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Kay, C., Tejera, N., Jennings, A., Halder, S., Bevan, D., Crossman, L. C., Li, S., Cassidy, A., & Minihane, A. M. (2025). Effect of age and sex on the urinary elimination of a single dose of mixed flavonoids: results from a single-arm intervention in healthy UK adults. *The American Journal of Clinical Nutrition*. Advance online publication. https://doi.org/10.1016/j.ajcnut.2025.05.006

Published in:
The American Journal of Clinical Nutrition

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

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Download date:11. Nov. 2025

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PII: S0002-9165(25)00258-8

DOI: https://doi.org/10.1016/j.ajcnut.2025.05.006

Reference: AJCNUT 908

To appear in: *The American Journal of Clinical Nutrition*

Received Date: 15 December 2024

Revised Date: 30 April 2025

Accepted Date: 7 May 2025

Please cite this article as: C.D Kay, N. Tejera, A. Jennings, S. Haldar, D. Bevan, L.C. Crossman, S. Li, A. Cassidy, A.-M. Minihane, Effect of age and sex on the urinary elimination of a single dose of mixed flavonoids: results from a single-arm intervention in healthy UK adults, *The American Journal of Clinical Nutrition*, https://doi.org/10.1016/j.ajcnut.2025.05.006.

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1 **Effect of age and sex on the urinary elimination of a single dose of mixed flavonoids:**
2 **results from a single-arm intervention in healthy UK adults**

3

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17 **Sources of Support:** The work was supported by a BBSRC, UK Programme Grant
18 (BB/J004545/1)
19 **Conflict of Interest**: The authors declare that they have no known competing financial
20 interests or personal relationships that could have appeared to influence the work reported in
21 this paper. Anne-Marie Minihane reports was provided by University of East Anglia Norwich
22 Medical School.
23 **Clinical Trial Registry:** NCT01922869

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27 **Running Head:** Age, sex and flavonoid absorption and metabolism

28

29 **Abbreviations:** absorption, distribution, metabolism, and elimination (ADME); maximum
30 urine excretion (Cmax); randomized control trials (RCTs); time of maximum urine excretion
31 (Tmax); total amount excreted in urine over 24 h (Aeu 0-24)

32

33 **ABSTRACT**

34 **Background:** Nutrition intervention trials demonstrate that increased flavonoid intake can
35 have clinically meaningful impacts on disease outcomes/biomarkers; however, high
36 variability in absorption and metabolism and large heterogeneity in biochemical and
37 physiological responses are observed. The etiology of this variability is poorly understood.
38 **Objective:** To explore the relationships between sex, age, and microbiota speciation on
39 mixed flavonoid elimination over 24 hour (h).
40 **Methods:** Healthy males and **females** (n=163) prospectively recruited on the basis of age
41 (18-30y or 65-77 y) and sex, consumed a standardized flavonoid-rich test meal providing
42 640mg of cocoa/chocolate flavan-3-ols, 340mg of citrus flavanones, and 390mg of
43 blackberry anthocyanins. Urinary samples collected at baseline (-24 h to 0 h), 0-3.5 h, >3.5h-
44 7 h and >7-24 h were analysed for flavonoids and their metabolites by Ultra high-
45 performance liquid chromatography-mass spectrometry (UPLC-MS/MS). Stool microbiome
46 speciation was determined via Illumina sequencing. Linear mixed-effect models were used to
47 assess differences in cumulative excretion across age and sex with time-by-group interaction
48 taken as the principal analysis of effect.

49 **Results:** There were no group (older females, older males, younger females, younger males)
50 differences in total 24 h urinary metabolite recovery, but there was a trend towards a higher
51 rate of cumulative recovery in older males at 24h (β (95% CI) -61.4 (-107, -16.0 younger
52 males compare to older males; P-group at 24hr=0.06). Of 76 metabolites, 20 had
53 significantly different Tmax by age and 9 by sex, with a later mean Tmax observed for older
54 participants (92% of instances). Associations with age were not mediated by BMI or
55 microbiome speciation. Significant differences in Cmax by sex were observed for only 6
56 metabolites and differences by age for 5 metabolites.

57 **Conclusion:** Total elimination recovery of (poly)phenols was relatively consistent across age
58 and sex groups, while elimination kinetics differed substantially; possibly resulting from
59 differences in intestinal transit time or kidney clearance. Assuming (poly)phenol metabolites
60 have varying biological activities, establishing dose response relationships, and defining
61 metabolite profiles in population subgroups is required to inform the future development of
62 dietary flavonoid/(poly)phenol recommendations.

63 **Clinical Trial Registry:** NCT01922869

64 **Keywords:** absorption, metabolism, excretion, flavonoids, polyphenols, genes, gut-
65 microflora

66

67 **INTRODUCTION**

68 Flavonoids are a diverse group of dietary phytochemicals which are structurally subclassified
69 into anthocyanins, flavonols, flavones, flavanones, flavan-3-ols, and isoflavones, and
70 includes their oligomeric (i.e., flavonoids) and polymeric (i.e., proanthocyanidins) forms(1,
71 2). Their bioactivity is in part responsible for the health benefits of fruits and vegetables, and
72 other plant-based foods, such as tea, fruit juice, wine, herbs and spices(3, 4). In prospective
73 cohort studies, a high flavonoid intake has been associated with a 10-40% reduction in all-

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74 cause mortality, dementia incidence and cognitive decline, cardiovascular events, and risk of
75 specific cancers and a lower body weight(5-9). An accumulating number of randomised
76 control trials (RCTs) are demonstrating, at a group level, clinically meaningful impacts of
77 increased flavonoid intake on chronic disease biomarkers, providing insight into the
78 underlying mechanisms of action at play(5, 6, 8, 10). The dose-response relationships
79 between flavonoid consumption and phenotype are however, highly variable, with large
80 heterogeneity in the concentration of flavonoid metabolites in biological samples following
81 intake of a given dose, as well as the biochemical and physiological responses observed(1, 8,
82 10-13). The molecular and physiological basis for this heterogeneity in dietary flavonoid
83 metabolism and responsiveness observed across studies is poorly understood.
84 As reviewed, although a small number of existing studies have investigated potential
85 modulators of (poly)phenol metabolism and elimination, most have had a limited number of
86 participants, with heterogeneity in response a secondary *post hoc* rather *a priori* aim, which
87 often results in a poorly powered analysis(14, 15).
88 Once ingested, the absorption, distribution, metabolism, elimination (ADME) of flavonoids is
89 influenced by human intestinal, liver and kidney functions, including phase 1 and phase 2
90 metabolising enzymes, many of which are also important in the metabolism of xenobiotics
91 and drugs(1, 4). Although not well characterised for flavonoid metabolism, there is emerging
92 evidence to suggest that the functionality of these enzymes may be influenced by such
93 physiological variables as sex and age(16-18). Furthermore, a substantial fraction of
94 flavonoid ADME is impacted by gut microbial metabolism, and flavonoid intake has also
95 been shown to influence microbiota speciation(19, 20). However, the role of gut microbiota
96 functional and metabolic diversity in flavonoid metabolism and bioavailability is still poorly
97 understood(1, 21).
98

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99 Here, using a prospective recruitment approach we conducted the first systematic
100 investigation of the impact of sex, age and microbiota speciation on flavonoid elimination
101 over 24 h following the consumption of a standardized cocoa/chocolate flavan-3-ol, citrus
102 flavanone, and blackberry anthocyanin rich test meal. Such establishment of metabolism and
103 elimination kinetics in population subgroups are needed to inform and refine the development
104 of dietary flavonoid recommendations(22).

105

106 **METHODS**

107 Study participants. The acute chocolate, orange/citrus, blackberry intervention study (COB
108 study) recruited prospectively on the basis of sex and age and aimed to include equal
109 numbers of males and females and those aged between 18-30y or 65-77y who were generally
110 healthy. Exclusion criteria were: BMI < 18.5 kg/m^2 or > 30 kg/m^2; smokers or nicotine users;
111 hypertension with either a systolic blood pressure >140mmHg or a diastolic blood pressure
112 >90mmHg; a medical condition or significant past medical history likely to affect study
113 measurements e.g., diagnosed type 2 diabetes, cardiovascular, renal, liver, thyroid or
114 gastrointestinal diseases; vaccinations or antibiotics use in the previous 3 months, (as these
115 will impact on the gut microflora composition and metabolism and also phase 1 and 2
116 metabolism); blood biochemistry (including alanine aminotransferase (ALT; >0-40IU/L),
117 alkaline phosphatase (ALP; >30-130 IU/L), bilirubin, albumin, urea and creatinine) outside
118 the normal ranges; prescribed medication use that could interact with the enzymes involved
119 in the metabolism of flavonoids (Supplementary Table S1); taking flavonoid containing
120 supplements or other dietary supplements for one month prior to the study; known allergies to
121 the intervention foods; consume more than 21 alcohol unit/week for males, or 14 units/week
122 for females; pregnant, lactating, or planning a pregnancy (or having fertility treatment);
123 unable to provide informed consent to participate in the study.

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124 Since excessively high intake of flavonoids has been shown to alter human metabolic
125 pathways(23, 24), individuals who are atypically high consumers of flavonoids as part of
126 their regular diet were precluded from participating. At the time of the COB trial delivery,
127 UK adults were reported to consume on average 10 servings of flavonoid-rich foods
128 (including tea, coffee, chocolate, fruit, vegetables and other plant-based products) per day
129 (National Diet and Nutrition Survey (NDNS; 2011)(25). Individuals were excluded if they
130 consumed more than 15 servings of flavonoid-rich foods per day. Flavonoid intake was
131 assessed using the EPIC food frequency questionnaire(26).
132
133 Study Design. During the dietary run-in period and acute test meal protocol, participants were
134 asked to adhere to a restricted diet to minimise the intake of (poly)phenols (including
135 flavonoids and phenolics) by avoiding the consumption of fruits, vegetables, chocolate,
136 spices, high-fibre products, tea, coffees, fruit juices and alcoholic beverages (Supplementary
137 Table S2) for 48 h before the study day and for the 24 h after the consumption of the
138 flavonoid-rich test meal (**Figure 1**). In addition to the medications listed in Supplementary
139 Table S1, participants were asked to avoid acetaminophen (paracetamol) or other
140 nonsteroidal anti-inflammatory drugs for the 10 h (fasting period) prior to the acute study day
141 and for the duration of the sample collection, unless it was a medical requirement.
142 Participants were provided with a standardized low-flavonoid ready meal, consisting of either
143 a vegetable-free fish pie or macaroni cheese, along with a bread roll and a portion of fruit-
144 free sponge cake to consume the evening prior to attending the clinic visit in order to control
145 for any inter-individual variability in background dietary (poly)phenols from their previous
146 meals, and then undergo an overnight fast (only water allowed during fasting period). The
147 participants attended the clinical trial unit (University of East Anglia, Norwich), after an
148 overnight fast (only water allowed) for the acute test-meal session, containing mixed-

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149 flavonoids. The standardized mixed flavonoid-rich test meal consisted of a bar of dark
150 chocolate (Barry Callebaut, Lebbeke, Belgium, 50g), and a freeze-dried orange extract
151 (Monteloeder, Alicante, Spain, 362mg) and blackberry (Nutra Ingredients Ltd, Brighton, UK,
152 37.5g) powder in water, known as the COB mixture, with the composition of the chocolate
153 provide by the manufacturer (Barry Callebut, Belgium) and the flavonoid composition of the
154 blackberry powder and citrus extracts established using an internal UHPLC-MS/MS based
155 analysis(10, 27, 28). This provided 640 mg of flavan-3-ols (~30% monomers, ~180mg (-)-
156 epicatechin), 340 mg of flavanones (89.3% hesperitin, 3.4% narirutin) and 390 mg of
157 anthocyanins (99+% cyanidin-3-*O*-glucoside). Urine samples were collected at baseline (0 h
158 a 24 h before the test meal), 0 – 3.5 h, 3.5 - 7 h, 7 - 24 h. Participants were free to leave the
159 clinical trial unit at 7 h and return at the 24 h points for the relevant urine sampling. During
160 the day of the intervention, two standardized low flavonoid meals (lunch and dinner), were
161 provided along with low-flavonoid snacks (Figure 1).
162 The study was approved by the National Health Service (NHS) Health Research Authority
163 (IRAS Project ID 1251207), followed the principles of the Declaration of Helsinki and was
164 conducted at the Clinical Research Facility, University of East Anglia. Informed consent was
165 obtained from all participants before study commencement. Recruitment occurred between
166 October 2013 and March 2015, with follow-up completed by April 2015. Clinical Trials
167 Registration # NCT01922869.
168 **Dietary assessment**
169 Participants completed a 131-item validated (26) FFQ which captured dietary habits over the
170 previous 12 months, from which nutrient intakes were determined using McCance and
171 Widdowson Food Tables(29, 30) and flavonoid intakes were calculated using the updated
172 USDA databases for the flavonoid and proanthocyanin content of food, as previously
173 described(31). If no values were available in the USDA database (USDA Database for the

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174 Flavonoid Content of Selected Foods Release 3.1) for foods in the FFQ, available data from
175 the phenol explorer database (www.phenol-explorer.eu)(32, 33) were included. Flavonoid
176 intakes were derived for the six main flavonoid subclasses habitually consumed: flavanones
177 (eriodictyol, hesperetin, and naringenin); anthocyanins (cyanidin, delphinidin, malvidin,
178 pelargonidin, petunidin, and peonidin); flavan-3-ols (catechins and epicatechins); flavonols
179 (quercetin, kaempferol, myricetin, and isorhamnetin); flavones (luteolin and apigenin); and
180 polymers (including proanthocyanidins, theaflavins, and thearubigins). Total flavonoid
181 intakes were estimated by summing the six component subclasses.

182

183 **Analytical methods**

184 Total urine voids were collected into light-protected collection bottles containing 1 g ascorbic
185 acid. Aliquots were stored at -80 °C until analysis. Urinary metabolites were purified from 50
186 µL human urine (1mL aliquots acidified with 40 µL formic acid) samples by 96-well plate
187 solid phase extraction (SPE; Strata™-X Polymeric Reversed Phase, microelution 2 mg/well).
188 Taxifolin was spiked into the urine and used as a SPE recovery reference standard and
189 scopoletin post SPE as a chromatography internal standard. The solid phase extraction treated
190 samples were chromatographically separated on an Exion ultra-high performance UHPLC
191 coupled to a SCIEX QTRAP 3200+ triple quadrupole mass spectrometer (MS/MS; SCIEX,
192 Framingham, MA, USA) with electrospray ionization source, as previously reported(10, 27,
193 28). The samples were injected into Kinetex® (Phenomenex®) 2.6 µm PFP 100 Å, LC
194 Column 100 x 4.6 mm (Part Number: 00D-4477-E0) with SecurityGuard™ ULTRA
195 cartridges for PFP, with oven temperature maintained at 37°C. Mobile phase A and B
196 consisted of 0.1% v.v. formic acid in water and 0.1% v.v. formic acid in acetonitrile
197 respectively, with a binary gradient ranging from 1% B to 90% B over 28min and flow rate at
198 1.5 mL/min. MS/MS scanning was accomplished using a targeted advanced scheduled MRM

199 (ADsMRM) assay using polarity switching between positive and negative ionisation mode in
200 Analyst (v.1.6.3, SCIEX) and with quantitation conducted using MultiQuant (v.3.0.2,
201 SCIEX) software platforms. If two metabolite isomers could not be resolved, they were
202 quantified relative to a single species. Finally, metabolites previously reported in nutrition
203 intervention studies feeding (poly)phenol-rich foods(27, 28, 34-45) were confirmed on the
204 basis of established retention times (using authentic and synthesised standards; Supplement
205 Table S3) and three or more precursor-to-product transition ions. In total, 82 (poly)phenol
206 were quantified via UPLC-MS/MS; 6 metabolites were below the limit of quantitation in all
207 samples, with statistical analysis completed for 76 analytes. All the metabolites where
208 quantified relative to their reference standard, with the exception of cyanidin-diglucuronide
209 which was quantified using the cyanidin-3-*O*-glucoside reference standard. Matrix matched
210 standard curves were prepared for quantification ranging from 0-10μM. Reference standards
211 scopoletin, taxifolin and phloridzin were used as quality controls and for internal standard
212 adjustment in the urine samples, with system blanks monitored for carryover effects and pre-
213 extracted urine as a reference blank.
214
215 Stool microbiome speciation. Stool specimens were collected on the day prior to the study
216 day using EasySampler® kits (GP Medical Devices, Nupark, Holstebro, Denmark),
217 transferred into sterile containers (Sarstedt, Leicester, UK), transported in cooled bags
218 provided by the research team and immediately transferred to a −20 °C freezer upon arrival.
219 Prior to DNA extraction, 200-300mg of faeces was transferred to a 2 mL safe lock tube
220 (Eppendorf, city, country) containing a 5mm steel ball bearing and was subjected to
221 mechanical disruption in 1.2 mL sterile PBS using a tissue lyser (Qiagen Tissue Lyser II, 30
222 Hz for 4 min at 4°C). Samples were centrifuged to remove insoluble material (1 min, 10,000
223 g at at 4°C). DNA was extracted from 800 μl of the supernatant using a QIAsymphony SP

224 automated platform (Qiagen) using the QIAsymphony DSP virus/bacteria midi kit and the
225 Complex 400 V6 DSP program. DNA was eluted in 110 µl buffer AVE containing carrier
226 RNA as provided in the kit. The DNA was quantitated to confirm a concentration between
227 5–20 ng/µl, and stored at -20°C until analysis. The region of the 16S gene was sequenced at
228 APHA, Surrey using paired-end Illumina MiSeq short read sequencing. The Qiime(46) 1.9.0
229 illumina workflow was used for joining the reads, demultiplex and filter samples and pick
230 operational taxonomic units (OTUs). Read counts varied across the samples from 70 in a
231 control sample to 112944 from sample COB354. Sequencing control samples were two
232 separate PBS samples and one sample with no template. Two samples were excluded based
233 on having a sequencing depth below the threshold. In addition, Qiime excluded two more
234 samples according to the quality control filter. This filter is described in Bokulich et al
235 (2013)(47). Qiime alpha diversity analyses (secondary analysis) involved testing a range of
236 multiple rarefactions run in parallel. The resulting graph from these technical analyses did not
237 indicate large-scale differences from using data at different sequencing depth levels (data not
238 shown).
239

240 **Statistical analysis**

241 Given the absence of established effect size estimates at the time the intervention was
242 designed, we justified our sample size based on prior empirical data from Czank et al.
243 2013(11), where significant changes in polyphenol metabolites were observed in 8
244 participants. Based on the observed confidence intervals in that study, we estimated that
245 group sizes of 30–40 participants would be sufficient for age and sex subgroup analyses. In
246 the analysis of metabolites, values above the signal to noise (S/N) but below the limit of
247 detection (LOD) were set as zero in the statistical analysis. If metabolites had the majority of
248 their values at 0, they were excluded from the analysis. Calibration curves were established

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249 between 1nM and 10uM. Values below the lower limit of quantitation (LLOQ) but above the
250 LOD were reported as a “near 0” value (0.0001) in the statistical analysis. Linear mixed-
251 effect models were used to assess differences in cumulative excretion by age (younger
252 compared to older), sex (males compared to females) and combined age by sex (younger
253 females, younger males, older females, older males) groups. Models included cumulative
254 excretion as the dependent variable, “participant” as a random effect, time (0 min, 210 min,
255 420 min and 1440 min) and groups as predictors, with the time x group interaction taken as
256 the principal analysis of effect. The linear combinations of coefficients between groups were
257 explored at the 24 h timepoint. Total flavonoid intake, as calculated from the FFQ, was
258 included as a covariate on a continuous scale (where indicated) to explore if habitual diet
259 markedly changed the results. Data were not transformed as generalized linear models have
260 shown to be tolerant of distribution assumptions and can provide valid inference regardless of
261 the distribution of the data(48). Total recovery of the metabolite mass in a urine sample is
262 calculated by converting its molar concentration (moles per volume) to mass (e.g., ng) using
263 the molecular weight of the metabolite, then multiplying by the total urine volume excreted
264 : ((Metabolite value [nmol/l] * molecular weight [ng/nmol] /1000000)*(urine volume
265 [L]))/time [h]. Where indicated in data tables, low concentrations are presented as 10^2 (for
266 example 0.01x10^{-}2 = 0.0001 ng). The UPLC-MS/MS standard mix contained 82
267 (poly)phenols (i.e., comprising both precursor polyphenols and phenolic metabolites;
268 Supplement S3), and 76 were above the lower limit of quantitation in the COB urine samples.
269 Six metabolites were found below the limit of detection in the majority of samples and were
270 therefore not included in the final statistical analysis. Elimination kinetic parameters were
271 calculated for each metabolite, Cmax (maximum urine concentration), Tmax (time of
272 maximum concentration, 0 h, 3.5 h, 7 h or 24 h), and total amount excreted in urine over 24 h
273 (Aeu 0-24). Differences in Cmax and Tmax were compared between groups using a linear

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274 regression model with the elimination parameter as the dependent variable, group as the
275 predictor and habitual total flavonoid intake on a continuous scale as a covariate (where
276 indicated). If metabolite data were missing at individual time points cumulative excretion was
277 calculated up to the missing data point. Cmax and Tmax were calculated if data were
278 available for at least one time-point. P-values <0.05 were considered statistically significant.
279 The Benjamini-Hochberg method for false discovery rate (using a false discovery rate Q-
280 value <0.20) was also used to explore the possible impact of multiple testing on observed age
281 and sex affects. Statistical analyses were performed with Stata statistical software version 16
282 (StataCorp, Texas, USA).
283

284 Linear Discriminant Analysis (LDA) was used to identify which combination of metabolites
285 separated the participants by group and how effective these metabolites were as predictive
286 discriminators of age. Metabolites included in the model were those with a significant time-
287 by-age group interaction in the primary analysis.
288 Differences in relative abundance of taxa at the Family level and microbial alpha diversity
289 (Shannon Index) were compared across age (younger compared to older), sex (males
290 compared tofemales) and combined age by sex (younger females, younger males, older
291 females, older males) groups using linear regression with group as the predictor. We
292 excluded taxa where relative abundance was < 0.01% in at least 10% of samples leaving 38
293 taxa in the final analysis.
294 Hierarchical regression analysis (using metabolite concentration as the dependent variable)
295 was used to examine if models including sex and microbial composition improved prediction
296 of metabolite concentrations over models including age. For microbial composition we
297 combined the taxa significantly associated with age using principal component analysis,
298 considering the first component defined. The metabolites included in the model were those

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299 with a significant time-by-group interaction in the primary analysis.

300

301 **RESULTS**

302 A total of 186 participants completed the COB protocol (**Figure 2**). Metabolite
303 concentrations in urine were quantified in 163 participants [44 older females, 46 older males,
304 42 younger females, 31 younger males] for 24 h and data on habitual diet was available for
305 161 participants (**Table 1**). Older males had the highest caloric intake (age and sex effect;
306 $p<0.04$ and 0.03 respectively) and BMI was higher in older participants and highest in older
307 males ($P<0.01$). Older participants generally consumed considerably more flavonoids
308 ($P<0.01$), with nearly three times higher habitual intakes than younger individuals, with
309 highest intakes reported in older females. Habitual intakes of flavonoids and flavonoid sub-
310 classes also differed by age, with lower intake reported by younger participants compared to
311 older participants, which was reflected in lower total fruit and vegetable and tea intakes. No
312 notable differences in flavonoid intakes were observed by sex, while females reported lower
313 total energy and carbohydrate intakes and a higher fruit and vegetable intake.

314 **Cumulative Excretion – sum of all (poly)phenols and metabolites.** There was a trend for
315 age-by-sex differences in cumulative (all urine "time bins") 24 h urinary metabolite recovery
316 ($P=0.06$; treatment effect, all metabolites and parent (poly)phenols; Supplement Table 4);
317 however, no significant time-by-group interactions were observed ($p=0.30$); with mean total
318 urinary recovery of 14.59±2.22 ng at baseline, 56.02±7.72 ng at 3.5h, 97.80±20.73 ng at 7 h;
319 and 122.32±25.34 ng at 24 h (mean±SD; data not shown). There were also no significant
320 group differences (mean, 95% CI) for maximum total excretion (Cmax, $p=0.20$) [(older
321 females 505ng (333, 678), older males 670 ng (502, 839), younger females 452 ng (276,
322 629), younger males 384 ng (179, 590); however, there was significant group differences in
323 Tmax ($P=0.04$) [older females 15.8 h (12.4, 19.2), older males 15.2 h (11.9, 18.5), younger

324 females 10.6 h (7.1, 14.1), and younger males 12.0 h (7.9, 16.0); (mean, 95% CI)].

325 **Individual (Poly)phenols and Metabolite Excretion.** Differences in cumulative 24 h
326 excretion were observed for 12 individual metabolites by age (time-by-treatment interaction,
327 P<0.05; 10 Q<0.2) and 5 metabolites by sex (1 Q<0.2)(**Table 2;** *Complete dataset found in*
328 Supplemental Table 4). Here the metabolites primarily comprised of small molecule
329 microbial metabolites of (poly)phenols, where younger participants, mainly males, generally
330 displayed higher cumulative excretion. Adjustment for habitual total flavonoid intake did not
331 materially change the age differences observed in urinary excretion over time or cumulative
332 24 h urinary recovery (Supplemental Table 5).

333 **Elimination Kinetics**

334 Differences in Cmax were observed for 7% of measured metabolites by age and 8% by sex,
335 while differences in Tmax were observed for 28% by age and 11% of metabolites by sex
336 (Supplement Table S6; 36 P<0.05; 14 Q<0.2), with the majority of differences seen as age
337 effects on Tmax.

338 Group differences in Cmax were observed for 4 metabolites (P<0.05; 2 Q<0.2), with the
339 highest Cmax observed most frequently in older males (**Table 3**; Complete dataset found in
340 Supplemental Table 6). Age differences were observed for 5 phenolic metabolites (0 Q<0.2)
341 and sex differences for 6 metabolites (0 Q<0.2). Metabolites recorded in highest
342 concentrations [(Cmax; mean (95% CI)] having age or sex differences were 3-
343 hydroxyhippuric acid 22.0 μM (14.7, 29.3) benzoic acid-4-sulfate 17.3 μM (12.7, 21.8) , 3-
344 methoxybenzoic acid-4-sulfate 4.4 μM (3.1, 5.8), 2,5-dihydroxybenzoic acid 0.85 μM (0.53,
345 1.2), 3-hydroxy-4-methoxybenzoic acid 0.60 μM (0.46, 0.74), hydroxy-methoxybenzoic acid
346 0.42 μM (0.32, 0.52), and 4-methylhippuric acid 0.03 μM (0.01, 0.04). Adjusting Cmax for total
347 flavonoid intake by age group had limited impact on the statistical output (**Supplemental**
348 **table 7**).

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349

350 The time to maximum urinary concentration (Tmax) varied considerably across metabolites
351 displaying group, age or sex differences, from 1h and 24 h, with a mean Tmax of 9.1±4.1h
352 (mean (95% CI); **Table 4**). Group differences were observed for 15 metabolites (4 Q>0.2).
353 Twenty-two of the metabolites displayed significant Tmax differences by age (14 Q<0.2) and
354 8 by sex (0 Q<0.2), with 92% of these metabolites reflecting significantly later Tmax in older
355 participants. Only two analytes, 4-hydroxybenzyl alcohol and 3-methylhippuric acid, had
356 greater Tmax in younger participants (*all analyte data provided in* **Supplement Table 6**).
357 Adjusting Tmax for total flavonoid intake by age group had limited impact on the statistical
358 output (**Supplemental table 7**).
359

360 **Group Prediction.** In LDA analysis 77% of younger participants and 62% of older
361 participants were classified into the correct age groups based on their scores on the
362 discriminant dimensions (**Table 5**). Hydroxy-methoxybenzoic acid, 2-hydroxycinnamic acid,
363 4-methoxybenzaldehyde (positive correlations), benzoic acid-4-*O*-glucuronide, and 3-
364 methylhippuric acid (negative correlations) were the metabolites with the largest beta
365 coefficients and therefore most likely to classify participants in the correct age group.
366 **Gut microbiome composition by age and sex.** Five taxa at the Family level -
367 *Bacteroidaceae, Christensenellaceae, Clostridiales (unclassified), Dehalobacteriaceae* and
368 *Rikenellaceae* displayed significant differences by age group (Q <0.20, **Supplemental Table**
369 **8**). Relative abundance of all taxa was higher in younger participants with the exception of
370 *Bacteroidaceae* which was higher in older participants. Relative abundance of
371 *Barnesiellaceae* differed according to sex, with higher abundance in males. There were no
372 significant differences in alpha diversity (Shannon Index) between older and younger
373 individuals (P= 0.12) or males and females (P= 0.84) (data not shown). Hierarchical

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374 regression analysis revealed that the addition of sex and microbial composition to the model
375 did not improve the prediction of metabolite concentrations over age (**Table 6)**
376

377 **DISCUSSION**

378 Overall, we observed no impact of age or sex on total cumulative 24 h urinary elimination of
379 (poly)phenols. This lack of effect of age is consistent with a smaller previous study (n=40)
380 which looked at flavan-3-ol metabolism in men only and observed no difference in total
381 'structurally related epicatchin metabolites' in plasma 0-6h or urine 0-24h. However, we did
382 observe that overall age was the main determinant of flavonoid metabolite excretion kinetics
383 over 24 h. Interestingly, adjustment for BMI and the gut microbial taxa had little effect on the
384 significance of the observed relationships, indicating that any age associated effects were not
385 mediated by differences in gut microbial speciation or BMI. Older participants consumed
386 considerably more flavonoids in their habitual diet, with nearly three times higher intakes
387 compared to younger individuals, and with the highest intakes reported in older females;
388 however adjusting for habitual intake only moderately reduced the number of metabolites
389 showing age affects (**Supplemental Table 5**).

390 The Cmax for individual microbial metabolites of (poly)phenols differed by either age or sex
391 for 9 metabolites, however, there were no overall differences in cumulative excretion or
392 Cmax for all polyphenol metabolites combined (i.e., total collective of (poly)phenol
393 metabolites). Differences in Tmax were more pronounced for many metabolites, including
394 both precursor (poly)phenols (hesperetin, naringenin, epicatechin, catechin and cyanidin
395 glucoside) and their microbial metabolites.

396 Metabolites displaying significant group, age or sex effects for Tmax included
397 phydroxybenzoic acids, 3-(phenyl)propionic acids, 5-(Hydroxyphenyl)-gamma-
398 valerolactones and hippuric acids. Hydroxybenzoic acids, 3-(phenyl)propionic acids, and

399 hippuric acids are reported to be derived from multiple (poly)phenol-rich food sources; while
400 the 5-(hydroxyphenyl)-gamma-valerolactones were most likely derived from cocoa flavan-3-
401 ols and procyanidins in cocoa and blackcurrant, hesperetin-3'-*O*-glucuronide from orange,
402 and ellagic acid from blackcurrant(2, 4, 21, 36). Interestingly, there were no clear patterns in
403 elimination kinetics (Cmax or Tmax) for precursor flavonoids relative to their conjugated
404 metabolites (i.e., methyl, sulfate, glucuronide conjugation) between age or sex groups.

405 A substantial fraction of flavonoid intake is subjected to gut microbial metabolism,
406 structurally altering the precursor flavonoids found in the diet, forming smaller molecules
407 such as phenolic and aromatic acids, which are more bioavailable and may be highly
408 bioactive(1, 4). Although flavonoid intake has been shown to influence microbiota
409 speciation(19, 20), the role of microbiota functional and metabolic diversity in flavonoid
410 bioavailability is poorly understood. In the present investigation five taxa at the family level
411 displayed age differences, however hierarchical regression revealed no effects of microbial
412 composition or sex on metabolite concentration. Within our microbial composition dataset
413 alpha diversity was not significantly higher in older than younger individuals, however, other
414 studies have presented a mixed picture of aging versus gut and microbial alpha diversity (49),
415 and whilst this finding needs to be confirmed in future studies it is noteworthy that we
416 observed higher habitual flavonoid intakes in older individuals at baseline.

417 Tmax differed by as much as 3-5h between the age groups and was typically later in older
418 individuals. This finding highlights the need for future studies to consider longer sampling
419 strategies when recruiting wide age ranges for exploring metabolite bioactivity and
420 attempting to establish correlations between peak blood concentration of flavonoid
421 metabolites and health/disease status biomarkers (e.g., glucose or lipoprotein homeostasis).
422 The etiology of differences in Tmax between older and younger individuals is unknown and
423 were not explained by differences in BMI or microbiome speciation. They may derive from

424 differences in glomerular filtration rates, liver metabolism, or gastric and intestinal transit
425 time (50, 51) affecting food digestibility and absorption and elimination of microbial
426 metabolites, variables which should be captured where possible in future studies.
427 Precursor flavonoids from citrus, cocoa, and berries (naringenin, epicatechin, cyanidin) and
428 their previously reported metabolites, including microbial metabolites (hippuric acids,
429 benzoic acids, and valerolactones)(2, 4, 21, 36) were graphically depicted (**Supplement**
430 **Figures 1-13**) to visualize if differences in rates of elimination of precursor flavonoid across
431 age or sex groups differed or were predictive of their metabolite elimination. Rates of
432 elimination (i.e., slopes of cumulative elimination curves) appeared consistent across age and
433 sex, with older individuals generally having higher Cmax and later Tmax than younger
434 individuals for these perceived biomarkers of flavonoid intake. This evidence indicates that
435 monitoring a limited number of perceived intake biomarkers in nutrition interventions is
436 likely to poorly reflect actual shifts in the dietary metabolomes of individuals and highlights
437 the importance of using more global metabolomics approaches in future interventions.
438 In the present study both standard ($P < 0.05$) and conservative statistical approaches ($Q < 0.20$;
439 Benjamini-Hochberg method for false discovery) were utilized to emphasise the potential
440 impact of multiple testing when characterizing large numbers of metabolites. Moving
441 forward, polyphenol interventions using untargeted or quantitative metabolomics approaches
442 will require considerations and consensus on the most appropriate statistical practices.
443 The present study design was relatively unique for flavonoid (and (poly)phenols in general)
444 interventions as participants were prospectively recruited by age and sex, allowing for the
445 first comprehensive investigation of the impact of sex, age and microbiota speciation on acute
446 flavonoid absorption, metabolism and elimination following the consumption of a flavonoid
447 enriched test meal. The elimination of flavonoids from the background diet 48 h prior to the
448 intervention was an additional strength as it standardized and minimized the contribution of

449 background diet derived metabolites detected in urine post test meal. The present study was
450 however, unable to pinpoint a possible mechanism behind the variability in elimination. The
451 lack of characterization of additional possible mediators, such as gastric emptying, intestinal
452 transit, liver and kidney function (glomerular filtration rate) and genetic polymorphisms in
453 phase 1 and 2 metabolism, is identified as a study limitation, along with a lack of capture of
454 metabolite concentrations beyond 24h, which would have resulted in gut derived metabolites
455 not being fully recovered. However, it was decided during the trial design stage to limit
456 further participant burden caused by the restricted diet and repeated sampling over an
457 additional 24h. Many phytochemical phase II metabolites are/were not commercially
458 available as references standards for use in quantitative analysis (e.g., glucuronide or sulfate
459 conjugates of flavan-3-ols, valerolactones, phenylvaleric acids, etc.) and therefore the
460 present total recovery is likely to be an underestimation of amount and diversity of
461 polyphenol metabolites excreted. Further, there is likely to be some minimal contribution of
462 endogenous and dietary aromatic amino acids to the phenolic acid metabolite pools
463 quantified in the urine in the present study, such as hippuric acids; however, all participants
464 were on the same intervention meals and the exclusion diet was essentially void of
465 (poly)phenols, minimizing this possible confounding of endogenous or dietary substrates.
466 Finally, the research was conducted in a healthy population, with >90% of our study
467 population being white British, and extrapolation of findings to clinical groups with
468 significant disease pathology and medication use, or other ethnic groups, should be done with
469 caution.

470 **Conclusion.**

471 Our study provides evidence of a large impact of age on elimination kinetics of (poly)phenol
472 metabolites, particularly Tmax, which occurs much later in older individuals. The age effects
473 observed on individual metabolite recovery does not appear to be substantially driven by

474 differences in background diet, BMI and microbiota speciation. Our results indicate that
475 evaluating blood or urine signatures of (poly)phenol metabolites at a single time point is
476 unlikely to capture the true absorption and elimination kinetics across age and sex groups.
477

478 **ACKNOWLEDGMENTS**

479 This work was funding by a Biotechnology and Biological Sciences Research Council
480 (BBSRC), Food and Health, Institute Strategic Programme Grant (ISP), who had no role in
481 the design or delivery of the study or in the data analysis and its interpretation.
482

483 **Declaration of interest:** The authors report no conflict of interest.
484

485 **Authors' responsibilities** were as follows: AMM and AC designed the study. SH, NT, BCD,
486 DB and SL were responsible for participant recruitment, delivery of the intervention protocol
487 and the collection, processing and storage of biological samples. NT, BCD and CDK
488 designed, delivered and interpreted the urinary flavonoid analysis. LCC and DM were
489 responsible for preparation of the faecal samples for microbiome speciation and the
490 interpretation of the data. AJ conducted all statistical analysis. CDK, AJ and AMM drafted
491 the paper, with all authors contributing to and approving the final manuscript.
492

493 Data sharing plan: Data described in the manuscript, including urine concentration, analytical
494 methodologies, and statistical analysis data files will be made available upon request and
495 approval from the corresponding author.
496

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649 **TABLES**

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650 **Table 1:** Participant characteristics Mean (95% CI) and habitual dietary intake stratified by age and sex group in 163 participants from the COB
651 study.

	Older females (n=44)	**Older males (n=46)**	**Younger females (n=42)**	**Younger males (n=31)**
Age (y)	66.9 (65.5, 68.2)	67.8 (66.5, 69.1)	22.6 (21.2, 24.0)	22.4 (20.8, 24.0)
BMI (kg/m^2)	24.2 (23.4, 25.0)	25.4 (24.6, 26.3)	23.4 (22.6, 24.3)	23.0 (22.0, 24.0)
Energy (kcal)	2063 (1861, 2265)	2229 (2036, 2422)	1811 (1609, 2013)	2079 (1844, 2315)
Protein (g)	89.7 (81.7, 97.7)	89.9 (82.3, 97.6)	78.2 (70.3, 86.2)	88.9 (79.6, 98.2)
Fat (g)	74.5 (65.9, 83.0)	78.5 (70.3, 86.7)	62.6 (54.1, 71.2)	75.5 (65.5, 85.5)
Carbohydrate (g)	258 (229, 286)	287 (259, 314)	241 (212, 269)	269 (236, 303)
Fibre (g)	25.5 (22.8, 28.3)	25.4 (22.8, 28.0)	22.9 (20.1, 25.6)	22.7 (19.5, 25.9)
Total flavonoids (mg/d)	1298 (1150, 1446)	1237 (1096, 1378)	509 (361, 657)	478 (306, 650)
Flavanones (mg/d)	41.1 (31.8, 50.4)	30.8 (22.0, 39.7)	23.4 (14.1, 32.7)	22.1 (11.3, 33.0)
Anthocyanins (mg/d)	34.7 (29.1, 40.3)	29.5 (24.2, 34.9)	19.2 (13.6, 24.8)	11.5 (5.0, 18.0)
Flavan-3-ols (mg/d)	254 (220, 289)	255 (222, 288)	102 (67.6, 136)	84.8 (44.8, 125)
Flavonols (mg/d)	57.1 (51.1, 63.0)	53.2 (47.5, 58.9)	29.7 (23.7, 35.6)	24.6 (17.7, 31.6)

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Flavones (mg/d)	1.9 (1.6, 2.2)	1.7 (1.4, 1.9)	1.6 (1.3, 1.8)	1.2 (0.89, 1.5)
Polymers (mg/d)	909 (801, 1017)	866 (763, 969)	333 (225, 441)	334 (208, 459)
Fruit and vegetables (g/d)	794 (696, 891)	653 (559, 746)	583 (486, 681)	481 (367, 594)
Tea (g/d)	763 (650, 877)	759 (650, 869)	204 (88.9, 319)	217 (76.1, 358)

652 Values are mean (95% CI). n=163 (data missing for n=2 participants for dietary data).

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Table 2: Cumulative excretion (ng) by age and sex group at 24h.

Metabolite (ng)	**Older females**		**Older males**		**Younger females**		**Younger males**				
	n=	**Mean (95% CI)**	**n=**	**Mean (95% CI)**	**n=**	**Mean (95% CI)**	**n=**	**Mean (95% CI)**	**p-group**	**p-age**	**p-sex**
3-(dihydroxyphenyl)propionic acid*	42	0.043 (0.034, 0.053)	45	0.035 (0.025, 0.044)	42	0.056 (0.046, 0.065)	28	0.046 (0.035, 0.058)	0.02[a]	0.01[a]	0.05
4-methylhippuric acid (x 10^2)	42	0.297 (0.173, 0.421)	45	0.629 (0.509, 0.749)	42	0.296 (0.171, 0.421)	29	0.412 (0.263, 0.562)	<0.01[a]	0.07	<0.01[a]
3-methoxycinnamic acid	42	0.033 (0.009, 0.056)	45	0.079 (0.056, 0.101)	42	0.049 (0.025, 0.072)	29	0.024 (-0.004, 0.052)	<0.01[a]	0.15	0.18
6-methoxysalicyclic acid (x 10^2)	42	0.091 (0.031, 0.150)	45	0.225 (0.166, 0.283)	41	0.147 (0.086, 0.208)	29	0.057 (-0.016, 0.129)	<0.01[a]	0.12	0.22
4-hydroxy-3-methoxyacetophenone	42	0.020 (0.013, 0.028)	45	0.029 (0.022, 0.037)	42	0.036 (0.028, 0.044)	29	0.037 (0.028, 0.046)	0.01[a]	<0.01[a]	0.28
Benzoylglutamic acid	42	0.012 (0.008, 0.016)	45	0.021 (0.017, 0.025)	42	0.013 (0.009, 0.018)	29	0.013 (0.007, 0.018)	0.01[a]	0.11	0.03
5-(Hydroxyphenyl)-gamma-valerolactone-sulfate	42	0.313 (0.192, 0.434)	45	0.585 (0.468, 0.703)	42	0.506 (0.385, 0.627)	29	0.186 (0.040, 0.331)	<0.01[a]	0.23	0.77
5-(hydroxyphenyl)-gamma-valerolactone (x 10^2)	42	2.4 (1.4, 3.4)	44	2.2 (1.3, 3.2)	42	4.1 (3.1, 5.0)	28	1.4 (0.207, 2.6)	<0.01[a]	0.19	0.01[a]
naringenin-7-*O*-glucuronide (x 10^2)	42	3.4 (2.5, 4.4)	45	4.2 (3.3, 5.1)	42	4.9 (4.0, 5.9)	29	2.4 (1.2, 3.5)	<0.01[a]	0.88	0.17
2,3-dihydroxybenzoic acid	42	0.059 (0.038, 0.080)	45	0.101 (0.081, 0.121)	42	0.093 (0.072, 0.114)	29	0.067 (0.042, 0.092)	0.01[a]	0.86	0.29
3-methylhippuric acid	42	0.006 (0.004, 0.009)	45	0.014 (0.012, 0.016)	42	0.006 (0.004, 0.009)	29	0.005 (0.002, 0.008)	<0.01[a]	<0.01[a]	<0.01[a]
3-methoxybenzoic acid-4-sulfate	41	0.616 (0.441, 0.791)	42	0.870 (0.697, 1.0)	41	0.525 (0.348, 0.701)	29	0.900 (0.691, 1.1)	<0.01[a]	0.50	<0.01[a]
Benzoic acid-4-*O*-glucuronide	42	0.034 (0.029, 0.040)	45	0.028 (0.023, 0.033)	42	0.028 (0.022, 0.033)	29	0.017 (0.011, 0.024)	<0.01[a]	0.01[a]	0.02[a]
2-hydroxycinnamic acid (x 10^2)	42	0.021 (0.007, 0.035)	46	0.018 (0.005, 0.032)	41	0.022 (0.008, 0.036)	30	0.065 (0.049, 0.082)	<0.01[a]	<0.01[a]	0.04
3-(4-hydroxy-3-methoxyphenyl)propionic acid	42	0.051 (0.026, 0.075)	46	0.081 (0.058, 0.105)	41	0.106 (0.081, 0.130)	30	0.106 (0.077, 0.135)	<0.01[a]	<0.01[a]	0.31
hesperetin-3'-*O*-glucuronide	42	0.517 (0.346, 0.689)	46	0.772 (0.607, 0.937)	41	0.567 (0.393, 0.741)	30	0.356 (0.152, 0.559)	0.02[a]	0.06	0.48
3-methoxy-4-hydroxyphenylacetic acid	42	0.922 (0.530, 1.3)	46	1.7 (1.3, 2.1)	41	1.0 (0.605, 1.4)	30	0.842 (0.378, 1.3)	<0.01[a]	0.06	0.06
3-methoxybenzoic acid-4-*O*-glucuronide	42	0.394 (0.236, 0.552)	46	0.625 (0.473, 0.777)	41	0.462 (0.302, 0.623)	30	0.285 (0.098, 0.471)	0.04[a]	0.13	0.46
hydroxy-methoxybenzoic acid*	42	0.065 (0.045, 0.085)	46	0.075 (0.056, 0.095)	41	0.090 (0.069, 0.110)	30	0.133 (0.109, 0.157)	<0.01[a]	<0.01[a]	0.06
Hydroxybenzoic acid*	42	0.010 (0.007, 0.013)	46	0.010 (0.008, 0.013)	40	0.013 (0.010, 0.016)	30	0.005 (0.002, 0.009)	<0.01[a]	0.78	0.05
3-hydroxyhippuric acid	42	1.8 (1.3, 2.2)	46	2.5 (2.0, 2.9)	41	1.5 (0.986, 1.9)	30	1.6 (1.1, 2.2)	0.02[a]	0.02[a]	0.04

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4-methoxybenzaldehyde	41	0.007 (0.005, 0.009)	46	0.005 (0.003, 0.007)	41	0.008 (0.007, 0.010)	29	0.011 (0.008, 0.013)	<0.01[a]	<0.01[a]	0.59
3-hydroxy-4-methoxybenzoic acid	42	0.096 (0.074, 0.119)	46	0.082 (0.061, 0.104)	41	0.140 (0.117, 0.163)	29	0.166 (0.139, 0.193)	<0.01[a]	<0.01[a]	0.82
Hydroxybenzoic acid-sulfate*	39	0.318 (0.188, 0.447)	44	0.544 (0.421, 0.667)	39	0.303 (0.173, 0.433)	26	0.404 (0.245, 0.563)	0.03[a]	0.18	<0.01[a]
Hippuric acid	34	58.1 (44.9, 71.3)	37	77.8 (65.1, 90.5)	34	58.7 (45.4, 72.0)	22	45.6 (29.2, 61.9)	0.02[a]	0.04	0.31
3-(phenyl)propionic acid	34	0.019 (-0.020, 0.058)	38	0.009 (-0.028, 0.046)	34	0.047 (0.008, 0.086)	22	0.092 (0.044, 0.140)	0.04[a]	0.01[a]	0.75

655 Values are mean (95% CI). Metabolites shown are those with a significant group, age or sex effect. P values are for group comparisons at 24
656 hours and calculated from linear mixed-effect models. [a] false discovery rate adjusted p values <0.2. *Full dataset providing p values for all*
657 *analytes is found in Supplement Table 4.* *Metabolite isomers which could not be resolved effectively by HPLC were quantified according to
658 one of the structural isomers. All urine metabolites values are presented as: ((Metabolite value [nmol/l] * molecular weight [ng/nmol]
659 /1000000)*(urine volume [L]))/time [h]. Where indicated in data tables, low concentrations are presented as 10^2 (for example 0.01x10^{-}2 =
660 0.0001 ng).

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661 **Table 3:** Maximum urinary elimination (Cmax) by age and sex group.

Metabolite (ng)	**Older females**		**Older males**		**Younger females**		**Younger males**				
	n =	**Mean (95% CI)**	**n=**	**Mean (95% CI)**	**n=**	**Mean (95% CI)**	**n=**	**Mean (95% CI)**	**p-group**	**p-age**	**p-sex**
2,5-dihydroxybenzoic acid	44	0.52 (0.26, 0.79)	46	0.83 (0.58, 1.1)	42	0.54 (0.27, 0.81)	31	0.85 (0.53, 1.2)	0.34	0.95	0.03
4-methylhippuric acid	44	0.02 (0.01, 0.03)	46	0.05 (0.04, 0.06)	42	0.02 (0.003, 0.03)	31	0.03 (0.01, 0.04)	0.53	0.03	<0.01a
3-methylhippuric acid	44	0.05 (0.02, 0.07)	46	0.11 (0.08, 0.13)	42	0.04 (0.01, 0.07)	31	0.04 (0.01, 0.07)	0.29	0.02	0.02
3-methoxybenzoic acid-4-sulfate	44	1.8 (0.68, 2.9)	46	2.7 (1.6, 3.8)	42	1.4 (0.29, 2.6)	31	4.4 (3.1, 5.8)	0.04	0.45	<0.01a
benzoic acid-4-*O*-glucuronide	44	0.16 (0.13, 0.20)	46	0.14 (0.10, 0.17)	42	0.13 (0.09, 0.17)	31	0.09 (0.05, 0.14)	0.02	0.07	0.13
benzoic acid-4-sulfate	44	9.7 (5.0, 14.3)	46	17.3 (12.7, 21.8)	41	8.1 (3.3, 12.9)	31	10.8 (5.3, 16.4)	0.62	0.09	0.02
hydroxy-methoxybenzoic acid*	44	0.17 (0.09, 0.26)	46	0.25 (0.17, 0.33)	41	0.26 (0.18, 0.35)	31	0.42 (0.32, 0.52)	<0.01a	0.01	0.02
3-hydroxyhippuric acid	44	17.5 (10.0, 25.0)	46	22.0 (14.7, 29.3)	41	10.5 (2.7, 18.2)	31	12.6 (3.6, 21.5)	0.14	0.03	0.30
3-hydroxy-4-methoxybenzoic acid	44	0.28 (0.16, 0.39)	46	0.29 (0.18, 0.41)	42	0.37 (0.25, 0.48)	31	0.60 (0.46, 0.74)	<0.01a	<0.01	0.12

662 Values are mean (95% CI). Metabolites shown are those with a significant group, age or sex effect; P values calculated from linear regression. [a] false

663 discovery rate adjusted p values <0.2. *Full dataset providing p values for all analytes is found in Supplement Table 6.* *Metabolite isomers which could

664 not be resolved effectively by HPLC were quantified according to one of the structural isomers. All urine metabolites values are presented as:

665 (Metabolite value [nmol/l] * molecular weight [ng/nmol] /1000000)*(urine volume [L]))/time [h].

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668 **Table 4: Time (hours) of Maximal urinary elimination** (Tmax) by age and sex group.

Metabolite	Older females		Older males		Younger females		Younger males				
	n=	Mean (95% CI)	n=	Mean (95% CI)	n=	Mean (95% CI)	n=	Mean (95% CI)	p-group	p-age	p-sex
3,4-dihydroxyphenylacetic acid	44	14.7 (11.4, 17.9)	46	15.7 (12.5, 18.8)	42	16.3 (12.9, 19.6)	31	20.9 (17.1, 24.8)	0.02	0.08	0.18
3-methoxycinnamic acid	44	8.1 (4.8, 11.4)	46	13.3 (10.0, 16.5)	42	6.9 (3.5, 10.3)	31	9.5 (5.6, 13.5)	0.76	0.12	0.02
4-hydroxybenzaldehyde	44	11.3 (8.0, 14.6)	46	13.5 (10.3, 16.7)	42	5.5 (2.1, 8.9)	31	9.3 (5.4, 13.2)	0.06	<0.01a	0.06
4-hydroxybenzyl alcohol	44	12.0 (8.6, 15.4)	46	11.2 (7.9, 14.5)	42	13.6 (10.1, 17.1)	31	17.5 (13.4, 21.5)	0.04	0.05a	0.60
3-caffeoylquinic acid	44	14.0 (10.7, 17.2)	46	16.8 (13.6, 20.0)	42	13.1 (9.7, 16.4)	31	17.7 (13.8, 21.6)	0.44	0.82	0.03
hesperetin	44	11.8 (9.0, 14.5)	46	9.7 (7.0, 12.4)	42	11.1 (8.2, 13.9)	31	5.7 (2.4, 9.0)	0.03	0.20	0.02
hesperetin-7'-sulfate	44	5.9 (3.4, 8.4)	46	5.5 (3.1, 8.0)	42	2.2 (-0.3, 4.8)	31	3.1 (0.15, 6.1)	0.04	0.02a	0.74
3,4-dihydroxybenzoic acid methyl ester	44	5.5 (2.8, 8.2)	46	8.3 (5.7, 10.9)	42	2.6 (-0.1, 5.4)	31	6.0 (2.8, 9.2)	0.45	0.05a	0.02
naringenin-7-*O*-glucuronide	44	11.1 (8.5, 13.8)	46	13.3 (10.7, 15.9)	42	8.5 (5.7, 11.2)	31	10.2 (7.1, 13.4)	0.22	0.03a	0.12
naringenin-7-*O*-glucuronide	44	11.1 (8.5, 13.8)	46	13.3 (10.7, 15.9)	42	8.5 (5.7, 11.2)	31	10.2 (7.1, 13.4)	0.22	0.03a	0.12
3,4-dihydroxybenzoic acid	44	12.4 (9.4, 15.3)	46	12.4 (9.5, 15.3)	42	9.4 (6.3, 12.4)	31	7.7 (4.2, 11.2)	0.02	0.02a	0.81
4-hydroxycinnamic acid	44	6.4 (3.9, 8.9)	46	9.7 (7.3, 12.2)	42	5.4 (2.9, 8.0)	31	7.7 (4.7, 10.7)	0.90	0.20	0.02
trihydroxybenzaldehyde*	44	6.9 (5.1, 8.8)	46	7.9 (6.1, 9.7)	42	4.4 (2.5, 6.3)	31	4.8 (2.6, 7.1)	0.03	<0.01a	0.34
3-methylhippuric acid	44	4.4 (1.3, 7.4)	46	4.2 (1.2, 7.2)	42	7.1 (4.0, 10.2)	31	9.3 (5.7, 12.9)	0.02	0.02a	0.74
2-hydroxy-4-methoxybenzoic acid	44	5.9 (3.3, 8.4)	46	5.5 (3.0, 8.0)	42	2.3 (-0.3, 4.9)	31	2.3 (-0.7, 5.4)	0.02	0.01a	0.93
3-(3-hydroxyphenyl)propionic acid	44	21.3 (18.5, 24.0)	46	19.6 (16.9, 22.3)	42	20.6 (17.8, 23.4)	31	14.0 (10.8, 17.3)	0.01a	0.08	0.02
(-)-epicatechin	44	9.6 (6.8, 12.3)	46	9.7 (6.9, 12.4)	42	6.9 (4.1, 9.7)	31	5.8 (2.5, 9.1)	0.04	0.03a	0.92
4-hydroxybenzoic acid-3-*O*-glucuronide	44	5.9 (3.8, 8.0)	46	8.7 (6.6, 10.8)	42	4.2 (2.1, 6.4)	31	7.6 (5.0, 10.1)	0.94	0.14	0.01
hydroxy-methoxybenzoic acid*	44	8.6 (5.9, 11.2)	46	12.1 (9.5, 14.7)	42	6.3 (3.6, 9.1)	31	5.9 (2.7, 9.1)	0.04	<0.01a	0.14
hydroxybenzoic acid*	44	15.4 (12.0, 18.7)	46	13.8 (10.6, 17.1)	42	11.3 (7.9, 14.7)	31	10.3 (6.3, 14.3)	0.03	0.04a	0.58

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3-hydroxybenzoic acid-4-*O*-glucuronide	44	9.6 (7.1, 12.0)	46	11.5 (9.0, 13.9)	42	6.3 (3.8, 8.8)	31	4.9 (2.0, 7.9)	<0.01a	<0.01a	0.52
cyanidin-3-*O*-glucoside	44	6.6 (4.5, 8.7)	46	6.7 (4.6, 8.7)	42	2.8 (0.60, 4.9)	31	6.4 (3.9, 8.9)	0.25	0.04a	0.10
ellagic acid	44	15.6 (12.7, 18.4)	46	15.1 (12.3, 17.9)	42	10.0 (7.1, 12.9)	31	13.4 (10.1, 16.8)	0.06	0.01a	0.29
3-hydroxy-4-methoxybenzoic acid	44	9.8 (6.8, 12.8)	46	11.4 (8.5, 14.3)	42	7.5 (4.4, 10.5)	31	7.1 (3.6, 10.7)	0.11	0.04a	0.51
catechin*	44	10.1 (7.0, 13.3)	46	12.3 (9.2, 15.4)	42	7.2 (4.0, 10.5)	31	8.6 (4.9, 12.4)	0.20	0.04a	0.21
hydroxybenzoic acid-sulfate*	44	9.6 (7.0, 12.1)	46	14.0 (11.5, 16.5)	42	5.6 (3.0, 8.3)	31	6.3 (3.2, 9.3)	0.01a	<0.01a	0.02
4-hydroxy-3-methoxybenzoic acid methyl ester	44	13.0 (9.8, 16.3)	46	9.3 (6.1, 12.4)	42	6.4 (3.1, 9.7)	31	6.2 (2.3, 10.1)	<0.01a	0.01a	0.32
3-methoxybenzoic acid	44	3.5 (1.0, 6.0)	46	3.9 (1.4, 6.3)	42	3.3 (0.71, 5.8)	31	4.7 (1.7, 7.6)	0.69	0.90	0.54
hippuric acid	44	11.8 (8.3, 15.3)	46	12.7 (9.3, 16.1)	42	7.6 (4.0, 11.2)	31	8.5 (4.4, 12.7)	0.07	0.02a	0.49

669 Values are mean (95% CI). Metabolites shown are those with a significant group, age or sex effect; P values calculated from linear regression. [a]

670 false discovery rate adjusted p values <0.2. *Full dataset providing p values for all analytes is found in Supplement Table 6.* *Metabolite isomers

671 which could not be resolved effectively by HPLC were quantified according to one of the structural isomers.

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Table 5: Classification tables obtained from linear discriminant analysis in 163 participants from the COB study

Metabolite	Coefficient[1]	P=
hydroxy-methoxybenzoic acid*	0.43	0.08
3-methylhippuric acid	-0.44	0.03
4-methoxybenzaldehyde	0.28	0.02
2-hydroxycinnamic acid	0.36	0.06
3-hydroxy-4-methoxybenzoic acid	0.17	0.01
3-(4-hydroxy-3-methoxyphenyl)propionic acid	0.27	0.13
4-Hydroxy-3-methoxyacetophenone	0.23	0.17
3-(phenyl)propionic acid	0.18	0.08
3-(dihydroxyphenyl)propionic acid*	0.15	0.22
benzoic acid-4-*O*-glucuronide	-0.50	0.13

[1] Standardized canonical discriminant function coefficients representing the effect of a 1 SD increase on metabolite concentration on the SD increase in the predicted values on the discriminant function. These values indicate the predictive ability of these metabolites to classify participants into the correct age group. P= calculated using ANOVA. *Metabolites without numbered nomenclature are isomers of unknown hydroxy, methoxy, sulfate or glucuronide structural orientation (no physical references standards were available for retention time conformation (but were matched for mass and MS/MS spectra).

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1 **Table 6: Hierarchal regression analysis of predictors of metabolite concentrations in COB participants**

Metabolite	**n=**	**M1**	**M2**	**M3**
		β (95% CI) for age	**P=**	**P=**
hydroxy-methoxybenzoic acid*	156	0.04 (0.01, 0.07)	0.06	0.67
3-methylhippuric acid(x 10^2)	155	-0.42 (-0.84, 0.00)	0.05	0.74
4-methoxybenzaldehyde	154	0.00 (0.05, 0.01)	0.94	0.97
2-hydroxycinnamic acid(x 10^2)	156	0.02 (0.00, 0.05)	0.10	0.58
3-hydroxy-4-methoxybenzoic acid	155	0.06 (0.02, 0.09)	0.69	0.56
3-(4-hydroxy-3-methoxyphenyl)propionic acid	156	0.04 (-0.15, 0.08)	0.36	0.73
4-Hydroxy-3-methoxyacetophenone	155	0.01 (-0.13, 0.02)	0.36	0.55
3-(phenyl)propionic acid	125	0.05 (-0.66, 0.11)	0.64	0.94
3-(dihydroxyphenyl)propionic acid*	154	0.01 (-0.75, 0.02)	0.39	0.58
benzoic acid-4-*O*-glucuronide(x 10^2)	155	-0.74 (-1.62, 0.00)	0.06	0.43

2 M1 = age; M2 = age and sex; M3 = age and PCA1. β (95% CI) for age were calculated from hierarchical regression with metabolite
3 concentration as the dependent variable and age, sex and PCA1 as predictors. P= p-value of the F-statistic indicating if the subsequent model
4 offered any significant improvement over M1. M= model; PCA1 = A linear combination of the gut microbiome variables associated with age

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5 (first principal component; 41 % of the variance). *Metabolite isomers which could not be resolved effectively by HPLC were quantified

6 according to one of the structural isomers.

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8 **FIGURE LEGENDS**

9 **Figure 1:** chocolate, orange juice and blackberry (COB) intervention to examine flavonoid

10 metabolism

11 **Figure 2:** Participant Flow Diagram.

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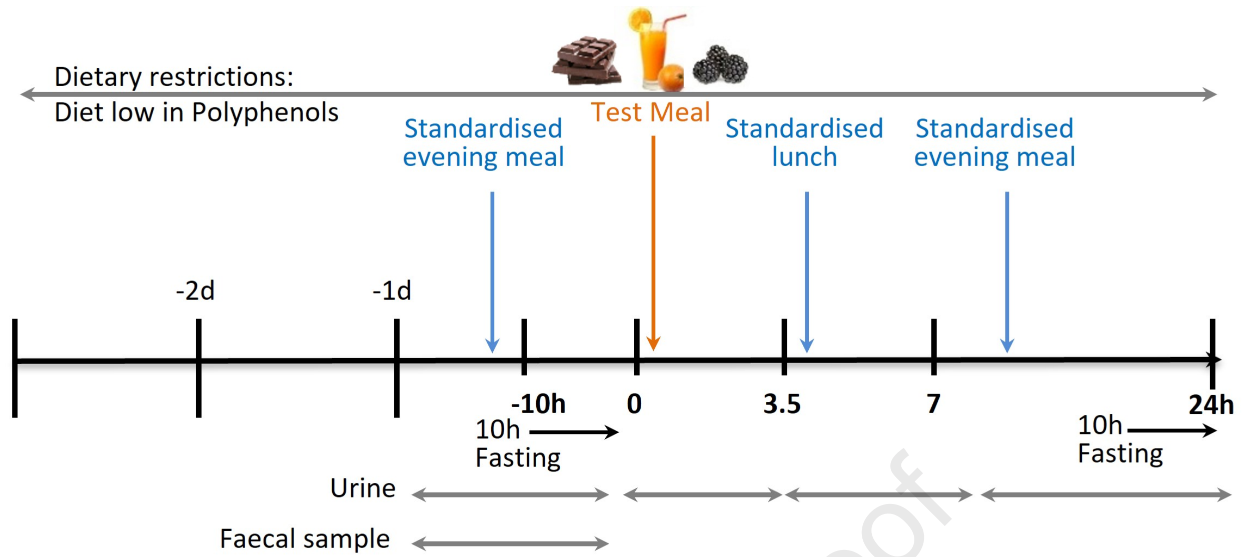

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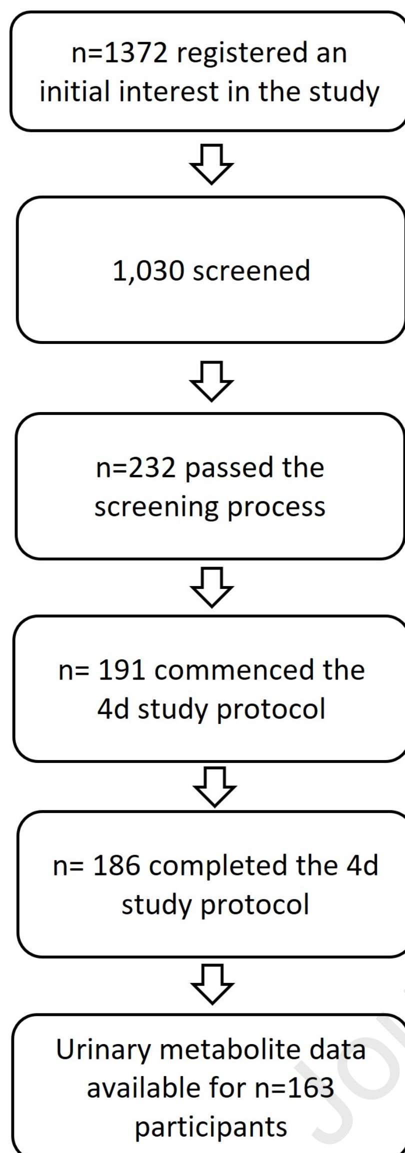

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Declaration of interests

x The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Anne-Marie Minihane reports was provided by University of East Anglia Norwich Medical School. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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