### Title

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### Journal

Journal of Agricultural and Food Chemistry, 69(3)

### ISSN

0021-8561



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### Publication Date

2021-01-27

### DOI

10.1021/acs.jafc.0c06144

Peer reviewed

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC

Article

# Measurement of Saccharin and *trans*-Resveratrol Metabolites in Urine as Adherence Markers for Small Quantity Lipid-Based Nutrient Supplement Consumption

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Cite This: *J. Agric. Food Chem.* 2021, 69, 1107−1114

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**ABSTRACT:** Saccharin and *trans*-resveratrol were incorporated into small quantity lipid-based nutritional supplements (SQ-LNS) to be evaluated as the markers of consumption for nutritional intervention studies. Forty-seven healthy women consumed a single supplement with either 8.6 mg of saccharin or 5 mg of *trans*-resveratrol, and urine was collected for 4 h. A rapid 11 min method employing multiple reaction monitoring and ultrahigh performance liquid chromatography coupled to a triple quadrupole mass spectrometer was developed to measure saccharin and resveratrol metabolites in urine simultaneously. The linear dynamic range of the method was from 3 to 1000 ng $mL^{-1}$, with the correlation coefficient of 0.999 and limits of quantification from 15.28 to 53.03 ng $mL^{-1}$. Sample preparation was simple dilution with an average recovery of 97.8%. Ion suppression was observed with urine concentrations >10%. Mean levels of saccharin and resveratrol-3-O-sulfate in urine were 5.481 ± 4.359 and 3.440 ± 4.160 nmol $L^{-1}$, respectively. We developed and validated a method to measure saccharin and *trans*-resveratrol metabolites in urine to objectively corroborate the consumption of SQ-LNS for the first time in nutrition intervention studies.

**KEYWORDS:** *saccharin, trans-resveratrol, adherence marker, mass spectroscopy, urine, small quantity lipid-based nutritional supplement*

## INTRODUCTION

Small quantity lipid-based nutrient supplements (SQ-LNS) are food-based supplements typically based on vegetable fat, skimmed milk powder, peanut paste, sugar, and a complex of minerals and vitamins, formulated to meet the needs of specific target populations.[1] SQ-LNS are used to supplement inadequate diets of young children (6−24 months of age) and pregnant/lactating women in low- and middle-income countries with the goal of promoting growth and, hence, preventing undernutrition. SQ-LNS are packaged in single-portion sachets (20 g) and can be eaten alone or mixed with food that is prepared in a household, such as porridge, soups, or stews. To adequately evaluate the impact of SQ-LNS on nutrition and health outcomes in intervention studies, information is required on whether or not the supplements are consumed as intended by the target population(s). Adherence to a supplementation protocol is usually assessed by the self-report or product disappearance rate in large community-based trials. Although these methods can be useful, they may not be reliable. An objective measure of adherence is needed to better estimate the consumption of SQ-LNS in intervention trials.

Although objective adherence markers that are quantitatively correlated to the intake of a nutrient or supplement are more accurate, they can be laborious and costly.[2] Objective adherence markers are measured in biological fluids such as blood, urine, saliva, and feces. A successful adherence marker for use in global community-based nutrition intervention studies must meet numerous criteria. The selected compound must have a safe dosage, consistent with the regulations set by international and/or national agencies such as Joint FAO/WHO Expert Committee on Food Additives (JEFCA), European Food Safety Authority (EFSA), and United States Food and Drug Administration (US FDA); be affordable; have a feasible dosage for SQ-LNS incorporation without compromising product acceptability; have minimal presence in the background diet of consumers; have complete excretion in 24−48 h; and have shelf-life stability conducive to warm climates/no refrigeration.[3] In addition, the adherence marker must be acceptable to the target population(s) in low- and middle-income countries, and the mode of sample collection for testing must be achievable in a community setting.

Based upon these criteria, sodium saccharin (saccharin) and *trans*-resveratrol were selected for evaluation as potential adherence markers for SQ-LNS. Both compounds are approved for consumption by EFSA,[4,5] the US FDA,[6,7] and JECFA.[8] In addition, both compounds are of low cost. Saccharin is a high-intensity sweetener that has been used safely in foods since the 1970s but is not likely to be found in the diets of the young children or pregnant/lactating women in low-income country settings. It is absorbed across gut epithelial

Received: September 24, 2020
Revised: December 22, 2020
Accepted: January 4, 2021
Published: January 13, 2021





https://dx.doi.org/10.1021/acs.jafc.0c06144
*J. Agric. Food Chem.* 2021, 69, 1107−1114

cells intact and is not metabolized. Approximately 85% of the dose of saccharin is excreted in urine within 48 h, with an excretion half-life of 1.2 and 6.6 h in men and women, respectively.[9,10] Saccharin is used in baking goods because of its ability to withstand heat; thus, it is an ideal candidate in terms of shelf-life stability for the nonrefrigerated SQ-LNS. *trans*-Resveratrol is a phenolic compound naturally found in wine[11] and grapes[12] and is not likely to be consumed in large quantities in the diets of young children and pregnant/lactating women with limited access to nutrient-rich foods containing resveratrol and commercial processed foods. Therefore, the amount of resveratrol in the diet is likely not high enough to interfere with quantifying adherence. *trans*-Resveratrol is absorbed across the gastrointestinal epithelium, and it is metabolized by uridine diphosphate (UDP)-glucuronyl transferases (EC 2.4.1.17) and sulfotransferases (EC 2.8.2.1) into a range of glucuronides and sulfate metabolites,[13] the latter being the most common pathway in humans.[14] Approximately 63% of *trans*-resveratrol consumed is excreted in urine over 48 h, with an excretion half-life of 7−18 h.[15−17] In the absence of light, *trans*-resveratrol is stable over time at pH < 7,[18] which is common for shelf-stable foods and urine samples.

Quantifying saccharin and *trans*-resveratrol metabolites from biological specimens, such as blood, saliva, and urine, can be challenging. Less invasive methods (e.g., saliva and urine) are always preferred, as blood sampling involves risk, is costly, and requires trained medical personnel. Herein, the focus was on assessing the adherence in urine, as the levels of these compounds have not been established in saliva, which is more difficult to collect from infants and young children.

Saccharin has been quantified in numerous beverages and other foods using ultrahigh performance liquid chromatography (UHPLC), in addition to having published methods available for quantifying saccharin in urine after the consumption of a beverage or food product.[19] Resveratrol has been successfully quantified in wine and other foods[20] and measured in urine to estimate wine consumption.[21] The common sample preparation techniques from food matrices include dilution with no further sample preparation,[22,23] ether extraction,[24,25] and solid-phase extraction (SPE).[26] Considerably fewer methods described the extraction of saccharin from biological fluids, and the existing methods rely on extraction with ether[27] or SPE.[28,29] Several methods exist for the extraction of *trans*-resveratrol metabolites from biological fluids.[30−33] Extraction techniques from urine include liquid−liquid extraction with or without the pretreatment of samples with β-glucuronidase and/or sulfatase,[31,34] or SPE, with hydrophilic−lipophilic balance (HLB), C18, or silica stationary phases.[16,21,30]

Saccharin and *trans*-resveratrol metabolites are stable in urine for up to 72 and 25 h, respectively, without boric acid preservation and have been quantified using UHPLC−electrospray ionization−triple quadrupole mass spectrometry (UHPLC(ESI)-MS/MS).[19,33] For example, Logue et al. (2017) optimized a method for the analysis of saccharin in urine by diluting each sample 10-fold with water and analyzing them with no further sample preparation.[19] The linear dynamic range (LDR) and limit of detection (LOD) for this method were 10−1000 ng $mL^{-1}$ and 0.06 ng $mL^{-1}$, respectively.[19] In another study, all *trans*-resveratrol metabolites were quantified using UHPLC(ESI)-MS/MS in urine after the consumption of 250 mL of Merlot wine. The LDR and LOD for this method were 1−1000 and 0.998 ng $mL^{-1}$, respectively.[33] None of these studies evaluate the effect of the matrix in which the compounds are contained. There is no study found that evaluated saccharin and resveratrol metabolites simultaneously in urine.

Herein, UHPLC(ESI)-MS/MS was used to measure saccharin in the urine of 23 women, after the consumption of a single dose SQ-LNS containing 8.6 mg of saccharin, and the *trans*-resveratrol metabolites (*trans*-resveratrol-3-O-β-D-glucuronide, *trans*-resveratrol-4-O-glucuronide, and resveratrol-3-O-sulfate) were measured in the urine of 24 women who consumed SQ-LNS containing 5 mg of *trans*-resveratrol. Confirmation of a quantification method for saccharin and *trans*-resveratrol as adherence markers will allow a novel way to directly measure compliance to nutritional intervention studies. The goals of this study were to (1) develop and validate a method to quantify saccharin and *trans*-resveratrol metabolites in urine and (2) show a proof of concept that saccharin and resveratrol metabolites can be quantified in urine after the consumption of SQ-LNS containing these compounds.

## MATERIALS AND METHODS (INCLUDING SAFETY INFORMATION)

**Chemical Reagents and Standards.** HPLC-grade methanol (>99.9%), glacial acetic acid (HPLC, > 99.7%), acetone (Optima, >99.9%), and acetonitrile (ACN; Optima LC/MS-grade) were obtained from Fisher Scientific (Hampton, NH, USA). *trans*-Resveratrol was purchased from Spectrum Chemical (New Brunswick, NJ, USA); *trans*-resveratrol-3-O-β-D-glucuronide, *trans*-resveratrol-4-O-glucuronide, and resveratrol-3-O-sulfate were purchased from Cayman Chemical (Ann Arbor, MI, USA); sodium saccharin was purchased from Sigma-Aldrich (St. Louis, MO, USA); and saccharin-$d_4$ was purchased from CDN Isotopes (Pointe-Claire, QU, Canada). Ultrapure water used for all experiments was obtained from a Milli-Q Academic system from Millipore Sigma (Burlington, MA, USA).

**Lipid-Based Nutritional Supplements.** Two types (SQ-LNS1 and SQ-LNS2) of SQ-LNS sachets were obtained from Nutriset (Malaunay, FR). The nutrient composition and ingredients of SQ-LNS1 and SQ-LNS2 were identical (Table S1), with the exception of the adherence marker added. SQ-LNS1 contained 8.6 mg of saccharin and SQ-LNS2 contained 5 mg of *trans*-resveratrol and 5 mg of sucralose. Sucralose was initially evaluated as an adherence marker; however, preliminary results indicated that sucralose concentrations in urine were very low, and therefore sucralose was eliminated from consideration as an adherence marker, and the data are not reported.

**Calibration Curve and Internal Standards.** Individual stock solutions of *trans*-resveratrol, *trans*-resveratrol-$d_4$, *trans*-resveratrol-3-O-β-D-glucuronide, *trans*-resveratrol-4′-O-D-glucuronide, resveratrol-3-O-sulfate, saccharin, and saccharin-$d_4$ were prepared by dissolving standards in methanol/water 1:1 at 4 mg $mL^{-1}$. All solutions were stored in the dark at −20 °C.

External 9-point calibration curves (3 ng $mL^{-1}$ to 1 μg $mL^{-1}$) were prepared from the stock solutions of *trans*-resveratrol, *trans*-resveratrol-3-O-β-D-glucuronide, *trans*-resveratrol-4′-O-D-glucuronide, resveratrol-3-O-sulfate, and saccharin. A 1 μg $mL^{-1}$ stock solution of saccharin-$d_4$ was used as an internal standard (IS).

**UHPLC(ESI)-MS/MS Analysis.** Quantification was performed using an Agilent 1290 Infinity UHPLC system interfaced to a 6460 triple-quadrupole mass spectrometer with ESI via Jet Stream technology (Agilent Technologies, Santa Clara, CA, USA). The UHPLC system was equipped with a binary pump with an integrated vacuum degasser (G4220A), an autosampler (G4226A), and a thermostated column compartment (G1316C). The column used was ZORBAX Extend-C18 (1.8 μm, 2.1 mm × 100 mm, Agilent Technologies, Memphis, TN, USA), and peaks were resolved using a mobile phase A (1% acetic acid in water) and a mobile phase B (1% acetic acid in 70:30 acetone/ACN). The gradient for elution was 0−4 min (3% B), 4−5 min (50% B), 5−7 min (80% B), 7−8 min (80% B),


https://dx.doi.org/10.1021/acs.jafc.0c06144
J. Agric. Food Chem. 2021, 69, 1107−1114

**Table 1. MRM Transitions and Parameters for Standards and Deuterated Standards**

| MRM transitions and parameters for standards and deuterated standards | | | | | | | |
|---|---|---|---|---|---|---|---|
| compound | chemical formula | molecular mass (g mol$^{-1}$) | $t_R$ (min) | precursor ion ($m/z$) | product ions ($m/z$) | fragmentor energy (V) | collision energy (V) |
| saccharin | $C_7H_5NO_3S$ | 183.18 | 1.101 | 182 | 106 | 105 | 16 |
| | | | | | 42[a] | | |
| saccharin $d_4$ | $C_7HD_4NO_3S$ | 187.21 | 1.105 | 186 | 106 | 115 | 18 |
| | | | | | 42[a] | | |
| *trans*-resveratrol | $C_{14}H_{12}O_3$ | 228.24 | 3.369 | 227 | 185 | 130 | 16 |
| | | | | | 89[a] | | |
| *trans*-resveratrol-3-O-$\beta$-glucuronide | $C_{20}H_{20}O_9$ | 404.37 | 3.107 | 403 | 227 | 110 | 22 |
| | | | | | 185[a] | 130 | 29 |
| *trans*-resveratrol-4′-O-D-glucuronide | $C_{20}H_{20}O_9$ | 404.37 | 2.926 | 403 | 227 | 110 | 22 |
| | | | | | 185[a] | 130 | 29 |
| resveratrol-3-O-sulfate | $C_{14}H_{12}O_6S$ | 308.31 | 3.196 | 307 | 227 | 105 | 16 |
| | | | | | 185[a] | 80 | 28 |

[a]The transition used as qualifier ion.

and 8−11 min (3% B) with a flow rate of 0.400 mL min$^{-1}$. The injection volume was 100 $\mu$L. The column temperature was held at 40 °C. All compounds were analyzed in negative mode using multiple reaction monitoring (MRM). The source conditions included the following: gas temperature at 350 °C; gas flow at 9 L min$^{-1}$; 30 psi for the nebulizer; sheath gas flow is 12 L min$^{-1}$ at 350 °C; capillary voltage at (−)5000 V, and electron multiplier voltage at (−)2000 V. The dwell time was 50 ms, and the cell acceleration was at 7 V for each transition.

**LOD and Limit of Quantification.** LOD and limit of quantification (LOQ) for each compound were determined using a 3−1000 ng mL$^{-1}$ calibration curve. The LOD was defined as 3.3 times the standard deviation of the $y$-intercept of the response divided by the slope of the curve, and the LOQ was defined as 10 times the standard deviation of the $y$-intercept of the response divided by the slope of the curve.

**Ion Suppression by Urine Matrix Effect.** Blank urine samples were mixed with methanol/water 1:1 v/v to achieve 2:98, 5:95, 10:90, 20:80, and 50:50 v/v dilutions. The samples were spiked with *trans*-resveratrol-3-O-$\beta$-D-glucuronide, *trans*-resveratrol-4′-O-D-glucuronide, resveratrol-3-O-sulfate, and saccharin at 100 ng mL$^{-1}$. The samples were prepared in triplicate and quantified by UHPLC(ESI)-MS/MS.

**Adherence Marker Recovery.** Blank urine samples were diluted 10:90 v/v with methanol/water 1:1 v/v and spiked at 10, 100, and 800 ng mL$^{-1}$ with *trans*-resveratrol-3-O-$\beta$-D-glucuronide, *trans*-resveratrol-4′-O-D-glucuronide, resveratrol-3-O-sulfate, and saccharin to study the recovery of each analyte. Deuterated saccharin (saccharin-$d_4$) was used as an IS, and a nonspiked sample was used as a control. Samples were prepared in triplicate and quantified by UHPLC(ESI)-MS/MS.

**Clinical Study.** A total of 47 healthy women (18−45 years old, 18−25 kg (m$^2$)$^{-1}$ body mass index) consumed one 20 g sachet of SQ-LNS containing either saccharin or *trans*-resveratrol. Twenty-three women consumed a single dose of SQ-LNS1 containing 8.6 mg of saccharin, and the remaining 24 women received a single dose of SQ-LNS2 containing 5 mg of *trans*-resveratrol. The amounts of saccharin and resveratrol were chosen based on JECFA ADIs, published excretion data, and potential method LOD. Based upon this, minimal amounts were used to refrain from changing the taste and/or need to modify the product formulation. All urine samples were collected for 0−4 h after a washout period of 3 days, which established the participants' blank urine sample. During the washout period, women were instructed to avoid any saccharin- or resveratrol-containing foods or food products, depending on their assigned SQ-LNS. SQ-LNS1 consumers were instructed to avoid diet sodas, low-calorie and diet food products, and saccharin, whereas SQ-LNS2 consumers were asked to avoid wine, grapes, blueberries, cranberries, pomegranates, pistachios, peanuts, and dark and baking chocolates. After SQ-LNS consumption, urine was collected for 48 h. Preliminary studies indicated that a time point of 4 h post consumption would be the appropriate time point to use to establish if this method could be used to monitor LNS consumption. Full details of the clinical trial (urine evaluated over 48 h) will be presented elsewhere.

**Sample Preparation.** The samples were acidified using 5 mg boric acid for preservation and kept at −80 °C and protected from light until analysis. A 20 $\mu$L of urine from participants who consumed SQ-LNS1 (containing saccharin) and 100 $\mu$L of urine from participants that consumed SQ-LNS2 (containing resveratrol) were added to 980 and 900 $\mu$L methanol/water 1:1 v/v, respectively. The samples were analyzed by UHPLC(ESI)-MS/MS as described above. Resveratrol metabolites were expressed as resveratrol equivalents, and total resveratrol metabolites were calculated by adding the three quantified metabolites (*trans*-resveratrol-3-O-$\beta$-D-glucuronide, *trans*-resveratrol-4′-O-D-glucuronide, and resveratrol-3-O-sulfate).

**Statistics.** Analysis of variance (ANOVA) and Student's $t$ test were used to compare the compound means using R (version 3.6.2, R Foundation for Statistical Computing, 2016) and Microsoft Excel (version 16.36, Microsoft, 2020). When significant mean differences ($p < 0.05$) were found with ANOVA, Tukey's test was applied to find the source of significance with a confidence level of 95%.

## RESULTS/DISCUSSION

**Summary of Method Optimization.** The fragmentation patterns for saccharin and resveratrol metabolites are given in Figure S1. A summary of MRM transitions and parameters can be found in Table 1. Examples of chromatograms are given in Figure S2. The predominant product ion and qualifier ion were chosen to increase the confidence in the identity of *trans*-resveratrol glucuronides and sulfate metabolites (Table 1). The fragmentor and collision energies were optimized for each transition and are given in Table 1. The fragmentor energy was monitored from 100 to 200 V at steps of 5 or 10 V to identify the best energy value to use. The fragmentor energy that provided the largest peak area, while maintaining an optimum peak shape, was chosen to quantify that compound. The collision energy (V) was optimized from 3−30 V at steps of 5 V. An example of the optimization curves for the fragmentation and collisions energies for saccharin can be found in Figure S3a,b. The optimal fragmentation energy was 115 V for saccharin; however, the fragmentation was robust with energies between 95 and 130 V (Figure S3a), whereas there was only one optimal collision energy of 16 V for saccharin (Figure S3b).

**LOD and LOQ.** The LOD and LOQ were identified for each standard by running concentrations at 3, 6, 10, 30, 60,


https://dx.doi.org/10.1021/acs.jafc.0c06144
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**Table 2. Transitions, Linearity Range, Correlation Coefficient, LOQ, and LOD for Saccharin and *trans*-Resveratrol Metabolites**

| | transitions, linearity range, correlation coefficient, LOQ, and LOD | | | | |
|---|---|---|---|---|---|
| Compound | transition | linearity range (ng mL$^{-1}$) | $R^2$ | LOQ (ng mL$^{-1}$) | LOD (ng mL$^{-1}$) |
| saccharin | 182 → 106 | 3−1000 | 0.99918 | 53.03 | 17.50 |
| *trans*-resveratrol-3-O-*β*-glucuronide | 403 → 227 | 3−1000 | 0.99966 | 15.28 | 5.04 |
| *trans*-resveratrol-4′-O-glucuronide | 403→ 227 | 3−1000 | 0.99977 | 20.52 | 6.77 |
| resveratrol-3-O-sulfate | 307 → 227 | 3−1000 | 0.9999 | 15.33 | 5.06 |

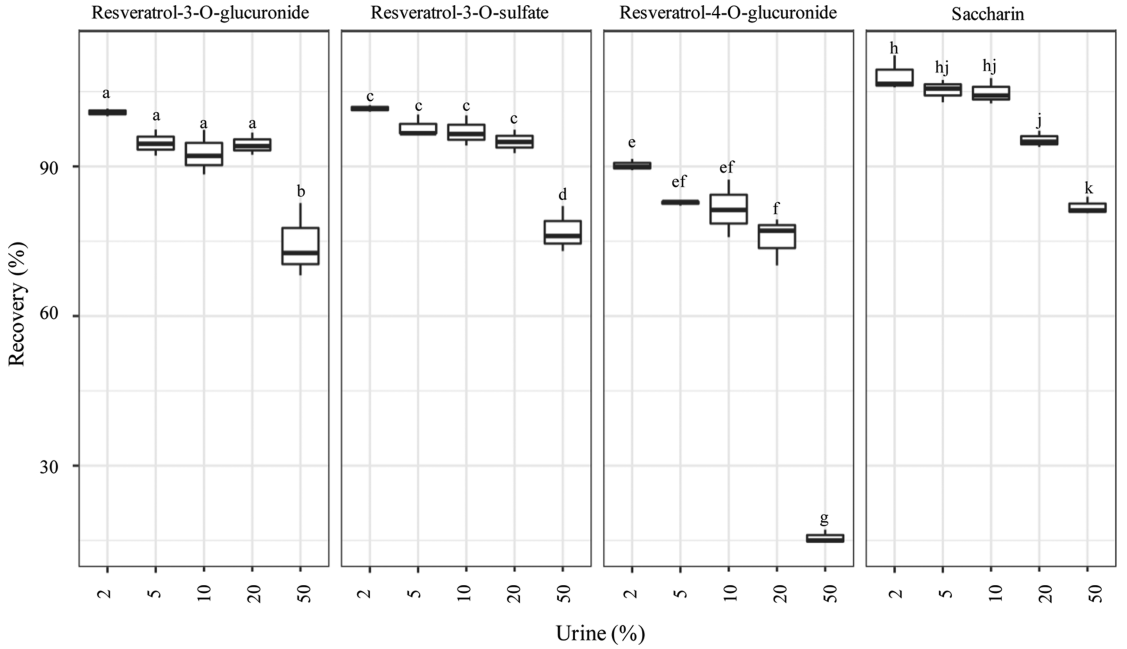


**Figure 1.** Percentage recovery of saccharin, *trans*-resveratrol-4′-O-D-glucuronide, *trans*-resveratrol-3-O-*β*-glucuronide, and resveratrol-3-O-sulfate at different urine concentration levels.

**Table 3. Percent Recovery of Saccharin and Resveratrol Metabolites from Blank Urine Spiked with Saccharin and *trans*-Resveratrol**

| | saccharin and resveratrol metabolite recoveries | | | |
|---|---|---|---|---|
| | recovery (%) | | | |
| expected concentration (ng mL$^{-1}$) | saccharin | *trans*-resveratrol-4-O-D-glucuronide | *trans*-resveratrol-3-O-*β*-glucuronide | resveratrol-3-O-sulfate |
| 10 | n.d.[a] | 108.2 ± 4.7 | 91.3 ± 10.1 | 111.1 ± 1.0 |
| 100 | 88.1 ± 1.7 | 91.9 ± 1.1 | 108.0 ± 1.5 | 103.2 ± 4.1 |
| 800 | 79.1 ± 1.4 | 92.6 ± 2.6 | 101.8 ± 0.5 | 99.0 ± 1.0 |

[a]Not determined (n.d.) because the values were below the LOD for saccharin.

100, 300, 600, and 1000 ng mL$^{-1}$ and measuring the response. The LOD and LOQ for saccharin were higher than the LOD and LOQ for resveratrol metabolites (Table 2).

**Matrix Effect on the Percentage Recovery of Markers.** To our knowledge, the effect of urine as a matrix has not been evaluated on the recovery of saccharin and resveratrol metabolites. However, it would be advantageous to analyze these compounds directly in urine to decrease sample preparation. Herein, the measurement of all compounds was strongly influenced by the percentage of urine in the sample, indicating a strong matrix effect. This was especially true for the resveratrol metabolite *trans*-resveratrol-4′-O-D-glucuronide, which showed a decrease from 90.2% recovery in 2% urine to 15.6% when 50% urine was used. Similarly, the percentage recovery decreased from 101.6 to 77.0% for resveratrol-3-O-sulfate, from 100.8 to 74.5% for *trans*-resveratrol-3-O-*β*-D-glucuronide, and from 108.2 to 81.9% for saccharin (Figure 1). ANOVA indicated that recovery of all compounds significantly ($p < 0.05$) decreased as the percentage of urine increased. The Tukey test showed a significant difference in recovery between 2, 5, 10, and 20% urine and 50% urine for all compounds (Figure 1). Based upon these observations, we recommend that the percent urine in the sample be ≤10% to avoid any significant urine matrix effect on recovery.

**Analyte Recovery Percentage.** Blank urine samples were diluted 10:90 v/v with methanol/water 1:1 v/v and spiked at 10, 100, and 800 ng mL$^{-1}$ of *trans*-resveratrol-3-O-*β*-D-glucuronide, *trans*-resveratrol-4′-O-D-glucuronide, resveratrol-3-O-sulfate, and saccharin to study the recovery. These concentrations were chosen to cover the range of expected concentrations in urine. Table 3 shows the recovery percentage of each compound. For all compounds, recoveries close to 100% were achieved at 100 and 800 ng mL$^{-1}$. For saccharin,


https://dx.doi.org/10.1021/acs.jafc.0c06144
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the recovery at the lowest concentration (10 ng $mL^{-1}$) was not determined because it was below the LOD.

**Human Consumption.** The urine concentrations of saccharin and resveratrol metabolites were measured in response to a single dose of SQ-LNS containing the marker compounds. All *trans*-resveratrol metabolites and saccharin were successfully detected and quantified in the urine samples from the study participants. Figure 2 shows the average saccharin content in pooled urine at baseline, before the administration of SQ-LNS1, and during the 4 h following consumption for 23 participants. At baseline, the average saccharin content was 1.909 ± 1.325 nmol $L^{-1}$. After consumption, the average saccharin content in urine was 5.481 ± 4.359 nmol $L^{-1}$.

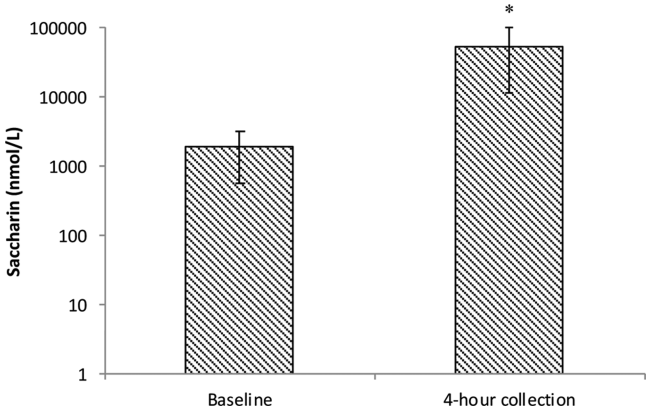


**Figure 2.** Quantification of saccharin in urine (nmol $L^{-1}$) at baseline and 0−4 h after the consumption of SQ-LNS containing 10 mg saccharin in 23 women. * Significant difference from baseline at $p < 0.05$ (baseline = 1909 ± 1325.63 nmol/L, 4 h collection = 54,812.04 ± 43,587.44 nmol/L).

Figure 3 shows the average content of *trans*-resveratrol-4′-O-D-glucuronide, *trans*-resveratrol-3-O-β-D-glucuronide, resveratrol-3-O-sulfate, and total resveratrol metabolites in urine of 24 women before and after the consumption of SQ-LNS2. The average content of resveratrol metabolites in urine at baseline was 0 ± 0, 15.50 ± 53.44, and 4.89 ± 22.39 nmol $L^{-1}$ for *trans*-resveratrol-4′-O-D-glucuronide, *trans*-resveratrol-3-O-β-D-glucuronide, and resveratrol-3-O-sulfate, respectively, which increased to 507.82 ± 768.80, 913.63 ± 927.51, and 3439.96 ± 4160.05 nmol $L^{-1}$ in the 4 h pooled sample after LNS2 consumption. A representative chromatogram of urine post SQ-LNS consumption can be found in Figure S4, where the largest peak represents resveratrol-3-O-sulfate.

A UHPLC(ESI)-MS/MS method was developed for the quantification of saccharin and *trans*-resveratrol metabolites in urine simultaneously, or independently, to monitor the consumption of SQ-LNS. This method was based upon the methods for saccharin quantification in soft drinks and urine from Logue et al. (2017)[19] and a method from Urpi-Sarda et al. (2007)[33] for the quantification of *trans*-resveratrol metabolites in urine after red wine consumption. Saccharin fragments into ions of $m/z$ 105.8 and 41.9.[36] The ion of $m/z$ 106.0 was chosen as the quantifier ion. A qualifier ion for saccharin was not measured as the fragmentation ion 41.9 is similar in mass to endogenous compounds including different ketones commonly found in human urine.[37]

Source parameters including sheath gas temperature (°C), drying gas temperature (°C), sheath gas flow (L $min^{-1}$), drying gas flow (L $min^{-1}$), capillary voltage (V), nozzle voltage (V), and nebulizer flow (psi) were optimized for maximum peak area for all compounds in the same run and are given in Table S2. The optimal conditions for each compound are given in Table S3, which is an excellent tool to visualize the optimum conditions for each compound in comparison to others. For example, the nebulizer flow was evaluated over 30−60 psi, but optimum ionization was obtained for all compounds at 30 psi,

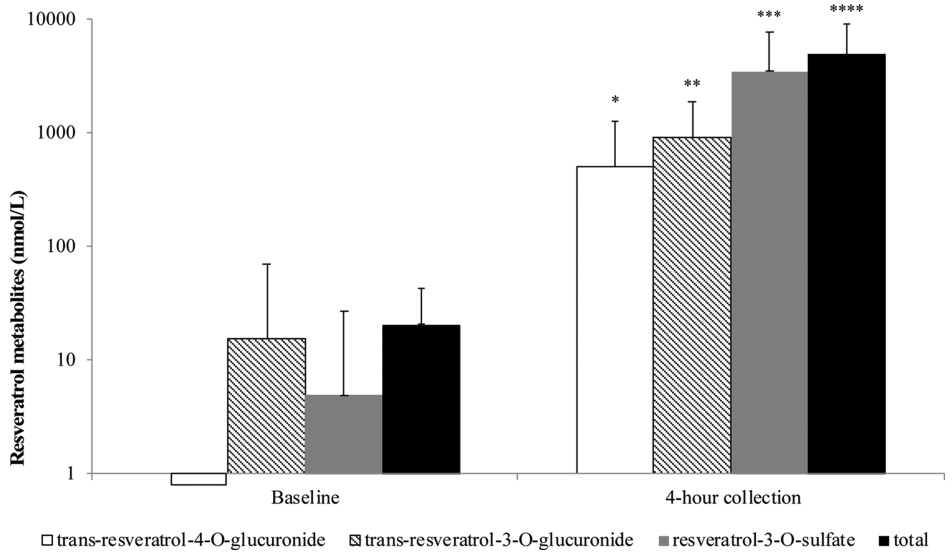


**Figure 3.** Quantification of *trans*-resveratrol-4′-O-D-glucuronide, *trans*-resveratrol-3-O-β-D-glucuronide, resveratrol-3-O-sulfate, and total resveratrol metabolites in urine (nmol $L^{-1}$) at baseline and 0−4 h after consumption of SQ-LNS with 5 mg *trans*-resveratrol in 24 women. * Significant difference from baseline at $p < 0.05$ (baseline = 0.00 ± 0.00 nmol/L, 4 h collection = 507.82 ± 768.80 nmol/L). **Significant difference from baseline at $p < 0.05$ (baseline = 15.50 ± 53.44 nmol/L, 4 h collection = 913.63 ± 927.51 nmol/L). ***Significant difference from baseline at $p < 0.05$ (baseline = 4.89 ± 22.39 nmol/L, 4 h collection = 3439.96 ± 4160.05 nmol/L). **** Significant difference from baseline at $p < 0.05$ (baseline = 15.50 ± 56.62 nmol/L, 4 h collection = 4861.40 ± 5284.61 nmol).


https://dx.doi.org/10.1021/acs.jafc.0c06144
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demonstrating that the nebulizer flow has only a small impact on the peak area of these compounds. In contrast, the sheath gas temperature varied significantly for each compound. Therefore, an average temperature of 350 °C was used to achieve the best peak area for all compounds, demonstrating that this parameter has a greater impact on the method.

Mobile phases can change the efficacy of quantification by affecting the peak shape and resolution. Three mobile phase combinations were explored: for mobile phase A, (1) 0.05% acetic acid in water and (2) 0.1% acetic acid in water were explored; for mobile phase B, (1) 0.05% acetic acid in ACN, (2) 0.1% acetic acid in ACN, and (3) 0.1% acetic acid in 70% acetone:30% ACN were explored. Mobile phase A (2) with mobile phase B (3) was the optimum combination for the separation of *trans*-resveratrol glucuronides of the same mass transitions and thus was selected to be the mobile phase system of choice for quantifying all compounds in urine samples.

As a proof of concept, a pilot study was performed in 47 women who consumed SQ-LNS containing either 5 mg of resveratrol or 8.6 mg of saccharin. The number of volunteers was chosen following the study design of Zamora-Ros, where resveratrol was validated as the urine marker for wine consumption.[35] Saccharin and resveratrol metabolites were measured in urine collected 0−4 h after the consumption of SQ-LNS. The variability measured for saccharin in urine (80% RSD) was similar to the variability reported by Logue et al. (2017) who reported RSD values of 85% to over 100%. Logue et al. (2017) had 21 participants consuming 0.1, 0.5, and 10% of the recommended acceptable daily intake of saccharin for a 70 kg person and collected total urine over a 24 h period.[19]

Herein, resveratrol-3-O-sulfate was the most abundant metabolite by far, constituting 69.16 ± 15.66% of the total measured resveratrol metabolites. As this translates into easier peak integration, resveratrol-3-O-sulfate was chosen as the signature metabolite for monitoring resveratrol consumption. The RSD for resveratrol-3-O-sulfate was 121%. Goldberg et al. (2003) demonstrated an RSD of 15−23% for resveratrol in urine, which is significantly lower than the RSD observed herein. However, in the study of Goldberg et al. (2003), a dose of 25 mg of resveratrol per 75 kg of body mass was given in 100 mL of vegetable juice, white wine, or white grape juice given in 20 s to participants that were fasted overnight.[15] Participants remained fasted 4 h after consuming the beverage, and resveratrol was measured in 24 h urine after the enzymatic hydrolysis of sulfate and glucuronide metabolites. Herein, a total dose of 8.6 mg, regardless of the body mass, was given in a lipid-rich food matrix (SQ-LNS), and the participants were not fasted, which would contribute to the higher RSD observed in our data. Our study was designed to mimic the consumption of SQ-LNS in the field where participants are not fasted and dosing periods are not controlled.

A rapid UHPLC(ESI)-MS/MS method was developed to measure saccharin and/or *trans*-resveratrol metabolites in urine and validated in 47 women who consumed a single dose of SQ-LNS containing saccharin or *trans*-resveratrol. The method was developed so that two adherence markers could be used, which is desirable for global studies employing SQ-LNS where background diets will vary. The method takes advantage of simple sample preparation (dilution) and minimal sample volumes.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06144.

Nutritional values of SQ-LNS; source conditions for UHPLC(ESI)-MS/MS and the condition levels evaluated; source conditions for compounds of interest by peak area; molecular structures and fragmentation patterns of compounds of interest; relevant chromatograms; and fragmentor and collision energies for saccharin (PDF)

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### Funding

This research was funded by The Bill & Melinda Gates Foundation Opportunity OPP1148410.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The authors thank Dr. Larry Lerno at the Food Safety and Measurement Facility UC Davis for technical support and Xin Dai, Shaine Robinson, and Sara Yeager for help with sample preparation and collection.


## ■ ABBREVIATIONS

SQ-LNS, small quantity lipid-based nutrient supplement; JEFCA, Joint FAO/WHO Expert Committee on Food Additives; EFSA, European Food Safety Authority; US FDA, United States Food and Drug Administration; UDP, uridine diphosphate; UHPLC, ultrahigh performance liquid chromatography; SPE, solid-phase extraction; HLB, hydrophilic−lipophilic balance; UHPLC(ESI)-MS/MS, ultrahigh performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry; LDR, linear dynamic range; LOD, limit of detection; ACN, acetonitrile; MRM, multiple

reaction monitoring; IDL, instrument detection limit; LOQ, limit of quantification; IS, internal standard; ANOVA, analysis of variance

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*J. Agric. Food Chem.* 2021, 69, 1107−1114