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Published in:
Journal of Nutrition

DOI:
10.1093/jn/nxz138

Publication date:
2019

Citation for published version (APA):
Wilson, T., Garcia-Perez, I., Posma, J. M., Lloyd, A., Chambers, E. S., Tailliart, K., Zubair, H., Beckmann, M., Mathers, J. C., Holmes, E., Frost, G., & Draper, J. (2019). Spot and Cumulative Urine Samples Are Suitable Replacements for 24-Hour Urine Collections for Objective Measure of Dietary Exposure in Adults Using Metabolite Biomarkers. *Journal of Nutrition*, *149*(10), 1692-1700. [nxz138]. https://doi.org/10.1093/jn/nxz138

Spot and cumulative urine samples are suitable replacements for 24-hour urine collections for objective measures of dietary exposure in adults using metabolite biomarkers

Thomas Wilson *[a], Isabel Garcia-Perez *[b], Joram M Posma [b], Amanda J Lloyd [a], Edward S Chambers [c], Kathleen Tailliart [a], Hassan Zubair [a], Manfred Beckmann [a], John C Mathers [d], Elaine Holmes [b], Gary Frost [c], John Draper [a]

Author Affiliations

[a] Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, SY23 3DA, UK

[b] Section of Biomolecular Medicine, Division of Integrative Systems Medicine and Digestive Diseases, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London, UK

[c] Nutrition and Dietetic Research Group, Division of Endocrinology and Metabolism, Department of Medicine, Imperial College London, London, UK.

[d] Human Nutrition Research Centre, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, NE24HH, UK

* These authors contributed equally to this research

Corresponding author: John Draper, Institute of Biological Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, SY23 3DA, United Kingdom. UK.

Phone number: +44 (0) 1970 622789. Fax number: +44 (0) 1970 621981. E-mail: jhd@aber.ac.uk

Conflict of Interest: None

Sources of Support: This study was funded by the Medical Research Council (MRC), grant number MR/J010308/1

Running head: Comparison of spot urine samples versus 24-hour urine collections.

Data Share: Data described in the manuscript, code book, and analytical code will be made available upon request pending publication

Abbreviations used:

^{1}H NMR, Proton Nuclear Magnetic Resonance; AGC, Automatic Gain Control; AUC, Area Under the Curve; BMI, Body Mass Index; CRF, Clinical Research Facility; CS1, Cumulative Sample 1; CS2, Cumulative Sample 2; CS3, Cumulative Sample 3; ESI, Electrospray Ionisation; FA, Fasting; FFQ, Food Frequency Questionnaire; FIE-HRMS, Flow Infusion Electrospray High Resolution Mass Spectrometry; FMV, First Morning Void; H_2O, Water; HPLC, High Performance Liquid Chromatography; WHO, World Health Organisation; LC-QqQ-MS, Liquid Chromatography Triple Quadrupole Mass Spectrometry; MeOH, Methanol; MRM, Multiple Reaction Monitoring; MCCV, Monte Carlo Cross-Validation; MDS, Multidimensional scaling; PB, Post Breakfast; PCA, Principal Component Analysis; PD, Post Dinner; pHILIC, polymeric Hydrophilic Interaction Chromatography; PL, Post Lunch; PLSDA, Partial Least Squares

Discriminant Analysis; ROC, Receiver Operator Characteristic; UHPLC, Ultra-High Performance Liquid Chromatography

Supplemental Data: Supplemental Tables 1–2, Supplemental Section 1 and Supplemental Figures 1-5 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.

Abstract

Background

Measurement of multiple food intake exposure biomarkers in urine may offer an objective method for monitoring diet. The potential of spot and cumulative urine samples that have reduced burden on participants as replacements for 24-hour urine collections has not been evaluated.

Objectives

The aim of this study was to determine the utility of spot and cumulative urine samples for classifying the metabolic profiles of people according to dietary intake when compared with 24-hour urine collections in a controlled dietary intervention study.

Design

19 healthy individuals (10 male, 9 female, aged 21 – 65, BMI 20 – 35 kg/m^2), each consumed four distinctly different diets, each for 1 week. Spot urine samples were collected approximately 2 h post meals on three intervention days each week. Cumulative urine samples were collected daily over three separate temporal periods. A 24-hour urine collection was created by combining the three cumulative urine samples. Urine samples were analysed using metabolite fingerprinting by both high-resolution flow infusion mass spectrometry (FIE-HRMS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). Concentrations of dietary intake biomarkers were measured using liquid chromatography triple quadrupole mass spectrometry (LC-QqQ-MS) and by integration of ^{1}H-NMR data.

Results

Cross-validation modelling using ^{1}H-NMR and FIE-HRMS data demonstrated the power of spot and cumulative urine samples in predicting dietary patterns in 24-hour urine collections. Particularly there was no significant loss of information when post – dinner (PD) spot or overnight cumulative samples were substituted for 24-hour urine collections (classification accuracies 0.891 and 0.938 respectively). Quantitative analysis of urine samples also demonstrated the relationship between post-dinner (PD) spot samples and 24-hour urines for dietary exposure biomarkers.

Conclusions

We conclude that PD spot urine samples are suitable replacements for 24-hour urine collections. Alternatively, cumulative samples collected overnight predict similarly to 24-hour urine samples and have a lower collection burden for participants.

Keywords

Spot urine; metabolomics; 24-hour urine; dietary intake; high resolution mass spectrometry; nuclear magnetic resonance spectroscopy

Introduction

The frequency and pattern of consumption of foods and beverages are major determinants of the risk of obesity and of multiple health outcomes (1,2). As a consequence, the World Health Organisation and many governments have introduced population-based policies, which aim to improve eating patterns and to reduce the burden of chronic diseases (3). However, evaluation of the impact of public health policies on dietary intake in populations is challenging. Traditionally, the assessment of dietary exposure has been wholly reliant on a range of self-reported measures such as Food Frequency Questionnaires (FFQs), recall and diet diaries. These methods are often inaccurate due to significant misreporting and bias by individuals (4). The data quality of these approaches can be improved upon if the collection is supervised by a trained researcher, however the cost of this will often be prohibitive for meaningful sample sizes in large scale epidemiological studies. Additionally, conventional tools based on dietary self-reporting can be tedious and time-consuming for both study participants and investigators. Whilst the advent of digital (including on-line) tools may assist with the challenge of daily dietary recording and reduce the workload for both respondents and researchers (5), this does not eliminate the subjectivity and biases, which is inherent for approaches based on self-reporting (6–8). With obesity and chronic disease levels continuing generally to rise each year there is an urgent need both for accurate and scalable technologies to assess diet at the level of both population and the individual.

Many foods contain characteristic, non-nutritive, secondary metabolites which after consumption undergo metabolism often at multiple sites prior to elimination from the body (9). Metabolomic analyses have been previously applied to biofluids in human studies, particularly to blood, saliva and urine, to discover novel metabolite biomarkers

of food intake (10–12) and more recently to detect patterns of overall dietary status (13–15). Urine can be considered the biological fluid of choice because of the ease of collection and, unlike blood, it provides an integrated estimate of exposure over several hours.

In studies evaluating the potential of urinary biomarkers to report dietary exposure, biomarker performance is often only examined in highly controlled dietary interventions, typically based on exposure to a single meal (16,17). In very few instances have such biomarker leads been validated and tested for specificity using dietary records and urine sample sets collected under epidemiological study conditions (18,19).

To have significant utility in the future it is essential that any emerging technology allows for the simultaneous measurement of multiple biomarkers, which can then be used to provide comprehensive coverage of dietary exposure in a real-world environment. A key requirement to achieve this objective is the development of a suitable approach for the sampling of urine to capture an accurate representation of eating behaviour, but which has minimal impact on an individual's daily activities. The assessment of dietary exposure using only urinary analysis is a challenging prospect. The frequency that foods are consumed varies between individuals and many foods are consumed typically within complex meals and as a variety of formulations and after different processing methods. Additionally, the presence and concentration of food biomarkers in any urine void will be modulated by the timing of dietary intake. In many research studies requiring accurate quantification of the daily excretion of a specific analyte the adopted 'gold standard' method for sampling demands the collection of all urine voids over a full day (24-hour urine) (20,21).

While a 24-hour urine can provide robust and accurate quantification of analytes its collection imposes a significant burden on study participants due to its adverse impact on normal daily activities (22). There is also a significant risk that measurements are misreported due to incorrect collections; in some studies it has been shown that up to 30 % of 24-hour collections are under-collections (23). In contrast to 24-hour urine, collection of spot urine samples has a reduced impact on normal daily activities and benefit from cheaper logistical costs. Against this background, if 24-hour collections could be substituted by spot samples or less burdensome cumulative/pooled urine samples representing specific temporal phases of the day, then the scale up of cohort size in nutritional epidemiological studies would be feasible and improve the accuracy of dietary assessment using urinary biomarkers at the population level. The main objective of the present study is to determine whether spot urines, or pooled urine samples (cumulative samples) representing specific temporal phases of the day, can adequately substitute for 24-hour urine samples in dietary exposure studies.

Materials and Methods

Study Design

Urine samples were obtained from 19 healthy individuals (10 male, 9 female, 21 – 65 years; BMI 20 – 35 kg/m^2) who participated in a randomised, controlled, crossover short-term food intervention (13) in which they were exposed to four diets with a stepwise degree of concordance with World Health Organisation's (WHO) dietary guidelines (24). Each diet differs in the contribution of macro and micro nutrients to total daily energy intake. Diet 1 (100%) was the most concordant, Diet 4 (25%) the least concordant, and Diets 2 (50%) and 3 (75%) were the intermediate diets.

Participants attended the Clinical Research Facility (CRF) for a 72-hour inpatient period on four separate occasions. Each inpatient stay was separated by a minimum of 5 days to ensure that any potential carryover from the dietary intervention periods was minimised. Adherence to the study protocol was strictly monitored during the inpatient stay. All food was weighed immediately before being given to the participants and any uneaten food was weighed. Physical activity was controlled; and participants were only allowed to engage in very light physical activity.

At each visit, the same menu plan was consumed every day for each of the 3 days within a single experimental period to ensure that a stable dietary exposure was established. The manipulated foods for each diet are detailed in Supplemental Table 1 of the following reference (13). During each 3 day dietary intervention, urine samples were constantly collected as previously described (13). Spot urines were collected in a fasted state (FA) each morning and approximately 2h after the consumption of each meal (post-breakfast, PB; post-lunch, PL and post-dinner, PD). Cumulative urine samples were collected to represent 3 temporal phases of each day. Cumulative sample 1 (CS1) was all urine from the 4h period post-breakfast to pre-lunch. Cumulative sample 2 (CS2) was all urine from the 5h period post-lunch to pre-dinner. Cumulative sample 3 (CS3) was all urine from the 13h period post-dinner to the fasting urine the following morning. A 24-hour (24HR) urine sample was prepared by pooling CS1, CS2 and CS3.

Sample extraction

Urine samples were thawed to room temperature, vortexed and 800 µL transferred to a clean 2 mL Eppendorf tube. Samples were centrifuged (EBA 12 R, Hettich) at 25,200 g for 5 minutes at 4 ºC. Following centrifugation, specific gravity of a 200 µL aliquot was measured using a hand-held refractometer (OpitDuo 38-53, Bellingham and Stanley). Specific gravity correction factors were calculated per participant as the fold change of individual sample specific gravity to the specific gravity of the sample in the whole sample set which recorded the minimum value (25). Extracts were then prepared as previously described (26).

Flow infusion metabolite fingerprinting and data pre – processing

All samples were analysed using high-resolution flow infusion mass spectrometry (FIE-HRMS). From each extracted sample, 20 µL was transferred to a glass HPLC vial containing a 200 µL flat bottom micro insert (Chromacol) and diluted with 80 µL of H_2O:MeOH (3:7) directly in the vial. Mass spectra were acquired on an Exactive Orbitrap (ThermoFinnigan, San Jose CA) mass spectrometer coupled to an Accela (ThermoFinnigan, San Jose CA) ultra-performance liquid chromatography system. 20 µL of sample was injected and delivered to the ESI source *via* a flow solvent (mobile phase) or pre-mixed HPLC grade MeOH (Fisher Scientific) and ultra- pure H_2O (18.2 Ω) at a ratio of 7:3. The flow rate was 200 $\mu L min^{-1}$ for the first 1.5 minutes, and 600 $\mu L min^{-1}$ for the remainder of the method. The total assay time was 3.0 minutes.

Positive and negative ionisation modes were acquired simultaneously. One scan event was used to acquire all mass spectra, 55.000 - 1000.000 *m/z* and 63.000 - 1000.000

m/z for positive and negative mode respectively. The scan rate was 1.0 Hz. Mass resolution was 100,000, with an automatic gain control (AGC) of 5 x 10^5 and maximum ion injection time of 250 ms, for both ionisation modes. Following data acquisition raw profile data (RAW, ThermoFinnigan) were converted to the mzML open file format and centroided (27). Conversion and centroiding was performed using msconvert (TransProteomicPipeline) (28). All further processing of mzML files was performed using the R Statistical Programming Language (29). Dimensionality reduction of the acquired mass spectra was performed by taking each *m/z* value from scans about the apex of the infusion profile and binning the *m/z* and intensity values at 0.01 amu intervals. The result was a *m x n* matrix, where *m* is the sample and *n* is the *m/z* feature and cells are the respective average intensity values. The result matrix was filtered to yield only variables which were present at an occupancy greater than or equal to 70 % in at least of one the specified biological classes. This resulted in 4574 and 4362 features for spot and cumulative (including 24 hour) urine samples respectively.

Quantification of dietary exposure biomarkers by targeted Liquid Chromatography- Mass Spectrometry.

Concentrations of selected biomarkers (see **Table 1**) were measured in selected urine samples using ultra-high-performance liquid chromatography (UHPLC) triple quadrupole (QQQ) mass spectrometry (MS) operating in Multiple Reaction Monitoring (MRM) mode. The dietary exposure biomarkers selected for quantification reflected the food components used in the diet interventions and included metabolites that were expected to eliminated from the body relatively quickly, as well as metabolites derived

as a result of gut microbiome activity that were expected to appear in urine at later time points. MRM chromatograms were acquired on a TSQ Quantum Ultra QQQ mass spectrometer (ThermoFinnigan, San Jose CA) equipped with a heated electrospray ionisation (HESI) source and coupled to an Accela UHPLC system. The UHPLC system was equipped with either a reverse phase (C_{18}) column or a polymeric Hydrophilic Interaction (pHILIC) column. Full chromatographic details are provided in **Supplemental Section 1.** Mass spectra were acquired using MRM acquisition, in positive and negative ionisation mode simultaneously. Collision energy and tube lens voltage values were optimised individually for each parent – product transition (**Supplemental Table 1**). All post-acquisition data processing was performed using Quan Browser (ThermoScientific) and Xcalibur (Thermo Scientific). Spearman rank correlations (**Table 4 and 5**) were performed using the R function; *cor. A*djusted coefficient of determination (R^2) values (**Table 4 and 5**) were calculated in R using the *lm* function.

Statistical analysis of flow infusion metabolite fingerprinting data

All statistical and classification analysis of flow infusion metabolite fingerprinting data was performed using the R Statistical Programming Language (29). Classification of metabolite fingerprint data was performed using the *randomForest* R package (Version 4.6) (30). For all classification models, the dietary intervention (i.e. 25, 50, 75 and 100% of WHO healthy eating guidelines) was the response variable. Models were constructed using all *m/z* variables 4574 and 4362, for spot and cumulative samples respectively. For each Random Forest model, the number of trees was 1000 and the number of variables considered at each internal tree node was the square root of the total number of available variables. Each model was assessed using classification

accuracy and multi-class area under the receiver operator characteristic (ROC) curve (AUC) (31). ROC-AUC values were calculated using the R package HandTill2001 (Version 0.2-12). For **Figure 2** the training proximity values and predicted proximity values were used to construct a dissimilarity matrix (1 – proximity) which was then scaled to two dimensions using multidimensional scaling (MDS).

^{1}H-NMR acquisition and processing for metabolic fingerprinting and quantitative data

Samples for ^{1}H-NMR spectroscopy analysis were prepared mixing 540 µL of urine with 60 µL of a pH 7.4 phosphate buffer as described previously (13). We analysed the samples at 300 K on a 600MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) using a standard one-dimensional pulse sequence with water-pre-saturation (32). As described previously (13), ^{1}H-NMR spectra were modelled with Partial Least Squares Discriminant Analysis (PLS-DA) in a repeated-measures Monte Carlo Cross-Validation (MCCV) framework. The mean prediction (T_{pred}) was estimated across the MCCV models where the sample was left-out of modelling. A positive T_{pred} indicated that the urinary metabolic profile of the sample resembled Diet 1 more than Diet 4, and vice versa for a negative T_{pred}. Variable importance was assessed based on bootstrap resampling of regression coefficients in each model, using the False Discovery Rate (q-value) of ≤0.01 to indicate significance. Skillings-Mack tests were used to assess differences between the four diets (multiple paired samples) (13). Subsequently, Wilcoxon's signed rank test was used to test pairwise differences and p values from post-hoc tests were adjusted for multiple testing with Hommel's adjustment.

Results

Dietary exposure is well discriminated by metabolite fingerprinting using spot, cumulative or 24-hour urine samples

Multidimensional scaling (MDS) of multinomial Random Forest classification models for diet differences in metabolome fingerprint data demonstrated excellent separation between diets with all three urine types from Day 3 (**Figure 1**). It is evident that urine composition reflective of the 4 different diets had already stabilised by Day 2 (**Supplemental Figure 1**). These findings were validated by the ^{1}H-NMR metabolic fingerprinting models (13) that showed good discrimination between dietary intervention on Day 1 (Skillings-Mack P=8.20 x 10^{-7}), Day 2 (Skillings-Mack P=7.11 x 10^{-10}) and Day 3 (Skillings-Mack P=7.21 x 10^{-9}) based on 24-hour samples, with the lowest significance values achieved using Day 1 urine samples. Classification performance of CS3 models was comparable with those obtained for PD spot models and 24-hour urine samples for both Day 3 (**Figure 1**) and Day 2 (**Supplemental Figure 1**) samples. Comparable results were obtained using ^{1}H-NMR metabolic fingerprinting modelling which demonstrated that PD spot urine and CS2 and CS3 models all significantly discriminated between dietary treatments, with the PD spot model having the lowest classification values (13).

Spot urine samples predicts diet discrimination in corresponding cumulative samples

Principal Component Analysis (PCA) was performed on an exemplar FIE-HRMS dataset to confirm that sample type was not a confounding factor (**Supplemental Figure 2**). The PCA model was constructed using spot samples only and the corresponding cumulative sample data were projected into the PCA model. This showed that sample type did not result in an observable change in model structure. Experimental cross validation was used to determine if discrimination between all diets measured in a spot urine sample type predicted diet correctly in unlabelled urines of a different type (i.e. cumulative or 24-hour). As an example, a Random Forest model was trained to discriminate between the 4 different diets using Day 3 PD spot urine fingerprint data and then diet was predicted in Day 3 24-hour urine samples. Multidimensional scaling (MDS) of predicted Random Forest proximity values demonstrated good correspondence of PD and 24-hour urine collection data following exposure to each of the 4 different diets. Classification performances for all urine type comparisons (using both FIE-HRMS and NMR data) based on Day 3 urine samples are shown in **Supplemental Table 1 and Supplemental Table 2**. In **Table 2** the results are shown where Day 3 PD spot samples only have been used to construct training models and predictions made concerning all samples types on both Day 2 and Day 3. Likewise, cumulative samples were used to predict diet discrimination in 24-hour urine samples, cumulative and spot samples (**Table 3**). The full dataset (**Supplemental Table 1**), in which PB and PL spot samples were used to construct a training model, demonstrates the power of spot urine samples to predict dietary patterns. In summary, 15 out of 30 FIE-HRMS models (**Supplemental Table 1**) yielded a classification accuracy of $\geq$ 0.9 and only10 models had performance of $< 0.9 \geq 0.8$ A key highlight from the classification modelling was confirmation of the ability of spot samples to predict accurately their corresponding temporal phase cumulative sample.

For example, PB-3 (training) and CS1-3 (test) gave an accuracy of 0.974, PL-3 (training) and CS2-3 (test) gave an accuracy of 0.975. In addition, PD-3 (training) and CS3-3 (test) yielded an accuracy of 0.918. Furthermore, the PD-3 spot sample predicted dietary exposure in the corresponding Day 3 24-hour (24S-3) sample, with an accuracy of 0.891. All of the FIE-HRMS models were validated by the ^{1}H-NMR analysis using the same sample model combinations. In all instances where classification accuracy was $\geq$ 0.9, Skillings-Mack significance testing (p-value) indicated strong coherence between the two independent, non-targeted analytical platforms (**Tables 2 and 3**). As expected, with both analytical platforms and modelling approaches, the best predictions were achieved when the sample collection of the training model was time-matched as closely as possible with the test samples.

Dietary biomarkers exhibit similar quantitative changes in both PD spot urines and 24-hour urine collections on exposure to different amounts of specific foods

Table 4 shows the correlations between the concentration of key dietary biomarkers in PD spot and 24-hour urine samples respectively. The biomarkers selected (see **Table 1**) included metabolites that were the product of biotransformation within the human body (e.g. 3,5-Dihydrophenylpropionoic acid 3-O-sulfate and D-L-Sulforaphane-N-acetyl-L-cysteine) and others which were eliminated from the body without biotransformation within a few hours after consumption (e.g. *L*-Anserine and tartrate). Spearman rank correlation coefficients (ρ) ranged from 0.447 to 0.959. The accuracy of each comparison was measured using the adjusted R^2 value from linear models between quantitative measures in PD and 24-hour urine samples (**Table 4**).

Adjusted R^2 values showed the same distribution in variability as the spearman rank correlation coefficients across the biomarkers. Dihydrophenylpropanoic acid (DHPPA) exhibited a weak correlation between PD and 24-hour urine samples (ρ = 0.147, R^2 = 0.451) and PD versus CS3 (ρ = 0.147, R^2 = 0.451). In this case, the relatively weak correlations between urine sample types is due to the fact that DHPPA, a biomarker of wholegrain consumption is eliminated from the body relatively slowly over several hours after wholegrain consumption (33,34). Therefore, because the wholegrain containing foods were consumed at breakfast and/or lunch DHPPA excretion is not captured adequately in the PD spot but is present in the 24-hour and CS3 samples. The range of correlation coefficients is reflective the unique absorption, metabolism and excretion dynamics of each of the biomarkers. While there were variations in correlation coefficients, the relative distribution of analytes between sample type remained consistent (see **Supplemental Figure 3** for Quantile-Quantile Plots). In all cases a right skew is visible indicative of the complex dynamic range of dietary biomarkers within urine.

Micturition characteristics supports sample homogeneity between different urine sample types

Inter-individual variation in water intake and micturition behaviour compounds the challenge of obtaining objective dietary information using urinary biomarkers. To have value for estimation of dietary intake it is essential that spot urine samples can be normalised to account for differences in inter-individual variation in micturition behaviour. In all three sample types the relationship between refractive index and creatinine concentration was consistent (**Supplemental Figure 4**). Wilcox-signed rank

test between PD urine creatinine concentrations and 24-hour urine creatinine concentrations showed no change in rank positions ($P = 0.586$). Similar results were also observed for the comparison of refractive index between sample types. A weak significant difference ($0.01<P<0.05$) was observed between refractive index values in CS3 and 24-hour urine samples. With no deviation between creatinine concentrations and refractive index across sample types there is no loss of accuracy in using pre-analytical physical measurements (refractive index) to determine sample concentration and normalise prior to analysis.

Discussion

The study design allowed us to compare the utility of spot, cumulative phase and 24-hour urine samples for quantifying biomarkers of dietary exposure. Because individuals consumed the same diet for three consecutive days in each experimental period, metabolic responses to each experimental diet stabilized within about 24-hours and the study design provided an opportunity for substantial replication, cross-validation and assessment of variability (13). In addition, we undertook non-targeted metabolite fingerprinting by two different analytical methods (FIE-HRMS and NMR) to provide a comprehensive representation of urine chemistry.

24-hour urine collection, for example for determination of sodium intake, is recognized as being expensive and burdensome (35). If urine is to be used for large-scale biomarker-based investigations of dietary exposure, then simpler, less burdensome methods of urine collection are needed. The collection of a single spot urine sample imposes much less burden on individuals compared with 24-hour urine collection and has a reduced impact on an individual’s daily activities. An essential criterion of any

urine sampling method is that it provides an accurate estimation of habitual eating behavior. Because hydration levels and micturition frequencies differ between individuals the utility of spot urines for dietary exposure assessment is dependent on simple methods to normalize concentration differences (36). Creatinine has been used widely as a measure for determining total urine concentration (37). Measurement of a physical attribute reflecting overall urine concentration, such as refractive index or osmotic concentration also provides a simple method to correct for differences in individual hydration status (25,38). An advantage of using a physical characteristic as a surrogate for sample concentration is that it accounts for the entire non-water content of the urine and is not dependant on the utility of any single analyte such as, creatinine, which itself can be confounded by intake of red meat or renal disease. In addition, pre-analytical normalisation has the advantage that it is independent of the analytical process for metabolite quantification (25).

From the present study, spot urine samples collected at least two hours post consumption of the main evening meal were found to be the best substitute for 24-hour urine collections. Although more burdensome to collect (compared to a spot urine), CS3 samples (collected from the 13-hour period after eating an evening meal to the fasting urine) followed by the CS2 samples (collected from the 5-hour period post-lunch to pre-dinner) were the best substitutes for 24-hour urine samples. Although dietary exposure biomarkers will be present at different concentrations in spot urine samples collected at different times after consumption of specific foods, the present study illustrates that exposure levels to several key foods of high public health interest can be assessed equally well in 24-hour, cumulative and spot urine samples. However, in order to minimize the limitations of spot sample variability, they must be collected in

an informative, relatively narrow time window. In situations where it is very difficult to obtain a spot sample in a narrow time window, cumulative samples have been demonstrated to predict similarly to 24-hour urine samples and have the advantage of being a lower collection burden for participants (13).

There have been relatively few studies describing targeted quantitative analyses of multiple biomarker chemical classes in urine samples. In the present study all participants were obliged to eat identical meals at the same time under supervision to ensure optimal compliance and enabling accurate alignment of biomarker excretion profiles in the different urine types. A recent review by *Dragsted et al* (39), stressed the importance of 'time response' as a characteristic to consider when choosing sample types for biomarker quantification. Although correspondence of biomarker levels between PD spot and 24-hour urine collections was generally good (**Supplemental Figure 5**), the presented data illustrates that weaker correlations between dietary intake and biomarker concentrations in both spot and 24-hour urine samples may also be expected; particularly in epidemiological studies where the time elapsed between eating and urine sampling is not tightly controlled biomarkers derived from colonic fermentation may not be highly represented in PD urine samples and so the use of First Morning Void (FMV) urine sampling in addition might be recommended. A typical example would be skewed distribution towards lower concentrations measured in PD spot urine for D-*L*-Suforaphane (a biomarker of exposure to cruciferous vegetables) (40,41) at higher dietary intake levels, which is derived from colonic fermentation and additionally may reflect the impact of metabotype differences within a specific population. For these hypotheses to be further investigated these methods first need scaling to the population level and testing in a representative

epidemiological contexts. Taking note of any possible complications arising, with regard to the behavior of specific biomarkers we suggest that in future PD spot or the CS3 urine voids in combination with FMV urines may provide ideal samples for epidemiological studies requiring assessment of dietary exposure. Their ease of collection by the participant and storage offer scope for assessing food intake on multiple days with little impact on the normal daily activities of individuals. We propose that dietary exposure biomarker technology in conjunction with traditional self-reporting tools should help improve the quality of future nutrition research.

Acknowledgements

This study was funded by the Medical Research Council (MRC), grant number MR/J010308/1.

The authors' responsibilities were as follows; IGP, EC, EH and GF designed and conducted the intervention study. JP, KT, HZ, MB analysed urine samples and generated raw data. TW, IGP and JP performed analysis of mass spectrometry and NMR data; and were responsible for interpretation of data. TW, IGP, JP, AL, JM, GF and JD wrote the manuscript and JD had primary responsibility for the final content. All authors read and approved the final version of the paper. The authors have no conflicts of interest to declare.

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Table 1: Analytical details of biomarkers used for absolute quantification in urine samples by LC-MS and ^{1}H-NMR

Biomarker	Dietary component	Parent ion [1]	Product ion [2]	Retention time (Rt) [3]	^{1}H-NMR Chemical shift (multiplicity) [4]
1 – Methylhistidine	Poultry / Fish	170.064 (+)	124.160	9.57	3.17 (2d), 3.22 (2d), 3.78 (s), 3.99 (dd), 7·17(s), 8·12 (s)
3 – Methylhistidine	Total meat	170.059 (+)	95.345	10.4	3.25 (2d), 3.30 (2d), 3.78 (s), 3.99 (dd), 7·23(s), 8·27 (s)
L-Anserine	Poultry / Fish	241.052 (+)	109.159	10.95	
Carnosine	Total meat	227.064 (+)	110.202	11.66	
Trimethylamine-*N*-oxide	Fish	76.188 (+)	58.517	8.55	3.27 (s)
3,5-Dihydrophenylpropionoic acid	Wholegrain	181.044 (-)	137.132	4.22 *	
3,5-Dihydrophenylpropionoic acid 3-O-sulfate	Wholegrain	260.950 (-)	95.225	3.89 *	
D-L-Sulforaphane	Cruciferous vegetables	178.034 (+)	114.155	5.77 *	
D-L-Sulforaphane-N-acetyl-L-cysteine	Cruciferous vegetables	299.003 (+)	114.103	3.92 *	
Tartaric acid	Grapes	149.016 (-)	87.191	13.15	4.34(s)
7-Methylxanthine	Cocoa	167.085 (+)	150.130	3.53 *	
3-Methylxanthine	Cocoa	167.096 (+)	94.22	3.67 *	
3-Methyluric acid	Cocoa	181.009 (-)	138.078	9.55	
Caffeine	Coffee / Caffeinated drinks	195.06 (+)	138.14	5.11 *	
Ferulic acid-4-*O*-sulfate	Polyphenol rich foods	273.021 (-)	193.087	4.28 *	
Quercitin-3-*O*-D-glucuoronide	Fruit / Vegetables	477.076 (-)	300.972	5.2 *	
Rhamnitol	Apple				1.28 (d)
O-acetylcarnitine	Red Meat				2.15 (s), 3.19 (s)
Carnitine	Red Meat				2.44 (dd), 3.23 (s), 3.43 (m)
Dimethylamine	Fish				2.72 (s)
N-acetyl-*S*-methyl-cysteinesulfoxide	Cruciferous vegetables				2.78 (s)
Glucose	Sugars				3.42 (m), 3.49 (m), 3.54 (dd), 3.74 (m), 3.84(m), 3.91 (dd)

N-acetyl-*S*-(1Z)-propenyl-cysteinesulfoxide	Onion				1.96 (dd), 2.03 (s), 6.49 (dq), 6.65 (dq)
N-methylnicotinate	Peas & Niacin				4.44 (s), 8.10 (t), 8.84 (d), 9.11 (s)
4-hydroxyhippurate	Fruits				3.95 (s), 6.97 (d), 7.76 (d)

[1]*:* All parent ions were detected as either the protonated (M+H) or deprotonated (M-H) form of the mono-isotopic mass (M) of each biomarker. Parent ions denoted with (+) or (-) indicates that the biomarker was detected in the protonated or deprotonated form respectively. **[2]*:*** For each parent ion a minimum of three product ions were detected and analysed. The product ions shown are the ones which demonstrated the greatest stability and were therefore used for quantification. The remaining product ions (not shown) were used as qualifying ions only. **[3]*:*** Retention times denoted with an * indicate that ultra-high-performance chromatography of the specified biomarker was performed on a RP-C18 column. All other biomarkers were analysed using a ZIC-pHILIC column. **[4]*:*** The chemical shifts and multiplicities are listed for peaks from significantly associated metabolites. Multiplicity key is as follows: s – singlet, d – doublet, t – triplet, q – quartet, dd –doublet of doublets, dq – doublet of quartets, 2d – two doublets, m – (other) multiplet.

Table 2: Predicted classification scores from Post-dinner (PD) day 3 spot samples and Cumulative / 24-hour (day 2 and day 3) urinary models

Model sample [1]	**Prediction data** [2]	**Skillings-Mack (*p*-value)** [3]	**Classification accuracy (95 % CI)** [4]
PD-3	PB-2	1.16×10^{-02}	0.613 (0.600,0.625)
PD-3	PB-3	8.89×10^{-04}	0.678 (0.664,0.691)
PD-3	PL-2	3.48×10^{-06}	0.814 (0.803,0.824)
PD-3	PL-3	2.30×10^{-10}	0.795 (0.784,0.806)
PD-3	PD-2	3.53×10^{-06}	0.976 (0.971,0.98)
PD-3	PD-3	1.99×10^{-11}	1.000 (1.000,1.000)
PD-3	CS1-3	9.43×10^{-08}	0.684 (0.671,0.697)
PD-3	CS2-3	1.29×10^{-11}	0.969 (0.964,0.973)
PD-3	CS3-3	2.06×10^{-10}	0.918 (0.911,0.926)
PD-3	24S-3	4.58×10^{-08}	0.891 (0.882,0.900)

[1] Sample type used for model training. [2] Sample type used for model prediction. [3] Skillings-Mack *p*-value are from Monte-Carlo cross validation (MCCV) of ^{1}H NMR data. [4] Classification accuracy is the resampled (n = 100) prediction accuracy of multinomial Random Forest classification models of FIE-HRMS data.

Table 3: Predicted classification scores from Cumulative day 3 (CS3) samples and spot sample (day 2 and day 3) urinary models

Model sample [1]	**Prediction data** [2]	**Skillings-Mack (*p*-value)** [3]	**Classification accuracy (95 % CI)** [4]
CS3-3	PB-2	1.05×10^{-04}	0.701 (0.688,0.714)
CS3-3	PB-3	2.92×10^{-03}	0.736 (0.725,0.747)
CS3-3	PL-2	2.94×10^{-06}	0.747 (0.735,0.759)
CS3-3	PL-3	8.34×10^{-08}	0.748 (0.735,0.760)
CS3-3	PD-2	4.31×10^{-06}	0.988 (0.986,0.991)
CS3-3	PD-3	2.12×10^{-11}	1.000 (1.000,1.000)
CS3-3	CS1-3	3.30×10^{-06}	0.754 (0.743,0.766)
CS3-3	CS2-3	7.32×10^{-11}	0.821 (0.810,0.833)
CS3-3	CS3-3	9.69×10^{-10}	1.000 (1.000,1.000)
CS3-3	24S-3	1.40×10^{-09}	0.938 (0.931,0.946)

[1] Sample type used for model training. [2] Sample type used for model prediction. [3] Skillings-Mack *p*-value are from Monte-Carlo cross validation (MCCV) of ^{1}H NMR data. [4] Classification accuracy is the resampled (n = 100) prediction accuracy of multinomial Random Forest classification models of FIE-HRMS data.

Table 4: Comparison of absolute quantitation values between Post-dinner (PD) spots and 24-hour urine samples

Biomarker	R^2 [1]	Spearman Rank (ρ) [2]
Creatinine	0.314	0.602
1-Methylhistidine	0.306	0.611
Anserine	0.879	0.931
Carnosine	0.440	0.690
Trimethylamine-*N*-Oxide	0.733	0.920
3-Methylhistidine	0.834	0.891
3-Methyluric-acid	0.528	0.846
Tartrate	0.500	0.871
3-Methyl-xanthine	0.797	0.941
7-Methyl-xanthine	0.891	0.959
Caffeine	0.399	0.837
DHPPA	0.147	0.451
DHPPA-3-sulfate	0.553	0.838
Ferulate-4-O-sulfate	0.369	0.731
Quercetin-3-O-D-glucuronide	0.198	0.474
D-L-Sulforaphane	0.788	0.820
D-L-Sulforaphane-N-acetyl-L-cysteine	0.735	0.858
Rhamnitol	0.251	0.539
O-acetylcarnitine	0.007	0.505
Carnitine	0.225	0.645
Dimethylamine	0.343	0.470
N-acetyl-*S*-methyl-cysteinesulfoxide	0.247	0.604
Glucose	0.182	0.456
N-acetyl-*S*-(1Z)-propenyl-cysteinesulfoxide	0.280	0.578
N-methylnicotinate	0.271	0.664
4-hydroxyhippurate	0.124	0.447

[1] R^2 is the adjusted coefficient of determination for linear models between biomarker quantifications in PD spot and 24-Hour urines samples. [2] ρ values are the correlation coefficients for Spearman-rank test between the two sample types

Table 5: Comparison of absolute quantitation values between Post-dinner (PD) spots and Cumulative sample (CS3)

Biomarker	R^2 [1]	Spearman Rank (ρ) [2]
Creatinine	0.284	0.526
1-Methylhistidine	0.348	0.673
Anserine	0.509	0.866
Carnosine	0.231	0.613
Trimethylamine-*N*-Oxide	0.806	0.935
3-Methylhistidine	0.808	0.860
3-Methyluric-acid	0.722	0.888
Tartrate	0.502	0.898
3-Methyl-xanthine	0.731	0.924
7-Methyl-xanthine	0.859	0.970
Caffeine	0.466	0.783
DHPPA	0.182	0.499
DHPPA-3-sulfate	0.609	0.838
Ferulate-4-O-sulfate	0.362	0.626
Quercetin-3-O-D-glucuronide	0.104	0.398
D-L-Sulforaphane	0.700	0.774
D-L-Sulforaphane-N-acetyl-L-cysteine	0.752	0.843
Rhamnitol	0.198	0.538
O-acetylcarnitine	0.361	0.608
Carnitine	0.522	0.675
Dimethylamine	0.381	0.589
N-acetyl-*S*-methyl-cysteinesulfoxide	0.531	0.765
Glucose	0.413	0.556
N-acetyl-*S*-(1Z)-propenyl-cysteinesulfoxide	0.775	0.806
N-methylnicotinate	0.373	0.710
4-hydroxyhippurate	0.167	0.632

[1] R^2 is the adjusted coefficient of determination for linear models between biomarker quantifications in PD spot samples and CS3 samples. [2] ρ values are the correlation coefficients for Spearman-rank test between the two sample types

Figure Titles and Legends

Figure 1 Multidimensional scaling (MDS) of multinomial Random Forest classification models for diet differences in metabolome fingerprint data representing three different urine sample types.

A; Post-dinner (PD) Day 3, B; Cumulative sample 3 (CS3)_Day 3 and C; 24-hour Day 3. Classification accuracies for A, B and C were 1.0, 0.93 and 0.89 respectively. Multi-class Area Under the ROC Curve (AUC) returned values of 1.0, 0.99 and 0.98 respectively.

Figure 2 Multidimensional scaling (MDS) of predicted Random Forest proximity values in metabolite fingerprint data representing Post-dinner and 24-hour urine samples following exposure to the four different diets

Blue symbols represent Post-dinner (PD) Day 3 samples which have been used to construct the training model. The red symbols are 24-hour urine Day 3 samples where diet (25, 50, 75 and 100) has been predicted using the PD spot training model.