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

Caomhan Logue [†*], Le Roy C. Dowey [†], J. J. Strain [†], Hans Verhagen [†, ‡, §], Stephen McClean [†], and Alison M. Gallagher [†]

[†] **Nutrition Innovation Centre for Food and Health (NICHE), Ulster University, Coleraine, Northern Ireland BT52 1SA**

[‡] **National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA, Bilthoven, The Netherlands (until 1 Nov 2015)**

[§] **European Food Safety Authority (EFSA), Parma, Italy (from 1 Nov 2015)**

*****Corresponding Author. Telephone: +44 (0)2870 124451. Email: c.logue@ulster.ac.uk.

Abstract

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

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

Introduction

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

LCSs are a chemically diverse group of intensely sweet, low energy, food additives that are non-cariogenic[7] and used in a wide range of dietary and non-dietary products to provide a desired sweet taste without increasing energy density or inducing adverse oral health outcomes. Intuitively, LCSs should be expected to offer health benefits when used in place of free sugars; indeed, a recent meta-analysis of RCTs (sustained over 4 weeks to 40 months) indicated that LCS consumption versus sugar led to reductions in body weight.[8] Despite such health benefits, debate around their long-term efficacy continues, particularly in relation to weight management,[9] type 2 diabetes[10,11] and other metabolic effects.[12] A number of mechanisms by which LCSs may adversely impact on health have been discussed and while a number of these putative mechanisms have been supported by some animal data, none have yet been demonstrated in humans.[13] Observational data, in particular, have been conflicting in relation to the health effects of LCSs, as highlighted in a recent

review of the evidence by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES);[14] however a major limitation of most cohort studies is that only low-calorie sweetened beverage (LCSB) consumption is considered when investigating associations between LCSs and health. LCSs are ubiquitous in today's society and LCSBs are only one of many sources of LCSs in the diet; indeed, some LCSs have recently been identified as potential environmental contaminants and detected in drinking-water sources[15] so exposure to some LCSs may actually occur inadvertently in certain populations. Furthermore, intakes of individual LCSs are very rarely estimated in cohort studies,[14] impairing the ability to properly explore relationships between these chemically diverse food additives and health. This is further compounded by the fact that blends of LCSs are now often used within the same product. With these factors in mind, conclusions drawn from current observational data are likely to be tenuous and therefore, an alternative approach which provides more objective and specific LCS intake data would be highly desirable in order to enhance research in the area of LCSs and human health.

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

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

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

Materials and Methods

Method development and dose-response studies

Reagents and reference materials

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

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

Individual stock solutions of 1 mg/ml were prepared in water/methanol (50:50) for acesulfame-K, sodium saccharin, sodium cyclamate, sucralose and steviol glucuronide and from these, a working solution containing all five compounds at concentrations of 0.1 mg/ml was prepared in water/methanol (50:50) and stored at 4 ^{0}C. For the internal standards (IS), individual stock solutions of 1 mg/ml were prepared in methanol for Ace-d4, Sac-d4 and Suc-d6. Warfarin sodium and Cyc-d11 were dissolved in water/methanol (50:50) at concentrations of 80 µg/ml and 40 µg/ml respectively. Prior to each batch, a working solution containing 5 µg/ml Ace-d4, Sac-d4, Suc-d6 and 4 µg/ml and 2 µg/ml warfarin sodium and Cyc-d11 respectively was prepared in mobile phase A (MP-A) (see below for MP-A composition).

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

Instrumentation

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

Method development and validation

Collection of LCS-free urine samples for method development

Twelve apparently healthy volunteers (6 females; 6 males; age range, 21-51 years) were recruited to provide blank urine samples for the purposes of method development and validation. Those with diabetes, renal impairment, taking or planning to take sulfonamide antibiotics during the study or with lactose intolerance were excluded from the study. Participants were asked to avoid foods and beverages known to contain acesulfame-K, saccharin, cyclamate, sucralose and steviol glycosides for a period of at least three days prior to collecting a 24-hour urine sample. To aid this, verbal advice and literature were provided to each participant. From each sample, ten 1 ml aliquots and two 30 ml aliquots were retained and stored at -80 ^{0}C for later use in method development. Ethical approval was granted for this method development study by the Ulster University Biomedical Sciences Research Ethics Filter Committee (Study No: FCBMS-13-058).

Calibration and Quality Control samples

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

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

Sample preparation

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

Liquid chromatography-tandem mass spectrometry conditions

Separation was accomplished using a Poroshell 120 EC-C18 (4.6 x 50 mm), 2.7 µm column equipped with a Poroshell 120 UHPLC EC-C18 guard column (Agilent Technologies, Santa Clara, US). HPLC conditions from an Agilent Technologies application note[31] were adapted for the purposes of this method. Flow rate was maintained at 0.6 ml/min throughout and a binary gradient program was used. MP-A consisted of 2 mM ammonium carbonate in water/methanol (95/5%)

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

The mass spectrometer was equipped with a Turbo-V ion source and ESI was operated in negative polarity with multiple reaction monitoring (MRM) scan mode used for detection. Scheduled scanning for specific compounds was carried out according to the expected time of elution, thus improving sensitivity and precision. Nitrogen gas, supplied by a Peak Scientific gas generator, was used as nebulizer, collision and desolvation gas. The two most intense MRM transitions were identified and compound specific parameters including declustering potential, collision energy and collision cell exit potential were optimized by directly infusing each compound at concentrations of 1 µg/ml in methanol into the mass spectrometer. Ion source parameters for each compound were optimized using flow injection analysis with the aim of maximizing signal intensity and stability. The source temperature was set at 600 ^{0}C and gas sources 1 and 2 were set at 35 psi and 40 psi respectively. Ion spray voltage was -4.5 kv and curtain gas was set at 40 psi.

Method validation

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

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

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

Stability

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

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

Dose-response study

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

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

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

All urine samples were processed within two hours of receipt and stored at -80 ^{0}C until analysis. Urinary biomarker analysis was carried out as described above and PABA analysis was carried out to assess completeness of the 24-hour urine samples as described elsewhere.[30] To standardize LCS concentrations in fasting spot urine samples, creatinine concentrations were determined using an ILab 650 (Instrumentation Laboratories, Massachusetts, USA).

Statistical analysis

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

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

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

Results and Discussion

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

Method performance

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

Good base peak separation was observed for the five compounds (Fig. 1). Previous methods have incorporated solid phase extraction[24,26] or liquid-liquid extraction[34] in sample preparation which may make the application of such methods less feasible for large numbers of samples, whereas the simple procedure described above lends itself better to a high throughput application. However, with minimal sample clean-up prior to introduction to the mass spectrometer, the integrated diverter valve was used to direct flow to waste outside the expected elution times and thereby protecting the mass spectrometer from excessive contamination. Various concentrations of methanol (2-15%) in MP-A were assessed and when the concentration was increased to 10% or above acesulfame-K eluted close to the void volume; therefore 5% was considered suitable.

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 environment[23] 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

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

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

Steviol glycosides also undergo bacterial hydrolysis in the gut and are absorbed into the body as steviol which subsequently undergoes conjugation before excretion via the urine as steviol glucuronide.[40] Previously it has been reported that approximately 34% of a dose is excreted in the urine over 24-hours[41] which is much lower than what was observed as part of this work which observed typical excretion of 47-86% over the same period. A possible explanation is the dose in the previous study was significantly larger than in the present work.

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

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

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

Abbreviations used

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

Acknowledgement

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

Supporting information description

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

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

Funding

This work was funded by the National Institute for Public Health and the Environment (RIVM) in The Netherlands.

References

1. Butland, B.; Jebb, S.; Kopelman, P.; McPherson, K.; Thomas, S.; Mardell, J.; Parry, V.; Foresight, Tackling Obesities: Future Choices- Project Report; 2nd Edition; UK Government for Science, 2007.
2. Finucane, M. M.; Stevens, G. A.; Cowan, M. J.; Danaei, G.; Lin, J. K.; Paciorek, C. J.; Singh, G. M.; Gutierrez, H. R.; Lu, Y.; Bahalim, A. N.; Farzadfar, F.; Riley, L. M.; Ezzati, M. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. *Lancet*. **2011,** *377*, 557-567.
3. Fryar, C.D.; Carroll, M.D.; Ogden, C.L. Prevalence of Overweight, Obesity, and Extreme Obesity among Adults: United States, 1960-1962 through 2011-2012; National Center for Health Statistics, Division of Health and Nutrition Examination Surveys. [Online] **2014,** http://www.cdc.gov/nchs/data/hestat/obesity_adult_11_12/obesity_adult_11_12.htm (accessed March 4, 2015).
4. World Health Organisation. Obesity and Overweight Fact sheet No 311. *WHO Media Centre* [Online] **2015,** http://www.who.int/mediacentre/factsheets/fs311/en/ (accessed May 15, 2015).
5. World Health Organisation Guideline: Sugars intake for adults and children. [Online] **2015,** http://apps.who.int/iris/bitstream/10665/149782/1/9789241549028_eng.pdf (accessed October 2, 2016).
6. Scientific Advisory Committee on Nutrition (SACN) (2015) Carbohydrates and Health. [Online] **2015,**

https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/445503/SACN_Carbohydrates_and_Health.pdf (accessed January 11, 2016).

7. Grenby, T.H. Update on low-calorie sweeteners to benefit dental health. *Int. Dent. J.* **1991,** *41*, 217-224.
8. Rodgers, P. J.; Hognkamp, P. S.; de Graaf, C.; Higgs, S.; Lluch, A.; Ness, A. R.; Penfold, C.; Perry, R.; Putz, P.; Yeomans, M. R.; Mela, D. J. Does low-energy sweetener consumption affect energy intake and body weight? A systematic review, including meta-analyses, of the evidence from human and animal studies. *Int. J. Obes.* **2016,** *40*, 387-394.
9. Swithers, S. E. Artificial sweeteners produce the counterintuitive effect of inducing metabolic derangements. *Trends Endocrinol. Metab.* **2013,** *24*, 431-441
10. Imamura, F.; O'Connor, L.; Ye, Z.; Mursu, J.; Hayashino, Y.; Bhupathiraju, S. N.; Forouhi, N. G. Consumption of sugar sweetened beverages, artificially sweetened beverages, and fruit juice and incidence of type 2 diabetes: systematic review, meta-analysis, and estimation of population attributable fraction. *Br. J. Sports Med.* **2016,** *50*, 496-504.
11. Romo-Romo, A.; Aguilar-Salinas, C. A.; Brito-Córdova, G.; Gómez Díaz, R. A.; Valentin D. V.; Almeda-Valdes, P. Effects of non-nutritive sweeteners on glucose metabolism and appetite regulating hormones: Systematic review of observational prospective studies and clinical trials. *PLoS ONE* **2016,** *11*, e0161264. Doi: 10.1371/journal.pone.0161264.
12. Gardner, C.; Wylie-Rosett, J.; Gidding, S. S.; Steffen, L. M.; Johnson, R. K.; Reader, D.; Lichtenstein, A. H. Nonnutritive sweeteners: current use and health perspectives. *Diabetes Care* **2012,** *35*, 1798-1808.
13. Burke, M. V.; Small, D. M. Physiological mechanisms by which non-nutritive sweeteners may impact body weight and metabolism. *Physiol. Behav.* **2015,** *152 (Pt B)*, 381-388.

14. French Agency for Food, Environmental and Occupational Health and Safety (ANSES) Opinion on the assessment of the nutritional benefits and risks related to intense sweeteners. [Online] **2015,** https://www.anses.fr/en/system/files/NUT2011sa0161RaEN.pdf (accessed February 23, 2015).
15. Lange, T.; Scheurer, M.; Brauch, H. J. Artificial sweeteners- a recently recognised class of emerging environmental contaminants: a review. *Anal. Bioanal. Chem.* **2012,** *403,* 2503-2518.
16. Bingham, S. A. Biomarkers in nutritional epidemiology. *Public Health Nutr.* **2002,** *5,* 821-827.
17. Landberg, R.; Aman, P.; Friberg, L. E.; Vessby, B.; Adlercreutz, H.; Kamal-Eldin, A. Dose-response of whole-grain biomarkers: alkylresorcinols in human plasma and their metabolites in urine in relation to intake. *Am. J. Clin. Nutr.* **2009,** *89,* 290-296.
18. Renwick, A.G. (1986) The metabolism of intense sweeteners. *Xenobiotica*, **1986,** *16 (10/11),* 1057-1071.
19. Wilson, A. L; Wilkinson, K.; Crews, H. M.; Davies, A. M.; Dick, C. S.; Dumsday, V. L. Urinary monitoring of saccharin and acesulfame-K as biomarkers of exposure to these additives. *Food Addit. Contam.* **1999,** *16 (6),* 227-238.
20. Roberts, A.; Renwick, A. G.; Sims, J.; Snodin, D. J. Sucralose metabolism and pharmacokinetics in man. *Food Chem. Toxicol.* **2000,** *38, Suppl. 2,* S31-S41.
21. Geuns, J. M. C.; Buyse, J.; Vankeirsbilck, A.; Temme, E. H. M.; Compernolle, F.; Toppet, S. Identification of steviol glucuronide in human urine. *J. Agric. Food Chem.* **2006,** *54,* 2794-2798.

22. Logue, C.; Dowey, L.C..; Strain, J.J.; Verhagen, H.; Gallagher, A.M. The potential application of a biomarker approach to investigate low-calorie sweetener exposure. *Proc. Nutr. Soc.* **2016,** *75(2),* 216-225.

23. Jenab, M.; Slimani, N.; Bictash, M.; Ferrari, P.; Bingham, S. A. Biomarkers in nutritional epidemiology: applications, needs and new horizons. *Hum. Genet.* **2009,** *125*, 507-525.

24. Scheurer, M.; Brauch, H. J.; Lange, F. T. Analysis and occurrence of seven artificial sweeteners in German waste water and surface water and in soil aquifer treatment (SAT). *Anal. Bioanal. Chem.* **2009,** *394 (6)*, 1585-1594.

25. Zygler, A.; Wasik, A.; Namiesnik, J. Analytical methodologies for determination of artificial sweeteners in foodstuffs. *TrAC, Trends Anal. Chem.* **2009,** *28 (9)*, 1082-1102.

26. Ordonez, E. Y.; Quintana, J. B.; Rodil, R.; Cela, R. Determination of artificial sweeteners in water samples by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A*, **2012,** *1256*, 197-205.

27. Ens, W.; Senner, F.; Gygax, B. Development, validation and application of a novel LC-MS/MS trace analysis method for the simultaneous quantification of seven iodinated X-ray contrast media and three artificial sweeteners in surface, ground, and drinking water. *Anal. Bioanal. Chem.* **2014,** *406*, 2789-2798.

28. Kuhnle, G. Nutritional biomarkers for objective dietary assessment. *J. Sci. Food Agric.* **2012,** *92*, 1145-1149.

29. Bingham, S.; Cummings, J. H. The use of 4-aminobenzoic acid as a marker to validate the completeness of 24 h urine collections in man. *Clin. Sci.* **1983,** *64*, 629-635.

30. Jakobsen, J.; Ovesen, L.; Fagt, S.; Pedersen, A. N. Para-aminobenzoic acid used as a marker for completeness of 24 hour urine: assessment of control limits for a specific HPLC method. *Eur. J. Clin. Nutr.* **1997,** *51*, 514-519.

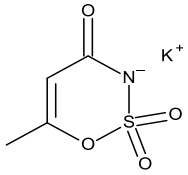
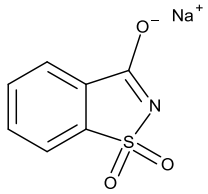
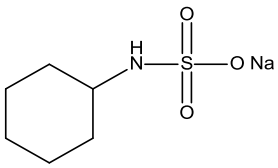
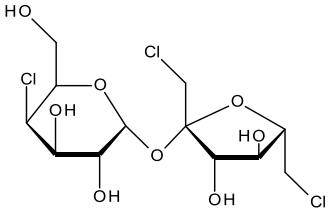
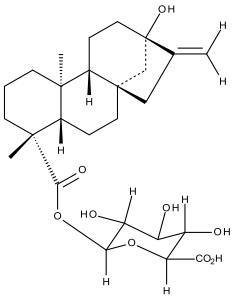
519 31. Agilent Technologies. Analysing Synthetic Sweeteners in Waste Water with Robust
520 Sample Preparation. Application Note (Environmental), [Online] **2011,**
521 http://www.chem.agilent.com/Library/applications/5990-8248EN.pdf. (accessed March
522 23, 2013).
523 32. US Food and Drug Administration (FDA) Guidance for Industry, Bioanalytical Method
524 Validation 2001.
525 33. Renwick, A. G. The intake of intense sweeteners- an update review. *Food Addit. Contam.*
526 **2006,** *23 (4)*, 327-338.
527 34. Yang, D. J.; Chen, B. Simultaneous determination of nonnutritive sweeteners in foods by
528 HPLC/ESI-MS. *J. Agric. Food Chem.* **2009,** *57*, 3022-3027.
529 35. Christ, O.; Rupp, W. Human experiments with Acetosulfam-^{14}C. Pharmacokinetics after
530 oral administration of 30mg to three healthy male probands. **1976,** Unpublished report. In
531 Acesulfame Potassium. WHO Food Additives Series 28. [Online]
532 http://www.inchem.org/documents/jecfa/jecmono/v28je13.htm (accessed June 3, 2015).
533 36. Ball, L. M.; Renwick, A. G.; Williams, A. G. The fate of [^{14}C] saccharin in man, rat and
534 rabbit and of 2-sulphamoyl[^{14}C]benzoic acid in the rat. *Xenobiotica,* **1977,** *7*, 189-203.
535 37. Sweatman, T. W.; Renwick, A. G.; Burgess, C. D. The pharmacokinetics of saccharin in
536 man. *Xenobiotica*, **1981,** *11*, 531-540.
537 38. Renwick, A. G.; Thompson, J. P.; O'Shaughnessy, M.; Walter, E. J. The metabolism of
538 cyclamate to cyclohexylamine in humans during long-term administration. *Toxicol. Appl.*
539 *Pharmacol.* **2004,** *196*, 367-380.
540 39. Bopp, B. A.; Sonders, R. C.; Kesterson, J. W. Toxicological aspects of cyclamate and
541 cyclohexylamine. *Crit. Rev. Toxicol.* **1986,** *16(3)*, 213-306.
542 40. Wheeler, A.; Boileau, A. C.; Winkler, P. C.; Compton, J. C.; Jiang, X.; Mandarino, D. A.
543 Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men.
544 *Food Chem. Toxicol.* **2008,** *46*, S54-S60.
545 41. Geuns, J. M. C.; Buyse, J.; Vankeirsbilck, A.; Temme, E. H. M. Metabolism of stevioside
546 by healthy subjects. *Exp. Biol. Med.* **2007,** *232*, 164-173.

Figure captions

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

Figure 2. Urinary excretion (a, 24-hour; b, fasting spot) of (i) acesulfame-K, (ii) saccharin, (iii) cyclamate, (iv) sucralose and (v) steviol following low, medium and high doses which were consumed in a randomized order. [a] Doses equated to 0.1% (low), 0.5% (medium) and 10% (high) 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]	-21	Ace-d4 (166/86)	0.01	10.0	0.9997
		162/78	-44				
Sodium saccharin	3.8	182/42[a]	-42	Sac-d4 (186/42)	0.06	10.0	0.9994
		182/106	-24				
Sodium cyclamate	4.7	178/80[a]	-42	Cyc-d11 (189/80)	0.02	10.0	0.9992
		178/96	-32				
Sucralose	6.7	395/35[a]	-32	Suc-d6 (401/35)	0.40	10.0	0.9969
		395/359	-14				
Steviol glucuronide	10.4	493/317[a]	-32	Warfarin Na (307/307)	0.01	10.0	0.9991
		493/113	-30				

[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 *n* 10	Females *n* 11	*P* 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	*P* value[b]
24-hour urine sample (mg/day)	*n* = 14	*n* = 13	*n* = 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)	*n* = 19	*n* = 19	*n* = 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] Freidman 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

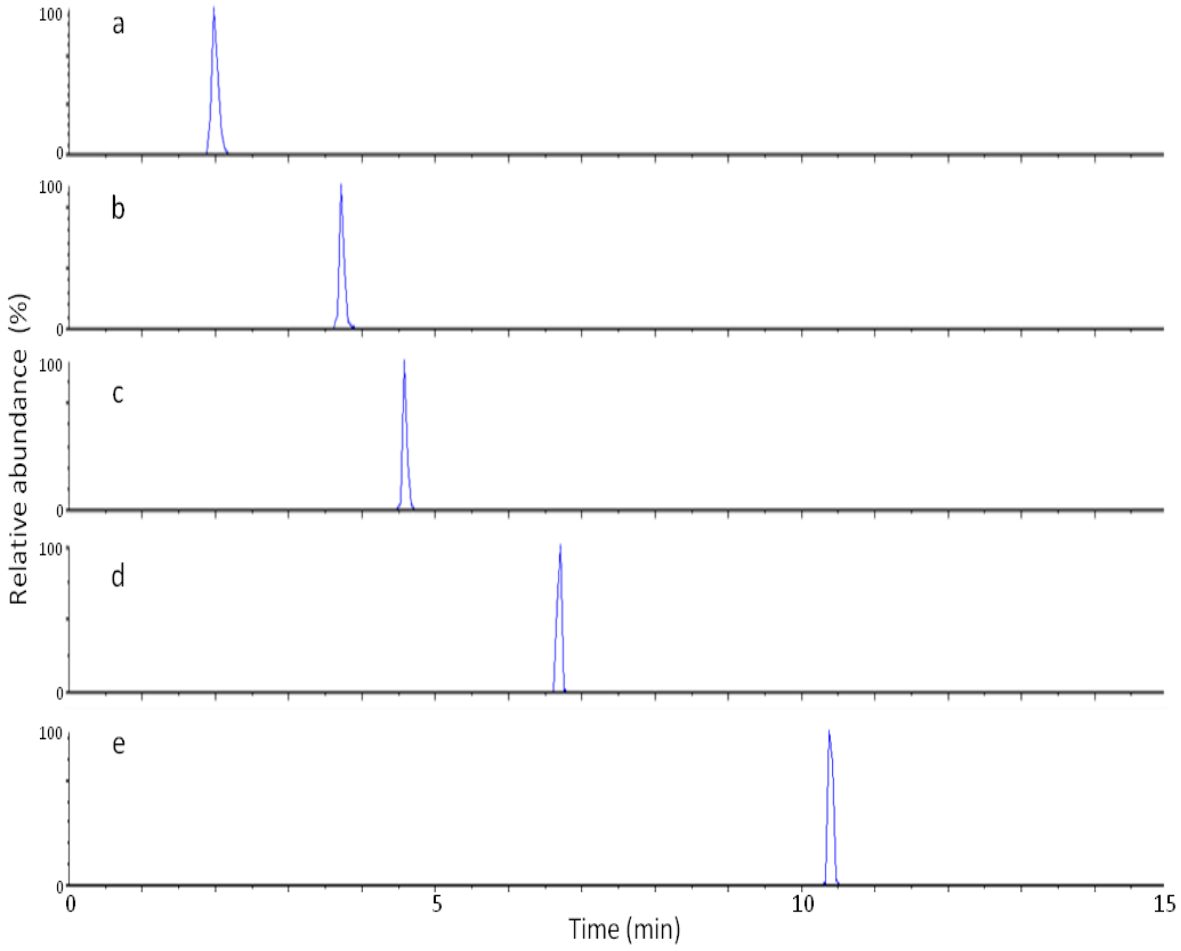
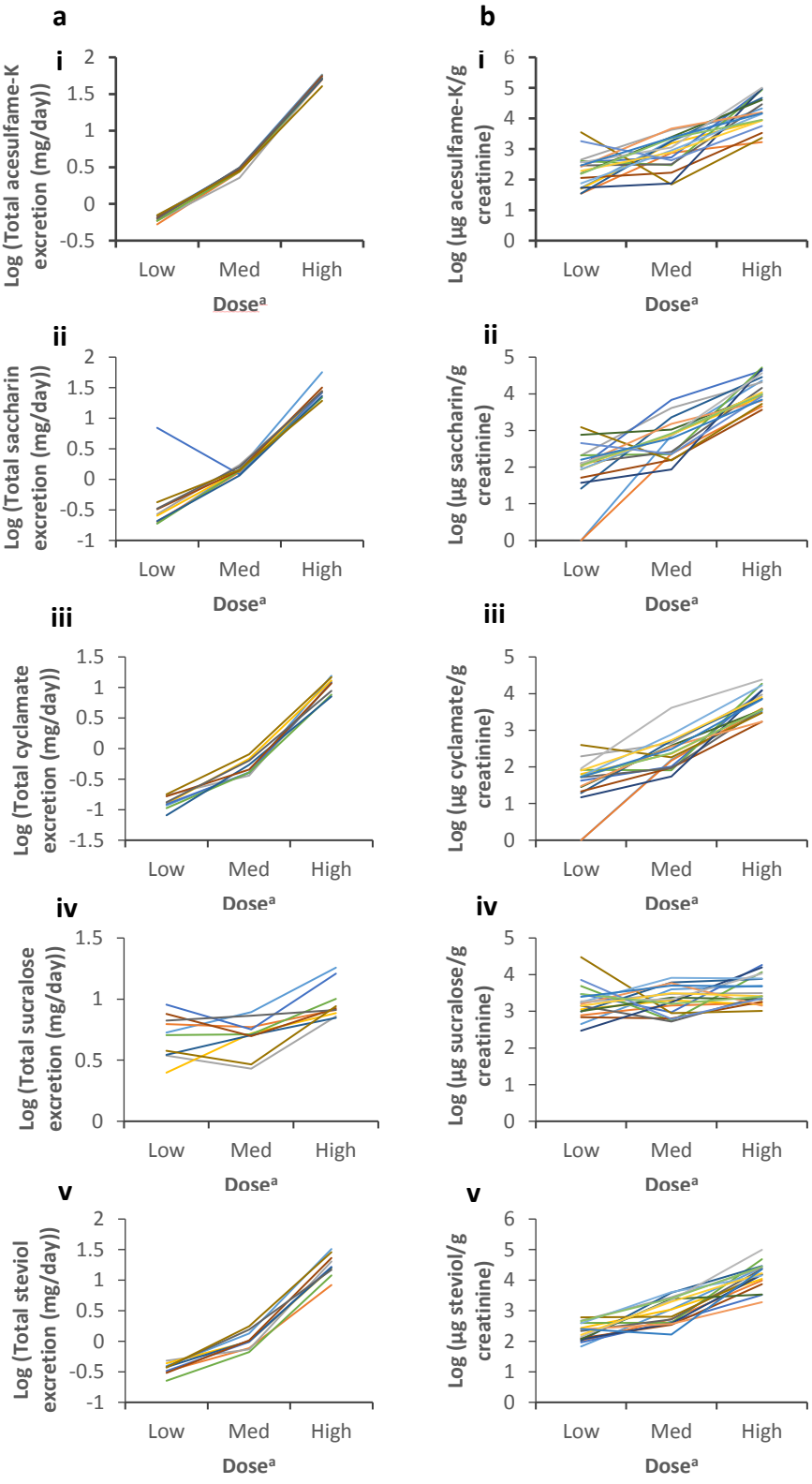


Figure 2


a
i
Log (Total acesulfame-K excretion (mg/day))
2
1.5
1
0.5
0
-0.5
Low
Med
High
Dose[a]
b
i
Log (µg acesulfame-K/g creatinine)
6
5
4
3
2
1
0
Low
Med
High
Dose[a]
ii
Log (Total saccharin excretion (mg/day))
2
1.5
1
0.5
0
-0.5
-1
Low
Med
High
Dose[a]
ii
Log (µg saccharin/g creatinine)
5
4
3
2
1
0
Low
Med
High
Dose[a]
iii
Log (Total cyclamate excretion (mg/day))
1.5
1
0.5
0
-0.5
-1
-1.5
Low
Med
High
Dose[a]
iii
Log (µg cyclamate/g creatinine)
5
4
3
2
1
0
Low
Med
High
Dose[a]
iv
Log (Total sucralose excretion (mg/day))
1.5
1
0.5
0
Low
Med
High
Dose[a]
iv
Log (µg sucralose/g creatinine)
5
4
3
2
1
0
Low
Med
High
Dose[a]
v
Log (Total steviol excretion (mg/day))
2
1.5
1
0.5
0
-0.5
-1
Low
Med
High
Dose[a]
v
Log (µg steviol/g creatinine)
6
5
4
3
2
1
0
Low
Med
High
Dose[a]

For table of contents only