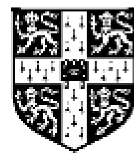
Public Health Nutrition



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Developing community-based urine sampling methods to deploy biomarker technology for assessment of dietary exposure

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Abstract:	Objective: Obtaining objective, dietary exposure information from individuals is challenging because of the complexity of food consumption patterns and the limitations of self-reporting tools (e.g. Food Frequency Questionnaires and Diet Diaries). This hinders research efforts to associate intakes of specific foods or eating patterns with population health outcomes. Design: Dietary exposure can be assessed by measurement of food-derived chemicals in urine samples. We aimed to develop methodologies for urine collection that minimised impact on the day-to-day activities of participants but also yielded samples that were data-rich in terms of targeted biomarker measurements. Setting and Participants: Urine collection methodologies were developed within home settings within different cohorts of free-living volunteers. Results: The home collection of urine samples using vacuum transfer technology was deemed highly acceptable by volunteers. Statistical analysis of both metabolome and selected dietary exposure biomarkers in spot urines collected and stored using this method showed that they were compositionally similar to urine collected using a standard method with immediate sample freezing. Even without chemicals preservatives, samples can be stored under different temperature regimes without any

	significant impact on the overall urine composition or concentration of 46 exemplar dietary exposure biomarkers. Importantly the samples could be posted directly to analytical facilities, without the need for refrigerated transport and involvement of clinical professionals. Conclusions: This urine sampling methodology appears to be suitable for routine use and may provide a scalable, cost-effective means to collect urine samples and to assess diet in epidemiological studies.

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1 **Abstract**

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16 sample freezing. Even without chemicals preservatives, samples can be stored under different
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23 epidemiological studies.

24 **Keywords:** Dietary exposure, metabolomics, biomarkers, home urine collection, population
25 monitoring, cost-effective diagnostic tool

26

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29 **Introduction**

30 Nutrition is a major determinant of health throughout the life-course and eating patterns
31 have a significant impact on the risk of developing common complex diseases including
32 cardiovascular disease, type 2 diabetes, dementia and several cancers [1, 2]. Consequently
33 healthy eating advice and interventions to improve dietary choices are at the core of many
34 public health information strategies internationally [3-5]. The measurement of habitual food
35 intake and the assessment of individual nutritional status provide core information for
36 monitoring population health, are used for exploring relationships between lifestyle choices
37 and health outcomes and in the design of clinical trials [6]. However, because of the complexity
38 of eating patterns and the conceptual and practical difficulties in recording or recalling the types
39 and amounts of foods and beverages consumed, errors in self-reporting of dietary intakes by
40 cognitively-able individuals is commonplace and substantial [7-9]. Such problems may be
41 exacerbated when individuals consume meals out of the home or eat ready-made meals and
42 other pre-prepared foods because they may not know the individual ingredients in these foods
43 or be able to estimate portion sizes accurately [10]. In addition, the instruments used for self-
44 reporting of diet, e.g. Food Frequency Questionnaires, 24h recalls or diet diaries, impose a
45 significant burden both on the individuals reporting their eating behaviour as well as on the
46 researchers subsequently calculating food and nutrient intake. Furthermore, the most
47 vulnerable members of society who are at greatest risk of malnutrition (very old individuals,
48 young children) encounter the most problems with self-reporting and thus alternative or
49 complimentary approaches to monitor diet would have substantial value [11-13].

50 To address these issues, there has been considerable recent interest in the discovery and
51 validation of metabolites derived from individual foods present in urine samples (or other
52 biofluids) that provide biomarkers of dietary exposure and whose measurement may mitigate
53 the limitations of traditional dietary assessment methodologies by providing objective
54 estimates of food consumption [14-16]. However, to provide robust evidence of dietary exposure,
55 such biomarker technology demands development of urine sampling methods that ensure high
56 compliance by populations. Urine collection and sampling kits need to be simple for
57 participants to use in their home-settings with minimal impact on their day-to-day activities
58 and which yield samples that allow comprehensive and reliable quantitation of the targeted
59 biomarkers. Many research studies requiring accurate measurements of exposure biomarkers
60 have adopted the 'gold standard' method of requesting participants to collect all urine over a
61 24-hour period [17]. However, spot urine samples are much less burdensome to collect than 24h
62 urine and there is also a risk that full 24-hr collection may not be achieved in all cases, leading

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63 to inaccurate and misleading results. Recently we have shown that spot urine samples
64 representing specific temporal phases of the day can substitute adequately for 24-hr urine
65 samples [18] for biomarker discovery and habitual dietary exposure measurements [19, 20].
66 Most common procedures for community-based urine sampling require either a
67 dedicated visit by participant/patients to a clinical research centre (CRC) to drop off urine
68 samples, a home visit by a research assistant [21], or a courier service to pick up samples [22].
69 As well as incurring significant costs for travel or transport, such approaches impose logistical
70 challenges, may interfere with the normal daily activities of study participants, and/or place
71 substantial time demands on CRC staff. Additionally, to avoid deterioration of the chemical
72 composition of urine during transport, cooling or refrigeration has been employed which adds
73 further cost. Although the use of chemical preservatives to inhibit the growth of contaminating
74 microbes in urine samples is commonplace [23-27], many of these compounds are strongly ionic
75 and may interfere with analytical methods based on mass spectrometry. In the present
76 manuscript, we report the outcomes of investigations of the feasibility and acceptability of
77 community-based procedures for collection, sampling, preservation and transport of urine
78 samples that are designed to be cost-effective, scalable and suitable for use in large
79 epidemiological studies or for national dietary surveys of populations.
80
81 **Methods**
82
83 ***Study design and urine sampling methods***
84 The overall study is comprised of three independent sub-studies, which aimed to
85 investigate the utility of vacuum transfer tubes for the sampling, in a home environment, of
86 spot urines for dietary biomarker analysis (**Fig 1**). ***Sub-study 1*** and ***Sub-study 2*** were
87 concerned with evaluating the compositional stability of urine samples under different
88 collection and storage conditions. Previous research indicated that analysis of 9 independent
89 urine samples would provide sufficient statistical power for metabolome comparisons [18, 28].
90 However, when dealing with people there is usually more chance of error, drop outs, non-
91 compliance etc, so therefore we aimed to recruit 12-15 individuals for both studies. ***Sub-study***
92 ***3*** recruited 122 free-living individuals and used an on-line questionnaire to assess the
93 acceptability in the home environment of an optimised spot urine sampling method using the
94 vacuum transfer system followed by posting to an analytical laboratory.
95

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96 ***Sub-study (1): Comparison of metabolite stability in vacuum tubes with and without***
97 ***preservative.***
98 The stability of urine chemistry when collected in a vacuum tube containing a
99 lyophilized preservative (Becton and Dickinson Vacutainer® urinalysis preservative tube;
100 chlorhexidine, ethyl paraben and sodium propionate) was compared with stability in a non-
101 coated vacuum tube. In an in-house study in Aberystwyth, 13 healthy individuals (8 male, 5
102 female; 1 smoker and 12 non-smokers; age: 25-60) were recruited and asked to continue
103 consuming their habitual diet. They collected a first morning void (FMV) urine at home and
104 dispensed this into 5 replicate preservative-containing vacuum tubes and 5 replicate non-coated
105 vacuum tubes using the vacuum transfer method utilising a plastic collection vessel (100 mL)
106 along with a separate transfer straw (Becton and Dickinson; as illustrated in **Supplementary**
107 **Material 1D**). These samples were stored at 4 °C in the participants' domestic fridges and then
108 transported to the research facility to be subjected to a series of storage treatments that
109 mimicked conditions likely to be encountered if samples remained for several days in a
110 domestic environment. A sample in each tube type was stored at -20ºC, deemed as 'control
111 storage' to mimic the conditions typical of a domestic freezer. A sample in each tube type was
112 subjected to the following 4 storage conditions: at 4 °C (2 days or 7 days) before freezing at -
113 80 °C; 2 or 7 days at room temperature (RT) before freezing at -80 °C.
114
115 ***Sub-study (2): Comparison of metabolite stability in preservative-free vacuum tubes***
116 ***versus a traditional spot urine collection method.***
117 Fifteen, free-living individuals (8 male, 7 female; non-smokers; age: 21-74) were recruited
118 from the Human Nutrition Research Centre, Newcastle University database. Each participant collected
119 FMV urine samples at home using the vacuum transfer system (**Supplementary Material 1C**) as well
120 as the traditional plastic jug and Universal tube method (**Supplementary Material 1A**). Samples were
121 collected using both methods on three consecutive days during which the participants consumed
122 different meals as part of a separate dietary intervention study (**Supplementary Material 2**), reported
123 elsewhere [29]. Written instructions on how to collect FMV urine samples using both methods were
124 provided for the 15 participants, but no verbal one-to-one guidance was given. All samples were stored
125 at home at 4 °C for up to 4 days and then brought to the research facility in Newcastle in a cooler bag
126 at the end of the study week. Universal tubes were stored immediately at -80 °C and the vacuum tubes
127 remained at 4 °C for a further 2 weeks before storage at -80 °C. Samples were then transported to the
128 analytical facility in Aberystwyth on dry ice for metabolite stability analysis.
129

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130 ***Sub-study (3): Evaluation of acceptability of vacuum transfer system for urine***
131 ***collection in the home environment.***

132 In a third study we recruited 122 healthy volunteers (28 male, 94 female; smokers and
133 non-smokers; age 18-64) by text message and e-mail invitation after a large scale survey on
134 eating habits. These volunteers were free-living and were asked to maintain their habitual diet.
135 A kit containing a urine collection container, transfer straw, vacuum tubes (as shown in
136 **Supplementary Material 1D**, where there was enough vacuum tubes to collect three randomly
137 spaced FMV urines over a week) and a Royal Mail Safebox^{TM} were posted to each individual.
138 Urine samples were collected and stored at home at 4 ºC and then posted back in the Royal
139 Mail Safebox^{TM}. The Safeboxes had prepaid first class postage, with the aim to arrive back at
140 the research centre within 1-2 working days. The volunteers were asked to complete an online
141 questionnaire about the acceptability of aspects of spot urine collection methodology
142 (**Supplementary Material 3**). The online questionnaire had 13 questions which asked
143 participants to rank the extent of their agreement with each statement on a five point scale from
144 "strongly disagree" to "strongly agree". Responses were analysed as % of overall feedback.
145

146 ***Optical density measurement to assess bacterial growth***

147 After storage treatments the urine samples were mixed via vortex and 100 µL aliquots,
148 in duplicate, were added to 96 well flat bottomed microtiter plates. The optical density of
149 samples was determined using a Hidex Sense Microplate Reader (model 425-301), with
150 absorbance set at 600 nm. Samples were read three times in Hidex PlateReaderSoftware
151 v0.5.11.0 at 37 °C, with agitation between readings.
152

153 ***Urine sample normalisation***

154 All urine samples were normalized by refractive index prior to analysis to ensure all
155 MS measurements were made within a similar dynamic range. Samples were defrosted
156 overnight in a 4 °C fridge. Once defrosted, samples were centrifuged (600 x g for 5 mins at 4
157 °C), placed on ice and aliquots of thawed urine (1000 µL) was transferred into labelled 2 mL
158 Eppendorf tubes. An OPTI Hand Held Refractometer (Bellingham Stanley™ Brix 54 Model)
159 was calibrated with de-ionised water (dH_2O) and dried with paper tissue according to the
160 manufacturer's instruction. Following this 220 µL of sample was transferred onto the
161 refractometer dish, the specific gravity (SG) value was recorded in triplicate and temperature
162 was noted. The refractometer was rinsed with dH_2O between samples and dried with tissue.

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163 Based on these figures, aliquots of the required amounts of urine were diluted with
164 dH_2O in 2 mL Eppendorf tubes to make up to a total volume of 500 µL. Extraction was
165 performed by adding 500 µL of pre – chilled MeOH (Extraction Grade, Fisher Scientific).
166 Samples were vortexed then placed on an orbital shaker (FATSM002, Favorgen Biotech Corp)
167 for 20 minutes at 1,400 rpm and 4 ºC in the dark. All extracted samples were stored at -80 ºC
168 until further analysis.

169

170 ***Non-targeted Flow Infusion-High Resolution Mass Spectrometry (FIE-HRMS)***
171 Urine samples were analysed using flow infusion electrospray ionisation (FIE) high
172 resolution (HR) mass spectrometry (MS) to generate a non-targeted metabolome fingerprint.
173 For this purpose, 20 µL of extracted sample was transferred to a glass HPLC vial containing a
174 200 µL flat bottom micro insert (Chromacol). All samples were diluted with 80 µL of
175 H_2O:MeOH (3:7) directly in the vial. Mass spectra were acquired on an Exactive Orbitrap
176 (ThermoFinnigan, San Jose CA) mass spectrometer coupled to an Accela (ThermoFinnigan,
177 San Jose CA) ultra-performance liquid chromatography system. 20 µL of diluted sample was
178 injected and delivered to the electrospray source *via* a flow solvent (mobile phase) of pre-mixed
179 HPLC grade MeOH (Fisher Scientific) and ultra-pure H_2O (18.2 Ω) at a ratio of 7:3. The flow
180 rate was 200 µL min^{-1} for the first 1.5 minutes, and 600 µLmin^{-1} for the remainder of the
181 analysis. The total run time was 3.0 minutes.
182 Positive and negative ionisation modes were acquired simultaneously. For each
183 ionisation mode; one scan event was used to acquire all mass spectra, 55.000 - 1000.000 *m/z*
184 and 63.000 - 1000.000 *m/z* for positive and negative mode respectively. The scan rate was 1.0
185 Hz. Mass resolution was 100,000, with automatic gain control 5×10^5 and maximum injection
186 time 250 ms, for both ionisation modes. Following data acquisition, raw profile data (.raw;
187 ThermoFinnigan) were converted to the .mzML open file format and centroided [(30)] using
188 msconvert (TransProteomicPipeline) [(31)]. All further processing of mzML files was performed
189 using the R Statistical Programming Language [(32)].
190 Dimensionality reduction of the acquired mass spectra was performed by taking each
191 *m/z* value from scans about the apex of the infusion profile and binning the *m/z* and intensity
192 values at 0.01 amu intervals. The result was a *n x p* matrix, where *n* is the sample and *p* is the
193 *m/z* feature and cells are the respective average intensity values.
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195 ***Targeted metabolite quantification using Ultra-High Performance Liquid Chromatography***
196 ***(UHPLC) and mass spectrometry***
197 Absolute concentrations of selected dietary exposure biomarkers (see table in
198 **Supplementary Material 4**) were measured using ultra-high performance liquid
199 chromatography (UHPLC) triple quadrupole (QQQ) mass spectrometry (MS) operating in
200 Multiple Reaction Monitoring (MRM) mode [20, 29]. MRM chromatograms were acquired on a
201 TSQ Quantum Ultra QQQ mass spectrometer (ThermoFinnigan, San Jose CA) equipped with
202 a heated electrospray ionisation source and coupled to an Accela UHPLC system.
203 The UHPLC system was equipped with either a (Thermo-Scientific Hypersil Gold
204 reverse phase (C_{18}) column (1.9 µm, 200 x 2.1mm) or a Merck ZIC-pHILIC column (polymeric
205 5 µm, 150 x 4.6 mm) (see **Supplementary Material 4** for details on the chromatography
206 column used for each dietary biomarker). Mass spectra were acquired using MRM acquisition,
207 in positive and negative ionisation mode simultaneously. Collision energy and tube lens voltage
208 values were individually optimised for each parent–product transition measured (see
209 **Supplementary Material 4** for optimised values for each measured transition). All post–
210 acquisition data processing was performed using Quan Browser (ThermoScientific) and
211 Xcalibur (Thermo Scientific).
212
213 ***Analysis of non-targeted metabolite fingerprint data***
214 Supervised classification of fingerprint data was performed using Random Forest (RF)
215 classification using the randomForest package [33], in R [32]. For all RF models, the number of
216 trees (*ntree)* used was 1000 and the number of variables considered at each internal node (mtry)
217 was the square root of the total number of variables. Accuracy, margins of classification and
218 area under the ROC (Receiver Operator Characteristic) curve (AUC) were all used to evaluate
219 the performance of classification models, as described previously [34]. RF classification models
220 were plotted following multi-dimensional scaling (MDS). Proximity measures for each
221 individual observation were extracted from RF models and scaled coordinates produced using
222 *cmdscale* on 1 – proximity.
223
224 ***Analysis of quantitative data from targeted metabolite profiling***
225 Kruskal-Wallis and paired *t*-test was used to determine significance difference between
226 the classes. All *p*-values are corrected for multiple testing using a Bonferroni correction.
227

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228 **Results**

229 In a preliminary experiment, we evaluated public perceptions of the three different
230 home-collection methods for spot urine sampling shown in **Supplementary Material 1A-C**.
231 The results showed that all three procedures were perceived to be acceptable by the general
232 public (results shown in **Supplementary Material 5**), with no significant differences observed
233 in the mean acceptability scores for each method (*p*-value 0.85, Kruskal- Wallis Test). In the
234 present study we evaluated vacuum tube technology utilising a separate transfer straw
235 (**Supplementary Material 1D)** which offered scope to collect multiple spot urines in a home
236 environment that could be posted to an analytical facility, potentially without the need for
237 refrigeration to preserve sample composition. The overall study was comprised of three
238 independent sub-studies (**Fig 1**). ***Sub-study (1)*** explored the need for chemical preservatives
239 in vacuum tubes, whilst ***Sub-study (2)*** tested the performance of vacuum tube technology to
240 maintain the compositional stability of urine samples in comparison to traditional methods for
241 urine collection, which required sample freezing; a range of storage treatments were evaluated
242 which mimicked conditions typically encountered in home environments. ***Sub-study (3)*** used
243 an on-line questionnaire in a free-living population to assess the acceptability of an optimised
244 spot urine sampling method potentially suitable for large-scale epidemiological studies.
245
246
247 ***Stability of urine metabolites after short to medium term storage in vacuum tubes maintained***
248 ***under different temperature regimes, with and without a preservative***
249
250 FIE-HRMS fingerprints were generated for each urine sample and multi-dimensional
251 scaling plots of RF proximity scores from supervised classification models were used to
252 determine whether the presence of preservative had an impact on overall urine chemical
253 composition following exposure to storage regimes likely to be encountered during home
254 collections spanning several days (**Fig 2**). Most participants had distinctly individual urine
255 metabolomes, with their urine samples after each different storage temperature regime
256 clustering together (**Fig 2A**). Inclusion of a preservative had little discernible impact on sample
257 clustering patterns (**Fig 2B**). RF classification analysis of FIE-HRMS fingerprint data was used
258 to quantify the overall compositional differences in binary comparisons between each treatment
259 and the -20 ºC control (which mimicked storage in a typical domestic freezer). Classification
260 accuracies and AUC values < 0.4 and RF margins < 0.2 indicated that storage temperature

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261 regimes in either the coated or non-coated tube had no significant impact on overall urine
262 composition (**Table 1**).

263 Absolute concentrations of selected biomarkers covering a wide range of foods were
264 measured in urine samples collected in the presence or absence of chemical preservatives after
265 exposure to range of temperature regimes (**Supplementary Material 6**). In line with the
266 previous results from metabolome fingerprinting, **Fig 3** shows for selected biomarkers that the
267 main source of variance was the individual participants, with only a small influence of storage
268 regime. Statistical analysis (Kruskal-Wallis) of this data comparing the effect of all storage
269 treatments on biomarker concentrations, in the presence and absence of a preservative
270 (**Supplementary Material 7**) revealed that only 4 of the 46 biomarkers (1-Methyl-histidine,
271 Daidzein, Ferulic acid and Tryptophan) had a p-value < 0.05 after correction for multiple
272 testing. In vacuum tubes with a lyophilized preservative there appeared to be degradation of 1-
273 Methyl-histidine after all storage conditions when compared to the -20 °C control. Daidzein
274 concentration specifically was affected by storage at room temperature for 7 days (**Fig 3**),
275 showing an increase in concentration in both the uncoated vacuum tube and vacuum tube with
276 a lyophilized preservative. Ferulic acid increased in the uncoated vacuum tube after 2 days at
277 4 °C. Tryptophan concentration significantly increased in the coated tubes after stoage. In
278 general, the presence of preservative had little additional impact on biomarker concentrations.
279 Small, but non-significant increases in optical density of urine samples were evident after 2
280 days incubation at room temperature (**Supplementary Material 8**) with little difference in
281 microbial growth in vacuum tubes containing a preservative compared with non-coated
282 vacuum tubes.

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283

284 ***Compositional analysis of spot urines collected and stored in a community setting using the*
285 *traditional jug and Universal tube method and the vacuum transfer system***

286

287 The chemical composition of urines collected in the home on three separate days using
288 either a traditional jug and Universal tube or a commercial vacuum transfer system (***Sub-study*
289 *2***) were compared using metabolite fingerprinting. The different diets consumed on each of the
290 three experimental days (**Supplementary Material 2**) were clearly evident in the FIE-HRMS
291 data following multi-dimensional scaling (MDS) of Random Forest (RF) proximity values (**Fig**
292 **4**). However, the chemical fingerprint data of the urines collected and stored using the two
293 sampling methods overlapped for each day, indicating compositional similarity. The summary

294 statistics for RF binary classification [34] of FMV spot urines samples collected on each food
295 intervention day (**Table 2**) indicate no detectable differences in overall chemical composition
296 of urines collected by the two different methods.
297 The stability of exemplar dietary exposure biomarkers was examined in urine derived
298 from both collection methods after absolute quantification using a targeted analysis method.
299 These previously published dietary exposure biomarkers (**Supplementary Material 4**)
300 represent a range of chemical classes for which standards were commercially available**.** The
301 paired *t*-test statistics (**Table 3**) revealed very few differences in biomarker concentration (only
302 seven biomarkers had an adjusted p-value ≤ 0.05; 1-Methyl-histidine, 4-Hydroxyhippuric-acid,
303 Hippuric-acid, Proline-betaine, Carnitine, Tryptophan and Ferulic acid-4-*O*-sulfate) between
304 urine samples collected and stored using the two collection methods.
305
306 ***Evaluation of study participants' acceptability of a postal method to collect urine samples in***
307 ***a community setting***
308
309 The demonstration that overall urine composition was stable when stored for up to a
310 week at 4 ºC in non-coated vacuum tubes and that the majority of dietary exposure biomarkers
311 concentrations were largely unaffected under these storage conditions, offered the opportunity
312 to explore the possibility of collecting urine samples in a community setting without the need
313 to visit a clinical research centre for sample drop-off. Free-living volunteers (n=122) were
314 asked to follow their usual diet and to collect three FMV urines on random days over a period
315 of a week using the vacuum tube and transfer straw method (**Supplementary Material 1D**).
316 Volunteers were asked to complete an online questionnaire assessing the acceptability of
317 various steps in the process of collecting, storing and posting urine samples (**Supplementary**
318 **Material 3**). Overall, the volunteers indicated a high acceptability of this method of home
319 collection, storage and posting of urine samples (**Fig 5**). The only question that showed a high
320 'neutral' response was 'I would have preferred to collect urine samples at a different time of
321 day'. In response to the last question (Q13) 'I think collecting a urine sample out of the home
322 environment is embarrassing', 38% of volunteers reported a negative answer (either agreeing
323 or strongly agreeing with the statement) compared with only 2% providing a negative response
324 for Q12; 'I think collecting urine samples in a home environment is embarrassing'. Although
325 Q13 recorded the largest negative response, 49% of recorded responses were positive with a
326 further 13% neutral.
327

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328 ## Discussion

329

330 A key observation in the present study is that spot urine samples collected using a
331 vacuum-transfer method are generally compositionally stable for several days in a domestic
332 fridge or at room temperature, even in the absence of a chemical preservative. From a practical
333 perspective the collection procedure was highly acceptable to study participants and the small
334 (6 mL) vacuum tubes could be posted via the domestic mail system in the UK, avoiding the
335 need for a visit to a clinical research centre.

336 Urine provides a rich source of objective information on dietary intake (and other
337 chemical exposures) [18, 20, 28, 35] and is a relatively non-invasive sample which participants can
338 collect in their home-settings. For high compliance to study protocols urine collection methods
339 need to be acceptable to participants, particularly with regard to hygiene and any adverse
340 impact on normal daily activities. In earlier work, we utilised a spot urine sampling
341 methodology by participants in home settings using the traditional plastic jug followed by
342 decanting of the sample into a smaller vessel, in this case a 30 mL Universal tube, for transport
343 to the laboratory [35, 36]. However, urine spillage may occur during the decanting process
344 resulting in potential contamination of both participant and the outside of the transport vessel,
345 and potentially exposing research staff and study participants/patients to microbial infection.
346 Bespoke kits that avoid contamination during decanting of urine are commercially available
347 including devices with a collection tube integrated into a 'flow through' collection vessel (e.g.
348 Peezy) and several alternative kits (e.g. Vacutest (Kima) and Vacutainer devices (Becton,
349 Dickinson and Company) that utilize a vacuum transfer system to draw up small volumes under
350 partial vacuum via a needle into transport tubes (**Supplementary Material 1**). Using an online
351 questionnaire, we demonstrated that members of the general public reported high, and similar,
352 acceptability for all three methods proposed for collection of spot urine samples
353 (**Supplementary Material 5)**. The vacuum transfer system was then explored in more detail
354 as it offered, additionally, an opportunity to evaluate whether storage under vacuum would help
355 preserve urine composition during storage in the home and transport without a need for
356 maintaining a 'cold chain', thus greatly increasing logistical flexibility as well as reducing
357 costs.

358 In ***Sub-study (1)*** we determined whether the use of vacuum tubes containing a
359 lyophilized preservative would further improve stability when urine samples were exposed to
360 range of conditions likely to be experienced during the collection and transport process. It is

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361 worth noting that the manufacturer states that the preservative stabilizes urine over 72 h without
362 the need for refrigeration, however we tested storage at -20ºC, refrigeration (2 and 7 days) and
363 RT (2 and 7 days) [37, 38]. Using triple quadrupole mass spectrometry multiple reaction
364 monitoring methodology we discovered that the concentration of a wide selection of dietary
365 exposure biomarker signals after each storage condition were very similar, irrespective of the
366 presence of preservative. The data suggest that the majority of concentration changes occurring
367 during storage were unlikely to be derived from bacterial activity. For example, degradation
368 of 1-Methyl histidine in the vacuum tube with and without a lyophilized preservative was
369 evident under all storage conditions when compared to the -20 °C control. Daidzein remained
370 stable at 4 °C with and without preservative but concentration increased after 2 and 7 days at
371 RT suggesting possible a breakdown of daidzein conjugates (glycines, glucuronides, sulfates
372 and sulfoglucuronides) into the aglycone [39], again unrelated to bacterial activity. In contrast,
373 ferulic acid a major microbial degradation product of dietary polyphenols [40], increased in the
374 uncoated vacuum tube which may reflect limited microbial activity. These observations
375 support our hypothesis that use of the vacuum tube (without preservative) and straw collection
376 method minimises dietary biomarker degradation by avoiding ingress of contaminating
377 microbes from the environment and through the low level of oxygen in the sample tubes which
378 limits microbial growth and oxidative degradation of the urine samples. The fact that inclusion
379 of a chemical preservative within the vacuum tubes provided little improvement in dietary
380 intake biomarker stability is an important consideration from an analytical perspective. This is
381 because as the lyophilized coating containing chlorhexidine, ethylparaben and sodium
382 propionate becomes solubilised the concentration of these compounds in the urine sample can
383 vary, depending on the volume of urine drawn into the tube. As a consequence, variable
384 amounts of these compounds can dominate compositional differences between samples and
385 can interfere with metabolome assessment. Other strongly ionic common urine preservatives,
386 such as boric acid, interfere with ionization behavior in MS and are to be avoided whenever
387 possible (as reviewed by [41]).

388 Previous research has documented the impact of sample collection and storage
389 conditions on the metabolic composition of human urine. In standard, non-vacuum tubes, high
390 resolution metabolic fingerprinting has demonstrated that the urinary metabolome is altered by
391 storage at room temperature from 24 to 72 h [26]. In contrast, urine samples stored at -20 °C
392 exhibited global stability over a long period when compared with urine stored at -80 °C [23, 24].
393 Other publications have reported no major changes in urinary metabolite fingerprints when
394 stored in non-vacuum tubes held at 4 °C for up to 72 h [26] but compositional modifications

395 have been observed with storage over longer periods [23-25]. Storage of urine in vacuum tubes
396 at -85 °C and then for up to 24 h at 4 °C did not affect metabolic profiles assessed by NMR or
397 GC-TOF-MS [42-44]. In ***Sub-study (2)*** we expanded research on the effects of storage conditions
398 on urine samples by simulating possible 'real-life' situations in which urine was stored 4 °C
399 for up to 4 days (simulating storage at home in a domestic fridge). Initial observations indicated
400 that the impact of storage conditions on the urinary metabolome was much smaller than the
401 distinctive inter-individual differences in urine metabolome when we compared specifically
402 the overall chemical composition of urine samples collected by the traditional jug and
403 Universal tube method (followed by immediate freezing at -80ºC) with a commercial vacuum
404 transfer system. The data indicated no major differences in overall chemical fingerprints of
405 urines between methods on each of the three intervention days during which very different
406 foods were consumed. Additionally, there were no concentration differences for the majority
407 of a large range of dietary exposure biomarkers covering multiple chemical classes. Overall,
408 this provided evidence that using the vacuum transfer system the patterns, and concentrations,
409 of key dietary biomarkers and chemical groups were stable over several days in a domestic
410 fridge without the need for immediate freezing.
411 It is unlikely that analysis of a single spot urine sample can provide robust data on
412 habitual dietary intake at the individual level and this issue is particularly acute for foods which
413 are eaten infrequently and/or at irregular intervals. Additionally, as excretion kinetics may also
414 differ between biomarkers [19] there may be a need to take samples at more than one time point
415 in a study day to ensure measurement sensitivity. Therefore, to provide data on habitual
416 exposure, multiple spot urine samples over several days would need to be collected and stored
417 by participants at home before transport to the analytical research facility. In ***sub-study (3)*** we
418 tested the acceptability of this idea by posting urine collection kits to free-living participants
419 (n=122) who were asked to follow their usual diet and to collect three FMV urines at home
420 (using the vacuum tube and transfer straw method) on random days over a period of a week
421 and then post samples back to the research centre. Acceptability of this protocol for urine
422 collection, storage and posting of urine samples was high and there was evidence that collecting
423 multiple samples in a home environment was preferable to visiting a clinical research facility
424 for sample collection.
425
426 To conclude we have developed and tested a spot urine collection methodology that is
427 intended to provide urine samples that are suitable for analysis of dietary exposure biomarkers.
428 We have demonstrated that this methodology is acceptable to members of the general public

429 for use at home and in community settings. To assist with evaluation of habitual dietary
430 exposure, multiple spot urine samples can be collected at home throughout a typical week and
431 stored in the fridge without significant degradation of the metabolite composition. In addition,
432 the vacuum tubes containing these urine samples can then be posted directly, without the need
433 for preservatives or refrigerated transport and without involvement of clinical professionals, to
434 a remote analytical facility for archiving and subsequent analysis.

435

436

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558 **Legends for figures**

559

560 **Fig 1**: Schematic of overall study design.

561

562 **Fig 2**: Multi-dimensional scaling (MDS) plots of Random Forest proximity scores from
563 supervised classification models of FIE-HRMS fingerprint data using storage treatment as the
564 response value. Storage treatments were as follows; Control, -20 ºC, T1, 2 Days at 4 ºC, T2, 7
565 Days at 4 ºC; T3, 2 Days at room temperature (RT); T4, 7 Days at RT. (**A)** preservative coated
566 tubes; **(B)** non- preservative coated tubes. Samples are coloured by individual and shapes
567 indicate treatment.

568

569 **Fig 3**: Box-plots of selected dietary biomarkers showing stability in vacuum tubes and impact
570 of preservative after exposure to various storage conditions. Where VT, non- preservative
571 coated vacuum tube; CVT, preservative coated vacuum tube (A) VT- 1-Methyl-histidine; (B)
572 CVT- 1-Methyl-histidine; (C) VT- Daidzein; (D) CVT- Daidzein; (E) VT- Ferulic acid; (F)
573 CVT- Ferulic acid; (G) VT- Tryptophan; (H) CVT- Tryptophan. Where: -20 (storage at -20
574 ºC); T1, 2 days at 4 °C; T2, 7 days at 4 °C; T3, 2 days at room temperature (RT); T4, 7 days at
575 RT.

576

577 **Fig 4**: Multi-dimensional scaling (MDS) plots of Random Forest proximity values from
578 supervised classification models of FIE-HRMS of two different first morning void (FMV)
579 urine collections (Universal tube and Vacuum tube) over three different dietary intervention
580 days.

581

582 **Fig 5**: Summary (as % of overall feedback) of responses to the 13 self-recorded urine collection
583 acceptability questions*. * See **Supplementary Material 5** for details.

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589 **Tables**

590
591
592 **Table 1**: Summary statistics for binary classification by Random Forest of FMV spot urines stored under different conditions within
593 preservative-coated and non-coated vacuum tubes

Pairwise	Preservative Coated Tube			Non-coated Tube		
	Accuracy	AUC	Margin	Accuracy	AUC	Margin
Control vs 2 Days at 4 ºC	0.30	0.24	-0.11	0.23	0.16	-0.16
Control vs 7 Days at 4 ºC	0.26	0.20	-0.13	0.20	0.15	-0.18
Control vs 2 Days at RT	0.30	0.26	-0.12	0.21	0.16	-0.16
Control vs 7 Days at RT	0.31	0.25	-0.10	0.27	0.20	-0.14
2 Days at 4 ºC vs 7 Days at 4 ºC	0.29	0.25	-0.11	0.20	0.13	-0.19
2 Days at 4 ºC vs 2 Days at RT	0.31	0.28	-0.11	0.24	0.16	-0.16
2 Days at 4 ºC vs 7 Days at RT	0.30	0.24	-0.12	0.27	0.20	-0.14
7 Days at RT vs 2 Days at RT	0.27	0.19	-0.15	0.25	0.18	-0.15
7 Days at 4 ºC vs 7 Days at RT	0.29	0.23	-0.12	0.25	0.19	-0.14
2 Days at RT vs 7 Days at RT	0.28	0.23	-0.12	0.22	0.16	-0.16

594

595 Where: Control (storage at -20 ºC); room temperature (RT); FMV, First Morning Void; Accuracy, classification accuracy; AUC, area under the ROC (receiver
596 operating characteristic) curve; Margin, RF classification margin

597

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598 **Table 2**: Summary statistics for pairwise comparisons between FMV urines collected using
599 the Universal and Vacuum transfer method by RF on three different food intervention days
600

Menu Day	Accuracy	AUC	Margin
Day 1	0.47 (0.45,0.50)	0.47 (0.44. 0.51)	-0.02 (-0.03, -0.01)
Day 2	0.38 (0.35, 0.40)	0.36 (0.33, 0.39)	-0.06 (-0.07, -0.05)
Day 3	0.48 (0.46, 0.50)	0.48 (0.46. 0.50)	-0.02 (-0.03, -0.01)

601

602 Where: RF, Random Forest; FMV, First Morning Void; Accuracy, classification accuracy; AUC, area
603 under the ROC (receiver operating characteristic) curve; Margin, RF classification 'margin'. Shown in
604 brackets are the 95% CI (confidence interval)
605

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606 **Table 3**: Paired *t*-tests were used to determine significance difference of biomarker
607 concentration between the standard Universal collection and Vacuum transfer method,
608 irrespective of menu or individual effects of 15 participants who collected first morning void
609 (FMV) urine on three separate days in a home setting.

Biomarker	*t*-Statistic	*p*-value*
1-Methyl-histidine	-4.725	<0.001
3-Hydroxyhippuric-acid	2.644	0.506
3-Methyl-histidine	-2.465	0.828
3-Methyl-xanthine	-0.659	1.000
4-Hydroxyproline-betaine	-3.414	0.064
4-Hydroxyhippuric-acid	4.075	0.009
7-Methyl-xanthine	1.703	1.000
Acesulfame-K	-0.128	1.000
Anserine	0.536	1.000
BOA-1-3-Benzoazol-2-one	-0.842	1.000
Caffeine	-2.564	0.636
Carnitine	-4.309	0.004
Carnosine	-0.664	1.000
Creatinine	-1.690	1.000
D-L-Sulforaphane-glutathione	-0.555	1.000
D-L-Sulforaphane-L-cysteine	1.632	1.000
D-L-Sulforaphane-N-acetyl-L-cysteine	1.412	1.000
Daidzein	0.761	1.000
DHBA	-0.506	1.000
DHBA-3-O-sulfate	2.360	1.000
DHPPA	-1.381	1.000
DHPPA-3-sulfate	0.056	1.000
Epicatechin	0.794	1.000
Ferulic-acid	1.912	1.000
Ferulic-acid-4-O-b-D-glucuronide	-1.140	1.000
Ferulic-acid-4-O-sulfate	3.611	0.036
Hippuric-acid	7.391	<0.001
Indoxyl-sulfate	1.187	1.000
L-Phenylalanine	-1.660	1.000
Tryptophan	-3.582	0.039
N-2-Furoyl-glycine	0.939	1.000
Naringenin	-1.583	1.000
p-Cresol-glucuronide	-2.677	0.478
p-Cresol-sulfate	1.622	1.000
Phenyl-acetyl-L-glutamine	0.933	1.000
Proline-betaine	-3.955	0.012
Protocatechuic-acid	-2.518	0.736
Quercetin	1.067	1.000
Quercetin-3-O-b-D-glucuronide	1.277	1.000

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Resveratrol	0.490	1.000
Rhamnitol	0.725	1.000
Sucrose	0.647	1.000
Tartarate	-1.574	1.000
Taurine	-1.561	1.000
Trigonelline	-2.414	0.920
Trimethylamine-N-oxide	-1.678	1.000

610

611 *All *p*-values are adjusted for multiple testing using Bonferroni correction.

612

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613 **Supplementary Material**

614

615 **Supplementary Material 1:** Assessing commonly used and novel methods of spot urine
616 sample collection and storage. (A) a standard 500 mL jug and aliquoting into a 30 mL
617 Universal tube; (B) the 'Peezy' urine collection device into a 30 mL Universal tube; (C)
618 vacuum transfer system using a 60 mL collection container with integral needle and a 6mL
619 vacuum tube; (D) vacuum transfer system using a 100 mL collection container, a separate
620 urine transfer straw and a 6 mL vacuum tube.

621

622 **Supplementary Material 2**: Meal plans in ***Sub-study (2)*** [29].

623

624 **Supplementary Material 3**: Vacuum transfer system questionnaire on acceptability of the
625 postal method for sample return.

626

627 **Supplementary Material 4**: Biomarkers used for absolute quantification in urine samples.

628

629 **Supplementary Material 5**: Perception of different spot urine collection methods.
630 (A) Online questionnaire to select spot urine collection methods acceptable for use in the home
631 environment; (B) Results of Questionnaire

632

633 **Supplementary Material 6**: Absolute quantification in selected first morning void (FMV)
634 urine samples stored in coated and non-coated vacuum tubes.

635

636 **Supplementary Material 7**: **S**tatistical analysis (Kruskal-Wallis) of absolute concentrations
637 of dietary exposure biomarkers in selected first morning void (FMV) urine samples stored in
638 coated vacuum tubes and vacuum tubes containing a lyophilized preservative.

639

640 **Supplementary Material 8:** Box-plot of Optical Density (OD) (at 600 nm) of storage
641 treatments.

642

643

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Figure 1

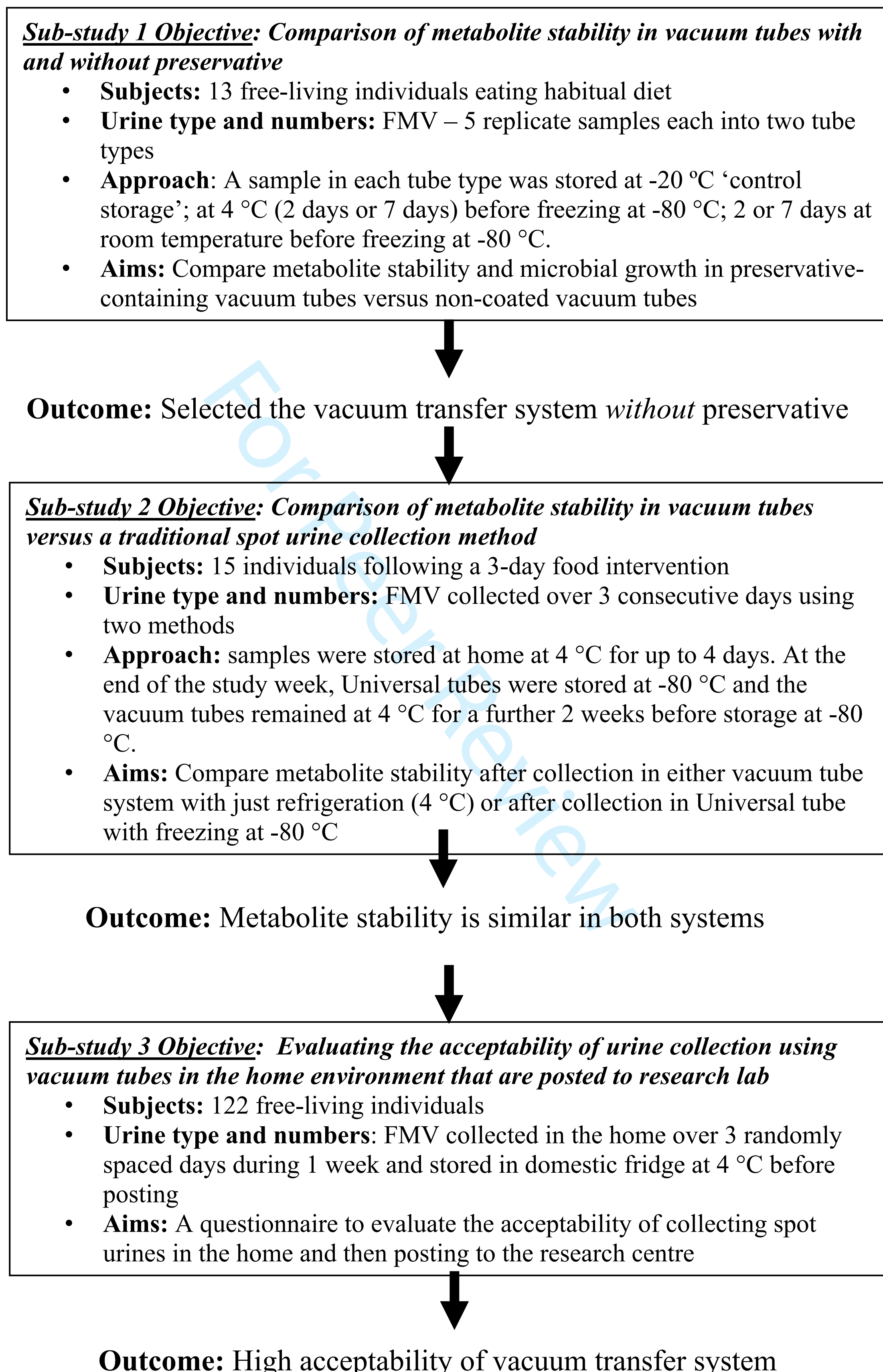

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Fig 2

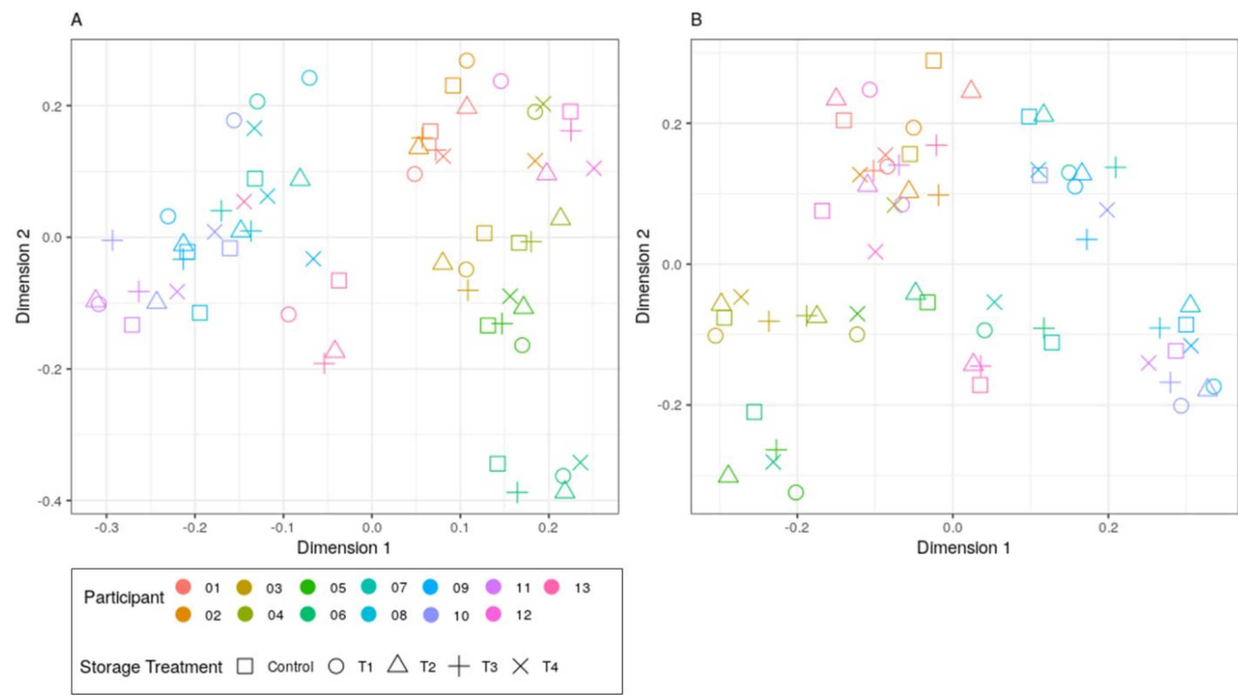


Fig 3:

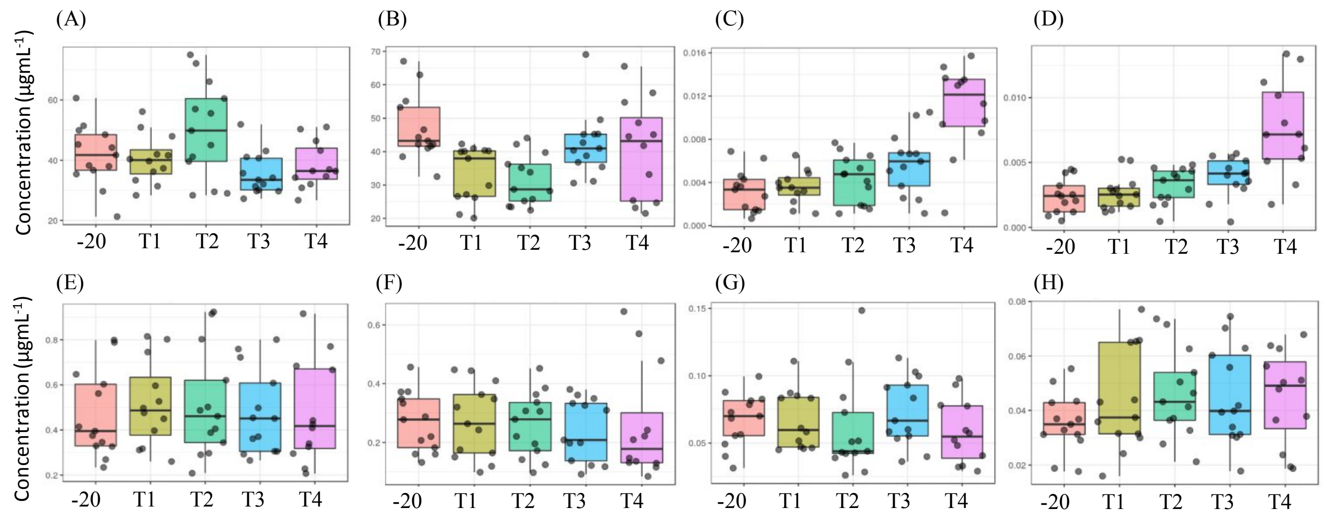


Fig 4

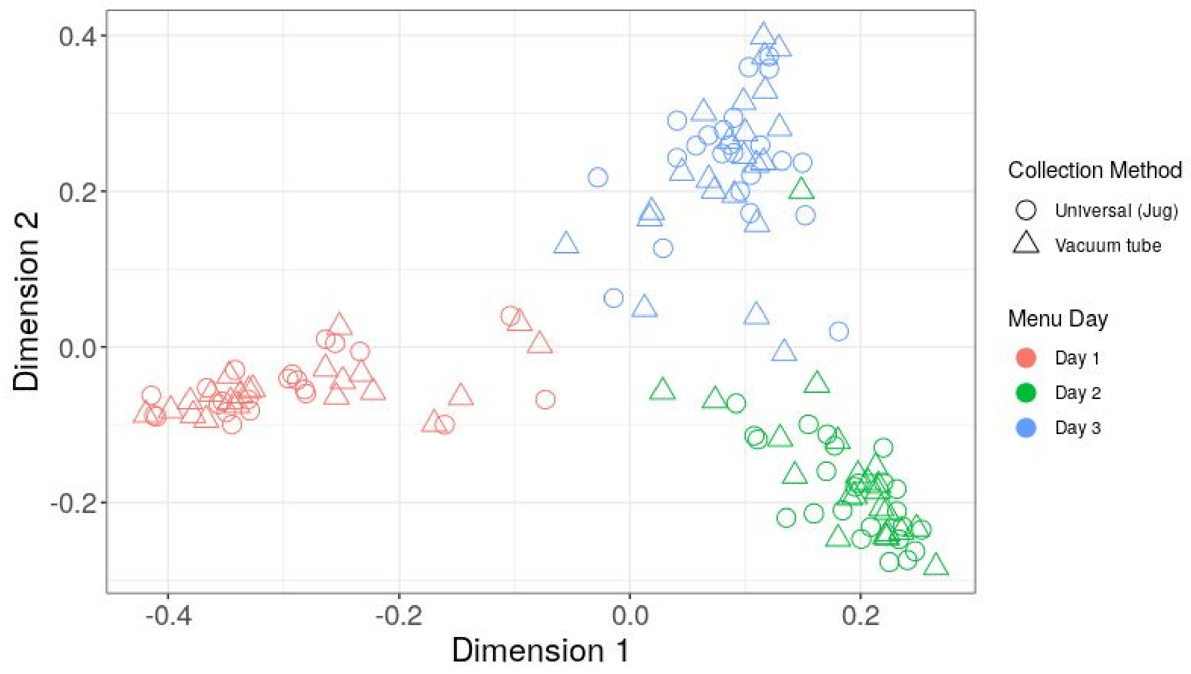
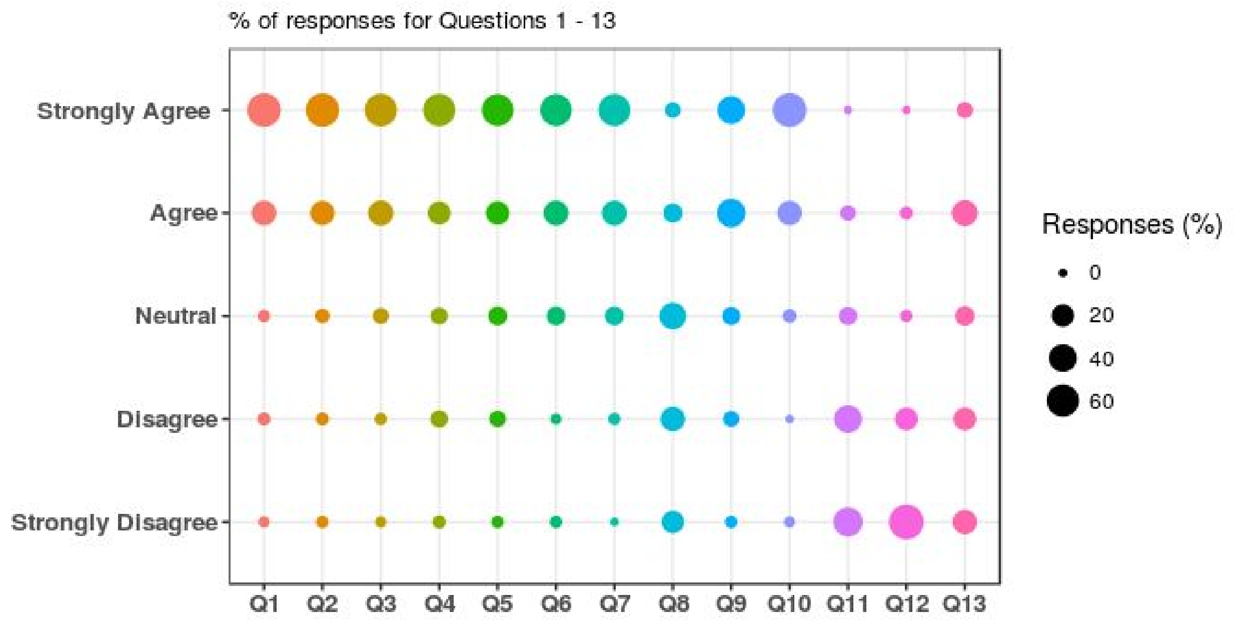


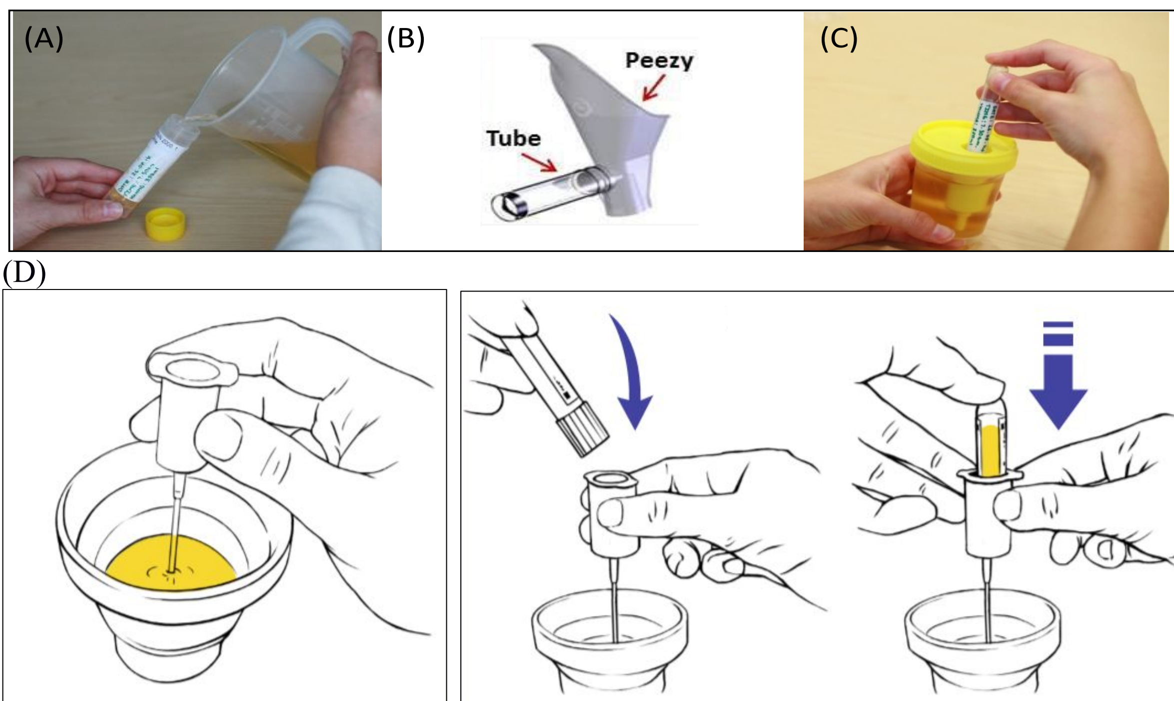
Fig 5



Supplementary Material 1: Assessing commonly used and novel methods of spot urine sample collection and storage

An internet search was carried out to identify commonly used and novel methods of spot urine sample collection and storage. Four methods were selected which could provide urine samples suitable for biomarker discovery/dietary exposure validation, based on their perceived suitability for use in the community and on the commercial availability of the collection devices. The first method (**A**) followed a standard practice of collecting urine in a 500 mL plastic jug followed by decanting a sample into a smaller vessel, in this case a 30 mL Universal tube, as described in previous studies . Two further methods utilised specific urine sampling tools including the 'Peezy' urine collection device (Peezy, Funnelly Enough Ltd, London, UK) which facilitated transfer of a sample into a 30 mL Universal tube (**B**), and a vacuum transfer system (**C**) using a 60 mL collection container with an integral transfer needle and a 6 mL additive-free vacuum tube (Vacutest, Kima). (**D**) A variant of (**C**) used a separate transfer straw to contact the urine sample.

Images of traditional jug and Universal tube, Peezy and vacuum transfer urine collection methods



(A) a standard 500 mL plastic jug and aliquoting into a 30 mL Universal tube; (B) the 'Peezy' urine collection device into a 30mL Universal tube; (C) vacuum transfer system using a 60 mL collection container with integral needle and a 6 mL vacuum tube. (D) Vacuum transfer system using a 100 mL collection container, a separate urine transfer straw and a 6 mL vacuum tube

Supplementary Material 2 Meal plans in ***Sub-study (2)*** [(29)]

Menu plan: Food intervention Day 1
Breakfast: Coffee, sourdough rye bread toasted, sweetened breakfast cereal and milk, banana
Lunch: Coffee, Tuna and sweetcorn salad on sourdough rye bread, banana
Dinner: Salmon, Broccoli and chips. Almonds and wine

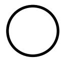
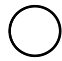
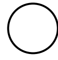
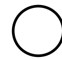
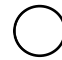
Menu plan: Food intervention Day 2
Breakfast: Tea, wholegrain bread toasted, red berries, milk
Lunch: Tea, wholegrain bread, cheese, ham, carrot, berries,
Dinner: Spinach, mushrooms, potato, steak pie, lager, raisins , milk

Menu plan: Food intervention Day 3
Breakfast: White bread, porridge, milk, egg, bacon, apple juice, cocoa
Lunch: White bread, salami, pepper, cocoa, apple
Dinner: Chicken curry, rice, peas, wine, cocoa

(29) Lloyd AJ, Willis ND, Wilson T *et al.* (2019) Developing a Food Exposure and Urine Sampling Strategy for Dietary Exposure Biomarker Validation in Free-Living Individuals. *Mol Nutr Food Res*. **63**(**14**), 1900062.

Supplementary Material 3: Vacuum transfer system questionnaire on acceptability of the postal method for sample return

The questions are asked in a way to rate your opinion towards a statement concerning the method on a scale from **strong agreement** to **strong disagreement** with the statement. Please estimate your opinion to the stated as best as you can and fill in the circle that is closest to match with it. Fill-in only one circle for each question.

				
Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree

Questions

1. I was successful in collecting urine using this method
2. It was easy to collect urine in the pot
3. I was confident collecting urine in the pot
4. It was easy to transfer urine from the pot into the capped tube using the straw
5. I felt confident transferring urine from the pot into the capped tube using the straw
6. I would be happy to write the collection date and time on the capped tube
7. I was happy collecting first morning void samples
8. I would have preferred to collect urine samples at a different time of day
9. I was happy storing several urine samples collected over a week in my fridge
10. I was happy to post urine samples in a pre-paid box
11. In general, I think collecting an urine sample is difficult
12. I think collecting an urine sample in a home environment is embarrassing
13. I think collecting an urine sample OUT of the home environment is embarrassing

Supplementary Material 4: Biomarkers used for absolute quantification in urine samples

Biomarker	Dietary component	Column	Ionisation mode[a]	Parent ion[b]	Product ion[c]	Retention time
1-Methyl-histidine	Striated muscle meat	pHILIC	Pos	170.064	124.160	9.9
3-Hydroxyhippuric-acid	Fruit and Vegetables	RP-C18	Neg	194.064	150.113	5.12
3-Methyl-histidine	Poultry and fish (no shellfish)	pHILIC	Pos	170.059	96.351	10.6
3-Methyl-xanthine	Cocoa (chocolate)	RP-C18	Pos	167.096	94.220	4.52
4-Hydroxyhippuric-acid	Fruit and Vegetables	RP-C18	Neg	194.073	100.240	4.84
4-Hydroxyproline-betaine	Citrus and Citrus fruit juice	pHILIC	Pos	160.167	88.367	9.4
7-Methyl-xanthine	Cocoa (chocolate)	RP-C18	Pos	167.085	124.185	4.36
Acesulfame-K	Low calorie drinks	RP-C18	Neg	161.947	82.233	4.32
Anserine	Poultry and fish (no shellfish)	pHILIC	Pos	241.052	109.159	11.1
BOA (1-3-Benzoazol-2-one)	Wholegrain rye	RP-C18	Pos	136.055	80.292	6.86
Caffeine	Cocoa (chocolate), Coffee, Tea, Caffeinated drinks	RP-C18	Pos	195.060	138.138	5.94
Carnitine	Striated muscle meat	pHILIC	Pos	162.153	103.301	9.9
Carnosine	Striated muscle meat	pHILIC	Pos	227.064	110.202	12
Creatinine	Striated muscle meat	pHILIC	Pos	114.129	44.695	8.5
Daidzein	soy, legumes	RP-C18	Pos	255.057	199.183	7.52
DHBA	Wholegrain	RP-C18	Neg	153.010	109.094	4.63
DHBA-3-O-sulfate	Wholegrain	RP-C18	Neg	232.880	152.988	3.99
DHPPA	Wholegrain	RP-C18	Neg	181.044	137.132	5.37
DHPPA-3-sulfate	Wholegrain	pHILIC	Neg	260.950	137.124	16.3
D-L-Sulforaphane-glutathione	Cruciferous vegetable e.g. Broccoli	RP-C18	Pos	485.049	355.919	5.18
D-L-Sulforaphane-L-cysteine	Cruciferous vegetable e.g. Broccoli	RP-C18	Pos	299.003	114.103	4.77
D-L-Sulforaphane-N-acetyl-L-cysteine	Cruciferous vegetable e.g. Broccoli	RP-C18	Pos	341.014	114.098	5.82
Epicatechin	Berries, Drupes, Apple, Cocoa, Coffee, Green/black tea, Vegetables e.g. beans.	RP-C18	Pos	291.084	139.070	5.74
Ferulic-acid	Coffee, polyphenol-rich foods	RP-C18	Pos	195.051	145.047	6.83
Ferulic-acid-4-O-b-D-glucuronide	Coffee, polyphenol-rich foods	RP-C18	Neg	369.054	193.042	5.44
Ferulic-acid-4-O-sulfate	Coffee, polyphenol-rich foods	RP-C18	Neg	273.021	193.087	5.59
Hippuric-acid	Fruit and Vegetables	RP-C18	Pos	180.075	105.180	5.92
Indoxyl-sulfate	Protein intake	RP-C18	Neg	212.012	132.155	5.28
Phenylalanine	Protein intake	pHILIC	Pos	166.131	120.279	8.9
Tryptophan	Protein intake	pHILIC	Pos	205.124	188.157	9.7
N-2-Furoyl-glycine	Strongly heated foods	RP-C18	Pos	170.092	95.296	4.9
Naringenin	Grapefruit, Citrus (orange, lemon, lime)	RP-C18	Neg	271.083	151.076	7.78
p-Cresol-glucuronide	Protein intake	pHILIC	Neg	283.033	107.163	8.1
p-Cresol-sulfate	Protein intake	RP-C18	Neg	187.026	107.228	6.31
Phenyl-acetyl-L-glutamine	Protein intake	pHILIC	Pos	265.105	130.233	6
Proline-betaine	Citrus and Citrus fruit juice	pHILIC	Pos	144.190	58.543	8.3
Protocatechuic-acid	Red wine and other plants sources	RP-C18	Neg	153.080	109.193	4.86
Quercetin	Fruits (e.g., apples, grapes, berries), Vegetables (e.g., onions, spinach, kale, broccoli, lettuce, and tomatoes), tea	RP-C18	Neg	301.002	151.032	7.58
Quercetin-3-O-b-D-glucuronide	Fruits (e.g., apples, grapes, berries) vegetables (e.g., onions, spinach, kale, broccoli, lettuce, and tomatoes), tea	RP-C18	Neg	477.076	300.972	6.66
Resveratrol	Red wine, grapes	RP-C18	Pos	229.089	107.199	7.49
Rhamnitol	Apple	pHILIC	Neg	165.146	59.139	8.6
Sucrose	High sugar intake	pHILIC	Neg	341.101	89.039	10.9
Tartarate	Grapes and wine	pHILIC	Neg	149.016	87.191	13.9
Taurine	Striated muscle meat	pHILIC	Pos	126.126	108.255	11.8
Trigonelline	Legumes: Beans, soya, peanuts, almonds, coffee, peas	pHILIC	Pos	138.023	92.259	8.9
Trimethylamine-N-oxide	Fish	pHILIC	Pos	76.188	58.517	9.7
4-Chloro-DL-phenylalanine	IS	pHILIC	Pos	200.042	154.112	8.5
Syringic acid	IS	RP-C18	Pos	199.060	140.047	6.12

a: ions denoted with (pos) or (neg) indicates that the biomarker was detected in the protonated or deprotonated form respectively.
b: 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.
c: 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.

Supplementary Material 5: Perception of different spot urine collection methods

Methods

In total, 31 individuals (20 female, 11 male; age: 20-73) were recruited from a database maintained at the Human Nutrition Research Centre, Newcastle University of individuals who had taken part previously, or had expressed an interest, in nutrition studies. These participants were asked to complete an online questionnaire consisting of six questions (below) about their perceptions of the three spot urine collection methods (**Supplementary Material 1, A-C**) which required them to rank the extent of their agreement with each statement on a five point scale from "strongly disagree" to "strongly agree". The Kruskal-Wallis Test was used to compare sample collection methods and to calculate *p*-values.

Online questionnaire

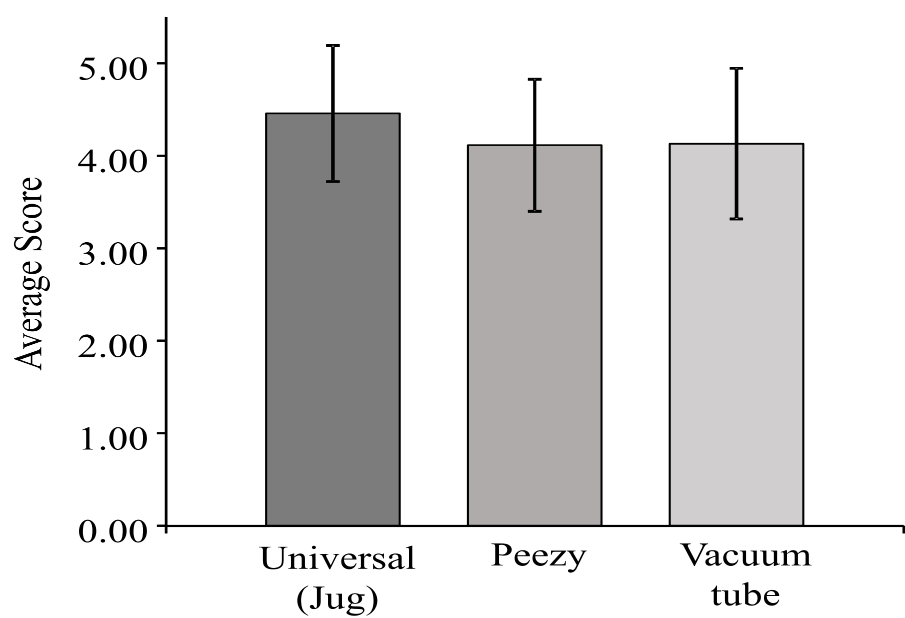
The questions are asked in a way to rate your opinion towards a statement concerning the method on a scale from **strong agreement** to **strong disagreement** with the statement. Please estimate your opinion to the stated as best as you can and fill in the circle that is closest to match with it. Fill-in only one circle for each question.

○ Strongly disagree ○ Disagree ○ Neither agree nor disagree ○ Agree ○ Strongly agree

Question 1: I understand how the collection of urine with Method x works
Question 2: I think it would be easy to collect urine with Method x
Question 3: I would feel confident collecting urine with Method x
Question 4: I think that the urine transfer step of Method x is easy
Question 5: I would feel comfortable performing the urine transfer with Method x
Question 6: I would be happy to label the tubes used in Method x

Results of Questionnaire

The results showed that all three procedures were perceived to be acceptable by the general public with a mean score greater than 4, where the maximum was 5. Although no significant differences were observed in the mean acceptability scores for each method (*p*-value 0.85, Kruskal- Wallis Test), there was some variance in the participant perception of the methods, depending on factors such as age and gender (data not shown).


5.00
4.00
3.00
2.00
1.00
0.00
Average Score
Universal (Jug)
Peezy
Vacuum tube

Supplementary Material 6: Absolute quantification in selected first morning void (FMV) urine samples stored in coated and non-coated vacuum tubes.

	Coated vacuum tubes					Non-Coated vacuum tubes				
Biomarker	Control	T1	T2	T3	T4	Control	T1	T2	T3	T4
1-Methyl-histidine	46.94	33.30	31.43	42.37	40.48	41.61	40.44	49.91	35.41	38.28
3-Hydroxyhippuric-acid	7.67	7.49	6.27	5.59	6.29	8.78	9.55	9.29	8.30	7.28
3-Methyl-histidine	62.17	53.80	51.69	59.59	55.63	65.45	46.09	66.73	53.51	36.97
3-Methyl-xanthine	2.61	2.63	2.42	2.47	2.67	3.18	3.15	3.27	3.13	3.58
4-Hydroxyproline-betaine	8.34	6.11	5.46	7.93	7.38	7.89	8.97	7.16	6.73	9.61
4-Hydroyhippuric-acid	9.33	9.91	9.15	8.69	7.89	9.99	10.80	10.57	10.04	11.67
7-Methyl-xanthine	7.38	7.49	7.58	7.17	7.37	8.32	8.13	8.39	8.14	8.96
Acesulfame-K	10.77	10.55	10.67	10.10	11.29	13.86	12.59	14.46	13.63	12.56
Anserine	3.97	3.82	3.82	4.31	3.75	4.52	3.46	4.06	3.82	2.77
BOA-1-3-Benzoazol-2-one	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Caffeine	0.35	0.37	0.35	0.33	0.34	0.38	0.38	0.38	0.38	0.47
Carnitine	19.50	15.45	14.21	17.84	21.33	21.29	20.01	23.03	16.00	22.13
Carnosine	1.08	1.11	1.18	1.25	1.33	1.35	1.40	1.32	1.36	1.45
Creatinine	2651.11	2768.50	2861.50	3266.37	2903.09	3589.09	3506.62	3516.80	3131.94	3397.63
D-L-Sulforaphane-glutathione	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01
D-L-Sulforaphane-L-cysteine	0.02	0.02	0.02	0.01	0.01	0.02	0.01	0.02	0.01	0.01
D-L-Sulforaphane-N-acetyl-L-cysteine	0.34	0.36	0.33	0.33	0.25	0.37	0.22	0.38	0.34	0.23
Daidzein	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	0.01	0.01
DHBA	0.47	0.49	0.48	0.52	0.48	0.50	0.51	0.53	0.53	0.55
DHBA-3-O-sulfate	1.98	2.02	1.83	1.77	1.74	2.76	2.85	2.80	2.76	3.12
DHPPA	0.25	0.27	0.26	0.29	0.27	0.30	0.30	0.30	0.28	0.30
DHPPA-3-sulfate	0.50	0.51	0.52	0.50	0.47	0.52	0.46	0.52	0.51	0.49
Epicatechin	0.02	0.02	0.03	0.02	0.02	0.02	0.03	0.02	0.02	0.02
Ferulic-acid	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.02
Ferulic-acid-4-O-b-D-glucuronide	0.27	0.27	0.26	0.24	0.26	0.47	0.52	0.51	0.48	0.48
Ferulic-acid-4-O-sulfate	0.96	1.02	1.01	0.93	1.01	1.31	1.33	1.40	1.37	1.49
Hippuric-acid	336.77	346.59	340.10	337.65	354.02	455.81	488.98	502.83	487.07	376.78
Indoxyl-sulfate	14.80	15.68	15.95	15.28	14.46	14.71	15.29	15.00	15.07	16.94
Phenylalanine	4.01	4.04	4.21	4.96	4.95	5.51	5.45	5.26	4.78	5.22
Tryptophan	7.36	5.41	4.85	6.97	6.89	6.69	7.71	7.98	6.22	7.01
N-2-Furoyl-glycine	3.19	3.34	3.52	3.15	0.91	4.07	4.06	4.24	3.87	4.11
Naringenin	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	0.01
p-Cresol-glucuronide	7.13	6.40	6.32	7.45	8.21	7.98	7.61	8.03	6.29	7.57
p-Cresol-sulfate	26.59	28.58	28.57	27.60	27.47	29.65	31.20	31.28	30.32	37.30
Phenyl-acetyl-L-glutamine	51.26	54.74	56.14	65.58	79.80	90.75	70.83	63.75	68.82	93.59
Proline-betaine	34.76	32.85	32.10	37.03	35.14	39.69	41.48	37.49	36.59	47.95

Protocatechuic-acid	0.12	0.13	0.13	0.14	0.12	0.13	0.13	0.13	0.14	0.13
Quercetin	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01
Quercetin-3-O-b-D-glucuronide	0.12	0.14	0.14	0.27	0.11	0.11	0.12	0.11	0.13	0.15
Resveratrol	0.04	0.04	0.05	0.05	0.04	0.07	0.07	0.06	0.07	0.06
Rhamnitol	1.93	2.14	2.12	2.44	2.16	2.63	3.19	2.62	2.64	3.17
Sucrose	4.92	4.89	4.57	5.33	5.06	4.89	5.40	5.37	4.80	3.48
Tartarate	36.29	30.95	29.16	42.50	38.77	49.03	48.49	45.41	40.89	40.24
Taurine	5.67	5.74	6.61	6.32	6.39	8.12	8.40	7.41	7.20	6.66
Trigonelline	36.74	42.87	43.06	48.86	48.12	54.45	48.17	52.13	47.86	53.29
Trimethylamine-N-oxide	18.44	17.01	16.60	27.68	23.62	30.58	29.68	39.96	34.53	28.74

Values are means (µg / mL) from 13 individuals.

Where: Control, -20 °C; T1, 2 days at 4 °C; T2, 7 days at 4 °C; T3, 2 days at room temperature (RT); T4, 7 days at RT.

Supplementary Material 7: Statistical analysis (Kruskal-Wallis) of absolute concentrations of dietary exposure biomarkers in selected first morning void (FMV) urine samples stored in coated vacuum tubes and vacuum tubes containing a lyophilized preservative*

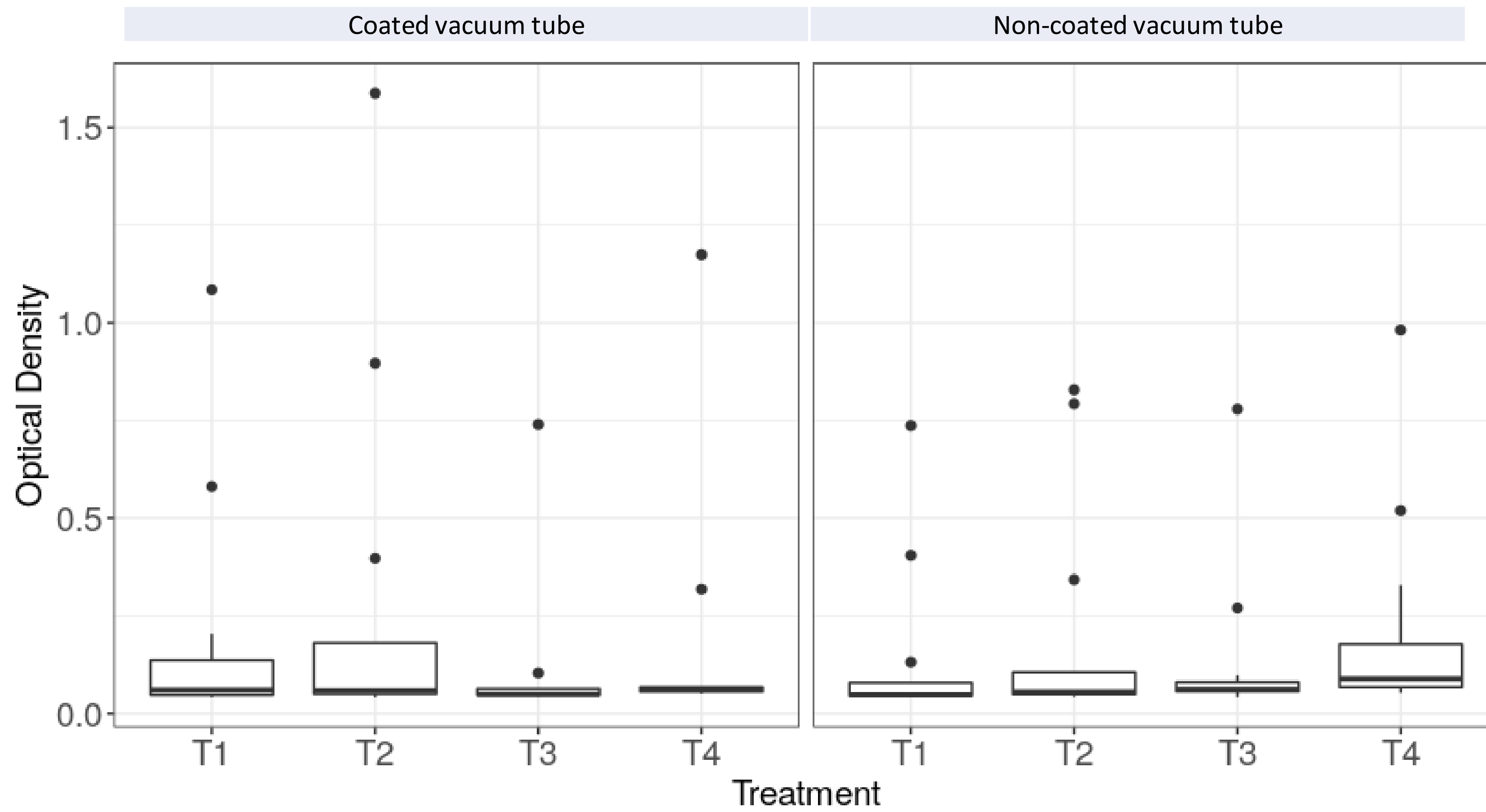
Biomarker	Coated vacuum tube			Non-coated vacuum tube		
	Statistic	*P*-value	*p*-value (corrected)	Statistic	*p*-value	*p*-value (corrected)
1-Methyl-histidine	18.03	**0.001**	0.056	8.02	0.091	1.000
3-Hydroxyhippuric-acid	0.58	0.965	1.000	0.41	0.982	1.000
3-Methyl-histidine	1.82	0.769	1.000	1.85	0.763	1.000
3-Methyl-xanthine	0.04	1.000	1.000	0.36	0.985	1.000
4-Hydroxyproline-betaine	0.26	0.992	1.000	0.10	0.999	1.000
4-Hydroyhippuric-acid	1.74	0.784	1.000	0.36	0.986	1.000
7-Methyl-xanthine	0.12	0.998	1.000	0.30	0.990	1.000
Acesulfame-K	0.12	0.998	1.000	0.25	0.993	1.000
Anserine	0.46	0.977	1.000	0.98	0.913	1.000
BOA-1-3-Benzoazol-2-one	9.13	0.058	1.000	1.66	0.797	1.000
Caffeine	0.17	0.997	1.000	0.39	0.983	1.000
Carnitine	2.25	0.689	1.000	0.96	0.917	1.000
Carnosine	1.55	0.817	1.000	0.41	0.981	1.000
Creatinine	1.98	0.739	1.000	1.11	0.893	1.000
D-L-Sulforaphane-glutathione	0.67	0.955	1.000	4.68	0.322	1.000
D-L-Sulforaphane-L-cysteine	3.99	0.407	1.000	2.95	0.565	1.000
D-L-Sulforaphane-N-acetyl-L-cysteine	0.64	0.959	1.000	0.45	0.978	1.000
Daidzein	22.62	**0.000**	**0.007**	23.26	**0.000**	**0.005**
DHBA	0.44	0.979	1.000	0.32	0.989	1.000
DHBA-3-O-sulfate	0.75	0.945	1.000	0.67	0.955	1.000
DHPPA	0.44	0.979	1.000	0.40	0.982	1.000
DHPPA-3-sulfate	0.52	0.972	1.000	0.16	0.997	1.000
Epicatechin	3.32	0.506	1.000	1.87	0.760	1.000
Ferulic-acid	4.05	0.399	1.000	17.59	**0.001**	0.068
Ferulic-acid-4-O-b-D-glucuronide	1.43	0.840	1.000	0.92	0.922	1.000
Ferulic-acid-4-O-sulfate	0.28	0.991	1.000	0.49	0.975	1.000
Hippuric-acid	0.31	0.989	1.000	3.34	0.502	1.000
Indoxyl-sulfate	0.39	0.983	1.000	0.30	0.990	1.000
Phenylalanine	7.00	0.136	1.000	2.02	0.731	1.00
Tryptophan	11.93	**0.018**	0.821	4.22	0.377	1.000
N-2-Furoyl-glycine	2.59	0.629	1.000	0.30	0.990	1.000
Naringenin	8.50	0.075	1.000	7.06	0.133	1.000

p-Cresol-glucuronide	1.62	0.806	1.000	0.82	0.935	1.000
p-Cresol-sulfate	0.26	0.992	1.000	0.70	0.952	1.000
Phenyl-acetyl-L-glutamine	6.50	0.165	1.000	5.53	0.237	1.000
Proline-betaine	0.19	0.996	1.000	0.19	0.996	1.000
Protocatechuic-acid	1.39	0.847	1.000	1.14	0.887	1.000
Quercetin	0.96	0.916	1.000	0.92	0.921	1.000
Quercetin-3-O-b-D-glucuronide	0.37	0.985	1.000	0.20	0.995	1.000
Resveratrol	3.35	0.502	1.000	5.16	0.271	1.000
Rhamnitol	0.78	0.941	1.000	1.24	0.871	1.000
Sucrose	1.00	0.910	1.000	4.26	0.372	1.000
Tartarate	0.46	0.977	1.000	0.23	0.994	1.000
Taurine	0.53	0.970	1.000	0.71	0.950	1.000
Trigonelline	1.08	0.898	1.000	0.34	0.987	1.000
Trimethylamine-N-oxide	5.62	0.229	1.000	4.59	0.332	1.000

- Becton and Dickinson Vacutainer® urinalysis preservative tube; chlorhexidine, ethyl paraben and sodium propionate.

All storage treatments: Control, -20 °C; T1, 2 days at 4 °C; T2, 7 days at 4 °C; T3, 2 days at room temperature (RT); T4, 7 days at RT as in **Supplementary Material 6**. Significant *p-values* highlighted in bold and yellow

Supplementary Material 8: Box-plots of Optical Density (OD) (at 600 nm) of urine samples after different storage treatments



Where: T1, 2 days at 4 °C; T2, 7 days at 4 °C; T3, 2 days at room temperature (RT); T4, 7 days at RT.