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Spot and Cumulative Urine Samples Are Suitable Replacements for 24-Hour Urine Collections for Objective Measures of Dietary Exposure in Adults Using Metabolite Biomarkers

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

4

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10

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31

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

33

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

36

37 **Abbreviations used:**

38 ¹H NMR, Proton Nuclear Magnetic Resonance; AGC, Automatic Gain Control; AUC,
39 Area Under the Curve; BMI, Body Mass Index; CRF, Clinical Research Facility; CS1,
40 Cumulative Sample 1; CS2, Cumulative Sample 2; CS3, Cumulative Sample 3; ESI,
41 Electrospray Ionisation; FA, Fasting; FFQ, Food Frequency Questionnaire; FIE-
42 HRMS, Flow Infusion Electrospray High Resolution Mass Spectrometry; FMV, First
43 Morning Void; H₂O, Water; HPLC, High Performance Liquid Chromatography; WHO,
44 World Health Organisation; LC-QqQ-MS, Liquid Chromatography Triple Quadrupole
45 Mass Spectrometry; MeOH, Methanol; MRM, Multiple Reaction Monitoring; MCCV,
46 Monte Carlo Cross-Validation; MDS, Multidimensional scaling; PB, Post Breakfast;
47 PCA, Principal Component Analysis; PD, Post Dinner; pHILIC, polymeric Hydrophilic
48 Interaction Chromatography; PL, Post Lunch; PLSDA, Partial Least Squares

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

51

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

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65

66 **Abstract**

67

68 **Background**

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

73

74 **Objectives**

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

78

79 **Design**

80 19 healthy individuals (10 male, 9 female, aged 21 – 65, BMI 20 – 35 kg/m²), each
81 consumed four distinctly different diets, each for 1 week. Spot urine samples were
82 collected approximately 2 h post meals on three intervention days each week.
83 Cumulative urine samples were collected daily over three separate temporal periods.
84 A 24-hour urine collection was created by combining the three cumulative urine
85 samples. Urine samples were analysed using metabolite fingerprinting by both high-
86 resolution flow infusion mass spectrometry (FIE-HRMS) and proton nuclear magnetic
87 resonance spectroscopy (¹H-NMR). Concentrations of dietary intake biomarkers were
88 measured using liquid chromatography triple quadrupole mass spectrometry (LC-
89 QqQ-MS) and by integration of ¹H-NMR data.

90

91 **Results**

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

99

100 **Conclusions**

101 We conclude that PD spot urine samples are suitable replacements for 24-hour urine
102 collections. Alternatively, cumulative samples collected overnight predict similarly to
103 24-hour urine samples and have a lower collection burden for participants.

104

105

106

107 **Keywords**

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

110 **Introduction**

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

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

135 of food intake (10–12) and more recently to detect patterns of overall dietary status
136 (13–15). Urine can be considered the biological fluid of choice because of the ease of
137 collection and, unlike blood, it provides an integrated estimate of exposure over
138 several hours.

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

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

159 While a 24-hour urine can provide robust and accurate quantification of analytes its
160 collection imposes a significant burden on study participants due to its adverse impact
161 on normal daily activities (22). There is also a significant risk that measurements are
162 misreported due to incorrect collections; in some studies it has been shown that up to
163 30 % of 24-hour collections are under-collections (23). In contrast to 24-hour urine,
164 collection of spot urine samples has a reduced impact on normal daily activities and
165 benefit from cheaper logistical costs. Against this background, if 24-hour collections
166 could be substituted by spot samples or less burdensome cumulative/pooled urine
167 samples representing specific temporal phases of the day, then the scale up of cohort
168 size in nutritional epidemiological studies would be feasible and improve the accuracy
169 of dietary assessment using urinary biomarkers at the population level. The main
170 objective of the present study is to determine whether spot urines, or pooled urine
171 samples (cumulative samples) representing specific temporal phases of the day, can
172 adequately substitute for 24-hour urine samples in dietary exposure studies.

173 **Materials and Methods**

174

175 **Study Design**

176

177 Urine samples were obtained from 19 healthy individuals (10 male, 9 female, 21 – 65
178 years; BMI 20 – 35 kg/m²) who participated in a randomised, controlled, crossover
179 short-term food intervention (13) in which they were exposed to four diets with a
180 stepwise degree of concordance with World Health Organisation's (WHO) dietary
181 guidelines (24). Each diet differs in the contribution of macro and micro nutrients to
182 total daily energy intake. Diet 1 (100%) was the most concordant, Diet 4 (25%) the
183 least concordant, and Diets 2 (50%) and 3 (75%) were the intermediate diets.

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

191 At each visit, the same menu plan was consumed every day for each of the 3 days
192 within a single experimental period to ensure that a stable dietary exposure was
193 established. The manipulated foods for each diet are detailed in Supplemental Table
194 1 of the following reference (13). During each 3 day dietary intervention, urine samples
195 were constantly collected as previously described (13). Spot urines were collected in
196 a fasted state (FA) each morning and approximately 2h after the consumption of each
197 meal (post-breakfast, PB; post-lunch, PL and post-dinner, PD). Cumulative urine
198 samples were collected to represent 3 temporal phases of each day. Cumulative
199 sample 1 (CS1) was all urine from the 4h period post-breakfast to pre-lunch.
200 Cumulative sample 2 (CS2) was all urine from the 5h period post-lunch to pre-dinner.
201 Cumulative sample 3 (CS3) was all urine from the 13h period post-dinner to the fasting
202 urine the following morning. A 24-hour (24HR) urine sample was prepared by pooling
203 CS1, CS2 and CS3.

204

205

206

207

208 **Sample extraction**

209

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

218

219 **Flow infusion metabolite fingerprinting and data pre – processing**

220

221 All samples were analysed using high-resolution flow infusion mass spectrometry
222 (FIE-HRMS). From each extracted sample, 20 μ L was transferred to a glass HPLC vial
223 containing a 200 μ L flat bottom micro insert (Chromacol) and diluted with 80 μ L of
224 H₂O:MeOH (3:7) directly in the vial. Mass spectra were acquired on an Exactive
225 Orbitrap (ThermoFinnigan, San Jose CA) mass spectrometer coupled to an Accela
226 (ThermoFinnigan, San Jose CA) ultra-performance liquid chromatography system. 20
227 μ L of sample was injected and delivered to the ESI source *via* a flow solvent (mobile
228 phase) or pre-mixed HPLC grade MeOH (Fisher Scientific) and ultra- pure H₂O (18.2
229 Ω) at a ratio of 7:3. The flow rate was 200 μ Lmin⁻¹ for the first 1.5 minutes, and 600
230 μ Lmin⁻¹ for the remainder of the method. The total assay time was 3.0 minutes.

231

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

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

248

249

250 **Quantification of dietary exposure biomarkers by targeted Liquid** 251 **Chromatography- Mass Spectrometry.**

252

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

259 as a result of gut microbiome activity that were expected to appear in urine at later
260 time points. MRM chromatograms were acquired on a TSQ Quantum Ultra QQQ mass
261 spectrometer (ThermoFinnigan, San Jose CA) equipped with a heated electrospray
262 ionisation (HESI) source and coupled to an Accela UHPLC system. The UHPLC
263 system was equipped with either a reverse phase (C₁₈) column or a polymeric
264 Hydrophilic Interaction (pHILIC) column. Full chromatographic details are provided in
265 **Supplemental Section 1**. Mass spectra were acquired using MRM acquisition, in
266 positive and negative ionisation mode simultaneously. Collision energy and tube lens
267 voltage values were optimised individually for each parent – product transition
268 (**Supplemental Table 1**). All post-acquisition data processing was performed using
269 Quan Browser (ThermoScientific) and Xcalibur (Thermo Scientific). Spearman rank
270 correlations (**Table 4 and 5**) were performed using the R function; *cor*. Adjusted
271 coefficient of determination (R^2) values (**Table 4 and 5**) were calculated in R using the
272 *lm* function.

273

274 **Statistical analysis of flow infusion metabolite fingerprinting data**

275 All statistical and classification analysis of flow infusion metabolite fingerprinting data
276 was performed using the R Statistical Programming Language (29). Classification of
277 metabolite fingerprint data was performed using the *randomForest* R package (Version
278 4.6) (30). For all classification models, the dietary intervention (i.e. 25, 50, 75 and
279 100% of WHO healthy eating guidelines) was the response variable. Models were
280 constructed using all *m/z* variables 4574 and 4362, for spot and cumulative samples
281 respectively. For each Random Forest model, the number of trees was 1000 and the
282 number of variables considered at each internal tree node was the square root of the
283 total number of available variables. Each model was assessed using classification

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

289

290 **¹H-NMR acquisition and processing for metabolic fingerprinting and** 291 **quantitative data**

292 Samples for ¹H-NMR spectroscopy analysis were prepared mixing 540 μL of urine with
293 60 μL of a pH 7.4 phosphate buffer as described previously (13). We analysed the
294 samples at 300 K on a 600MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany)
295 using a standard one-dimensional pulse sequence with water-pre-saturation (32). As
296 described previously (13), ¹H-NMR spectra were modelled with Partial Least Squares
297 Discriminant Analysis (PLS-DA) in a repeated-measures Monte Carlo Cross-Validation
298 (MCCV) framework. The mean prediction (T_{pred}) was estimated across the MCCV
299 models where the sample was left-out of modelling. A positive T_{pred} indicated that the
300 urinary metabolic profile of the sample resembled Diet 1 more than Diet 4, and vice
301 versa for a negative T_{pred} . Variable importance was assessed based on bootstrap
302 resampling of regression coefficients in each model, using the False Discovery Rate
303 (q-value) of ≤ 0.01 to indicate significance. Skillings-Mack tests were used to assess
304 differences between the four diets (multiple paired samples) (13). Subsequently,
305 Wilcoxon's signed rank test was used to test pairwise differences and p values from
306 post-hoc tests were adjusted for multiple testing with Hommel's adjustment.

307

308

309 **Results**

310

311 **Dietary exposure is well discriminated by metabolite fingerprinting using spot,** 312 **cumulative or 24-hour urine samples**

313

314 Multidimensional scaling (MDS) of multinomial Random Forest classification models
315 for diet differences in metabolome fingerprint data demonstrated excellent separation
316 between diets with all three urine types from Day 3 (**Figure 1**). It is evident that urine
317 composition reflective of the 4 different diets had already stabilised by Day 2
318 (**Supplemental Figure 1**). These findings were validated by the ¹H-NMR metabolic
319 fingerprinting models (13) that showed good discrimination between dietary
320 intervention on Day 1 (Skillings-Mack $P=8.20 \times 10^{-7}$), Day 2 (Skillings-Mack $P=7.11 \times$
321 10^{-10}) and Day 3 (Skillings-Mack $P=7.21 \times 10^{-9}$) based on 24-hour samples, with the
322 lowest significance values achieved using Day 1 urine samples. Classification
323 performance of CS3 models was comparable with those obtained for PD spot
324 models and 24-hour urine samples for both Day 3 (**Figure 1**) and Day 2
325 (**Supplemental Figure 1**) samples. Comparable results were obtained using ¹H-
326 NMR metabolic fingerprinting modelling which demonstrated that PD spot urine and
327 CS2 and CS3 models all significantly discriminated between dietary treatments, with
328 the PD spot model having the lowest classification values (13).

329

330 **Spot urine samples predicts diet discrimination in corresponding cumulative** 331 **samples**

332

333 Principal Component Analysis (PCA) was performed on an exemplar FIE-HRMS
334 dataset to confirm that sample type was not a confounding factor (**Supplemental**
335 **Figure 2**). The PCA model was constructed using spot samples only and the
336 corresponding cumulative sample data were projected into the PCA model. This
337 showed that sample type did not result in an observable change in model structure.
338 Experimental cross validation was used to determine if discrimination between all diets
339 measured in a spot urine sample type predicted diet correctly in unlabelled urines of a
340 different type (i.e. cumulative or 24-hour). As an example, a Random Forest model
341 was trained to discriminate between the 4 different diets using Day 3 PD spot urine
342 fingerprint data and then diet was predicted in Day 3 24-hour urine samples.
343 Multidimensional scaling (MDS) of predicted Random Forest proximity values
344 demonstrated good correspondence of PD and 24-hour urine collection data following
345 exposure to each of the 4 different diets. Classification performances for all urine type
346 comparisons (using both FIE-HRMS and NMR data) based on Day 3 urine samples
347 are shown in **Supplemental Table 1 and Supplemental Table 2**. In **Table 2** the
348 results are shown where Day 3 PD spot samples only have been used to construct
349 training models and predictions made concerning all samples types on both Day 2 and
350 Day 3. Likewise, cumulative samples were used to predict diet discrimination in 24-
351 hour urine samples, cumulative and spot samples (**Table 3**). The full dataset
352 (**Supplemental Table 1**), in which PB and PL spot samples were used to construct a
353 training model, demonstrates the power of spot urine samples to predict dietary
354 patterns. In summary, 15 out of 30 FIE-HRMS models (**Supplemental Table 1**) yielded
355 a classification accuracy of ≥ 0.9 and only 10 models had performance of $< 0.9 \geq 0.8$
356 A key highlight from the classification modelling was confirmation of the ability of spot
357 samples to predict accurately their corresponding temporal phase cumulative sample.

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

369

370

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

373

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

383 Adjusted R^2 values showed the same distribution in variability as the spearman rank
384 correlation coefficients across the biomarkers. Dihydrophenylpropanoic acid (DHPPA)
385 exhibited a weak correlation between PD and 24-hour urine samples ($\rho = 0.147$, $R^2 =$
386 0.451) and PD versus CS3 ($\rho = 0.147$, $R^2 = 0.451$). In this case, the relatively weak
387 correlations between urine sample types is due to the fact that DHPPA, a biomarker
388 of wholegrain consumption is eliminated from the body relatively slowly over several
389 hours after wholegrain consumption (33,34). Therefore, because the wholegrain
390 containing foods were consumed at breakfast and/or lunch DHPPA excretion is not
391 captured adequately in the PD spot but is present in the 24-hour and CS3 samples.
392 The range of correlation coefficients is reflective the unique absorption, metabolism
393 and excretion dynamics of each of the biomarkers. While there were variations in
394 correlation coefficients, the relative distribution of analytes between sample type
395 remained consistent (see **Supplemental Figure 3** for Quantile-Quantile Plots). In all
396 cases a right skew is visible indicative of the complex dynamic range of dietary
397 biomarkers within urine.

398

399

400 **Micturition characteristics supports sample homogeneity between different** 401 **urine sample types**

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

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

416

417 **Discussion**

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

426

427 24-hour urine collection, for example for determination of sodium intake, is recognized
428 as being expensive and burdensome (35). If urine is to be used for large-scale
429 biomarker-based investigations of dietary exposure, then simpler, less burdensome
430 methods of urine collection are needed. The collection of a single spot urine sample
431 imposes much less burden on individuals compared with 24-hour urine collection and
432 has a reduced impact on an individual's daily activities. An essential criterion of any

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

446

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

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

462

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

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

491

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495

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500 and JD wrote the manuscript and JD had primary responsibility for the final content.
501 All authors read and approved the final version of the paper. The authors have no
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Table 1: Analytical details of biomarkers used for absolute quantification in urine samples by LC-MS and ¹H-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- <i>N</i> -oxide	Fish	76.188 (+)	58.517	8.55	3.27 (s)
3,5-Dihydrophenylpropionic acid	Wholegrain	181.044 (-)	137.132	4.22 *	
3,5-Dihydrophenylpropionic 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- <i>N</i> -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 *	
Quercetin-3-O-D-glucuronide	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)
<i>N</i> -acetyl- <i>S</i> -methylcysteinesulfoxide	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)

<i>N</i> -acetyl-S-(1 <i>Z</i>)-propenyl-cysteinesulfoxide	Onion				1.96 (dd), 2.03 (s), 6.49 (dq), 6.65 (dq)
<i>N</i> -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	Skilings-Mack (<i>p</i> -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 Skilings-Mack *p*-value are from Monte-Carlo cross validation (MCCV) of ¹H 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 (<i>p</i> -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 ¹H 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- <i>N</i> -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- <i>N</i> -acetyl- <i>L</i> -cysteine	0.735	0.858
Rhamnitol	0.251	0.539
<i>O</i> -acetylcarnitine	0.007	0.505
Carnitine	0.225	0.645
Dimethylamine	0.343	0.470
<i>N</i> -acetyl- <i>S</i> -methyl-cysteinesulfoxide	0.247	0.604
Glucose	0.182	0.456
<i>N</i> -acetyl- <i>S</i> -(1 <i>Z</i>)-propenyl-cysteinesulfoxide	0.280	0.578
<i>N</i> -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- <i>N</i> -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- <i>O</i> -sulfate	0.362	0.626
Quercetin-3- <i>O</i> - <i>D</i> -glucuronide	0.104	0.398
<i>D</i> - <i>L</i> -Sulforaphane	0.700	0.774
<i>D</i> - <i>L</i> -Sulforaphane- <i>N</i> -acetyl- <i>L</i> -cysteine	0.752	0.843
Rhamnitol	0.198	0.538
<i>O</i> -acetylcarnitine	0.361	0.608
Carnitine	0.522	0.675
Dimethylamine	0.381	0.589
<i>N</i> -acetyl- <i>S</i> -methyl-cysteinesulfoxide	0.531	0.765
Glucose	0.413	0.556
<i>N</i> -acetyl- <i>S</i> -(1 <i>Z</i>)-propenyl-cysteinesulfoxide	0.775	0.806
<i>N</i> -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.