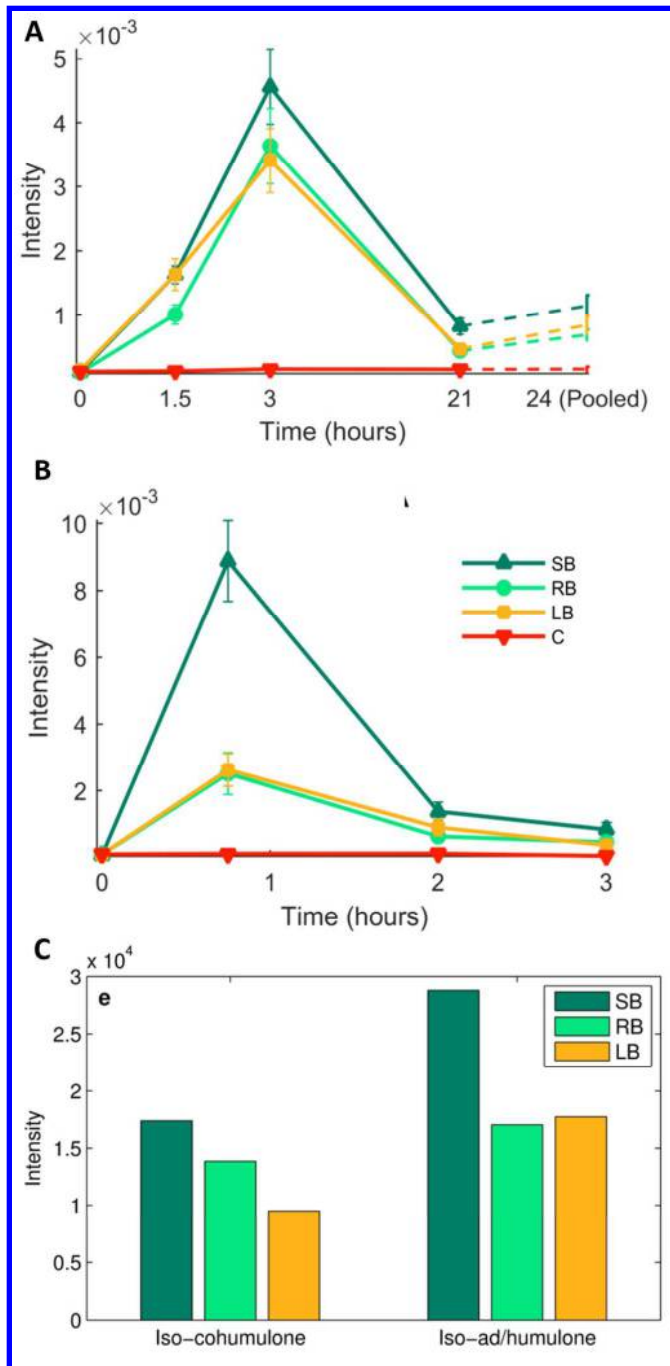41
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**Figure 2.** Iso-cohumulone excretion profiles in urine (A) plasma (B) and iso-cohumulone levels in test beers (C).

Metabolites originating from human metabolism: Major metabolites were related to IAA biotransformation products and the same conjugates were recorded both for iso-cohumulone and iso-ad/humulones (Table 2, Metabolites 12 to 23). Three related groups of conjugates were observed with addition of 29.98 Da (equal to addition of NO or CH_2O; metabolites 12 and 13), 45.98 Da (equal to NO_2 or CH_2O_2; metabolites14 and 15) and 166.99 Da (metabolites 16 and 17) to iso-cohumulone and iso-ad/humulone. We speculate that these metabolites are oxidation and methylation products as well as cysteine adducts of isocohumulone and iso/adhumulone. Urinary excretion profiles of the IAA conjugates were similar to those of IAAs (Figure 2). Metabolites 15 and M16 were still present at high levels in urine collected between 3 and 21 h, indicating that the peak of excretion of those metabolites was later than 3 h after intake (Figure S-8).

Metabolites 18 to 22 (Table 2) were products of phase II metabolism (glucuronides and sulfates) based on their fragmentation patterns, yet the identity of the biotransformed beer components remains unknown. Metabolites 20 to 22 were phase II (glucuronide and sulfate) metabolites of the same compound and their fragmentation pattern revealed the presence of a thiol group. Furthermore, one cysteine mercapturic acid conjugate (Metabolite 23) is detected as a urinary marker. In terms of excretion profiles, all of them peaked in urine collected within 1.5 to 3 h. The products were not specific to the beer intake as they were also present after intake of C, albeit at much lower levels (Figure S-8).

Metabolites originating from wort and fermentation: The identified metabolites are given in Table 2 (Metabolites 24 to 30). The only metabolite originating from wort that is detected in both urine and plasma was pGlu-Pro. NMR based structure elucidation of pGlu-Pro is described in Figure S-9. The urinary excretion profile is given in Figure 3A with the highest excretion level around 3 h following

beer intake. The pGlu-pro was also detected in urine samples prior to the test meals and did not fluctuate following the consumption of C drink, indicating that it probably is a constituent of foods other than beer/wort or even that it is formed endogenously, but levels after beer intake are significantly higher. Hordenine, maltose (or an isomer) and NMT sulfate are identified as markers of beer intake in urine. Hordenine had already reached its maximum excretion level 0-1.5 h after intake, whereas maltose and NMT sulfate peaked in urine collected between 1.5-3 h and 3-21 h, respectively (**Figure 3B**, Figure S-10). Hordenine and NMT were present in the urine of the a few subjects prior to beer intake albeit with lower levels.
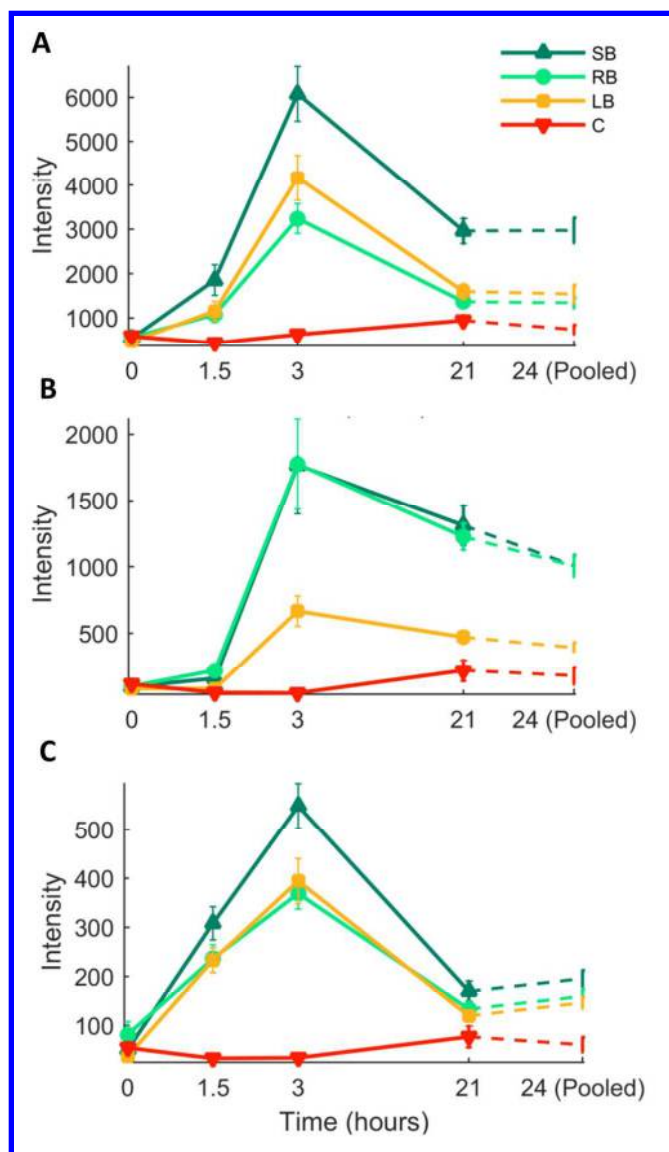
Figure 3. Urinary excretion profiles of pGlu-pro (A), NMT sulfate (B) and 2-ethyl malate (C).

In plasma, isoleucine, leucine and tyrosine are identified as metabolites originating from wort and associated with beer intake. The excretion profile of tyrosine is shown in Figure S-11, in agreement with tyrosine concentration in beer. Both tyrosine and isoleucine/leucine were observed as rapidly absorbed metabolites.

In relation to fermentation, 2-ethyl malate is identified as a metabolite associated to beer intake in urine. It is excreted rather late compared to metabolites from hops and wort, with a reasonable high excretion between 3-21 h (Figure 3C).

Selection of metabolites for the aggregated beer biomarker

None of the beer intake markers discovered in this study was sufficiently specific or sensitive as single biomarkers to assess beer intake. The major issue with metabolites originating from hops (e.g. IAAs) is not specificity (see baseline levels, Figure 2) but sensitivity because they are not stable and several degradation products were also identified in urine at varying amounts. The issue with metabolites originating from wort or fermentation was that they were observed as markers of effect rather than exposure in the sense that they were present already at baseline but increased following beer intake. Hence none of the markers identified are specific to beer intake *per se* (see baseline levels, Figure 3). For the aggregated beer intake biomarker we selected one metabolite from each raw material (hops and barley) and production process (malting and fermentation) and established a compliance biomarker model for detection of beer intake based on MSt1. To overcome the issue with variable degradation of IAAs as representative of hops derived metabolites we used the sum of iso-cohumulone and iso-ad/humulones and their major degradation products, tricyclocohumol and tricyclohumol. NMT sulfate was selected representing barley and pGlu-pro to represent heat treatment during malting. Fermentation was represented by 2-ethyl malate since this compound is not removed in de-alcoholization processes to produce light and non-alcoholic beers.

Figure 4 demonstrates how the calibration model performs in terms of distinguishing samples after beer intake from the control time points and/or periods for calibration and validation sets. The scores plot of

the first component (Figure 4A) demonstrates that the calibration model provides correct prediction of

the classes as control and beer groups for the samples from MSt2 (open circles in Figure 4A) for urine

collected up to 12 h. Testing the same calibration model on urine samples collected from 12 to 24 h

(MSt2), resulted in 0 sensitivity such that all the samples collected more than 12 hours after beer intake

were wrongly classified as control samples (Figure S-12). This suggests that metabolites associated

with beer intake were already excreted at 12 h. Therefore, the calibration model is valid for detection of

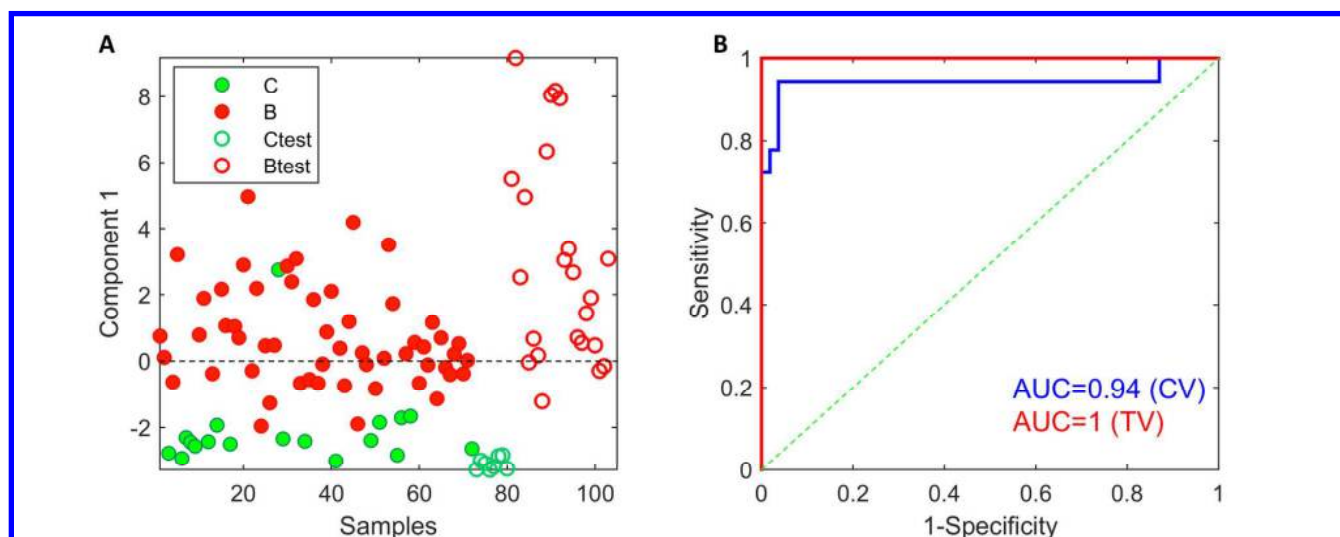beer intake up to 12 h post consumption as well as in 24 h pooled urine samples.

Figure 4 (A) PLSDA scores plot of the first component. The calibration model is developed using the

measurements of four metabolites for samples from the meal study 1 (filled circles) and the model is

tested with samples from meal study 2 (open circles). C and B refer to control samples and 24 h pooled

samples from the meal the meal study 1, respectively. Ctest and Btest refer to the samples before and

collected up to 12 h following beer intake from meal study 2 (B) ROC curve describing the

performance of the PLSDA model in terms of predicting beer intake for calibration (CV) and test set

(TV) samples, respectively.

In order to assess the importance of each metabolite, we removed one of the four metabolites at a time and the calibration model still performed efficiently in terms of separating beer drinkers from control, thus none of the metabolites is individually crucial for the prediction.

Discussion

Accurate assessment of food intake is central in nutrition research and identification of specific food intake biomarkers is therefore a promising way ahead. Finding single biomarkers for complex foods like beer is difficult because each component in the food may be used also in other foods. The strategy described in the current study is therefore to derive aggregated biomarkers for complex foods adding the best markers for each component and processing step to improve the resulting biomarker specificity and sensitivity. Our cross-over meal study provided many markers associated with recent beer intake, particularly in urine, reflecting the metabolomes of beer, as well as metabolites originating from their human metabolism (Table 2). Beer metabolome is characterized by compounds originating from its raw materials, barley and hops, and their transformation products derived upon malting and fermentation. Each of the raw materials and processes flag specific compounds in urine and plasma. However, these are either present in other foods or unstable. Therefore individually they lack the specificity and sensitivity needed for intake biomarkers.

IAAs from hops have been previously suggested as biomarkers of beer intake as they are specific to beer[11, 12, 31]. Although IAAs provided high sensitivity and specificity for detection of beer intake up to 21 h for MSt1, they were not even detected after intake of low hops beer (MSt2). This is attributed not only to the low hops content of the test beer but also to formation of several oxidative degradation products of IAAs[32, 33] underlining their instability. Tentatively identified degradation products of *trans*-IAA in urine were tri- and tetra-cyclohumenes and tri-cyclohumols[14, 34] whereas, mono- and di-

hydroxylated humulones and humulinones appeared as oxygen-dependent degradation products of both *cis*- and *trans*-IAAs[33]. Indeed, only tricyclohumols were detected in low hops beer (MSt2), which has also been shown previously to be one of the major degradation products[34]. Therefore, using IAAs as single biomarkers of beer intake is not feasible since consumption of longer-term stored beers and beer varieties with low hops content may result in total degradation and therefore to low sensitivity for assessment of beer intake. Instead, the sum of IAAs and their major degradation products (e.i. tri-cyclohumols) as a single biomarker provides a better predictor of beer intake as potential loss of IAAs is partially compensated by formation of tri-cyclohumols. Although validating this biomarker in MSt2 provided high sensitivity and specificity (AUC =1), further studies including diverse types of beer and different storage periods are required for final validation. Our results also suggest that after ingestion IAAs are oxidized into metabolites, which could be tentatively identified based on fragmentation patterns (Table 2). The oxidized IAA metabolites are compounds similar to products of degradation during storage[35]. It is difficult to discriminate between phase I metabolism and storage-induced oxidation of IAA as the products have very similar structural characteristics but IAA oxidation is much accelerated after ingestion indicating enzyme-mediated formation. Oral administration of IAAs to rabbits lead to recovery of less than 6% of the dose as such metabolites, suggesting that IAAs may also be subject to other kinds of metabolism[16]. Indeed, we observed unidentified conjugation products of IAAs formed from oxygenated IAAs by methylation and cysteine conjugation. Further isolation and structure elucidation is required to verify these structures. Some beer brands use hydrogenated hops extracts, which renders the IAAs more stable, however it is uncertain whether these are used exclusively since their organoleptic properties do not exactly match those of the normal hops extract[36]. Adding markers for these compounds might be needed to cover some more unusual brands of beer.

The wort-derived beer intake markers were present in barley or formed during the malting/boiling process; therefore they may also be found in other barley containing foods, limiting their specificity. pGlu-pro was increased after beer intake, potentially due to a high content of proline in barley. pGlu-pro may be formed upon heating of the barley sprouts during malting or wort boiling. Pyroglutamyl peptides have been suggested to act on inflammatory diseases[16, 37, 38] and have been shown to exhibit an antidepressant-like effect in mice[39]. To our knowledge, pGlu-Pro has not previously been observed in beer samples or associated with an increase in blood or urine after beer intake. The sulfate conjugate of NMT is another wort-derived compound, potentially formed through phase II metabolism in the plant or during the malting process. NMT is produced by N-methylation of tyramine, which is further methylated to hordenine[40]. Hordenine is suggested as a qualitative marker of beer intake[41], yet its excretion profile after intake of other barley containing foods should be investigated since it may not be possible to discriminate beer intake from intake of other barley containing diets.

Malic acid, particularly in the form of di-ethyl malate is widely described as a constituent of alcoholic beverages[42, 43], yet according to our knowledge, 2-ethyl malate has not previously been described in beer. We could also confirm its presence in wine and cider samples (data not shown), indicating that it is not specific to beer but to fermented beverages. 2-ethyl malate is probably formed through esterification of malic acid with ethanol, followed by partial rearrangement[44] indicating that non-alcoholic food fermentation is probably not a source of its formation.

NMT sulfate, pGlu-pro and 2-ethyl malate were each detected in the urine of some subjects prior to intake of the test drink, suggesting intake from other food sources. Therefore, none of the single metabolites were sufficiently sensitive and specific for assessment of beer intake. Yet an aggregated biomarker characterizing the beer metabolome (i.e. its raw materials and the malting and brewing processes) provided a unique pattern. The four metabolites - NMT sulfate originating from barley, 2-

ethyl malate originating from fermentation, pGlu-Pro originating from malting, and the sum of IAAs and their major degradation products (tricyclo-co- and -ad-humols) originating from hops – were used to establish an aggregated biomarker. It is crucial to validate new biomarkers with independent controlled trials as well as samples from observational studies[45]. Therefore, we utilized MSt2 for validation of the calibration model. The calibration model efficiently predicted beer intake up to 12 h (AUC = 1, Figure 4B); this set of four metabolites therefore provides an excellent aggregated biomarker for detection of beer intake. When we removed any one of the four metabolites from the aggregated biomarker, the calibration models performed equally well, suggesting that none of the individual metabolites are crucial for the model and that the aggregated approach is very robust.

Other compounds originating from hops, such as reduced derivatives of IAAs and prenylated phenols have been suggested previously as urinary and blood biomarkers of beer intake [13, 31, 46]. Isoxanthohumol was shown to be stable even after 10 years of storage, making it a suitable biomarker candidate. In our study, isoxanthohumol was only identified in hops extracts and beer samples (level 1), but not in urine and plasma (data not shown). This may be related to the low concentration of isoxanthohumol in urine and plasma; prenylated hops phenol glucuronide conjugates accounted for only 2% of the dose [47, 48]. The gut microbial biotransformation of xanthohumol to prenylnaringenin in humans has been shown to vary from 0-100% between individuals[49]. This may also explain the relatively low sensitivity observed previously for this marker [46].

Conclusions

The concept of aggregated biomarkers proposed here may find widespread use to solve the problems of overlapping markers for intakes of many foods and especially for processed foods and whole diets. A recent study demonstrated that aggregated biomarkers provide higher sensitivity and specificity for intake of sugar sweetened beverages compared to single biomarkers[50]. Further development of this

concept will be to move it from the stage of providing qualitative markers to actually estimating the amount ingested and the time elapsed since ingestion. This should eventually be possible based on the different kinetics of the individual metabolites included in the aggregated biomarkers.

In conclusion, we were able to segregate metabolites that are increased following beer intake into compounds originating from hops, wort, fermentation, and human metabolism. The majority of beer-specific compounds originate from hops, but many of these are chemically unstable limiting their usefulness as single biomarkers. Many of the suggested biotransformation products of IAAs are novel, yet still need to be fully structurally characterized. We propose that wort and fermentation products may provide signature markers of beer intake in combination with one or more hops-specific marker. Therefore, we have shown here that an aggregated biomarker model based on a set of three out of four of these signature biomarkers was able to completely discriminate between samples collected after beer intake in an independent study with high sensitivity and specificity. It still remains to be shown that the aggregated biomarker of beer intake proposed herein is sensitive and specific in an observational setting and whether adjustments may be needed, e.g. adding markers for optional beer components such as reduced IAAs, caramel, wheat, corn, etc.

Author Contributions

GG, MGJ, EL and LOD designed the research; LOD conducted the study; MGJ, SM, LB and EL provided essential materials and advice; GG and MGJ analyzed the data; GG, SM, LOD interpreted the results GG and LOD wrote the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information

Figure S-1. SSQ distribution calculated by permuting the class assignments and the SSQ calculated for original class assignments for time and test drink effects.

Figure S-2. SSQ distribution calculated by permuting the class assignments and the SSQ calculated for original class assignments for time–test drink interaction.

Text S-1. Identification of significant factors in the model

Figure S-3. ASCA decomposition of urine LC-MS profiles. SCA scores plots for (a) test drink, (b) time and (c) interaction of test drink and time for PC1.

Figure S-4. ASCA decomposition of plasma LC-MS profiles. SCA scores plots for (a) test drink, (b) time and (c) interaction of test drink and time for PC1.

Table S-1. Unknown metabolites associated with consumption of test beers.

Table S-2. Unknown metabolites associated with consumption of test beers.

Text S-2. Identification of hops-derived metabolites

Figure S-5. Extracted ion chromatogram for iso-cohumulone in ICS-I3, hops extract stored for 4 months, fresh hops extract, test beer (SB) and urine sample 3 hours after consumption of the test beer (SB)

Figure S-6. LC-MS/MS spectrum at 20V (ESI-) of iso-cohumulone and iso-/tricyclocohumene.

Figure S-7. Urinary excretion profiles of metabolites originating from hops.

Figure S-8. Urinary excretion profiles of unknown metabolites originating from human biotransformation observed after beer intake.

Figure S-9. NMR identification of pyroglutamyl-proline with ^{1}H and ^{13}C chemical shifts for the aliphatic CH groups in UPLC fraction containing several molecular species. .

Figure S-10. Urinary excretion profiles of maltose originating from wort

Figure S-11. Tyrosine excretion profiles in plasma (a) and their corresponding levels in the test beers (b).

Figure S-12. PLSDA model developed including pooled 24 h samples from MSt1 and the performance of the model in terms of detection of beer intake between 12 and 24 h for MSt2.

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Figure Legends
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Figure 1. SCA score plot of the test drink matrix for both the urine (top) and plasma (bottom)
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Table Legends

Table 1. Participant characteristics of MSt1 and MSt2 at the time of enrollment

Table 2. Identified plasma and urine metabolites originating from beer raw materials hops and wort,

fermentation process and human and/or microbial biotransformation of beer components.

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