The Investigation of Sucrose and Fructose in Spot Versus 24-hour Urine As Biomarkers of Sugars Intake

by

Annalisa Averill

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved December 2017 by the
Graduate Supervisory Committee:

Natasha Tasevska, Chair
Christina Shepard
Carol Johnston

ARIZONA STATE UNIVERSITY

May 2018

ABSTRACT

Background: Twenty-four hour urinary sucrose and fructose (24uSF) has been developed as a dietary biomarker for total sugars intake. Collection of 24-h urine is associated with high costs and heavy participant burden, while collection of spot urine samples can be easily implemented in research protocols. The aim of this thesis is to investigate the utility of uSF biomarker measured in spot urine. Methods: 15 participants age 22 to 49 years completed a 15-day feeding study in which they consumed their usual diet under controlled conditions, and recorded the time each meal was consumed. Two nonconsecutive 24-hour urines, where each urine void was collected in a separate container, were collected. Four timed voids (morning, afternoon, evening, and next day) were identified based on time of void and meal time. Urine samples were measured for sucrose, fructose and creatinine. Variability of uSF excretion was assessed by coefficient of variation (%CV) and variance ratios. Pearson correlation coefficient and multiple linear regression were used to investigate the association between uSF in each timed void and corresponding 24uSF excretion. Results: The two-day mean uSF was 50.6 mg (SD=29.5) for the 24-h urine, and ranged from 4.5 to 7.5 mg/void for the timed voids. The afternoon void uSF had the lowest within-subject variability (49.1%), and lowest within- to between-subject variance ratio (0.2). The morning and afternoon void uSF had the strongest correlation with 24-h uSF for both mg/void (r=0.80 and r=0.72) and mg/creatinine (r=0.72 and r=0.67), respectively. Finally, the afternoon void uSF along with other covariates had the strongest predictive ability of 24-h uSF excretion (mg/void) (Adjusted R^2= 0.69; p=0.002), whereas the morning void had the strongest predictive

ability of 24-h uSF excretion (mg/g creatinine) (adjusted R^2= 0.58; p=0.008). Conclusions: The afternoon void uSF had the most favorable reproducibility estimates, strong correlation with 24uSF excretion, and explained greatest proportion of the variability in 24uSF. USF in mg/void may be better to use than uSF in mg/g creatinine as a biomarker in spot urine. These findings need to be confirmed in a larger study, and in a study population with a wide range of sugars intake.

DEDICATION

To my father, Roderick Averill, I dedicate this thesis to you. I would not have made it to the finish line without all your assistance. He has encouraged me from the front line and been the behind the scenes throughout the whole journey. His subtle check ins fueled my drive to tackle each step. Thank you for instilling knowledge seeking in me.

ACKNOWLEDGMENTS

Dr. Natasha Tasevska, I would like to send you my gratitude for being an unending teacher and rigorous mentor throughout my graduate education at the School of Nutrition and Health Promotion at ASU. Her one-on-one support has pushed me in my academic endeavors and I would not be the student I am now without her guidance throughout every step of the way.

I would like to thank Dr. Virag Sagi-Kiss for teaching me the ropes in the laboratory and for countless check ins along the way, always encouraging me during my mistakes and victories.

To my committee, thank you for helping me achieve my dreams of completing my thesis. From the many emails and meetings, I so appreciate your unfailing support and assistance.

Thank you to my family, although you may be states away you continually kept my morale high. This thesis could not have been completed without their many phone calls, emails, and messages. To my friends that I have met along the way and everyone in my program, thank you for reminding me to step back and enjoy the process and encourage me.

TABLE OF CONTENTS

CHAPTER Page

APPENDIX Page

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Added sugars consumption has increasingly contributed to total energy intake in the U.S., over the last several decades. The most significant increase in calories from added sugars was found to be from 1977 to 2003 (Powel et al., 2016). In 2011-2012, children and adults in the U.S. consumed 17% and 14% of their total energy from added sugars, respectively (Powell et al, 2016). While added sugars are a large component of the American diet, the implications of high sugars consumption are not explicitly understood. So far, there has been sufficient evidence to conclude that sugars are a significant determinant of body weight likely due to providing excess energy (Te Morenga et al, 2012), and increase the risk of metabolic disease (Te Morenga et al., 2014). Yet, the evidence on sugars in relation to type 2 diabetes (Lean & Te Morenga, 2016) and cancer risk (Tasevska et al., 2012) is largely inconclusive. Gaining better understanding of sugars-disease associations is very important to help make stronger recommendations to build healthier communities.

Associations between sugars and risk of disease are difficult to assess due to unreliability of dietary intake, which is an important obstacle in determining true diet-disease associations (Freedman et al., 2011). Self-reporting dietary instruments, such as food frequency questionnaires (FFQ), 24 hour dietary recalls, and diet records are main methods of measuring intake in population studies; however, they are flawed with measurement error (Kipnis et al., 2003). Memory issues, inability to estimate portion size and to conceptualize intake over an extended periods of time (Thompson &

Subar, 2012), as well as social desirability, varying interpretation of questions, and body dissatisfaction can cause people to misreport their food intake (Tooze et al., 2004). Additionally, it has been found that energy dense foods are more likely to be underreported (Subar et al., 2003). Foods containing added sugars are particularly prone to being misreported as they are perceived to be unhealthy (Krebs-Smith et al., 2000; Price et al., 1997). The underreporting of sugars intake may seriously affect the ability to confirm disease and sugars associations, and more objective measures of diet are needed (Tasevska et al., 2011).

Biomarkers have been identified as one tool to overcome the problem of measurement error in self-reported dietary intake (Bingham, 2002), and can help strengthen diet-disease associations (Freedman et al., 2010; Freeman, 2014). The sum of sucrose and fructose measured in 24-hour urine has been developed as a biomarker of total sugar intake in highly controlled feeding studies conducted in the UK (Tasevska et al., 2005). The sum of the 30-d mean of sucrose and fructose measured in 24-h urine in 13 participants consuming their usual diet was found to be highly correlated with 30-d mean total sugars ($r_{SUG} = 0.84$; $P < 0.001$) and sucrose intake ($r_{SUC} = 0.77$; $P = 0.002$). Although characteristics and performance of this biomarker are highly promising (Tasevska, 2015), collection of 24-h urine samples is not always feasible in large population studies. Collection of spot samples can be easily implemented in research protocols compared to 24-h samples; however, the utility of the sugars biomarker measured in spot urine has never been investigated. Spot urine samples as a matrix for biomarker measurement are being increasingly investigated due to ease of collection

(Wang et al., 2013) and could have major implications in population research.

This thesis aims to investigate the use of spot urine for the measurement of urinary sucrose and fructose excretion as a proxy to the gold standard 24-h urine collection.

Aim 1: To investigate reproducibility (i.e., describe the within-subject and between-subject variability) of sucrose and fructose excretion in spot urine;

Aim 2: To investigate the correlation of sucrose and fructose from each spot urine with the corresponding 24-h sucrose and fructose excretion, and to determine which spot urine will be most indicative of 24-h urinary excretion.

Aim 3: To investigate if age, gender, body mass index (BMI), time of last meal, and other characteristics will improve the relationship between sucrose and fructose measured in spot and 24-h urine.

Purpose statement:

This was the first controlled feeding study to investigate the performance of sucrose and fructose in spot urine as a biomarker of sugars intake in the US. Using the spot urine measure for assessing sugars that can be easily incorporated in protocols of population studies can have a great impact on elucidating the association between sugars intake and chronic disease risk. Given the American diet is high in added sugars (Ervin & Ogden, 2013), reliably determining these associations is very important to help make stronger dietary guidelines and reinforce recommendations for the public. When sugar-disease associations are better understood, policies can be created to make healthier communities.

Participants were committed to a fifteen-day highly controlled feeding study where they collected 24-hour urine samples on eight alternating days. On two of these eight 24-hour urine collection days, each urine void was collected in a separate container during the 24-hour urine collection period as a multiple spot urine collection. Sucrose and fructose measured in spot urines were compared to sucrose and fructose in the corresponding 24-hour urine collection to determine their relationship. Lastly, other variables such as age, gender, BMI, time of last meal, and physical activity that may have an effect on the investigated association were measured, and their effect was explored. While participants' dietary intake can be determined from the highly controlled feeding study, dietary intake data were not utilized in this investigation. Instead, 24-h urinary sucrose and fructose were measured and used as a surrogate measure of intake.

The recovery of orally administered para-amino benzoic acid (PABA) was assessed to determine the completeness of 24-h urine collection (Bingham & Cummings, 1983). Measuring completeness of urine collections is essential, because incomplete collections can affect estimates of the biomarker and characterizing its performance as a dietary biomarker.

Definition of terms:

- 24 hour urine- composite urine collected for 24 hours, starting with the collection of the second morning void, and finishing with the first morning void of the following day.
- Spot urine- single urine sample collected by a participant, which can be untimed

or timed (i.e., collected at a specific time during the day, e.g., first morning void)

- Biomarker- a compound measured in any biological sample that can reflect intake sufficiently, acting as an objective measure of true intake.
- Sucrose- a disaccharide by chemical structure composed of one molecule of glucose and one molecule of fructose.
- Fructose- fruit sugar, a monosaccharide by chemical structure.
- Total sugars- the sum of all free monosaccharides (glucose, fructose and galactose) and disaccharides (lactose, sucrose and maltose), naturally-occurring or added to foods and beverages, regardless of the food source (Cummings & Stephen, 2007; Vos et al., 2016).
- Added sugars- the sum of any monosaccharide and disaccharide used as ingredients in processed and prepared foods, soft and alcohol drinks, jams and jellies, candies, and ice cream as well as sugars eaten separately or added to foods at the table (Bowman, 2017).
- Free sugars – a term used by the World Health Organization for added sugars, however this term includes sugars naturally present in fruit juices and juice concentrates.
- Measurement error -the difference between measured and true value.

<u>Limitations (problems inherent to the design):</u>

- The spot urine collection protocol is rather complex as each spot urine void will be collected in a separate container during the 24-hour urine collection period,

thus mislabeling or incomplete collections could occur.

- This biomarker is excreted between 2-6 hours postprandially, therefore the timing of the last meal consumed before the spot urine is collected is expected to affect the excretion of the biomarker.
- Dietary intake data were not available for this analysis, therefore the association between sucrose and fructose in spot urine and intake could not be investigated. Instead, sum of 24-h urinary sucrose and fructose excretion was be used as a surrogate measure of intake.

Delimitations (narrow the scope of the study):

- The total sugars intake of our study population may not be generalizable to the US population.
- Inclusion: non-smoking, age 18 and 70 years old, having a BMI of less than 35 kg/m^2, having no known allergy to sunscreen or PABA, be willing to refrain from taking dietary supplements over the 10-week study period, and residing in the Phoenix Metropolitan Area.
- Exclusion: presence of diseases that can affect nutrient absorption or metabolism, such as autoimmune diseases (e.g., type 1 diabetes, inflammatory bowel disease, celiac disease), endocrine (e.g., type 2 diabetes, hyper or hypothyroidism), stomach disorders (e.g., ulcers, gastrointestinal bleeding), diseases that affect urine excretion (e.g., kidney disease, urinary incontinence), or require any dietary restrictions (e.g., celiac disease); participation in any diet related research study or

trying to lose weight over the last four months; known allergy to sunscreen; if their fasting blood glucose or HbA1c were greater or equal to 100 mg/dl and 5.7 % respectively, based on the screening blood draw; and for women, being pregnant, planning to become pregnant in the next 15 weeks or breastfeeding.

CHAPTER 2

LITERATURE REVIEW

Definition of sugars

Sugars, a type of carbohydrate, include three subgroups: monosaccharides, disaccharides, and polyols (sugar alcohols). Monosaccharides are the building blocks of di-, oligo- and polysaccharides (Cummings & Stephen, 2007). Monosaccharides are sweet tasting/water-soluble molecules, which include glucose, galactose, and fructose. Disaccharides include sucrose, composed of one molecule of glucose and one molecule of fructose; lactose, composed of glucose and galactose; and maltose, composed of two molecules of glucose. Fructose is naturally found in honey, fruits, vegetables, and other plants; however, it is also frequently derived from corn to produce high fructose corn syrup (Cummings & Stephen, 2007), which is commonly used as added sugar in processed foods and sugar sweetened beverages. Lactose is naturally found in dairy products. Sucrose is naturally found in sugar cane and sugar beets, which are used to produce table sugar, a common added sugar in the American diet, but is also a naturally occurring sugar in fruits and vegetables. Polyols are sugar alcohols, including sorbitol and xylitol, and are often used as a sugar replacement (Cummings & Stephen, 2007; IFIC Foundation, 2016). Though, they are found naturally in some fruits, they can also be manufactured (Cummings & Stephen, 2007). Their structure differs slightly from monosaccharides and therefore the digestion, absorption, and metabolism is dissimilar. Additionally, polyols taste sweet but they are not calorically equal to monosaccharides (IFIC Foundation, 2016).

The United States Federal Drug Administration (FDA) defines **sugars or total sugars** as "the sum of all free mono and disaccharides" which include glucose, fructose, galactose, lactose, sucrose, and maltose, naturally occurring or added to foods and beverages, regardless of the food source (Cummings & Stephen, 2007; Vos et al., 2016).

Added sugars (Bowman, 2017) are the sum of any monosaccharide and disaccharide used as ingredients in processed and prepared foods, soft and alcohol drinks, jams and jellies, candies, and ice cream as well as sugars eaten separately or added to foods at the table. The World Health Organization uses the term 'free sugars' for added sugars, however this term also includes sugars naturally present in fruit juices and juice concentrates. Added sugars are commonly added to sweeten a food product, improve palatability, or as fillers in processed food, such as in low fat items (Cummings & Stephen, 2007; Erickson & Slavin, 2015; Wittekind & Walton, 2014). Because of this, some fat free dairy or grain products have higher amounts of added sugars. Although added sugars are frequently consumed (Powell et al., 2016), the implications of excess consumption are not well understood.

Naturally occurring sugars, implied by their name, are naturally occurring in foods. They are sometimes regarded as 'healthier' than added sugars; however, this is controversial because the chemical structure of added and naturally occurring sugars is identical (World Health Organization, 2015). Nonetheless, added sugars may have more adverse metabolic effects as they are more rapidly available for absorption and metabolism due to disrupted food matrix of processed foods, whereas naturally-occurring sugars are incorporated into the food's cellular structure and therefore slower to enter

metabolism (Johnson et al., 1996).

Sugars absorption and metabolism

Carbohydrates are chemically and mechanically digested in the gastrointestinal tract until they are hydrolyzed into monosaccharides (Brody, 1994). Simple carbohydrates (disaccharides) are digested quicker and therefore can be absorbed in the duodenum, whereas complex carbohydrates (polysaccharides) take longer to fully break down and may be absorbed in the jejunum and ileum. Final digestion occurs by brush boarder enzymes, maltase, lactase, and sucrase; sucrase hydrolyses sucrose into glucose and fructose, lactase hydrolyses lactose into glucose and galactose, and maltase hydrolyzes maltose into two glucose molecules.

Absorptive epithelial cells, enterocytes, are lined with transporters to assist absorption of glucose, galactose, and fructose across the phospholipid membranes. Glucose and galactose are absorbed across the apical membrane via co-transport with sodium (GLUT1) (Brody, 1994). In the absence of sodium, the driving force for glucose absorption is lacking. Fructose is absorbed across the apical member via the GLUT5 transporter. These molecules then cross the basolateral membrane by facilitated (GLUT2) or simple diffusion via the concentration gradient into the capillaries and enter the blood stream. Glucose, fructose and galactose are transported to the liver by the hepatic portal vein, where fructose and galactose are rapidly converted into glucose (Mann & Truswell, 2007).

Absorbed glucose directly affects blood glucose levels and is controlled by uptake

in different tissues. Cells in these tissues absorb glucose via GLUT 1. Some tissues, skeletal muscle, heart muscle, and adipocytes use insulin dependent transporter GLUT 4 for the uptake of glucose. Regardless of the type of transporter, these cells immediately convert glucose molecule into glucose-6-phosphate (G6P). The brain specifically uses GLUT 1 and GLUT 3. This phosphorylation traps the glucose inside the cell. Cells perform glycolysis, the breakdown of glucose to produce energy. When the cells reach saturation of glucose, they convert the excess glucose into glycogen or fatty acids (Brody, 1994). Gluconeogenesis is performed when not enough glucose is present in the blood, providing an energy source to sustain cells and tissues (Mann & Truswell, 2007).

Fructose on the other hand does not directly impact blood glucose levels as it must reach hepatic cells to be metabolized. It is absorbed by GLUT 5 and then it is phosphorylated by fructokinase into fructose-1-phosphate (F1P). Further metabolism results in intermediates for the glycogenesis pathway and triglyceride synthesis. Sugars provide 4 kcal/g similar to all digestible carbohydrates. As glucose, fructose, and galactose are metabolized, energy is stored. Energy dense foods, such as foods high in added sugars, are characterized as having high caloric value, versus nutrient dense foods, which have high nutrient value. Energy dense diets may promote excess adiposity from the storage of excess energy, leading to weight gain (World Health Organization, 2015). There is a debate as to whether fructose and glucose have similar affects. They are calorically equivalent but have prominently different metabolic fate, as glucose directly impacts blood glucose levels and fructose does not (Vos et al., 2016).

Sugars guidelines

Well-defined nutritional guidelines regarding sugars consumption have long been lacking. In 2002, the Institute of Medicine (IOM) proposed a recommendation that less than 25% of total energy intake should be from added sugars (IOM, Food and Nutrition Board 2002). In 2009, the American Heart Association (AHA) recommended reducing added sugars consumption to 'no more than half of the discretionary calories allowance', which for most women is ≤100 kcal/d (6 teaspoons) and for most men is ≤150 kcal (9 teaspoons) per day from added sugars (Johnson et al., 2009).

Most recently, the World Health Organization (WHO) released recommendations stating that adults and children should consume less than 10% of their energy intake from free sugars. Additionally, the WHO advises cutting free sugars to less than 5% of total energy, which is equivalent to six teaspoons or 25 grams per day for an adult with a healthy BMI, to further reduce the risk of chronic diseases (World Health Organization, 2015). Decreasing added sugar intake to recommended levels will reduce energy content of the diet without compromising nutrient adequacy (Vos et al., 2016; World Health Organization, 2015). The most recent 2015-2020 Dietary Guidelines for Americans, published by the USDA, recommend limiting added sugars consumption to less than 10% of allotted calories per day (US Department of Health & Human Services and USDA, 2016).

Added sugars intake in the U.S.

Sugars intake in the U.S. remains above the recommend levels (US Department of

Health & Human Services and USDA, 2016; Powell et al., 2016). A study by Powell et al. (2016) examined added sugars intake in US children and adults between 1977 and 2012, using nationally representative data. They found that in 2003-2004, children consumed 18% and adults 15% of their total energy intake from added sugars, a rise from 14% and 12% in 1977-1978, respectively. Although in 2011-2012, added sugars intake somewhat decreased to 17% in adults and 14% in children, it still remains above the recommended 10% of the total energy intake.

Park et al. (2016) investigated socio-demographic and behavioral factors that contribute to added sugars consumption among US adults using data from the 2010 National Health Interview Survey data (n=24,967). The median sugars intake in this population was 17.6 tsp/day (296 kcal) for men and 11.7 tsp/day (197 kcal) for women. Based on findings from the National Health and Nutrition Examination Survey (NHANES) 2005-2010, on average men consumed 335 kcals from added sugars, whereas women consumed 239 kcals (Ervin & Ogden, 2013).

Park et al. (2016) also discussed factors associated with sugars intake greater than 22 tsp /day for men (equivalent to 92.4 g or 18.5% EI on a 2000 kcal diet) and 14.6 tsp /day for women (equivalent to 61.3 g or 12.2% EI on a 2000 kcal diet). They identified that being a smoker, less educated, and less physically active, having low income, and infrequently consuming alcohol were associated with increased added sugars consumption. Based on NHANES 2005-2010 data, Ervin & Ogden (2013) reported that added sugars contributed to a larger percentage of their total energy intake for Non-Hispanic black men (14.5%) compared to non-Hispanic white (12.8%) and Mexican-

American men (12.9%). Similar trends were observed in women with 15.2%, 13.2%, and 12.6% of total energy from added sugars consumed by non-Hispanic black, non-Hispanic white and Mexican-American women, respectively. The differences in percentage of calories was not significant between non-Hispanic white and Mexican-American men and women; however, these results were noted in adult populations only.

Ervin & Ogden (2013) further reported that more calories from added sugars came from foods (67%) than from beverages (33%). Additionally, these calories were found to be largely consumed at home, suggesting that food items high in added sugars are typically found in the home. Total calories from added sugars were found to have an inverse relationship with age and income. Added sugars consumption across ages 20-39, 40-59, and ≥ 60 years was 397, 338, and 224 kcals in men, and 275, 236, and 182 kcals in women, respectively. In summary, while added sugars intake is elevated in the U.S. population, the intake varies based on gender, age, ethnicity, income level, education level, and smoking status.

Sugars and disease risk

The association between sugars intake and dental caries has long been established (Freeman, R., 2014; Vos et al., 2016), while the association with other disease outcomes has been less understood (Key et al., 2002). More recently, the WHO commissioned a systematic review that included 30 randomized controlled trials (RCTs) and 38 cohort studies to investigate the association between dietary sugars and body weight (Te Morenga et al., 2012). The analysis of the RCTs showed that limiting sugar-containing

foods in individuals following an ad libitum diet led to significant reduction in body weight (-0.80 kg, 95% CI = 0.39-1.21; $p<0.001$) (Te Morenga et al., 2012). Analysis of the cohort studies revealed that those with highest sugar sweetened beverage (SSB) intake were 55% more likely to become obese (HR = 1.55, 95% CI = 1.32 – 1.82) compared to those with the lowest intake.

Diet is believed to be the second influential factor on disease development, specifically cancer (Key et al., 2002). However, although sugars consumption is a highly prevalent dietary behavior, its association with cancer remains largely unknown. Potential mechanisms for this association include increased insulin and insulin-growth growth factor-I (IGF-I) synthesis that may promote caricinogenesis (Kaaks & Lukanova, 2001), induce oxidative stress (Ceriello et al., 1999) and weight gain (Renehan et al., 2015; Te Morenga et al., 2012). In their prospective investigation of sugars in relation to 24 cancer sites, in approximately half a million participants of the NIH-AARP cohort, Tasevska et al. (2012) reported an increased risk of esophageal adenocarcinoma and small intestinal cancer with high intake of added sugars ($HR_{Q5\ vs.\ Q1}$: 1.62, 95% CI: 1.07–2.45; $P_{trend} = 0.01$) and added fructose ($HR_{Q5\ vs.\ Q1}$: 2.20, 95% CI: 1.16–4.16; $P_{trend} = 0.009$), respectively, but found no association with any of the highly prevalent types of cancer, such as breast or colorectal cancer. In an analysis of the same cohort, the authors found total sugars and fructose but not added sugars intake associated with an increased risk of total mortality (total sugars, $HR_{Q5\ vs.\ Q1}$: 1.13, 95% CI: 1.06 - 1.20; $P_{trend} =0.0001$); fructose, $HR_{Q5\ vs.\ Q1}$: 1.10, 95% CI: 1.04 - 1.17, $P_{trend} =0.0001$, respectively). Nonetheless, in a recent prospective analysis of NHANES III (1988–2006), Yang et al.

(2014) found that US adults consuming 17–21% and >21% of energy from added sugars had significantly increased risk for cardiovascular disease (CVD) mortality compared to those consuming ≤8% of energy from added sugars (HR: 1.38, 95% CI: 1.11-1.70; and 2.03, 95% CI: 1.26-3.27, respectively). This association was consistent across age, sex, race, ethnicity, education, physical activity level, health eating index (HEI) score, and BMI. They also reported that total caloric intake from added sugar rose from 15.7% to 16.8% and then decreased to 14.9% over the respective years of NHANES data collection (1988-1994; 1999-2004; 2005-2010). During 2005-2010, 71.4% of adults consumed more than the recommended calorie intake (10%) from added sugar and 10% of adults consumed more than 25% of their daily calories from added sugar. The range of added sugars intake in this NHANES analysis was rather wide, i.e., the 10^{th} and 90th percentile were at 7.5 and 25% of energy intake (Yang et al., 2014), while in the NIH-AARP study, which included mainly educated 50- to 69-y-old adults, at 4 and 18% of energy intake, respectively (Tasevska et al., 2012). Lower intake of added sugars in the later cohort may have prevented an observation of higher risks with respect to added sugars and CVD mortality risk.

The American Heart Association reviewed and graded scientific evidence on added sugars intake in relation to CVD risk and CVD related outcomes, such as elevated blood pressure, uric acid levels, dyslipidemia, insulin resistance, diabetes mellitus, nonalcoholic fatty liver disease, and obesity among children (Vos et al., 2016). They found that added sugars intake far below the current consumption levels significantly increased risk for CVD disease, which association was mediated by increased energy

intake, adiposity and dyslipidemia. Their findings supported the recommendation that children age ≥ 2 years should limit their added sugars intake to ≤ 25 g/day (100 kcal or 6 tsp), while children < 2 years should avoid added sugars. This means that at levels of common consumption, the risk for these outcomes is likely higher, increasing awareness to the high risk even at low levels of consumption.

Te Morenga et al. (2014) conducted a systematic review and meta-analysis of RCTs that examine effects of dietary free sugars on blood pressure and lipids (minimum trial duration was 2 weeks). Out of 39 trials included, 37 trials reported lipid outcomes and 12 trials reported blood pressure outcomes. They observed that higher sugars consumption was associated with an increase in triglycerides, cholesterol, low density lipoprotein (LDL) and high density lipoprotein (HDL), independent of body weight. The reported mean difference in these cardiometabolic risk outcomes between participants on high and low free sugars diet was 0.11 mmol/L (95% CI = 0.07- 0.15) for triglycerides, 0.16 mmol/L (95% CI = 0.10-0.24) for cholesterol, 0.12 mmol/L (95% CI = 0.05-0.19) for LDL, and 0.02 mmol/L (95% CI = 0.00-0.03) for HDL, all of which were statistically significant. The strongest association between sugars intake and blood pressure was found in eight week long trials with a 6.9 mm Hg increase in systolic blood pressure (95% CI = 3.4-10.3) and 5.6 mm Hg increase in diastolic blood pressure (95% CI = 2.5-8.8) for a higher sugars intake diet (Te Morenga et al., 2014). Based on evidence from systematic reviews and individual studies that investigated dietary sugars, specifically SSBs, and type 2 diabetes mellitus (T2DM) incidence, Lean & Te Morenga (2016) concluded that the evidence directly linking sugars to T2DM is still unconvincing. While

there was a consistent association between SSBs and T2DM, the association was weakened when data were adjusted for BMI. Excess caloric intake contributes to weight gain, regardless of the origin of the calorie, which promotes T2DM, suggesting that sugar reduction as the only intervention will not likely impact diabetes incidence. The evidence on sugars intake and glycemia showed that high sugar diets can still maintain good glucose control. Yet, more research is needed to fully understand the role of sugars in etiology of T2DM and the effect of isocaloric exchange between sugar and non-sugar carbohydrates. Whereas the evidence that high sugars intake increases the risk of macrovascular complications of T2DM is more established, some studies point out that SSBs may promote kidney disease among individuals with T2DM, but not necessarily from sugars but from other components of SSBs, such as caramel coloring or phosphoric acid (Lean & Te Morenga, 2016). They concluded that the true hazard lies in weight gain and obesity, as foods beyond just sugar can contribute to these rising rates.

Little research has been done to establish a threshold for the potential adverse effects of sugars on health. The National Institutes of Health (NIH) and WHO, among other organizations, are leading the frontier in investigating effects of added sugars intake on health. The consumption of processed foods increases the intake of sugars and with food sources of sugars being diverse, it is difficult to assess their intake (Jenab et al., 2009). The level of dietary misreporting, which is greater among overweight and obese (Lara et al., 2004) who are commonly at higher risk of disease, further complicates the investigation of the etiology role of sugars and invalidates the evidence on sugars and disease risk.

Dietary assessment and issues with self-reported diet

Self-reported food consumption by the individual is the primary way the nutritional science community approaches measuring people's dietary intake. Yet, random and systematic errors plague self-reporting. Daily variation of intake, inability to estimate portion size, and misreporting the amount and type of food all contribute to these errors (Bingham, 2002). The most commonly used self-reporting dietary instruments in population studies include food diary or food record, food frequency questionnaire (FFQ), and 24-hour dietary recall (24HRs). Unfortunately measuring dietary intake is challenging, while researchers continue to rely on these methods.

FFQ, a major technique for assessing dietary intake in epidemiologic studies, measures usual or long-term intake over weeks, months, or a year (Willett, 2012). It uses a food list and a selection of frequency each food item was consumed. Questions on quantity can also be included. This tool captures the usual exposure to nutrients or foods of interest, rather than intake over a few days. Benefits of FFQ include that it is self-administered, low cost, and it assesses long-term diet (Willet, 2012). Yet, it has inherent flaws, including errors due to misestimated intake averaged over extended periods of time, or unwarranted correlations between frequencies and weights of the foods. Validity of FFQs can be determined against multiple food records and recalls, or dietary biomarkers in a representative sample of the population (Willet, 2012).

A 24-hour dietary recall is a dietary assessment instrument that measures short-term intake. It asks individuals to recall foods and beverages they consumed in the twenty-four hours prior to the interview. A food record is a dietary assessment

instrument where participants are asked to record all foods and beverage consumed over a certain period of time, ranging from a few days to a week. Food records and 24-hour recalls are open ended, and assess diet consumed on specific days. Food records are prospective, whereas 24-h recalls are retrospective methods. These can be repeated multiple times to collect enough data to approximate usual intake. Day to day variability in intake contributes to random measurement error (ME) particularly when using 24-h recalls to measure intake (Willet, 2012). Foods that are infrequently consumed can be missed by 24-recalls, yet combining dietary recalls with FFQ can be beneficial in eliminating the issue (Willet, 2012).

All self-reporting instruments rely on the information given by the subject, hence the potential for ME in self-reported diet is high (Bingham et al., 2007). These methods, particularly the FFQs, rely on the participant's memory, which can contribute to ME. Also, estimates of food intake may not be objective as people rely on perception and conception of portions sizes and averaging intake over time to determine the amount consumed (Willet, 2012). Lastly, people seek social acceptance, thus estimates of intake can be biased or skewed because of social desirability issues, especially in face-to-face interviews. Underreporting dietary intake is very high when self-reporting methods are used (Subar et al., 2003), which results in underestimation of energy and nutrient intakes (Newens & Walton, 2016).

Prentice et al. (2011) used the Nutrition and Physical Activity Assessment study (NPASS), a calibration sub-study nested in the Women's Health Initiative (WHI), to evaluate the validity of FFQ, 4-day food record and 24-h recall against dietary

biomarkers for energy and protein intake. Each self-reported method considerably under estimated energy intake by 20-27% and to a lesser extent protein by 4-10%. They discovered that FFQ provided the poorest estimate of energy and protein intake, with 24-h recall next, while that 4-day food record was found to be most accurate among the three instruments. In the calibrated equations from the regression of the biomarker on each of the self-reporting instruments along with BMI, age and ethnicity, the percentage biomarker explained was 71.1%, 76.2%, and 71.8% for energy intake, and 39.7%, 63.8%, and 55.6%, for protein intake, for the FFQ, 4-day food record, and 24-h recall, respectively. This suggests that calibrated equations for these instruments may provide useful estimates of energy and protein intake in epidemiological studies.

The Observing Protein and Energy Nutrition (OPEN) study was another validation study that evaluated the extent of dietary misreporting in FFQ and 24-h recalls by use of dietary biomarkers (Subar et al., 2003). The study population (n=484) was recruited from a suburban area in Maryland. They found that men and women underreported their energy intake by 12-14% and 16-20% when using 24-h recalls, and by 31-36% and 34-38% when using FFQs, respectively. The corresponding percentages for under-reporting protein intake in men and women were 11-12% and 11-15% for 24-h recall, and 30-34% and 27-32% for FFQ, respectively. This ME needs to be accounted for in diet disease association studies, and many researchers agree that dietary biomarkers may be effective in identifying and quantifying the error. The correlation between FFQ and true intake of protein and energy for women, measured by biomarkers were 0.30 and 0.10 in women, and 0.32 and 0.20 in men, respectively (Kipnis et al., 2003). When 24-h

recall was used as a reference instrument to assess validity of the FFQ, these correlations were largely overestimated (women: 0.33 and 0.26 for protein and energy; men: 0.31 and 0.44 for protein and energy). Hence, using FFQ to assess diet in epidemiologic studies may weaken the observed effect of diet on disease risk (Kipnis et al., 2003). Furthermore, the use of 24-h recall as a reference instrument can seriously overestimate the validity of FFQ, and invalidate interpretation of findings. The lack of reliable dietary intake data has made establishing diet-disease associations very challenging.

Freedman et al. (2010) explains how biomarkers can be used in addition to self-reporting measures to strengthen diet disease associations. While one method by itself may not be sufficient, together dietary biomarkers and self-reports may be able to detect important diet-disease relationships.

Dietary biomarkers

Dietary biomarkers are defined as compounds measured in any biological sample that can reflect intake sufficiently, acting as an objective measure of true intake (Bingham, 2002). So far, four classes of dietary biomarkers have been identified: recovery, concentration, replacement, and predictive.

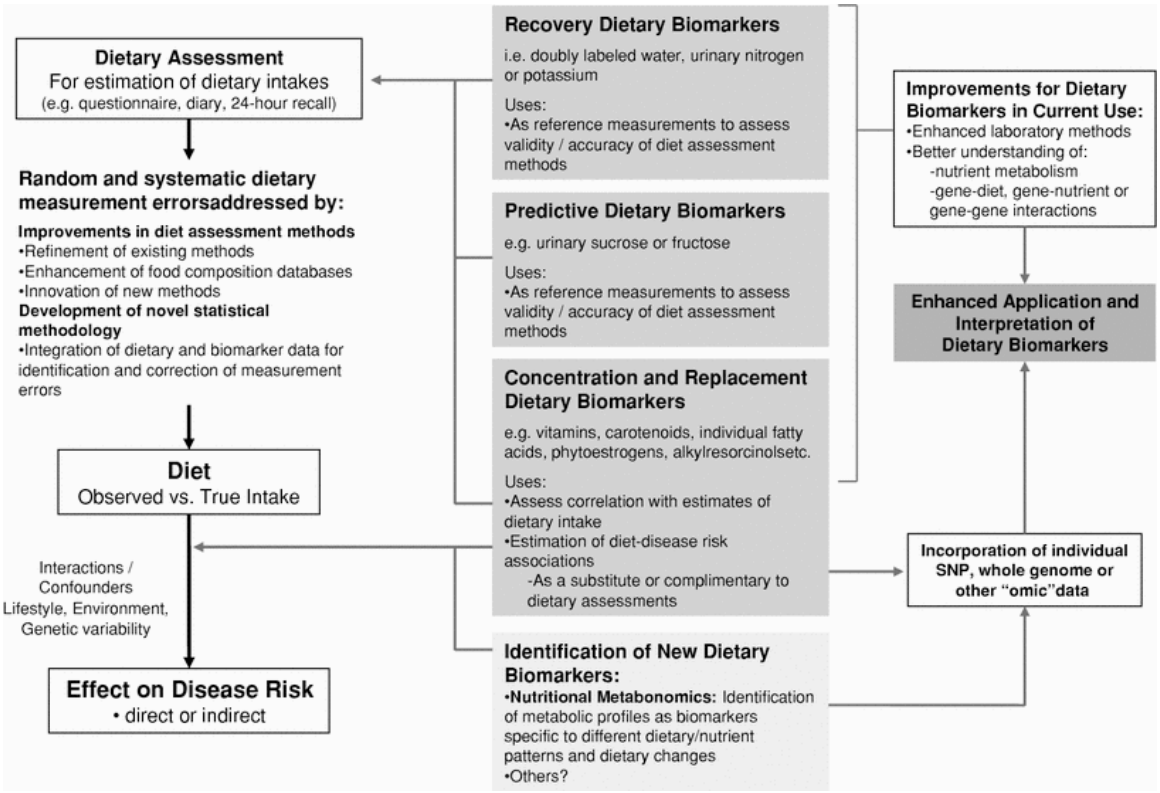


Figure 1: Classifications of dietary biomarkers (Jenab et al., 2009)

Recovery biomarkers are based on the metabolic balance between intake and excretion over a fixed period of time, and can provide an estimate of absolute intake (Kaaks et al., 1997). For example, doubly labeled water, which measures total energy expenditure (Schoeller, 1999), and urinary potassium and nitrogen which measure potassium (Tasevska et al., 2006) and protein intake (Bingham & Cummings, 1985), respectively.

Concentration biomarkers correlate with intakes levels of the food or nutrients but total levels cannot be determined (Kaaks et al., 1997). These include carotenoids, vitamin C, fatty acids, etc. in plasma or serum (Figure 1). **Replacement biomarkers** are type of biomarkers that replace estimates of nutrients for which databases are inadequate

or unavailable. **Predictive biomarkers** are type of biomarkers that can predict intake after being adjusted for certain level of error in the biomarker, with the assumption that the error is stable across populations (Tasevska et al., 2011). The predictive biomarker category is relatively new, and 24-h urinary biomarker to measure sucrose and fructose is the only member of this category so far. Only small fractions of the total sugars consumed are recovered in urine and because of this, this category is different compared to recovery biomarkers (Willet, 2012).

Use of dietary biomarkers do not come without challenges, however. Individual factors, metabolic and genetic, can affect the association between biomarker and dietary intake (Jenab et al., 2009). Additional aspects that affect biomarkers include sensitivity to intake, specificity and non-dietary determinants, as well as biological sample collection, processing, analysis and storage (Willet, 2012).

The usefulness of different biomarkers varies depending on the nutrient being measured (Willet, 2012). In order for a biomarker to be valuable, it must measure what it is supposed to measure, suggesting sensitivity to intake is a substantial factor. Homeostasis, bioavailability, and time integration all play a role in the biomarkers sensitivity to intake. Due to homeostatic mechanisms, concentrations of some nutrients/compounds can be well controlled in the body, making the use of these measurements as biomarkers of intake difficult. This in particular refers to the class of concentration biomarkers. Because of the homeostatic mechanisms, the relationship between dietary components and the biomarker is usually not linear. For many nutrients, the association may be linear across a certain range and then plateaus. If the plateau phase

is large and it is within the normal range of intake (i.e., serum retinol), then the biomarker will not be informative of intake, making the measurement impractical (Willet, 2012). If the association is linear across the normal range of intake for the nutrient, then biomarker measurements can distinguish between low, average and high intakes, making the measurement useful. Another component to consider is bioavailability, or intestinal absorption, transport, and metabolism. Bioavailability can increase variation of the measured biomarker. Both chemical structure and the food matrix can influence bioavailability. The information on time integration of the biomarkers is essential, as it determines the application of the biomarker. Biomarker measuring a nutrient stored in adipose tissue will reflect long-term dietary intake, versus biomarkers in urine or plasma, which typically reflect recent dietary changes. Short-term fluctuations in intake may be seen as large within-person variation of the biomarker. If within-person variation is large, it is difficult to predict a long-term intake based on a single measure. Repeated sampling and analysis may alleviate the problem of high within-person variability in the biomarker, and provide long-term estimate of intake.

Specificity is another component that can affect biomarkers of interest. Some biomarkers have low specificity, as some nutrient concentrations will correlate with other nutrient levels, making it difficult to measure a specific compound (Willet, 2012). This is because dietary nutrients have shared food sources. Additionally, some biomarkers are the result of the metabolism of other dietary components, making confounding variables difficult to identify (Willet, 2012). Non-dietary determinants also play a role, such as genetics, environment, and lifestyle. These factors must be considered when classifying

subjects according to their biomarker level.

Lastly, methodological aspects can affect dietary biomarkers. These include laboratory measurements, methods to process, preserve and store samples (i.e., contamination and stability) (Willet, 2012). Timing of sampling and proper storage techniques is essential to ensure the validity of the biomarker measurements. The goal is to maintain the concentration through the collection, processing, and storage steps.

Dietary biomarker for total sugars intake

The newly developed biomarker of total sugars intake, sucrose and fructose in 24-h urine (24uSF) belongs to the class of predictive biomarkers. It is based on the fact that in healthy individuals, very small measureable amounts of sucrose and fructose are excreted in urine, which were found to be highly predictive of total sugars intake (Tasevska et al., 2005). Sucrose occurs in urine probably because it escapes enzymatic hydrolysis in the duodenum (Kawabata et al., 1998; Luceri et al., 1996; Tasevska, 2015). Intact sucrose, hence, passes through the intestinal wall, and once in the blood stream, is filtered into urine by the kidneys. However, gastrointestinal damage can also increase permeability and promote leakage of intact sucrose through damaged gastrointestinal wall (Kawabata et al., 1998). Fructose occurrence in the urine is probably due to fructose escaping hepatic metabolism and being excreted in the urine (Tasevska, 2015). Nakamura & Tamura (1972) observed 9.54 mg of fructose in urine 4 hours after taking 10 g of sucrose mixture. Although some amount of glucose can be measured in urine, glucose excretion appears to be person-specific and is not reflective of dietary intake due to insulin-controlled glucose reabsorption occurring in the kidneys (Tasevska, 2015).

Luceri et al. (1996) first discovered urinary excretion of sucrose and fructose as a marker of sucrose intake. In this study, nine participants consumed their habitual diet for one week, followed by a low sucrose diet for three days, while keeping a daily diet log over the entire study 10-d study period. On the last day of their one week habitual diet period and the 3-d low sucrose diet period, they provided a fasting urine sample, and another 3 voids collected within 2 hours of breakfast, lunch and dinner (i.e., at 8:00 am, 10:00 am, 3:00 pm, and 10:00 pm). This study expressed urinary sugars concentrations per gram urinary creatinine. They observed a reduction in urinary excretion of sucrose ($p<0.05$), glucose ($p<0.001$), and fructose ($p<0.001$) when participants followed the low sucrose diets. Yet, when they averaged the urinary excretion levels measured in each of the four timed urines for the habitual and low sucrose diet, and related those with sucrose intake of the collection day, they found sucrose intake highly significantly correlated with sucrose ($r=0.7$; $p<0.01$) and fructose ($r=0.82$; $p<0.01$), but not glucose excretion ($r=0.35$; $p>0.05$). This suggested that urinary sucrose and fructose may be reflective of sucrose intake (Luceri et al., 1996)

As a tool to more accurately measure sugars intake, urinary sucrose and fructose were then investigated under highly controlled conditions in a feeding study design in participants collecting 24-h urine collections (Tasevska et al., 2005). Two studies were conducted in a volunteer suite to investigate whether sucrose and fructose in 24-h urine can be used as biomarkers of total sugars intake. The first study (n=12) investigated whether the amount of sucrose and fructose detectable in urine is dose dependent to total sugars intake. Participants received a low sugars diet (63 ± 5 g/day), medium sugars diet

(143 ± 5 g/day), or a high sugars diet (264 ± 3 g/day) for 10 days each. The percentage of total sugars from sucrose were reported at 34.4%, 37.4%, and 48.9% respectively. Out of the 10 days, participants provided 24-hour urine samples on day 4 to 7. The mean sucrose and fructose excretion increased in a dose-response manner across low, medium and high sugars intake periods (Tasevska et al., 2005). Although, the within-subject variation for urinary sugars excretion was high while subjects were on the same level of sugars, the mean urinary excretion of both sucrose and fructose were statistically significantly different between the periods of different sugars intake (urinary sucrose: Wilks' Lambda = 0.082, $F_{2,9} = 50.1$, $P < 0.001$, Partial eta squared = 0.918; and fructose: Wilks' Lambda = 0.077, $F_{2,9} = 54.3$, $P < 0.001$, Partial eta squared = 0.923) (Tasevska et al., 2005).

The second feeding study (n=13) investigated the performance of the biomarker with participants consuming their normal diet varying in sugars intake day to day, while maintaining their occupational and recreational activities. For 30 days, participants consumed their habitual diet in a controlled environment in a residential volunteer suite (Tasevska et al., 2005). Consumed food was carefully measured and the UK food composition tables were used to determine dietary intake (McCance & Widdowson, 2002). Participants collected 24-h urine daily over the 30-d study period, which were analyzed for sucrose and fructose. Participants total sugars intake ranged from 95 to 323 g/day, averaging around 202 g/day or 29.2% of total energy intake. The sum of the 30-d mean of urinary sucrose and fructose was highly correlated with 30-d mean total sugars ($r = 0.84$; $P < 0.001$) and sucrose intake ($r = 0.77$; $P = 0.002$).

From these two feeding studies, 24uSF was identified as a biomarker for total

sugars intake in free-living individuals. Because this study found the recovery of sugars to be low, i.e., 0.02-0.05% of total sugars intake, the 24uSF biomarker was not classified as a recovery biomarker, but a new category of predictive biomarkers was proposed. 24uSF is not considered a concentration biomarker because it is related to intake in a dose-dependent and time sensitive manner (Tasevska et al., 2005).

Following these findings, Tasevska et al. (2011) used the OPEN study (n=484) to investigate the validity of the National Cancer Institute Diet History Questionnaire (DHQ) and 24-h recall to assess total sugars intake. From 1999 to 2000, 261 men and 223 women completed an FFQ (DHQ), and 24-h recall two times within 14 days, and provided two 24-h urine collections. The sum of fructose and sucrose amount measured in 24-h urines was used as a predictive biomarker for total sugars intake, which was then used to estimate the ME of the FFQ and 24-h recall. The correlation between true and self-reported total sugars density was 0.5 for the FFQ and 0.6 for the average of two 24-h recall in men, and 0.2 and 0.3 in women, respectively, supporting the need for using this biomarkers in diet-disease association studies (Tasevska et al., 2011).

Collection of 24-h urine samples for biomarker measurement

Urinary excretion of various compounds can provide valuable information on recent dietary intake. Typically, these compounds are measured in 24-h urine. 24-h urine samples are all urine voids collected over a 24-hour period, and although they can be cumbersome, they are feasible in motivated participants. Although 24-h biomarker measurements are useful in establishing total dietary intake of a nutrient, there is a high likelihood for incomplete 24-h collections. To measure the completeness of a 24-h

urine sample or compliance by a subject, para-aminobenzoic acid (PABA) can be used (Bingham & Cummings, 1983). PABA is absorbed and completely excreted with little variation between individuals within 6 hours of oral administration. When three doses of PABA are taken spread out on the urine collection day (240 mg total), recovery of ≥85% of PABA in urine indicates a complete urine collection (Bingham & Cummings, 1983). This method is often used in 24-h urine studies to identify complete versus incomplete urine collections.

Collection of spot urine samples for biomarker measurement

Due to large participant burden, complex logistics and high cost, collection of 24-h samples for measurement of biomarkers is not always feasible in large population studies, whereas collection of spot urines is more easily implemented. Therefore, investigation of the performance and utility of biomarkers measured in spot urine would have major implications in population based research. Spot urine is a single urine sample collected by a participant, which can be untimed or timed (i.e., collected at a specific time during the day, e.g., first morning void). If certain spot urine reflects 24-hour urine composition, then those spot urines can be used for biomarker measurement instead of using the 24-hour collection sample. The collection of spot urines is much more feasible to obtain, and is less prone to collection errors, as seen in 24-hour urine collections (Wang et al., 2013; Willet, 2012).

While spot urine measurement in theory is ideal, an important challenge to address for this measurement is diurnal variation in excretion of the biomarker.

Diurnal effects can influence biomarker measurements and its use (Willet, 2012). A study by Wang et al. (2013) explored the diurnal variation of sodium, potassium, chloride, and iodine. The study included 481 free living participants 18-39 years of age collecting one 24-h urine where each void was collected in a separate container. They investigated the use of spot urines for measurement of sodium, chloride, potassium, creatinine, and iodine intake against the biomarker measured in the same day 24-h urine collection. Authors report diurnal patterns for sodium, potassium, and chloride. They found that overnight urine samples had the lowest concentrations of these nutrients compared to urine samples collected during the day. Furthermore, they report consistent excretion of iodine and creatinine across different voids. While diurnal variation has been identified in the excretion of these nutrients, it has yet to be investigated for the sugars biomarker.

As the biomarker concentration in a spot collection depends on spot urine volume, in order to appropriately study diurnal variation of the biomarker, it is important that its concentration is adjusted for the water content of the urine void. To adjust for volume differences in spot urine collections, creatinine is most commonly used. Creatinine is excreted in the urine at a relatively constant rate throughout the day, hence it can be used to normalize biomarker concentration in a void, i.e., express the concentration per gram creatinine (Barr et al., 2005). Creatinine is excreted in the urine as a product of muscle metabolism, and it is highly dependent on muscle mass, but has been also shown to be associated with BMI, age, gender and ethnicity (Barr et al., 2005). Furthermore, meat consumption (Cross et al., 2011) can cause between-person variation in creatinine excretion, therefore the use of creatinine to adjust for urine dilution has some limitations.

In the investigation of the performance of the biomarker measured in spot urine, the concern is whether the spot urine measurement will sufficiently reflect true dietary intake compared to the biomarker measure in 24-h urine collections. In order for a biomarker measure in spot urine to be considered a good tool for measurement of dietary intake, the spot urine measurement should have a high correlation to 24-h urine measurement of the biomarker, for established 24-h urine biomarkers. This will be investigated for the sugars biomarker. The following studies found spot urine estimates to be indicative of 24-hour urine estimates of the biomarker, and therefore indicate that spot urine is an appropriate tool.

A study by Perrine et al. (2014) measured urinary iodine excretion (UIE) and urine iodine concentration (UIC) to estimate population iodine intake levels. They then compared UIE and UIC in 24-h and spot urine samples to determine if the spot urine sample is a sufficient tool to assess iodine intake. Participants (n=400) collected morning, afternoon, evening, and overnight voids and these samples were analyzed for iodine. They measured urinary iodine excretion (UIE) and urine iodine concentration (UIC) in the urine samples. Median 24-h UIE was reported as 173.6 μg/day and median 24-h UIC was reported as 144.8 μg/L. From the timed spot collections, 24-h UIC was estimated and ranged from 147.3 to 156.2 μg/L (Perrine et al., 2014) and 24-h UIE estimates ranged from 145.7 to 163.3 μg/day. They were able to estimate 24-h UIE by multiplying I/Cr ratio by predicted 24-h creatinine excretion (Perrine et al., 2014). Interestingly, measured UIC and UIE do not fall within the estimated ranges, however they are very close in numbers. They concluded that the UIC in spot samples reasonably estimated UIC from

24-h samples; however, both were observed to be lower than UIE from 24-h urine samples. On average, iodine concentrations in spot urine samples did not differ compared to iodine concentrations from 24-h urine samples (Perrine et al., 2014).

Doenyas-Barak et al. (2015) evaluated the use of multiple timed spot urine collections for measurement of daily sodium and potassium excretion. They found that sodium and potassium measured in spot collections can adequately estimate 24-h urine excretion. Participants (n=50) collected 24-h urine and were instructed to provide a 10 ml urine sample at 12:00 pm (spot 1), 4:00 pm (spot 2), 8:00 pm (spot 3), and from the final void collection the following morning (spot 4). Sodium, potassium, chloride, and creatinine excretion levels were measured in 24-h urine. The mean electrolyte level in each spot sample was expressed per gram creatinine. Expected creatinine excretion per day was calculated by the following equations: men = [28 - (age in years/6)] x weight (kg) and women = [22 - (age in years/9)]. Estimated sodium and potassium excretions were calculated for each spot and then correlated with the 24-h urine sodium and potassium excretion. They found a statistically significant linear correlation between 24-hour urine excretion and the excretion estimated by any of the scheduled spot urine collections (sodium r = 0.51 to 0.68; potassium r = 0.53 to 0.75; chloride r = 0.34 to 0.51; for all $p<0.05$). Yet, the average of the four spot urines was best correlated with the 24-hour urine excretion. They reported that two to four spots collected throughout the day may strengthen the correlation to the 24-h urine excretion (Doenyas-Barak et al., 2015). Interestingly, sodium concentrations in morning spot samples had the highest correlation to the 24-h samples; whereas, potassium and chloride concentrations in afternoon spot

samples were better correlated. They suggested collecting four spot samples as a convenient method for estimation of 24-hour excretion of sodium and potassium.

These findings demonstrate the practical implementation of spot urine samples for biomarker analysis. Because 24-hour urine collection is associated with high participant burden, finding an alternative way to measure excretion of sugars as a measure of intake will have great implications for the scientific community. No study thus far has investigated the uSF biomarker in spot urine. Determining the association between sugars biomarker in spot urine and sugars biomarker in 24-hour urine is the first step in validating the sugars biomarker in spot urine as a biomarker of intake. Using the spot urine measure in studying sugars-disease associations may have a great impact on elucidating the obscured association of sugars intake with risk of cancer, cardiovascular disease, type 2 diabetes, and other chronic disease. Reliably determining these associations is very important to help make stronger guidelines and reinforce recommendations. When sugar-disease associations are better understood, policies can be created to make healthier communities, disparities in consumption can be tackled, and education can be provided to parents to create a healthier environment for their children.

CHAPTER 3

METHODS

Participants

The study population included 15 participants age 22 to 49 years from an ongoing feeding study aimed to develop biomarkers of sugars intake (March 2016-July 2019) conducted in the Phoenix Metropolitan Area. Participants of the current study were recruited between March and August 2016. *Inclusion criteria* for study participation included being a non-smoker, age 18 and 70 years old, having a body mass index (BMI) of less than 35 kg/m^2, having no known allergy to sunscreen or para-amino benzoic acid (PABA), being willing to refrain from taking dietary supplements over the 10-week study period, and residing in the Phoenix Metropolitan Area. Participants living or working within close proximity to the study center were given priority for participation, as there was a need for frequent visits to the study center. *Exclusion criteria* included presence of diseases that can affect nutrient absorption or metabolism, such as autoimmune diseases (e.g., type 1 diabetes, inflammatory bowel disease, celiac disease), endocrine (e.g., type 2 diabetes, hyper or hypothyroidism), stomach disorders (e.g., ulcers, gastrointestinal bleeding), diseases that affect urine excretion (e.g., kidney disease, urinary incontinence), or requiring any dietary restrictions (e.g., celiac disease); participation in any diet related research study or trying to lose weight over the last four months; known allergy to sunscreen; and for women, being pregnant, breastfeeding, or planning to become pregnant in the next 15 weeks. Furthermore, people were considered ineligible if their fasting blood glucose or HbA1c were greater or equal to 100 mg/dl and 5.7%

respectively, based on the screening blood draw. The study was approved by Arizona State University Institutional Review Board (IRB) (Appendix A).

Study recruitment

Participants were recruited through various approaches. Recruitment tactics included posting flyers in ASU buildings (see Appendix B), advertising the study via the website www.sugarsbio.org and Facebook (https://www.facebook.com/asusugarsbio/?fref=ts), and through word of mouth.

Prospective participants completed a preliminary screening questionnaire (see Appendix C) online or over the phone to determine eligibility based on the outlined inclusion and exclusion criteria. If potential participants passed the initial screening test, they were contacted by the project coordinator who then scheduled an in-person screening visit at the Arizona State University School of Nutrition and Health Promotion (ASU SNHP) study center in downtown Phoenix. During the screening in-person visit, the project coordinator described the study to the prospective participant in more detail using the information sheet (Appendix D). If the participant was still interested in participation, he/she then signed the consent form (see appendix E). During this visit, a fasting blood sample was collected for measurement of fasting blood glucose levels and HbA1c, and participant's weight and height were taken for calculation of BMI. Once the fasting blood glucose and HbA1c were available, potential participants who met all the eligibility requirements, were scheduled for a baseline visit described below.

A total of 34 potential participants were screened for the study between March

and August 2016. Eleven were deemed ineligible due to unacceptable fasting blood glucose or HbA1c levels (n=8), participation in other diet related studies over the last 4 months (n=1), supplement use (n=1), and thyroid disorder (n=1). Out of 23 eligible individuals, one did not show up for the baseline visit, one participant was dropped from the study during the first food diary week because of non-compliance, and one withdrew because he was no longer interested in participating. Therefore, 15 participants completed the entire protocol between March and August 2016, while the remaining five eligible participants were scheduled to complete the feeding protocol at a later date.

The recruitment of participants in the original ongoing feeding study was stratified by age (18-30, 31-43, 44-56, and 57-70 years), gender (male and female), and BMI (<25, 25-29.9, and 30-34.9 kg/m^2) to ensure inclusion of participants with a wide range of sugars intake. For a final sample of 107 participants in the ongoing feeding study, four participants need to be recruited in each age/gender/BMI stratum, and eleven randomly recruited in any of these strata. The stratification of the 15 participants of the current study has been reported in Table 1.

Table 1. Stratification of the study population (n=15)

Age groups	BMI (kg/m^2)					
	< 25		25-29.9		30-34.9	
	Men	Women	Men	Women	Men	Women
18-30 yrs	3	1	1			1
31-43 yrs	2	3				1
44-56 yrs		2		1		
57-70 yrs						

Study design

This was a highly controlled 15-day feeding study in which participants consumed their normal diet and collected eight, nonconsecutive, 24-hour urine samples (i.e., every other day) (see Appendix F for Study figure). On two out of the eight 24-h urine collection days, each urine void was collected in a separate container, resulting in a multiple spot urine collection. Additionally, three fasting blood samples were collected from each participant before the start, upon completion, and 5 weeks following completion of the feeding period. During the 15-day study, participants only consumed food and beverages provided to them by the study center, except black tea, black coffee, and alcohol.

Baseline visit

The baseline visit took approximately one and a half hours. During this visit, a baseline questionnaire was administered gathering information on demographics, lifestyle habits, and personal medical history (see appendix G). Upon completion of the questionnaire, a detailed overview of the study was given by one of the research team members, including detailed instructions for completion of the urine collection and feeding protocols. All study forms (urine collection logs, meal checklist and physical activity log) were reviewed and explained in detail. Any questions or concerns regarding the study protocol or the policy for consuming no outside foods or beverages were addressed at this visit.

To assess and replicate participants' habitual diet, participants completed two consecutive 7-day food records prior to the commencement of the feeding protocol. In

two 7-day increments, participants wrote down all foods and beverages they consumed over 7 days, providing details on quantities, brand information, cooking methods, and recipes. During the baseline visit, the research chef trained participants on how to keep a food diary over the following two weeks (see appendix H), and provided them with measuring tools and a Food Model Booklet (FMB) (Beltsville Human Nutrition Research Center, July 2007) to help them determine and record quantities. The USDA FMB contains life size, two-dimensional drawings of foods and utensils, to help participants more accurately estimate the amount of food consumed. Participants were instructed to maintain their usual dietary behavior during this two-week recording period.

Upon completion of each 7-day food diary, a meeting with the research chef was scheduled to review the food diary and obtain more detail on the foods, participants' dietary habits, and cooking practices. These two 7-day food diaries were then used to replicate participants' usual diet, and mimic their habitual eating patterns during the 15-day feeding period. One week after the second food diary was completed, which was used to purchase all the food and start preparing the daily meals, the participants began the 15-day feeding period.

Feeding protocol

The 15-day feeding period was the highly controlled portion of the study wherein participants received all their foods from the metabolic kitchen in the study center. No consumption of outside foods or beverages was allowed over the 15-d feeding period, besides black coffee, tea, and alcohol (wine, beer or hard liquor only). During the 15

days, participants came to the study center Monday to Friday to consume their breakfast, and to collect the rest of the meals for the day. To lessen participants' burden, all the meals for the weekend were collected on Fridays and consumed at home. Upon arrival at the study center Monday to Friday, participants weight was measured and recorded before consumption of breakfast, and monitored throughout the study to ensure that participants were weight stable and in energy balance.

All meals, snacks, and beverages, besides water, for that day were prepared, carefully weighed, labeled with participant ID, and provided to the participant in a cooler trolley for ease of transport. Instructions on how to reheat the meals along with food safety information were also given to participants. While participants were free to eat as much as they wanted from the provided food, they were asked to keep all leftovers and unconsumed foods in their respective container and return them to the study center the following day. All the returned food was then carefully weighed to the nearest gram, which allowed the metabolic kitchen to determine the exact amount of food consumed.

For each of the 15 days, energy and nutrient intake were calculated using the Nutrition Data System for Research (NDS-R), Version 2016, Nutrition Coordinating Center [NCC], Minneapolis, MN. The NDS-R food composition information database draws from the USDA Nutrient Data Laboratory composed of over 18,000 foods, almost half of which are brand name items (University of Minnesota, Nutrition Coordinating Center (NCC). However, no dietary intake data were used for the purposes of the thesis.

Meal checklist

To monitor compliance to the feeding protocol, participants were asked to complete a meal checklist daily, throughout the 15-day feeding period (see Appendix I). Participants checked a box for each meal and snack consumed, recorded the time of each meal/snack, noted any deviation from the feeding protocol (amount of any unconsumed food that was not returned, or food that was consumed that was not provided by the metabolic kitchen), and recorded type and amount of alcohol, tea and coffee consumed.

Blood collection

Three fasting blood samples were taken during the 10-week study period; on the first day of the feeding period, the morning following the completion of the feeding period, and five weeks after the last day of the feeding period. The blood samples will be used to measure another biomarker of sugars intake, which is outside the scope of this thesis.

Physical activity assessment

During the 15-day feeding period, participants recorded their physical activity daily using a validated physical activity (PA) log (see Appendix J). The log contains 48 items of occupational, transportation, home, conditioning, sports, and leisure activities. Participants were instructed to record the number of minutes and hours doing each activity and the time the activity began in this PA log.

Urine collection protocol

During the 15-day feeding period, participants collected 24-h urine every other day starting with day 1 (i.e., day 1, 3, 5, 7, 9, 11, 13, 15 of the study). Participants were instructed to discard the first morning urine void, and from there on to collect all subsequent voids, and end the collection with the first morning void of the following day.

The day preceding the collection of urine, participants were given a urine collection kit comprised of *Directions for Collecting 24-hour Urine Sample* (see appendix K), a 24-h urine collection log (see appendix L), and a trolley cooler, which included two three-liter urine collection containers containing approximately 4 g of boric acid each as a urine preservative (for a final concentration of boric acid of 2 g/L assuming collection of 2 L urine per container); collection hat (for females) or urinal (for males); three POTABA tablets and a funnel. Because the sugars in the urine are sensitive to higher than room temperature, participants were asked to keep the collection container(s) in the provided cooler with ice packs, in order to keep the urine under 22°C. Temperature above 22°C may promote microbial growth and compromise sucrose and fructose integrity in the urine samples (Tasevska N, personal communications). A temperature logger was kept in the cooler to track any changes in temperature.

To ease participants' burden, a courier service was organized to collect urine samples from participants' homes. After participants collected their last urine void on the following morning, they were asked to place their cooler trolley with urine samples outside their place of residence to be picked-up and delivered directly to the study center. During weekends, the specimens were kept at the courier's facilities under refrigeration, and delivered to the study center on the next business day.

During each 24-h urine collection day, participants kept a *24-hour Urine Collection log* (see Appendix L), logging the time of the first morning void they discarded, the time of the second morning void (i.e., the first void of the 24-h urine collection), and the first morning void of the following day (i.e., the last void of the 24-h urine collection); time when the POTABA capsules were taken; and information on any missed urine (approximate amount ant time), and medications taken during the collection period (name, brand and dose).

Multiple spot urine collection protocol

On two of the eight 24-hour urine collection days, participants were asked to collect each urine void in a separate container. These urine voids collected over a 24-h period are called multiple spot urine collections. Similar to a 24-hour urine collection day, on the multiple spot urine collection day, participants discarded the first morning void, collected all urine voids thereafter, and ended the collection with the first morning void of the following day; however, each void was collected into a separate container. For this purpose, participants were given twelve small containers, six one-liter (containing 1 g boric acid) and six 500 ml (containing 600 mg boric acid) in a trolley cooler with ice packs. They also received *Directions for Collecting Multiple Spot Urine Sample* (see Appendix M), *Multiple Spot Urine Collection log* (see Appendix N), collection hat or urinal, a funnel, and three POTABA tablets (see below). On these two days, participants were instructed to record the time of each individual void on the urine collection container, as well as on the *Multiple Spot Urine Collection log.* In addition,

they logged the time when the POTABA capsules were taken; time of the discarded first morning void; and information on any missed urine (approximate amount and time), and medications taken during the collection period (name, brand and dose). Containers were kept in a cooler with frozen ice packs at all time, in order to ensure storage temperature of under 22°C. Upon collection of the last spot urine void on the following morning, participants were asked to place their cooler trolley with samples outside their place of residence for the courier to pick up.

Marker for 24-hour urine completeness

To assess completeness of the 24-hour urine collections, participants were asked to take three 102 mg tablets of potassium para-amino benzoate (POTABA) on each urine collection day. These capsules contain a daily dose of 240 mg of PABA (80 mg PABA per capsule), which is a known marker for 24-hour completeness (Bingham & Cummings, 1983) commonly used in dietary biomarker studies (Subar et al, 2013). Given pharmaceutical-grade PABA is not available in the U.S., research has been performed to determine if POTABA, the potassium salt of PABA, can be used (Sharma et al., 2014). This study (n=20) analyzed 24-h urine collections and determined the recovery of PABA after POTABA administration was 95%, indicating that POTABA can be used as an alternative (Sharma et al., 2014). Participants were instructed to take the tablets spread out during the day, within scheduled time intervals (with breakfast, lunch and dinner). Reminders were sent to the participants via email or text, to ensure that they did not forget or delay taking of the tablets. Minimal side effects, such as skin allergy and

upset stomach, have been reported from taking PABA. To prevent the occurrence of adverse effects, participants with known allergies to PABA or sunscreen (as PABA used to be a common ingredient of sunscreen) were not be eligible to participate. Because some medications, such as acetaminophen (Tylenol), sulphonamides, furosemide (Lasix), have similar structure to PABA and may interfere with measurement of PABA, participants were instructed to refrain from taking these medications, if possible. If any medications had to be taken by the participant, they were asked to record the type of medication, brand and the amount in the *24-h Urine Collection* or *Multiple Spot Urine Collection log*.

For urine to be considered a complete collection, it is expected that 85-110% of the orally administered PABA will be recovered in the 24-hour urine collection. PABA was measured on Gentek Synergy H1 spectrophotometer using colorimetric technique as previously described (Bingham & Cummings, 1983). Urine collections with PABA recovery <85% were excluded from the analysis as incomplete. PABA recovery >110% often indicates that the participant took medications that have similar structure to PABA, which cannot be distinguished by the colorimetric method. In such cases, urine collections were reanalyzed using a High Performance Liquid Chromatography (HPLC) method (Jakobsen et al., 1997), which is more specific and can separate PABA from similar compounds. Urine with PABA recoveries lower than 78% as measured by the HPLC were considered incomplete (Jakobsen et al., 2003). Percent PABA recovery was calculated as concentration of PABA in urine mg/L x 24-h urine volume (L)/2.4.

Urine Processing Protocol

The day following the urine collection day, the urine was delivered to the study center by the courier and was stored for maximum of 4 hours at 4°C until processing. For processing, 24-hour urine samples were weighed, thoroughly mixed at least twenty times with rotation and inversion, immediately aliquoted into multiple vials, and stored at -20°C and -80°C for further analyses.

The multiple spot urine collections were similarly processed; each separate void was weighed, thorough mixed, aliquoted into multiple vials, and stored at -20°C and -80°C for further analyses. In addition, a composite of a 24-hour urine was created from the multiple spot voids, by mixing 10% of each void volume in a single container. The container was then thoroughly mixed, aliquoted, and vials stored at -20°C and -80°C.

Laboratory analysis of sucrose and fructose in urine

Urinary sucrose and fructose (uSF) were measured in multiple spot urine voids and the corresponding 24-h composite urine (Spot Day 1 and Spot Day 2). USF concentrations (mg/L) were measured on a Beckman DU 730 Life Sciences UV/Vis spectrophotometer, using a colorimetric enzymatic assay (Sucrose/D-Glucose/D-Fructose; Biochemica Mannheim, R-Biopharm, Roche; http://www.r-biopharm.com/ catalogue no. 10716260035) (Appendix O).

The colorimetric enzymatic assay for analysis begins with sucrose being hydrolyzed by fructosidase to D-glucose and D-fructose. D-glucose is then phosphorylated by ATP to D-glucose-6-phosphate (G-6-P), and D-fructose is phosphorylated by ATP to D-fructose-6- phosphate (F-6-P), both reactions catalyzed

by hexokinase. Phosphoglucose isomerase (PGI), which converts F-6-P to G-6-P, is then added. In the presence of nicotinamide adenine dinucleotide phosphate (NADP), G-6-P is oxidized, while NADP is reduced to NADPH. The Beckman DU 730 Life Sciences UV/Vis spectrophotometer set at 340 nm measures the amount of NADPH by light absorbance proportional to the amount of D-glucose. This value is then the basis to determine the concentrations of sucrose, fructose and glucose. D-glucose is determined before and after the enzymatic hydrolysis of sucrose. Finally, the sucrose concentrations can be calculated from the difference of the D-glucose concentration before and after β-fructosidase is added, whereas fructose from the difference of the D-glucose concentration before and after PGI is added.

Samples to be analyzed were retrieved from the freezer, and thawed out within one hour of analysis. Next, standards of 5 mg/L, 50 mg/L, and 100 mg/L of sucrose, fructose, and glucose were prepared with Milli-Q (MQ) water using fructose (Sigma D-Fructose), sucrose (R-Biopharm Sucrose assay control material), and glucose (Sigma D-Glucose, minimum 99.5%). All standards were labeled, kept in the refrigerator and remade on a monthly basis.

Ten ml of MQ water was added to Bottle 1 from the kit to create Solution 1 (fructosidase), mixed well, and left out to reach room temperature. Forty-five ml of MQ water was added to Bottle 2 to create Solution 2 (NADP). Bottle 3, containing 1.1 ml suspension of hexokinase (320 U) and glucose-6-phosphate dehydrogenase (160 U), and bottle 4, 0.6 ml phosphoglucose isomerase suspension (420 U), were kept at 4°C.

Samples were ran in duplicates and each participant's samples were analyzed in

the same run. Up to 14 samples can be ran in one run, however samples were organized to keep the runs at an ideal range of 7-10 samples per run. Depending on how many multiple spot voids each participant had, on average it took two runs per participant, keeping samples from the same multiple spot collection day in the same run.

In preparation to run the urine samples, cuvettes were prepared for each sample. Cuvettes in duplicates were labeled, one set for sucrose determination and another set for glucose/fructose determination. This totals to four cuvettes for each sample (two sucrose and two glucose/fructose cuvettes), the blank, 5 mg/L, 50 mg/L, and 100 mg/L standards, and a quality control (QC). A QC was created from a pooled urine sample preserved with boric acid from volunteers who had taken 3 x 102 mg POTABA tables, stored as single-use aliquots at -80°C, and included in every run.

The solutions were added in order as shown in Table 2.

Table 2: Protocol for colorimetric determination of sucrose, glucose and fructose

	Blank/Sucrose sample (ml)	**Blank/Glucose/Fructose sample (ml)**
Solution 1*	0.2	
Sample	0.5	0.5
Mix, incubate for 15 min. ***Bring Solution 1 and 2 to room temperature◦C before use.** Then add		
Solution 2*	1.0	1.0
MQ Water	1.3	1.5
Mix, and after 3 min read absorbance (A) 1		
Bottle 3	0.02	0.02
Mix, and after 15 min read A2		

Bottle 4		0.02
Mix, and after 15 min read A3		

*Solution 1 (fructosidase), solution 2 (NADP), solution 3 (suspension of hexokinase, 320 U, and glucose-6-phosphate dehydrogenase, 160 U), and solution 4 (phosphoglucose isomerase suspension 420 U).

To begin the analysis, 0.2 ml of fructosidase was added to the cuvettes prepared for sucrose determination. Next, 0.5 ml of each standard and each urine samples, were pipetted into the respective cuvettes. Plastic mixing sticks were placed in all sucrose cuvettes and the content was thoroughly mixed. The timer was set for 15 minutes. Then, using the repeater pipette, 1.0 ml of Solution 2 (NADP) was added to all the cuvettes. This was repeated adding 1.3 ml of MQ water to the sucrose cuvettes and 1.5 ml to the fructose cuvettes. The content was mixed again using the mixing sticks. The timer was set for 3 minutes, after which the first absorbance (A1) was read on 340 nm. Next, 0.02 ml of Bottle 3 (hexokinase) was added to each cuvette, using the pipette tip to mix the content. Fifteen minutes following the addition of hexokinase, second absorbance (A2) was read. Lastly, 0.02 ml of Bottle 4 (PGI) was added to only the glucose/fructose determination cuvettes, mixed, and left for 15 minutes. Final absorbance (A3) was then read and recorded. All absorbance data were entered into an excel sheet and using the equations shown below, concentrations of sucrose, fructose, and glucose in the urine were calculated.

$\Delta A_{sucrose} = [(A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}]$

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A$$

C = concentration (mg/L)

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of the substance to be measured (g/mol)

d = light path of the instrument (cm), which equals the distance from bottom of the well to the upper surface of the final volume.

ε = extinction coefficient of NADPH, which for 340 nm = 6.3 [l x $mmol^{-1}$ x cm^{-1}]

$$c_{suc} = \frac{3.02 \times 342.3}{6.3 \times 1 \times 0.5} \times (\Delta A_{suc} - \Delta A_{glu})$$

$\Delta A_{glucose} = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$

$\Delta A_{fructose} = (A_3 - A_2)_{sample} - (A_3 - A_2)_{blank}$

$$c_{glucose} = \frac{3.02 \times 180.16}{6.3 \times 1 \times 0.5} \times \Delta A_{glucose}$$

$$c_{fructose} = \frac{3.04 \times 180.16}{6.3 \times 1 \times 0.5} \times \Delta A_{fructose}$$

The limit of detection for this method is 0.4 mg for D-glucose and D-fructose, and 2 mg of sucrose.

The mean ± SD of sucrose and fructose concentration of the QC was 15.9 ± 0.75 mg/L and 10.48 ± 0.25 mg/L, respectively, based on nine QC samples analyzed in a single run. The acceptable range for the QC was set at +/- 10% of the mean, and it was 14.27-17.47 mg/L for sucrose, and 9.43-11.53 mg/L for fructose. One QC in duplicate was included in each run. If any of QCs produced sucrose or fructose concentrations

outside of the acceptable range, the run was repeated. Additionally, samples were reanalyzed if the % Coefficient of Variation (CV) for the duplicates (SD/mean x 100) fell within the following criteria: sucrose concentration (mg/L) of 5-10 mg/L with a %CV>20%, 10-20mg/L with a %CV>15%, 20-30 mg/L with a %CV>10%, and greater than 30 mg/L with a %CV>5%; fructose concentration (mg/L) of less than 10 mg/L with a %CV>20%, 10-15 mg/L with a %CV>10%, and greater than 15 mg/L with a %CV>5%.

The mean inter-assay %CV for the QC was 10.9% for sucrose and 4.0% for fructose. The mean intra-assay %CV for the QC was 5.3% for sucrose and 2.6% for fructose.

Creatinine determination

Creatinine was measured in each urine void (g/L) on a Cobas C111 analyzer, using a kinetic colorimetric assay using the Jaffe method, CREJ2 Roche (Roche Diagnostics; Catalog Number: 06407137190). Determination of creatinine is based on the rate of dye formation, as the yellow-orange complex is proportional to the amount of creatinine in the urine sample. To minimize the quantification of similar compounds, including proteins and ketones, creatinine concentrations are adjusted by 0.2 mg/dL. Using this method, the lower detection limit is 0.31 mg/dL. Each urine sample was loaded into the systems cuvettes and ran in duplicates. The mean %CV for the duplicates was 1.71%. Any samples with a %CV greater than 5% were reran.

Statistical analysis

The amount of sucrose and fructose excretion in 24-h urine (mg/day) was calculated by multiplying the measured concentration in mg/L with the 24-h urine volume (L). Similarly, sucrose and fructose excretion per void was calculated by multiplying measured concentration in mg/L with the volume of each spot urine (L). Urinary sucrose and fructose per void was also expressed per gram creatinine, and was calculated as sucrose and fructose concentrations (mg/L) divided by the respective creatinine value (g/L). Sucrose and fructose amounts in urine were summed and denoted as uSF thereafter. 24-h uSF concentrations were initially measured in the corresponding 24-h composite urine; however, we used 24-h uSF (mg/day) calculated by summing uSF excretion in the multiple spot voids (mg/void) for each spot day (i.e., spot day 1 and spot day 2).

Due to the variability in the number of multiple spot voids per day each participant had, four timed spots were identified in order to standardize their number between subjects and run statistical analyses. The definition previously used in NHANES (Wang et al., 2013) to identify four timed spots out of all collected voids was modified to include meal time. The first void collected after breakfast or between 8:30 am-12:30 pm was identified as the morning void; afternoon void was the first void collected after lunch or between 12:31 pm-5:30 pm; evening void was the first void after dinner or between 5:31 pm-12:00 am; next day void was the first morning void and between 4:00 am-12:00 pm.

The distributions of all continuous variables were checked by the Shapiro-Wilk

test. If variables had skewed distributions, each variable was either log 10 or square root transformed to achieve normal distribution (Table 3).

Table 3: Normality distribution check of variables

Variable	**Spot day 1**	**Spot day 2**	**Two day mean**
24-h excretion of sucrose and fructose (mg/day)	ND*	ND*	ND*
24-h urine volume (ml)	ND*	ND*	ND*
24-h PABA % recovery (HPLC)	ND*	ND*	ND*
24-h PABA % recovery (Spec)	ND*	Log10	ND*
Number of voids per subject (n)	ND*	ND*	ND*
Morning void sucrose and fructose excretion (mg/void)	Sqrt	Log10	Sqrt
Afternoon void sucrose and fructose excretion (mg/void)	Log10	Log10	Log10
Evening void sucrose and fructose excretion (mg/void)	Log10	Log10	Log10
Next day sucrose and fructose excretion (mg/void)	Log10	Log10	Log10
Morning void sucrose and fructose excretion (mg/g creatinine)	Log10	Log10	ND*
Afternoon void sucrose and fructose excretion (mg/void)	Log10	Log10	Log10
Evening void sucrose and fructose excretion (mg/void)	ND*	Log10	Log10
Next day void sucrose and fructose excretion (mg/void)	Log10	Log10	Log10
Morning void volume (ml)	ND*	ND*	ND*
Afternoon void volume (ml)	Log10	ND*	Log10
Evening void volume (ml)	Log10	ND*	ND*
Next day void volume (ml)	ND*	Log10	ND*
Morning void minutes since last meal (minutes)	ND*	ND*	ND*
Afternoon void minutes since last meal (minutes)	ND*	Log10	ND*
Evening void minutes since last meal (minutes)	Log10	Log10	Log10
Next day void minutes since last meal (minutes)	ND*	ND*	ND*

* ND - normal distribution

Mean and standard deviations (SD) were calculated for normally distributed continuous variables, and medians and interquartile range (IQR) for continuous variables with skewed distributions.

To ensure energy balance was maintained during the feeding period, participants' body weights (kg) were measured and recorded daily. A paired samples t test was run to determine if there was a difference in body weights (kg) before and after the 15-day feeding period, indicating energy balance or imbalance.

To investigate if there is a difference in uSF excretion (mg/void and mg/g creatinine) between the four timed urine voids, a Wilcoxon non-parametric test was performed. To control for multiple comparisons (6 comparison tests), we used Bonferroni correction, which sets a more conservative significance cut-off at α/n, where α is Type 1 error (0.05) and n is number of tests/comparisons; the cut off was $p<0.008$.

Within and between subject variability in uSF excretion (mg/void) and uSF excretion (mg/g creatinine) were calculated for the identified timed urine voids using %CV (CV% = SD/mean x 100) and variance ($VAR = SD^2$). Within subject (WS) variability measures day-to-day variability in uSF excretion within participants, whereas between subject (BS) variability measures variability in uSF excretion between participants in a group. Each participant's mean values were calculated by averaging spot day 1 and spot day 2 values, then SD_{WS}, $\%CV_{WS}$ and VAR_{WS} were calculated from the same values. The participants' mean values were averaged to create the group mean. A SD_{BS} was calculated from these means, then allowing $\%CV_{BS}$ and VAR_{BS} to be calculated. A ratio of within subject and between subject variance (VAR_{WS}/VAR_{BS}) was

used to determine if within subject variance is greater or smaller than between subject variance in excretion.

Pearson correlation coefficient was used to determine if there is an association between spot void uSF excretion and 24-h uSF excretion; correlations were determined between timed urine voids and 24-h urine collection for uSF excretion (mg/void) and uSF excretion (mg/g creatinine).

Lastly, multiple linear regression models were run to determine the predictive ability of sucrose and fructose excretion in timed spot collection and other covariates on 24-h uSF excretion. The dependent variable was 24-h sucrose and fructose excretion (mg/day), whereas uSF in timed spot voids (mg/void or mg/g creatinine) was run as the independent variable. Multiple covariates in addition to uSF excretion in timed spots (mg/void and mg/g creatinine) were included in the models. These included age (years), BMI (kg/m^2), gender, time since last meal (minutes), and void volume (ml). Time since last meal was calculated by counting the minutes between the time of the identified void and the time of the last meal recorded in the meal checklist. Covariates were included in the final model only if they were statistically significant predictors of 24-h uSF excretion or they strengthened the adjusted R^2 value of the final model. Models were run for spot day 1, spot day 2, and two-day mean 24-h uSF excretion. We report adjusted R^2 for the final model, and beta coefficient and standard error (SE) for each independent variable. A higher R^2 means that the dependent variable, 24-h uSF is better predicted or had a higher proportion of its variability explained by the independent variables, the predictors.

All the analyses were conducted using SPSS Statistical Software (version 24.0;

Armonk, NY: IBM Corp). P values less than 0.05 were considered statistically significant.

CHAPTER 4

RESULTS

Fifteen participants completed the entire protocol between March and August 2016. Twelve participants collected complete 24-h urine collections during the two multiple spot urine collection days (multiple spot urine collection day 1 and 2). One participant self-reported one missed void for multiple spot urine collection day 2. Based on PABA, multiple spot urine collection 2 was found to be incomplete for one participant, and for one participants, both multiple spot urine collections were incomplete. Hence, one participant was excluded from all the analyses, and for two participants, only data from one multiple spot urine collection day were used.

Table 4 describes the study population demographics (n=14). Study population included eight females and six males with mean age of 34.3 years (SD=9.4 years).

Table 4: Study population demographics (n=14)

Demographic characteristic		Means ± SD (range) or n (%)
Gender	Male	6 (42.9%)
	Female	8 (57.1%)
Age	Years	34.3 ± 9.4 (23.0-53.0)
BMI	kg/m^2	24.2 ± 3.5 (17.9-30.7)
Ethnicity	White	11 (78.6%)
	Hispanic/Latino	2 (14.3%)
	Asian	1 (7.1%)
Income	Less than $44,999	9 (64.3%)
	$45,000-104,999	3 (21.4%)
	More than $105,000	2 (14.3%)
Occupation	Student	8 (57.1%)
	Academic fields	4 (28.6%)
	Tech and lab	2 (14.3%)

Marital status	Single	8 (57.2%)
	Married/living with partner	5 (35.7%)
	Divorced	1 (7.1%)

The mean BMI (kg/m^2) was 24.2 kg/m^2 (SD=3.5); ten participants were normal weight (BMI of < 25 kg/m^2), two participants were overweight (BMI of 25-29.9 kg/m^2), and two participants were obese (BMI of 30-34.9 kg/m^2). Further study population demographics are presented in Table 4. **Table 5** reports the study participants' body weight at the beginning and at completion of the 15-d feeding period. The mean difference in body weight between pre- and post-feeding was 0.57 kg, and was not statistically significant (t=1.76; p=0.102).

Table 5: Participants' body weight (BW) measured before and after the 15-day feeding period (n=14)

Participant	**Pre- feeding BW (kg)**	**Post- feeding BW (kg)**	**BW difference***
SB001	67.8	68.2	0.4
SB002	66.0	64.8	-1.2
SB003	60.8	58.5	-2.3
SB004	71.4	70.5	-0.9
SB005	63.2	63.2	0
SB007	90.4	92.5	2.1
SB008	82.7	83.0	0.3
SB009	69.0	68.6	-0.4
SB010	54.5	54.0	-0.5
SB011	66.7	66.3	-0.4
SB012	64.5	63.0	-1.5
SB013	79.0	79.3	0.3
SB014	81.2	78.6	-2.6
SB015	79.0	77.7	-1.3
Mean	71.2	70.6	-0.57

* Difference between pre and post body weight was investigated using a paired t test: t=1.76 ; p= 0.102.

Table 6 reports descriptive statistics of spot void collections made during multiple spot urine collection day 1 and 2. The two-day mean 24-h urine volume collected by the study population was 2825.4 ml (SD= 1291.7 ml), whereas the average void volume per participant was 365.3 ml (SD=48.7 ml). On average, 7.2 urine voids/day were collected per participant (range: 3 - 10).

Table 6: Descriptive statistics of spot void collections made during multiple spot urine collection day 1 and 2 (n=14)

	n	Spot day 1	n	Spot day 2	Two-day Mean
Total 24-h urine volume (ml)	14	2973.4 ± 1405.0* (726.2 – 5059.5)†	12	2580.0 ± 1168.8* (1165.1 – 4484.5)	2825.4 ± 1291.7* (1078.1 – 4811.5)
Number of voids per subject (n)	14	7.0 ± 2.0* (4.0 – 10.0)	12	7.5 ± 1.9* (3.0 – 10.0)	7.2 ± 1.7 * (3.0 – 10.0)
Void volume per subject (ml)	14	399.7 ± 173.8*	12	330.8 ± 111.1*	365.3 ± 48.7*
Timed void volume (ml)					
Morning	14	350.7 ± 236.2* (114.6 – 847.8)	11	313.1 ± 171.4* (55.8 – 584.9)	314.1 ± 195.5* (90.2 – 716.4)
Afternoon	14	428.0 ± 385.4 (197.8 – 1016.0)	11	270.7 ± 163.8* (100.7 – 605.8)	359.8 ± 270.5 (159.7 – 1016.0)
Evening	14	331.4 ± 372.9 (135.9 – 128.5)	12	353.2 ± 159.0* (136.4 – 682.7)	409.8 ± 216.1* (175.6 – 826.1)
Next day	14	508.7 ± 177.8* (268.0 – 843.3)	12	400.4 ± 423.0 (255.6 – 1230.6)	509.1 ± 192.9* (286.7 – 980.5)
Time since last meal (min)					
Morning void	14	87.4 ± 53.0* (0.0 – 195.0)	11	85.2 ± 57.7* (1.0 – 210.0)	88.2 ± 45.0* (22.5 – 197.0)
Afternoon void	14	111.5 ± 83.8* (0.0 – 330.0)	11	59.0 ± 105.0 (6.0 – 285.0)	93.1 ± 45.2* (15 – 170.5)
Evening void	14	60.5 ± 83.0 (9.0 – 229.0)	12	41.0 ± 56.3 (5.0 – 335.0)	55.3 ± 84.3 (11.5 – 229.0)

Next day void	14	606.8 ± 134.9* (385.0 – 872.0)	12	598.8 ± 91.0* (415.0 – 717.0)	603.9 ± 109.7* (425.0 – 794.5)
Para amino benzoic acid (PABA) recovery in 24-h urine					
HPLC measurements (%)	14	93.9 ± 8.0* (78.6 – 107.5)	14	84.9 ± 10.1* (60.9 – 99.6)	89.4 ± 6.9* (75.1 – 99.8)
SPEC measurements (%)	14	103.9 ± 10.5* (87.6 – 128.1)	14	103.0 ± 25.0 (57.0 – 200.2)	108.6 ± 17.4* (74.5 – 147.7)

*Indicated values are means ± SD, and all other values are median ± IQR
† Range all such values.

The volume of individual spot voids ranged from 22.8 to 1280.5 ml, and among timed voids, it was largest for the next day void (Table 6). Time (minutes) since last meal were determined for each of the timed urine voids. The two-day mean minutes since last meal were 88.2, 93.1, 55.3 and 603.9 minutes for morning, afternoon, evening, and next day void, respectively. The two-day mean PABA recovery was 89.4% (SD=6.9%) by the HPLC method, and 108.6% (SD=17.4%) by the spectrophotometric method (Table 6).

The uSF excretion in 24-h urine (mg/day) and timed spot collections (mg/void) during spot day 1 and spot day 2, and respective two-day mean values, are reported in **Table 7**. The two-day mean uSF was 50.6 mg (SD=29.5) for 24-h urine, and 7.5 (IQR=14.0), 4.5 (IQR=8.5), 5.1 (IQR=7.1), and 4.6 (IQR=12.3) mg for morning, afternoon, evening, and next day void, respectively. There were no statistically significant differences in uSF excretion level between the timed spot voids for spot day 1, spot day 2, and the two-day mean as tested by the Wilcoxon non-parametric test (Table 7).

Table 7: Sum of sucrose and fructose excretion (uSF) in 24 hour urine (mg/day) and timed spot urine collections (mg/void) during multiple spot urine collection day 1 and 2 (n=14)†

	n	Spot day 1	n	Spot day 2	n	Two day mean
uSF in 24 hour urine (mg/day)	14	43.5 ± 28.5[*1]	12	62.5 ± 44.3[*1]	14	50.6 ± 29.5[*1]
uSF in morning void (mg/void)	14	6.4 ± 5.7[1]	11	4.4 ± 9.6[1]	14	7.5 ± 14.0[1]
uSF in afternoon void (mg/void)	14	3.6 ± 7.2[1]	11	4.7 ± 11.7[1]	14	4.5 ± 8.5[1]
uSF in evening void (mg/void)	14	3.6 ± 10.1[1]	12	3.7 ± 9.4[1]	14	5.1 ± 7.1[1]
uSF in next day void (mg/void)	14	5.0 ± 9.1[1]	12	3.8 ± 10.7[1]	14	4.6 ± 12.3[1]

[*]Indicated values are means ± SD, and all other values are median ± IQR.
[†] Wilcoxon test performed with Bonferroni adjustment ($p<0.008$) to test the difference in excretion by column. Values marked with the same numeric superscript are not statistically significantly different from each other.

Table 8 describes the uSF excretion in timed spot collections during spot day 1 and spot day 2, and two-day mean values expressed per gram creatinine. The two-day mean uSF was 60.2 (SD=39.9), 25.5 (IQR=40.2), 32.6 (IQR=38.2), and 12.3 (IQR=18.9) mg/g creatinine for morning, afternoon, evening, and next day void, respectively. Using the Wilcoxon non-parametric test, we found no statistically significant difference in uSF excretion (mg/gram creatinine) between any of the timed spot voids for spot day 1 and spot day 2. When we compared the two-day mean uSF excretion per gram creatinine among the four timed spot voids, we only found a statistically significant difference between uSF excretion in the morning and next day void ($Z = -2.794$, $p=0.005$).

Table 8: Sum of sucrose and fructose excretion (uSF) per gram creatinine in timed spot urine collections during multiple spot urine collection day 1 and 2 (n=14)[†]

	n	Spot day 1	n	Spot day 2	n	Two day mean
uSF in morning void (mg/g creatinine)	14	37.3 ± 69.5[*1]	11	56.1 ± 80.6[*1]	14	60.2 ± 39.9[1]

uSF in afternoon void (mg/g creatinine)	14	23.3 ± 23.2[*1]	11	40.7 ± 76.8[*1]	14	25.5 ± 40.2[*1,2]
uSF in evening void (mg/g creatinine)	14	33.6 ± 29.4[1]	12	22.3 ± 33.3[*1]	14	32.6 ± 38.2[*1,2]
uSF in next day void (mg/g creatinine)	14	12.3 ± 20.2[*1]	12	8.7 ± 22.6[*1]	14	12.3 ± 18.9[*2]

[*] Indicated values are median ± IQR, and all other values are means ± SD.
[†] Wilcoxon test performed with Bonferroni adjustment ($p<0.008$), to test the difference in excretion by column. Values marked with the same numeric superscript are not statistically significantly different from each other.

The uSF excretion by spot void for each participant by spot day 1 and 2 are shown in Appendix P. No particular trends in uSF excretion by spot void were observed. Overall, the majority of participants had low uSF excretion. Eleven out of the 14 participants had less than 45 mg/void uSF excretion on both spot collection days. USF excretion ranged from 0.07 to 38.1 mg/void for spot day 1 and 0.3 to 82.6 mg/void for spot day 2.

The within and between subject variability calculated for uSF excretion in timed spot urine collections (mg/void) and 24-h urine (mg/d) are presented in **Table 9**. Within-subject (WS) and between-subject (BS) %CV for uSF in 24-h urine was 38.4% and 58.3%, respectively. Within-subject variability in uSF was lowest in the afternoon timed void (CV_{WS}= 49.1%) and highest in the morning timed void (CV_{WS}=81.8%). Between-subject variability was lowest in the next day timed void (CV_{BS}= 103.4%) and highest in the evening timed void (CV_{BS}=138.9%). The ratio of within to between subject variance of uSF excretion (var_{ws}/var_{bs}) was 1.3 for 24-h urine, and it ranged from 0.2 for the afternoon timed void to 2.9 for the morning timed void (Table 9).

Table 9: Variability estimates for two-day mean sum of sucrose and fructose excretion (uSF) in 24 hour urine (mg/day) and time 62spot urine collections (mg/void) made

during multiple spot urine collection day 1 and 2 (n=14)

	%CV_{WS}	**Var_{WS}**	**%CV_{BS}**	**Var_{BS}**	**Var_{WS} / Var_{BS}**
uSF in 24h urine	38.4	1170.4	58.3	868.3	1.3
uSF in morning void	81.8	458.8	104.9	157.9	2.9
uSF in afternoon void	49.1	18.9	125.6	96.8	0.2
uSF in evening void	60.5	273.2	138.9	158.4	1.7
uSF in next day void	65.8	49.6	103.4	85.0	0.6

%CV_{WS}: Within-subject coefficient of variation; **%CV_{BS}** : Between-subject coefficient of variation; **Var_{WS}**: Within-subject variance; **Var_{BS}**: Between-subject variance

The within and between subject variability for uSF excretion (mg/g creatinine) have been reported in **Table 10**. Within-subject variability in uSF excretion (mg/g creatinine) was lowest in the afternoon timed void (CV_{WS}= 49.6%) and highest in the morning timed void (CV_{WS}=73.3%). Between-subject variability in uSF excretion (mg/g creatinine) was lowest in the morning timed void (CV_{BS}= 66.3%) and highest in the afternoon timed void (CV_{BS}=111.4%). The ratio of within to between subject variance for excretion mg/g creatinine for the timed spot collections ranged from 0.3 for the afternoon timed void to 2.5 morning timed void.

Table 10: Variability estimates for two-day mean sum of sucrose and fructose excretion (uSF) in time spot urine collections (mg/g creatinine) made during multiple spot urine collection day 1 and 2 (n=14)

	%CV_{WS}	**Var_{WS}**	**%CV_{BS}**	**Var_{BS}**	**Var_{WS} / Var_{BS}**
uSF in morning void	73.3	3913.6	66.3	1588.6	2.5
uSF in afternoon void	49.6	767.5	111.4	2408.0	0.3
uSF in evening void	62.7	2460.3	82.3	1053.9	2.3

uSF in next day void	60.0	149.5	94.5	327.1	0.5

%CV$_{WS}$: Within-subject coefficient of variation; **%CV$_{BS}$**: Between-subject coefficient of variation; **Var$_{WS}$**: Within-subject variance; **Var$_{BS}$**: Between-subject variance

Table 11 and **12** present the correlation coefficients for the association between uSF excretion in 24-h urine and timed urine voids in mg/void (Table 11) or mg/g creatinine (Table 12).

Table 11: Correlation between the sum of sucrose and fructose excretion (uSF) in timed urine voids (mg/void) and 24-h urine collection (mg/day)

	Spot day 1			Spot day 2			2-day means	
	n	**r***	**p value**	**n**	**r***	**p value**	**r***	**p value**
uSF in morning void vs. 24-h urine	**14**	0.76	0.002	**11**	0.70	0.017	0.80	0.001
uSF in afternoon void vs. 24-h urine	**14**	0.79	0.001	**11**	0.69	0.020	0.72	0.004
uSF in evening void vs. 24-h urine	**14**	0.45	0.103	**12**	0.69	0.013	0.65	0.011
uSF in next day void vs. 24-h urine	**14**	0.67	0.009	**12**	0.42	0.173	0.58	0.029

*Pearson Correlation Coefficient

The two-day mean uSF excretion in all four timed urine voids was statistically significantly correlated with 24-h uSF excretion. The two-day mean uSF excretion in morning timed spot showed the strongest correlation with 24-h uSF excretion (mg/void: r=0.80; p<0.001; mg/g creatinine: r=0.72; p=0.003) followed by the uSF excretion in afternoon timed spot (mg/void: r=0.72; p=0.004; mg/g creatinine: r=0.67; p=0.009). Correlations based on multiple spot urine collection day 1 and 2 ranged from r=0.42 (p=0.173) for uSF excretion (mg/void) in the next day void to r=0.79 (p<0.001) for

uSF excretion in the afternoon timed void (Table 11). For uSF excretion in mg/g creatinine, the correlations based on multiple spot urine collection day 1 and 2 ranged from r=0.43 (p=0.158) for uSF excretion (mg/void) in the next day void to r=0.79 (p=0.002) for uSF excretion (mg/void) in the evening void (Table 12).

Table 12: Correlation between the sum of sucrose and fructose excretion in timed urine voids (mg/g creatinine) and 24h urine collection (mg/day)

	Spot day 1			Spot day 2			2-day means	
	n	**r***	**p value**	**n**	**r***	**p value**	**r***	**p value**
uSF in morning void vs. 24-h urine	**14**	0.53	0.053	**11**	0.60	0.053	0.72	0.003
uSF in afternoon void vs. 24-h urine	**14**	0.78	0.001	**11**	0.58	0.063	0.67	0.009
uSF in evening void vs. 24-h urine	**14**	0.52	0.058	**12**	0.79	0.002	0.64	0.014
uSF in next day void vs. 24-h urine	**14**	0.64	0.014	**12**	0.43	0.158	0.57	0.034

*Pearson Correlation Coefficient

Table 13 presents findings from various linear regression models with 24-h uSF as a dependent variable, using uSF mg/void in timed spot voids as a predictor.

Table 13: Linear regression models findings from regressing the sum of sucrose and fructose excretion (uSF) in 24-h urine (mg/day) on the sum of sucrose and fructose excretion (uSF) in timed spot voids (mg/void) along with other covariates for spot day 1, spot day 2 and two-day mean

	Spot day 1		Spot day 2		Two-day mean	
	Beta coefficient; SE	**p value**	**Beta coefficient; SE**	**p value**	**Beta coefficient; SE**	**p value**
Model 1:						
USF in *morning void*	12.8; 3.1	0.002	62.0; 16.3	0.005	13.3; 2.9	0.001
Gender (male)	-	-	-38.1; 19.1	0.082	-11.5; 9.8	0.268

Adjusted R^2	0.54		0.57		0.62	
p value	0.002		0.014		0.002	
Model 2:						
USF in *afternoon void*	55.2; 11.7	0.001	25.4; 15.0	0.134	50.8; 10.3	0.001
Age (years)	0.9; 0.6	0.178	1.8; 1.0	0.111	1.7; 0.5	0.010
Time since last meal (mins)	-	-	37.5; 18.9	0.088	0.1; 0.1	0.307
Adjusted R^2	0.63		0.64		0.69	
p value	0.002		0.016		0.002	
Model 3:						
USF in *evening void*	28.0; 12.1	0.043	50.4; 16.7	0.013	33.2; 11.1	0.011
Age (years)	-1.2; 0.8	0.143	-	-	-	-
Void volume (ml)	-31.0; 23.4	0.214	-	-	-	-
Adjusted R^2	0.30		0.42		0.38	
p value	0.090		0.013		0.011	
Model 4:						
USF in *next day void*	21.1; 10.2	0.069	28.1; 19.5	0.193	39.3; 11.5	0.008
Age (years)	-1.3; 0.7	0.079	3.9; 1.5	0.30	1.5; 0.6	0.033
Gender (male)	-	-	-61.4; 29.9	0.079	-31.6; 11.4	0.021
Time since last meal (mins)	-0.1; 0.1	0.048	-	-	-	-
Void volume (ml)	-0.1; 0.0	0.107	-74.5; 60.8	0.260	-0.1; 0.0	0.021
Adjusted R^2	0.57		0.38		0.63	
p value	0.018		0.122		0.009	

Other covariates, including BMI (kg/m^2), age (years), gender, void volume (ml), and time since last meal (minutes) were investigated. BMI did not improve the predictability of any of the models and was therefore not included. In the linear regression models predicting two-day mean 24-h uSF excretion, the model with the afternoon void uSF along with age and time since last meal (Table 13; model 2) generated the largest adjusted R^2 value of 0.69 (p=0.002). The adjusted R^2 for the model with the afternoon void uSF excretion as the only independent variable was 0.38 (p=0.011). However,

adding age and time since last meal significantly improved the predictive ability of the final model (adjusted R^2 = 0.69; p=0.002).

For the remaining two-day mean models, uSF in the morning, evening, and next day void were all statistically significant, with adjusted R^2 values ranging from 0.38 to 0.63. Adding gender as a covariate improved the two-day mean model for the morning void (Table 13; model 1) from adjusted R^2 of 0.47 (p=0.004) to 0.62 (p=0.002). No covariates improved the evening void model (Table 13; model 3), whereas adding age, gender, and void volume improved the next day void model (Table 13; model 4) from adjusted R^2 of 0.61 (p=0.001) to 0.63 (p=0.009).

This trend was similar in spot day 1 and spot day 2 models, with the afternoon void uSF excretion being the strongest predictor of 24-h uSF. All models for spot day 1 and spot day 2 were statistically significant except for the evening void uSF (Table 13; model 3) on spot day 1 (R^2=0.30, p=0.090) and the next day void (Table 13; model 4) on spot day 2 (R^2=0.38, p=0.122). The predictability of the models improved when using the two-day mean void compared to single spot voids for morning, afternoon and next day void, but not for the evening void.

Table 14 presents the results from linear regression models ran for 24-h uSF using uSF mg/g creatinine in timed spots as predictors.

Table 14: Linear regression models findings from regressing the sum of sucrose and fructose excretion (uSF) in 24-h urine (mg/day) on the sum of sucrose and fructose excretion (uSF) in timed spot voids (mg/g creatinine) along with other covariates, for spot day 1, spot day 2 and two-day mean

	Spot day 1		Spot day 2		Two-day mean	
	Beta coefficient; SE	**p value**	**Beta coefficient; SE**	**p value**	**Beta coefficient; SE**	**p value**

Model 1:						
USF in *morning void*	21.3; 10.0	0.053	73.2; 25.0	0.019	0.6; 0.1	0.001
Gender (male)	-	-	-39.9; 22.7	0.117	-19.3; 10.7	0.101
Void volume (ml)	-	-	-	-	0.1; 0.0	0.123
Adjusted R^2	0.22		0.42		0.58	
p value	0.053		0.047		0.008	
Model 2:						
USF in *afternoon void*	51.4; 12.0	0.001	16.4; 18.5	0.403	46.5; 13.6	0.008
Age (years)	-	-	1.4; 1.1	0.251	1.1; 0.6	0.112
BMI (kg/m^2)	-	-	-	-	3.9; 2.1	0.104
Time since last meal (mins)	-	-	49.4; 19.0	0.035	0.4; 0.2	0.047
Adjusted R^2	0.57		0.55		0.56	
p value	0.001		0.036		0.021	
Model 3:						
USF in *evening void*	0.8; 0.2	0.005	80.4; 19.7	0.002	43.6; 15.1	0.014
Age (years)	-2.2; 0.8	0.022	-	-	-	-
Gender (male)	13.7; 13.1	0.321	-	-	-	-
Adjusted R^2	0.46		0.59		0.36	
p value	0.028		0.002		0.014	
Model 4:						
USF in *next day void*	21.3; 11.5	0.097	25.8; 21.3	0.265	38.7; 13.9	0.021
Age (years)	-1.5; 0.7	0.048	3.7; 1.5	0.043	1.2; 0.7	0.105
Gender (male)	-	-	-60.6; 31.1	0.092	-32.4; 12.7	0.031
Time since last meal (mins)	-0.1; 0.1	0.068	-	-	-	-
Void volume (ml)	-0.1; 0.0	0.112	-63.8; 62.4	0.341	-0.1; 0.0	0.037
Adjusted R^2	0.54		0.33		0.54	
p value	0.024		0.151		0.023	

Other covariates, including BMI (kg/m^2), age (years), gender, void volume (ml), and time since last meal (minutes) were investigated. For the two-day mean linear regression

models, the morning void uSF (Table 14; model 1) along with gender and void volume was the strongest predictor of 24-h uSF with adjusted R^2 value of 0.58 (p=0.008). Adding these as covariates, strengthened the model from an adjusted R^2 of 0.48 (p=0.003) to 0.58 (p=0.008), and was thus included.

The remaining two-day mean models (afternoon void, evening void, and next day void) were all statistically significant, with adjusted R^2 values ranging from 0.36 for the evening void to 0.56 for the afternoon void. Age, BMI and time since last meal improved the afternoon void model (Table 14; model 2) from adjusted R^2 of 0.40 (p=0.009) to 0.56 (p=0.021). No covariates improved the evening void model (Table 14; model 3). Age, gender, and void volume, when added as covariates improved the predictive ability of the next day void uSF excretion (Table 14; model 4) from adjusted R^2 of 0.35 (p=0.015) to 0.54 (p=0.023).

For spot day 1 and spot day 2 models, the adjusted R^2 values ranged from 0.22 for the morning void to 0.59 for the evening void. All models for spot day 1 and spot day 2 were statistically significant, except the morning void model (Table 14; model 1) on spot day 1 (R^2=0.22, p=0.053) and the next day void model (Table 14; model 4) on spot day 2 (R^2=0.33, p=0.151).

CHAPTER 5

DISCUSSION

The purpose of this analysis was to investigate the utility of sucrose and fructose in spot urine as a biomarker of sugars intake in US adults. Previously, two feeding studies conducted in the United Kingdom investigated this biomarker in 24-h urine, and found a dose-response relationship between total sugars intake and excretion of sucrose and fructose, and determined good performance of the biomarker (Tasevska et al., 2005). However, high cost, heavy participant burden, and complex logistics associated with 24-h urine collections justifies the investigation of this biomarker measured in spot urine.

Data were collected from 15 participants, age 22 to 49 years, residing in the Phoenix Metropolitan Area, who completed a 15-day feeding study in which they consumed their normal diet, and collected eight, nonconsecutive, 24-hour urine samples (i.e., every other day for 15 days). On two of the eight days, participants collected each urine void in a separate container, resulting in a multiple spot urine collection. Data from these two urine collection days were analyzed and were the focus of this thesis analyses.

The mean 24-h sucrose and fructose excretion in our population, while following their normal diet provided by the metabolic kitchen, was 50.6 mg/day (SD: 29.5; range: 5.0 - 110.6 mg/day), which was lower compared to other studies. In their 13 participants, using a similar study design to ours, Tasevska et al. (2005) reported a 30-day mean 24-h uSF excretion of 98 mg/day with a range of 25.4 to 267.5 mg/day, which is nearly double the excretion level we measured. This implies that the total sugars intake in our study population (n=14) was at the lower end of the intake range.

Upon analysis of the urine collections, we found that among four timed voids, the uSF excretion in the afternoon void - first void collected after lunch or between 12:31-5:30 pm, had the lowest day-to-day (i.e., within subject) variation and the lowest ratio of within to between subject variance. This suggests that less afternoon voids would be needed to characterize individuals with regard to their excretion level. Secondly, two-day mean uSF excretion for all four timed urine voids were significantly correlated with 24-h uSF excretion, yet the morning and afternoon void exhibited the strongest associations. Lastly, all timed voids along with other covariates were found to be significant predictors of 24-h uSF excretion. The afternoon void was found to have the strongest predictive ability of 24-h uSF excretion when measured in mg/void, especially when age and time since last meal were included as covariates in the models explaining 69% of the variability in 24-h uSF excretion. In comparison, uSF in the afternoon void expressed in mg/g creatinine explained 56% of the variability in 24-h uSF excretion. This suggests that creatinine may be introducing an error, and therefore it may be better to use uSF in mg/void when possible.

In the analyses, uSF biomarker was expressed in mg/void and mg/gram creatinine, as urinary creatinine is commonly used to adjust biomarker concentrations for urine dilution. For example, if a urine sample is significantly diluted, indicating that the participant is overly hydrated, or concentrated, indicating dehydration, albeit identical amount of the biomarker in the sample, the biomarker concentration measured under those two conditions will be very different, and thus it would not reflect the actual exposure. While measuring the biomarker in mg/gram creatinine may be advantageous

because it corrects for urine volume, meaning the volume of the urine void would not need to be known, creatinine may possibly introduce an error. Barr et al. (2005) report differences in creatinine excretion by gender, age and race. For example, Non-Hispanic blacks compared to all other racial groups, and adult men compared to females had significantly greater concentrations of urinary creatinine. Kidney dysfunction and red meat intake can also effect urinary creatinine concentrations, though presence of any kidney disease was an exclusion criterion for our study. While requiring the urine void volume to be known makes the utilization of this biomarker in population studies more difficult to implement, the risk of creatinine possibly introducing ME may not outweigh the benefits of using it.

We found no statistically significant difference in uSF excretion level (mg/void) between any of the timed voids. However, when using uSF excretion per g creatinine, there was a statistically significantly higher uSF excretion in the morning compared to next day void. Given our limited sample size, data from more participants are needed to gain a better understanding of diurnal variation in uSF excretion.

Studies of other biomarkers have investigated diurnal variation for these biomarkers. In a study with 481 free-living participants, researchers found that the next day void had the lowest concentrations (mmol/L) of sodium, potassium, and chloride compared to morning, afternoon and evening voids (Wang et al., 2013). The same study discovered concentrations of iodine and creatinine to be consistent across the four timed voids. Another study investigated urinary creatinine concentrations from participants in the third NHANES from 1988-1994 (Barr et al. 2005). They established reference ranges

for ages, gender, race/ethnicity, etc., and they reported a small but statistically significant increase in creatinine concentrations in the morning compared with the afternoon and evening voids. The author discussed that the significant difference could be in part due to morning voids being more concentrated, reflecting lengthy overnight periods; however, the notion that the significant difference could possibly indicate a diurnal variation should not be overlooked (Barr et al., 2005). While a greater sample size is needed to further confirm the lack of diurnal variation in the uSF biomarker, even a low variation would support the use of spot urines over 24-h urine.

The first aim of this study was to describe the within-subject (WS) and between-subject (BS) variability of uSF in 24-hour and multiple spot urine collections, both of which are measured as percent coefficient of variation ($\%CV_{WS}$ or $\%CV_{BS}$) and as variance estimates (VAR_{WS} and VAR_{BS}). WS variability indicates participant's day-to-day variability in uSF excretion. High $\%CV_{WS}$ means that a higher number of urine voids would be needed to obtain usual uSF excretion level, whereas a low $\%CV_{WS}$ suggests that individuals' uSF excretion levels do not vary greatly from day to day, meaning a lower number of urine voids would be needed to obtain the usual uSF excretion level (Bingham, 2002; Tasevska et al., 2005). BS variability reports the variability in excretion between participants. Lower WS than BS variability, notated $VAR_{WS}/VAR_{BS} <1$ allows researchers to more reliably rank participants according to their excretion level in a group, using fewer urine samples. Less urine void collections reduce the participant burden and associated cost.

In our study population, day-to-day variability of uSF excretion based on two 24-

h urine collection was 38.4%. Tasevska et al. (2005) investigated 24-h uSF as a biomarker in the UK with participants consuming their normal diet and collecting 24-h urine daily for 30 days (n=13) and found a similar $\%CV_{WS}$ for 24-h uSF (36.9%). Yet, the ratio of WS/BS variance (VAR_{WS}/VAR_{BS}) in this study was lower (0.49) compared to what we found (1.3). This may have been due to the much higher number of repeated measurements of 24-h uSF they had (n = 30) allowing them to better characterize individuals' excretion levels within the group. In another study (n=12), the same investigators examined the dose-response of uSF to constant levels of low-sugars (63±5 g/d, 9.5% of energy intake-EI), medium-sugars diet (143± 5 g/d, 21.8% EI), and high-sugars diet (264± 3 g/d, 40.2% EI) (Tasevska et al., 2005). Based on four 24-h urine collections per dietary period, they found that the within-subject variability of urinary sucrose and urinary fructose to be high in participants while on low sugars diet ($\%CV_{WS}$ for urinary sucrose = 61.4%; $\%CV_{WS}$ for urinary fructose = 60.7%), whereas the $\%CV_{WS}$ was lower during medium and high sugars diets ($\%CV_{WS}$ was approximately 30% for urinary sucrose and 20% for urinary fructose). Although, in the latter study authors reported variability estimates separately for urinary sucrose and urinary fructose, this suggests that the variability of 24-h uSF excretion is higher at lower levels of excretion. However, in our study, participants were not assigned to a constant level of sugars intake, rather, they consumed their normal diet with varying levels of intake. Our study found uSF excretion in the timed spots to be low (3.6-7.5 mg/void) but the WS variability estimates were high ($\%CV_{WS}$ = 49.1-81.8%). WS variability was lowest in the afternoon timed void ($\%CV_{WS}$ = 49.1-49.6%) and highest in the morning timed void ($\%CV_{WS}$ =

73.3-81.8%) for both uSF mg/void and mg/g creatinine.

Similar to what was observed for uSF excretion, Wang et al. (2013) found that the WS variability of sodium and potassium excretion was lower for the 24-h urine excretion than for the timed spot urines. For sodium, 24-h $\%CV_{WS}$ was reported to be 19-23%, whereas for the timed spot urines, $\%CV_{WS}$ ranged from 21-39%. For potassium, 24-h $\%CV_{WS}$ was reported to be 17%, whereas for the timed spot urines $\%CV_{WS}$ ranged from 22-40%. Ultimately, it appears that the $\%CV_{WS}$ variability was highest in the evening voids for all of the investigated nutrients (Na, K, Cl, creatinine, and iodine) ranging from 28-41%.

In our study population, we found high BS variability for all the timed spots for both uSF mg/void and mg/g creatinine. Yet, the BS variability was higher for uSF mg/void ($\%CV_{BS}$ range = 103.4-138.9%) than for mg/g creatinine ($\%CV_{BS}$ range = 66.3 – 111.4%). More importantly, the ratios of VAR_{WS}/VAR_{BS} ranged from 0.2 to 2.9 for uSF mg/void and from 0.3 to 2.5 for mg/g creatinine. Favorable variance ratios were identified for afternoon and next day voids for uSF excretion mg/void (0.2 and 0.5, respectively) and mg/g creatinine (0.3 and 0.6, respectively). For the morning and evening voids, unfavorable VAR_{WS}/VAR_{BS} >1was noted. Overall, the uSF in the afternoon spot from our study was identified to be the most reproducible having had the lowest day-to-day variability and high BS variability, resulting in VAR_{WS}/VAR_{BS} ratios of less than one. In comparison to what we found for uSF, the ratios of within to between subject variance for sodium, potassium and chloride were much lower (Wang et al., 2013); for sodium, the ratio was lowest in the afternoon void among blacks (0.6), and

overnight void among other races (0.3); for potassium, in the afternoon void for both blacks and other races (0.3); and for chloride, in the morning void for blacks (0.4), and the overnight void for other races (0.3). The results for the majority of the timed voids were all favorable with ratio <1. This may be due to their larger samples size, and a higher number of repeated measurements, or due to the metabolic turnover rate of the nutrients themselves.

The second aim was to investigate the correlation of uSF excretion in spot urine with the corresponding 24-h excretion. All timed void uSF excretion levels were significantly correlated with 24-h uSF excretion. The strongest associations were found in the morning (r= 0.80) and the afternoon void (r=0.72) for mg/void, and for mg/g creatinine (r=0.72 and r=0.67, respectively). While the morning timed void was identified as having the strongest association with 24-h uSF excretion, the unfavorable VAR_{WS}/VAR_{BS} ratio suggests that this void is less reproducible. Given the afternoon timed void had a strong association with 24-h uSF excretion and VAR_{WS}/VAR_{BS} ratio <1, it may provide a more reliable estimate of uSF. Though no other study has compared the uSF in spot urine to 24-h uSF excretion, other studies have investigated the correlation between spot and 24-h urine of other biomarkers.

In a study by Patil et al. (2014), participants (n=241) were asked to collect a 24-h urine and a random spot sample, either before or after completing the 24-h urine collection, which were analyzed for protein and creatinine. The urinary protein to creatinine ratio in the random (untimed) spot sample was highly correlated with 24-h urine protein (r=0.98, p <0.05).

Several other studies have compared the excretion of other analytes in timed spot voids to 24-h excretion. A study by Doenyas-Barak et al. (2015) evaluated the use of multiple timed spot urine collections as a new method to estimate 24-h urinary sodium, potassium and chloride compared to measuring the electrolytes in 24-h urine, i.e., the gold standard. Participants consumed their usual diet and collected a 24-h urine and four timed 10 ml spot urine collections. Investigators determined the electrolyte to creatinine ratio for each timed spot collection, and averaged the electrolyte to creatinine concentrations from 2, 3 or 4 timed voids. They then used these values to estimate 24-h electrolyte excretion based on the expected creatinine excretion in mg per day. They discovered statistically significant correlation between estimated 24-hour urine solute excretion (based on a spot measurement) and measured 24-h urinary solute excretions. Furthermore, they found that averaging electrolyte-to-creatinine excretion from two to four spots rather than using a single spot improved the correlation with measured 24-h electrolyte excretion; the correlations for a single spot collection were 0.68, 0.75, and 0.51, and for the average of 3 spots 0.85, 0.78, and 0.56 for Na, K, and Cl, respectively (Doenyas-Barak et al., 2015). They concluded that average sodium and potassium concentrations across four scheduled spots may better approximate daily excretion level. As these analytes are affected by other variables rather than intake only, collecting several spot voids over a 24-h period and averaging the excretion amount can help improve estimate 24-h excretion and help reduce diurnal variation.

In another study, Perrine et al. (2014) investigated the use of spot urine samples to estimate iodine status of a population. Four hundred participants collected all the voids

over 24-hours, and morning, afternoon, evening, and overnight voids as well as a 24-h urine composite were measured for iodine and creatinine. They investigated excretion versus concentration of iodine and explored different equations to estimate 24-h iodine excretion from iodine measured in spot urine. They calculated urine iodine concentration (UIC) for each time spot sample as well as the iodine-to-creatinine ratio (I/Cr) for each spot. They also estimate 24-h urinary iodine excretion (UIE) based on predicted 24-h creatinine, and reported no statistically significant difference between estimated and observed 24-h UIE (Perrine et al., 2014). Furthermore, they concluded that UIC in spot samples can reasonably estimate UIC for 24-h samples, but estimates of UIC, I/Cr, and estimated 24-h UIE from spot samples should not be used interchangeably (Perrine et al., 2014).

As demonstrated, several analytes (Na, K, I) measured in spot urine samples can adequately predict or estimate 24-h excretion using different approaches. While there is growing evidence that multiple spot urine collections may be able to serve as a proxy for estimating 24-h urine excretion, the protocol for this will be analyte-dependent.

Lastly, linear regression models were fit to determine the ability of spot void uSF to predict 24-h uSF excretion, and how the dependent variable (24-h uSF excretion) changes based on the independent variable (uSF in timed spot void). The adjusted R^2 value indicates the percentage of variability in the outcome variable that can be explained by the independent variable. The adjusted R^2 was considered in the analyses because unadjusted estimate may be too optimistic due to multiple predictors in the models and the small sample size. The addition of other covariates to these models, including BMI

(kg/m^2), age (years), void volume (ml), gender, and time since last meal (minutes), were investigated to see if they would improve the predictability of the models. These were investigated because if these variables improve the prediction of 24-h uSF, then they can be used in addition to the spot urine uSF excretion.

In all timed spot models, but the evening void, the investigated covariates improved the model. USF excretion in the afternoon void along with age and time since last meal was the strongest predictor of 24-h uSF excretion, with an adjusted R^2 value of 0.69 (mg/void). This means that nearly 69% of the variability in 24-h uSF excretion can be explained by this model. Whereas the morning void had the strongest predictive ability for uSF excretion in mg/g creatinine with an adjusted R^2 value of 0.58, the afternoon void was nearly as strong with an adjusted R^2 value of 0.56. Even though the afternoon void model did not generate the strongest adjusted R^2 for uSF excretion (mg/g creatinine), this void had the lowest estimated day-to-day variability and the most favorable VAR_{WS}/VAR_{BS} ratio, which indicates good reproducibility of the measure. We can thus assume that this void may be best to use estimate 24-h uSF excretion. Yet, this should be further investigated in a larger sample size, and participants with a wide range of sugars intake representative of the US diet.

A study by Robinson Cohen et al. (2014) investigated the precisions and accuracy of spot urine phosphate measurements compared to 24-h measurements. Participants (n=148) collected 24-h collections and spot urine collections on the same day. They used multiple linear regression to explore the effect of various covariates in model prediction (age, sex, height, weight, collection time, and last meal time). They found that age, sex,

and weight significantly improved the prediction of 24-h urinary phosphate, and suggested that this novel equation provides a reasonable estimate of 24-h phosphate excretion that can be used for clinical purposes and future research studies.

This highly controlled feeding study designed to mimic participants' habitual diet was the first of its kind to investigate the utility of uSF biomarker in spot urine. Participants maintained their body weight throughout the feeding period implying that they were in energy balance, which is essential for investigating performance of dietary biomarkers. Another strength of this analysis is that we were able to assess completeness of 24-h urine collections using the PABA marker. Complete collections are essential when measuring urinary biomarkers, as any missing urine could introduce bias in biomarker estimates (Bingham, 2002). Lastly, multiple spot urine collections were made on two days of the 15-day feeding period, which allowed us to assess day-to-day variability in uSF excretion in the identified timed spot voids. One of the major limitations of our analysis is the small samples size, which may have led to overestimation of the within subject and between subject variability estimates for uSF in timed spot voids. Additionally, dietary data were not available for this analysis, hence 24-h uSF excretion was used as a surrogate measure of total sugars intake. As a next step, the utility of uSF in spot urine needs to be investigated against true sugars intake.

CHAPTER 6

CONCLUSION

In this highly controlled feeding study investigating the utility of spot urine for uSF biomarker measurement, uSF in each of the four timed voids (morning, afternoon, evening and next day void) was significantly correlated with 24-h uSF excretion. We found that uSF in the afternoon timed void had the best reproducibility, and along with other covariates, explained the highest proportion of variability in 24-h uSF excretion. Due to our limited sample size and limited range of sugars intake in the study population, these findings have yet to be confirmed in a larger study.

While 24-h urine collections are the gold standard for urinary biomarkers, they are cumbersome and costly. The findings of this study suggest that spot urine samples can be utilized for sugars biomarker measurement instead of relying on burdensome 24-h urine collections. More feasible methods to objectively measure sugars intake can lead to better understanding of sugars and disease associations that previously have been difficult to establish.

REFERENCES

Ainsworth, B. E., Bassett, D. R.,Jr, Strath, S. J., Swartz, A. M., O'Brien, W. L., Thompson, R. W., et al. Comparison of three methods for measuring the time spent in physical activity. Medicine and Science in Sports and Exercise, 32(9 Suppl) 2000, S457-64.

Barr, D. B., Wilder, L. C., Caudill, S. P., Gonzalez, A. J., Needham, L. L., & Pirkle, J. L. Urinary creatinine concentrations in the U.S. population: Implications for urinary biologic monitoring measurements. Environmental Health Perspectives 2005; 113(2), 192-200.

Beltsville Human Nutrition Research Center. The U.S. department of Agriculture's food model booklet, FMB. Agricultural Research Service. July 2007.

Bingham, S. A., Cassidy, A., Cole, T. J., et al. Validation of weighed records and other methods of dietary assessment using the 24 h urine nitrogen technique and other biological markers. The British Journal of Nutrition, 1995; 73(4), 531-550. DOI:S0007114595000584

Bingham, S. A., & Cummings, J. H.. The use of 4-aminobenzoic acid as a marker to validate the completeness of 24 h urine collections in man. Clinical Science, 1983; 64(6), 629-635.

Bingham, S. A., & Cummings, J. H.. Urine nitrogen as an independent validatory measure of dietary intake: A study of nitrogen balance in individuals consuming their normal diet. The American Journal of Clinical Nutrition, 1985; 42(6), 1276-1289.

Bingham, S., Luben, R., Welch, A., Tasevska, N., Wareham, N., & Khaw, K. T. Epidemiologic assessment of sugars consumption using biomarkers: Comparisons of Obese and Nonobese Individuals in the European Prospective Investigation of Cancer Norfolk. Cancer Epidemiol Biomarkers Prev, 2007; 16: 1651-1654.

Bingham, S. A. Biomarkers in nutritional epidemiology. Public Health Nutrition, 2002; 5(6A), 821-827. DOI: 10.1079/PHN2002368.

Bowman, Shanthy A. "Added sugars: Definition and estimation in the USDA Food Patterns Equivalents Databases." Journal of Food Composition and Analysis, 2017.

Brody, T.. Digestion and absorption. Nutritional biochemistry (Second edition ed., pp. 57-131). San Diego USA: Academic Press. 1994.

Ceriello, A., Bortolotti, N., Motz, E., Pieri, C., Marra, M., Tonutti, L., et al. Meal-induced oxidative stress and low-density lipoprotein oxidation in diabetes: The

possible role of hyperglycemia. Metabolism: Clinical and Experimental, 1999; 48(12), 1503-1508.

Cogswell ME, Wang CY, Chen TC, et al. Validity of predictive equations for 24-h urinary sodium excretion in adults aged 18-39 y. Am J Clin Nutr. 2013;98(6):1502-1513.

Cross, A. J., Major, J. M., & Sinha, R. Urinary biomarkers of meat consumption. Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 2011; 20(6), 1107-1111. DOI:10.1158/1055-9965.EPI-11-0048.

Cummings, J. H., & Stephen, A. M.. Carbohydrate terminology and classification. European Journal of Clinical Nutrition, 2007; 61 Suppl 1, S5-18. DOI:1602936 .

Doenyas-Barak, K., Beberashvili, I., Bar-Chaim, A., Averbukh, Z., Vogel, O., & Efrati, S.. Daily sodium and potassium excretion can be estimated by scheduled spot urine collections. Nephron, 2015; 130(1), 35-40. DOI:10.1159/000430105.

Erickson, J., Sadeghirad B., Lytvyn L., Slavin J., Johnston BC. The scientific basis of guideline recommendations on sugar intake: A systematic review. Annals of Internal Medicine, 2016; DOI:10.7326/M16-2020.

Erickson, J., & Slavin, J.. Total, added, and free sugars: Are restrictive guidelines science-based or achievable? Nutrients, 2015; 7(4), 2866-2878. DOI: 10.3390/nu7042866.

Ervin, R. B., & Ogden, C. L.. Consumption of added sugars among U.S. adults, 2005-2010. NCHS Data Brief, 2013; (122)(122), 1-8.

Freedman, L.S., Kipnis, V., Schatzkin, A., Tasevska, N., Potischman, N. Can we use biomarkers in combination with self-reports to strengthen the analysis of nutritional epidemiologic studies? Epidemiol Perspect Innov. 2010;7(1):2-5573-7-2.

Freedman, L.S., Schatzkin, A., Midthune, D., Kipnis, V. Dealing with dietary measurement error in nutritional cohort studies. J Natl Cancer Inst. 2011;103(14):1086-1092.

Freedman, L. S., Tasevska, N., Kipnis, V., Schatzkin, A., Mares, J., Tinker, L., & Potischman, N.. Gains in statistical power from using a dietary biomarker in combination with self-reported intake to strengthen the analysis of a diet-disease association. American Journal of Epidemiology, 2010; 172(7), 836-842. DOI:10.1093/aje/kwq194.

Freeman, R.. Moderate evidence support a relationship between sugar intake and dental caries. Evidence-Based Dentistry, 2014; 15(4), 98-99. DOI:10.1038/sj.ebd.6401055.

Freudenheim, J. L., & Marshall, J. R.. The problem of profound mismeasurement and the power of epidemiological studies of diet and cancer. Nutrition and Cancer, 1988; 11(4), 243-250. DOI:10.1080/01635588809513994.

Han, S. Y., Hong, J. W., Noh, J. H., & Kim, D. J.. Association of the estimated 24-h urinary sodium excretion with albuminuria in adult koreans: The 2011 korea national health and nutrition examination survey. PloS One, 2014; 9(10), e109073. DOI:10.1371/journal.pone.0109073.

Han, W., Sun, N., Chen, Y., Wang, H., Xi, Y., & Ma, Z.. Validation of the spot urine in evaluating 24-hour sodium excretion in chinese hypertension patients. American Journal of Hypertension, 2015; 28(11), 1368-1375. DOI:10.1093/ajh/hpv037.

Hedrick, V. E., Dietrich, A. M., Estabrooks, P. A., Savla, J., Serrano, E., & Davy, B. M.. Dietary biomarkers: Advances, limitations and future directions. Nutrition Journal, 2012; 11, 109-2891-11-109. DOI:10.1186/1475-2891-11-109.

Hess, J., Latulippe, M. E., Ayoob, K., & Slavin, J. The confusing world of dietary sugars: Definitions, intakes, food sources and international dietary recommendations. Food & Function, 2012; 3(5), 477-486. DOI:10.1039/c2fo10250a.

"IFIC Foundation" International Food Information Council. (24 August, 2016). Retrieved from <http://www.foodinsight.org/>

Institute of Medicine, Food and Nutrition Board (IOM). Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids, National Academies Press, Washington, DC, 2002.

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. European Journal of Clinical Nutrition, 1997; 51(8), 514-519.

Jakobsen J, Pedersen AN, Ovesen L. Para-aminobenzoic acid (PABA) used as a marker for completeness of 24 h urine: effects of age and dosage scheduling. Eur J Clin Nutr 2003; 57: 138–142.

Jenab, M., Slimani, N., Bictash, M., Ferrari, P., & Bingham, S. A.. Biomarkers in nutritional epidemiology: Applications, needs and new horizons. Human Genetics, 2009; 125(5-6), 507-525. DOI:10.1007/s00439-009-0662-5.

Johnson, I.T., Southgate, D.A., Durnin, J.V. Intrinsic and non-milk extrinsic sugars: does

the distinction have analytical or physiological validity? *Int J Food Sci Nutr* 1996;47(2):131-40.

Johnson, R., Appel, L., & Brands M, e. a.. Dietary sugars intake and cardiovascular health: A scientific statement from the american heart association. Circulation, 2009; 120(11):1011-1020.

Johnson, R. J., Segal, M. S., Sautin, Y., Nakagawa, T., Feig, D. I., Kang, D. H., . . . Sanchez-Lozada, L. G.. Potential role of sugar (fructose) in the epidemic of hypertension, obesity and the metabolic syndrome, diabetes, kidney disease, and cardiovascular disease. The American Journal of Clinical Nutrition, 2007; 86(4), 899-906. DOI:86/4/899.

Kaaks, R., & Lukanova, A. Energy balance and cancer: The role of insulin and insulin-like growth factor-I. The Proceedings of the Nutrition Society, 2001; 60(1), 91-106. DOI:S002966510100012X.

Kaaks, R., Riboli, E., & Sinha, R. Biochemical markers of dietary intake. IARC Scientific Publications, 1997; (142)(142), 103-126.

Kawabata, H., Meddings, J. B., Uchida, Y., Matsuda, K., Sasahara, K., & Nishioka, M.. Sucrose permeability as a means of detecting diseases of the upper digestive tract. Journal of Gastroenterology and Hepatology, 1998; 13(10), 1002-1006.

Key, T. J., Allen, N. E., Spencer, E. A., & Travis, R. C.. The effect of diet on risk of cancer. Lancet, 2002; 360(9336), 861-868. DOI:S0140-6736(02)09958-0.

Kipnis, V., Subar, A. F., Midthune, D., Freedman, L. S., Ballard-Barbash, R., Troiano, R. P., Carroll, R. J.. Structure of dietary measurement error: Results of the OPEN biomarker study. American Journal of Epidemiology, 2003; 158(1), 14-21; discussion 22-6.

Krebs-Smith, S. M., Graubard, B. I., Kahle, L. L., Subar, A. F., Cleveland, L. E., & Ballard-Barbash, R.. Low energy reporters vs others: A comparison of reported food intakes. European Journal of Clinical Nutrition, 2000; 54(4), 281-287.

Kuhnle, G. G., Joosen, A. M., Wood, T. R., Runswick, S. A., Griffin, J. L., & Bingham, S. A.. Detection and quantification of sucrose as dietary biomarker using gas chromatography and liquid chromatography with mass spectrometry. Rapid Communications in Mass Spectrometry, 2008; RCM, 22(3), 279-282. DOI:10.1002/rcm.3355.

Lara, J. J., Scott, J. A., & Lean, M. E. Intentional mis-reporting of food consumption and its relationship with body mass index and psychological scores in women. Journal of Human Nutrition and Dietetics, 2004; 17(3), 209-218. DOI:10.1111/j.1365-

277X.2004.00520.

Lean, M. E., & Te Morenga, L. Sugar and type 2 diabetes. British Medical Bulletin, 2016; 120(1), 43-53. DOI:ldw037.

Luceri, C., Caderni, G., Lodovici, M., Spagnesi, M. T., Monserrat, C., Lancioni, L., & Dolara, P.. Urinary excretion of sucrose and fructose as a predictor of sucrose intake in dietary intervention studies. Cancer Epidemiol Biomarkers Prev 1996; 5(3), 167-171.

Mann, J., & Truswell, S.. Essentials of human nutrition (Third Edition ed.). New York, United States: Oxford University Press. 2007.

McCance and Widdowson. Food standards agency, research IoF. McCance and widdowson's the composition of foods. (6th summary ed.). Cambridge: Royal Society of Chemistry, 2002.

Nakamura, H., & Tamura, Z.. Gas chromatographic analysis of mono- and disaccharides in human blood and urine after oral administration of disaccharides. Clinica Chimica Acta, 1972; International Journal of Clinical Chemistry, 39(2), 367-381.

"National Health and Nutrition Examination Survey." National Center for Health Statistics, Centers for Disease Control and Prevention, 2017; www.cdc.gov/nhanes/index.htm.

Newens, K. J., & Walton, J.. A review of sugar consumption from nationally representative dietary surveys across the world. Journal of Human Nutrition and Dietetics : The Official Journal of the British Dietetic Association, 2016; 29(2), 225-240. DOI:10.1111/jhn.12338.

Park, S., Thompson, F. E., McGuire, L. C., Pan, L., Galuska, D. A., & Blanck, H. M. Sociodemographic and behavioral factors associated with added sugars intake among US adults. Journal of the Academy of Nutrition and Dietetics, 2016; 116(10), 1589-1598. DOI:10.1016/j.jand.2016.04.012.

Patil, P., Shah, V., & Shah, B.. Comparison of spot urine protein creatinine ratio with 24 hour urine protein for estimation of proteinuria. The Journal of the Association of Physicians of India, 2014; 62(5), 406-410.

Perrine, C. G., Cogswell, M. E., Swanson, C. A., Sullivan, K. M., Chen, T. C., Carriquiry, A. L., et al. Comparison of population iodine estimates from 24-hour urine and timed-spot urine samples. Thyroid : Official Journal of the American Thyroid Association, 2014; 24(4), 748-757. DOI:10.1089/thy.2013.0404.

Powell, E. S., Smith-Taillie, L. P., & Popkin, B. M.. Added sugars intake across

the distribution of US children and adult consumers: 1977-2012. Journal of the Academy of Nutrition and Dietetics, 2016; 116(10), 1543-1550.e1. DOI:10.1016/j.jand.2016.06.003.

Prentice, R. L., Mossavar-Rahmani, Y., Huang, Y., Van Horn, L., Beresford, S. A., Caan, B., et al. Evaluation and comparison of food records, recalls, and frequencies for energy and protein assessment by using recovery biomarkers. American Journal of Epidemiology, 2011; 174(5), 591-603. DOI:10.1093/aje/kwr140.

Price, G. M., Paul, A. A., Cole, T. J., & Wadsworth, M. E.. Characteristics of the low-energy reporters in a longitudinal national dietary survey. The British Journal of Nutrition, 1997; 77(6), 833-851. DOI:S0007114597000846.

Renehan, A. G., Zwahlen, M., & Egger, M. Adiposity and cancer risk: New mechanistic insights from epidemiology. Nature Reviews.Cancer, 2015; 15(8), 484-498. DOI:10.1038/nrc3967.

Robinson-Cohen, C., Ix, J. H., Smits, G., Persky, M., Chertow, G. M., Block, G. A., & Kestenbaum, B. R.. Estimation of 24-hour urine phosphate excretion from spot urine collection: Development of a predictive equation. Journal of Renal Nutrition, 2014; 24(3), 194-199. DOI:10.1053/j.jrn.2014.02.001.

Schoeller, D. A. Recent advances from application of doubly labeled water to measurement of human energy expenditure. The Journal of Nutrition, 1999; 129(10), 1765-1768.

Sharma, R. S., Joy, R. C., Boushey, C. J., Ferruzzi, M. G., Leonov, A. P., & McCrory, M. A. Effects of para-aminobenzoic acid (PABA) form and administration mode on PABA recovery in 24-hour urine collections. Journal of the Academy of Nutrition and Dietetics 2014; 114(3), 457-463. DOI:10.1016/j.jand.2013.07.045.

Subar, A. F., Kipnis, V., Troiano, R. P., Midthune, D., Schoeller, D. A., Bingham, et al. Using intake biomarkers to evaluate the extent of dietary misreporting in a large sample of adults: The OPEN study. American Journal of Epidemiology, 2003; 158(1), 1-13.

Tasevska, N., Midthune, D., Potischman, N., Subar, A. F., Cross, A. J., Bingham, S. A.. Use of the predictive sugars biomarker to evaluate self-reported total sugars intake in the observing protein and energy nutrition (OPEN) study. Cancer Epidemiol Biomarkers Preven 2011; 20(3), 490-500. DOI:10.1158/1055-9965.EPI-10-0820.

Tasevska, N., Runswick, S. A., & Bingham, S. A. Urinary potassium is as reliable as urinary nitrogen for use as a recovery biomarker in dietary studies of free living individuals. The Journal of Nutrition, 2006; 136(5), 1334-1340. DOI:136/5/1334.

Tasevska, N., Runswick, S. A., McTaggart, A., & Bingham, S. A. Urinary sucrose and fructose as biomarkers for sugar consumption. Cancer Epidemiol Biomarkers Preven 2005; 14(5), 1287-1294. DOI:14/5/1287.

Tasevska, N. Urinary sugars-A biomarker of total sugars intake. Nutrients, 2015; 7(7), 5816-5833. DOI:10.3390/nu7075255.

Te Morenga, L., Mallard, S., & Mann, J.. Dietary sugars and body weight: Systematic review and meta-analyses of randomised controlled trials and cohort studies. BMJ (Clinical Research Ed.), 2012; 346, e7492. DOI:10.1136/bmj.e7492.

Te Morenga, L. A., Howatson, A. J., Jones, R. M., & Mann, J.. Dietary sugars and cardiometabolic risk: Systematic review and meta-analyses of randomized controlled trials of the effects on blood pressure and lipids. The American Journal of Clinical Nutrition, 2014; 100(1), 65-79. DOI:10.3945/ajcn.113.081521.

Thompson, F.E., Subar, A.F. Dietary assessment methodology. in: A.M. Coulston, C.J. Boushey, M.G. Ferruzzi (Eds.) Nutrition in the prevention and treatment of disease. 3rd ed. Academic Press, New York, 2012:5–46.

Tooze, J. A., Subar, A. F., Thompson, F. E., Troiano, R., Schatzkin, A., & Kipnis, V.. Psychosocial predictors of energy underreporting in a large doubly labeled water study.The American Journal of Clinical Nutrition, 2004; 79(5), 795-804.

University of Minnesota, Nutrition Coordinating Center (NCC). Food and Nutrient Database. Nutrient Completeness. Internet: http://www.ncc.umn.edu/products/databasenutrientcompleteness2.html. (accessed 2017).

US Department of Agriculture. USDA National Nutrient Database for Standard Reference, Release 27. Agricultural Research Service; 2014. Internet: http://www.ars.usda.gov/Services/docs.htm?docid=8964

US Department of Agriculture and US Department of Health and Human Services. Dietary Guidelines for Americans, 2010. 7th ed. Washington, DC: US Government Printing Office, 2010.

US Department of Health & Human Services and USDA. 2016. New dietary guidelines to encourage healthy eating patterns to prevent chronic diseases.

Vos, M. B., Kaar, J. L., Welsh, J. A., Van Horn, L. V., Feig, D. I., Anderson, C. A. Added sugars and cardiovascular disease risk in children: A scientific statement from the american heart association. Circulation, 2016; 134:00-00. DOI:CIR.0000000000000439.

Wang, C. Y., Carriquiry, A. L., Chen, T. C., Loria, C. M., Pfeiffer, C. M., Liu, K., et al. Estimating the population distribution of usual 24-hour sodium excretion from timed urine void specimens using a statistical approach accounting for correlated measurement errors. The Journal of Nutrition, 2015; 145(5), 1017-1024. DOI:10.3945/jn.114.206250.

Wang, C. Y., Cogswell, M. E., Loria, C. M., Chen, T. C., Pfeiffer, C. M., Swanson, C. et al. Urinary excretion of sodium, potassium, and chloride, but not iodine, varies by timing of collection in a 24-hour calibration study. The Journal of Nutrition, 2013; 143(8), 1276-1282. DOI:10.3945/jn.113.175927.

Willet, W. Nutritional epidemiology (3rd ed.). United States of America: Oxford University Press. 2012.

Wittekind, Anna, & Walton, Janette. Worldwide trends in dietary sugars intake. Nutrition Research Reviews, 2014; DOI: 10.1017/S0954422414000237.

World Health Organization. Guideline: Sugars intake for adults and children. 2015. ISBN 978 92 4 154902 8.

Yang, Q., Zhang, Z., Gregg, E. W., Flanders, W. D., Merritt, R., & Hu, F. B. Added sugar intake and cardiovascular diseases mortality among US adults. JAMA Internal Medicine, 2014; 174(4), 516-524. DOI:10.1001/jamainternmed.2013.13563.

APPENDIX A

IRB APPROVAL FORM

ASU Knowledge Enterprise Development

APPROVAL: EXPEDITED REVIEW

Natasha Tasevska
SNHP - Nutrition
602/827-2485
Natasha.Tasevska@asu.edu

Dear Natasha Tasevska:

On 5/22/2015 the ASU IRB reviewed the following protocol:

Type of Review:	Initial Study
Title:	Investigation of Biomarkers for Sugars Intake – A Controlled Feeding Study
Investigator:	Natasha Tasevska
IRB ID:	STUDY00002695
Category of review:	(3) Noninvasive biological specimens, (2)(a) Blood samples from healthy, non-pregnant adults, (4) Noninvasive procedures, (7)(b) Social science methods, (7)(a) Behavioral research
Funding:	Name: NCI: National Cancer Institute, Grant Office ID: FP00001446 , Funding Source ID: 1R01CA197902-01
Grant Title:	FP00001446 ;
Grant ID:	FP00001446 ;
Documents Reviewed:	• Appendix 7, Category: Participant materials (specific directions for them); • Appendix 12 - Consent Form, Category: Consent Form; • Appendix 3 - Screening Q, Category: Recruitment Materials; • Appendix 6, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • Appendix 7a, Category: Participant materials (specific directions for them); • Appendix 11, Category: Measures (Survey questions/Interview questions /interview guides/focus

	group questions); • Appendix 10, Category: Other (to reflect anything not captured above); • Appendix 4, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • Appendix 2 - Information Sheet, Category: Recruitment Materials; • FP 1446 Tasevska FP.pdf, Category: Sponsor Attachment; • Sugars Biomarkers Protocol, Category: IRB Protocol; • Appendix 1 - Study Advertisement, Category: Recruitment Materials; • Appendix 9, Category: Other (to reflect anything not captured above); • Appendix 8a, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • Appendix 5, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • IRB Exemption letter.pdf, Category: Off-site authorizations (school permission, other IRB approvals, Tribal permission etc); • IRB clarification requested May12.pdf, Category: Other (to reflect anything not captured above); • Appendix 8, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions);

The IRB approved the protocol from 5/22/2015 to 5/21/2016 inclusive. Three weeks before 5/21/2016 you are to submit a completed Continuing Review application and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 5/21/2016 approval of this protocol expires on that date. When consent is appropriate, you must use final, watermarked versions available under the "Documents" tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

cc:
Carol Johnston
Kate Zemek

APPENDIX B

RECRUITMENT FLYER

Investigation of Biomarkers for Sugars Intake

ARIZONA STATE UNIVERSITY
425 N 5rd Street
Phoenix, AZ 85004

www.Sugarsbio.org

602-827-2545
Sugarsbio@asu.edu

ASU School of Nutrition & Health Promotion

Want to earn $599?

Bored with cooking for yourself?

Are you healthy and 18-70 years old?

Then, we need your help for a 15-day feeding study conducted by ASU researchers. You will be consuming your "usual" diet and all meals will be provided for you. Participation is voluntary.

If you are interested, please call, email or check our website!

Sugarsbio@asu.edu 602-827-2545 www.Sugarsbio.org

APPENDIX C

SCREENING QUESTIONAIRE

SCREENING QUESTIONNAIRE

We are inviting you to participate in a study to help researchers at Arizona State University identify urine and blood biomarkers that can predict how much sugars people are consuming. You will be prompted with up to 19 questions to determine if you would be eligible to participate in the study. Please, allow approximately **10 minutes to complete the questionnaire**.

Participation in this questionnaire is completely voluntary and you may exit the questionnaire at any time. This questionnaire will only be used to determine if you are eligible to enter the study. The data will not be published in any way and will only be used for screening purposes. Please feel free to contact our Project Coordinator Wondra Lee at 602-827-2545 or sugarsbio@asu.edu for any questions on the study or if you prefer to complete the questionnaire on paper or over the phone.

Thank you for your interest in our study!

Q1 What is your gender?
- ❍ Male (1)
- ❍ Female (2)

Q2 Please select your age group:
- ❍ 18-34 years of age (1)
- ❍ 35-44 years of age (2)
- ❍ 45-54 years of age (3)
- ❍ 55-70 years of age (4)
- ❍ None of the above (5)

If answer is None of the above (5), then END OF SURVEY.

Q3 Zip Code of residence over the next 3 months:

Q4 Do you use tobacco products (e.g., cigarettes, chewing tobacco, cigars)?
- ❍ Yes (1)
- ❍ No (2)

If answer is YES, then END OF SURVEY.

Answer If: What is your gender? Female Is Selected

Q5 Are you pregnant or planning on becoming pregnant in the next 15 weeks?
- ❍ Yes (1)
- ❍ No (2)

If answer is YES, then END OF SURVEY.

Answer If: What is your gender? Female Is Selected

Q6 Are you currently breastfeeding?

- ❍ Yes (1)
- ❍ No (2)

If answer is YES, then END OF SURVEY.

Q7 Have you participated in any diet-related research study over the past 4 months?

- ❍ Yes (1)
- ❍ No (2)
- ❍ Unsure (3)

If answer is YES, then END OF SURVEY.

Q8 Are you currently trying to lose weight or have you been trying to lose weight at any point over the past 4 months?

- ❍ Yes (1)
- ❍ No (2)

If answer is YES, then END OF SURVEY.

Q9 Do you currently take supplements (vitamins, minerals, herbal supplements, etc.)?

- ❍ Yes (1)
- ❍ No (2)

Answer If: Do you currently take supplements (vitamins, minerals, herbal supplements, etc.)? Yes Is Selected

Q10 Would you be willing to restrict supplement use during the 10-week study period?

❍ Yes (1)
❍ No (2)

If answer is NO, then END OF SURVEY.

Q11 Do you have a refrigerator and freezer at your residence to store food?

❍ Yes (1)
❍ No (2)

If answer is NO, then END OF SURVEY.

Q12 Has a doctor every told you that you have any of the following conditions?

	Yes (1)	No (2)
Kidney Disease (1)	❍	❍
Bladder Disorder/Urinary Incontinence (2)	❍	❍
Type 2 Diabetes (3)	❍	❍
Autoimmune Diseases (e.g., Type 1 Diabetes, Inflammatory bowel disease, etc.) (4)	❍	❍
Celiac Disease (5)	❍	❍
Stomach Disorder (e.g., ulcers, GI bleeding) (6)	❍	❍

Answer If: Has a doctor every told you that you have any of the following conditions? Autoimmune Diseases (i.e., Type 1 Diabetes, Inflammatory bowel disease, etc.) - Yes Is Selected

Q13 Please specify which autoimmune disorder, if known (if unknown, please write unknown):

Answer If: Has a doctor every told you that you have any of the following conditions? Stomach Disorder (e.g., ulcers, GI bleeding) - Yes Is Selected

Q14 Please specify which stomach disorder, if known (if unknown, please write unknown):

_____

_____

Q15 Are you on any kind of dietary restriction due to a medical condition? (e.g., inflammatory bowel disease, celiac disease, etc.)

- ❍ Yes (1)
- ❍ No (2)

Answer If: Are you on any kind of dietary restriction due to a medical condition? (e.g., inflammatory bowel disease, celiac disease, etc.) Yes Is Selected

Q16 Please give details to the dietary restriction here:

_____

_____

Q17 Please provide your height:

Feet (1) _____

Inches (2) _____

Q18 Please provide your weight in pounds:

Pounds (1) _____

Q19 Have you ever experienced an allergic reaction to sunscreen, amino benzoate potassium (POTABA) or para-amino benzoic acid (PABA)?

- ❍ Yes (1)
- ❍ No (2)

If answer is YES, then END OF SURVEY.

(EXIT SURVEY message)

Thank you for taking the time to complete the questionnaire. Unfortunately, based on the answers you have provided you are not, at this point, a candidate for this study. The answers you have provided will not be retained under any circumstances.

After this message is displayed the participant will be presented with an 'exit survey' link.

The following question, which asks about contact information will only be displayed if the respondent is eligible to participate, i.e., has not been skipped to the end of the screening questionnaire after answering any of the 7 exclusion questions. Only respondents who may be eligible to participate in the study will be asked to provide identifying information.

Thank you for filling out the questionnaire. Based on your answers, you may be eligible to take part in this study. Please provide your contact information, so we can tell you more about the study, and schedule a study visit, if you agree to participate. The study will take place at the Arizona Biomedical Collaborative Bldg.1 located on 5th St. and Van Buren in downtown Phoenix.

Q20 Contact information so we may have the opportunity to follow up with you:

Last Name (1)
First Name (2)
Email (3)
Phone (numbers only) (4)

APPENDIX D

INFORMATION SHEET

STUDY INFORMATION SHEET

INVESTIGATION OF BIOMARKERS FOR SUGARS INTAKE

You are invited to participate in our new study. Please, take a few minutes to read about the study and to consider if you would like to take part in it. Your participation is absolutely on a voluntary basis and you may refuse without giving any explanation.

WHAT IS THE PURPOSE OF THE STUDY?

Sugars are thought to play very important role in developing many diseases such as diabetes, cancer and cardiovascular disease. To see if this is true we need to measure the food people eat accurately, and if type of food people eat relates to the sort of diseases they develop. This study will test how accurately urine and blood biomarkers can predict how much sugars people are consuming.

WHY HAVE I BEEN INVITED TO TAKE PART IN THE STUDY?

You are invited to participate because we wish to recruit healthy, non-smoking volunteers, like yourself.

DO I QUALIFY?

- YES, if you (i) are 18-70 years of age, (ii) have a BMI <35 kg/m^2, and (iii) reside in the Phoenix metropolitan area;
- NO, if you (1) suffer from kidney disease or bladder incontinence; (ii) are or have been on any kind of dietary restriction due to a weight loss at any point over the last 4 months; (iii) are on any kind of dietary restriction due to a medical condition; (iv) participated in a diet-related research study over the past 4 months; (v) have type 2 or type 1 diabetes or your fasting blood glucose ≥100 mg/dl or HbA1c ≥5.7% (checked at screening); (vi) smoke, or (vii) are allergic to sunscreen, or any part of aminobenzoate potassium (POTABA) or para-amino benzoic acid (PABA).

WHO IS ORGANISING THE STUDY?

The study is organised by the School of Nutrition and Health Promotion at Arizona State University. Our office is situated in the Arizona Biomedical Collaboration Building 1 on the corner of 5th Street and E Van Buren Street in downtown Phoenix, AZ. Here, in the School of Nutrition and Health Promotion, we are interested in the influence of food on human health.

WHAT WILL BE ASKED FROM ME?

If you decide to take part in the study:

- A **screening fasting blood sample** will be taken to check your plasma glucose and HbA1c level. If your fasting blood glucose <100 mg/dl and HbA1c <5.7%, you will be scheduled for a baseline visit.
- **Your body weight and height will be measured.**
- **You will complete a questionnaire**, with questions on your demographics, lifestyle habits, and personal and family medical history.
- **You will record all the foods and drinks you consume over two weeks**. You will be given a food diary with set of instructions and pictures to help you record your diet. Following each

week, you will meet with our Research Kitchen Coordinator and Chef to discuss what you have recorded in your food diary and help us gather more information.

- A week after you have completed the 2-wk food diary, **you will participate in a 15-day feeding study**. During the feeding period, you will be provided with all your food on a daily basis. This is the food that you would usually eat, which we have purchased and prepared for you based on the food diaries you kept over the previous 2 weeks. You will come to our kitchen daily Monday-Friday where you will eat your breakfast or lunch, and then collect your dinner, snacks and breakfast or lunch for the following day. On Fridays, you will collect your food for the entire weekend. We will provide you with cooler bags on wheels to ease the transport of meals to your home. You will be free to eat as much as you like from the food prepared for you, and you will NOT be allowed to consume any foods or drinks prepared outside of our kitchen, besides water, alcohol, and black coffee and tea (no added sugar, sweetener, milk, creamer, etc.). If you drink alcohol, you will record the type and amount consumed; you are allowed to drink wine, beer or spirits (i.e., hard liquor, such as whisky, vodka, tequila, gin, etc.) ONLY. Please note that any alcohol beverages that contain added sugars, fruits, cream, spices, herbs, flowers or nuts, such as liqueurs (e.g., Grand Marnier, schnapps) or cocktails are not allowed. We ask you to keep your intake of coffee and tea consistent throughout the 15-day feeding study. You will keep the unconsumed food/drinks in the respective container/bottle and return them to the metabolic kitchen on your next visit. Please note that no one else is allowed to eat the leftovers, and you have to return all leftovers to the metabolic kitchen, so we can calculate exactly how much food you have consumed.
- You will **collect nine breath samples during the 15-day feeding study** (three samples per day on three randomly selected days; on the breath collections days, you will collect one breath sample before breakfast, and two others at randomly selected time points during the day).
- **We will collect 3 blood samples from you:** before and at the end of the 15-day feeding study and 5 weeks later.
- **You will collect 24-hour urine every other day during the 15-day feeding study (8 in total)**. On two urine collection days, you will collect each of your urine voids in a separate container. We will give you a trolley bag for carrying urine bottles when away from home. To alleviate your burden, we will organize a pick-up service to collect the 24-h urine from your home the morning after the urine collection day (including weekend and holiday). In order to determine whether the collections are complete, you will be requested to take a capsule of aminobenzoate potassium (POTABA) with your breakfast, lunch and dinner (three capsules per day) on the urine collection days. POTABA is commonly used as a marker for urine completeness in research studies, as it is nearly completely excreted in the urine soon after taking a tablet of POTABA.
- **You will keep study logs during the 15-day feeding study**: a brief physical activity log (<5 minutes to complete), and a meal checklist daily, and a urine collection log on the urine collection days.
- **You will be asked to refrain from taking any dietary supplements** during the feeding study and until collection of the final blood sample.

ARE THERE ANY RISKS OF TAKING PART?

There is no risk related to the participation in the study. Very few side effects have been associated with taking POTABA capsules, the marker for ensuring 24-h urine completeness. Reported side effects include upset stomach and skin allergy. An allergic reaction to sunscreen may indicate that side effects from POTABA may occur. If you are allergic to sunscreens, you may not qualify to participate in the study.

HOW WILL CONFIDENTIALITY BE MAINTAINED?

All details held by us will be treated with strict confidentiality. In all publications resulting from the study, a study number will be used to refer to volunteers. Your identity will be known only by the people conducting the study. At the end of the study we will be happy to explain individual results.

WILL I BE REMUNERATED?

You will receive $10 a day for keeping a record of your diet during the two weeks; $20 per day during the 15-day feeding period; and an extra $159 for completion of the whole study to a total of $599. You may withdraw from the study, without explanation, at any time. If you decide to withdraw, you will only be compensated for the portions of the protocol you completed.

INTERESTED IN PARTICIPATING IN THE STUDY?

Please contact Cassandra Kettenhoven at sugarsbio@asu.edu or 602-827-2545 to complete a brief 2-page screening questionnaire, so we can determine if you are eligible to participate. You can also complete the screening questionnaire through our website at www.sugarsbio.org. The information from the screening questionnaire will be used to determine if you are eligible to enter the study, and to describe the recruitment process in reports, however, you will not be identified in any way and under no circumstances. You may also schedule a visit to our study center, located at 425 N 5th St, Phoenix, AZ 85004, to hear more about the study and to ask questions.

APPENDIX E

CONSENT FORM

CONSENT FORM

What is the purpose of this form?

The purpose of this form is to provide you (as a prospective research study participant) with information that may affect your decision as to whether or not you would want to participate in this study and to record your consent that you agree to take part in the study.

Who are the researchers?

Dr. Natasha Tasevska, an Assistant Professor at the Arizona State University (ASU) School of Nutrition and Health Promotion, is inviting you to participate in a research study that will be conducted over 11 weeks.

Why am I being invited to take part in a research study?

We are asking you to take part in this research study because we wish to recruit healthy, non-smoking volunteers 18-70 years, like yourself.

Why is this research being done?

Sugars are thought to play very important role in developing many diseases such as diabetes, cancer and cardiovascular disease. To see if this is true we need to measure the food people eat accurately and see if type of food people eat relates to the sort of diseases they develop. This study will test how accurately urine and blood biomarkers can predict the usual consumption of sugars.

How long will the research last?

While the study will run over 11 weeks, individuals will spend one month actively participating in the proposed activities.

How many people will be studied?

We plan to recruit 107 people in this research study.

What happens if I say yes, I want to be in this research?

It is up to you to decide whether or not to participate. If you decide to take part in the study:

- A **screening fasting blood sample** will be taken to check your plasma glucose and HbA1c levels. If your fasting blood glucose <100 mg/dl and HbA1c <5.7%, you will be scheduled for a baseline visit.
- **Your body weight and height will be measured.**
- **You will complete a questionnaire** with questions on your demographics, lifestyle habits, and personal medical history.
- **You will record all the foods and drinks you consume over two weeks**. For that purpose, you will be given a food diary in which you will find set of instructions to help you record your diet, and measuring

cups, spoons, and a food model booklet to help you record quantities. Following each week, you will be invited to meet with our Research Kitchen Coordinator and Chef to discuss what you have recorded in your food diary and help us gather more information.

- A week after you have completed the 2-wk food diary, **you will participate in a 15-day feeding study**. During the feeding period, you will be provided with all your food on a daily basis. This is the food that you would usually eat, which we have purchased and prepared for you based on the food diaries you kept over the previous 2 weeks. You will come to our kitchen daily Monday-Friday where you will eat your breakfast or lunch and then collect your dinner, snacks and breakfast or lunch for the following day. On Fridays, you will collect your food for the entire weekend. We will provide you with cooler bags on wheels to ease the transport of meals to your home. You will be free to eat as much as you like from the food prepared for you, and you will NOT be allowed to consume any foods or drinks prepared outside of our kitchen, besides water, alcohol, and black coffee and tea (no added sugar, sweetener, milk, creamer, etc.). If you drink alcohol, you will record the type and amount consumed; you are allowed to drink wine, beer or spirits (i.e., hard liquor, such as whisky, vodka, tequila, gin, etc.) ONLY. Please note that any alcohol beverages that contain added sugars, fruits, cream, spices, herbs, flowers or nuts, such as liqueurs (e.g., Grand Marnier, schnapps) or cocktails are not allowed. We ask you to keep your intake of coffee and tea consistent throughout the 15-day feeding study. You will keep the unconsumed food/drinks in the respective container/bottle and return them to the metabolic kitchen on your next visit. Please note that no one else is allowed to eat the leftovers, and you have to return all leftovers to the metabolic kitchen, so we can calculate exactly how much food you have consumed.
- You will **collect nine breath samples during the 15-day feeding study** (three samples per day on three randomly selected days; on the breath collections days, you will collect one breath sample before breakfast, and two samples at randomly selected time points during the day).
- **We will collect 3 blood samples from you:** before and at the end of the 15-day feeding study and 5 weeks later.
- **You will collect 24-hour urine every other day during the 15-day feeding study (8 in total).** On two urine collection days, you will collect each of your urine voids in a separate container. We will give you a trolley bag for carrying urine bottles when away from home. To alleviate your burden, we will organize a pick-up service to collect the 24-h urine from your home the morning after the urine collection day (including weekend and holiday). In order to determine whether the collections are complete, you will be requested to take a capsule of aminobenzoate potassium (POTABA) with your breakfast, lunch and dinner (three capsules per day) on the urine collection days. POTABA is commonly used as a marker for urine completeness in research studies, as it is nearly completely excreted in the urine soon after taking a tablet of POTABA.
- **You will keep study logs during the 15-day feeding study**: a brief physical activity log (<5 minutes to complete), and a meal checklist daily, and a urine collection log on the urine collection days.
- **You will be asked to refrain from taking any dietary supplements** during the feeding study and until collection of the final blood sample.

Samples will be stored and may be used at a later date to see if we can find other dietary biomarkers.

Participant Timeline:

Visit Timeline	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 12
Screening Visit	Day 1							
Baseline Visit	Day 4							
Food Diary		All Days	All Days					
Meeting with Chef	Day 4		Day1	Day 1				
Feeding Study					All Days	All Days	Day 1	
24-hour Urine Collection					Day 1, 3, 5, 7	Day 2, 4, 6	Day 1	
Blood Draw[†]	Day 1				Day 1[‡]		Day 2[‡]	Day 1[‡]
Breath Sample					Randomly selected day (3x)	Randomly selected day (3x)		

[†] 6 ml blood.

[‡] 24 ml per blood draw (3x).

What happens if I say yes, but I change my mind later?

Even if you say "yes" now, you are free to say "no" later, and withdraw from the study at any time. Your decision will not affect your relationship with Arizona State University or otherwise cause a loss of benefits to which you might otherwise be entitled. If you decide to leave the research, you should contact the investigator so that the investigator can notate your departure in our database. If you stop being in the research, already collected data may not be removed from the study database. If it becomes evident that you are not complying with the feeding, urine collection or blood collection protocol, the research staff may remove you from the study without your consent. If this occurs, you will only be compensated for the portions of the protocol you completed.

Is there any way being in this study could be bad for me?

There are no risks associated with the feeding portion of the study. All food safety precautionary measures will be taken to ensure safe food handling and prevention of food borne illnesses. You may experience slight pain from the blood draws (4 in total, including the blood draw at screening). Although unlikely, some bruising and/or infection can occur from the blood draws. You may be inconvenienced by collecting 24-h urines (8 in total) and by not being able to eat or drink anything prepared outside of our kitchen (except for water, alcohol, coffee and tea) during the 15-day feeding study. On the urine collection days, you will be asked to take three 102 mg capsules of POTABA, one with each main meal, as a marker for 24-h urine completeness. Only few instances of side effects, such as upset stomach, nausea, loss of appetite, fever and skin allergy (rash), have been reported following intake of POTABA, and in doses much larger than the dose in this study. If you experience these symptoms, please notify the research staff, and taking of the capsules will be discontinued. An allergic reaction

Signature of person obtaining consent | Date

Printed name of person obtaining consent

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided (offered) the subject/participant a copy of this signed consent document."

Signature of investigator | Date

Printed name of investigator

to sunscreen may indicate that side effects from POTABA can occur. At screening, you have informed us that you have never experienced an allergic reaction to sunscreens.

Will being in this study help me in any way?

You will be given the results on your fasting blood glucose and HBA1c level from your screening blood collection. We cannot promise any other benefits from taking part in this research to you directly. However, the potential benefit to others is large, due to long-term public health impact of this project. This study will help in determining the role of sugars in risk of obesity, cardiovascular disease, cancer, type 2 diabetes, and other chronic diseases.

What happens to the information collected for the research?

All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but the researchers will not identify you. In order to maintain confidentiality of your records, we will assign you a participant number at study entry, which will be used on all forms, meals and specimens. Your name will not appear anywhere aside from this consent form. This form will be kept in a locked cabinet in Dr. Natasha Tasevska's office to maintain your confidentiality.

What else do I need to know?

This research is being funded by the National Institutes of Health (NIH).

If you agree to take part in this research study, we will pay you up to $599: $10/day for keeping food diary for 2 weeks, $20/day during the 15-day dietary study and an additional $159 as an incentive for completing the entire study protocol. If you agree to participate in the study, this consent does not waive any of your legal rights. However, no funds have been set aside to compensate you in the event of injury.

At the end of this research project, we will be happy to explain individual results.

Who can I talk to?

If you have questions, concerns, or complaints, or think the research has hurt you, please contact **Natasha Tasevska,** at Natasha.Tasevska@asu.edu or 602 827-2485 or **Cassandra Kettenhoven**, Project Coordinator, at Cassandra.Kettenhoven@asu.edu or 602-827-2545.

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk; you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at 480-965 6788 or research.integrity@asu.edu.

Signature Block for Capable Adult

Your signature documents your permission to take part in this research.

Signature of participant	Date

Printed name of participant

Signature of person obtaining consent | Date

Printed name of person obtaining consent

INVESTIGATOR'S STATEMENT

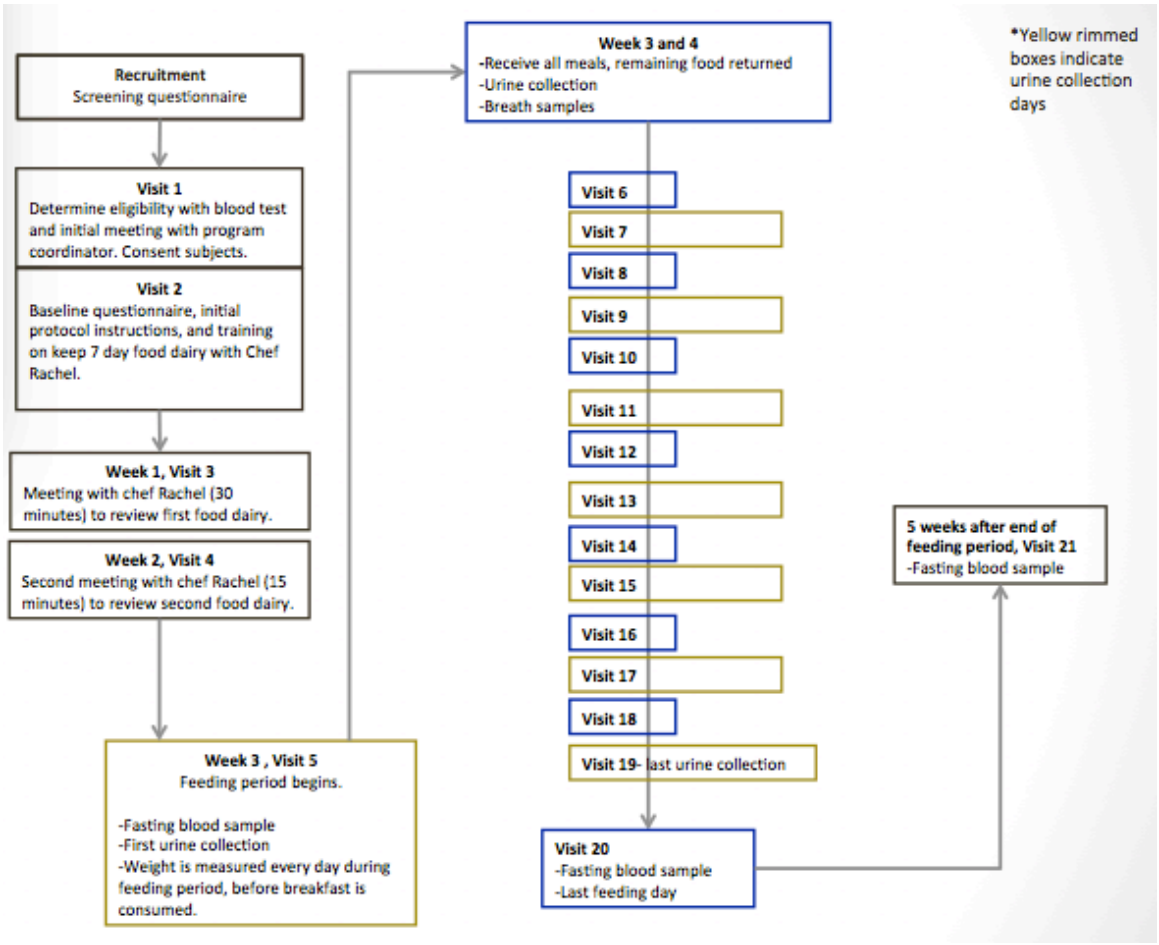
"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided (offered) the subject/participant a copy of this signed consent document."

Signature of investigator | Date

Printed name of investigator

APPENDIX F

STUDY FIGURE


Recruitment
Screening questionnaire
Visit 1
Determine eligibility with blood test and initial meeting with program coordinator. Consent subjects.
Visit 2
Baseline questionnaire, initial protocol instructions, and training on keep 7 day food dairy with Chef Rachel.
Week 1, Visit 3
Meeting with chef Rachel (30 minutes) to review first food dairy.
Week 2, Visit 4
Second meeting with chef Rachel (15 minutes) to review second food dairy.
Week 3 , Visit 5
Feeding period begins.
-Fasting blood sample
-First urine collection
-Weight is measured every day during feeding period, before breakfast is consumed.
Week 3 and 4
-Receive all meals, remaining food returned
-Urine collection
-Breath samples
Visit 6
Visit 7
Visit 8
Visit 9
Visit 10
Visit 11
Visit 12
Visit 13
Visit 14
Visit 15
Visit 16
Visit 17
Visit 18
Visit 19- last urine collection
Visit 20
-Fasting blood sample
-Last feeding day
5 weeks after end of feeding period, Visit 21
-Fasting blood sample
*Yellow rimmed boxes indicate urine collection days

APPENDIX G

BASELINE QUESTIONAIRE

BASELINE QUESTIONNAIRE

Q1.1 Study ID: _____

Q1.2 General Information

Title
Last Name
First Name
Address
City
State
Zip Code
Email

Q1.3 Date of Birth:

|__|__| |__|__| |__|__|__|__|
MO DAY YEAR

Q1.4 Please provide contact phone numbers:

	Area Code (XXX)	XXX	XXXX
Mobile Phone			
Home Phone			
Work Phone			

Q1.5 Preferred method of contact:

- ❍ Email
- ❍ Mobile Phone
- ❍ Home Phone
- ❍ Work Phone

Q1.6 Which method do you prefer to receive your reminder notification to begin the 24-hour urine collection?

- ❍ Email
- ❍ Mobile Phone
- ❍ Home Phone
- ❍ Work Phone

Q1.7 Occupation _____

Q1.8 Gender:
- ❍ Male
- ❍ Female

Q1.9 What is your current marital status?
- ❍ Single (never married)
- ❍ Living with a partner
- ❍ Married
- ❍ Divorced
- ❍ Widowed
- ❍ Other (please specify): _____

Q1.10 What is your education level?
- ❍ Less than high school
- ❍ High school/GED
- ❍ Some college
- ❍ Associate's Degree
- ❍ Bachelor's Degree
- ❍ Master's Degree
- ❍ Terminal Degree (PhD, MD, etc.)

Q1.11 What is your annual family income?
- ❍ < $15,000
- ❍ $15,000-$24,999
- ❍ $25,000-$44,999
- ❍ $45,000-$64,999
- ❍ $65,000-$84,999
- ❍ $85,000-$104,999
- ❍ >$105,000

Q1.12 What is your ethnicity?
- ❍ White/Non-Hispanic/Caucasian
- ❍ Hispanic/Latino
- ❍ African American
- ❍ Pacific Islander (Native Hawaiian, Guamanian, Samoan, Polynesian, Micronesian or Melanesian, etc.)
- ❍ Native American
- ❍ Asian
- ❍ Other: (Please specify) _____

YOUR LIFESTYLE

Q2.2 Have you smoked in the past?

- ❍ Yes
- ❍ No

Answer If Have you smoked in the past? Yes Is Selected

Q2.3 If yes, how often did you smoke? Choose the best answer that describes your average past smoking habits.

- ❍ Less than once a day
- ❍ Once a day
- ❍ Half a pack a day
- ❍ One pack a day
- ❍ Two packs a day
- ❍ Greater than two packs a day

Answer If Have you smoked in the past? Yes Is Selected

Q2.4 How long did you smoke?

	Please List Years and Months
	(e.g., 1 year 6 months)
Years	
Months	

Answer If Have you smoked in the past? Yes Is Selected

Q2.5 When did you quit smoking?

	Please type full month and year
	(e.g., August 2011)
Month	
Year	

Q2.6 Do you drink alcohol?

- ❍ Yes
- ❍ No

Answer If Do you drink alcohol? Yes Is Selected

Q2.7 How frequently do you drink alcohol?

- ❍ Everyday
- ❍ Twice a week
- ❍ Once a week
- ❍ Every other week
- ❍ Once a month
- ❍ Less than once a month

Answer If Do you drink alcohol? Yes Is Selected

Q2.8 On average, how many units of alcohol do you consume per week? (One unit = 12 oz of beer, 5 oz of wine, or a 1.25 oz of liquor)

Units per week: _____

Q2.9 Do you have any special dietary requirements or food allergies?

- ❍ Yes
- ❍ No

Answer If Do you have any special dietary requirements or food allergies? Yes Is Selected

Q2.10 If you answered yes to special dietary requirements or food allergies, please specify:

Q2.11 Please indicate the total time you spend in each activity category for an average week: (0-10 = hours per week)

	0	1	2	3	4	5	6	7	8	9	10+
Light activities (slow walking, golf, easy swimming, gardening, etc.)	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍
Moderate activities (moderate walking, cycling, swimming, weight lifting, etc.)	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍
Vigorous activities (fast walking or cycling, jogging, heavy weight lifting, etc.)	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍	❍

YOUR MEDICAL HISTORY

Q3.2 Has a doctor every told you that you have any of the following conditions?

	Yes	No
Heart Disease	❍	❍
Kidney Disease	❍	❍
Bladder Disorder/Urinary Incontinence	❍	❍
Autoimmune Diseases (e.g., Type 1 Diabetes, Inflammatory bowel disease, etc.)	❍	❍
Stomach Disorder (e.g., ulcers, GI Bleeding)	❍	❍
Type 2 Diabetes	❍	❍
Celiac Disease	❍	❍
Thyroid Problem	❍	❍
Cancer	❍	❍
Liver Disease	❍	❍
High Blood Pressure	❍	❍
Other Chronic Conditions (Please Specify)	❍	❍

Q3.3 Have you taken any medications, including sulfonamides (e.g., Azulfidine, Diamox, Sequels, Sulfazine, Truxazole), **acetaminophen** (Tylenol, Acephen, Anacin, Feverall etc.), **Furosemide** (Lasix, Furocot), **painkillers, aspirin, steroids, birth control, etc.** (prescribed or over the counter) **over the last 4 weeks?**

❍ Yes
❍ No

Answer If Have you taken any medications any medications, including... Yes Is Selected

Q3.4 If so, please list:

	Medication name and Brand	Frequency	Dose
Rx/OTC 1			
Rx/OTC 2			
Rx/OTC 3			
Rx/OTC 4			
Rx/OTC 5			

Answer If Have you taken any medications any medications, including... Yes Is Selected

Q3.5 Do you need to list any additional medications, including sulfonamides, acetaminophen, painkillers, aspirin, steroids, birth control, etc. (prescribed or over the counter)**?**

- ❍ Yes
- ❍ No

Answer If Do you need to list any additional medications, including…? Yes Is Selected

Q3.6 If so, please list:

	Medication name and Brand	Frequency	Dose
Rx/OTC 1			
Rx/OTC 2			
Rx/OTC 3			
Rx/OTC 4			
Rx/OTC 5			

Q3.7 Are you currently taking any dietary supplements, such as vitamins, minerals, bioactive compounds, fatty acids, herbal supplements - does NOT include herbal tea, etc. (prescribed or over the counter)**?**

- ❍ Yes
- ❍ No

Answer If Are you currently taking any dietary supplements, such as vitamins, minerals, bioactive compounds... Yes Is Selected

Q3.8 If so, please list:

	Supplement name and Brand	Frequency	Dose
Dietary supplement 1			
Dietary supplement 2			
Dietary supplement 3			
Dietary supplement 4			
Dietary supplement 5			

Answer If Are you currently taking any dietary supplements, such as vitamins, minerals, bioactive compounds... Yes Is Selected

Q3.9 Do you need to list any additional dietary supplements, such as vitamins, minerals, bioactive compounds, fatty acids, herbal supplements - does NOT include herbal tea, etc.?

❍ Yes
❍ No

Answer If Do you need to list any additional dietary supplements, such as vitamins, minerals, bioactive compounds... Yes Is Selected

Q3.10 If so, please list:

	Supplement name and Brand	Frequency	Dose
Dietary supplement 1			
Dietary supplement 2			
Dietary supplement 3			
Dietary supplement 4			
Dietary supplement 5			

Q3.11 Is there anything else that you would like to tell us or you think we should know that may affect your ability to participate in this study (i.e., pregnancy, infections, travel vacations, etc.)?

❍ Yes
❍ No

Answer If Is there anything else that you would like to tell us or think we should know that may affect... Yes Is Selected

Q3.12 If yes, please specify:

YOUR FAMILY DOCTOR AND EMERGENCY CONTACT DETAILS

Q4.1 Do you have a family doctor?

- ❍ Yes
- ❍ No

Answer If Do you have a family doctor? Yes Is Selected

Q4.2 Family doctor contact information:

Family doctor name
Address
City
State
Zip
Phone (use numbers only, no other spaces or characters. e.g., 8005551212)

Q4.3 Emergency contact information:

Name of emergency contact
Relationship to contact
Address
City
State
Zip
Phone (use numbers only, no other spaces or characters. e.g., 8005551212)

APPENDIX H

FOOD DIARY

FOOD DIARY

ARIZONA STATE UNIVERSITY

Investigation of Biomarkers for Sugars Intake

Instructions

We would like you to keep a record of everything you eat and drink over the next 7 days.

It's extremely important that you don't make changes from what you'd normally eat or drink when completing this.

As you will see, each day is marked in sections, beginning with the first thing you eat when you get up in the morning, and ending with an evening snack before bedtime. For each part of the day write down all food and drink consumed, the amounts and a description if necessary. If nothing is eaten or drunk during a part of the day, draw a line through that section or include N/A. <u>It is important that you record everything immediately after or at the time of eating, and not from memory at the end of the day.</u>

On the next 4 pages is a list of popular foods and drinks. Next to each item is the information we need to know so we can tell what the food item is made of and how much you had. This list cannot cover all foods and drinks, so try to relate to a similar item if any items that you have eaten are missing. Please give as much detail as you can.

Use provided measuring cups and spoons to help you estimate amounts. For some foods you may find it easier to describe how much you had by using the pictures in the food models booklet you received. Use one or the other approach to estimate amount.

Drinks and many packaged foods have weights printed on them, so please use these to show how much you consumed.

At the end of each day there is a list of snacks and drinks that can easily be forgotten. Please write down any extra items in here, if you have not already included them in some other part of the day.

FOR EACH ITEM THAT YOU EAT OR DRINK PLEASE READ THE FOLLOWING FOR DETAILS WE NEED:

Eat as you normally would.

Always state what sort of **oil** or **fat** was used for baking, frying, etc.

Give **brand and full name** of products where possible.

For **meals/snacks eaten away from home**, please note where these items were eaten, giving name and/or type of restaurant, café, bar, etc. where appropriate.

Record **serving sizes of meats in ounces** or by the piece (1 thigh and 1 breast of chicken).

Measure or estimate the volume of fluid in your glass or cup (4 fl oz, 8 fl oz). It's okay to estimate serving sizes for all foods.

Don't forget to record all the little "extras" like sugar in your coffee or on your cereal, fruit on your cereal, butter on your vegetables or bread, honey in tea, mustard, mayo or ketchup on sandwiches, cream in coffee and so forth.

Fully describe the food e.g., 2% milk, water-packed tuna, low-fat cottage cheese, low-salt Wheat Thins, tomato soup made with whole milk, sugar-free Jell-O, canned peaches in heavy syrup, breast of chicken with or without skin.

Check food labels for weights, etc., such as candy bars, individually wrapped cheeses, cookies and juices – write it down and/or take photos.

Please remember to provide us with as much detail as you possibly can

WHERE POSSIBLE, ALWAYS STATE WHAT SORT OF OIL OR FAT WAS USED FOR BAKING, FRYING, ETC.			
Food/Drink	**Description & Preparation**	**Amount**	**USDA Food Models**
Home-made dishes	Please say what the dish is called and give recipe or ingredients, including amounts	Ounces	Mounds, grid, block, wedges
Ready-made meals	What type; e.g. ,pizzas, microwave dishes, brand name. Please give main ingredients and nutritional information on packet, and enclose label and bar code if possible.	Weight from package	
Meals eaten away from home	What type; e.g., pizzas, Chinese, Indian, fish and chips, hamburgers, hot dogs. Please say what the dish is called and give ingredients where possible. Give the name of the restaurant.	Ounces	Mounds, grid, blocks, wedges
Cider	Sweet, dry, vintage, low alcohol, % alcohol	Ounces; number of cans/bottles, including size	Cups, mugs, glasses
Coffee	With/without milk, what sort; half and half; 1%, 2%, whole milk; ground, instant, decaffeinated/caffeinated; strong, average or weak, sweetened or unsweetened	Ounces	Cups, mugs
Spices	Pepper, salt, or other	½ or ¼ teaspoon, pinch, etc.	
Cooking oil	Type, brand name	Teaspoons	
Cream	Half, single, sour, whipping, double, clotted, low fat; fresh or substitute; sweetened or unsweetened	Tablespoons	
Chips	Brand name; baked or low-fat; low salt	Packet weight or number of chips	
Egg	How was it cooked: boiled, fried, scrambled, poached, omelette, etc.; full egg, egg white only, yolk only, etc; any oils or fat used to cook it	Number	

Food/Drink	Description & Preparation	Amount	USDA Tools
Fish	What sort, fried, boiled, grilled, poached, microwaved, pickled, smoked or salted; with batter or breadcrumbs; tinned with oil or tomato sauce; size; give brand name where applicable	Ounces	Blocks, grid
Fish sticks	What sort; large, medium or small size; fried or grilled; give brand name if possible	Number	Circles, blocks, inches
Fruit – fresh	What type and variety e.g. Granny Smith apple; cored, with or without skin, size	Number or cups	
Fruit – frozen or canned	What type and variety; with or without sugar; in fruit juice or syrup	Size, cups or can size	
Fruit – juice	What type; sweetened or unsweetened	Ounces	Glasses, cups
Gravy	Thick or thin, instant or packet, made with or without dripping, meat juices, etc.	Tablespoons	
Herbs	Type, fresh or dried	Teaspoons	
Honey, jam	Type, specify if low sugar	Teaspoons	
Ice-cream	Dairy or non-dairy, flavor or variety, brand name if possible	Cups	
Liver, Kidney	Pig, lamb, ox; fried or stewed	Picture	
Margarine	Hard, soft, polyunsaturated, low fat, very low fat; give name and brand, if possible	Tablespoons	Mounds
Mayonnaise	Give name and brand; state if low fat	Teaspoons	
Meat pie, pasty, pastry	What type; individual or helping; size; fat used for pastry; give brand name, where possible	Number	Mounds, circles
Meats	What type; lean or fatty; fried, microwaved, grilled, roast, barbequed; well done, rare, etc.; with or without gravy, cut used; pickled, smoked, breaded, salted, etc.	Ounces, slices, helping	Grid, blocks

Food/Drink	Description & Preparation	Amount	USDA Tools

APPENDIX I

MEAL CHECKLIST

Study ID: _____

Starting with _____, please track all of the meals and snacks that you eat. **Please do not eat anything not provided to you by the metabolic kitchen.** However, if you did eat something outside of the food provided to you, please record it on this checklist. Make sure to check the meals off as you eat them and not wait until the end of the day.

- You will need to consume **1 meal per day (breakfast or lunch) Monday-Friday in the metabolic kitchen.**
  - During this visit, you will pick up any remaining meals or snacks for the day and the next day's meal(s) to be consumed prior to your next visit.
- On Fridays, you will collect all of your meals and snacks for the weekend and the meals and snacks to be consumed prior to Monday's visit.
  - You will be provided with a cooler bag on wheels to ease the transportation of the meals to your home.
- You are free to eat as much as you want from the foods provided for you. **Please keep any uneaten portions in the respective container and return them to the metabolic kitchen on your next visit.**
- All meals are categorized on your Menu Plan. **Use the Menu Plan to identify which "meal" you are consuming**. Mark the correct time for each meal for example:
  - Grilled Chicken Salad is listed as "Lunch" on the menu plan, but you eat it for dinner at 7:30pm. Mark 7:30 pm next to "Lunch" on your meal checklist.
  - Pita with Hummus is listed as "afternoon snack" on the menu plan, but you eat it for your morning snack at 10am. Mark 10am next to "afternoon snack" on the meal checklist.
- If you consume one component of a meal or snack with another meal or snack please indicate that in the notes section. For example:
  - Chips and a Coke are listed as your afternoon snack, and you have the Coke with lunch at 12:00pm. Write in the notes section next to "Lunch" had Coke from afternoon snack.
  - Fish with rice, black beans, and a salad is listed as your dinner, and you have the rice (or some amount of rice) for afternoon snack at 3pm. Write in the notes section next to "afternoon snack" had rice from dinner (note estimated amount if different from the total amount given to you).
- Check Yes, No, and N/A according to your Menu Plan
  - No means meal was provided on Menu Plan but was not eaten
  - N/A meals meal was not provided on Menu Plan
- **In the notes section**, please specify type and amount of any **unconsumed food that you did not return to us** for any given reason:
  - Forgot to eat a meal,
  - Threw any of it away,
  - Failed to return some of the food for any given reason, or
  - Someone else consumed it.
- Please record your **alcohol consumption** throughout the day. Indicate type and amount of alcohol consumed. You are allowed to drink wine, beer or spirits (i.e., hard liquor, such as whisky, vodka, tequila, gin, etc.), only. Please note that any alcohol beverages that contain added sugars, fruits, cream, spices, herbs, flowers or nuts, such as liqueurs (e.g., Grand Marnier, schnapps) or cocktails ARE NOT ALLOWED.
- Please record your **coffee and tea consumption** throughout the day. Indicate type and amount of consumed. Please keep your coffee and tea intake consistent during the feeding study. **DO NOT add** sugar, any other sweetener, milk, creamer, etc., to your coffee and tea – those will be provided by the metabolic kitchen.
- Please record any consumed food and/or beverage that was not provided by the metabolic kitchen.
- Please do not take any dietary supplements (vitamins, minerals, bioactive compounds, fatty acids, herbal supplements, etc.) during the 15-day feeding study and 5 weeks following the completion of the feeding study until the 3rd blood collection is collected!!!!

Body Weight (kg): 65.5

Date	Meal	Consumed? (Check the appropriate box when you eat your meal)	Time of Meal:	Notes - specify variations from Menu Plan	Notes - specify type and amount of any unconsumed food that you did not return to us	Alcohol Consumption (Indicate type of drink and amount consumed in ounces)		Tea and coffee consumption (Indicate type of drink and amount consumed in cups)	Did you consume any food and/or beverage that was not provided by the metabolic kitchen? (If yes, please specify the food and the approximate amount)
						Type of drink (i.e., beer, wine, liquor)	Ounces		
07/12/2016 Monday	Pre Breakfast	☒ Yes ☐ No ☐ N/A	5:30 AM / PM						
	Breakfast	☒ Yes ☐ No ☐ N/A	7:30 AM / PM					1 single espresso	
	Morning Snack	☒ Yes ☐ No ☐ N/A	9:30 AM / PM		½ apple				
	Lunch	☒ Yes ☐ No ☐ N/A	12:00 AM/ PM						1 Hershey's Dark Chocolate Kiss
	Afternoon Snack	☐ Yes ☒ No ☐ N/A	: AM/PM						
	Dinner	☒ Yes ☐ No ☐ N/A	6:00 AM/ PM	Coke from Morning Snack		Red Wine	10 oz		
	Evening Snack	☒ Yes ☐ No ☐ N/A	10:00 AM/ PM					I cup of chamomile tea	
	Late Night Snack	☐ Yes ☐ No ☒ N/A	: AM/PM						

Complete these questions the following morning:
How long did you sleep last night? (hours:minutes) 7:15
Yesterday, how long did you sleep/nap during the day? (if you did not, select 0) (hours:minutes) 0:45

Body Weight (kg):_____

Date	Meal	**Consumed?** (Check the appropriate box when you eat your meal)	**Time of Meal:**	**Notes -** specify variations from Menu Plan	**Notes -** specify type and amount of any unconsumed food that you did not return to us	**Alcohol Consumption** (Indicate type of drink and amount consumed in ounces)		**Tea and coffee consumption** (Indicate type of drink and amount consumed in cups)	**Did you consume any food and/or beverage that was not provided by the metabolic kitchen?** (If yes, please specify the food and the approximate amount)
						Type of drink (i.e., beer, wine, liquor)	**Ounces**		
Day 1: Day of Week: _____ ___/___/___	Pre Breakfast	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						
	Breakfast	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						
	Morning Snack	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						
	Lunch	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						
	Afternoon Snack	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						
	Dinner	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						
	Evening Snack	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						
	Late Night Snack	☐ Yes ☐ No ☐ N/A	___:___ AM/PM						

Complete these questions the following morning:
How long did you sleep last night? (hours:minutes) _____
Yesterday, how long did you sleep/nap during the day? (if you did not, select 0) (hours:minutes) _____

APPENDIX J

PHYSICAL ACTIVITY LOG

Designed based on Ainsworth BE, Bassett DR Jr, Strath SJ, et al. Comparison of three methods for measuring the time spent in physical activity. Med Sci Sports Exerc 2000;32(9 Suppl):S457-64.

Contact Us

We are located on the corner of N 5th Street and Van Buren in building 1 of the Arizona Biomedical Complex.

425 N 5th Street
Phoenix, AZ 85004

Phone: 602-827-2545
Email: SugarsBio.asu.edu
Web: SugarsBio.org

ASU School of Nutrition & Health Promotion

Physical Activity Log Book

ARIZONA STATE UNIVERSITY

Investigation of Biomarkers for Sugars Intake

Table of Contents

Instructions

We would like you to keep a log of all physical activities you engage in over the next 15 days.

It's extremely important that you don't make any changes in your physical activity when you are completing the log.

You will complete a log for each day throughout the 15-day feeding study. Fill the log out at the end of the day.

You will be asked about number of popular activities you may have done each day, including home, transportation, occupational, conditioning, sports and leisure activities.

For each activity, circle *yes* if you did the activity and *no* if you did not do the activity.

For each activity you did, write down the number of hours and/or minutes you were actually moving and the time you began the activity (am or pm).

If you did an activity many times during the day, write down the total time you did that activity during the day.

If you did any activities that are not on this list, please write them on the line labeled "other," circle *yes* and write in the hours and/or minutes.

Remember to record only the hours and/or minutes you were actively engaged in the activity.

Day 1: Date ___/___/20__ Day of the Week: _____				
Did you do this activity today?	**Yes** (circle one)	**No**	**How Long?** Hours: Minutes	Time started activity **AM or PM**
Home Activities				
Sweep, scrub floors, vacuum, washing clothes, etc	Yes	No	____:____	_____ AM/PM
Carpentry	Yes	No	____:____	_____ AM/PM
Gardening or Yard Work	Yes	No	____:____	_____ AM/PM
Transportation				
Walk to work, school, shopping	Yes	No	____:____	_____ AM/PM
Bicycle to work, school, shopping	Yes	No	____:____	_____ AM/PM
Occupation				
Sitting at work	Yes	No	____:____	_____ AM/PM
Standing at work	Yes	No	____:____	_____ AM/PM
Walking at work	Yes	No	____:____	_____ AM/PM
Lift or carry 10-20 lbs at work	Yes	No	____:____	_____ AM/PM
Lift or carry 20+ lbs at work	Yes	No	____:____	_____ AM/PM
Other: _____	Yes	No	____:____	_____ AM/PM
Conditioning Activities				
Aerobic Exercise, Aerobic Dance	Yes	No	____:____	_____ AM/PM
Bicycling	Yes	No	____:____	_____ AM/PM
Calisthenics or gymnastics	Yes	No	____:____	_____ AM/PM
Jogging or running	Yes	No	____:____	_____ AM/PM
Hiking with pack or in mountains	Yes	No	____:____	_____ AM/PM
Martial arts (judo, karate, tai chi)	Yes	No	____:____	_____ AM/PM
Rowing a boat, canoeing	Yes	No	____:____	_____ AM/PM
Swimming	Yes	No	____:____	_____ AM/PM
Walking for exercise	Yes	No	____:____	_____ AM/PM
Weight lifting, body building	Yes	No	____:____	_____ AM/PM
Other: _____	Yes	No	____:____	_____ AM/PM
Other: _____	Yes	No	____:____	_____ AM/PM
Sports Activities				
Baseball or softball	Yes	No	____:____	_____ AM/PM
Basketball, European Handball	Yes	No	____:____	_____ AM/PM
Surfing	Yes	No	____:____	_____ AM/PM
Cross-country skiing	Yes	No	____:____	_____ AM/PM
Handball, racquetball, or squash	Yes	No	____:____	_____ AM/PM
Ice or roller skating, ice-hockey	Yes	No	____:____	_____ AM/PM
Rugby, football	Yes	No	____:____	_____ AM/PM
Soccer	Yes	No	____:____	_____ AM/PM
Tennis	Yes	No	____:____	_____ AM/PM
Volleyball	Yes	No	____:____	_____ AM/PM
Other: _____	Yes	No	____:____	_____ AM/PM
Other: _____	Yes	No	____:____	_____ AM/PM
Leisure Activities				
Bowling	Yes	No	____:____	_____ AM/PM
General Dancing	Yes	No	____:____	_____ AM/PM
Golf	Yes	No	____:____	_____ AM/PM
Fishing	Yes	No	____:____	_____ AM/PM
Table Tennis	Yes	No	____:____	_____ AM/PM
Walking for pleasure or social	Yes	No	____:____	_____ AM/PM
Yoga	Yes	No	____:____	_____ AM/PM
Watching television	Yes	No	____:____	_____ AM/PM
Other: _____	Yes	No	____:____	_____ AM/PM
Other: _____	Yes	No	____:____	_____ AM/PM
End of Day 1				

APPENDIX K

DIRECTIONS FOR COLLECTING 24-HOUR URINE SAMPLE

DIRECTIONS FOR COLLECTING 24-HOUR URINE SAMPLE

As part of our study, we are asking you to collect EIGHT 24-hour urine samples over the 15-d feeding period (Day 1, 3, 5, 7, 9, 11, 13, and 15). Please note that on two out of eight days, we ask you to collect each of your urine voids in a separate container (see "*Directions for Collecting Multiple Spot Urine Sample"* for more details). In this kit, you will find all of the materials needed to collect your 24-hour urine sample (six in total) and temporarily store it until it is retrieved by a courier.

This URINE COLLECTION KIT contains the following:

- Two 3.0 L containers each containing 4 g boric acid powder in a drawstring plastic bag.
- Urinal for males or collection 'hat' for females.
- POTABA Tablets (3 x 102 mg)
- Directions for Collecting 24-Hour Urine Sample
- 24-Hour Urine Collection Log
- Safety Pin
- Trolley Cooler bag (with HOBO Temperature Data Logger included)
- Seven ice packs
- Seven ziploc bags
- Sharpie pen

IF YOU MUST TAKE ANY MEDICATIONS CONTAINING ACETAMINOPHEN (TYLENOL), SULPHONAMIDES, FUROSEMIDE (LASIX) OR ANY OTHER PRESCRIPTION OR NON-PRESCRIPTION MEDICATIONS DURING THE 24-HOUR URINE COLLECTION PERIOD, PLEASE RECORD THIS IN YOUR 24-HOUR URINE COLLECTION LOG.

TO COLLECT THE 24-HOUR URINE SAMPLE, PLEASE FOLLOW THE DIRECTIONS BELOW:

1. Please place the seven ice packs in your freezer the evening before you start your 24-hour urine collection.
2. When you first get up in the morning on the day of the 24-hour urine collection, **DISCARD** your first urine. Enter the **DATE** and **TIME** of this first morning void for Question #1 and Question #2 on the 24-Hour Urine Collection Log, respectively.
3. At this point, please take out three frozen ice packs from your freezer and place them upright on three sides of your urine containers in the cooler bag. Please, keep the lid of the cooler closed at all times. Please do not remove the HOBO Temperature Data Logger from the cooler bag at any time.
4. Take one **POTABA** tablet with one full glass of water at breakfast time or within one hour after you wake up, whichever occurs first. Please ensure that you take the first POTABA tablet AFTER you have voided your first morning urine. Enter the time you took this first POTABA tablet for Question #4 on the 24-Hour Urine Collection Log.
5. Pin the safety pin to your undergarments. This is a visual cue to remind you to collect your urine each and every time you use the bathroom during the 24-hour collection period.
6. Record the **TIME** of your second morning urine of the day, which is the first urine of your 24-h urine collection. For females: place the hat on the toilet seat and collect the entire amount. For males: urinate directly into the urinal, collecting the entire amount. If possible, pass urine before passing stool. Pour the urine from the hat/urinal into the 3.0 L container, avoiding any

spillage. Please ensure that the lid of the urine container is in closed position, and that the container remains in the cooler between voids at all times.

7. For the next 24-hour time period, you must collect ALL of your urine.
8. If you accidentally miss collecting a sample or part of a sample, please make a note for Question #5 and enter the time and approximate amount of the missed amount for Question #5a on the 24-Hour Urine Collection Log. It is very important that we know if any urine has been missed. Continue your collection.
9. Take the **second POTABA** tablet with one full glass of water sometime between 12 - 2 pm (with lunch). Enter the time you took this second POTABA tablet for Question #4 on the 24-Hour Urine Collection Log.
10. Take the **third POTABA** tablet with one full glass of water sometime between 5 – 7 pm (with dinner). Enter the time you took this second POTABA tablet for Question #4 on the 24-Hour Urine Collection Log.
11. In the evening, please take out another two frozen ice packs from your freezer and insert them upright on two sides of your urine containers in the cooler bag. Please, make sure that the lid of the cooler is kept closed at all times.
12. Upon awakening in the morning the next day, COLLECT your first morning urine. This will be your last collected void of this 24-hour urine collection. Enter the **TIME** of this final urine void for Question #6 on the 24-Hour Urine Collection Log.
13. Please take out the last two frozen ice packs from your freezer and add them upright on two sides of your urine containers in the cooler bag.

14. Please record all prescription and nonprescription medications you took during the 24-hour urine collection period and the previous day in Question #7 on the Log. Please specify the name, brand and dose of medication, and day you took it.

15. To prepare your urine collection for pick-up, please make sure that the urine collection containers have been secured in a tightly closed bag in the trolley bag with all six ice packs. You do not need to return the hat/urinal. If you do use the paper form of the 24-Hour Urine Collection Log, please bring it with you next time you visit the metabolic kitchen. A courier will be scheduled to pick-up the cooler bags from your home (please talk with the Project Coordinator to make arrangements).

Tips to help you remember to collect all of your urine:

- Attach the safety pin provided to your underclothes.
- When at home, leave the hat/urinal on top of the toilet seat.
- When away from home, keep your supplies close by at all times.

If you have any questions or concerns, please contact

Cassandra Kettenhoven, Project Coordinator at 602-827-2545 or sugarsbio@asu.edu

THANK YOU FOR PROVIDING THIS SAMPLE!

APPENDIX L

24-HOUR URINE COLLECTION LOG

24-HOUR URINE COLLECTION LOG

SUBJECT ID _____

24-HOUR URINE COLLECTION _____

INSTRUCTIONS:
- Please collect all urine for the entire 24-hour period into the urine bottles. Make sure that all collections are complete (see "Directions for Collecting 24-hour urine sample" for more details).
- When you first get up in the morning on the day of the 24-hour urine collection, **DISCARD** your first urine, and record the TIME you did this. Then, collect all the urines up to **AND INCLUDING** the first urine you pass on the following morning, and record the TIME.
- Take three POTABA tablets at evenly spaced intervals throughout the 24-h urine collection day, starting after discarding the first urine (see "Directions for Collecting 24-hour urine sample" for more details).
- Keep the urine bottles cool at all times.

1. Please enter the DATE when you start this 24-h urine collection.

   Date: |___|___| |___|___| |_2_|_0_|_1_|__|
   MO DAY YEAR

2. Please enter the TIME of your first morning urine that you have discarded.

   Time: |___|___|:|___|___| AM/PM

3. Please enter the TIME of your second morning urine of the day, which is the first urine of your 24-h urine collection.

   Time: |___|___|:|___|___| AM/PM

4. Please record the time you take each POTABA tablet.

		Did you forget to take a tablet? Check box if YES
Tablet 1:	Time: |___|___|:|___|___| AM/PM	☐
Tablet 2:	Time: |___|___|:|___|___| AM/PM	☐
Tablet 3:	Time: |___|___|:|___|___| AM/PM	☐

5. Did you miss collecting any urine during this 24-hour period?

   |___| Yes |___| No → Skip to Question #6

5a. If you missed collecting any urine, please record the time and approximate amount of the missed void.

Time: |___|___|:|___|___| AM/PM estimated amount: _____ oz.

Time: |___|___|:|___|___| AM/PM estimated amount: _____ oz.

6. Please enter the TIME of your first morning urine you pass the following day, which is the last urine of your 24-h urine collection.

Time: |___|___|:|___|___| AM/PM

7. Please record all prescription and nonprescription medications you took during the 24-hour urine collection period and the previous day in the space provided below. Please specify the name, brand and dose of medication, and day you took it:

_____

_____

_____

THANK YOU FOR PROVIDING THIS SAMPLE!

APPENDIX M

DIRECTIONS FOR COLLECTING MULTIPLE SPOT URINE SAMPLE

DIRECTIONS FOR COLLECTING MULTIPLE SPOT URINE SAMPLE

As part of our study, on two out of eight 24-h urine collection days, we are asking you to collect all your urine voids in a separate container. Please remember that you will again need to collect ALL URINE for the entire 24-hour period. In each kit, you will find all of the materials needed to collect your urine sample and temporarily store it until it is retrieved by a courier.

This URINE COLLECTION KIT contains the following:

- Six 1L containers and six 500 mL containers each containing 1 g and 600 mg boric acid powder, respectively, in a drawstring plastic bag.
- Urinal for males or collection 'hat' for females
- POTABA Tablets (3 x 102 mg)
- Directions for Collecting Multiple Spot Urine Sample
- Multiple Spot Urine Collection Log
- Safety Pin
- Trolley Cooler bag (with HOBO Temperature Data Logger included)
- Seven ice packs
- Seven ziploc bags
- Sharpie pen

IF YOU MUST TAKE ANY MEDICATIONS CONTAINING ACETAMINOPHEN (TYLENOL), SULPHONAMIDES, FUROSEMIDE (LASIX) OR ANY OTHER PRESCRIPTION OR NON-PRESCRIPTION MEDICATIONS DURING THE MULTIPLE SPOT URINE 24-HOUR COLLECTION PERIOD, PLEASE RECORD THIS IN YOUR MULTIPLE SPOT URINE COLLECTION LOG.

TO COLLECT THE MULTIPLE SPOT URINE SAMPLE, PLEASE FOLLOW THE DIRECTIONS BELOW:

1. Please insert the seven ice packs in provided ziploc bags each and place them in your freezer the evening before you start your multiple spot urine collection.
2. When you first get up in the morning on the day of the 24-hour multiple spot urine collection, **DISCARD** your first urine. Enter the date and time of this first morning void for Question #1 and Question #2 on the Multiple Spot Urine Collection Log, respectively.
3. At this point, please take out three frozen ice packs from your freezer and place them upright on three sides of your urine containers (outside of the plastic bag) in the cooler. Please, keep the lid of the cooler closed at all times, and do not remove the HOBO Temperature Data Logger from the cooler bag at any time.
4. Take one POTABA tablet with one full glass of water at breakfast time or within one hour after you wake up, whichever occurs first. Please ensure that you take the first POTABA tablet AFTER you have voided your first morning urine. Enter the time you took this first POTABA tablet in Question #3 on the Multiple Spot Urine Collection Log.
5. Pin the safety pin to your undergarments. This is a visual cue to remind you to collect your urine each and every time you use the bathroom during the 24-hour collection period.
6. For the next 24-hour time period, you must collect each of your urine in a separate container. For females: place the hat on the toilet seat and collect the entire amount. For males: urinate directly into the urinal, collecting the entire amount. If possible, pass urine before passing stool. Please check the amount of passed void using the graduation on the urinal/hat. If the void is less than 300-350 ml, use a smaller 500 ml container, otherwise use one of the 1L

containers. Pour the urine from the hat/urinal into one the container, avoiding any spillage. Please ensure that the lid of the urine container is in the closed position, and that the container remains in the cooler between voids at all times.

7. Use the Sharpie provided in the kit to record the DATE and TIME of the void on the container label. Next, record the TIME of the void in Question 4 on your Multiple Spot Urine Collection Log.

   Repeat steps under 6) and 7) for each subsequent void you pass throughout the 24-h urine collection period.

8. If you accidentally miss collecting a sample or part of a sample, please make a note for Question #5 and enter the time and approximate amount of the missed amount for Question #5a on the Multiple Spot Urine Collection Log. It is very important that we know if any urine has been missed. Continue your collection, recording the date and time on each container and the time in Question #4 of your Multiple Spot Urine Collection Log.
9. Take the second POTABA tablet with one full glass of water sometime between 12 - 2 pm (with lunch). Enter the time you took this second POTABA tablet for Question #3 on the Multiple Spot Urine Collection Log.
10. Take the third POTABA tablet with one full glass of water sometime between 5 – 7 pm (with dinner). Enter the time you took this third POTABA tablet for Question #3 on the Multiple Spot Urine Collection Log.

11. In the evening, please take out another two frozen ice packs from your freezer and insert them upright on two sides of your urine containers (outside of the plastic bag) in the cooler. Please, make sure that the lid of the cooler is kept closed at all times.

12. Upon awakening in the morning the next day, COLLECT your first morning urine. This will be your last collected void of this multiple spot urine collection. Enter the date and time of this final urine on the container label, and the time in Question #6 on the Multiple Spot Urine Collection Log.

13. Please take out the last two frozen ice packs from your freezer and add them upright on two sides of your urine containers (outside of the plastic bag) in the cooler.

14. Please record all prescription and nonprescription medications you took during the 24-hour multiple spot urine collection period and the previous day in Question #7 on the Log. Please specify the name, brand and dose of medication, and day you took it.

15. To prepare your urine collection for pick-up, please make sure that the lid of all urine containers is in closed position, and the urine collection containers have been secured in a tightly closed bag in the trolley cooler with all six ice packs. You do not need to return the hat/urinal, however you may want to keep it to use for your next 24 hour urine collection. If you are using the paper format of the Multiple Spot Urine Collection Log, please bring it with you next time you visit the metabolic kitchen. A courier will be scheduled to pick-up the cooler bags from your home (please talk with the Project Coordinator to make arrangements).

Tips to help you remember to collect all of your urine:

* Attach the safety pin provided to your underclothes.

- When at home, leave the hat/urinal on top of the toilet seat.
- When away from home, keep your supplies close by at all times.

If you have any questions or concerns, please contact

Cassandra Kettenhoven, Project Coordinator at 602-827-2545 or sugarsbio@asu.edu

THANK YOU FOR PROVIDING THIS SAMPLE!

APPENDIX N

MULTIPLE SPOT URINE COLLECTION LOG

SUBJECT ID _____
MULTIPLE SPOT URINE COLLECTION _____

MULTIPLE SPOT URINE COLLECTION LOG

INSTRUCTIONS

- Please collect all urine voids in a **SEPARATE CONTAINER** over the entire 24-hour collection period. Make sure that all collections are complete (see "Directions for Multiple Spot Urine Collection" for more details). Please check the amount of passed void using the graduation on the urinal/hat. If the void is less than 300-350 ml, use a smaller 500 ml container, otherwise use one of the 1L containers.
- When you first get up in the morning on the day of your multiple spot urine collection, **DISCARD** your first urine, and record the TIME you did this. Then, collect all the urines up to **AND INCLUDING** the first urine you pass on the following morning in a separate container, and record the TIME on each container. Please remember that you again need to collect ALL URINE for the entire 24-hour period.
- Take three POTABA tablets at evenly spaced intervals throughout the 24-h urine collection day, starting after discarding the first urine (see "Directions for Multiple Spot Urine Collection" for more details).
- **Please RECORD THE DATE AND TIME OF EACH VOID on the container label.**

1. Please enter the DATE when you start this 24-hour multiple spot urine collection.

   Date: |__|__| |__|__| |2|0|1|__|
   MO DAY YEAR

2. Please enter the TIME of your first morning urine that you have discarded.

   Time: |__|__|:|__|__| AM/PM

3. Please record the time you take each POTABA tablet.

		Did you forget to take a tablet? Check box if YES
Tablet 1:	Time: |__|__|:|__|__| AM/PM	☐
Tablet 2:	Time: |__|__|:|__|__| AM/PM	☐
Tablet 3:	Time: |__|__|:|__|__| AM/PM	☐

SUBJECT ID _____
MULTIPLE SPOT URINE COLLECTION _____

4. Please enter below the container NUMBER and the TIME of each void you pass today. Remember to collect each of your voids in a separate container, and to record the date and time of each void on the container label.

Void #1 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #2 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #3 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #4 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #5 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #6 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #7 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #8 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #9 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #10 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #11 – Container #____: Time: |___|___|:|___|___| AM/PM

Void #12 – Container #____: Time: |___|___|:|___|___| AM/PM

5. Did you miss collecting any urine during this 24-hour period?

|___| Yes |___| No → Skip to Question #6

5a. If you missed collecting any urine, please record the time and approximate amount of the missed voided.

Time: |___|___|:|___|___| AM/PM estimated amount: _____ oz.

Time: |___|___|:|___|___| AM/PM estimated amount: _____ oz.

6. Please enter the TIME of your first morning urine you pass the following day.

SUBJECT ID _____
MULTIPLE SPOT URINE COLLECTION _____

Time: |___|___|:|___|___| AM/PM

7. Please record all prescription and nonprescription medications you took during the 24-hour urine collection period and the previous day, in the space provided below. Please specify the name, brand and dose of medication, and day you took it:

_____
_____
_____

THANK YOU FOR PROVIDING THIS SAMPLE!

APPENDIX O

COLORIMETRIC ENZYMATIC ASSAY PROCEDURE

Sucrose/D-Glucose/ D-Fructose

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

UV method

for the determination of sucrose, D-glucose and D-fructose in foodstuffs and other materials

For *in vitro* use only

Store at 2-8°C

Cat. Nr. 10 716 260 035
Test-Combination for 22 assays each

For recommendations for methods and standardized procedures see references (A 2, B 2, C 2, D 2)

Principle (Ref. A 1)

The D-glucose concentration is determined before and after the enzymatic hydrolysis of sucrose; D-fructose is determined subsequently to the determination of D-glucose.

Determination of D-glucose before inversion:
At pH 7.6, the enzyme hexokinase (HK) catalyzes the phosphorylation of D-glucose by adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1).

$$(1)\ \text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

In the presence of glucose-6-phosphate dehydrogenase (G6P-DH), the D-glucose-6-phosphate (G-6-P) formed is specifically oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).

$$(2)\ \text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The NADPH formed in this reaction is stoichiometric to the amount of D-glucose and is measured by means of its light absorbance at 334, 340 or 365 nm.

Determination of D-fructose:
Hexokinase also catalyzes the phosphorylation of D-fructose to D-fructose-6-phosphate (F-6-P) with the aid of ATP (3).

$$(3)\ \text{D-Fructose} + \text{ATP} \xrightarrow{\text{HK}} \text{F-6-P} + \text{ADP}$$

On completion of the reaction (3) F-6-P is converted by phosphoglucose isomerase (PGI) to G-6-P (4).

$$(4)\ \text{F-6-P} \xrightarrow{\text{PGI}} \text{G-6-P}$$

G-6-P reacts again with NADP with formation of D-gluconate-6-phosphate and NADPH (2). The amount of NADPH formed now is stoichiometric to the amount of D-fructose.

Enzymatic inversion:
At pH 4.6, sucrose is hydrolyzed by the enzyme β-fructosidase (invertase) to D-glucose and D-fructose (5).

$$(5)\ \text{Sucrose} + \text{H}_2\text{O} \xrightarrow{\beta\text{-fructosidase}} \text{D-glucose} + \text{D-fructose}$$

The determination of D-glucose after inversion (total D-glucose) is carried out according to the principle outlined above.

The sucrose content is calculated from the difference of the D-glucose concentrations before and after enzymatic inversion.

The Test-Combination contains

1. Bottle 1 with approx. 0.5 g lyophilizate, consisting of: citrate buffer, pH approx. 4.6; β-fructosidase, approx. 720 U
2. Bottle 2 with approx. 7.2 g powder mixture, consisting of: triethanolamine buffer, pH approx. 7.6; NADP, approx. 110 mg; ATP, approx. 260 mg; magnesium sulfate
3. Bottle 3 with approx. 1.1 ml suspension, consisting of: hexokinase, approx. 320 U; glucose-6-phosphate dehydrogenase, approx. 160 U
4. Bottle 4 with approx. 0.6 ml phosphoglucose isomerase suspension, approx. 420 U
5. Bottle 5 with sucrose assay control material for assay control purposes (measurement of the assay control material is not necessary for calculating the results.) Expiry date: see pack label
6. Bottle 6 with D-glucose assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) The assay control solution does not contain sucrose and D-fructose because of their insufficient stability in aqueous solutions. Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Dissolve contents of bottle 1 with 10 ml redist. water.
2. Dissolve contents of bottle 2 with 45 ml redist. water.
3. Use contents of bottle 3 undiluted.
4. Use contents of bottle 4 undiluted.

Stability of reagents

The contents of bottles 1, 2, 3 and 4 are stable at 2-8°C (see pack label). Solution 1 and solution 2 are stable for 4 weeks at 2-8°C, or for 2 months at −15 to −25°C.
Bring solutions 1 and 2 to 20-25°C before use.

Procedure

Wavelength[1]: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.020 ml (3.040 ml, determination of D-fructose)
Read against air (without a cuvette in the light path) or against water
Sample solution: 4-150 µg sucrose + D-glucose + D-fructose/assay[3] (in 0.100-1.800 resp. 2.000 ml sample volume)

Pipette into cuvettes	Blank sucrose sample	Sucrose sample	Blank D-glucose/ D-fructose sample	D-Glucose/ D-fructose sample
solution 1*	0.200 ml	0.200 ml	-	-
sample solution**	-	0.100 ml	-	0.100 ml
Mix*, incubate for minimum 15 min at 20-25°C or for minimum 5 min at 37°C (before pipetting, warm up solution 1 to 37°C). Addition of:				
solution 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml
redist. water	1.800 ml	1.700 ml	2.000 ml	1.900 ml
Mix***, read absorbances of the solutions after approx. 3 min (A_1). Start reaction by addition of:				
suspension 3	0.020 ml	0.020 ml	0.020 ml	0.020 ml
Mix***, wait for completion of the reaction (approx. 10-15 min) and read absorbances of the solutions (A_2). If the reaction has not stopped after 15 min, continue to read the absorbances at 2 min intervals until the absorbance increases constantly over 2 min. Addition of:				
suspension 4	-	-	0.020 ml	0.020 ml
Mix***, read absorbances of the solutions after 10-15 min (A_3).				

* Pipette solution 1 and sample solution each, onto the bottom of the cuvette and mix by gentle swirling. When using a plastic spatula, remove it from the cuvette only directly before measuring absorbance A_1.
** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.
*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

If the absorbance A_2 increases constantly, extrapolate the absorbances A_2 to the time of the addition of suspension 3 (HK/G6P-DH).

Determine the absorbance differences (A_2-A_1) for both, blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample.

$$\Delta A = (A_2\text{-}A_1)_{\text{sample}} - (A_2\text{-}A_1)_{\text{blank}}$$

The difference between $\Delta A_{\text{total D-glucose}}$ (from the sucrose sample) and $\Delta A_{\text{D-glucose}}$ (from the D-glucose sample) yields $\Delta A_{\text{sucrose}}$.

It follows for the determination of D-fructose:

Determine the absorbance differences (A_3-A_2) for both, blank and sample (D-glucose/D-fructose sample). Subtract the absorbance difference of the blank from the absorbance difference of the sample. This results in $\Delta A_{\text{D-fructose}}$.

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt.4).

1 The absorption maximum of NADPH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 See instructions for performance of assay

0114.10716 936001 ⑦

Calculation

According to the general equation for calculating the concentrations:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = extinction coefficient of NADPH at
340 nm = 6.3 [l × mmol^{-1} × cm^{-1}]
Hg 365 nm = 3.5 [l × mmol^{-1} × cm^{-1}]
Hg 334 nm = 6.18 [l × mmol^{-1} × cm^{-1}]

It follows for sucrose:

$$c = \frac{3.020 \times 342.3}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{sucrose} = \frac{10.34}{\varepsilon} \times \Delta A_{sucrose}$$

[g sucrose/l sample solution]

for D-glucose:

$$c = \frac{3.020 \times 180.16}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-}glucose} = \frac{5.441}{\varepsilon} \times \Delta A_{glucose}$$

[g D-glucose/l sample solution]

for D-fructose:

$$c = \frac{3.040 \times 180.16}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-}fructose} = \frac{5.477}{\varepsilon} \times \Delta AE_{fructose}$$

[g D-fructose/l sample solution]

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$Content_{Sucrose} = \frac{c_{sucrose}\text{ [g/l sample solution]}}{weight_{sample}\text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

$$Content_{D\text{-}glucose} = \frac{c_{D\text{-}glucose}\text{ [g/l sample solution]}}{weight_{sample}\text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

$$Content_{D\text{-}fructose} = \frac{c_{D\text{-}fructose}\text{ [g/l sample solution]}}{weight_{sample}\text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

1. Instructions for performance of assay

The amount of sucrose + D-glucose + D-fructose present in the assay has to be between 8 µg and 150 µg (measurement at 365 nm) or 4 µg and 80 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a sucrose + D-glucose + D-fructose concentration between 0.10 and 1.5 g/l or 0.05 and 0.8 g/l, respectively.

Dilution table

Estimated amount of sucrose + D-glucose + D-fructose per liter measurements at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.8 g	< 1.5 g	-	1
0.8-8.0 g	1.5-15.0 g	1 + 9	10
8.0-80 g	15.0-150 g	1 + 99	100
> 80 g	> 150 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml (D-glucose and D-fructose sample), or up to 1.800 ml (sucrose sample). The volume of water added must then be reduced so as to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

If the estimated amount of sucrose is below 0.2 g/l, the incubation time stated in the assay scheme, when sucrose is splitted by β-fructosidase, may be reduced from 15 min to 5 min.

2. Technical information

If the ratio D-glucose to sucrose (D-glucose to D-fructose) in the sample is higher than e.g. 10:1, the precision of the sucrose and D-fructose determination is impaired. In this case, as much as possible of the D-glucose should be removed by means of glucose oxidase in the presence of oxygen from the air. (For details see pt. 11: Determination of sucrose, D-glucose and D-fructose in honey).

3. Specificity

β-Fructosidase hydrolyzes the β-fructosidic bonds in sucrose and other glycosides. If the sample only contains sucrose it will be measured specifically via D-glucose. Even in the presence of fructosanes, sucrose can be measured specifically if after enzymatic hydrolysis with β-fructosidase, D-glucose and D-fructose are determined and the ratio of these monosaccharides is 1:1. If the D-fructose part dominates the sample contains 2 β-fructosanes.

The measuring of the D-glucose and D-fructose is specific.

In the analysis of commercial sucrose results of 100% have to be expected. In the analysis of commercial water-free D-glucose (molecular weight 180.16), D-glucose monohydrate (molecular weight 198,17) and of D-fructose results of < 100% have to be expected because the materials absorb moisture. (Commercial D-fructose may also contain D-glucose.)

4. Sensitivity and detection limit

The smallest differentiating absorbance for the procedure in the determination of D-glucose or D-fructose is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml and measurement at 340 nm of a D-glucose or D-fructose concentration of 0.2 mg/l sample solution (if v = 0.100 ml, this corresponds to 4 mg/l sample solution).

The detection limit of 0.4 mg D-glucose or D-fructose/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume v = 2.000 ml.

The smallest differentiating absorbance for the procedure in the determination of sucrose (in the presence of D-glucose in the sample) is 0.010 absorbance units. This corresponds to a maximum sample volume v = 1.800 ml and measurement at 340 nm of a sucrose concentration of 1 mg/l sample solution (if v = 0.100 ml, this corresponds to 15 mg/l sample solution).

The detection limit of 2 mg sucrose/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume v = 1.800 ml.

5. Linearity

Linearity of the determination exists from 4 µg sucrose + D-glucose + D-fructose/assay (2 mg sucrose + D-glucose + D-fructose/l sample solution; sample volume v = 1.800 ml) to 150 µg sucrose + D-glucose + D-fructose/assay (1.5 g sucrose + D-glucose + D-fructose/l sample solution; sample volume v = 0.100 ml).

6. Precision

In a double determination of D-glucose or D-fructose using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a D-glucose or D-fructose concentration of approx. 4-8 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.04-0.08 g/ 100 g can be expected.)

In a double determination of sucrose using one sample solution, a difference of 0.010 to 0.015 absorbance units may occur in the presence of D-glucose in the sample. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a sucrose concentration of approx. 15-25 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.15-0.25 g/100 g can be expected.)

The following data have been published in the literature:

Liquid whole egg:

D-Glucose:
x = 0.44 g/100 g — r = 0.073 g/100 g, $s_{(r)} = \pm 0.026$ g/100 g; R = 0.106 g/100 g, $s_{(R)} = \pm 0.037$ g/100 g

D-Fructose:
x = 6.72 g/100 g — r = 0.587 g/100 g, $s_{(r)} = \pm 0.207$ g/100 g; R = 0.748 g/100 g, $s_{(R)} = \pm 0.264$ g/100 g

Sucrose:
x = 43.32g/100g — r = 1.722 g/100 g, $s_{(r)} = \pm 1.033$ g/100 g; R = 4.268 g/100 g, $s_{(R)} = \pm 1.501$ g/100 g

For further data see references (Ref. A 2.4)

Fruit juice:

Sucrose: r = 1.9 + 0.033 × ($c_{sucrose}$ in g/l) g/l; R = 3.3 + 0.061 × ($c_{sucrose}$ in g/l) g/l (Ref. B 2.6)

D-Glucose: r = 0.42 + 0.027 × ($c_{D\text{-}glucose}$ in g/l) g/l; R = 1.0 + 0.042 × ($c_{D\text{-}glucose}$ in g/l) g/l

D-Fructose: r = 0.15 + 0.033 × ($c_{D\text{-}fructose}$ in g/l) g/l; R = 1.05 + 0.045 × ($c_{D\text{-}fructose}$ in g/l) g/l (Ref. C 2.9)

Wine:

$r = 0.056 \times x_i$
$R = 0.12 + 0.076\ x_i$
x_i = D-glucose- resp. D-fructose content in g/l (Reg. C 2.17, 2.18)

7. Recognizing interference during the assay procedure

7.1 If the conversion of D-glucose and D-fructose has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

7.2 On completion of the reaction, the determination can be restarted by adding D-glucose or D-fructose (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

The reaction cannot be restarted with sucrose as, subsequent to altering the reaction conditions from pH 4.6 to pH 7.6 ("change of the buffer"), sucrose is no longer cleaved.

7.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

The use of "single" and "double" sample volumes in double determinations is the simplest method of carrying out a control assay in the determination of sucrose.

7.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

7.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

8. Reagent hazard

The reagents used in the determination of sucrose, D-glucose and D–fructose are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulacion 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

9. General information on sample preparation

In carrying out the assay:
Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml (D-glucose, D-fructose), resp. 1.800 ml (sucrose);
Filter **turbid solutions**;
Degas **samples containing carbon dioxide** (e.g. by filtration);
Adjust **acid samples** to approx. pH 8 by adding sodium or potassium hydroxide solution (determination of D-glucose and D-fructose);
Adjust **acid and weakly colored samples** to approx. pH 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min (determination of D-glucose and D-fructose);
Measure **"colored" samples** (if necessary adjusted to pH 8) against sample blank (= buffer or redist. water + sample), adjust the photometer to 0.000 with the blank in the beam;
Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyvinylpolypyrrolidone (PVPP) or with polyamide, e.g. 1 g/100 ml;
Crush or homogenize **solid or semi-solid samples,** extract with water or dissolve in water and filter if necessary; resp. remove turbidities or dyestuffs by Carrez clarification;
Deproteinize **samples containing protein** with Carrez reagents;
Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter; alternatively clarify with Carrez reagents after the extraction with hot water.

Carrez clarification:
Pipette the liquid sample into a 100 ml volumetric flask which contains approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g K_4 [$Fe(CN)_6$] × 3 H_2O/100 ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g $ZnSO_4$ × 7 H_2O/100 ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

Samples containing protein should not be deproteinized with perchloric acid or with trichloroacetic acid in the presence of sucrose and maltose as these disaccharides are fully or partially hydrolized with the release of D-glucose. The Carrez clarification is recommended for normal use.

10. Application examples

Determination of sucrose, D-glucose and D-fructose in fruit juices and similar beverages
Filter turbid juices (alternatively clarify with Carrez reagents) and dilute sufficiently to yield a sucrose + D-glucose + D-fructose concentration of approx. 0.1-1.5 g/l. The diluted sample solution can also be used for the assay if it is colored. Only strongly colored juices which are used undiluted for the assay because of their low sucrose + D-glucose + D-fructose content are to be decolorized. In that case proceed as follows:

Mix 10 ml of juice and approx. 0.1 g of polyamide powder or polyvinylpolypyrrolidone, stir for 1 min and filter. Use the clear, slightly colored solution for the assay.

Determination of sucrose, D-glucose and D-fructose in beer
To remove the carbonic acid, stir approx. 5-10 ml of beer in a beaker for approx. 30s with a glass rod or filter through a fluted filter paper. The largely CO_2-free sample can be used undiluted for the assay.

Determination of sucrose, D-glucose and D-fructose in sweetened condensed milk
Accurately weigh approx. 1 g of sample into a 100 ml volumetric flask, add approx. 60 ml water and incubate for 15 min at approx. 70°C; shake from time to time. For clarification, add 5 ml Carrez-I-solution (3.60 g potassium hexacyanoferrate(II), $K_4[Fe(CN)_6]$ x 3 H_2O/100 ml), 5 ml Carrez-II-solution (7.20 g zinc sulfate, $ZnSO_4$ x H_2O/100 ml) and 10 ml NaOH (0.1 M), mix after each addition, adjust to room temperature and fill up to the mark with water, mix and filter. Use the clear, possibly slightly opalescent solution diluted according to the dilution table for the assay.

Determination of sucrose, D-glucose and D-fructose in jam and ice cream
Homogenize approx. 10 g sample in a mixer. Accurately weigh approx. 0.5 g of the homogenized sample into a 100 ml volumetric flask, mix with water, dilute to the mark and filter. Discard the first 5 ml of the filtrate. Use the clear filtrate diluted according to the dilution table, if necessary, for the assay.

Determination of sucrose, D-glucose and D-fructose in potatoes
Homogenize 50 g peeled potatoes with 50 ml water in a homogenizer for 3 min. Transfer quantitatively into a 250 ml beaker. Fill up to approx. 150 ml with water. Add successively 5 ml Carrez-I-solution (preparation see pt. 9) and 5 ml Carrez-II-solution (preparation see pt. 9) mix after each addition. Adjust to pH 7.0 to 7.5 (pH-meter) with sodium hydroxide (0.1 M). Transfer quantitatively into a 250 ml volumetric flask, rinse with water, add 0.3 ml n-octanol and shake until the foam has disappeared. Fill up to 250 ml with water, mix and filter.
Use the light yellow, occasionally yellow-green solution with v = 0.100 ml or 0.200 ml, if necessary, immediately for the assay.

Determination of sucrose, D-glucose and D-fructose in tobacco (Ref. A 3.7)
Accurately weigh approx. 0.3 g dried, finely ground and sieved tobacco leaves (grain size approx. 0.2 mm) into a 100 ml volumetric flask, add approx. 70 ml water and stir for 1 h (magnetic stirrer). Fill up to the mark with water, mix and filter.
In a 25 ml volumetric flask add successively 1.25 ml Carrez-I-solution and 1.25 ml Carrez-II-solution (preparation see pt. 9) to 10 ml of the filtrate, mix, and subsequently add 2.5 ml sodium hydroxide (0.1 M) and mix again. Fill up to the mark with water, mix and filter. Use the clear solution diluted, if necessary, for the assay.

11. Special sample preparation for the determination of sucrose and D-fructose in the presence of excess D-glucose

Determination of sucrose, D-glucose and D-fructose in honey
Thoroughly stir the honey with a spatula. Take approx. 10 g of the viscous (or crystalline) honey, heat in a beaker for 15 min at approx. 60°C, and stir occasionally with a spatula (there is no need to heat liquid honey). Allow to cool. Accurately weigh approx. 1 g of the liquid sample into a 100 ml volumetric flask. Dissolve first with only a small portion of water, and then fill up to the mark.

a) Determination of D-glucose and D-fructose
Dilute the 1% honey solution 1:10 (1 + 9) and use for the assay.

b) Determination of sucrose
If the estimated sucrose content in the honey lies between 5 and 10%, dilute the 1% solution 1:3 (1 + 2) and use for the assay.

If the estimated sucrose content in the honey lies between 0.5 and 5%, as much as possible of the excess of D-glucose should be removed before sucrose is determined, otherwise the precision of the sucrose determination will be impaired. D-Glucose is oxidized to D-Gluconate in the presence of glucose oxidase (GOD) and oxygen from the air:

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{D-gluconate} + H_2O_2$$

The hydrogen peroxide is destroyed by catalase:

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

Reagents
Glucose oxidase (GOD) from Aspergillus niger, 200 U/mg (25°C; D-glucose as substrate), amylase and β-fructosidase < 0.01 % each
Catalase
Triethanolamine hydrochloride
$MgSO_4 \times 7\,H_2O$
NaOH, 4 M

Preparation of solutions for 10 determinations
Enzyme solution:
Dissolve 5 mg (≙ approx. 1000 U) GOD with 0.750 ml redist. water, add 325 KU catalase (from bovine liver, 25°C; H_2O_2 as substrate), and mix.

Buffer solution:
Dissolve 5.6 g triethanolamine hydrochloride and 0.1 g $MgSO_4 \times 7\,H_2O$ in 80 ml redist. water, adjust to pH 7.6 with sodium hydroxide (4 M), and fill up to 100 ml with redist. water .

Stability of solutions
The enzyme solution must be prepared freshly daily.
The buffer solution is stable for 4 weeks at 2-8°C.

Procedure for D-glucose oxidation

Pipette into a 10 ml volumetric flask	
buffer solution sample solution (up to approx. 0.5% D-glucose) enzyme solution	2.000 ml 5.000 ml 0.100 ml
Pass a current of air (O_2) through the mixture for 1 h; during the oxidation process **check the pH with indicator paper and, if necessary, neutralize the formed acid with NaOH.**	

To inactivate the enzymes GOD and catalase, place the volumetric flask in a boiling water-bath for 15 min, allow to cool, and dilute to the mark with water. Mix and filter, if necessary. Use 0.500 ml of the clear solution for the determination of sucrose. Determine the residual D-glucose in a parallel assay and subtract as usual.

12. Further applications

The method may also be used in the examination of pharmaceuticals, paper (Ref. B 2.2) and in research when analyzing biological samples.

Determination of sucrose, D-glucose and D-fructose in fermentation samples and cell culture media
Place the sample (after centrifugation, if necessary) in a waterbath at 80°C for 15 min to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with Carrez reagents. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

D-Glucose assay control solution (Bottle 6)

Concentration*: see bottle label

D-Glucose assay control solution is a stabilized aqueous solution of D-glucose. It serves as assay control solution for the enzymatic determination of D-glucose in foodstuffs and other materials.

Application:

1. *Addition of D-glucose assay control solution to the assay mixture D-glucose/D-fructose sample:*
   Instead of sample solution the assay control solution is used for the assay.
2. *Restart of the reaction, quantitatively:*
   After completion of the reaction with sample solution and measuring of A_3, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_4 after the end of the reaction (approx. 15 min). Calculate the concentration from the difference of $(A_4\text{-}A_3)$ according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by addition of the assay control solution, the result differs insignificantly from the data stated on the bottle label.
3. *Internal standard:*
   The assay control solution can be used as an internal standard in order to check the determination of D-glucose for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
solution 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml
Mix, and read absorbances of the solutions (A_1) after approx 3 min. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.				

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$

Note:
An assay control solution of sucrose and D-fructose is not contained in the Test-Combination because an aqueous solution of sucrose and D-fructose is not stable enough.

* Stated as anhydrous D-glucose

A. References for the determination of sucrose, D-glucose and D-fructose

A 2.1 Fuchs, G. & Wretling S. (1979) Bestämning av fruktos, glukos och sackaros i livsmedel, Vär Föda **31,** 435-439

A 2.2 Bundesverband der Deutschen Feinkostindustrie e.V. Bonn; Analysenmethoden: Bestimmung des Zuckergehaltes in Tomatenmark (enzymatisch), IV/61 (Dezember 1979)

A 2.3 Norme Française Homologuée NF V 76-106 (Octobre 1980) Jus Fruits et Jus de Légumes: Détermination de la Teneur en Saccharose, D-Glucose, D-Fructose (Méthode enzymatique)

A 2.4 Amtliche Sammlung von Untersuchungsverfahren nach § 64. LFGB; Untersuchung von Lebensmitteln: Bestimmung des Zuckergehalts in Tomatenmark (enzymatische Methode), 26.11.03-8 (Mai 1983); Bestimmung des Zuckergehalts in Tomatenketchup und vergleichbaren Erzeugnissen (enzymatische Methode), 52.01.01-8 (November 1983); Bestimmung von Saccharose, Glucose und Fructose in teiladaptierter Säuglingsnahrung auf Milchbasis, 48.01-3 (Mai 1985); Bestimmung des Gesamtzuckergehaltes in Speisesenf, 52.06-5 (Dezember 1991); Bestimmung von Glucose, Fructose und Saccharose in Eiern und Eiprodukten, 05.00-10 (Dezember 2003)

A 2.5 Office International du Cacao, du Chocolat et de la Confiserie, IOCCC Method number 113-1989: Determination of Glucose, Fructose and Sucrose in Chocolate and Sugar Confectionery Products by Means of Enzymes, Draft Standard Method, 1 st Edition

A 2.6 Österreichisches Lebensmittelbuch (Codex Alimentarius Austriacus), Kapitel B29: Senf; Erlaß vom 27. Oktober 1989 (s. ERNÄHRUNG/NUTRITION **14,** 168-170 (1990))

A 3.1 Drawert, F. (1964) Enzymatische Analyse von Glucose, Fructose, Saccharose und Sorbit in Weinen und Traubenmosten, VITIS **4,** 185-187

A 3.2 Trautner, K. (1969) Enzymatische Zuckerbestimmungen, Zeitschrift für Ernährungswissenschaft, Suppl. 8, S. 40-45

A 3.3 Somogyi, J.C. & Trautner, K. (1974) Der Glucose-, Fructose- und Saccharosegehalt verschiedener Gemüsearten, Schweiz.Med.Wochenschrift **104,** 177-182

A 3.4 Trautner, K. & Somogyi, J.C. (1979) Zuckergehalte von Obst und Gemüse - Einflüsse von Reifegrad, Sorte und Lagerung, Mitt.Gebiete Lebensm.Hyg. **70,** 497-508

A 3.5 Zürcher, K. & Hadorn, H. (1976) Veränderungen des Zuckerspektrums eines Sirups während der Lagerung, Mitt.Gebiete Lebensm.Hyg. **67,** 136-139

A 3.6 Zürcher, K. & Hadorn, H. (1976) Vergleichende Zuckerbestimmungen mit gaschromatographischen, enzymatischen und reduktometrischen Methoden, Deutsche Lebensmittel-Rundschau **72,** 197-202

A 3.7 Sekin, S. (1978) Enzymatic Determination of Glucose, Fructose and Sucrose in Tobacco, Tobacco Sci. **23,** 75-77; Tobacco International **181,** 27-29

A 3.8 Polascsek-Rácz, M., Pauli, M. P., Horváth, G. & Vámos-Vigyázó (1981) Enzymatic determination of the sugars in red pepper, Z. Lebensm. Unters. Forsch. **172,** 115-117

B. References for the determination of sucrose and D-glucose

B 1.1 Bergmeyer, H. U. & Bernt, E. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H.U., Hrsg.) 3. Aufl., Bd. 2, S. 1221-1224; Verlag Chemie, Weinheim, and (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd ed., vol. 3, p. 1176-1179, Verlag Chemie Weinheim/Academic Press, Inc., New York and London

B 2.1 Handbuch für diätetische Lebensmittel. Analytik - Reinheitsanforderungen - (1973) des Bundesverbandes der diätetischen Lebensmittelindustrie e.V., Bad Homburg, herausgegeben von der Fördergesellschaft Diätetische Lebensmittel mbH, S. 57-60

B 2.2 Untersuchung von Papieren, Kartons und Pappen für Lebensmittelverpackungen (gem. Empfehlungen XXXVI der Kunststoffkommission des Bundesgesundheitsamtes) Kapitel 8 (Methoden), Pkt. 3.5.2 (März 1979)

B 2.3 Schweizerisches Lebensmittelbuch, Kapitel 61B (Enzymatische Bestimmungen)/1.3 (1981), Kap.2A (Milchmischgetränke)/10 (1980), Kap. 2B (Sauermilchprodukte)/10 (1980), Kapitel 4 (Milchdauerwaren)/5.2 (1993), Kap. 9 (Speiseeis)/4.3 (1983), Kap. 22

(Diätetische Lebensmittel und Speziallebensmittel)/6.3 (1991), Kap. 28A (Frucht- und Gemüsesäfte u.a.)/5.4 (1988), Kapitel 30A (Wein aus Trauben)/4.4 (1993), Kapitel 36A (Kakao, Kakaomasse, Kakaopulver und Schokoladenpulver)/7.2 (1992)

B 2.4 Gombocz, E., Hellwig, E., Vojir, F. & Petuely, F. (1981) Deutsche Lebensmittel-Rundschau **77,** 3 (Glucose), 11 (Saccharose)

B 2.5 Brautechnische Analysenmethoden, Band III, S. 586-589 (1982), Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK), herausgegeben von F. Drawert im Selbstverlag der MEBAK, Freising

B 2.6 Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB; Untersuchung von Lebensmitteln: Bestimmung von Saccharose in Fleischerzeugnissen, 07.00-24 (Mai 1983); Bestimmung von Saccharose in Wurstwaren, 08.00-25 (Mai 1983); Bestimmung des Gehaltes an Saccharose und Glucose in Milchprodukten und Speiseeis; Enzymatisches Verfahren, 02.00-12 (Juni 2009); Bestimmung des Gehaltes an Saccharose und Glucose in Käse; Enzymatisches Verfahren, 03.00-12 (Mai 1986); Bestimmung des Gehaltes an Saccharose und Glucose in Speiseeis; Enzymatisches Verfahren, 42.00-5 (Juni 2009); Enzymatische Bestimmung des Saccharosegehaltes in Frucht- und Ge-müsesäften, 31.00-13 (September 1997); Enzymatische Bestimmung des Saccharosegehalte in Gemüsesäften, 26.26-17 (September 1997)

B 2.7 Deutsche Norm DIN 10326 (Februar 1986) Bestimmung des Gehaltes an Saccharose und Glucose in Milchprodukten und Speiseeis; Enzymatisches Verfahren

B 2.8 International Federation of Fruit Juice Producers (IFU, Methods of Analysis, no. 56-1985); contained in "Code of Practice for Evaluation of Fruit and Vegetable Juices" (1996) edited by Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community (A.I.J.N.)

B 2.9 Niederlande: Warenwet, Uitvoeringsvoorschriften (CII-6), Regeling Onderzoekings-methoden voor brood; Methode 14: De Bepaling van het Suikergehalte als de Som van Saccharose en Invertsuiker, de laatste herleid tot Saccharose (Oktober 1986); Dit voorschrift betrijft een methode voor de enzymatische bepaling van de som van saccharose en invertsuiker, de laatste berekend als saccharose, in brood

B 2.10 Österreichisches Lebensmittelbuch (Codex Alimentarius Austriacus), Kapitel B8 (Essig), Erlass vom 16. Juni 1986 (s. ERNÄHRUNG/NUTRITION **11,** 49-53 (1987); Kapitel B15 (Kakao, Kakaoerzeugnisse, Lebensmittel mit Kakao oder Schokolade, Nougat, Nougatmassen) (1983)

B 2.11 RSK-Values, The Complete Manual, Guide Values and Ranges of Specific Numbers for Fruit Juices and Nectars, Including the Revised Methods of Analysis (1987), 1st ed., Verlag Flüssiges Obst/ Liquid Fruit, D-56370 Eschborn, pp. 151-154

B 2.12 Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA (1988) Methodenbuch Band VI: Enzymatische Bestimmung des Glucose- und Saccharosegehaltes von Milchprodukten; C20.3

B 2.13 Nederlandse Norm NEN 2858 (1e druk, oktober 1989) Vruchtesappen: Bepaling van het saccharosegehalte; Enzymatische methode (Fruit juices - Determination of the sucrose content - Enzymatic method)

B 2.14 Europäische Norm/European Standard EN 12146 (Okt. 1996) Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Saccharosegehaltes - Spektralphotometrische Verfahren mit NADP (Fruit and vegetable juices - Enzymatic determination of sucrose content - NADP spectrophotometric method)

B 2.15 Deutsche Norm DIN EN 12146 (Okt. 1996) Frucht- und Gemüsesäfte, Teil 4: Enzymatische Bestimmung des Saccharosegehalts; Spektralphotometrisches Verfahren mit NADP

B2.16 Nederlandse Norm NEN-EN 12146 (October 1996) Vruchten- en groentesappen. Enzymatische Bepaling van het gehalte aan sacharose. NADP-spectrometrische methode

B2.17 Standard der Russischen Föderation / Standard of the Russian Federation / GOS-STANDART ROSSII GOST R 51258-99 (1999) Milk and milk products. Method for determination of sucrose and glucose content

C. References for the determination of D-Glucose und D-Fructose

C 1.1 Schmidt, F.H. (1961) Die enzymatische Bestimmung von Glucose und Fructose nebeneinander, Klinische Wochenschrift **39,** 1244-1247

C 1.2 Bergmeyer, H.U., Bernt, E., Schmidt, F. & Stork, H. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., Hrsg.) 3. Aufl., Bd. 2, S. 1241-1246; Verlag Chemie, Weinheim and (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd ed., vol. 3, pp. 1196-1201; Verlag Chemie, Weinheim/Academic Press, Inc., New York and London

C 1.3 Bernt, E. & Bergmeyer, H.U. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H.U., Hrsg.) 3. Aufl., Bd. 2, S. 1349-1352; Verlag Chemie, Weinheim and (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 2nd ed., vol. 3, pp. 1304-1307; Verlag Chemie, Weinheim/Academic Press, Inc., New York and London

C 1.4 Kunst, A., Draeger, B. & Ziegenhorn, J. (1984) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 3rd ed., vol. VI, pp. 163-172, Verlag Chemie, Weinheim, Deerfield Beach/Florida, Basel

C 1.5 Beutler, H.-O. (1984) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 3rd ed., vol. VI, pp. 321-327, Verlag Chemie, Weinheim, Deerfield Beach/Florida, Basel

C 2.1 Handbuch für diätetische Lebensmittel. Analytik - Reinheitsanforderungen - 1973 des Bundesverbandes der diätetischen Lebensmittelindustrie e.V., Bad Homburg, herausgegeben von der Fördergesellschaft Diätetische Lebensmittel mbH, S. 27-30

C 2.2 Lafon-Lafourcade, S., Lafitte, M. & Joyeux, A. (1977) Dosage du Glucose et du Fructose Residuels dans les Vins par Methode Enzymatique, Office International de la Vigne et du Vin, n600/F.V. 634

C 2.3 Deutsche Norm (April 1979) Untersuchung von Stärke und Stärkeerzeugnissen; Bestimmung von D-Glucose und D-Fructose in derselben Untersuchungsprobe (Enzymatisches Verfahren), DIN 10381

C 2.4 Methodenbuch für Weinanalysen in Österreich (1980), herausgegeben von Arbeitsgemeinschaft der Landw. Versuchsanstalten in Österreich (ALVA)

C 2.5 Schweizerisches Lebensmittelbuch, Kapitel 61B (Enzymatische Bestimmungen)/1.1, 1.2 und 1.6 (1981), Kapitel 2A (Milchmischgetränke)/11 (1980), Kapitel 9 (Speiseeis)/4.3 (1983), Kapitel 22 (Diätetische Lebensmittel und Speziallebensmittel)/6.3 (1991), Kapitel 23A (Honig)/8.2 (1995), Kapitel 28A (Frucht- und Gemüsesäfte u.a.)/ 5.4 (1988), Kapitel 30A (Wein aus Trauben)/4.4 (1993), Kapitel 34 (Gärungsessig)/8.1 (1994)

C 2.6 Gombocz, E., Hellwig, E., Vojir, F. & Petuely, F. (1981) Deutsche Lebensmittel-Rundschau **77,** 3 (Glucose) und 9-10 (Fructose)

C 2.7 Brautechnische Analysenmethoden, Band III, S. 580-586 (1982), Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK), herausgegeben von F. Drawert im Selbstverlag der MEBAK, Freising

C 2.8 Österreichisches Lebensmittelbuch (Codex Alimentarius Austriacus), Kapitel B15 (Kakao, Kakaoerzeugnisse, Lebensmittel mit Kakao oder Schokolade, Nougat, Nougatmassen) (1983); Kapitel B22 (Zucker und Zuckerarten) (1983)

C 2.9 Amtliche Sammlung von Untersuchungsverfahren nach § 64. LFGB; Untersuchung von Lebensmitteln: Bestimmung von Glucose und Fructose in Kinder-Zwieback und Zwiebackmehl, 48.02.07-1 (Mai 1985); Enzymatische Bestimmung der Gehalte an D-Glucose und D-Fructose in Frucht- und Gemüsesäften, 31.00-12 (Januar 1997); Enzymatische Bestimmung der Gehalte an D-Glucose und D-Fructose in Gemüsesäften, 26.26-11 (Januar 1997)

C 2.10 Henniger, G. & Mascaro, L. (1985) Enzymatic-Ultraviolet Determination of Glucose and Fructose in Wine: Collaborative Study, J. Assoc. Off. Anal. Chem. **68,** 1021-1024

C 2.11 Official Methods of Analysis of the Association of Official Analytical Chemists (1990), 15th ed., vol. 2, pp. 741-742 (985.09)

C 2.12 The method is approved for official wine inspection in Rhenish-Palatinate (1985; Chamber of Agriculture, Bad Kreuznach) and in Hesse (1986; Ministry for Agriculture and Forestry).

C 2.13 Ministero dell'Agricoltura e delle Foreste (1986) Approvazione dei "Metodi ufficiali di analisi per i mosti, i vini, gli agri di vino (aceti) e i sottoprodotti della vinificazione". Gazzetta Ufficiale della Repubblica Italiana, n. 161 del 14 luglio 1986

C 2.14 Methodensammlung der Internationalen Fruchtsaft-Union (IFU-Analysen-Methode Nr. 55-1985); contained in "Code of Practice for Evaluation of Fruit and Vegetable Juices" (1996) edited by Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community (A.I.J.N.)

C 2.15 RSK-Values, The Complete Manual, Guide Values and Ranges of Specific Numbers for Fruit Juices and Nectars, Including the Revised Methods of Analysis (1987), 1st ed., Verlag Flüssiges Obst/ Liquid Fruit, D-56370 Eschborn, pp. 110-114

C 2.16 Nederlandse Norm NEN 2857 (1e druk, oktober 1989) Vruchtesappen: Bepaling van het glucose- en fructosegehalte; Enzymatische methode (Fruit juices - Determination of the glucose and fructose content - Enzymatic method)

C 2.17 Recueil des méthodes internationales d'analyse des vins et des moûts, Complément n° 1 à l'édition officielle de juin 1990, OFFICE INTERNATIONAL DE LA VIGNE ET DU VIN, S. 97-100

C 2.18 Amtsblatt der Europäischen Gemeinschaften L272 (3. Oktober 1990), Rechtsvorschriften: Verordnung (EWG) Nr. 2676/90 der Kommission vom 17. September 1990 zur Festlegung gemeinsamer Analysenmethoden für den Weinsektor (S. 61-63)

C 2.19 Deutsche Norm DIN EN 1140 (1994) Frucht- und Gemüsesäfte; Enzymatische Bestimmung der Gehalte an D-Glucose und D-Fructose; Spektralphotometrische Bestimmung von NADPH (Fruit and vegetable juices; Enzymatic determination of D-glucose and D-fructose content; NADPH spectrophotometric method)

C 2.20 European Standard EN 1140 (Dec. 1994) Fruit and vegetable juices; Enzymatic determination of D-glucose and D-fructose content by the NADPH spectrometric method

C 2.21 Standard der Russischen Föderation / Standard of the Russian Federation / GOS-STANDART ROSSII GOST R 51240-98 (1998) Fruit and vegetable juices. Determination of D-glucose and D-fructose content

C 3.1 Weichel, H.H. (1965) Die quantitative Bestimmung von Fructose neben anderen Kohlenhydraten in Lebensmitteln durch enzymatische Analyse, Deutsche Lebensmittel-Rundschau **61,** 53-55

C 3.2 Tschersich, J. & Mauch, W. (1968) Enzymatisch-photometrische Bestimmung von D-Glucose und D-Fructose in Verbraucherzucker, Zeitschrift für die Zuckerindustrie **18,** 107-110

C 3.3 Baumann, G. & Gierschner, K. (1971) Die Bestimmung von Zucker in Fruchtsäften - ein Vergleich der enzymatischen mit der Luff-Schoorl-Methode, Ind. Obst- und Gemüseverwertung **56,** 165-170

C 3.4 Kubadinow, N. (1974) Die enzymatische Bestimmung von D-Glucose und D-Fructose in Zuckerrüben und Betriebssäften der Zuckerproduktion und Vergleich ihrer Ergebnisse mit den Werten der Berliner Institutsmethode, Zucker 72-78

C 3.5 Schiweck, H. & Büsching, L. (1974) Das Verhalten von Glucose und Fructose während der Zuckerfabrikation, Zucker **27,** 122-128

C 3.6 Schultze, K.W. & Rummel, M. (1975) Beispiel für die Anwendung von Enzymen bei der pharmazeutischen Analyse, Acta Pharmaceutica Technologica **21,** 167-170

C 3.7 Promayon, J., Barel, M., Fourny, G. & Vincent, J.-C. (1976) Essais de Détermination de la Teneur en Chicorée dans les Mélanges Solubles de Café et de Chicorée, Café Cacao Thé **20,** 209-217

C 3.8 Motz, R.J. (1976) Enzymatische Zuckerbestimmung, Zucker und Süsswarenwirtschaft **29,** 75-77

C 3.9 Wagner, K. & Kreutzer, P. (1977) Zusammensetzung und Beurteilung von Auslesen, Beeren- und Trockenbeerenauslesen, Die Weinwirtschaft **10,** 272-275

C 3.10 Henniger, G. & Boos, H. (1978) Anwendung der enzymatischen Analyse bei der Untersuchung kosmetischer Präparate - dargestellt an einigen Beispielen, Seifen - Öle - Fette - Wachse **104,** 159-164

C 3.11 Klingebiel, L., Grossklaus, R. & Pahlke, G. (1979) Erfahrungen mit der enzymatischen Bestimmung von Zucker und Zuckeraustauschstoffen in Diabetikerwaren, Z. Lebensm. Unters. Forsch. **169,** 359-360

C 3.12 Wucherpfennig, K., Otto, K. & Yueh-Chyng Huang (1986) Aussagekraft des Glucose-Fructose-Verhältnisses, Die Weinwirtschaft-Technik **122,** 254-260

C 3.13 Millies, K. D., Sponholz, W.R. & Eberle, C. (1988) Methoden zur Zuckerbestimmung, Weinwirtschaft - Technik, Heft 6, S. 6-11

C 3.14 Plessi, M., Monzani, A., & Coppini, D. (1988) Determination of the Monosaccharide and Alcohol Content of Balsamic and Other Vinegars by Enzymatic Methods, Agric. Biol. Chem. **52,** 25-30

D. References for the determination of D-Glucose

D 2.1 Brautechnische Analysenmethoden, Band II, S. 400-402 (1979), Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK), herausgegeben von F. Drawert im Selbstverlag der MEBAK, Freising

D 2.2 Schweizerisches Lebensmittelbuch, Kapitel 61B (Enzymatische Bestimmungen)/1.1 (1981)

D 2.3 Gombocz, E., Hellwig, E., Vojir, F. & Petuely, F. (1981) Deutsche Lebensmittel-Rundschau **77,** 3 (Glucose)

D 2.4 Amtliche Sammlung von Untersuchungsverfahren nach § 64. LFGB; Untersuchung von Lebensmitteln: Bestimmung von Glucose in Fleischerzeugnissen, 07.00-22 (Mai 1983); Bestimmung von Glucose in Wurstwaren, 08.00-23 (Mai 1983); Bestimmung der belastenden Kohlenhydrate in Diätbier für Diabetiker (als Gesamtglucose), 49.01.05-1 (Mai 1984)

D 2.5 Ministero dell'Agricoltura e delle Foreste (1986) Approvazione dei "Metodi ufficiali di analisi per i mosti, i vini, gli agri di vino (aceti) e i sottoprodotti della vinificazione". Gazzetta Ufficiale della Repubblica Italiana, n. 161 del 14 luglio 1986

D 3.1 Henniger, G. & Schütz, A. (1987) Methods for the Enzymatic Determination of D-Glucose, Poster-Präsentation bei Association of Official Analytical Chemists Annual International Meeting, San Francisco, CA, USA

Note

for Test-Combination Sucrose/D-Glucose/D-Fructose

Sucrose is supplied in this pack as assay control material (see Bottle 5). It may be used for the preparation of an assay control solution (concentration e.g. 1 g/l) which is pipetted (v = 0.100 ml) instead of the sample according to the pipetting scheme.

Furthermore, the sucrose may also be used for performing the Swiss Sucrose Test in order to check performance of the assay.

The Swiss Sucrose Test
An assay control solution is prepared and the concentration is measured enzymatically. The results are used for the evaluation of accuracy and precision.

Reagents
Prepare solutions according to the instructions in the Test-Combination.

Sample solution (assay control solution)
Weigh 1.6 g of sucrose (accuracy 0.1 mg) and dissolve with redist. water in a 1 liter volumetric flask, fill up to the mark and mix thoroughly.

Procedure
For details of performing the assays and calculating the results see instructions in the Test-Combination.

Run 2 blank and 6 sample assays.

Pipetting scheme

Pipette into cuvettes	Blanks		Samples					
	blank 1	blank 2	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6
solution 1*	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml
sample solution*	-	-	0.100ml	0.100ml	0.100ml	0.100ml	0.100ml	0.100ml
Mix**, incubate for 15 min at 20-25C°. Mix in:								
solution 2	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml
redist. water	1.800ml	1.800ml	1.700ml	1.700ml	1.700ml	1.700ml	1.700ml	1.700ml
Mix, read absorbances of the solutions after approx. 3 min (A_1). Start reaction by addition of:								
suspension 3	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml
Mix, incubate for 15 min at 20-25C°. Read the absorbances of the solutions (A_2).								
Readings								
A_1:								
A_2:								
$A_2 - A_1$:								
Mean of the blanks' absorbance differences								
$(A_2 - A_1)_{sample} - (A_2 - A_1)_{mean\ of\ the\ blanks} = \Delta A$:								

* Pipette the solution onto the bottom of the cuvettes.
** Mix by gentle shaking the cuvettes. If a mixing spatula is used, remove the spatula from the cuvette before reading A_1, not earlier.

Calculation
Calculate the absorbance differences (A_2-A_1) for each blank and sample assay. Subtract the mean absorbance difference of the blanks from the absorbance differences of the samples. It follows:

$\Delta A_{sample\ 1, 2, \ldots . 6}$

Calculate the concentration of sucrose in g/l:

$$c = \frac{V \times MG}{\varepsilon \times d \times v \times 1000} \times \Delta A_{sample\ 1, 2, \ldots . , 6}$$

$$c = \frac{3.020 \times 342.3}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta E_{sample\ 1, 2, \ldots . , 6}$$

$$c = \frac{10.34}{\varepsilon} \times \Delta E_{sample\ 1, 2, \ldots . , 6}$$

$c_{sucrose}$ (g/l):							

From the 6 results $c_1, c_2, \ldots . , c_6$ calculate the mean $\bar{c}$ and the standard deviation s_c:

mean $c_{sucrose}$ (c): g/l

standard deviation s_c: g/l

Calculation of the mean yield $\bar{Y}$ and its standard deviation s_A:

$$\bar{Y} = \frac{(\bar{c}\ ) [g/l] \times 100}{\text{weighed sucrose [g/l]}} = \frac{\quad \times 100}{\quad} = \quad g/100\ g$$

$$s_Y = \frac{s_c [g/l] \times 100}{\text{weighed sucrose [g/l]}} = \frac{\quad \times 100}{\quad} = \quad g/100\ g$$

Evaluation of the standard deviation
Standard deviation $s_Y \leqq 0.79$ g/100g:
The precision of the determination is ideal.

Standard deviation $s_Y > 0.79$ g/100g:
The standard deviation is too high. This may result either from the use of unsuitable equipment (photometer, cuvettes, pipettes) or from their wrong handling. Something should be done to overcome these difficulties (e.g. control of photometer, cuvettes and pipettes).

Evaluation of yield
Deviation of the mean yield $\bar{Y}$ from the theoretical yield ($\triangleq$ 100 g/100 g) = ΔY

$\Delta Y = |\ 100 - \bar{Y}\ | \leqq 0.42$ g/100 g:
The accuracy of the determination is ideal.

$\Delta Y = |\ 100 - \bar{Y}\ | = 0.43$ to 1.42 g/100 g:
Systematic errors are evident. This has to be accepted because they lie within the specifications of most photometers.

$\Delta Y = |\ 100 - \bar{Y}\ | > 1.42$ g/100 g:
The deviation of the mean yield from the theoretical yield is too high. The reason is also either the use of unsuitable equipment (balance, photometer, cuvettes, pipettes) or due to their wrong handling. Something should be done to overcome these difficulties (e.g. control of balance, photometer, cuvettes and pipettes).

References

1 Walter, E. (1980) Zur Prüfung der Laborausrüstung für enzymatische Bestimmungen, alimenta **19,** 159-164

2 Schweizerisches Lebensmittelbuch/Swiss Food Manual (1981), 5. Auflage, Kapitel 61: Enzymatische Bestimmungen, Enzymatic determinations, Allgemeiner Teil, General part, pp. 17-18 (Elaborated from the members of the 26th Sub-Commission, edited by the "Eidgenössische Drucksachen- und Materialzentrale", CH-Bern)

Also available:

Test-Combination D-Glucose	**Cat.No. 10 716 251 035**
Test-Combination D-Glucose/ D-Fructose	**Cat.No. 10 139 106 035**
Test-Combination Maltose/Sucrose/ D-Glucose	**Cat.No. 11 113 950 035**
Test-Combination Sucrose/ D-Glucose	**Cat.No. 10 139 041 035**
Test-Combination Sorbitol/Xylitol	**Cat.No. 10 670 057 035**
Test-Combination Starch	**Cat.No. 10 207 748 035**

R-BIOPHARM AG
An der neuen Bergstraße 17
D-64297 Darmstadt
Phone + 49 61 51 / 81 02-0
Fax + 49 61 51 / 81 02-20
www.r-biopharm.com

Roche

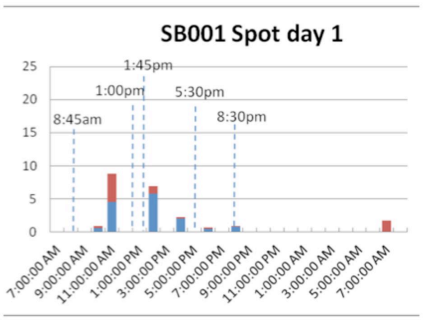
APPENDIX P

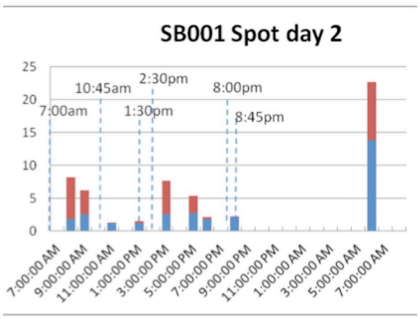
URINARY SUCROSE AND FRUCTOSE (mg/void) BY VOID IN MULTIPLE SPOT URINE DAY 1 AND 2 BY PARTICIPANT

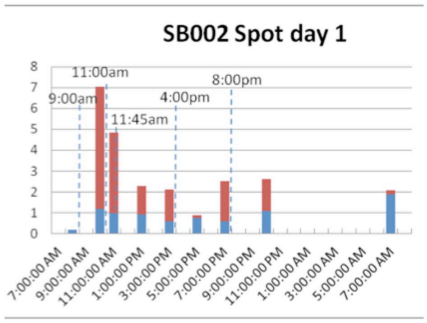
Legend

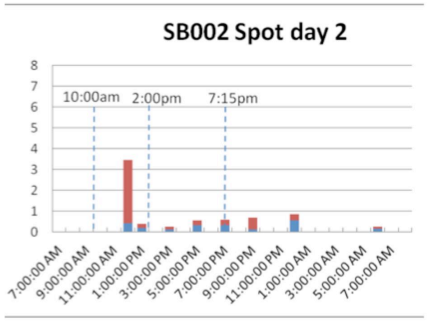
Y axis: uSF excretion (mg/void)
X axis: timeline (24-h)
Blue dashed lines: participant reported meal times
Red bars: urinary void fructose excretion
Blue bars: urinary void sucrose excretion

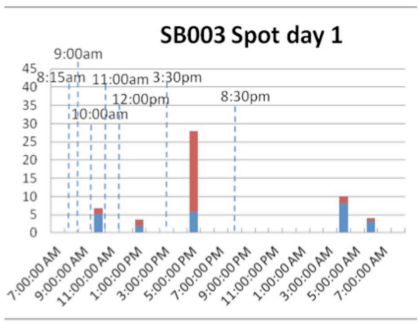
Fructose
Sucrose

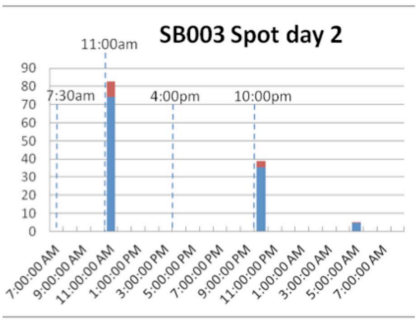


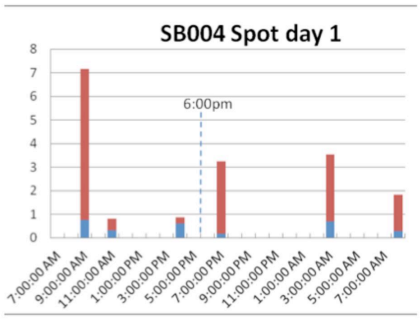


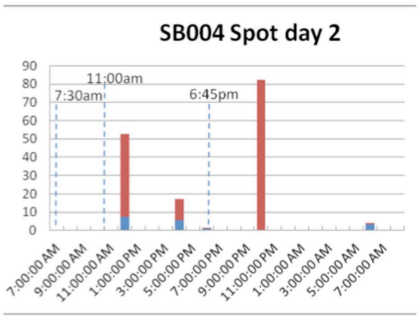


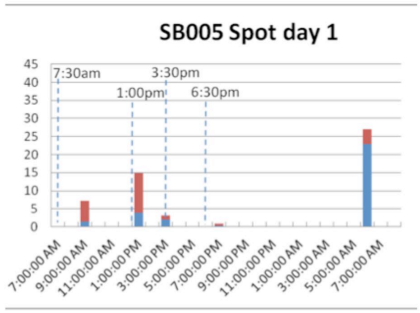


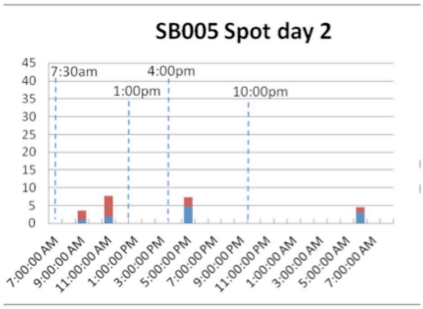


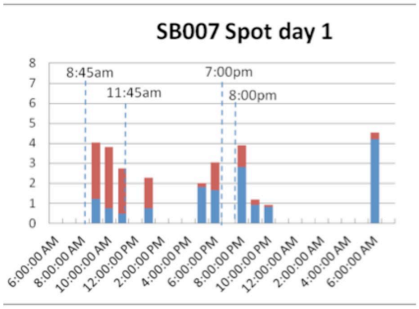


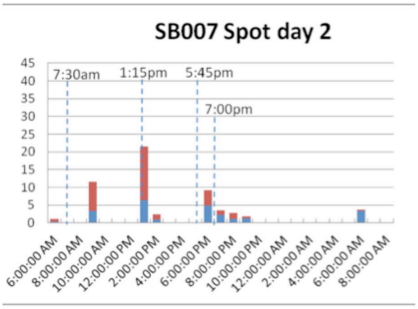

SB004 Spot day 1
6:00pm

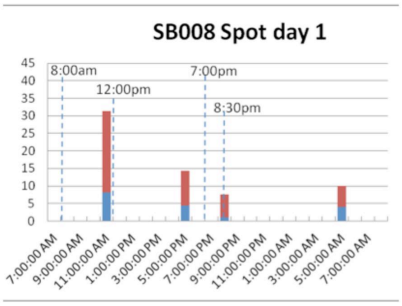

SB004 Spot day 2
11:00am
7:30am
6:45pm

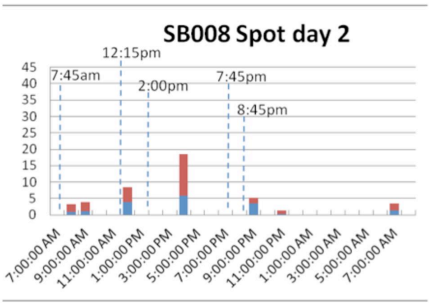

SB005 Spot day 1
7:30am
3:30pm
1:00pm
6:30pm

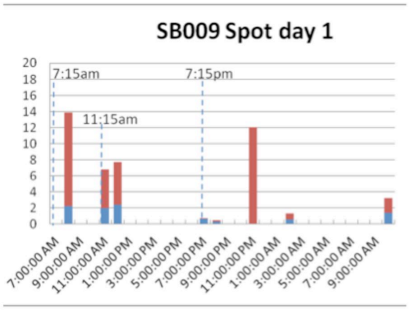

SB005 Spot day 2
7:30am
4:00pm
1:00pm
10:00pm

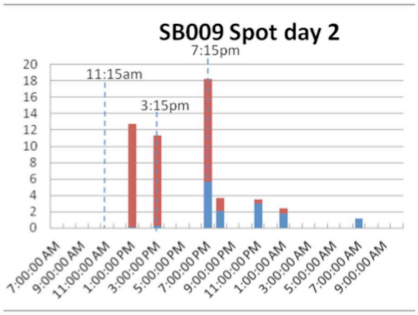

SB007 Spot day 1
8:45am
7:00pm
11:45am
8:00pm

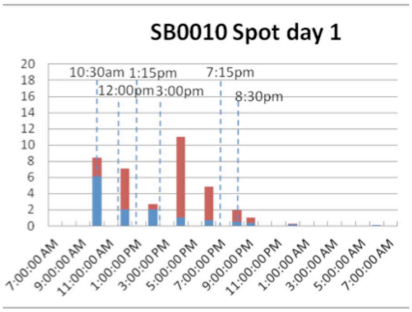

SB007 Spot day 2
7:30am
1:15pm
5:45pm
7:00pm

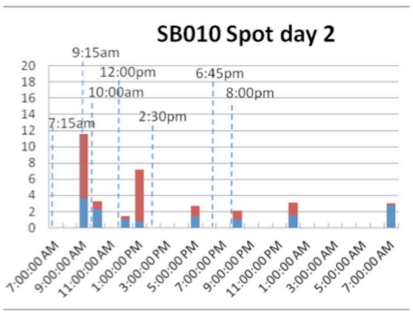

SB008 Spot day 1
8:00am
12:00pm
7:00pm
8:30pm

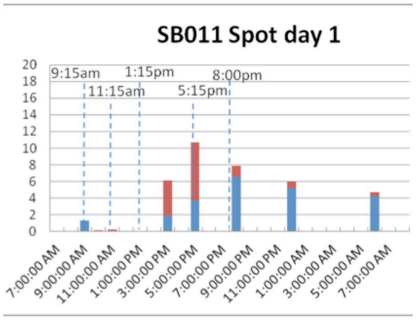

SB008 Spot day 2
12:15pm
7:45am
2:00pm
7:45pm
8:45pm

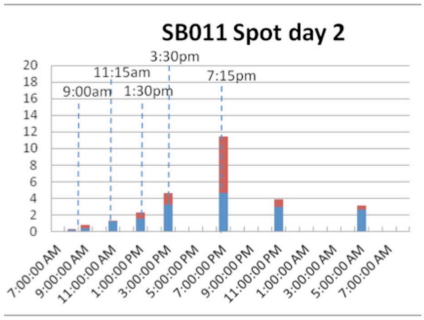

SB009 Spot day 1
7:15am
7:15pm
11:15am

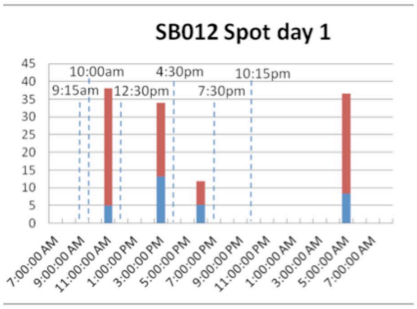

SB009 Spot day 2
7:15pm
11:15am
3:15pm

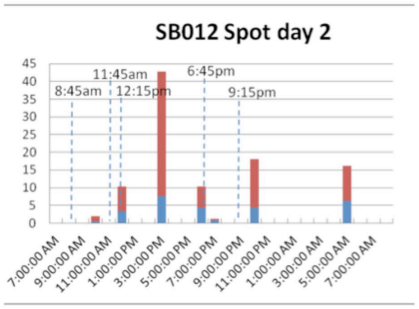

SB0010 Spot day 1
10:30am 1:15pm
7:15pm
12:00pm 3:00pm
8:30pm

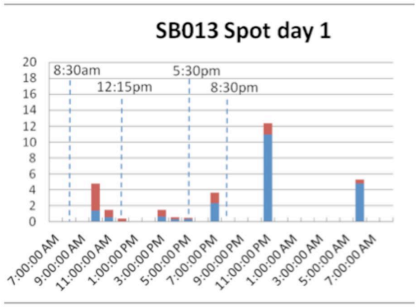

SB010 Spot day 2
9:15am
12:00pm
6:45pm
10:00am
8:00pm
7:15am
2:30pm

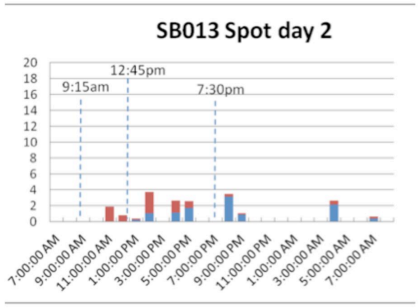

SB011 Spot day 1
9:15am
1:15pm
8:00pm
11:15am
5:15pm

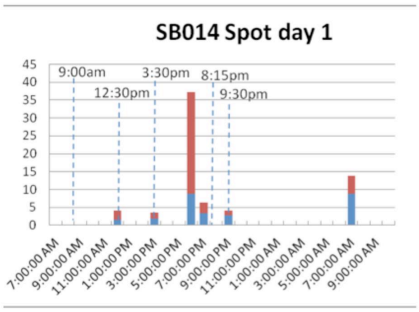

SB011 Spot day 2
3:30pm
11:15am
7:15pm
9:00am
1:30pm

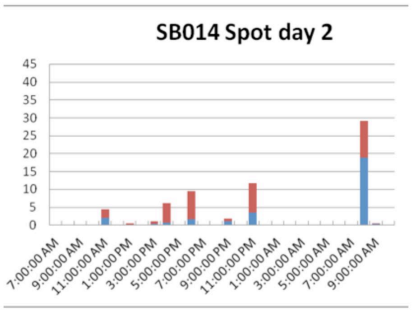

SB012 Spot day 1
10:00am
4:30pm
10:15pm
9:15am
12:30pm
7:30pm

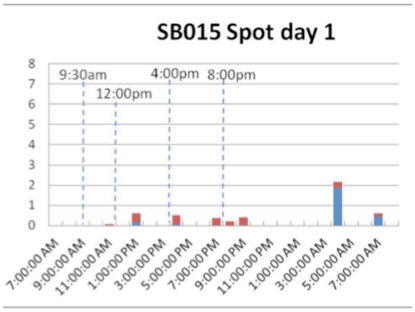

SB012 Spot day 2
11:45am
6:45pm
8:45am
12:15pm
9:15pm

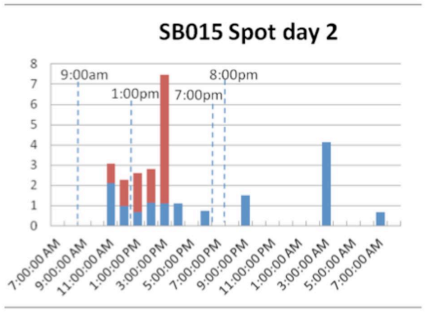

SB013 Spot day 1
8:30am
5:30pm
12:15pm
8:30pm


SB013 Spot day 2
12:45pm
9:15am
7:30pm


SB014 Spot day 1
9:00am
12:30pm
3:30pm
8:15pm
9:30pm


SB014 Spot day 2


SB015 Spot day 1
9:30am
12:00pm
4:00pm
8:00pm


SB015 Spot day 2
9:00am
1:00pm
7:00pm
8:00pm