

1 **Application of liquid chromatography-tandem mass spectrometry to determine urinary**
2 **concentrations of five commonly used low-calorie sweeteners: a novel biomarker approach**
3 **for assessing recent intakes?**

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12 Abstract

13 Although the use of low-calorie sweeteners (LCSs) is widespread, methods of assessing
14 consumption within free-living populations have inherent limitations. Five commonly consumed
15 LCSs, namely acesulfame-K, saccharin, sucralose, cyclamate and steviol glycosides are excreted
16 via the urine and therefore a urinary biomarker approach may provide more objective LCS intake
17 data. A LC-ESI-MS/MS method of simultaneously determining acesulfame-K, saccharin,
18 sucralose, cyclamate and the excretory metabolite of steviol glycosides, steviol glucuronide, in
19 human urine was developed and validated. Linearity was observed over a concentration range of
20 10-1000 ng/ml with coefficients of determination ranging from 0.9969 to 0.9997. Accuracy ranged
21 from 92 to 104% and intra-batch and inter-day precision were within acceptable limits with % CV
22 below 8% for all compounds. A double-blind, randomized cross-over dose-response study was
23 conducted to assess the usefulness of urinary LCS excretions (from both fasting spot and a full 24-
24 hour urine collection) for investigating recent intakes. Both modes of sampling were useful for
25 distinguishing between the three short-term intakes of acesulfame-K, saccharin, cyclamates and
26 steviol glycosides ($p < 0.001$) while for sucralose, urinary concentrations were useful for
27 distinguishing between low (0.1% ADI) and high doses (10% ADI) only ($p < 0.001$). In summary,
28 this biomarker approach may be useful for assessing intakes of five commonly consumed LCSs.

29 **Keywords:** low-calorie sweeteners; intense sweeteners; biomarkers; urinary biomarkers;
30 exposure; food additives; human urine.

31 **Introduction**

32 The prevalence of obesity, a major risk factor for the development of chronic conditions such as
33 cardiovascular disease, type 2 diabetes mellitus and hypertension,¹ has increased significantly over
34 recent decades.²⁻⁴ Although its cause at the population level is multi-factorial,¹ central to its
35 occurrence at the individual level are lifestyles characterized by sedentary behaviors and unhealthy
36 dietary practices. One dietary practice that has been implicated in contributing to overweight and
37 obesity, as well as other adverse health outcomes, is the over-consumption of free sugars,
38 particularly from sugar-sweetened beverages.^{5,6} As such, recently published guidelines
39 recommend that intakes of free sugars should not exceed 5%⁶ or 10%⁵ of total energy intake. Given
40 that current free sugar intakes within the population largely exceed these recommendations,⁶
41 strategies that support a reduction in intake, while maintaining diet palatability, include the
42 substitution of free sugars with low-calorie sweeteners (LCSs).

43 LCSs are a chemically diverse group of intensely sweet, low energy, food additives that are non-
44 cariogenic⁷ and used in a wide range of dietary and non-dietary products to provide a desired sweet
45 taste without increasing energy density or inducing adverse oral health outcomes. Intuitively, LCSs
46 should be expected to offer health benefits when used in place of free sugars; indeed, a recent
47 meta-analysis of RCTs (sustained over 4 weeks to 40 months) indicated that LCS consumption
48 versus sugar led to reductions in body weight.⁸ Despite such health benefits, debate around their
49 long-term efficacy continues, particularly in relation to weight management,⁹ type 2 diabetes^{10,11}
50 and other metabolic effects.¹² A number of mechanisms by which LCSs may adversely impact on
51 health have been discussed and while a number of these putative mechanisms have been supported
52 by some animal data, none have yet been demonstrated in humans.¹³ Observational data, in
53 particular, have been conflicting in relation to the health effects of LCSs, as highlighted in a recent

54 review of the evidence by the French Agency for Food, Environmental and Occupational Health
55 and Safety (ANSES);¹⁴ however a major limitation of most cohort studies is that only low-calorie
56 sweetened beverage (LCSB) consumption is considered when investigating associations between
57 LCSs and health. LCSs are ubiquitous in today's society and LCSBs are only one of many sources
58 of LCSs in the diet; indeed, some LCSs have recently been identified as potential environmental
59 contaminants and detected in drinking-water sources¹⁵ so exposure to some LCSs may actually
60 occur inadvertently in certain populations. Furthermore, intakes of individual LCSs are very rarely
61 estimated in cohort studies,¹⁴ impairing the ability to properly explore relationships between these
62 chemically diverse food additives and health. This is further compounded by the fact that blends
63 of LCSs are now often used within the same product. With these factors in mind, conclusions
64 drawn from current observational data are likely to be tenuous and therefore, an alternative
65 approach which provides more objective and specific LCS intake data would be highly desirable
66 in order to enhance research in the area of LCSs and human health.

67 A nutritional biomarker approach involving the measurement of components of the diet, or their
68 metabolites, as indicators of intake may offer an opportunity to more objectively and specifically
69 assess intakes of LCSs and therefore address a number of fundamental limitations with some
70 current research approaches into the health impacts of LCS use. Such an approach has previously
71 been utilized for investigating intakes of other dietary components such as salt, protein and
72 wholegrains.^{16,17} A number of LCSs, once absorbed into the body, are excreted via the urine
73 following little or no metabolism.¹⁸ Urinary excretions of acesulfame-K and saccharin were
74 previously investigated as potential biomarkers of intakes and were found to be useful.¹⁹ Of the
75 other commonly used LCSs, sucralose and cyclamate may also be suitable for a urinary biomarker
76 approach as the absorbed proportion is excreted largely unchanged via the urine.^{18,20} Steviol

77 glycosides, in contrast, undergo bacterial hydrolysis to steviol in the large intestine and, following
78 absorption, are excreted in the urine as steviol glucuronide;²¹ as such, this metabolite may prove
79 to be a useful biomarker of intake. To determine the feasibility of such a biomarker approach, it is
80 first necessary to develop a suitable and reliable analytical method for measuring the compounds
81 of interest.^{22,23} One of the most commonly reported techniques for simultaneously analyzing LCSs
82 in foods, beverages, and in water samples, is liquid chromatography with mass spectrometry using
83 electrospray ionization (LC-ESI-MS/MS).^{15,24-27} Validation of the biomarker should also be
84 conducted to characterize the relationship between the candidate marker and the dietary
85 component of interest.²⁸

86 The present work consisted of two studies with distinct aims: firstly, to develop and validate a LC-
87 ESI-MS/MS method of simultaneously determining acesulfame-K, saccharin, sucralose and
88 cyclamate and steviol glucuronide in human urine and secondly, to assess whether urinary
89 excretions of these compounds are useful for investigating short-term intakes of the respective
90 LCS. It was hypothesized that urinary excretions of the compounds of interest would be useful for
91 distinguishing between three levels of intake relevant to the free-living population.

92 **Materials and Methods**

93 **Method development and dose-response studies**

94 *Reagents and reference materials*

95 High performance liquid chromatography (HPLC) grade water and HPLC grade methanol were
96 purchased from Fisher Chemicals (Loughborough, UK). Ammonium carbonate, along with
97 acesulfame-K, sodium saccharin, sodium cyclamate and sucralose, were purchased from Sigma-
98 Aldrich (St. Louis, MO, USA). Steviol glucuronide (96.4% purity by HPLC) was kindly supplied

99 by The Coca-Cola Company (Atlanta, US). Acesulfame-d4 potassium salt (Ace-d4), sucralose-d6
100 (Suc-d6) and saccharin-d4 (Sac-d4) were purchased from Toronto Research Chemicals Inc.
101 (Toronto, Canada) while sodium cyclamate-d11 (Cyc-d11) and warfarin sodium were purchased
102 from QMX Laboratories (Thaxted, UK) and Sigma-Aldrich (St. Louis, MO, USA) respectively.
103 All reference materials were of analytical standard.

104 Individual stock solutions of 1 mg/ml were prepared in water/methanol (50:50) for acesulfame-K,
105 sodium saccharin, sodium cyclamate, sucralose and steviol glucuronide and from these, a working
106 solution containing all five compounds at concentrations of 0.1 mg/ml was prepared in
107 water/methanol (50:50) and stored at 4 °C. For the internal standards (IS), individual stock
108 solutions of 1 mg/ml were prepared in methanol for Ace-d4, Sac-d4 and Suc-d6. Warfarin sodium
109 and Cyc-d11 were dissolved in water/methanol (50:50) at concentrations of 80 µg/ml and 40 µg/ml
110 respectively. Prior to each batch, a working solution containing 5 µg/ml Ace-d4, Sac-d4, Suc-d6
111 and 4 µg/ml and 2 µg/ml warfarin sodium and Cyc-d11 respectively was prepared in mobile phase
112 A (MP-A) (see below for MP-A composition).

113 Boric acid, purchased from Sigma-Aldrich (St. Louis, MO, USA), was used as a preservative in
114 urine samples by adding 10 g to each 3 liter container. Completeness of 24-hour urine collections
115 was assessed using the paraminobenzoic acid (PABA) method.²⁹ During each 24-hour urine
116 collection period, participants were asked to consume one tablet containing 80 mg PABA at three
117 specified times (8am, 12pm and 6pm; 240 mg in total) and a recovery of at least 187 mg (78% of
118 the dose) signified a complete sample.³⁰ PABA tablets were purchased from the Medical Research
119 Council (Cambridge, UK). If participants forgot to take all three PABA tablets yet reported
120 collecting a complete 24-hour sample, the urine sample was also considered complete.

121 *Instrumentation*

122 Method development and validation was conducted on a HPLC-MS system consisting of a Thermo
123 Separation Products HPLC system (Waltham, US) interfaced with a QTrap 3200 hybrid
124 quadrupole-linear ion trap mass spectrometer (AB Sciex, Warrington, UK). Urine analysis in the
125 dose-response study was conducted on a HPLC-MS system consisting of a Shimadzu UHPLC
126 system (Milton-Keynes, UK) and an API 4000 triple quadrupole mass spectrometer (AB Sciex,
127 Warrington, UK) following method transfer and optimization of MS conditions.

128 **Method development and validation**

129 *Collection of LCS-free urine samples for method development*

130 Twelve apparently healthy volunteers (6 females; 6 males; age range, 21-51 years) were recruited
131 to provide blank urine samples for the purposes of method development and validation. Those
132 with diabetes, renal impairment, taking or planning to take sulfonamide antibiotics during the study
133 or with lactose intolerance were excluded from the study. Participants were asked to avoid foods
134 and beverages known to contain acesulfame-K, saccharin, cyclamate, sucralose and steviol
135 glycosides for a period of at least three days prior to collecting a 24-hour urine sample. To aid this,
136 verbal advice and literature were provided to each participant. From each sample, ten 1 ml aliquots
137 and two 30 ml aliquots were retained and stored at -80 °C for later use in method development.
138 Ethical approval was granted for this method development study by the Ulster University
139 Biomedical Sciences Research Ethics Filter Committee (Study No: FCBMS-13-058).

140 *Calibration and Quality Control samples*

141 All urine samples (n = 12) were screened for the presence of the compounds of interest and those
142 found with no or negligible concentrations (n = 6), were used for the purposes of method
143 development and validation. Calibration and quality control (QC) samples were prepared by
144 fortifying blank urine samples with known concentrations of the compounds of interest.

145 For the preparation of calibration standards, fortification solutions at concentrations of 1.0, 12.5,
146 25.0, 37.5, 50.0, 75.0 and 100.0 µg/ml were prepared from the respective LCS working solution.
147 Fortification solutions were also prepared at concentrations of 12.5, 55.0 and 93.0 µg/ml and these
148 were used for the preparation of QC samples which were run during each batch. Fortification of
149 the blank urine samples resulted in a 10-fold dilution of these concentrations.

150 *Sample preparation*

151 A simple preparation procedure was used in which fortified urine samples underwent a further 10-
152 fold dilution and were filtered. Each sample was vortexed for a minimum of 10 seconds to ensure
153 homogenization and 100 µl was mixed with 20 µl of IS working solution in a 1.5 ml tube and made
154 up to 1 ml using MP-A (see below for composition of MP-A). The mixture was again vortexed for
155 a minimum of 10 seconds and filtered using a 0.22 µm mixed cellulose ester filter membrane
156 (Merck KGaA, Darmstadt, Germany). From the filtrate, 100 µl was retained for analysis.

157 *Liquid chromatography-tandem mass spectrometry conditions*

158 Separation was accomplished using a Poroshell 120 EC-C18 (4.6 x 50 mm), 2.7 µm column
159 equipped with a Poroshell 120 UHPLC EC-C18 guard column (Agilent Technologies, Santa Clara,
160 US). HPLC conditions from an Agilent Technologies application note³¹ were adapted for the
161 purposes of this method. Flow rate was maintained at 0.6 ml/min throughout and a binary gradient
162 program was used. MP-A consisted of 2 mM ammonium carbonate in water/methanol (95/5%)

163 (pH 8.8) and mobile phase B (MP-B) consisted of 2 mM ammonium carbonate in methanol (pH
164 8.9). The gradient program was as follows: 0 min, 100% A, 7.0 min, 25% A, 9.0 min, 25% A, 9.1-
165 15.0 min, 100% A. Sample injection volume was 20 µl and the needle was flushed with 500 µl of
166 a water/methanol (60/40%) solution following each injection. Column temperature was
167 maintained at 30 °C throughout using an integrated column oven.

168 The mass spectrometer was equipped with a Turbo-V ion source and ESI was operated in negative
169 polarity with multiple reaction monitoring (MRM) scan mode used for detection. Scheduled
170 scanning for specific compounds was carried out according to the expected time of elution, thus
171 improving sensitivity and precision. Nitrogen gas, supplied by a Peak Scientific gas generator, was
172 used as nebulizer, collision and desolvation gas. The two most intense MRM transitions were
173 identified and compound specific parameters including declustering potential, collision energy and
174 collision cell exit potential were optimized by directly infusing each compound at concentrations
175 of 1 µg/ml in methanol into the mass spectrometer. Ion source parameters for each compound were
176 optimized using flow injection analysis with the aim of maximizing signal intensity and stability.
177 The source temperature was set at 600 °C and gas sources 1 and 2 were set at 35 psi and 40 psi
178 respectively. Ion spray voltage was -4.5 kv and curtain gas was set at 40 psi.

179 *Method validation*

180 Method validation was conducted by assessing method performance in relation to linearity, limits
181 of detection (LOD), lower limits of quantification (LLOQ), precision and accuracy, and finally
182 matrix effects (ME). Linearity was assessed across a concentration range of 10-1000 ng/ml by
183 generating eight-point calibration curves for each compound of interest. The ratios of the peak
184 areas of the target analytes and their corresponding IS were plotted versus concentration following

185 duplicate analysis and a weighting of 1/x was applied owing to the large working range. LOD and
186 LLOQ, defined as signal to noise ratio of 3:1 and the lowest concentration of the linear regression
187 respectively,³² were assessed after conducting duplicate analysis of blank urine samples fortified
188 to concentrations ranging from 0.001-5ng/ml. Precision and accuracy were assessed at three
189 concentrations within the working range; 12.5 ng/ml (low), 550 ng/ml (medium) and 930 ng/ml
190 (high) for all five compounds. Intra-batch precision was assessed by calculating the % co-efficient
191 of variation (% CV) following six repetitions at each concentration. Accuracy was assessed by
192 calculating the mean % accuracy of the same samples. To assess inter-day precision, % CV were
193 calculated following six repetitions at each concentration on six days over a three week period.
194 ME, a commonly observed source of error in LC-ESI-MS/MS bioanalysis, were assessed by
195 comparing the analyte peak areas of a neat solution containing 500 ng/ml of each analyte with
196 those of urine samples, obtained from six different volunteers and fortified to the same
197 concentrations. The % ME were then calculated as:

$$198 \quad \% \text{ ME} = 100 \times \left(\frac{\text{peak area of fortified urine sample}}{\text{peak area of the neat solution}} \right)$$

199 *Stability*

200 The optimal mode of sampling in the application of a biomarker approach for assessing LCS
201 intakes may involve the collection of 24-hour urine samples and therefore samples are likely to be
202 kept in potentially sub-optimal storage conditions for longer periods of time prior to final storage
203 and subsequent analysis. Therefore, the stability profiles of the compounds of interest was assessed
204 over a period of 72-hours. To do this, two 1.5 ml aliquots of blank urine samples were fortified to
205 approximately 500 ng/ml with each of the five compounds of interest and transferred to two amber
206 glass vials. Both were stored in the dark with one sample stored at room temperature while the

207 other was kept refrigerated at 4 °C. Aliquots of each sample were taken at five time-points (0, 18,
208 24, 48 and 72 hours) and stored at -80 °C until analysis.

209 **Dose-response study**

210 A double-blind, randomized crossover trial was conducted to assess the usefulness of using urinary
211 excretions to distinguish between different short-term intakes of the five LCSs. A total of 21
212 participants were recruited to the study. Participation lasted three weeks, during which participants
213 were asked to consume three doses of the five LCSs via water-based drinks and to collect both a
214 fasting spot and 24-hour urine sample during each dosing period. The study was approved by the
215 Ulster University Research Ethics Committee (Study No. BMS 014-0095) and a comprehensive
216 description of the study protocol can be found within the supplementary information.

217 In brief, participants' height and weight were measured and they were asked to avoid all of the
218 LCS of interest from at least three days prior to commencing the study to completion, again
219 receiving written literature and verbal advice to aid this. Three doses of the five LCSs of interest,
220 representing 0.1% (low), 0.5% (medium) and 10% (high) of the respective acceptable daily intake
221 (ADI) based on a 70 kg person, were consumed in a randomized order via two water-based drinks.
222 These doses are relevant to intakes within the free-living population.³³ Randomization (using
223 Sealed Envelope™) and blinding (by labelling each dose A, B or C) was conducted by a local
224 clinical trials manager who was not part of the current research team. The drinks (500 ml each)
225 were consumed over two consecutive days at specified times during the three week period and, to
226 blind participants to the dose, 75 ml of an orange cordial (Sainsbury's, UK) was added to each
227 drink during preparation. Participants were purposely advised to consume the test drinks as they
228 preferred across the day to reflect consumption patterns in free-living individuals as much as

229 possible, with the only stipulation that they consumed one drink (500 ml) per 24-hour period. On
230 the second day of each dosing period, participants collected a morning fasting spot urine sample
231 and a 24-hour urine sample and returned these to the university.

232 All urine samples were processed within two hours of receipt and stored at -80°C until analysis.
233 Urinary biomarker analysis was carried out as described above and PABA analysis was carried
234 out to assess completeness of the 24-hour urine samples as described elsewhere.³⁰ To standardize
235 LCS concentrations in fasting spot urine samples, creatinine concentrations were determined using
236 an ILab 650 (Instrumentation Laboratories, Massachusetts, USA).

237 **Statistical analysis**

238 All data related to method development and validation were acquired and statistically analyzed
239 using Analyst Software Version 1.4.2 (AB Sciex, Warrington, UK) while statistical analysis of the
240 dose-response data was performed using the Statistical Package for Social Sciences (SPSS)
241 Version 22.0 (SPSS UK Ltd, Chersey, United Kingdom). Total LCS excretions were calculated
242 by multiplying the 24-hour urinary concentrations (mg/ml) by the volume of the total sample (ml)
243 and expressed as mg/day. Creatinine concentrations in fasting spot urine samples were used to
244 standardize LCS concentrations and values were expressed as $\mu\text{g/g}$ creatinine. Steviol glycosides
245 are excreted via the urine as steviol glucuronide and therefore values were converted to steviol
246 equivalents; therefore, based on their molecular weights, factors of 0.643 and 0.39 were applied to
247 steviol glucuronide and steviol glycoside values respectively.

248 The distribution of continuous data was assessed using the Shapiro-Wilk test and data which were
249 not normally distributed were log-transformed or non-parametric alternatives were employed.
250 Urinary excretions of all LCSs were skewed and therefore transformation of the data was attempted

251 prior to carrying out statistical analysis. It was not possible to normalize the distribution of the
252 data; therefore Freidman Tests were used to assess mean differences in excretions across all
253 treatments. Statistically significant results were followed up with Wilcoxon Signed Rank Tests to
254 identify where the differences were. Bonferroni adjustment was applied to the alpha values in post-
255 hoc Wilcoxon Signed Rank Tests to control for Type 1 errors as a result of multiple comparisons
256 and therefore an alpha level of 0.017 (0.05/number of comparisons) was considered significant.
257 Spearman correlations were carried out to assess co-linearity between the dose of LCS ingested
258 and urinary excretions (both 24-hour urine and fasting spot sample). Standard multiple regression
259 was then used to assess the ability of 24-hour urinary excretion of the five compounds of interest
260 to predict intake after controlling for age, gender and body mass index (BMI). A P-value of <0.05
261 was considered statistically significant unless otherwise stated.

262 **Results and Discussion**

263 Improved assessment of LCS intakes is necessary to properly investigate relationships between
264 LCS use and health. To this end, a LC-ESI-MS/MS method was developed and validated for the
265 simultaneous determination of five commonly used LCSs in human urine. Although numerous
266 methods have been published describing the simultaneous determination of various combinations
267 of LCSs in matrices such as foods, beverages and water sources,^{15,24-27} the method described here
268 is the first which simultaneously determines these particular LCSs as they are excreted via urine.
269 As an initial biomarker validation step, the method was applied in a dose-response study to assess
270 the usefulness of using urinary excretions to assess recent intakes.

271 **Method performance**

272 The general characteristics, including the chemical structures of the five compounds of interest,
273 are presented in Table 1. Single spectral peaks corresponding to $[M-K]^-$ for acesulfame-K, $[M-$
274 $Na]^-$ for sodium saccharin and sodium cyclamate and $[M-H]^-$ for sucralose and steviol glucuronide
275 were observed. Table 2 presents the two MRM transitions used for quantitative and qualitative
276 purposes, the corresponding IS and the collision energy for each MRM transition. The most intense
277 transition for each compound was used for quantification. In contrast to a previous study by Yang
278 and Chen,³⁴ which utilized MS only, the utilization of MS/MS in this study allowed for increased
279 specificity and sensitivity which is important in bioanalysis. As has been reported elsewhere in
280 the literature, the signal intensity for sucralose was lower than for the other analytes and post-
281 column infusion of TRIS-buffer has previously been used to enhance the signal for sucralose.²⁴
282 However for the purposes of our method, and the desired working range, the signal was deemed
283 adequate without the need for further enhancement.

284 Good base peak separation was observed for the five compounds (Fig. 1). Previous methods have
285 incorporated solid phase extraction^{24,26} or liquid-liquid extraction³⁴ in sample preparation which
286 may make the application of such methods less feasible for large numbers of samples, whereas the
287 simple procedure described above lends itself better to a high throughput application. However,
288 with minimal sample clean-up prior to introduction to the mass spectrometer, the integrated
289 diverter valve was used to direct flow to waste outside the expected elution times and thereby
290 protecting the mass spectrometer from excessive contamination. Various concentrations of
291 methanol (2-15%) in MP-A were assessed and when the concentration was increased to 10% or
292 above acesulfame-K eluted close to the void volume; therefore 5% was considered suitable.

293 *Method validation*

294 Results for linearity, LOD and LLOQ, and precision and accuracy are presented in Table 2.
295 Excellent linearity was observed for all five compounds over the desired concentration range of
296 10-1000 ng/ml with coefficients of determination (r^2) ranging from 0.9969 to 0.9997. Mean
297 accuracy ranged from 98% to 104% at low concentrations while at high concentrations, accuracy
298 ranged from 92% to 102%. The use of stable isotopes as IS for acesulfame-K, sodium saccharin,
299 sodium cyclamate and sucralose resulted in excellent levels of accuracy (99-103%) and precision
300 with % CV below 7.7% at all three concentrations assessed (i.e. 15 ng/ml, 550 ng/ml and 930
301 ng/ml). For steviol glucuronide, warfarin sodium was used as IS as this has been previously used
302 for LC-MS analysis of LCSs.³⁴ Accuracy and precision for steviol glucuronide fell within
303 acceptable limits, albeit the use of the stable isotope of this compound as IS in future analyses
304 would likely improve these figures further.

305 Prior to the assessment of the presence of ME, screening of 'blank' urine samples revealed traces
306 of several of the compounds of interest and therefore duplicate runs of all six samples were carried
307 out prior to analysis of the fortified samples so that corrections could be applied. The average area
308 of each observed peak in the pre-fortified samples was recorded and the results were subsequently
309 corrected. Mean % ME ranged from 89% to 99% for acesulfame-K, 80% to 100% for saccharin,
310 89% to 104% for cyclamate, 87% to 99% for sucralose and 94% to 107% for steviol glucuronide.
311 These results suggest the existence of interfering compounds within some urine samples,
312 potentially introducing a source of inaccuracy and/or imprecision if neat solutions were used as
313 calibration and QC standards. However, with the use of IS, % CV within urine samples were below
314 5.5% for all five compounds and therefore all calibration and QC samples were prepared using
315 blank urine samples. As such, the potentially deleterious effects of ME were largely minimized.

316 *Stability*

317 Acesulfame, saccharin, cyclamate and sucralose have recently been identified as potential aquatic
318 contaminants owing to their persistence in the environment²³ and therefore good stability was
319 expected over the 72-hour test period. However the stability profile of steviol glucuronide was less
320 well known. Indeed, acesulfame-K, saccharin, cyclamate and sucralose did not undergo any
321 detectable degradation over the 72-hour period either when stored at room temperature or when
322 refrigerated. A small, yet non-significant ($p = 0.312$), degree of degradation (~8%) was observed
323 for steviol glucuronide when stored at room temperature for up to 72-hours.

324 **Dose-response study**

325 *Participants and urine collections*

326 No significant differences were observed between males and females with respect to age, weight,
327 BMI or volume of 24-hour urine samples (Table 3). During the dose-response study, participants
328 consumed three different amounts (0.1%, 0.5% and 10% of the ADI based on a 70 kg person) of
329 the five LCSs comparable to what has been observed within the free-living population,^{22,33} while
330 avoiding the five LCSs in their diet. A total of 84 24-hour urine samples were collected (four per
331 participant) and of these, 55 (65.5%) were considered complete based on PABA excretion and/or
332 participant reporting. Mean urinary concentrations (based on fasting spot samples) and daily
333 excretion (based on 24-hour samples) following each dosing period are presented in Table 4.

334 *Biomarker validation*

335 Mean urinary concentrations of all five compounds were significantly correlated with intakes of
336 the respective LCS; the correlations for 24-hour urinary acesulfame-K, saccharin, cyclamate,
337 sucralose and steviol glucuronide were 0.909 ($p < 0.001$), 0.888 ($p < 0.001$), 0.942 ($p < 0.001$),
338 0.512 ($p = 0.001$) and 0.942 ($p < 0.001$) respectively. Correlations for fasting spot urinary

339 concentrations of acesulfame-K, saccharin, cyclamate, sucralose and steviol glucuronide were
340 0.823 ($p < 0.001$), 0.874 ($p < 0.001$), 0.818 ($p < 0.001$), 0.410 ($p = 0.007$) and 0.887 ($p < 0.001$)
341 respectively. Until now, the dose-response relationship between intake and urinary excretion has
342 only been investigated for acesulfame-K and saccharin when strong correlations were also
343 observed between 24-hour urinary excretion and intakes ($r^2 = 0.9912$ for acesulfame-K and $r^2 =$
344 0.9963 for saccharin) during an acute intake/excretion study.¹⁹

345 In relation to 24-hour urine samples, mean recoveries from the low, medium and high doses were
346 98%, 89% and 85% for acesulfame-K, 100%, 79% and 86% for saccharin, 24%, 28% and 25% for
347 cyclamate and 86%, 52.7% and 47% for steviol glycosides (see Table 4 for absolute recoveries).
348 Higher than expected levels of recovery of sucralose were observed at the lower doses (450%,
349 100% for low and medium respectively) as it was present in the cordial used in the LCS drinks
350 and this is discussed in more detail later. For the high dose of sucralose, mean recovery was 8 %
351 while previous work investigating the pharmacokinetics of sucralose reported an average excretion
352 of 13% of a dose in urine within the first 24-hours post-ingestion.²⁰ For acesulfame-K and
353 saccharin the majority of the dose was recovered which is in agreement with results reported
354 elsewhere,³⁵⁻³⁷ although one study reported an average recovery of 68% of an acesulfame-K dose
355 in 24-hour urine samples.¹⁹ In contrast to acesulfame-K and saccharin, only partial recoveries were
356 observed in the 24-hour urinary samples for cyclamate (24-28%) and steviol glycosides (47-86%).
357 Previous pharmacokinetic investigations of cyclamate primarily focused on its metabolite,
358 cyclohexylamine, as approximately 20% of the population have the ability, to varying degrees, to
359 convert cyclamate to cyclohexylamine via bacterial hydrolysis. Chronic exposure has been shown
360 to enhance conversion in some individuals;³⁸ however, conversion only occurs to the unabsorbed
361 proportion reaching the colon and therefore does not affect the absorbed proportion of cyclamate.³⁹

362 Steviol glycosides also undergo bacterial hydrolysis in the gut and are absorbed into the body as
363 steviol which subsequently undergoes conjugation before excretion via the urine as steviol
364 glucuronide.⁴⁰ Previously it has been reported that approximately 34% of a dose is excreted in the
365 urine over 24-hours⁴¹ which is much lower than what was observed as part of this work which
366 observed typical excretion of 47-86% over the same period. A possible explanation is the dose in
367 the previous study was significantly larger than in the present work.

368 Both modes of sampling were useful for distinguishing between all three intakes tested in the
369 present study for acesulfame-k ($p < 0.001$), saccharin ($p < 0.001$), cyclamate ($p < 0.001$) and steviol
370 glycosides ($p < 0.001$) (Table 4). It is particularly noteworthy that fasting spot urine samples are
371 useful as the collection of such samples would be less invasive for participants, making the
372 application of a biomarker approach in larger-scale population based studies more feasible.
373 However, further validation work with a larger sample size would help to confirm the usefulness
374 of fasting spot samples for distinguishing between different intakes. In relation to sucralose, it was
375 possible to use both the fasting spot and 24-hour urinary sucralose excretions to distinguish the
376 high dose from both the low and medium doses ($p < 0.001$) but not the low and medium doses (p
377 $= 0.198$). The presence of sucralose in the cordial used for the LCS drinks is likely to have biased
378 the results at the lower doses and therefore further work, with proper wash-out periods, would
379 facilitate more comprehensive investigations of the dose-response relationship for sucralose.

380 Given that 24-hour urinary excretions allow for calculation of overall daily excretion, regression
381 analysis using the LCS dose as a dependent variable and 24-hour urinary concentrations as an
382 independent variable were conducted and 24-hour excretions explained 99% of the variability for
383 acesulfame-K ($F(1, 39) = 2302.32$, adjusted $r^2 = 0.987$, $p < 0.001$), 87% of the variability for
384 saccharin ($F(1, 39) = 261.75$, adjusted $r^2 = 0.870$, $p < 0.001$), 91% of the variability for cyclamate

385 (F (1, 39) = 419.23, adjusted $r^2 = 0.913$ $p < 0.001$), 35% of the variability for sucralose (F (1, 39)
386 = 22.59, adjusted $r^2 = 0.350$ $p < 0.001$) and 75% of the variability for steviol glycosides (F (1, 39)
387 = 118.09, adjusted $r^2 = 0.745$ $p < 0.001$). Such findings would suggest that 24-hour urinary
388 excretions may be useful for estimating absolute intakes of the respective LCS.

389 A number of limitations with the present work should be acknowledged. The presence of sucralose
390 in the cordial used in the LCS drink prevented a comprehensive investigation of the relationship
391 between sucralose consumption and urinary excretion. The cordial was chosen as no LCS-free
392 cordial could be sourced at the time of conducting the study and therefore measures were taken to
393 control for this by maintaining a consistent dose of cordial added to the LCS drink. However, no
394 account was taken of the possible variation in sucralose concentrations within the product which
395 may have contributed to variation in the results. Despite the presence of sucralose in the cordial, a
396 high intake of sucralose was shown to result in higher mean excretions as compared with the low
397 or medium intakes in both modes of sampling; as such, urinary excretions of sucralose may be
398 useful in distinguishing between low and high consumers which, nevertheless, may be of most
399 clinical interest. A further consideration is that the present studies only investigated urinary
400 concentrations following short-term intakes (over two consecutive days) and in individuals who
401 were actively avoiding LCSs. Further work to assess the usefulness of a biomarker approach in
402 regular and potentially high LCS consumers, such as those with diabetes mellitus or children, is
403 warranted. Moreover, investigations of the long-term reproducibility of these biomarkers,
404 specifically aiming to establish the required number of samples to determine habitual intakes are
405 warranted to better characterize the relationship between intakes and urinary excretions.

406 A novel urinary biomarker approach for assessing recent intakes of five commonly consumed
407 LCSs has been presented. Such an approach will help generate more objective LCS intake data

408 when applied in population-based studies, representing an opportunity to significantly enhance our
409 understanding of the relationship between LCSs and human health.

410 **Abbreviations used**

411 Ace-d4, acesulfame-d4 potassium salt; ADI, acceptable daily intake; Cyc-d11, cyclohexyl-d11;
412 ESI, electrospray ionization; EU, European Union; FFQs, food frequency questionnaires; HPLC,
413 high performance liquid chromatography; IS, internal standard; LCS, low-calorie sweetener; LC-
414 ESI-MS/MS, liquid chromatography tandem-mass spectrometry; LC-MS, liquid chromatography
415 mass spectrometry; MRM, multiple reaction monitoring; PABA, paraminobenzoic acid; Sac-d4,
416 saccharin-d4; Suc-d6, sucralose-d6.

417 **Acknowledgement**

418 We would like to thank Eddie O'Kane for technical assistance throughout method development.

419 **Supporting information description**

420 The Supporting Information is available free of charge on the ACS Publications website at DOI:
421 xxxx

422 Description of the dose-response study protocol; the doses administered to participants in the dose-
423 response study (Table S-1).

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426 The Netherlands.

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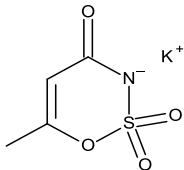
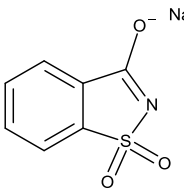
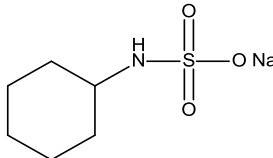
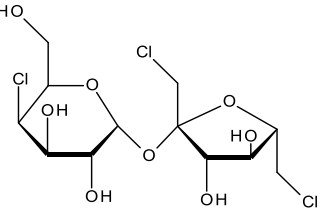
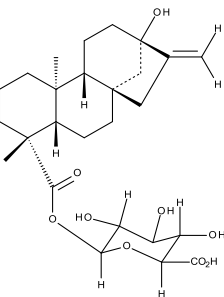
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547 **Figure captions**

548 **Figure 1.** LC-ESI-MS/MS chromatogram of a single analysis of spiked urine sample depicting the
549 most intense MRM transition for each compound. (a) acesulfame-k, m/z 162/82, (b) sodium
550 saccharin, m/z 182/42, (c) sodium cyclamate, m/z 178/80, (d) sucralose, m/z 395/35, (e) steviol
551 glucuronide, m/z 493/317.

552 **Figure 2.** Urinary excretion (a, 24-hour; b, fasting spot) of (i) acesulfame-K, (ii) saccharin, (iii)
553 cyclamate, (iv) sucralose and (v) steviol following low, medium and high doses which were
554 consumed in a randomized order. ^a Doses equated to 0.1% (low), 0.5% (medium) and 10% (high)
555 of the acceptable daily intake based on a 70 kg person.

Table 1. General characteristics of acesulfame-K, sodium saccharin, sodium cyclamate, sucralose and steviol glucuronide.

Compound	Cas No	Sweetness ^a	Structure	Monoisotopic mass (Da)
Acesulfame-K	55589-62-3	200		200.95
Sodium saccharin	82385-42-0	300-500		204.98
Sodium cyclamate	139-05-9	30		201.04
Sucralose	56038-13-2	600		396.01
Steviol glucuronide	N/A ^b	N/A ^b		494.57 ^c

^a Sweetness relative to sucrose. ^b N/A, not applicable. ^c Average molar mass. Da, daltons.

Table 2. Method characteristics, limits of detection and quantification and coefficients of determination.

Compound	Retention time (min)	MRM transitions (m/z)	Collision energy (eV)	Internal standard (m/z)	LOD (ng/ml)	LLOQ (ng/ml)	r^2
Acesulfame-K	2.0	162/82 ^a 162/78	-21 -44	Ace-d4 (166/86)	0.01	10.0	0.9997
Sodium saccharin	3.8	182/42 ^a 182/106	-42 -24	Sac-d4 (186/42)	0.06	10.0	0.9994
Sodium cyclamate	4.7	178/80 ^a 178/96	-42 -32	Cyc-d11 (189/80)	0.02	10.0	0.9992
Sucralose	6.7	395/35 ^a 395/359	-32 -14	Suc-d6 (401/35)	0.40	10.0	0.9969
Steviol glucuronide	10.4	493/317 ^a 493/113	-32 -30	Warfarin Na (307/307)	0.01	10.0	0.9991

^a Transition used for quantification. Limit of detection (LOD) defined as a signal to noise ratio of 3:1. Lower limit of quantification (LLOQ) defined as lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. r^2 for concentration ranges of 10-1000ng/ml. MRM, multiple reaction monitoring; Ace-d4, acesulfame-d4 potassium salt; Sac-d4, saccharin-d4; Cyc-d11, cyclamate-d11; Suc-d6, sucralose-d6; Warfarin Na, Warfarin sodium.

Table 3. General characteristics of participants taking part in the dose-response study.^a

	Overall	Males <i>n</i> 10	Females <i>n</i> 11	<i>P</i> value ^b
Age (years)	25.7 (4.9)	26.3 (3.7)	25.1 (5.8)	0.173
Height (m)	1.7 (0.09)	1.78 (0.05)	1.64 (0.07)	< 0.001
Weight (kg)	71.4 (11.9)	77.5 (10.6)	65.8 (10.6)	0.021
Body mass index (kg/m ²)	24.7 (3.4)	24.7 (2.8)	24.6 (4.0)	0.947
Urine volume (ml)				
Time-point 1 ^c	2024 (759)	1859 (736)	2174 (783)	0.355
Time-point 2 ^c	1957 (865)	1784 (708)	2114 (994)	0.396
Time-point 3 ^c	2086 (834)	1831 (556)	2319 (995)	0.188

^a Values are mean (SD).

^b Statistical analyses were carried out to investigate differences between males and females. Age was assessed with Mann U Whitney test; height, weight, body mass index and urine volume were assessed with Independent Samples t-test.

^c Time-points refer to 24-urine collections after each dosing period.

Table 4. Mean urinary excretions of acesulfame-K, saccharin, cyclamate, sucralose and steviol following low, medium and high doses of the respective low-calorie sweetener.^a

	0.1% ADI	0.5% ADI	10% ADI	<i>P</i> value ^b
24-hour urine sample (mg/day)	<i>n</i> = 14	<i>n</i> = 13	<i>n</i> = 12	
Acesulfame-K	0.59 (0.09)	2.85 (0.30)*	52.56 (5.33)*	<0.001
Saccharin	0.40 (0.32)	1.34 (0.37)*	29.51 (9.72)*	<0.001
Cyclamate	0.12 (0.05)	0.54 (0.13)*	10.99 (2.92)*	<0.001
Sucralose	5.40 (2.07)	5.20 (1.58)	9.23 (3.92)*	<0.001
Steviol	0.23 (0.04)	0.72 (0.29)*	12.70 (5.54)*	<0.001
Spot urine sample (µg/g creatinine)	<i>n</i> = 19	<i>n</i> = 19	<i>n</i> = 19	
Acesulfame-K	455 (836)	1396 (1331)*	31983 (34562)*	<0.001
Saccharin	210 (304)	1171 (1650)*	18408 (15562)*	<0.001
Cyclamate	71 (89)	470 (904)*	7569 (6494)*	<0.001
Sucralose	3453 (6644)	2668 (2210)	5493 (5159)*	<0.001
Steviol	146 (106)	905 (799)*	13913 (13956)*	<0.001

^a Values represent means (SD); ADI, acceptable daily intake. Low, medium and high doses represent 0.1%, 0.5% and 10.0% of the ADI for a 70 kg person.

^b Friedman Tests carried out to compare means urinary concentrations across all time-points. Wilcoxon Signed Rank tests then carried out as post-hoc analysis to determine where the differences were with Bonferroni adjustment applied to control for multiple comparisons; as such *P* value of <0.017 was considered as significant.

* denotes a statistically significant difference with the immediate lower dose.

Figure graphics

Figure 1

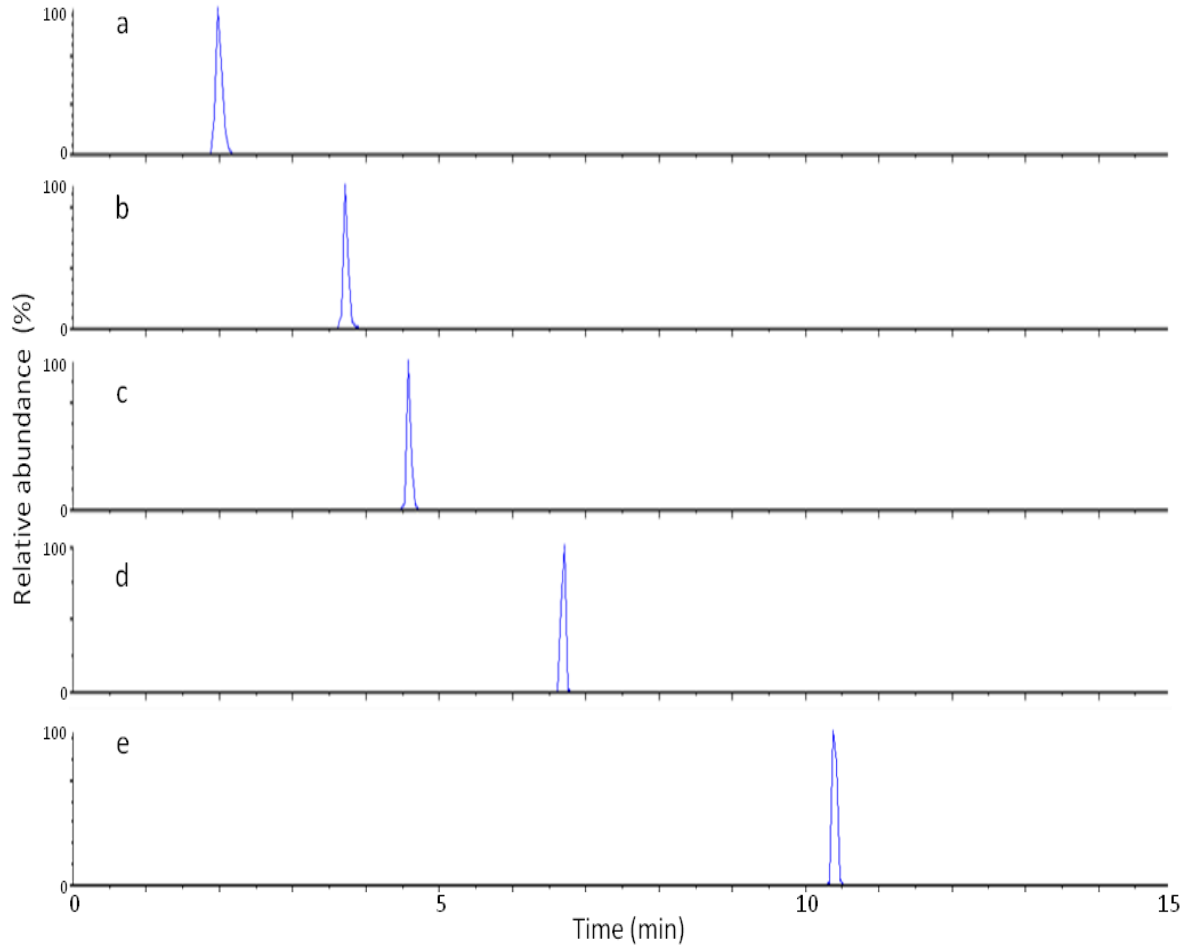
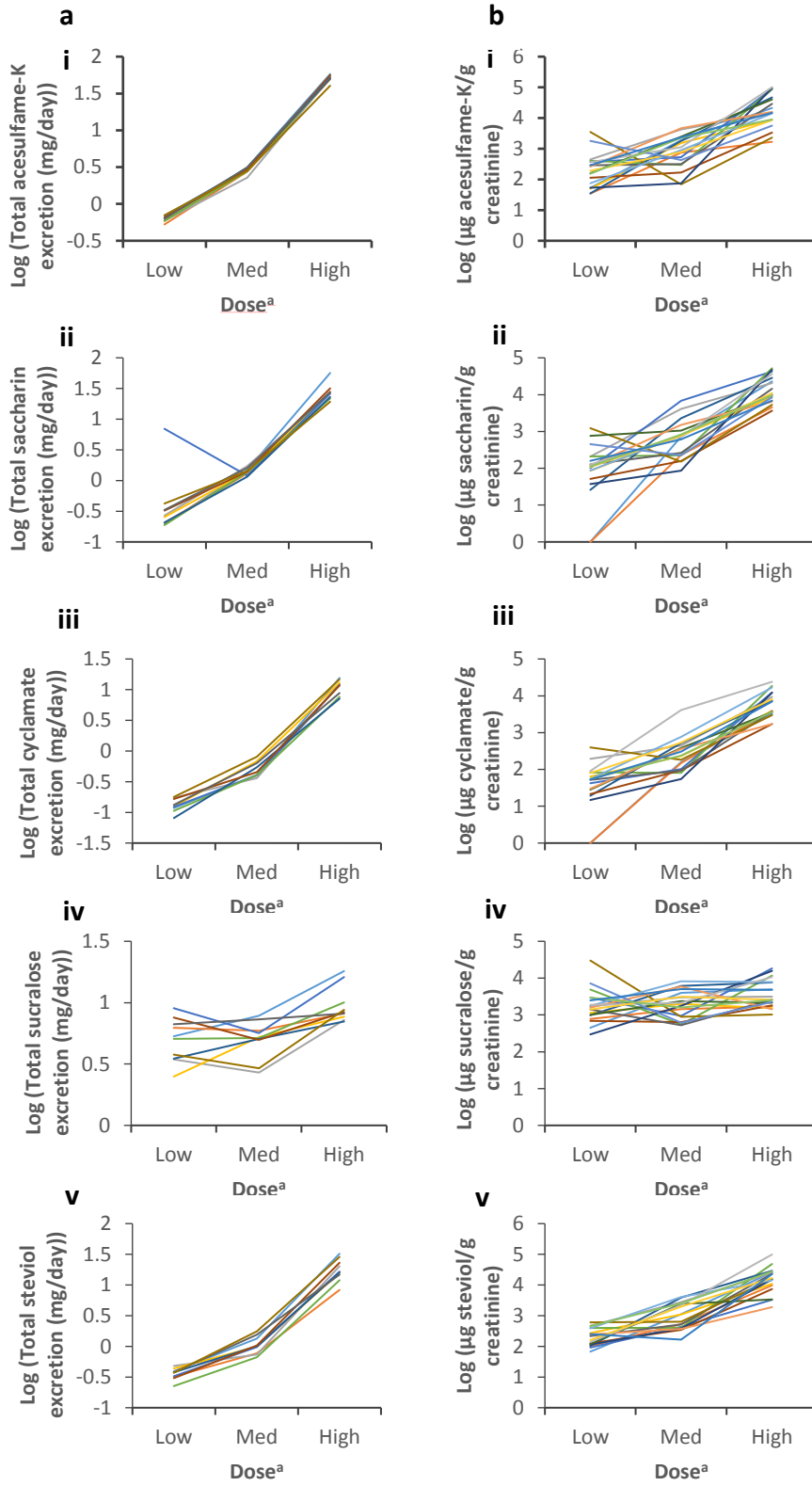


Figure 2



For table of contents only

