University of Alberta

Preliminary Analysis of Dietary Sugar Consumption During Pregnancy Using a Potential Biomarker of Urinary Fructose Excretion

by

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Abstract

A 24 hour urinary fructose excretion correlates with total sugar intakes. However, whether or not a random ("spot") urinary fructose measurement is a reliable biomarker of fructose intake is not known. This study was done to determine the extent to which it is possible to estimate fructose intake in women using a biomarker of urinary fructose. Pregnant women were recruited from the Sweet Moms project (n=135) and provided a spot urine sample. Urinary fructose was enzymatically measured using a kit for measuring glucose and fructose. No correlation between fructose concentrations in urine sample and the fructose consumption in pregnant women. A validation study was performed on non pregnant women (n=9) after four hours to examine the validity of a urinary biomarker. Fructose excretion by non-pregnant women was positively associated with fructose ingestion. Fructose intake in pregnant women continues to be difficult to measure with currently available biomarkers.

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Chapter1: Introduction

1. Rationale

Dietary recommendation in pregnancy

Meeting the recommended nutritional intake in pregnancy is important to maintaining a healthy pregnancy and supporting appropriate fetal growth. The Institute of Medicine (IOM) estimated additional energy requirements during pregnancy is 340 kcal/d in the second trimester and 452 kcal/d in the third trimester, with no additional calories recommended in the first trimester (Alavi et al., 2013). Nutrition recommendations aimed at pregnant women suggest that their needs are different than those for healthy non-pregnant women. For example, pregnant women have higher requirements for folic acid, vitamin D and iron than non-pregnant women (Lim et al., 2013). According to Canada's Food Guide to Healthy Eating (CFG), pregnant women should meet their increased caloric needs by adding 1-2 servings/day from any food group (i.e. ,from fruit and vegetables, grain products, milk and alternatives and meat and alternatives (Health Canada, 2007). The increased caloric intake can be eaten as snacks or added to meals; however, increasing caloric intake by eating sugary foods is not recommended by CFG due to health concerns and their potential to increase disease risk. It is suggested that fruit and vegetables should be substituted for sugary drinks and baked goods (Garriguet et al., 2007). Many pregnant women are willing to quit smoking, eliminate alcohol consumption and improve their dietary habits for the health of the fetus, yet find sugar consumption during pregnancy difficult to abandon. Not only is the intake of sugary food items

considered socially acceptable during pregnancy (Graham et al., 2013), but many women consume such foods to alleviate physical symptoms such as cravings, fatigue and nausea, and because they have an increased appetite (Graham et al., 2013).

Dietary intake assessments methods in pregnancy

It is important to estimate dietary quality during pregnancy, since it may differ from pre-pregnancy. A UK-based study by Crozier et al. (2009) compared the dietary intake pattern in non-pregnant women (n=12,572), women in early pregnancy (n=2,270) and women in late pregnancy (n=2,649) using a food frequency questionnaire (FFQ). Participants were aged 20-34 y with a gestational age of 11–34 weeks. The study reported that some foods such as white bread, breakfast cereals, cakes and biscuits, processed meat, crisps, fruit and fruit juices, sweet spreads, confections, hot chocolate drinks, puddings, cream, milk, cheese, spread, cooking fats and salad oils, red meat, and soft drinks increased in pregnancy, while other food such as rice and pasta, liver and kidney, vegetables, nuts, diet cola, tea and coffee, boiled potatoes, and crackers decreased in pregnancy. While there were shifts in the food choices women made, the study concluded that of 48 food items that analyzed, 21 were increased during pregnancy and 10 were decreased. Women were influenced by public health messages for healthy food intake, as demonstrated by a reduction in caffeinated beverage consumption (Crozier et al, 2009).

In pregnancy, dietary assessment tends to result in under-reported energy intake and over-reported fruit and vegetable intake (Wen et.al, 2010). Data from

dietary assessment methods such as FFQs and self-reported dietary records contribute to systematic measurement errors and bias. Investigators are aware that there are always errors in dietary assessments; there is a desire to estimate and eventually reduce these errors by using biomarkers derived from biological specimens such as blood, urine and hair (Bingham, 2002). Dietary biomarkers are essential to reducing the errors of self-reported dietary intake data. It has been suggested that it may be possible to measure the true dietary intake of an individual using urine biomarkers (Bingham, 2002). Urinary biomarkers may also be used to validate dietary assessment methods, or to detect relative risks of certain diseases. Studies carried out by Bingham et al. (2002) have reported that urinary fructose and sucrose biomarkers predict sugar intake and could be helpful in studies aiming to estimate intake of simple sugars in a number of different populations (Bingham et al., 2002). Reliable biomarkers that reflect nutrient intake or status are also useful in the assessment of the impact of nutrients on health (Comb et al., 2013).

Recent studies using a biomarker to assess sugar or fructose intake

A small quantity of dietary sucrose escapes hydrolysis by sucrase in the small intestine; similarly, some fructose escapes hepatic metabolism (Tappy et al., 2010). These sugars are then removed by the circulation and excreted in the urine (Joosen et.al, 2008). A number of studies investigated whether the sugar content in a 24 hour urine collection predicted sugar intake of healthy and obese adults (Joosen et. al, 2008), and children (Johner et.al, 2010). When sugar in the diet was controlled or daily consumption reported using traditional dietary intake

assessment methods, urinary biomarkers were predictive of dietary sugar intake (Binghum et al., 2007). Dietary intake of 200 g of total sugar resulted in 100 mg of sucrose and fructose in a 24 hour urine collection (Tasevska et al., 2009). Urinary sugar biomarkers are strongly associated with dietary intake and are related to time and sample collection method (Tasevska et al., 2005). However, urinary glucose does not reflect dietary intake (Binghum et al., 2007). The use of urinary sugar measurements as predictive biomarkers is grouped into categories that could be different to the existing 3 categories of biomarkers: recovery, concentration and replacement (Tasevska et al., 2005). Urine biomarkers can potentially differentiate between sugar types and may be preferable for estimating sugar intake, instead of dietary assessment methods which are associated with unintentional errors and under-reporting (Johner et al., 2010). Earlier studies have suggested that urinary sugars may predict total sugar intake in different populations including healthy lean and obese individuals and children, but has not been evaluated during pregnancy. We therefore examined the utility of urinary fructose as a biomarker in pregnancy to estimate dietary fructose intake. We also examined whether spot urine samples could be used, in contrast to 24 hour urine collection methods that have been previously validated in the non-pregnant population.

2. Research objectives and hypothesis:

Objective (1)

To examine the relationship between a urinary biomarker method and self-reported dietary recall in estimating sugar intake in pregnant women, for potential

use of a biomarker as a complementary measurement to standard estimates of sugar intake using dietary assessment questionnaires.

Hypothesis (1)

It is hypothesized that urinary fructose concentrations will be associated with dietary fructose consumption estimated using a self-reported recall in healthy pregnant women.

Objective (2)

To validate the use of a fructose biomarker in a spot urinary sample as a tool for estimating fructose consumption in healthy non-pregnant women, and more specifically to determine urinary fructose concentration changes in response to 2 different intakes of fructose in healthy non-pregnant women.

Hypothesis (2)

It is hypothesized that measuring fructose concentration in spot urine samples from subjects consuming a controlled amount of sugar will predict the amount of fructose consumed over a 4 hour period.

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Chapter 2: Literature review

Section 1: Fructose

1. What is fructose?

Fructose is a monosaccharide. It is the strongest natural sweetener known and is markedly sweeter than glucose (Whitney, 2011). The chemical structure of fructose consists of six carbon atoms (hexose) and a hydroxyl group to form $C_6H_{12}O_6$ (Figure 1). While the chemical structure of fructose is the same as glucose, it differs in the arrangement of atoms. This difference in atomic arrangement contributes to the distinctive sweet flavor of fructose (Whitney, 2011).

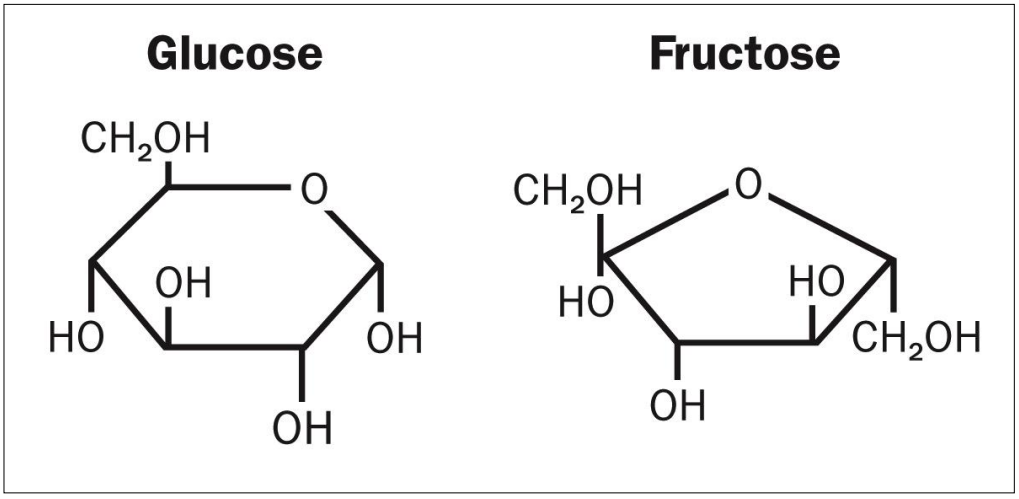

Figure 2. 1: The chemical structure of D-fructose and D-glucose

2. Fructose sources

Free fructose molecules can be found naturally in in varying concentrations in foods such as apples, pears, cherries and dates (Tappy et al, 2006). The amount of fructose in these sources is normally small and has no adverse side effects on metabolism (Tasevska et al, 2009). The fact that fructose is amongst the sweetest

tasting monosaccharides has led to its use as a sweetener; the use of high fructose corn syrup (HFCS) in food processing and baked goods has increased substantially in recent decades (Marriotte et al, 2009). Some surveys have reported as much as a 46% increase in fructose consumption over a 10 to 16 year period (Tappy et al, 2010). Much of this increase is due to the use of HFCS, which is produced using an enzymatic process. Briefly, cornstarch is extracted from corn and then enzymatically hydrolyzed into glucose and fructose (Tappy et al., 2010). HFCS has been widely used in food processing because in addition to adding sweetness, it also helps to moisturize foods, especially baked goods, and helps to extend shelf life. In addition, HFCS has replaced the use of sucrose as a food sweetener because of its lower manufacturing cost resulting from government subsidies to corn production (Tappy et al., 2009). HFCS-55 contains 55% fructose, 42% glucose and 3% water, and is the ratio most commonly used to sweeten manufactured foods (Tappy et al., 2010).

0.1 Fructose content of variety of food products and their contribution to energy intake listed from Canadian nutrition file (CNF)

Products	Portion size	Total energy (kcal)	Fructose content
Concentrated apple Juice	250 ml	121 kcal	15.02 g
Carbonated beverage: cola	335 ml	153 kcal	22.70 g
Applesauce, canned, sweetened	125 ml/135g	92 kcal	9.79 g
Granola bar, cereal bar fruit flavored	1 bar (37g)	143kcal	4.81 g

3. Fructose consumption

A high-fructose diet, especially from sugar-sweetened beverages (SSBs), is now common around the world. In 2005-2006 about 175 kcal per day came from SSBs in American adults, and in children, 172 kcal per day came from SSBs (Brownell, 2009). The proportion of total energy from soft drinks among Americans has increased from about 2.8% in 1977 to 7.0% in 2001 (Nielsen et al., 2004). Ludwig et al (2001), in their study on increased consumption of SSBs in school-aged children and its association with obesity, reported that children who consumed 265 ml of soft drinks (equal to 835 kJ) per day consumed more calories than children who did not consumed SSB (Ludwig et al., 2001). As the effect of fructose consumption has become a major public health concern due to the prevalence of obesity and metabolic syndrome, the American Heart Association has advised that added sugar intake should not exceed 100 kcal/day for women and 150 kcal/day for men (Stanhope et al., 2013). Being at a healthy weight does not protect individuals from diseases connected to the consumption of high levels of fructose. In fact, more than 40% of normal-weight individuals suffer from diabetes, hypertension, and heart disease (Lustig et al., 2012). For this reason, the effect of diet on disease outcomes has become the focus of numerous epidemiological studies.

4. Fructose absorption and metabolism

Fructose has different absorptive and metabolic pathways than other monosaccharides such as glucose (Angelopoulos et al., 2009). These differences are likely a key factor in the development of metabolic syndrome, as fructose has

the capacity to stimulate hepatic de novo lipogenesis. Fructose consumption has increased in parallel with obesity, suggesting fructose may have a role in the development of metabolic syndrome (Anne Le et al., 2009). In contrast to glucose, fructose does not enhance insulin secretion probably because of the lack of fructose transporters and GLUT5 on pancreatic cells (Teff et al, 2004). According to observation of some studies, the obesity rate greater than 10% of total fructose intake was increased when the amount is greater than 50g/day. Thus, exceeding 50 g/day is defined as excessive fructose intake (Johnson et al., 2009). However, a moderate intake of fructose is defined as 25g/day, as the small intestine is capable of completely absorbing this amount (Frieling et al., 2011)

Intestinal absorption of fructose

Dietary fructose is mostly derived from fruits, vegetables, some grains, as well as from sugars added to processed or prepared foods and sweetened beverages. Sources of fructose that are currently commercially used are crystalline fructose, HFCS, inverted sugar syrup, sucrose, liquid fructose (Park et al., 1993). In the normal diet of healthy individuals, monosaccharide and disaccharide absorbance occurs in the upper regions of the intestine (Southgate, 1995). In fact, when free fructose intake exceeds 35 g/d, the absorbance appears to be incomplete taking into account individuals' variation (Southgate, 1995). However, fructose derived from sucrose or maltose enters the intestine as disaccharides, then glucose from the breakdown of disaccharides is absorbed by glucose co-transporters, and fructose is absorbed independently of sodium by the duodenum and jejunum (Bray et al., 2004). Fructose is absorbed into enterocytes

in the gut by glucose transporter GLUT5 (Tappy et al.,2010), then enters the blood vessels via GLUT2 (Rizkalla et al., 2010). Subsequently, absorbed fructose and glucose enter portal circulation and are either taken up by the liver and converted to glucose or enter the circulation (Bray et al., 2004).

Excessive fructose intake from sucrose, HFCS and sweetened beverage remains unabsorbed in the intestine because it exceeds the capacity of the intestine to absorbed fructose. The amount of fructose remaining in the intestine can lead to gastrointestinal difficulties due to the fermentation of fructose by intestinal bacteria (Fireling et al., 2011).

Fructose metabolism in the liver

Fructose is rapidly metabolized by the liver or kidneys by three main enzymes: fructokinase, aldolase B, and triokinase. Fructokinase converts fructose to fructose-1-phosphate using ATP. Aldolase B breaks down fructose-1-phosphate to glyceraldehyde and dihydroxyacetone phosphate, which are part of glycolysis. Triokinase uses ATP to phosphorylate glyceraldehyde into glyceraldehyde-3-phosphate. This conversion is an intermediate step in glycolysis. As a result of fructose metabolism, glucose, glycogen or lactate are generated and can be used for energy production. In the starved state, 66% of fructose is converted to glucose, 25% to lactate and 8% to glycogen (Mayes, 1993). However, excessive consumption of fructose rapidly stimulates the liver to activate lipogenesis and increases circulating VLDL and triglyceride concentrations (Basciano et.al., 2005). Triglyceride accumulation leads to decreased insulin sensitivity and glucose intolerance in the liver due to the hepatic

exposure to excessive fructose. In contrast, low fructose intake improves glucose tolerance by reducing the glycemic index of glucose (Basciano et al, 2005). This is due to the inability of the human intestine to convert fructose to glucose with the enzyme glucose-6-phosphatase. Since glucose-6-phosphatase is present in the liver and kidney, fructose metabolism can occur in these organs (Mayes, 1993).

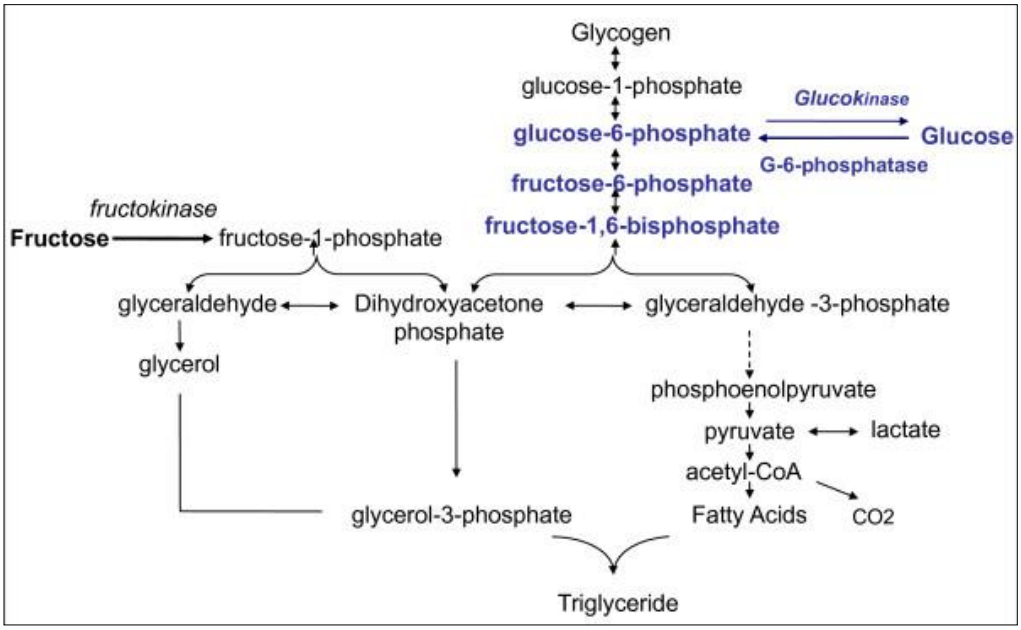

Figure 2.2:Fructose metabolism in the liver.

Glucose conversion to fructose-1,6-biphosphate is regulated by phosphofructokinase, which is inhibited by the presence of ATP and citrate. In addition, glucose uptake into cells and its metabolism is regulated by insulin. In contrast, fructose uptake by the liver and its conversion to triose-phosphate proceeds without the need for ATP molecules and this process is not under the control of insulin. After ingestion of large amounts of fructose, a portion of the fructose is converted to glucose and stored as glycogen, and some is converted to

lactate. The remaining fructose is converted to fatty acids for triglyceride synthesis Adapted from Rizkalla et al, 2010.

Insulin response to fructose intake

Unlike glucose, fructose does not stimulate insulin secretion, which plays a role in energy regulation, due to the lack of fructose transporters GLUT5 in pancreatic β cells. In fact, consuming a high fructose meal leads to reduced insulin and leptin secretion during the meal (Elliott et al., 2002).

Fructose impact on lipid metabolism

Research investigating the impact of high fructose intake on lipid metabolism is still underway. An earlier study published by Tappy et al. (2006) investigated the effect of a moderate intake of fructose on lipid metabolism and insulin sensitivity in 7 healthy male subjects (mean age 24 ± 1.3 and BMI 19-25 kg/m^2). The investigators measured insulin sensitivity (IS), intrahepatocellular lipids (IHCL), and intramyocellular lipids (IMCL) before and after 1 and 4 weeks of a 1.5 g fructose per kg body weight per day. A significant increase in fasting plasma concentrations of triacylglycerol (36%), VLDL-triacylglycerol (72%), lactate (49%), glucose (5.5%), and leptin (48%) was observed ($p < 0.05$). No significant changes were observed in body weight, IHCL, or IMCL. The study also reported a negative correlation between IHCL and plasma triacylglycerol after 4 weeks of the high-fructose diet ($r = -0.78$, $p < 0.05$). These findings indicate that a high intake of fructose over 4 weeks causes increases in plasma triacylglycerol and glucose concentrations with no adverse effects on ectopic lipid deposition or insulin resistance in healthy adults. The authors suggested that

adverse effects of fructose intake may not be exhibited due to adaptation of hepatic cells, skeletal muscle, or adipose cell to metabolic changes in those healthy subjects (Ann Le et al., 2006). However, more recent studies suggested that regular consumption of large amounts of dietary fructose could be a concern due to increases in circulating triglycerides and fat accumulation in the liver (Tappy et al., 2012). A substantial intake of fructose, up to 150 g of fructose per day by healthy subjects for 1 week up and to 6 months, resulted in a significant increase in body weight (Tappy et al., 2012). The increase in fat deposition among subjects was explained by excess energy intake (Tappy et al., 2012). The fact that fructose overconsumption increased fat deposition could be because of its ability to stimulate hepatic de novo lipogenesis. In addition, hepatic inflammation and non-alcoholic fatty liver disease may be a consequence of excessive fructose intake (Tappy et al., 2012). Evidence from animal studies regarding fructose metabolism suggests that a diet containing 32% fructose or sucrose led to weight gain in rats, and increased energy intakes compared to chow-fed controls. In addition, fructose-or sucrose fed rats had higher TG levels than chow-fed rats (Basciano et al., 2005).

5. Fructose intakes and health risks

A number of epidemiological studies have examined the effect of high sugar intake on human health (Lustig et al., 2012). The western diet now contains more prepared foods containing added sugar than in the past (Tappy et al., 2010). Consumption of added fructose is estimated to be between 85 and 100 g per day in adults (Basciano et al., 2005). Fructose is often hidden in foods so actual

consumption may exceed perception of intake. The American Heart Association recommendation for added sugar intake suggests it should be limited to 80 calories/day; this is equivalent to 5 teaspoons per day for women, and 144 calories or 9 teaspoons per day for men (Johnson et al., 2009). Controversy exists regarding the contribution of dietary fructose from added sugars, in particular sweetened beverages, to obesity, cardiovascular diseases, diabetes and metabolic syndrome (Stanhope et al., 2013). Unfortunately, the inconsistent results from fructose consumption trails have caused confusion about the adverse effects of fructose on health (Jones et al., 2009). Some studies and intervention trials have demonstrated the adverse effects of high fructose intake on health whereas other studies have not shown any effects. The varied results observed in the literature may due to the use of pure fructose in feeding trials while humans consumed fructose as HFCS (Jones et al., 2009), and relatively short trials with small numbers of participants (Sievenpiper et al., 2012). For example, in hypercaloric trials, an effect of fructose on body weight was demonstrated, while isocaloric trials have not demonstrated any changes (Sievenpiper et al., 2012). Pure fructose has been demonstrated to induce hyperuricemia in animal studies; this may contribute to metabolic syndrome and high triglyceride levels (Angelopoulos et al., 2009). Further studies are required to clarify the differential effects of HFCS and purified fructose (Angelopoulos et al., 2009). The impact of increased dietary fructose on health remains controversial (Angelopoulos et al., 2009).

Fructose effects on metabolic disorder

While the definition of metabolic syndrome differs among organizations in terms of criteria and details, there is agreement that the essential elements of metabolic syndrome include glucose intolerance, obesity, hypertension and dyslipidemia (Eckel et al., 2005). Through the investigation of the contribution of dietary fructose to obesity and weight gain, the association between dietary fructose and metabolic syndrome has also captured scientific attention. This was closely examined in studies examining the relationship between intake of SSB and risk of metabolic syndrome. SSB refers to soft drinks, fruit drinks, energy and vitamin water drinks which included caloric sweeteners such as sucrose, HFCS or fruit-juice concentrates (Hu et al., 2010). A prospective cohort analysis of 50,000 women in the Nurses' Health Study II conducted over a period of 8 years from 1991 to 1999 investigated the association between intake of SSB and weight change and risk of type 2 diabetes. Semi-quantitative food frequency questionnaires (FFQ) were used to assess dietary intake of all food and beverages over a 1 year period. Participants' age ranged from 24 to 44 years. The study reported that the average weight gain observed in women who had stable intake of SSB was 2.8 kg. However, weight gain of 8 kg was observed in women who had intakes of SSB that increased from "1 or fewer drinks per week" to "1 or more drinks per day" over the 8 year study period. In addition, the study reported an association between high SSB intake and risk of type 2 diabetes compared to women whose intake was less than 1 SSB per month (relative risk, 1.83; P<001) (Schulze et al., 2004). A recent study by de Koning et al. (2012) investigated the

role of sugar-sweetened and artificially-sweetened beverage intake on coronary heart disease (CHD). The study was part of a large prospective cohort, the "Health Professionals Follow-up Study" of 42,883 men. Participants' usual intake was assessed using a FFQ. Over 22 years, 3,683 men developed CHD. A higher relative risk of CHD was observed in the top quartile of SSB consumption compared with the bottom quartile (RR=1.20; $P< 0.001$). In contrast, no association was found with artificially sweetened beverages (P=0.28). The association between CHD and SSB was weaker after adjusting for smoking, alcohol consumption, physical activity, multivitamin intake and family history; however, the association between CHD and artificially sweetened beverage was strengthened by those adjustments. Positive associations were identified between SSB and plasma triglycerides, C-reactive protein, tumor necrosis factor receptors 1 and 2 and negative associations were observed between SSB intake and lipoprotein and leptin concentrations (P<0.02). In general, this study suggests that eliminating SSB may help to prevent CHD (Koning et al., 2012). Another study examined the associations between diet soda intake, risk of metabolic syndrome and the occurrence of type 2 diabetes, controlling for multiple lifestyle factors, demographics and dietary intake (Nettleton et al., 2009). This study was part of the Multi-Ethnic Study of Atherosclerosis (MESA) which recruited 6,814 Caucasian, African American, Hispanic, and Chinese adults, aged 45–84 years. FFQ were used to assess the consumption of diet soda. Diet soda referred to diet soft drinks and unsweetened mineral water, while sugar sweetened soda referred to regular soft drinks, soda, sweetened mineral water and non-alcoholic beer.

Three follow-up examinations were carried out to identify the incidence of type 2 diabetes (2002-2003, 2004-2005, and 2005-2007). The study demonstrated that those who consumed ≥ 1 serving per day of diet soda had a greater relative risk of developing metabolic syndrome (RR=1.36) compared to those who did not consume diet soda, and greater relative risk of developing type 2 diabetes (RR=1.67) compared to non-consumers, after adjustment for demographics and lifestyle.

Effect of fructose on weight gain

Evidence suggests that consuming SSB and carbonated drinks that contain HFCS contributes to energy imbalance and the risk of developing obesity. A study by Teff et al. (2004) investigated the effect of fructose-sweetened beverages versus a glucose-sweetened beverages consumed with a controlled diet, in 12 normal weight women (aged 19-33 y, BMI ranges 19.8-26.7 kg/m^2). Participants were randomly assigned to each experimental beverage one month apart. Each study lasted for two days with 24 hours of controlled diet. Three meals containing 55% carbohydrates, 33% fat, and 15% protein of total kcal were provided, in addition to 30% of total kcal as either glucose-sweetened drinks or fructose-sweetened drinks. The gastroenteric hormone ghrelin was suppressed by approximately 30% 1–2 h after consumption of high glucose beverages ($P < 0.01$); however, the postprandial suppression of ghrelin was less pronounced after consumption of high fructose beverages ($P<0.05$). Leptin reduction was associated with decreased insulin concentration after the fructose-containing meals (Teff et al., 2004). The study indicated that high intake of fructose-

sweetened beverages results in increased plasma triglycerides compared to glucose-sweetened beverages ($p < 0.005$). As insulin, leptin and ghrelin function as key signals for the regulation of energy balance (Porte et al., 2002), the observed decrease in circulating insulin and leptin and increased ghrelin concentration could increase caloric intake and thus may contribute to weight gain (Teff et al., 2004).

The role of fructose in the development of cardiovascular diseases

The prevalence of diets high in fructose throughout the world appears to be a risk factor for hypertension and diabetes, which can lead to coronary vascular diseases (Johnson et al., 2007*)*. An increased blood pressure was observed in healthy subjects after six weeks of consuming a 33% sucrose diet, but not after a 5% or 18 % sucrose diet (Johnson et al., 2007). Other studies have suggested that a diet high in either fructose or sucrose leads to insulin resistance and impaired glucose tolerance (Johnson et al., 2007). Fructose consumption elevates blood uric acid concentration which may contribute to cardiovascular disease. When fructose is metabolized by fructokinase, it prevents the decrease of ATP molecules. As a result of this mechanism lactic and uric acids are produced (Johnson et.al, 2007). Uric acid increases 1-4 mg/day when a high fructose diet is consumed (Johnson et.al, 2007). Evidence from animal studies has suggested that reducing uric acid concentration produced by a high fructose diet may prevent the development of metabolic syndrome by reducing weight gain, hypertriglyceridemia, hypertension and insulin resistance (Nakagawa et al., 2006).

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Section 2: Dietary Assessment Methods

The role of dietary fructose assessment methods

Monitoring fructose intake is important to understanding the associated risk of chronic and obesity, since fructose may contribute to these conditions. As fructose in the diet is derived from natural sources and is added to foods as sweeteners, a database of fructose-containing foods would be useful for gathering information about the amount of fructose that is consumed and its epidemiological role (Park, 1993).

1. Commonly used methods for assessing dietary fructose intake

The most common approaches that have been used to assess fructose intake have been per capita disappearance data and individual food intake reports (Tappy et al., 2010). Understanding the strengths and weaknesses of these methods is important for the interpretation of results and can also help provide practical guidance in developing new methods for fructose assessment.

Per capita disappearance data

This method was established to provide information on the availability of agricultural commodities and has provided important information to parties involved in food production, marketing, and consumption (Park.1993). It has been used to estimate food intakes of a certain country in a specific period of time. Information collected regarding a particular food includes production, warehousing, import and exports, and distribution (Gibson, 2005). These data are very useful in the study food consumption for industrial purposes and to help ascertain individual consumption (Tappy et al., 2010) (USDA).

Fructose consumptions using per-capita disappearance data

Fructose intake is calculated according to its sources, either naturally occurring or from added sweeteners. The disappearance data available for sweeteners includes sucrose, HFCS, honey and syrup (Tappy et al., 2010). Sucrose and HFCS represent added fructose while disappearance data for fruit and vegetables represents naturally occurring or free fructose (Park, 1993).

Strengths and limitations

The measurements of food availability in per-capita disappearance data remain consistent when data are collected over time. The accuracy of per-capita disappearance data depends on the accuracy of food availability sources and does not provide reports of actual consumption. For example, disappearance data does not take into account food wastage and spoilage in the store or at the consumer's home, which leads to overestimation of true intakes (Jeffery et al., 2007). Moreover, disappearance data provide basic information on food availability in the early stages of production (farm production or at the first level of processing). These data are incomplete when reporting on highly processed foods such as baked goods, frozen meals and prepared soups (USDA)**.** Therefore, fructose intake cannot accurately be estimated from disappearance data due to its presence in highly processed foods, and losses and waste at the consumer level (Tappy et al., 2010).

2. Individual food intake reports

There are two methods for estimating total intake of a particular nutrient by individuals. The first measures intake over a 24-hour period using food recalls or diet records. The second method is used to obtain information about food patterns over a long period of time such as by FFQ.

Twenty four hour recalls

For the 24 h recall method, subjects are asked by a trained researcher to report exactly the foods and beverages they consumed in the previous 24 h period. Reporting actual intake using 24 h recalls is carried out using a standardized method called the multi-pass method, and is conducted in five stages. First, subjects are asked to list all food and beverages that were consumed on the previous day, without interruption from the interviewer. Second, participants are asked about forgotten foods and beverages from nine food categories. Third, the timing of meals and snacks is reported. Fourth, detailed information on food that was consumed and its portion size is collected (Willett, 2012). Portion sizes can be determined using household measurements. For example, the use of cups, spoons and food models can help subjects recall the portion size they consumed. Last, the reported food is reviewed to ensure all items are included (Willett, 2012). To improve and enhance the 24 h recall, an Automated Multiple-Pass Method (AMPM) using a computerized program to support the 5 step method was developed (Moshfegh et al, 2008). The AMPM improves the recalls by providing standard questions and a variety of answers in response to different food intake. Each option is designed to lead to the next question. This approach helps to

reduce participants' response burden and keep the interview consistent (Moshfegh et al, 2008).

A single 24 h recall is not effective in estimating the habitual food intake of an individual; however, multiple 24 h recalls for several days can be used to provide valid measurements of actual intake. The validation of a 24 h recall can be measured under controlled conditions in which actual dietary intake of protein, carbohydrates, energy and fat is determined after observing subjects' intake of food offered in a research setting. Conway and colleagues employed this method to assess the accuracy of 24 hour dietary recall in 42 obese, overweight and normal weight men within two weeks period. Variety of meals and snack was provided in the study facility for subjects to select and consume for 1 day. By observation and 24hour dietary recall, the study determines the actual and recall of energy, carbohydrates, and protein intake. The findings indicate there was no significant differences between the actual intake and the recall , and the accuracy of recall was not related to BMI (Conway et al, 2004).

Strengths and limitations

The 24 h recall is a low cost tool that requires relatively little time, since the interview usually lasts between 30-45 minutes. This method is a very useful approach to assessing dietary intake in a study population, because the interviewer manages the interview which leaves relatively little burden on subjects (Coulston et al., 2012). Detailed information about both the type of food consumed and portion sizes is provided in the 24 h recall method, which may reduce systematic errors (Dodd et al., 2006). Moreover, the 24 h recall method interferes less with

dietary behavior than the food record method, because the food that is reported by the 24 h recall method has already been consumed (Coulston et al., 2012). However, within-subject variables such as dietary variation from day to day and measurement errors derived from variability in cooking methods, recipes and food composition databases, indicate that a single 24 h recall is not suitable for estimating intake over long periods (Dodd et al., 2006). The primary limitation of the 24 h recall method is that it relies on the subject's memory to report intake, which depends on a variety of factors including age, gender, intelligence, mood, attention, and consistency of eating pattern (Willett, 2012). Thus, a 24 h recall is not suitable for subjects who use medications or who have loss of memory because psychological factors affect recall. In addition, subjects may underestimate their intake, especially of high calorie foods and the amount they consumed, and may overestimate foods perceived to be healthy (Johansson et al., 2001). The degree of error can be reduced by collecting information about the individual's activities on the day before the interview. This approach helps to refresh memory and facilitate more accurate reporting (Willett, 2012).

Dietary record method

This method involves collecting detailed information on foods consumed on one or more days by recording this information in a journal. Recording must be completed immediately after the food is consumed in order to reduce errors related to memory (Gibson, 2005). When multiple dietary records are needed, food records must be carried out consecutively within a 7 day period (Coulston et al., 2012). To increase accuracy of this method, participants must receive detailed

instructions about recording techniques. Dietary assessment by recording foods eaten prospectively can involve a number of different methods. Weighed food records can be obtained by determining the weight of each portion of a food or ingredient measured in grams, and measuring volumes. In addition, using household measurements such as cups, spoons and rulers facilitates recording of measurements. Calibrated scales and measuring tools can be made available for participants for use through the recording period (Willett, 2012).

Strengths and limitations

One of the major advantages of dietary record methods is that participants are well trained to precisely record their intake, including time of consumption of food which minimizes memory errors and promotes healthy eating habits (Willett, 2012). However, the method has high participant burden and can result in fatigue and therefore less reliability in recording (Biro et al., 2002). When subjects are not eating at home or delay their recording, details about ingredients or cooking methods may be missed. Prospective recording also leads to changes in dietary behavior which may not represent the participant's true intake. In fact, this approach may lead to "reactivity" that causes participants to select healthier or more socially acceptable foods (Willett, 2012).

Food frequency questionnaire (FFQ)

The food frequency questionnaire (FFQ) is used to estimate food that is consumed based on dietary components over a longer term (weeks, months, or years; Willett, 2012). The FFQ is commonly used for epidemiological studies, as

the logistics of this method are compatible with estimating general consumption of a large cohort of subjects, unlike diet records or 24 h recalls (Willett, 2012). Further, the method facilitates the ranking of subjects as high or low consumers (Brio et al., 2002). The questionnaire lists commonly consumed foods and asks the participants to record the frequency of intake of each food item. In addition, composition and quantity of food are included. The food types are listed clearly so that subjects can respond easily (Willett, 2012). When portion size estimation is required, a semi-quantitative FFQ is performed. There are many options to enable participants to specify their portion size, for example, the participant is asked how many glasses of milk were consumed instead of how often milk is consumed. Another possibility is to request a description of the portion size of each food. Generally, this approach involves multiple choice questions that describe the portion as small, medium and large using food model or pictures or units (Willett, 2013).

Strengths and limitations

Since the FFQ is affordable, easy to use and self–administered, it is very commonly used to estimate usual intake in large epidemiological studies (Willett, 2012). The FFQ is designed to estimate changes in dietary intake due disease or other temporary conditions. The responses on the FFQ may be used to distinguish between individuals according to nutrient or food group intake, especially when portion size is reported (Coulston et al., 2012). However, one limitation of the FFQ is that measurements are only estimates, which are often are not as accurate as recording the diet or 24 h recall. Neglecting to report or choosing to omit foods

from the list are two examples of these errors. The FFQ may lead to overestimates or underestimates of usual intake depending on the length of the questionnaire. There is debate about whether the FFQ is a suitable method to reflect population intake (Coulston et al., 2012). Some studies suggested combining a FFQ with 24 h recall or dietary record would be the ideal method to estimate true intake.

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Section 3: Dietary Biomarkers

1. Introduction of dietary biomarker

As sugar consumption continues to rise, the task of developing accurate assessment methods of sugar intake becomes more important (Tappy et al., 2010). Assessment methods commonly used include FFQ, food diaries, and 24 h recalls. These methods rely on participants' self-reporting of foods and ingredients consumed, portion sizes, frequency of intake, and cooking details, which are all potentially subject to substantial error and bias (Jenab et al., 2009). Furthermore, these methodologies generally do not capture differences in dietary habits due to variation in culture and lifestyle. Other psychosocial and environmental factors may affect dietary assessment accuracy, including underestimation of the frequency of food consumption and portion sizes, omission of ingredients in recipes and cooking method, lifestyle confounders, and variation in daily consumption (Jenab et al., 2009). Since dietary assessment methods are always associated with errors, understanding and reducing these errors is important and necessary. As a result of the errors and biases inherent in self-reported assessment methods, there is considerable interest in the use of biomarkers as a means of assessing true dietary intake. Typically, a biomarker is a compound measured in biological specimens such as urine or blood that is specific for a particular nutrient (Bingham et al., 2002).

2. Applications of a urinary biomarker

The ideal dietary biomarker is sensitive, accurate and reflects the intake levels of particular nutrients. A true biomarker functions as an indicator of food consumption through different periods and may relate to chronic or acute effects of the nutrient (Jenab et al., 2009). Since biomarkers are quantitative measures which do not have the same measurement errors as those related to self-reported dietary assessment errors and reporting bias, they may be appropriate for use among populations or individuals. There are four types of dietary biomarker: recovery, predictive, concentration, and replacement. These types of biomarker have been used either to validate dietary assessment methods or to estimate intake levels (Jenab et al., 2009).

The recovery biomarker

This type of biomarker is considered the most important because it helps to assess the extent of measurement errors associated with dietary assessments methods (Jenab et al., 2009). It is described as a compound demonstrating a "quantitative, time-associated relationship between dietary intake and recovery (excretion) in human waste" (Neuhouser et al., 2008). In other words, there is a strong association between excretion levels and total intake at a fixed period in time. Three examples of a recovery biomarkers are: doubly labeled water, which is used to measure energy expenditure (Bingham et al., 2002), urinary nitrogen, which is used to estimate dietary protein, and urinary potassium, which is used to measure the total intake of potassium (Jenab et al., 2009). However, recovery biomarkers are not extensively used in epidemiological studies because they

generally require complex analysis systems and are currently very expensive (Jenab et al., 2009).

Concentration and replacement biomarkers

These are two types of biomarkers that have similar purposes in that they are used to estimate the relationship between diet and the risk of certain diseases. Concentration biomarkers are used in validation studies of dietary self-reports since these biomarkers are involved in complex metabolic processes. Examples of a concentration biomarker are plasma ascorbate for vitamin C, serum carotenoids for fruit and vegetable intake, and n-3 fatty acid (FA) for fish intake (Vandevijvere et al., 2012). To examine the use of ascorbate as an estimate of vitamin C intake, a study by Drewnowski et al. (1998) assessed dietary intake of 361 males and 476 females aged 18-94 y from France, using the diet history method. An automated method was employed to measure vitamin C in plasma samples. The study reported there was high correlation between fruit intake and serum ascorbate ($P<0.001$). When multiple regression analysis was carried out, a negative association was identified between age, smoking, and body mass, and serum vitamin C (Drewnowski et al., 1998). Some limitations have arisen regarding the use of concentration biomarkers. Plasma samples must be preserved immediately once collected to avoid degradation of ascorbate; even when samples were preserved with metaphosphoric acid, 0.1% of total ascorbic acid was lost in the samples. Secondly, the concentration of ascorbate varied according to the current intake of vitamin C, therefore this biomarker would not be useful for an intermediate or long period of time. In addition, the fluctuation of ascorbate in

plasma samples requires a fasting blood sample. While plasma ascorbate can be predicted at low intakes of vitamin C between 50-90 mg/d, when the intake exceeds 200 mg/d, plasma vitamin C is not predictive of intake due to increases in renal clearance (Mayne et al., 2003). Therefore, this biomarker does not reflect the actual amount of dietary intake and is usually used in addition to dietary questionnaires (Vandevijvere et al., 2012).

Predictive biomarkers

This class of biomarker has been recently developed to estimate the degree of error arising from dietary assessment methods. It is defined as a sensitive marker that shows a dose-response relationship to the intake of a nutrient, over a specific period (Jenab et al., 2009). For instance, 24 h urinary fructose excretion has been used to predict the total sugar intake of an individual. A study by Tasevska et al. (2005) demonstrated a dose–response association between sugar (fructose/sucrose) intake and excretion in 12 healthy volunteers given a diet for 10 days that contained either low, medium or high amounts of sugar per day (63, 143, and 264 g/day respectively). There was a significant correlation between total sugar intake and sucrose and fructose excretion in urine collected over a 24 h period ($r=0.89$; $p < 0.001$). The study also examined the use of 24 h urinary fructose and sucrose concentrations as biomarkers to predict sugar intake in healthy participants while they consumed their usual diet. In this case, 41% of total sugar came from sucrose and the average total sugar intake was 202 ± 69 g/day .The average urinary sucrose and fructose excretion in the subjects was 36.6 ± 16.6 mg/day and 61.8 ± 61.3 mg/day, respectively. A significant correlation was

observed between sugar consumption and the sum of fructose and sucrose in the urine (r=0.841; $P< 0.001$). The authors concluded that the measurement of total fructose and sucrose in the urine are correlated with total sugar intake, which suggested that 24 h urinary fructose and sucrose excretion could be a potential biomarker for sugar consumption (Tasevska et al., 2005).

3. Factors that affect the utility of urinary biomarkers

Understanding the utility of biomarkers for estimating intake of particular nutrients such as vitamins, minerals, proteins and other foods not categorized as nutrients (e.g., sugar), would allow greater precision in assessing health outcomes of a variety of diets (Potischman & Freudenheim, 2003). However, interference with the concentration of a nutrient due to metabolism, absorption, nutrient-nutrient interactions and variation in excretion rates may influence the validation of a dietary biomarker (Potischman & Freudenheim, 2003). It is also necessary to take into account other factors that contribute to the accuracy of biomarkers in biological samples such as storage, collection and treatment. The accuracy, precision, and technical aspects of analytical methods used to measure biomarkers vary from one laboratory to another and may influence the utility of dietary biomarkers (Jenab et al., 2009). These variables should all be considered when a new biomarker is investigated and assessed, in order to obtain greater accuracy.

4. Evidence supporting the use of urinary fructose as a biomarker of sugar intake

The validity of using urinary fructose as a biomarker of sugar intake has recently been evaluated. Although the investigation is ongoing and more evidence is needed to understand the validity of this biomarker, several studies have demonstrated the possibility that urinary fructose and sucrose excretion may be reflective of the amount of sugars consumed by individuals.

Urinary biomarker of intrinsic and extrinsic sugars intake

Extrinsic (added sugars) may be absorbed or metabolized differently than intrinsic (naturally occurring) sugars (Tasevska et al., 2009). A total of 13 healthy individuals participated in an observational study with controlled conditions over a 30-day period to investigate the use of urinary fructose and sucrose as biomarkers to measure dietary extrinsic vs. intrinsic sugar intake. Participants recorded their usual diet in 7-day food diaries for 4 weeks before moving to the metabolic suite, where they lived for 30 days. From UK food composition tables, dietary sugar was classified according to its sources: extrinsic sugar such as "breakfast cereals, biscuits, cakes, sweet, buns, pies, flans, pastries, scones, cereal-based puddings and fruit juice" and sugar from fruit and vegetables, labeled as intrinsic sugar. Milk sugar was considered part of extrinsic sugar but was grouped separately because it has no cariogenic effects. All urinary output was collected every 24 h from each individual over the 30-day study period. The study reported a mean total sugar intake of 202 ± 69 g/day. Extrinsic sugar represented 60.1% of the total sugar intake, intrinsic sugars represented 34.1%,

and 5.5% of the sugars were from lactose. The study reported a significant correlation between individuals' 30-day mean sugar excretion and the 30-day mean intake of extrinsic sugar ($r=0.84$; $p< 0.001$). There was no correlation between urinary fructose and sucrose excretion and intrinsic sugar intake ($r=0.43$; $p<0.144$). By using regression models, a significant proportion of the variability in sugar excretion was attributed to extrinsic sugar intake (adjusted $R^2=0.64$; $p=0.001$). The unadjusted regression equation indicated that 100 mg of sucrose and fructose excreted per day in the urine predicted a daily consumption of 124 g of total extrinsic sugars. In addition, the study also investigated the use of a single 24 h urinary sugar assessment and a 4-day diet. A strong association was observed between urinary sugar excretion and extrinsic sugar intake ($r=0.63$, $P>0.001$). The authors concluded that urinary sugars correlate better with extrinsic sugar intake than the intrinsic sugar intake. This finding was suggested to be attributed to the high rate of extrinsic sugar absorption in the gut and consequent failure in hepatic capacity to metabolize excess sugar, which leads to faster gastric emptying and subsequently more sugar excretion in the urine. However, intrinsic sugar is present in smaller amounts and is associated with the absorption of a variety of nutrients, leading to slower absorption and more efficient uptake by the liver. Thus, there is less gastric emptying and less sugar in the urine. Another possibility is that the high intake of extrinsic sugar may interfere with biomarker correlation with intrinsic sugar, since both are measured as independent variables. As these findings were determined from a small group of volunteers, larger studies are

needed to confirm the use of this biomarker and validate the correlation between extrinsic sugar intake and urinary sugar (Tasevska et al., 2009).

Urinary biomarker in assessing sugar intake of normal weight and obese individuals

A study by Joosen et al. (2008) investigated the utility of urinary fructose and sucrose biomarkers in a randomized, crossover, dietary intervention study with two groups of volunteers who were classified according to their BMI: normal weight (BMI≤ 25 kg/m2, n=10), or obese (BMI≥30 kg/m2, n=9).The participants were provided three isocaloric diets containing a low, medium or high sugar content (13, 30 and 50% of total energy) over 3 separate 4-day periods. The study reported that over the three dietary periods, 24 h urinary fructose and sucrose excretion increased as the consumption of sugar increased, in both BMI groups. In addition, there were significant differences between dietary sucrose and dietary fructose. No significant interaction between the BMI groups and mean urinary fructose and sucrose excretion was observed. Thus, the study indicated that urinary fructose and sucrose excretion could be used as a biomarker because of its sensitivity to sugar intake, rapid reaction to dietary changes, and lack of impact on the biomarker by BMI class (Joosen et al., 2008).

Urinary biomarker in assessing sugar consumption in children

As recent studies have demonstrated, urinary fructose can be used as a biomarker in healthy adults to estimate their sugar intake. A study by Johner et al (2010) investigated the validity of urinary fructose as a biomarker for dietary sugar (added and total) intake in children. A total of 114 healthy children were

recruited from Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD), a study that involved the assessment of dietary intake, growth and metabolism from infancy to adulthood. From dietary intake assessment from 3-day weighed dietary records, added sugar intake was determined as the amounts of sucrose, maltose, lactose, glucose and dextrin, including honey and sugar from prepared food. Total sugar referred to the sum of both added sugar and intrinsic sugar and included fruit and fruit juices. The study reported a significant association between both added and total sugar, and 24 h urinary fructose excretion. Linear regression models revealed a stronger correlation between total sugar and urinary fructose values (R^2=0.181, $p<0.001$) compared with added sugar and urinary fructose value (R^2=0.055, $p=0.01$). A possible reason is that total sugar intake consists of natural and added sugar which included a considerable amount of fructose. Most urinary excretion of fructose originates from fructose consumption and it was reported that subject's diet contained a mixed amount of fructose-containing fruit and fruit juice. Thus, the study suggested that 24 h urinary fructose excretion could be used as a biomarker to estimate sugar consumption in children, regardless of its sources (Johner et al., 2010).

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Section 4: Nutrient Metabolism and Normal Physiological Changes of Pregnancy

1. Introduction

Physiological changes during normal pregnancy are essential as the mother's body is challenged to adapt and support the development of a growing fetus. Several factors affect the body's ability to respond to pregnancy including maternal age, the number of times she has conceived, and the number of fetuses being supported (i.e., multiple gestations) (Carlin et al., 2008).

2. The concepts of nutrient metabolism in pregnancy

The physiological adjustments that occur in pregnancy as a result of the demands of a growing fetus have an impact on maternal metabolism, and are essential for fetal growth and maternal homeostasis. Maternal metabolism adjustments happen gradually through the pregnancy and consist of complex interactions between the mother, the fetus, and the placenta (King et al., 2000). Several factors promote these adjustments which may vary from woman to woman since they are affected by factors such as pre-pregnancy nutritional intake, maternal health and lifestyle behaviors, and the size of the fetus (King et al., 2000).

The affects of hormones changes in pregnancy on metabolism

The secretion of the hormones from the placenta and corpus luteum are elevated in order to support the maintenance of the pregnancy, which has an effect on metabolism. Human chorionic gonadotropin is found in the serum and urine shortly after fertilization and rapidly increases through early pregnancy. A

decrease in the secretion of this hormone is observed later in pregnancy. Human placental lactogen also increases during pregnancy. Although the main function of this hormone remains unclear, it is suggested that it plays a role in carbohydrate and lipid metabolism. The biological structure of placental lactogen is similar to growth hormones, which also suggests it could play a direct role in the growth of the fetus and placenta (King et al., 2000). Estrogen hormones also have an impact on the mother, placenta, fetus, uterus and some of the reproductive organs, suggesting that estrogen stimulates uterine growth by enhancing uterine blood flow. In addition, changes in carbohydrate, lipid, and bone metabolism may be influenced by high levels of estrogen and other hormones which are secreted into maternal circulation and influence metabolism (King, 2006). Finally, the concentration of progesterone increases during pregnancy. This hormone helps relax the smooth muscles of the gastrointestinal and urinary tracts (King et al., 2000).

Fetal demands for certain nutrients

While fetal demand for nutrients increases in the last half of gestation, maternal adjustments to nutrient metabolism begin within the first gestational weeks. By approximately 10 weeks gestation, serum triacyglycerol was shown to be 20% higher than in non-pregnant women (King et al., 2000). In addition, phospholipids, cholesterol, glycerol, and fatty acids are increased in the serum of pregnant women but are not higher than triacyglycerol (King et al., 2000). The circulating concentration of nutrients decreases after the first 10 weeks of pregnancy to below the level observed in non-pregnant women (King et al.,

2000). The reduction of blood nutrient concentrations occurs prior to the rise of blood volume. For instance, an 8-10% decline in serum albumin at 10 weeks of gestation has been observed, and often drops to concentrations that are lower than in non-pregnant women (King et al., 2000). The decrease in serum albumin concentration is partially responsible for the decline in circulating nutrients since albumin functions as a protein carrier for many nutrients (King et al., 2000).

There are many aspects of nutrition in pregnancy which need to be considered, such as pre-pregnancy nutrition, adequate food intake through the pregnancy and birth weight (King et al., 2000). Therefore, assessment of nutrient intake in pregnancy should be treated with caution and precision.

3. Overview of the renal system

During pregnancy, several renal adaptations occur. The enlarged uterus causes the kidneys to increase in size and contributes to ureteral distension (Ind et al, 2007). In addition, hormones influence the renal pelvis, calyces and uterus. For example, high levels of estrogen and progesterone induce hyperplasia of the organs of the renal system. Moreover, an increase in blood volume also contributes to the increased kidney size observed in pregnancy. Studies of maternal physiology reviewed by Carline et al. (2008) suggest that kidney length in pregnant women is increased by approximately 2 cm (Carlin, 2008). The renal physiological changes start by the third month of gestation and remain until the twelfth week postpartum (Ind et al., 2007).

The functional changes in the renal system during pregnancy

During early pregnancy, increased renal blood flow (RBF) is accompanied by an increase in cardiac output (Ind et al., 2007), which results in increased renal capacity (Carlin et al., 2008). The increased RBF promotes a 40-50% increase in the glomerular filtration rate (GFR) at the beginning of the second trimester and this increase remains until the delivery (Carlin et al., 2008). As a result of increased GFR, excretion of creatinine remains constant, but serum creatinine concentration decreases. Creatinine clearance in pregnancy ranges from 100-150 ml/min and 91-130 ml/min in non-pregnant women (Jeyabalan et al, 2007). Uric acid excretion is increased during pregnancy due to dramatic changes in renal function; the elevation of GFR in early pregnancy may increase urinary uric acid later in pregnancy (Dunlop and Davison ,1977).

Nutrients excretion in pregnancy

Nutrient excretion patterns in pregnancy may change relative to the non-pregnant state due to the high nutrient load filtered by the glomeruli exceeding the reabsorption capacity of kidney (Ind et al., 2007). Excretion patterns of protein, amino acids, water soluble vitamins, glucose and sugars are discussed briefly below**.**

Protein

As pregnancy progresses, it is normal to detect a small amount of protein in the urine, particularly after 20 weeks of gestation. Urine may contain a trace amount of protein with an average of 300 mg per 24 h period (Jeyabalan et al., 2007). Urinary albumin excretion increases in pregnancy with concentrations

of 20 mg per 24 h excretion (Higby et al., 1994) compared to non-pregnant women whose excretion rates are approximately 10 mg per 24 h. However, higher levels of urinary protein excretion indicate a risk of renal disease. Preeclampsia is a pregnancy complication in which a significant amount of protein is detected in the urine. (Jeyabalan et al., 2007). The consequences of preeclampsia include placental abruption, fetal growth restriction, and maternal renal and liver failure. The cause of this condition remains unknown, but most studies refer to it as hypertensive or chronic illness (Seow et al., 2005). Recent studies have demonstrated that high energy intake including high added sugar and/or fat intake are also associated with increased risk of preeclampsia (Brantsaeter et al., 2009).

Amino acids

There are several amino acids that are excreted in the urine during pregnancy as a result of the increased GFR (Ind et al., 2007). The excretion of glycine, histidine, serine, threonine and alanine increases rapidly beginning at approximately 16 weeks gestation, compared to non-pregnant conditions. Other amino acids such as lysine, cystine, taurine, tyrosine, phenylalanine, valine and leucine also show a rapid increase in urinary excretion during early pregnancy and as pregnancy advances. The excretion patterns of amino acids in the urine are believed to have no association with physiological function or biochemical structures (Hytten et al., 1973).

Water soluble vitamins

Water soluble vitamins can be excreted in the urine when consumed at high levels in the diet (Shibata et al., 2013). When large amounts of these vitamins are consumed, they are absorbed by the digestive tract, stored in the liver and ultimately appear in the urine (Fukuwatari et al., 2011). A recent study by Shibata et al. (2013) investigated the potential of urinary excretion of water soluble vitamins as biomarkers in pregnancy. The study compared the excretion and intake of vitamin B_1, B_2, B_6, B_{12}, pantothenic acid, niacin, folate, biotin and vitamin C in pregnant women in all trimesters (first trimester, n=54; second trimester, n=24; and third trimester, n=32) and in lactating women (n=49) to a control group of non-pregnant and non-lactating women (n=37) in Japan. The average age among participants was 30 years. Dietary intake of water soluble vitamins was approximately equal to the recommended amounts, consistent with the DRI in Japan for pregnant and non-pregnant groups, excluding folate intake, which was higher in pregnancy. There were no differences in urinary excretion of vitamin B_2, B_6, B_{12}, biotin, or vitamin C among the groups. Urinary excretion of folate and niacin were higher in pregnancy than in the control group. Vitamin B_1 excretion decreased in pregnancy and pantothenic acid decreased in both pregnant and lactating women (Shibata et al., 2013). However, another study reported the

excretion of urinary niacin increased while urinary folate excretion decreased in pregnancy (Ladipo et al., 2000). The authors suggested that this could be due to a decrease in intestinal absorption, inadequate intake, or increased demand by the fetus (Ladipo et al., 2000).

Glucose

Changes in carbohydrate metabolism in pregnancy occur to ensure that the growing fetus receives an adequate supply for development (Mazze et al., 2012). Eventually, the increase in plasma volume that occurs during pregnancy leads to increased GFR and tubular flow rate. As a result of high tubular flow rate, the ability of the proximal tubule to reabsorb glucose is limited, which causes glucosuria in pregnancy (Jeyabalan et al., 2007). Approximately 50% of pregnant women exhibit glucosuria due to increased GFR, despite normal blood sugar (Alto et al., 2005). However, when plasma glucose is elevated over 140 mg/dl, glucose appears in the urine, and thus glucosuria may be result of gestational diabetes mellitus (Sacks et al., 2002).

Other sugars

Various types of sugars such as lactose, ribose, xylose and fructose are excreted in the urine during pregnancy (Hytten et al., 1973). The renal excretion of these sugars is not well understood. The mechanism of excretion and changes in the metabolism of these sugars remain unknown and further investigation is required.

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Chapter 3: Observational Study

Study (1): Spot urinary fructose analysis as a predictive tool for recent fructose intake during pregnancy

1. Introduction

High fructose intakes have been shown to increase the risk of diabetes, cardiovascular disease and insulin resistance (Mukai et al., 2012). Likewise, high sugar intake in pregnancy is associated with an increased risk of gestational diabetes mellitus (GDM) for the mother, and risk of obesity and other chronic conditions for the child in later life (Santiago et al., 2013). Inadequate intake and poor nutrition during gestation are also associated with poor pregnancy outcomes. A protein-restricted diet during pregnancy has been shown to lead to long-term metabolic effects for the offspring such as hypertension, glucose intolerance and kidney dysfunction in adulthood (Mukai et al., 2012). A maternal high fat diet may contribute to obesity in the child which is associated with the development of insulin resistance and hyperleptinemia (Mukai et al., 2012). Lastly, sugar (fructose) consumption during pregnancy has been shown to be detrimental to the health of offspring. This was shown in animal model which revealed that a maternal high-fructose diet resulted in hyperinsulinemia and altered carbohydrate metabolism in the offspring of pregnant rats (Mukai et al., 2012). High fructose intake through pregnancy may also affect the level of triglycerides in the liver and SREBP-IC expression (a transcription factor that regulates hepatic fatty acids and triglycerides biosynthesis) in both dams and fetuses (Mukai et al. 2012), which

may affect carbohydrate and lipid metabolism and contribute to the increased risk of metabolic syndrome and obesity. SREBP-IC is a regulator of lipid metabolism in the liver, and upregulation of this transcription factor may lead to insulin resistance, which contributes to the risk of metabolic syndrome and obesity (Mukai et al., 2013).

Better assessment methods for both sugar and fructose intake during pregnancy are required, as there are many shortcomings of dietary assessment methods such as 24 h recall or dietary food records. The development of a biomarker of fructose intake would be complementary to the traditional dietary questionnaires and food reports (Bingham et al., 2007). Since pregnancy is a state of physiological and dietary changes, this study investigates the use of a urinary biomarker to estimate fructose intake in the usual diet of pregnant women participating in the Sweet Moms Study.

2. Objective

To examine the relationship between a urinary fructose biomarker method and dietary sugar (fructose) intake estimated by self-reported dietary recall in pregnant women, for potential use as a complementary measurement to dietary assessment questionnaires.

3. Method

Study design and participants

Women living in the greater Edmonton area were recruited to the Sweet Moms study from maternal clinics, local hospitals, prenatal classes, and by word-of-mouth, between 2010 and 2012. The overall purpose of the project was to

examine the influence of sugar intake during pregnancy on mothers' and infants' health at birth. The inclusion criteria of the Sweet Moms study were: being pregnant without any pre-existing chronic medical condition, greater than 16 years of age, able to write and read in English, and living in the Edmonton area.

The data analyzed for this project included dietary and demographic information as well as urinary concentrations of fructose (described in detail below).

Demographics and anthropometrics

Women were asked to come to the research unit in any trimester of pregnancy for a single visit to complete a FFQ, a 24 h online dietary recall, a short questionnaire specifically targeting sugar intake in the past 7 days and to provide a urine sample. In addition, participants were asked about their weight before being pregnant and their height and current weight were measured. Wearing light clothes, women were weighed (to the nearest 0.1 kg) using a digital scale (Health O meter, China, capacity 0.1- 227 kg), and height was measured using a stadiometer (Digikit, WA, USA) to the nearest to 0.01 cm. Pre-pregnancy BMI was calculated from the information obtained (kg/m^2). Demographic information including age, educational level, marital status, ethnicity and personal income were also collected.

Dietary assessment questionnaire

A FFQ was used to estimate participants' dietary intake over the past year before pregnancy, and an online, 24h dietary recall tool was used to assess

participants' dietary intake on the previous day. Finally, a short sugar screening questionnaire (SSQ) was used to estimate dietary sugar intake from specific high-sugar foods over the past week (Appendix 3). The term "added sugars" included sugars that were added to food during processing or cooking and the term "natural sugars" referred to sugars that occur naturally in fruits, vegetables, dairy products, and grains (USDA, 2010).Total dietary fructose was calculated by analyzing the amount of fructose in each item or food that is either listed or reported by participants using a nutrient database such as Canadian nutrition file (CNF).

Urine collection

Participants were asked to provide a spot urine sample during their visit with no specific time in collection. Total urine volume was not recorded. Each participant was given a plastic sterile cup to collect the urine and samples were aliquoted into 3 small tubes (5.0 ml) and stored at -80°C until analysis.

Analytical method

Fructose concentration was measured in spot urine samples using a kit for glucose and fructose (D-glucose, D-fructose R-biopharm Mannheim enzymatic bioanalysis, Mannheim) using a spectrophotometer method (Spectra max 190, Molecular Devices, USA). Creatinine was measured using a biochemical urinary creatinine assay (Cayman, No.500701, Michigan, USA) and was used to correct for urine volume by calculating the ratio of fructose over creatinine (Barr et al., 2004).

Sample Treatment

Frozen urine samples were thawed at room temperature and centrifuged for 2 minutes in order to clear the samples from any solutes. Samples were checked for pH (within normal range 6.0) and clarity before analysis to ensure there was no interference with fructose measurements. Urine samples were aliquoted (30 µl) into a 96-well, UV plate. Buffer solution (100 µl) and ddH_2O (170 µl) were added to the samples and the plate was shaken for 3 minutes. Absorbance at 340 nm was determined using the spectrophotemeter and designated as A_1. Hexokinase was added (2.0 µl) to each well and the plate was incubated on the shaker for 10-15 minutes, after which the absorbance was read at 340 nm designated as A_2. Finally, phosphoglucose isomerase was added to each well (2.0 µl) and the plate was incubated for 10-15 minutes. This reading was designated as A_3. By subtracting (A_3 - A_2) in order to determine the absorbance difference of the blank and the sample. The final amount was calculated using the following equation: ($c = \frac{V \times MW}{\epsilon \times d \times v \times 100} \times \Delta A$) (Appendix 1). For the creatinine assay, urine samples were diluted 1:10, and the assay was performed according to the kit instructions. Creatinine concentration was calculated with the equation (creatinine (mg/dl) $= \frac{Sample\ absorbance - (y\ intercept)}{Slope} \times sample\ dilution$).

4. Statistical analysis

Urinary fructose values were $\log_{10}$ transformed to normalize the distribution as described by Bingham et al. (2005, 2007, and 2010). Correlation analyses were carried out by calculating Pearson's correlation coefficients to examine the

association between sugar intake and urinary fructose concentrations. Multiple regression analysis was used to estimate the relationship between several independent variables (maternal age, gestational weeks, pregnancy weight, pre pregnancy BMI and sugar intake) and dependent variables (urinary fructose excretion). In addition, a paired t-test was used to compare fructose consumption before and during pregnancy. P-values less than 0.05 were considered statistically significant.

Estimating Dietary intakes of Sugars

A SSQ was used to determine the amount of sugar consumed per day from commonly consumed sugary foods. Total sugar intake was assessed by using the values for total sugars available for each food in a sugar database that was constructed in our lab and combined information from the Canadian Nutrition File (CNF), the United States Department of Agriculture Nutrient Database (USDA), the Minnesota Food and Nutrition Database, and recipes. Added and naturally-occurring sugars were assessed by constructing a database that detailed this information.

5. Results

Women in this study were 16 to 41 years old (mean = 30 years) and had a pre-pregnancy BMI within the normal range (mean = 23.5 kg/m^2, Table 3.1). Gestational age at the time of recruitment ranged from 9 to 40 weeks. Based on the SSQ the average total sugar intake was approximately 86 g/day (603.2 g/week), while total dietary fructose intake was 284.7 g/week. The average of

daily energy derived from total sugar was 344.7 kcal. Urinary excretion of fructose ranged from 1.9 to 689 mg/L.

Table 3.1: Participant characteristics in Sweet Mom Study

Characteristic	n	Mean± SD	Range
Maternal age (yrs)	115	30.4 ± 4.8	16, 41
Pre-pregnancy weight (kg)	142	64.8 ± 14.3	43.6, 118.2
Pregnancy weight(kg)	148	75.7 ± 14.1	52.2, 128.2
Pre-pregnancy BMI (kg/m²)	140	23.5 ± 4.4	17.4, 40.6
week gestation (mean)	150	29.9 ± 6.6	9, 40
Pre-pregnancy sugar intake (FFQ)			
Total fructose (g)	156	48.7 ± 30.3	1.8,149.18
Weekly sugar intake (g/week)			
Total sugars g/week(SSQ)	168	603.2 ± 291.9	111.9, 1730
Natural sugars g/week (SSQ)	168	272.9 ± 131.9	57.8, 694.5
Added sugars g/week(SSQ)	168	390.3 ± 221.5	46.5, 1267.3
total fructose /week (SSQ)	168	284.7 ± 139	56.1 , 882.8
Previous day's sugar intake			
Sugar from 24 hr recall (g)	87	105 ± 46.8	18 , 268.1
Total energy from total sugar (kcal)	168	344.7 ± 167	63 , 988
Urine excretion			
Urine fructose mg/L	135	92.8 ± 114.7	1.9 , 689
Urine creatinine mg/L	135	673 ± 599.8	11.1 , 2730
Fructose creatinine ratio	135	0.81 ± 1.79	0 , 10.50

There was a significant difference between fructose intake in pregnancy (41.9 g ±19.8) and pre-pregnancy fructose intake (30.6 g±20.3) when a paired t-test was carried out (P<0.01) (Table 3.2).

Table 3.2: Comparison of fructose intake from FFQ and SSQ before pregnancy and during pregnancy

	n	Pre-Pregnancy (Mean ± SD)	During Pregnancy (Mean ± SD)	P
Total sugar intake g/d	**134**	**63.1 ± 40.5**	**87.04 ±40.8**	**<0.01**
Total fructose intake g/d	**134**	**30.6 ± 20.3**	**41.9 ± 19.8**	**<0.01**
Natural sugar intake g/d	**134**	**30.5 ± 23.2**	**39.1 ± 19.2**	**<0.01**
Total energy from sugar kcal	**134**	**252.5 ± 162**	**348.2 ± 163**	**<0.01**

Figure 3.1 describes the amount of fructose from foods and beverages in the food categories derived from the SSQ. These foods and beverages represent those that are commonly consumed sources of dietary sugar in Alberta.

Fruit is the most highly-consumed sugar-containing food among participants; 77% of participants consumed fruit 7 or more times per week (Table 5.3). Fruit juice consumption was reported to be consumed by 27% of women 1 to 2 times per week; 36.5% reported they did not consume any sweet drink such as pop, sport drinks, and slushes during the previous week. Also, 28% of participants reported they had “treats” such as chocolate bars, candies and granola bar about 3 to 4 times per week and 12.9% of participants consumed dessert 5 to 6 times per

week.

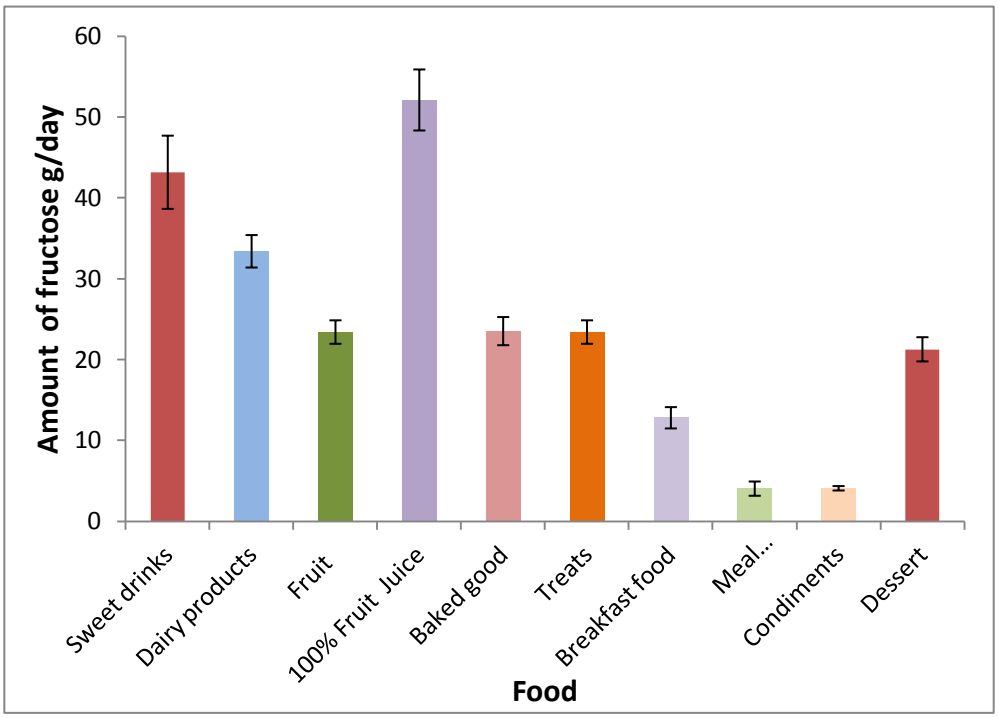

Figure 3.1: The amount of fructose from foods and beverages in the food categories listed in the SSQ

Sweet drinks: regular Pop, Sports Drinks, Sugar Sweetened Drinks and Slurpees/Slushies

Dairy products: chocolate milk, hot chocolate, flavored cappuccinos, frappuccinos, sweetened coffee drinks, flavored yogurt and milkshakes

Fruit: fresh fruit, canned fruit in syrup, fruit smoothies

Fruit juice: 100% orange juice, 100% fruit juice

Baked goods: cake, brownie, pie, fruit crisp, cobbler, strudel, donut, sweet rolls, Danish, pop-tart and cookies

Treats: chocolate bar, M&M's, smarties, Chocolate chips, candies and granola bar

Breakfast food: jam, honey and high sugary cereal

Meal replacements: meal replacement bar, meal replacement drink

Condiments: ketchup, sweet and sour sauce.etc

Dessert: ice cream, frozen yogurt, sorbet, sherbet, flavored ices, pudding, custard, jello, freezies, popsicles

Table 0.3The proportion of participants who reported consuming foods and beverages from different categories listed on the SSQ

	None		1-2 time/week		3-4 times/week		5-6 times/week		7 + more times/ week	
	n	%	n	%	n	%	n	%	n	%
Sweetened drink	65	36.5	56	31.5	27	15.2	16	9.0	14	7.9
Dairy products	17	9.6	31	17.4	36	20.2	37	20.8	57	32
Fruit			4	2.2	16	9.0	21	11.8	137	77
Fruit juice	28	15.7	48	27.0	34	19.1	27	15.2	41	23
Baked goods	21	11.8	45	25.3	48	27	24	13.5	40	22.5
Treats	24	13.5	45	25.3	51	28.7	30	16.9	28	15.7
Breakfast food	53	29.8	52	29.2	37	20.8	20	11.2	16	9
Meal replacement	153	86	12	6.7	4	2.2	4	2.2	5	2.8
Condiments	38	21.3	63	35.4	55	30.9	9	5.1	13	7.3
Dessert	25	14	61	34.3	45	25.3	23	12.9	24	13.5

*The total number of participants who completed the SSQ was 178

Based on the responses to the SSQ, 44% of total sugar intake comes from natural sources, and 56% comes from added sugar intake (Figure 3.2).

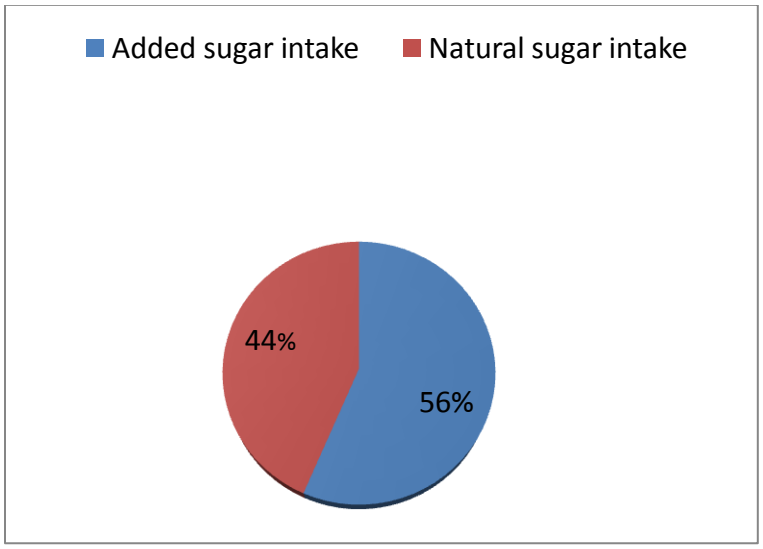

Figure 3.2:Proportion of added and naturally occurring sugars consumed daily by Sweet Mom participants

The distribution of urinary fructose excretion was positively skewed (Figure 3.3). Therefore, urinary fructose values were $\log_{10}$ transformed in order to normalize the distribution (Bingham et al., 2005). No significant association was observed between sugar intake and urinary fructose concentrations among pregnant women (Table 3.4). Linear regression analysis was used to determine the relationship between urinary fructose concentrations and sugar intake assessed using the SSQ, after adjusting the data according to pre-pregnancy BMI, maternal age and age of gestation. No significant interrelationship was found between those variables (Table 3.5).

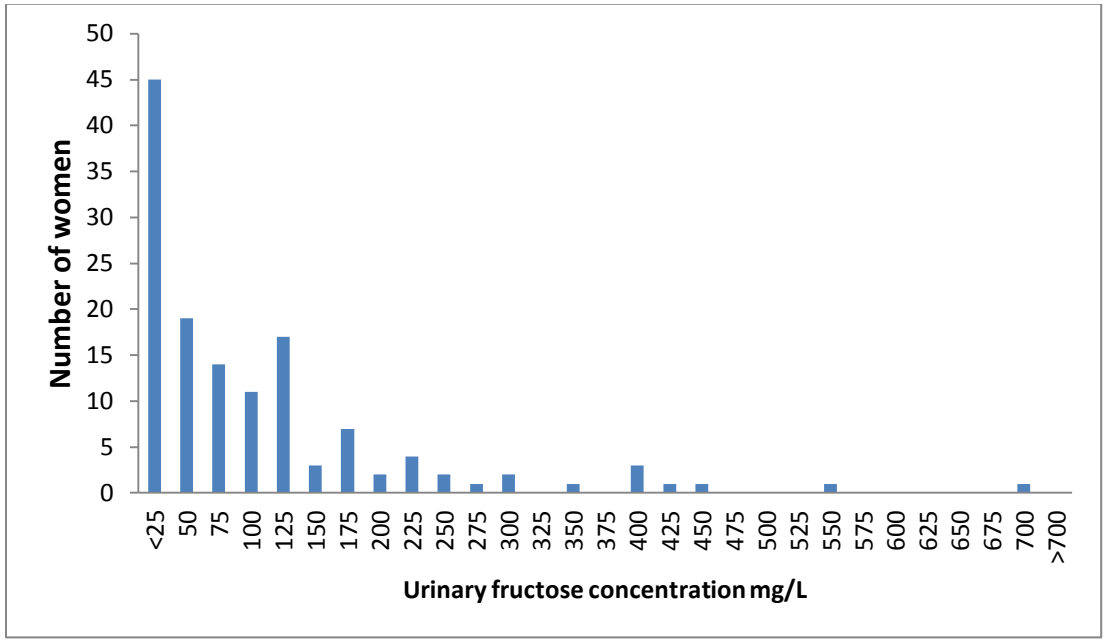

Figure 3.3: Frequency distribution of urinary fructose concentrations of pregnant women in the Sweet Moms study.

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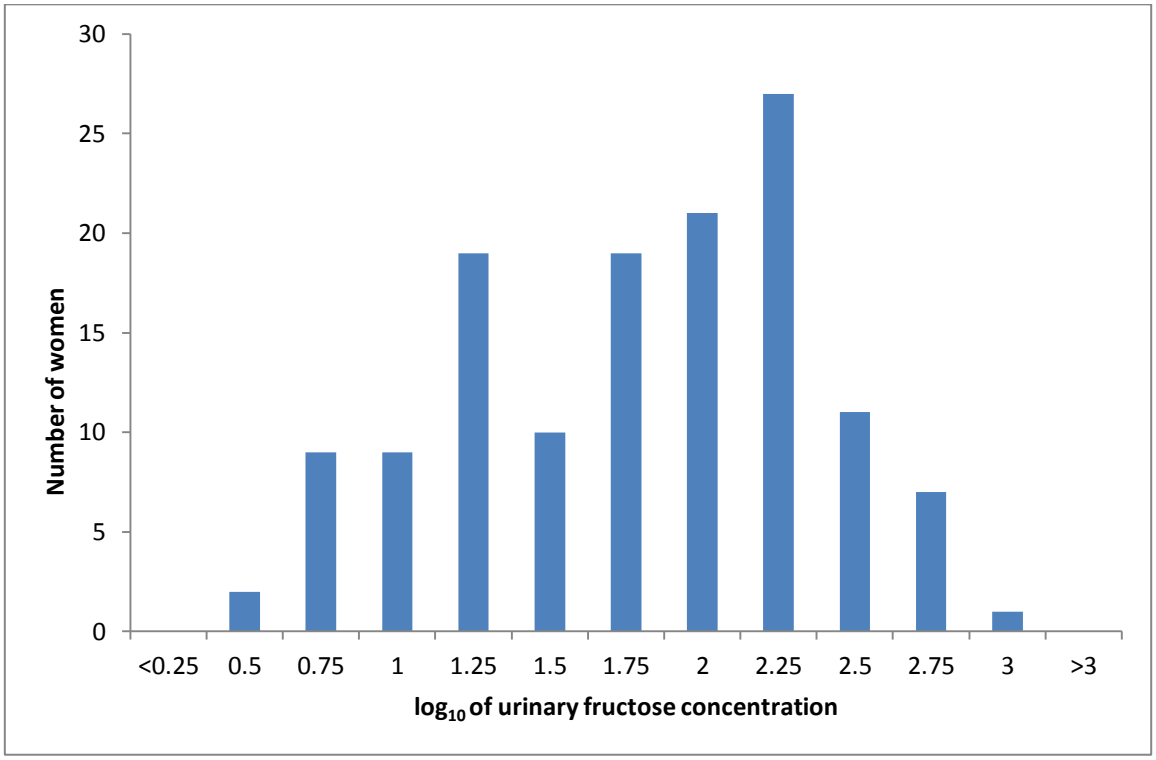
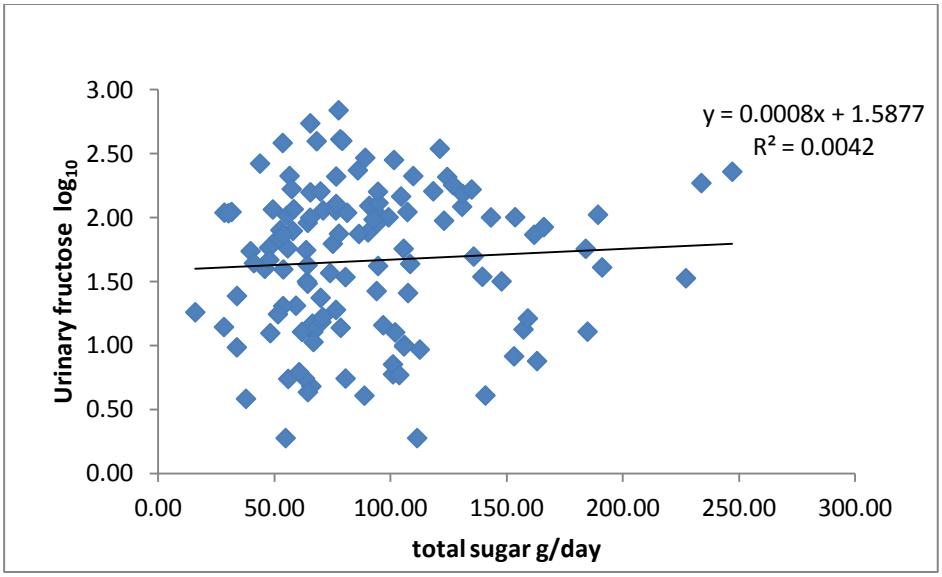


Figure 3.4. Frequency distribution of urinary fructose.



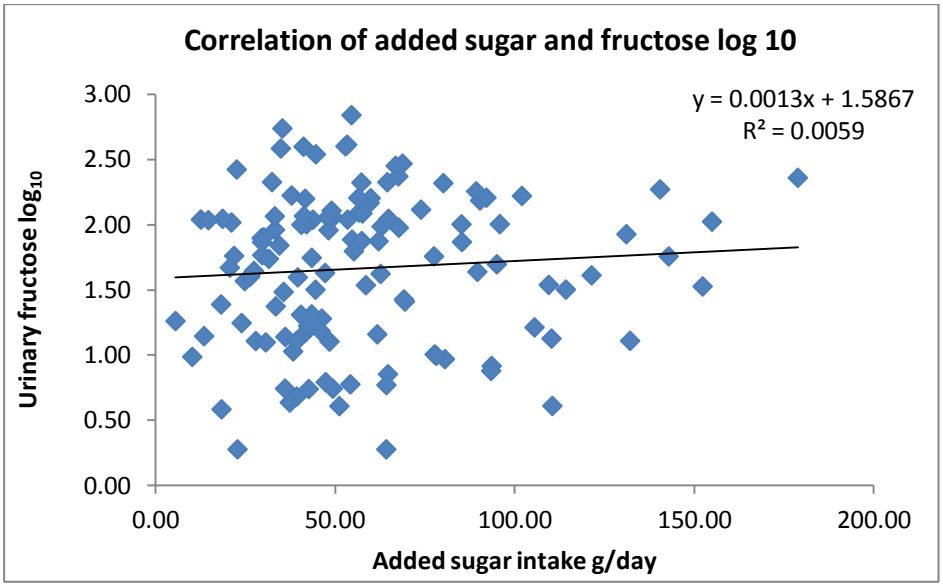


Figure 0.4:Dietary sugar intake (total, added) vs. urinary fructose concentrations (log10)

Table 0.4: Association between urinary fructose concentration and participant characteristic or dietary sugar intake

Variables	R	P value
Pre-pregnancy weight kg	0.11	0.20
Weeks of gestation	-0.01	0.87
Total sugar intake g/day	0.06	0.44
Added sugar intake g/day	0.07	0.40
Natural sugar intake g/day	0.01	0.84
Total fructose g/ day	0.05	0.54
Total fructose from FFQ	-0.08	0.34
Total sugar from 24hour recall	0.08	0.48

Table 3.5:Linear regression analysis of urinary fructose concentration with sugar intake assessed using the Sugar Screening Questionnaire (SSQ)

Sugar intake assessed using the SSQ	Urinary Fructose Excretion*			
	Coefficient (β)	p	95% confidence	
Natural sugar (g/day)	0.005	0.587	-0.26	0.14
Added Sugar (g/day)	-0.0011	0.86	-0.014	0.012
Sugar from Sweet drinks (g/day)	-0.0004	0.96	-0.019	0.018
Urinary creatinine excretion (mg/L)	0.00051	0.045	0.000011	0.001

*Multi linear regression, data was adjusted for pre-pregnancy BMI, maternal age, gestational age

6. Discussion

The objective of this study was to examine the relationship between urinary fructose and dietary sugar intake estimated by self-reported dietary recall in pregnant women, to determine whether sugar intake correlated with the concentration of urinary fructose. There was no association between these variables. This is somewhat surprising given that others have used similar methods and reported strong correlations between these variables in other populations, including healthy normal-weight and obese adults, and children (Bingham et al., 2007; Tasevska et al., 2005 & Johner et al., 2010). The extent to which the low degree of association between dietary intake and urinary excretion of fructose may reflect physiological differences between pregnant women and populations of non-pregnant adult women, men, and children, or reflects limitations in the current study that were not clear.

Fructose was detected in the urine samples from these women. This suggests that the biochemical assay was working and that the fructose concentrations were within detectable limits. The precision of the assay was determined by calculating the average CV% of urine concentration (19.4%). We expected that fructose would be found in the urine in very low concentrations as some fructose escapes hepatic hydrolysis and is excreted in the urine (Tasevska et al., 2009). The high urinary fructose excretion observed (meand 92.8 mg/L) was indicative of a high intake of dietary fructose. Other studies have suggested that intake of 200 g of sugar yields 100 mg of fructose and sucrose in 24 h urine excretion (Tasevska et all, 2009). Possible reasons for the lack of association

between dietary fructose intake and urinary fructose concentrations could be related to the urine collection method used, the dietary intake collection and analysis methods selected, and potential changes in the mechanisms related to sugar absorption and excretion in pregnancy. A wide range of fructose excretion in the urine (1.88-689 mg/L) was observed in this study. This may be related to the fact that urinary excretion of fructose was determined in spot urine samples. Spot urine samples are known to be influenced by recent intake (Barr et al., 2005). Spot urine samples were collected at a single study visit, based on the reports of other investigators who have demonstrated a strong relationship between spot urine samples and 24 hour total urine collection samples in non-pregnant participants (adult men and women) (Luceri et al., 1996). In the current study, urine samples were not obtained at any particular time of the day. The wide variation in the timing of urine collection could explain some of the variation in urinary fructose excretion observed. In pregnancy, spot urine samples may not reflect the true intake of sugar compared with the 24 h urine collection. Twenty-four h urine excretion is believed to predict the actual intake of sugar and may potentially be used as a predictive biomarker (Tasevska et al., 2005). Further, one study demonstrated the accurate use of a spot urine sample as a biomarker to estimate sugar intake in obese and non-obese individuals (Bingham et al., 2007).

Another possibility explaining why no significant association was observed between intake and fructose excretion in this study could be related to the variation in gestational age at the time of sample collection. Since the average gestational weeks was 29.9 ± 6.6 (Table 5.1), the majority of Sweet Moms

participants (n=95) were recruited in their third trimester of pregnancy. It has been suggested that fetal growth in the third trimester causes the bladder capacity of the mother to decrease, which results in an increase of the frequency of urination, urinary urgency, and incontinence (Gabbe et al., 2012). These physiological changes could affect the excretion of nutrients, including fructose. A creatinine assay was used to correct for variation in dilution among samples, and surprisingly this correction did not improve the association between dietary and urinary fructose. It is possible that the excretion of creatinine was inconsistent because of subject variability related to age and dietary intake (Miller et al., 2004) and thereby any relationship that exists between these variables in the pregnant state was masked. The presence of creatinine in the urine indicates proper kidney function since creatinine is produce as a waste product of muscle activity and it has been used to correct for variability in urine (Millers et al., 2004). The urinary excretion of creatinine is higher in pregnancy than in non-pregnancy, due to elevated blood volume and GFR. The normal value of urinary creatinine excretion is 25- 400 mg/dl (from creatinine kit assay datasheet). Excretion of creatinine in this study ranged from 11.1 to 2730 mg/L. Low value of creatinine in urine samples indicates that the samples was too diluted .In this case, fructose concentration in the urine are more likely to results in errors. In fact, the correction for creatinine in urine samples to indicate fructose level may lead to false reading due to within-subject variability. (Alessio et al., 1985).

This study also examined the validity of reported sugar intake during pregnancy. The results of a t-test indicated a significant difference between

fructose intakes prior to and during pregnancy, with a higher fructose intake during pregnancy than fructose intake before pregnancy. A study by Graham et al. (2013) demonstrated that pregnant women tend to increase their sugar intake as a substitute for caffeinated beverages and alcoholic drinks, or to meet the dietary recommendation of certain nutrients by adding a sweet taste to certain food items, such as chocolate milk (Graham et al., 2013). The FFQ is commonly used in cross sectional studies and provides general aspects about dietary intake. However, the FFQ that was used in the Sweet Moms study provided information about dietary intake in the year prior to pregnancy. A correlation between FFQ data and spot urine excretion would be impractical since the dietary data and samples are obtained from different periods of time. In this study, a sugar screener questionnaire (SSQ) indicated that high amounts of natural sugar were consumed by participants; this accounted for about 44% of total sugar intake. The major sources of this sugar were fresh fruit and 100% fruit juice. As 77% of participants reported their weekly consumption of fruit as 7 times or greater, this suggests that the intake of natural sugar is relatively high. Fruit contains a variety of nutrients and different type of fibers. Foods high in fiber tend to have slow rates of fructose absorption (Englyst and Englyst, 2005), thus, it is possible that there could be less sugar in the urine as a result of this process. This matter deserves further investigation, perhaps using alternate methods to measure dietary intake of fruits, vegetables and other foods containing naturally occurring sugars. Another possible limitation of the study was that the SSQ and other methods of dietary assessment relied on participants' memory. Therefore, low reliability in reporting,

errors in estimating intake, and bias in the assessments methods could lead to an under- or overestimation of fructose intake. In this study, 24 h dietary recalls were completed only once, which may not be sufficient to obtain a reliable estimate of sugar intake in this population (Willett et al., 2012). As well, this study had a relatively small number of participants which could be a limitation for detecting a statistically significant correlation. Determining the ideal sample size depends on the study objective and the expected results (Gibson, 2005) and can be determined by population size, confidence interval and standard deviation.

In this study, additional data analysis was carried out to demonstrate whether or not gestational age has an impact on sugar intake and excretion. In this case, urine samples were classified into three groups according to pregnancy trimesters: the first trimester (9-13 weeks), second trimester (14-28 weeks) and third trimester (29-40 weeks). No significant association was observed between sugar intake and spot urinary fructose between the groups. As mentioned earlier, the physiological adjustments in the renal system are substantial in the second and third trimester of pregnancy (Haas et al., 2003) and could interfere with fructose excretion.

7. Conclusion

Although the results of the study indicate no significant association between urinary fructose and sugar intake, further studies are required to investigate the potential use of spot urinary fructose as a biomarker of sugar intake during pregnancy. There were several challenges that we faced in the Sweet Moms project that need to considered, particularly the sugar intake assessments (SSQ)

and sample collection (random spot urine). In general, sugar intake was difficult to estimate since sugar is part of many food items and can be naturally occurring or added to foods during processing, cooking or at the table. Thus, true intake of sugar is difficult to estimate from self-reported intake assessments. The concept that sugar intake is highly correlated with urinary excretion may not be applicable to free-living participants. A previous study demonstrated that a urinary sugar biomarker correlated with sugar intake of healthy subjects under precise conditions. A controlled diet study could be more applicable for developing biomarkers (Bingham et al., 2007). The random collection of urine was the main limitation in the analysis, indicating that using a single random collection of urine does not yield a potentially useful biomarker. In addition, the lack of reporting of the size, time and fructose content of the most recent meal is a limitation of the study. Moreover, a larger sample size is needed to investigate the potential use of spot urinary fructose biomarker and verify the correlation with added sugar intake. Further research in this area is required to predict maternal sugar intake by using a urinary fructose biomarker considering all factors associated with the biomarker, dietary sugar assessment method and pregnancy status.

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Chapter 4: Validation study

Study 2: A urinary fructose biomarker to estimate dietary fructose intake in non-pregnant healthy individuals

1. Introduction

Due to increasing dietary fructose consumption, many research studies have focused on the effects of high fructose intake on the human body. Whether or not fructose is a contributor to the epidemic of obesity and risk of chronic disease is a controversial topic due to variation in methodologies employed in feeding trials, the small number of subjects and poor analysis methods (Sievenpiper et al., 2012). High fructose intake could increase circulating lipid levels (Havel, 2005), since hepatic fructose metabolism produces glycerol and acetyl CoA, which result in increased lipid synthesis (Angelopoulos et al., 2009). As the consumption of fructose has become significantly increased among the general population (Taylor et al., 2008), more accurate assessment methods of dietary fructose intake are needed. Although fructose is difficult to estimate from dietary intake due to selective misreporting and the likelihood of overestimated fruit intake and underestimated processed food and added sugar intake, a biomarker of urinary fructose excretion could potentially be useful for estimating fructose intake (Tasevska et.al, 2009).

2. Purpose

As the Sweet Moms study results (Chapter 3) were inconclusive in determining the validity of a urinary fructose biomarker in sugar assessment in

pregnancy, a validation study of non-pregnant healthy individuals was designed to assess the accuracy of a fructose biomarker under more precise settings. This validation study was a randomized, controlled, crossover dietary trial of non-pregnant women to validate the use of a fructose biomarker in spot urine samples. Due to limited research in this area in pregnancy, in addition to the confounding factors of hormonal and physiological changes during pregnancy, we hypothesized that non-pregnant women would be more suitable for a validation study, and allow an improved understanding of fructose excretion in urine.

3. Objective

a) To validate the utility of a fructose biomarker in spot urinary samples as a tool for estimating fructose consumption in healthy non-pregnant women.

b) To determine urinary fructose concentration changes in response to high and low intakes of fructose in healthy, non-pregnant women.

4. Study Protocol

Participants

A total of nine healthy non-pregnant women ages 22 to 29 (26.4 ± 2.13) from the University of Alberta were recruited for the study as they met the inclusion criteria of the study. Participants had different social and cultural backgrounds but all lived in Edmonton, Alberta, and were graduate or undergraduate students or research assistants. Participants' weight and height were measured at the research unit and body mass index (BMI, kg/m^2) was calculated. For weight measures, participants were asked to step on a digital scale (Health O Meter with capacity of 227-0.1 kg, China) 3 times consecutively and

the average was recorded. This approach was used to reduce errors in measuring. Height measurements were obtained by the same method using a stadiometer scale (Digi kit, WA, USA). Information about subjects' sugar intake was obtained via a short questionnaire that asked about their consumption of sugar during the previous week (SSQ). In addition, a 24 h online dietary recall was completed by participants to estimate sugar (fructose) intake on the previous day. A FFQ was also used to estimate dietary intake of the past 6 months. All participants were verbally informed about the study and signed the consent form. This study was approved by the Human Research Ethics Board at the University of Alberta.

Breakfast protocol

Participants were asked to visit the Human Nutrition Research Unit in the Alberta Diabetes Institute twice for a maximum 4 h per visit. During their visit which was typically early in the morning, participants consumed either a low or high-fructose breakfast, after an overnight fast of at least 12 h. In addition to the breakfast, a snack was provided. The breakfast consisted of a whole wheat cereal (Cheerios), 1% skim milk and blackberries with raw almonds as a snack (Appendix 2). For the high fructose meal, 450 mL of concentrated sweetened apple juice was served immediately after breakfast. By using The Canadian Nutrition File (CNF) for food labeling, we determined the amount of sugar and in particular, fructose that was contained in the breakfast (Appendix 2). Total fructose from the high-fructose breakfast was approximately 17 g. However, for the low fructose group, apple juice was replaced with water and total fructose was calculated as 3 g. The portion size and the amount of calories in the breakfasts

were calculated for each participant according to their basal metabolic rate (BMR). BMR was determined according to the following equation: (655.1+(weight(kg)× 9.56)+(height(cm)× 1.85)- (age(yrs) ×4.7). Then BMR was multiplied by physical activity to determine total energy intake. For determining energy intake for breakfast, we divided BMR by 4 since fructose intake was only examined in one meal, which represents ¼ of total daily intake. Energy intake for each participant remained constant through the study. Water was served freely during both study periods.

Experimental design

Participants were recruited by word-of-mouth. To meet the study criteria, participants had to be non-pregnant, aged 16-40, able to speak and write English, and live around University campus. All of the information about the study, such as purpose, protocol, measurements, dietary assessment, and sample collection, was explained briefly by a trained researcher. Once participants understood the study and agreed to participate, they signed the consent form and had their weight and height measured at the research unit. Accordingly, dates were assigned to each participant for the high and low fructose meals. Study meals were scheduled to be approximately 1 week apart. The breakfast was served once participants arrived to the research unit. While they consumed their prepared meal, they were asked to complete the following: FFQ, 24 h online dietary recall and SSQ. The dietary assessment questionnaire and recall were carried out at the first visit, and the SSQ was completed at each visit. After breakfast, participants were instructed about urine sample collection methods (described below) and waited in the unit

for 4 h. A snack and water were also offered within the study period. Table 4.1 provides an example of breakfast contents and energy intake of several study participants, and the study is summarized in Figure 4.1.

Table (4.1): Selected participants' breakfast portion size and calorie intake.

ID	High Fructose Meal			Low fructose Meal		
SM500	Breakfast content	Portion	Kcal	Breakfast content	Portion	Kcal
	Cereal	13.5 g	55	Cereal	27 g	110
	1% skim milk	125 ml	55	1% skim milk	250 ml	110
	Raw black berries	50 g	13	Raw black berries	76 g	33
	Almonds	1/3 cup	47.5	Almonds	1/8 cup	95
		Total	391		Total	348
SM418	Cereal	13.5 g	55	Cereal	54 g	220
	1% skim milk	125 ml	55	1% skim milk	250 ml	110
	Raw black berries	152 g	66	Raw black berries	152 g	66
	Almonds	18.5 g	107	Almonds	19.7 g	114
		Total	503		Total	510

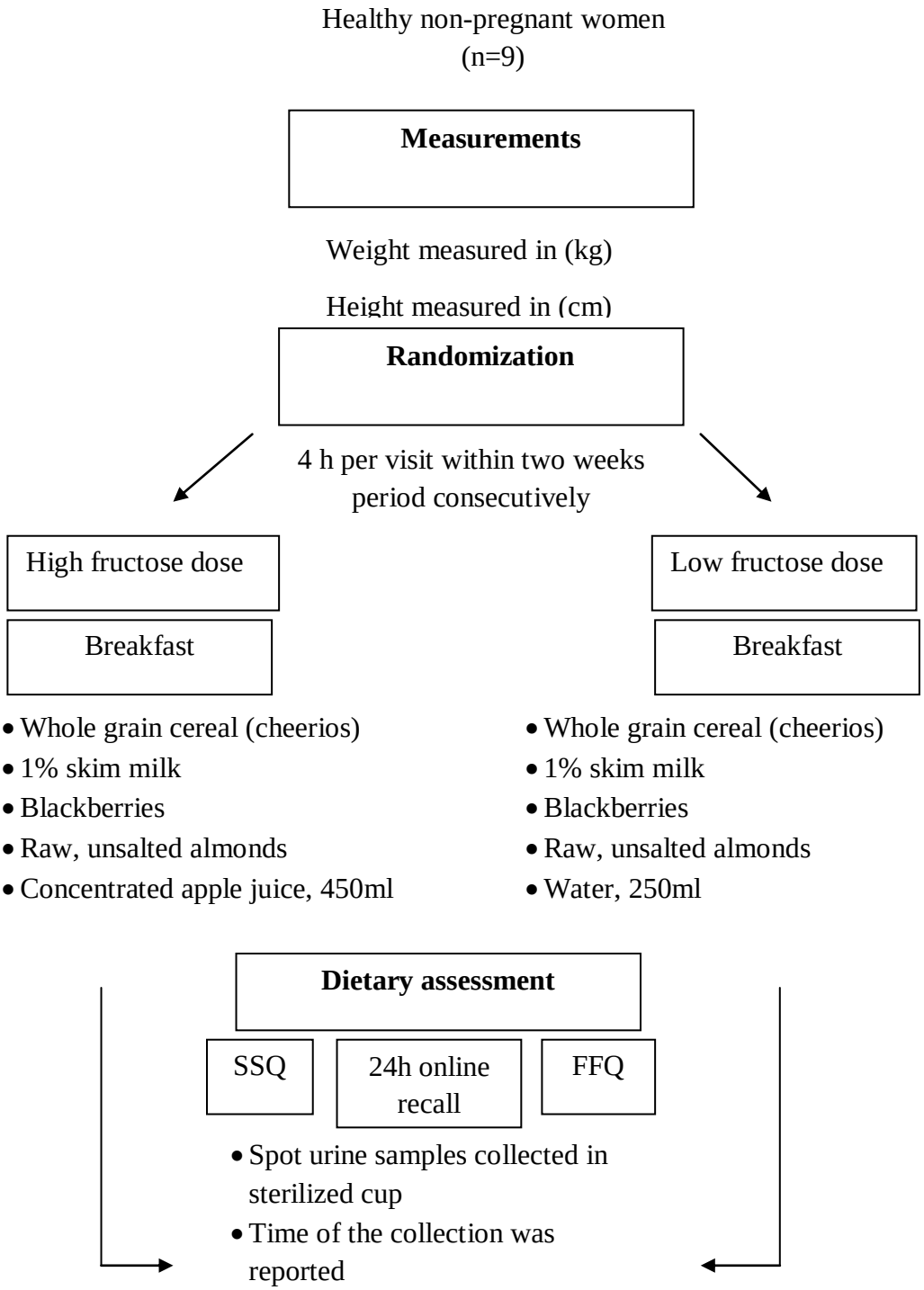

Figure 4.1: Study design for validation study

Participants were asked to provide a spot sample during the 4 h period after having the breakfast in order to measure the fructose amount in the sample. Participants were asked to provide at least one sample during their time in the study and record the time of the excretion. Some of the samples were collected using a urine hat in order to determine the volume of urine (which was recorded). This approach was not taken for all participants because the urine hat was not available to use at the time some participants started the study. Aliquots from each urine collection were stored at -80ºC until samples were analyzed. Fructose and creatinine in the urine were measured as described in the Sweet Moms study (Chapter 3).

5. Statistical analysis

Data were analyzed using Microsoft Excel for Windows 2010. Demographic data and dietary sugar intake data were presented as mean and range. The variability in intake and excretion levels within subjects was performed by calculating the coefficient of variation (%CV; SD/mean*100). A paired t-test was used to compare urinary fructose excretion after the high fructose meal and low fructose meal. Statistical significance was observed when $P < 0.05$.

6. Results

Participants characteristics

Participant characteristics are shown in Table 4.2 and were measured only once at the beginning of the study. The average BMI for the group was 21.8 ± 2.24. Daily total sugar consumption was 62.6 ± 24.4 g/day with added and natural sugars being 38.5 ± 19.3 g/day and 26.4 ± 10.5 g/day, respectively.

Table 4.2: Participants characteristics in validation study of non pregnant women

Parameter	n	Mean (Range)
Weight (kg)	9	57.1 (49.7-67.1)
Height (m^2)	9	2.6 (2.4 -2.8)
BMI (kg/m^2)	9	21.8 (19.1-25.9)
Age (yrs)	9	26.5 (22-33)
Dietary sugar intake		
Total sugar intake (g/day)	9	62.6 (38.03-112.6)
Added sugar intake (g/day)	9	38.5 (23.3-84.4)
Natural sugar intake (g/day)	9	26.4 (12.7-45.8)
Total sugar from beverages (g/day)	9	12.4 (0-28.6)

Dietary sugar intake

Data from the SSQ were used to calculate the total sugar intake from the most common foods in the Western diet. Natural sugar, which is derived from fruit, vegetables and dairy products was also calculated. In this study, the term "added sugar" referred to sugars that have been added to processed foods such as sucrose and fructose (mainly HFCS). Added sugar intake contributed 44-75% of total sugar intake with a group mean of 63% and natural sugar contributed 26-60% with a group mean 40% (Figure 4.1).

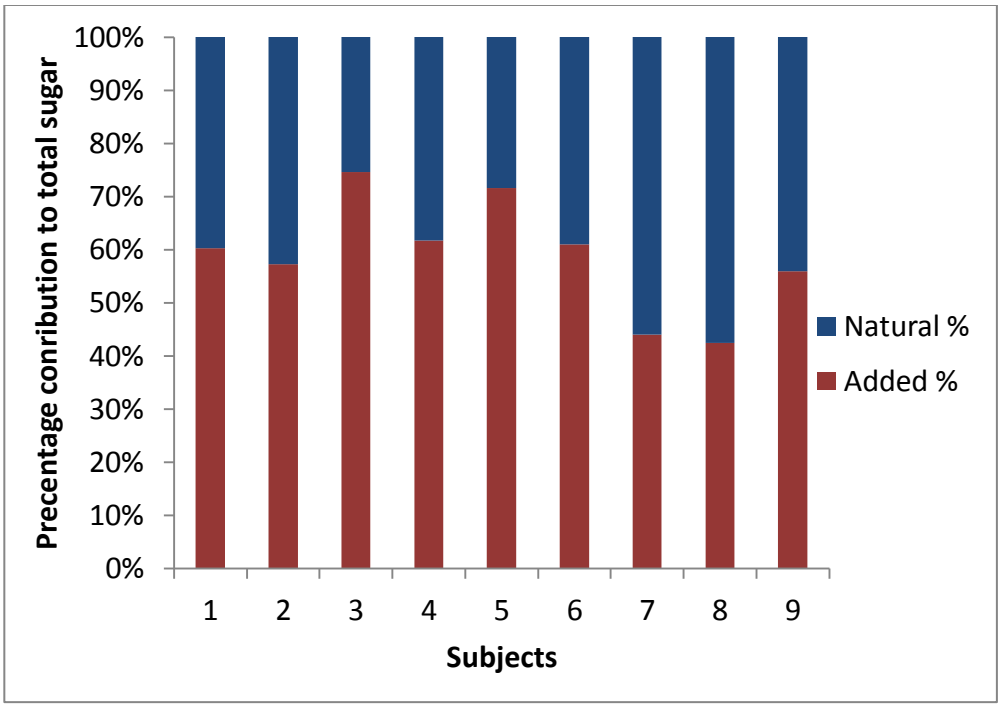

Figure 4.1: Contribution of added and natural sugar to total sugar intake derived from the SSQ, of 9 healthy non-pregnant subjects. Each bar represents the subject's total sugar intake.

Food Frequency Questionnaire

Data from the FFQ were analyzed to estimate the subjects' dietary fructose intake over the previous 6 months. Participants responded to the questions by selecting one of the following options: never, 1 time or less per month, 2-3 times per month, 1-2 time per week, 3-4 time per week, 5-6 time per week, 1-2 time per day, 3-4 times per day, 5-6 times per day and 6 or more times per day. Although data from FFQ may not be reflective of urinary sugar in a spot urine sample, it may be useful to determine subjects' overall consumption of sugar as to

categorize them as high, moderate or low sugar consumers. Figure 4.2 describes participants' energy from sugar consumption as determined by the FFQ data.

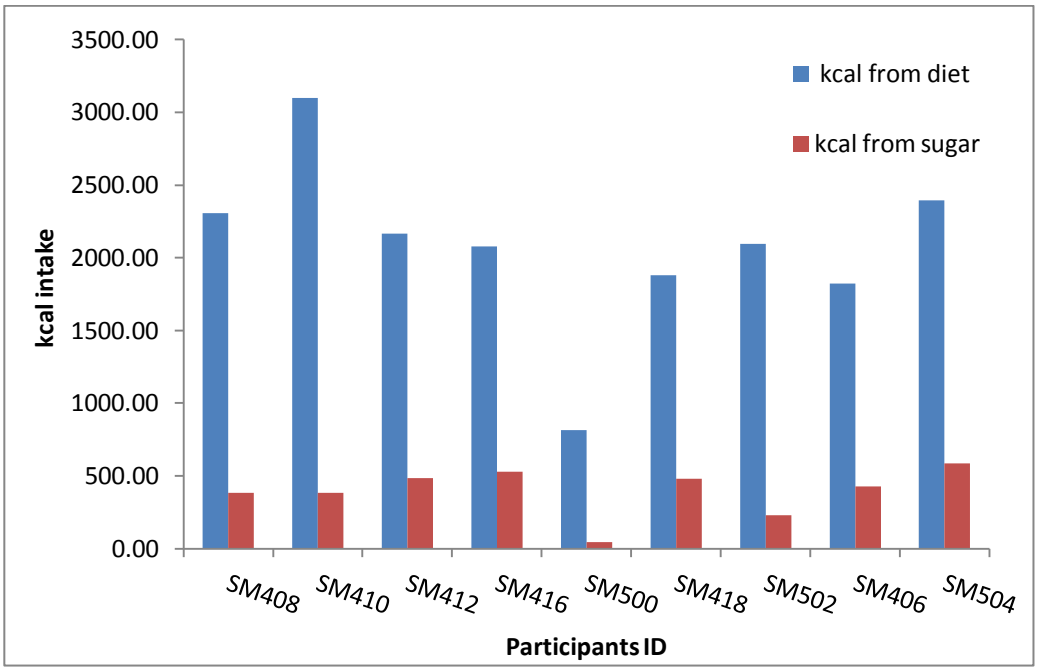

Figure 4.2: Energy from total dietary intake and dietary sugar intake derived from the FFQ for each participant. Total energy consumption from their diet (blue bar) and of energy consumption from sugar intake (red bar).

Urine analysis

Fructose concentrations in spot urine samples collected in the 4 h following the consumption of either a high or low fructose breakfast are shown in Figures 4.3 and 4.4. Each point in the graph represents the concentration of urinary fructose in the first, second and third (if collected) time point of urine collection. After the high-fructose meal, the initial urine samples provided had a higher fructose concentration than the initial samples collected after the low-fructose meal (Figures 4.3 and 4.4). Further, a dramatic decrease in urinary fructose was

observed between the first sample obtained after breakfast and the second sample (Figure 4.3). After the low-fructose meal, only a slight decrease was observed between the first and second samples, except for one participant (Figure 4.4).

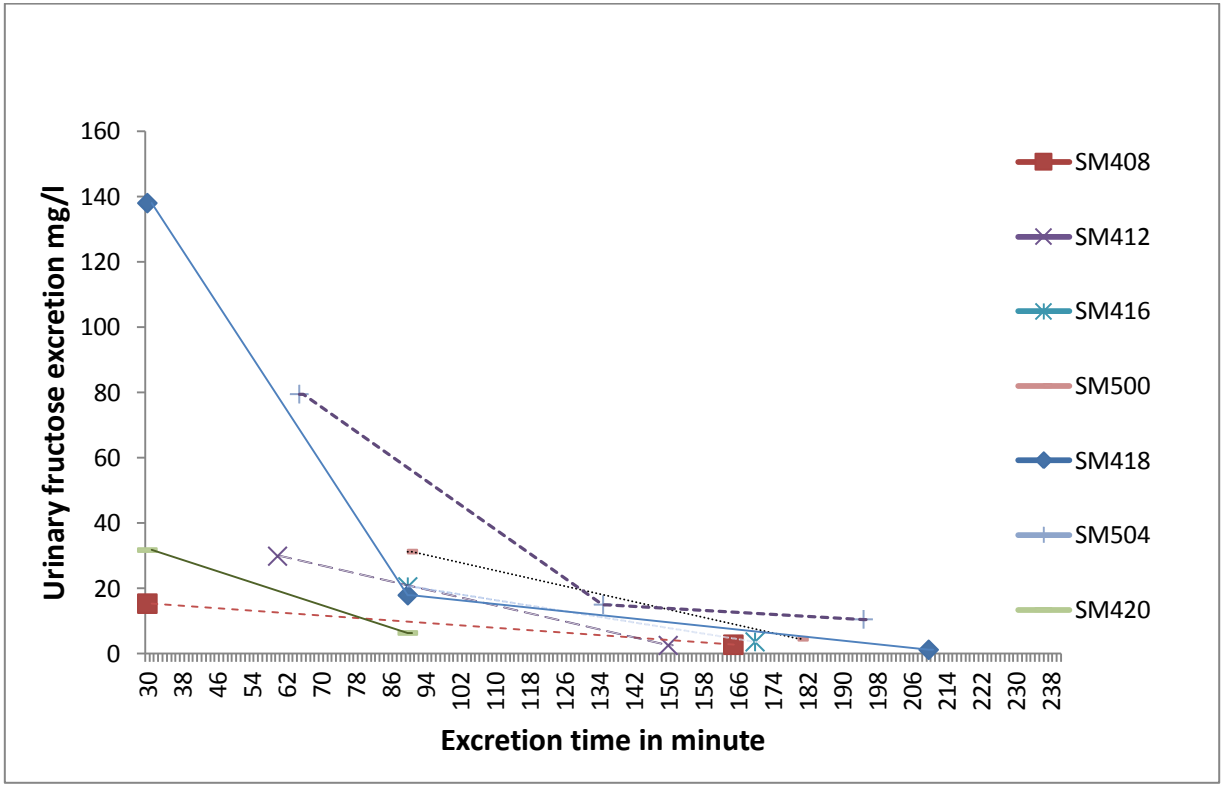

Figure 4.1:Individual urinary fructose excretion collected at different times through the study period after high fructose meal

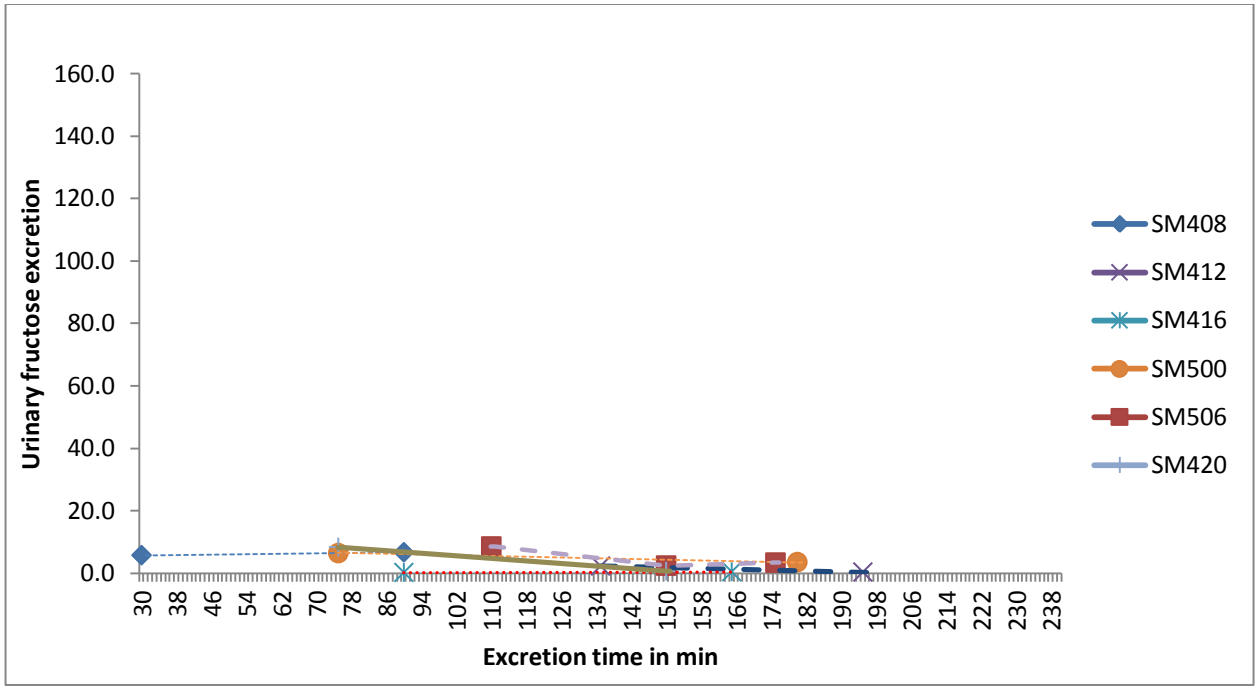

Figure 4.2:Individual urinary fructose excretion collected at different times through the study period after a low-fructose meal

Determination of urine volume

Tables 4.3 and 4.4 detail the amount of fructose excreted in urine samples following high and low fructose meals. By multiplying the urine volume by urinary fructose excretion, the amount of fructose excreted is observed, and a cumulative amount excreted is calculated. The rate of excretion was determined as a ratio of amount excreted to time length of the interval. An example was presented for one participant in Figure 4.5, demonstrating the cumulative amount excreted vs. time of urine collection during the study period for both high and low fructose meals. However, this approach was not taken for all participants since there was a delay in getting the urine hat and urine volume was not measured in all samples for all participants.

Table 4.3: Determination of the amount of fructose excreted by urine volume after a high fructose meal

ID	Time	Time interval (h)	Urine Volume (ml)	Urine fructose concentration (mg/ml)	Amount excreted (mg)	Cumulative excretion (mg)	Rate of excretion (mg/h)
SM408	9:00						
	10:30	0-1.5	350	0.02	5.37	5.3	3.58
	11:45	1.5-2.75	375	0.00	1.02	6.3	0.82
		2.75-4				6.3	
	9:15						
SM410	10:15	0-1	500	0.07	34.59	34.6	34.6
	12:15	1- 3	450	0.01	5.87	40.5	2.9
		3-4				40.5	
	9:00						
SM416	10:30	0-1.5	600	0.02	12.28	12.3	8.19
	11:30	1.5-2	475	0.00	1.70	14.0	3.41
		2-4				14.0	
	9:00						
SM418	9:30	0-0.5	50	0.14	6.90	6.90	13.80
	10:30	0.5-1.5	325	0.02	5.81	12.9	5.81
	12:30	1.5-3.5	175	0.00	0.20	12.7	0.10
		3.5-4				12.7	

Table4.4: Determination of fructose excreted within a 4 h period after a low fructose meal

ID	time	Time interval	Volume ml	urine fructose concentration mg/ml	Amount excreted mg	Cumulative amount excreted	Rate of excretion mg/hr
	9:00						
SM408	9:30	0-0.5	75	0.006	0.4	0.4	0.86
	10:30	0.5-1.5	50	0.007	0.3	0.7	0.33
		1.5-4				0.7	
	9:00						
SM412	11:15	0-2.25	350	0.002	0.8	0.8	0.36
	12:15	2.25-3.25	200	0.000	0.1	0.9	0.07
		3.25-4				0.9	
	9:00						
SM416	9:30	0-0.5	450	0.004	1.8	1.8	3.69
	11:45	0.5-2.75	550	0.000	0.1	1.9	0.06
		2.75-4				1.9	

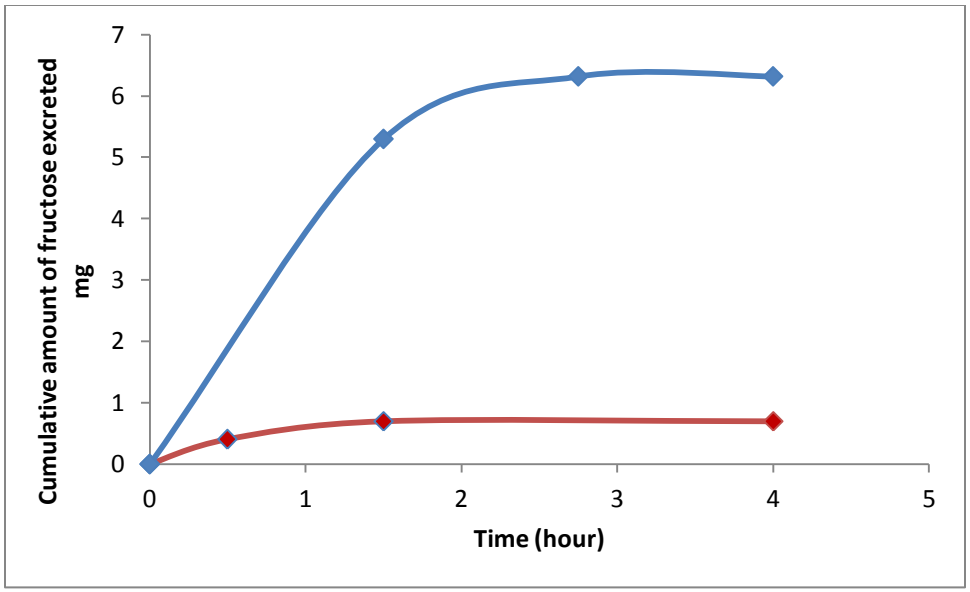

Figure 4.3:Total fructose excretion in the 4 h period after high and low fructose meals for participant SM 408.

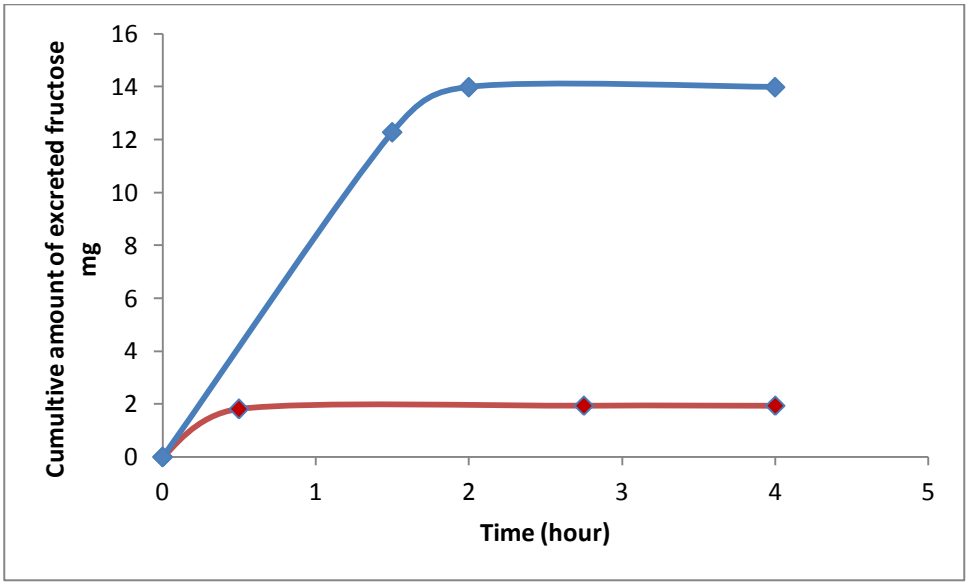

Figure 4.4:Total fructose excretion in the 4 h period after high and low fructose meals for participant SM 416

Within-subject measurements

As participants provided 2 or 3 samples of urine in both meals during the study, it is important to determine the variation of urinary fructose excretion within subjects. By calculating the coefficient of variance CV% (standard deviation /mean *100), we determined how different one sample of urinary fructose was from another. Table 4.5 presents the means and CV% of urinary fructose excretion for each participant when consuming the two different fructose meals (high and low). Overall, variation of fructose excretion in the urine was high, in particular when participants consumed the low fructose meal. Additionally, a calculation of the CV of the dietary sugar intake reported from SSQ was calculated, as participants completed 2 SSQ (Table 4.6).

Table 0.5: Within-subject variation of urinary fructose excretion after consumption of a high- or low-fructose meal

High fructose dose				Low fructose dose			
ID	**Time of collection**	**Mean**	**CV%**	**ID**	**Time of collection**	**Mean**	**CV%**
SM500	9:59	0.1	21.2	SM500	9:45	0.02	19.4
	11:30	0.0	8.0		11:30	0.01	43.0
SM504	9:05	0.0	11.9	SM504	N	0.04	128.0
	10:15	0.3	15.1				
	11:15	0.0	19.7				
SM420	9:30	0.1	16.6	SM420	9:15	0.02	4.5
	10:30	0.0	18.5		10:30	0.03	53.4
SM408	9:30	0.1	25.0	SM408	9:30	0.02	17.1
	11:45	0.0	12.1		12:30	0.02	24.5
SM416	10:30	0.1	6.4	SM416	9:30	0.01	20.1
	11:50	0.0	27.9		10:30	0.00	350.2
						0.02	43.8
SM412	11:00	0.1	10.0	SM412	11:15	0.32	18.5
	12:30	0.0	44.9		1:00	0.01	38.3
Mean			20.4				72.4

Table 4.6 : Within-subject variation in dietary sugar intake from SSQ

ID	total sugar (g/d)	CV%	Natural sugar (g/d)	CV%	added sugar (g/d)	CV%	total fructose (g/d)	CV%
SM 408	38.0	3.0	15.4	25.7	23.3	1.3	10.2	4.4
SM 410	56.2	13.0	25.8	8.3	34.5	14.2	18.5	1.6
SM 416	85.2	8.1	33.7	12.4	54.2	9.3	24.8	2.5
SM 500	45.2	68.5	12.7	80.9	32.0	61.3	21.3	64.2
SM 418	40.6	4.6	17.4	32.6	27.3	27.6	11.5	5.9
SM 406	75.5	11.7	45.8	23.8	33.9	7.0	25.5	25.4
SM 504	50.6	39.3	24.2	41.9	30.7	33.0	17.3	40.0
Mean		21.2		32.2		21.9		20.6

High versus low fructose dose

As this study employed a repeated measure design, we used a paired t-test to compare fructose excretion for each participant after the low and high fructose meals. A significant difference was observed between urinary fructose (log_{10} transformed, p=0.01) and the ratio of fructose:creatinine (p=0.03, Figure 4.7). However, no significant difference was observed in sugar intake (Table 4.7). In the high fructose meal group there was a significant difference between first sample and the second sample that was collected (t=,-2.88 p>0.02); no significant was observed in the low fructose meal group between the two samples (t=1.20, p=0.2).

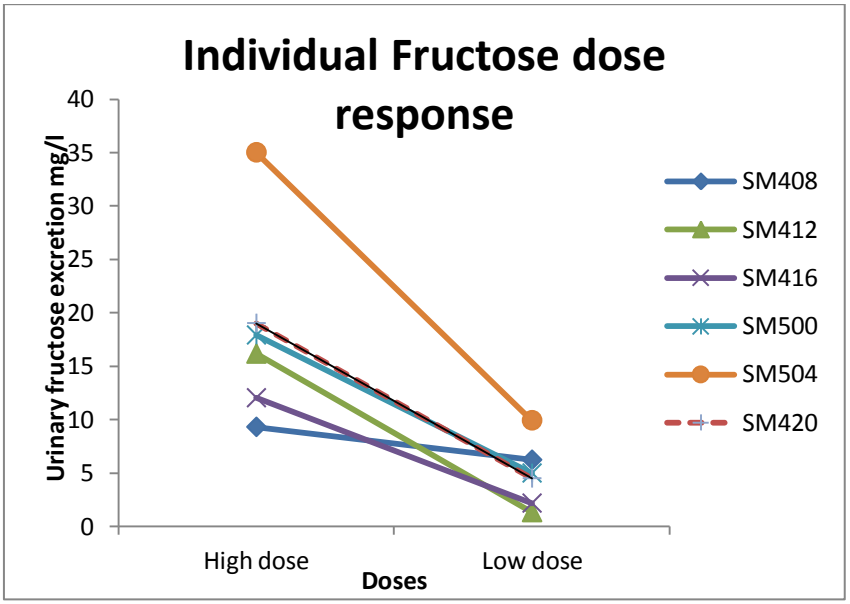

Figure 4.7: Comparison of mean urinary fructose excretion after consumption of a low and high fructose meal.

Table 4.7: Comparison of urinary fructose excretion after a high vs. low fructose meal

	n	High fructose dose mg/l	Low fructose dose mg/l	Mean difference	t value	P value
Urinary fructose excretion	7	21.5±11.9	11.4±18.2	10.02	2.30	0.06
Urinary creatinine excretion	7	327.7±82.1	647.8±440.0	-320.10	-2.13	0.08
Urinary fructose creatinine ratio	7	0.07±0.06	0.017±0.018	0.06	2.92	0.03*
log10 urinary fructose	7	1.28±0.23	0.73±0.52	0.54	3.60	0.01*

*statistically significant

6. Discussion

A validation study for the potential use of urinary fructose as a biomarker in estimating fructose intake was carried out in 9 subjects who consumed two prepared breakfast diets (high and low fructose) at two different time points. By measuring fructose excretion in the urine through a dose response study, we observed that urinary fructose excretion was significantly different between high and low fructose ingestion. The fact that urinary sugar can reflect sugar intake relies on the mechanism of absorption and metabolism of dietary sugars. Recent studies illustrate the difference in absorption and metabolism between extrinsic and intrinsic sugar (Taseveska et al., 2009). Extrinsic sugar comes from added or processed food and is rapidly released from the intestine, causing a high influx of fructose into the portal vein which is then metabolized in the liver (Gaby, 2005). Once hepatic fructose exceeds liver metabolic capacity a portion of the fructose escapes hepatic hydrolysis and is release into circulation (Taseveska et al., 2009). Some studies have suggested that fructose appears in the urine as a fraction of dietary fructose or sucrose (Taseviska et al, 2005). In a study of healthy individuals aged 23-33 y administered 50 g of sucrose orally, a considerable amount of fructose appeared in the urine, but not the blood (Nakamura et al, 1972). In our validation study, fructose concentration in the urine was elevated after consumption of a 450 ml juice beverage high in fructose, which contained 49 g of sugar. The mean excretion of fructose after the high-fructose meal was 21.5 ± 11.9 mg/L and after the low-fructose meal was 11.4 ± 18.8 mg/L, which indicates that urinary fructose is affected by the intake of fructose as was hypothesized.

Although the excretion of fructose after the high-fructose meal varied among subjects, the first sample collected in the 4 h period after consumption exhibited higher fructose concentration compared to the second sample, in all subjects. Participants provided their first sample in the first 90 minutes of the study period (average collection of the first sample was 57 minutes). This indicated that fructose metabolism occurred rapidly when fructose was present from an added-sugar source, and appeared in the urine. When participants consumed a low-fructose meal, there was no difference in urinary fructose concentration between the first and second time point. Differences in urinary fructose in samples obtained after the high-fructose meal indicated that urinary fructose is a potential biomarker of fructose intake. The CV of urinary fructose excretion was calculated as a measurement of precision, to indicate the performance of the assay. In this study, the mean CV% of the low fructose group was higher than the CV% of the high fructose group. This suggests that the fructose assay may not be sufficiently sensitive at low fructose concentrations. According to the kit instructions, the amount of fructose must be 1-50 µg/ml. The CV from the 2 SSQ indicated how much change occurred and the validity in reporting sugar intake over two weeks. The average CV% was 21%, 32%, 22%, 21% for total sugar, natural sugar, added sugar and total fructose, respectively. As the CV% of natural sugar is higher than added sugar, participants may have overestimated their fruit intake.

FFQ data were used to assess dietary intake over the 6 months prior to the study, and to estimate total calories from sugar. As the recommendation by the

Institute of Medicine states that added sugar intake should not exceed 25% of total daily energy intake (calories), we calculated energy intake derived from sugar consumption to determine how much energy is consumed from dietary sugar among study participants. In the study findings, data showed non of participants have exceed 25% of total calories per day.

The capacity of the human bladder to hold urine is up to 600 ml, however urgency to urinate may be experienced at as little as 150 ml (Hole et.al., 1981). The normal range of urination is between 800-2000 ml per day. In this study, participant urine volume in the 4 h study period ranged from 50 – 600 ml which is within the specified normal range.

Overall, urinary fructose may be useful as a biomarker to evaluate fructose.

7. Conclusion

As the validation study was performed in a small group of subjects, a larger study with validated dietary fructose assessments is needed to verify the use of this urinary biomarker. Further investigation is required to provide more information of this potential biomarker in more thorough validation studies that investigate the time and dose relationships between fructose intake and excretion. Although our findings in the validation study did not indicate a significant correlation between dietary fructose intake and the urinary biomarker (data not presented), a fructose excretion response to different fructose intakes was determined in this study. In conclusion, fructose is rapidly excreted in the urine and it is associated with fructose intake and time of excretion.

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Chapter5: Summary and Conclusion

Summary

Excessive fructose intake in pregnancy has been associated with increased risk of metabolic syndrome and may contribute to obesity. The assessment of dietary fructose using self-reported dietary recalls and diet history are inaccurate due to measurement errors. Therefore, in this study, a urinary fructose biomarker was evaluated for use in pregnant women, employing subjects enrolled in the Sweet Moms study, to estimate fructose consumption. The results indicated a wide variation in urinary fructose excretion, likely due to the variation in sugar intake. No significant association between the biomarker and fructose intake was observed. This could be due to a number of factors associated with pregnancy such as variation in gestational weeks or changes in metabolic status. To investigate the validity of a fructose biomarker in spot urine samples, a validation study was carried out in healthy, non-pregnant women by ingestion of a high fructose and low fructose meal, and observing urinary fructose excretion in each participant over the 4 h period following the meal. The validation study demonstrated that measuring fructose in spot urine sample was possible and related to dose response.

Research implications

As urinary fructose has been used recently to estimate sugar intake of healthy lean or obese individuals and children, we proposed that this existing biomarker could potentially be useful in estimating the sugar consumption of pregnant women. Since the literature review demonstrated that excessive fructose

intake during pregnancy increases the risk of metabolic disorders and pregnancy complications, an estimation of the true intake of fructose is essential to further evaluate these risks. Overall, urinary biomarkers that have been used to measure sugar consumption have shown significant correlations with sugar intake, and thereby it is possible to use this biomarker as a complementary measure to dietary assessments. Added sugar intake in the Western diet comes from sodas, sport drinks, sugar sweetened fruit beverages, candy and desserts and the major source of fructose in this food comes from sucrose and HFCS (Tappy et. al, 2012). Most food analysis tables used to assess intake have relied on total sugar rather than one type of sugar (fructose) (Tasevska et al., 2005). Thus, it is difficult to fructose intake from added sugar (Tappy et al., 2012). Errors from dietary fructose assessment methods frequently occur, since these methods (dietary history and self-reported recall) depend on subjects reporting accurately; individuals commonly overestimate healthy food and underestimate high-sugar foods. In general, a biomarker is measured in biological specimens such as urine or blood and eliminates the errors which come from self-reported dietary survey methods (Bingham, 2002). Based on our findings, the use of urinary fructose may have some potential as a biomarker in spot urine samples during pregnancy to estimate dietary fructose intake in pregnant women rather than the use of self-reported and dietary questionnaires in assessing fructose. Although our results from both studies did not show the correlation that was expected between fructose intake and urinary biomarker due to some limitations, further studies in this area are

required as evidence from the literature indicates a significant correlation exists between fructose intake and urinary excretion in healthy obese and lean subjects.

Strength and limitations

In spite of variation**,** the enzymatic method of fructose analysis is an effective technique to determine fructose concentration in urine samples. Fructose is stable in frozen urine with no preservation, up to 1 year (Tee Khaw et al., 2004), making the procedure applicable. As observed in this study, urinary fructose is sensitive to the intake of different amounts of fructose and may provide increased accuracy in assessment. In terms of collection, spot urine collection has been suggested to be potentially useful in determining a biomarker for fructose intake (Bingham et al., 2007). However, it is ideal to collect several spot samples to correlate with sugar intake. In fact, some studies have demonstrated that the correlation between biomarker and dietary intake would improve if repeated measures of biological samples were obtained (collection of several spot urine samples over the day) (Kaaks et al, 2002). Thus, this approach was the main limitation of Sweet Moms study, since urine samples were collected only once and at different times during the day. This may explain why no significant correlation was observed between fructose intake and urinary fructose.

Regardless of its sensitivity, this biomarker is time-related since fructose from added sources is rapidly metabolized in the liver and excreted in the urine. In the analysis, the kit that was used to measure fructose concentration in the urine as described in studies by Bingham et al. (2005-2010) and our study is mainly designed for food analysis. It is possible that substances in the urine may have

interfered with the assay (Song et.al, 2013). In addition, the assay was performed with three enzymes that were applied in three steps, which may cause a variation in the assay (Song et al., 2013).

Future direction

Pregnant women tend to increase their sugar consumption, especially from soda and sweet flavored beverages, either to meet diary recommendation such as increasing calcium intake or relieve physical symptoms such as nausea and craving (Graham et al., 2013). The use of a urinary fructose biomarker is increasingly needed by nutrition scientists as evidence of negative health impacts of high fructose intake on the mother and offspring is accumulating. Since dietary assessment methods such as FFQ and self-reported recalls are associated with measurement and systematic errors, a urinary biomarker in pregnancy that is objective would help identify these errors and provide more precision in predicting true intake of dietary sugar. Moreover, distinguishing fructose sources as added to processed food or naturally occurring in fruit is important in understanding their absorption, metabolism and ability to appear in the urine. We anticipated urinary fructose would be an accurate biomarker in pregnancy, as an association between sugar intake and urine fructose excretion has already been documented in different populations; however, the study findings did not support our hypothesis. More precise settings and consideration of pregnancy-related variables would likely yield better results. Time of excretion should be recorded, as should the most recently consumed food, and two or more urine sample should be collected. Further studies are needed to establish a fructose biomarker in

pregnancy using spot urine sample to have a better estimation of fructose intake and help identify health risks of excessive fructose intake on the mother and newborn.

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D-Glucose/D-Fructose

UV-method
for the determination of D-Glucose and D-fructose in food-stuffs and other materials
Fast method for analysis of wine: see under pt. 10

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For *in vitro* use only

Store at 2-8°C

Cat. No. 10139106035

Test-Combination for 27 determinations each

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

Principle (Ref. A 1)

D-Glucose and D-fructose are phosphorylated to D-glucose-6-phosphate (G-6-P) and D-fructose-6-phosphate (F-6-P) by the enzyme hexokinase (H K) and adenosine-5' -triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1,2).

(1) D-Glucose + ATP —HK→ G-6-P + ADP

(2) D-Fructose + ATP —HK→ F-6-P + ADP

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (3).

(3) G-6-P + NADP+ —G6P-DH→ D-gluconate-6-phosphate + NADPH + W

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

On completion of reaction (3), F-6-P is converted to G-6-P by phospho-glucose isomerase (pGI) (4).

(4) F-6-P ~ G-6-P (PGI)

G-6-P reacts in turn with NADP forming D-gluconate-6-phosphate and NADPH. The amount of NADPH obtained in this reaction is stoichiometric to the amount of D-fructose. The increase in NADPH is measured by means of its light absorbance.

The Test-Combination contains

1. Bottle 1 with approx. 5 g of powder mixture, consisting of: triethanolamine buffer, pH approx. 7.6; NADP, approx. 64 mg; ATP, approx. 160 mg; magnesium sulfate
2. Bottle 2 with approx. 0.7 ml suspension, consisting of: hexokinase, approx. 200 U; glucose-6 phosphate dehydrogenase, approx. 100 U
3. Bottle 3 with approx. 0.7 ml suspension phosphoglucose isomerase, approx. 490 U
4. Bottle 4 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 D-fructose because of its 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 in 27 ml of redist water.
2. Use contents of bottle 2 undiluted.
3. Use contents of bottle 3 undiluted.

Stability of reagents

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

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:

Pipette into cuvettes	Blank	Sample
solution 1	1.000 ml	1.000 ml
sample solution'	-	0.100 ml
redist water	2.000 ml	1.900 ml
MiX*', and read absorbances of the solutions (A_l) after approximately 3 min and start reaction by addition of:		
suspension 2	0.020 ml	0.020 ml
Mix", wait for the end of the reaction (approx. 10-15 min), and read the absorbances of the solutions (Az). If the reaction has not stopped after 15 min, continue to read the absorbances at 2 min intervals until the absorbances increase constantly over 2 min···. Add		
suspension 3	0.020 ml	0.020 ml
Mix", read absorbances of the solutions after 10-15 min (AJl.		

D-glucose 3.020 ml
D-fructose 3.040 ml

Read against air (without a cuvette in the light path) or against water
Sample solution: 1-100 fig of D-glucose and D-fructose per assay3

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 dosing the cuvette with Parafilm (trademark of the American Can Company, Greenwich. Ct. USA)

- "Creep reactions" occur very occasionally. They are mostly brought about by the contents of the sample solution, such as enzymes or coloring agents. These interfering substances may be removed during sample preparation.

If the absorbance A2 increases constantly, extrapolate the absorbances to the time of the addition of suspension 2 (H KlG6P-DH).

Determine the absorbance differences (A_2-A_l) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining LIAO-9lucose'

Determine the absorbance differences (ArAz) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining LIAo-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).

c Calculation

According to the general equation for calculating the concentration:

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

V = final volume [ml]

v = sample volume [mil

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 x mmor' x em"]
Hg 365 nm = 3.5 [l x mmor' x cm-l
Hg 334 nm = 6.1S [l x mmor' x cm']

It follows for D-glucose:

$$c = \frac{3.020 \times 1000 \times 0.100 \times 1000 \times MG\text{-glucose}}{6.3 \times 1.00 \times 1.900 \times 1000} \times \Delta A \text{ D-glucose} = \frac{5.441}{\varepsilon} \times \Delta A \text{ D-glucose}$$

[g D-glucose/l sample solution]

for D-fructose:

$$c = \frac{3.040 \times 1000 \times 0.100 \times 1000 \times MG\text{-fructose}}{6.3 \times 1.00 \times 1.900 \times 1000} \times \Delta A \text{ D-fructose} = \frac{5.477}{\varepsilon} \times \Delta A \text{ D-fructose}$$

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

$$\text{Contento\_glUCOSe} = \frac{\text{CD-glucose } [g/l \text{ sample solution})}{\text{weightsample in } g/l \text{ sample solution}} \times 100\ [g/100\ \text{g})$$

$$\text{Contento-fructose} = \frac{\text{cD-fructose } [g/l \text{ sample solution})}{\text{weightsample in } g/l \text{ sample solution}} \times 100\ [g/100\ \text{g})$$

1. Instructions for performance of assay

The amount of D-glucose + D-fructose present in the assay has to be between 2 fJ-g and 100 fJ-g (measurement at 365 nm) or 1 fJ-g and 50 fJ-g (measurement at 340. 334 nm). respectively. In order to get a sufficient absorbance difference. the sample solution is diluted to yield aD-glucose + D-fructose concentration between 0.15 and 1.0 *gll* or 0.08 and 0.5 *gll* respectively.

Dilution table

Estimated amount of D-glucose + D-fructose per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365nm		
< 0.5g	< 1.0g	-	1
0.5-5.0 g	1.0-10.0 g	1 + 9	10
5.0-50 g	10.0-100 g	1+ 99	100
> 50g	> 100g	1 + 999	1000

If the measured absorbance difference (M) 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 pi petted into the cuvette can be increased up to 2.000 ml. 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.

2. Technical information

If the ratio D-glucose to D-fructose in the sample is higher than e.g. 10:1. the precision of the 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.)

If the concentration of D-fructose in the sample solution is higher or much higher than the concentration of D-glucose. both. D-fructose and D-glucose can be determined with high precision when the determinations are done in separate assays with different sample solutions. For details see the dilution table.

3. Specificity (Ref. A 1)

The method is specific for D-glucose and D-fructose.

In the analysis of commercial water-free D-glucose (molecular weight 180.16). D-glucose monohydrate (molecular weight 198.17) and D-fructose. results of < 100CAl have to be expected because the materials absorb moisture. (Commercial D-fructose may also contain D-glucose.)

4. Sensitivity and detection limit (Ref. A 1.4, A 1.5)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml and measurement at 340 of a D-glucose or D-fructose concentration of 0.2 *mgll* sample solution (if v = 0.1 00 mi. this corresponds to 4 mg/l sample solution). The detection limit of 0.4 mg D-glucose. resp. 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.

5. Linearity

Linearity of the determination exists from approx. 1 fJ-g D-glucose + D-fructoselassay (0.4 mg D-glucose + D-fructose/l sample solution; sample volume v = 2.000 mt) to 100 fJ-g D-glucose + D-fructosel assay (1 g D-glucose + D-fructose/l sample solution; sample volume v = 0.100 ml).

6. Precision

In a double determination 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 *mgll.* Of the sample is diluted during sample

D-Glucose	CV= 1.2	blood	(Ref. A 1.2)
	CV=1.8	blood	
D-Fructose:	CV=1.5	chocolate	(Ref. A 1.4)
	CV=1.5	drinks. juices. honey	
Fruit juice:	D-glucose:	r = 0.42 + 0.027 x (CO.glucose in *gll*) *gll*	(Ref. A 1.3)
		R = 1.0 + 0.042 x (CD-glucose in g/l) g/l	
	D-fructose:	r = 0.15 + 0.033 x (CD-fructose in g/O g/l	(Ref. A 1.5)
For further data see references		R = 1.05 + 0.045 x (CD-fructose in g/l)	(Ref. A 2.9)
Wine:	r= 0.056 x Xi R = 0.12 + 0.076 x;		(Ref. A 2.17, 2.18)
Xi = D-glucose resp. D-fructose content in			
r = 0.030 gl1 00 ml		s(r) = ± 0.011 g/100 ml	
R = 0.122 g/100 ml			
For further data see references		s(R) = ± 0.043 g/100 ml	
Liquid whole egg:			
	D-glucose: X = 0.44 g/100 9	r = 0.073 g/100 9	s(r) = ± 0.026 g/l 00 9
	D-fructose: x = 6.72 gl1 00 9	R = 0.106 g/100 9	s(R)= ± 0.037 gl100 9
For further data see references		r = 0.587 g/100 9	s(r) = ± 0.207 gl1 00 9

7. Recognizing interference during the assay procedure

7, 1 If the conversion of D-glucose and of D-fructose has been completed according to the time given under "Procedure". it can be concluded in general that no interference has occurred.

Z2 On completion of the reaction. the determination can be restarted by adding D-glucose *andlor* 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.

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 mt): 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.

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.

S. Reagent hazard

The reagents used in the determination of D-glucose and D-fructose are not hazardous materials in the sense of the Hazardous Substances Regulations. the Chemicals Law or EC Regulation *671* 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;

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;

Adjust acid and weakly colored samples to pH 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;

Measure "colored" samples (if necessary adjusted to pH 8) against a

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-solutions after the extraction with hot water.

Carrez clarification:
Pipette the liquid sample into a 100 ml volumetric flask containing 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 hexacyanoferrateOO (ferrocyanide), 85 mM = 3.60 g K [Fe(CN)al x 3 H O/l00 rnl) and 5 ml Carrez II solution (zinc sulfate, 250 mM = nO g ZnS04 x 7 H_2O/100 rnl), 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 only be deproteinized with perchloric acid or with trichloroacetic acid in the absence of sucrose and maltose as these disaccharides are fully or partically hydrolized with the release of D-glucose. The Carrez clarification is recommended for normal use.

10. Application examples
Determination of D-glucose and D-fructose in fruit juices and similar beverages
Filter turbid juices (alternatively, clarify with Carrez reagents). Dilute the filtrate or clarified fruit juice until the D-glucose + D-fructose concentration is approx. 0.1-1.0 g/1. Decolorization of colored fruit juice is usually not required. Strongly colored juices, which are used undiluted for the assay, are decolorized as follows: add approx. 0.1 g of polyamide powder, gelatine or polyvinylpolypyrrolidone (PVPP) to approx. 10 ml of juice, stir for 1 min, and filter. Use the clear solution (which may still be slightly colored) for the assay.

Determination of D-glucose and D-fructose in wine (Ref. A 2)
Proceed as described for fruit juices. Red wine may also be used directly for the assay without further decolorization.
Fast method: Determination of D-glucose + D-fructose (without differentiation) in white wine with > 5 g total sugar
Dissolve contents of bottle 1 of the Test-Combination with 80 ml redist water. Add contents of the bottles 2 and 3 and mix carefully.
The solution is stable for 8h at 20-25°C, or for 3 days at 2-8°C.
Place 3.000 ml of the reaction mixture, brought to 20-25°C, into a cuvette (e.g. with a dispenser) and measure absorbance A_1. Start reaction by addition of 0.100 ml of the sample, that has previously been diluted according to the dilution table. Mix and after the end of the reaction (approx. 10-15 min) read absorbance A_2. Calculate absorbance difference (Az-A_1) = M.
Calculate concentration of D-glucose + D-fructose:
c = M x 1.596 x F (at Hg 365 nm) [g D-glucose + D-fructose/l sample)
c = M x 0.9037 x F (at Hg 334 nm) [g D-glucose + D-fructose/l sample)
c = M x 0.8865 x F (at 340 nm) [g D-glucose + D-fructose/l sample)
(F = dilution factor)

Determination of D-glucose and D-fructose in beer
To remove the carbonic acid, filter the sample or stir approx. 5-10 ml of beer in a beaker for approx. 30 s with a glass rod. Use the largely COz-free sample of beer directly for the assay.
Determination of D-glucose and D-fructose in preserves, specifically dietetic jam, and other vegetable and fruit products
Homogenize about 10 g of sample in a mixer. Accurately weigh approx. 0.5 g of the sample into a 100 ml volumetric flask, mix with water, make up to the mark, mix, and filter. Discard the first 5 ml of filtrate. Use the clear undiluted filtrate directly for the assay (0.100-2.000 mf),

Determination of D-glucose and D-fructose in honey
Stir honey thoroughly with a spatula. Transfer approx. 5-10 g of viscous (or crystalline) honey to a beaker and heat for 15 min at approx. 60°C, stirring ocassionally with the spatula (there is no need to heat liquid honey). Allow to cool. Pour approx. 1 g of the liquid sample, accurately weighed, into a 100 ml volumetric flask, dissolve at first with only a small portion of water, and then dilute to the mark and mix. Prepare a 1:10 (1 + 9) dilution of the 1 honey solution. Use 0.100 ml for the assay.

Determination of D-glucose and D-fructose in desserts and ice-cream
Accurately weigh approx. 1 g of sample into a 100 ml volumetric flask, add about 60 ml water and incubate for 15 min at approx. 70°C; shake from time to time. For clarification, add one after the other and mix after each addition: 5 ml Carrez-I-solution (3.60 g potassium hexacyanoferrate(II), ~[Fe(CN)61 x 3 H_2O/l00 ml), 5 ml Carrez-Ii-solution (7.20 g zinc sulfate, ZnS04 x 7 HzO/ 100 rnl) and 10 ml NaOH (0.1 M). Adjust to 20-25°C, fill up to the mark with water, mix and filter. Use the clear, possibly slightly opalescent solution for the assay, diluted according to the dilution table.

11. Special preparation of sample for the determination of D-fructose in the presence of a large excess of D-glucose
The precision of the D-fructose determination is impaired if the ratio of D-glucose to D-fructose is higher than e.g. 10:1. In this case, the D-glucose should be as much as possible removed. In the presence of glucose oxidase (GOD) and oxygen from the air, D-glucose is oxidized to D-gluconate:

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

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 J3-fructosidase < O.Ol°Aleach
Catalase
Triethanolamine hydrochloride
$MgSO_4$ x 7 HzO
NaOH,4M

Preparation of solutions for 10 determinations
Enzyme solution: Dissolve 5 mg (approx. 1000 U) GOD in 0.750 ml redist. water, add 325 KU catalase (from bovine liver, 25°C; HzOz as substrate), and
mix.
Buffer solution: Dissolve 5.6 g triethanolamine hydrochloride and 0.1 g $MgSO_4$ x 7 HzO in 80 ml redist. HzO adjust to pH 7.6 with NaOH (4 M), and make 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 when stored at 2-8°C.

Performance of D-glucose oxidation

Pipette into a 10 ml volumetric flask	
buffer solution	2.000 ml
sample solution (up to approx. 0.5 D-glucose)	5.000 ml
enzyme solution	0.100ml
Pass a current of air (Oz) 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, keep the volumetric flask in a boiling water-bath for 15 min, allow to cool, and fill up to the mark with water. Mix and filter, if necessary. Use the clear solution for the determination of D-fructose. In a parallel assay, determine the residual D-glucose and consider in the calculation as usual.

12. Further applications
The method may also be used in the examination of pharmaceuticals (Ref. A 3.6), cosmetics (Ref. A 3.10), paper (Ref. D 2.2) and tobacco (Ref. C 3.7). Carry out sample preparation and assay as described for analysis of foodstuffs.
The method may also be used, for example, in research when analyzing biological samples. For details of sampling, treatment and stability of the sample see Ref. A 1.
Determination of D-glucose and D-fructose in fermentation samples and cell culture media
Place the sample (after *centrifugation,* if necessary) in a water-bath at ao°c for 15 min to stop the enzymatic reactions. *Centrifuge* and use the supernatant (diluted according the dilution table, if necessary) for the assay. (Alternatively, deproteinization can be carried out with perchloric acid, however only in the absence of disaccharides, or with Carrez-solutions. See

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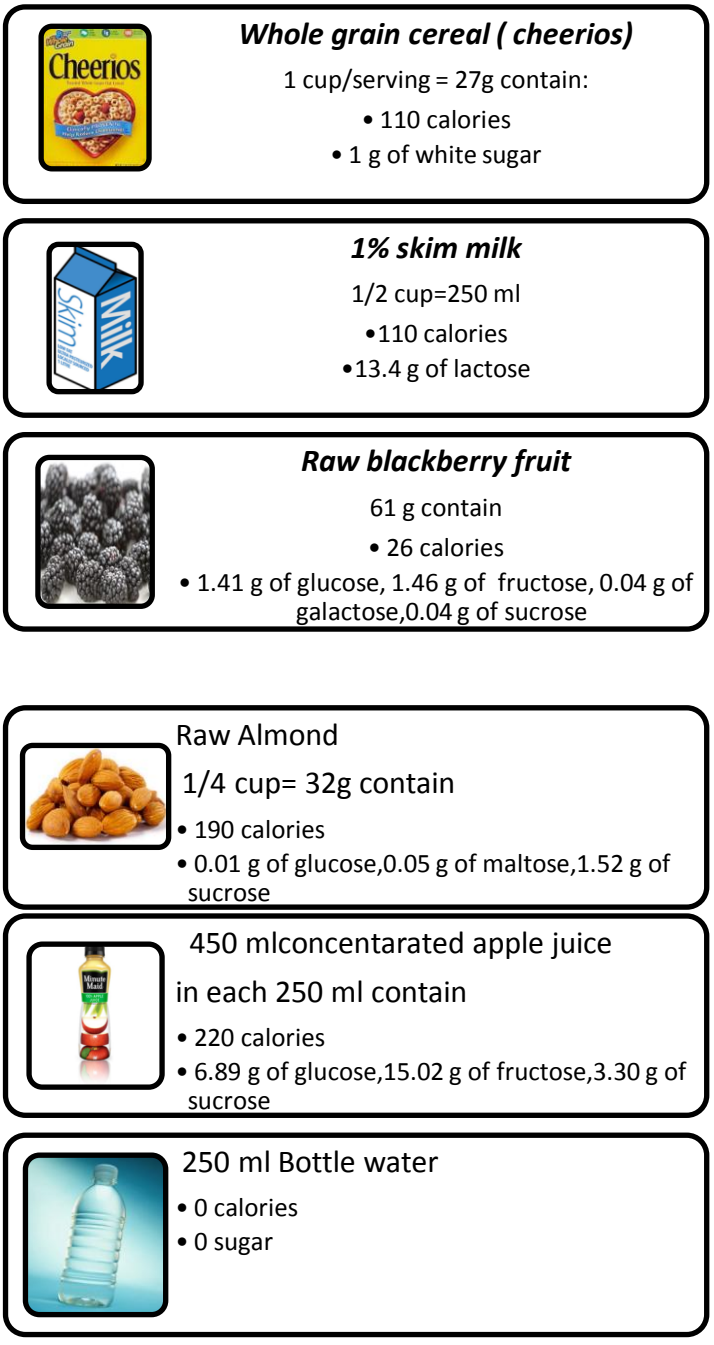
Appendix 2

The breakfast components of validation study



Appendix (3)

The sugar screener questionnaire

Study ID: _____

Sweet Moms - Sugar Screener

Instructions: This questionnaire will ask you for information about your current diet. Answer each question as best as you can. If you are not sure, please estimate as a guess is better than leaving a blank. You may fill in the answers with an 'x' or by filling in the circle.

You may use the following 'handy' portions to help with portion sizes:

A fist of cupped hand = 1 cup

Handful = 1-2 oz. of snack food

Thumb tip = 1 teaspoon

How often have you consumed the following beverages during the past week? *For example, if you drank 2 cups of chocolate milk on two different days last week, you would fill in the circle for 4 times per week.(Note: If the item was sugar free and made with Artificial Sweetener, please leave the circle blank)*

	Serving Size	None	1 time/ week	2 times/ week	3 times/ week	4 times/ week	5 times/ week	6 times/ week	7 times/ week	More than 7 times/ week
BEVERAGES										
Chocolate Milk	1 Cup	○	○	○	○	○	○	○	○	○
Hot Chocolate, Flavoured Cappuccinos, Frappuccinos, Sweetened Coffee Drinks	1 Cup	○	○	○	○	○	○	○	○	○
100% Apple Juice	1 Cup	○	○	○	○	○	○	○	○	○
100% Fruit Juice (Orange, Grapfruit, Peach)	1 Cup	○	○	○	○	○	○	○	○	○
Sugar Sweetened Drinks (Lemonade, Iced tea)	1 Cup	○	○	○	○	○	○	○	○	○
Regular Pop	1 Can (355ml)	○	○	○	○	○	○	○	○	○
Sports Drinks (Powerade, Gatorade)	1 Bottle (591 ml)	○	○	○	○	○	○	○	○	○
Slurpees/Slushies	Small (12 oz)	○	○	○	○	○	○	○	○	○
Milkshakes	Small (12 oz)	○	○	○	○	○	○	○	○	○

	Serving Size	None	1 time/ week	2 times/ week	3 times/ week	4 times/ week	5 times/ week	6 times/ week	7 times/ week	More than 7 times/ week
FOODS										
Ice Cream, Frozen Yogurt, Sorbet, Sherbet, Flavored Ices	1 Cup	O	O	O	O	O	O	O	O	O
Freezies, Popsicles	1 (120 mL)	O	O	O	O	O	O	O	O	O
Jam, Jelly	1 Tbsp	O	O	O	O	O	O	O	O	O
Honey	1 Tbsp	O	O	O	O	O	O	O	O	O
High-Sugar Cereals (Corn-pops, Fruit-Loops, etc.)	1 1/4 Cup	O	O	O	O	O	O	O	O	O
Flavored Yogurt	3/4 Cup	O	O	O	O	O	O	O	O	O
Dried Fruit	1/4 Cup	O	O	O	O	O	O	O	O	O
Canned Fruit in Syrup	1/2 Cup	O	O	O	O	O	O	O	O	O
Chocolate Bar, M&M's, Smarties, Chocolate	1 Bar (50g)	O	O	O	O	O	O	O	O	O
Candies (Jujubes, Wine-gums, etc.)	10 Pieces (50g)	O	O	O	O	O	O	O	O	O
Cookies	1 (3"Diameter)	O	O	O	O	O	O	O	O	O
Granola Bar (Special K, Nature Valley, Quaker)	1 Bar (35g)	O	O	O	O	O	O	O	O	O

	Serving Size	None	1 time/ week	2 times/ week	3 times/ week	4 times/ week	5 times/ week	6 times/ week	7 times/ week	More than 7 times/ week
FOODS										
Meal Replacement bar (Slimfast, Powerbar, Cliff)	1 Bar (56g)	O	O	O	O	O	O	O	O	O
Cake, Brownie	1 Piece of Cake/Brownie (1/8 of an 9" Cake)	O	O	O	O	O	O	O	O	O
Pie, Fruit Crisp, Cobbler, Strudel	1 Slice of Pie (1/8 of a 9"	O	O	O	O	O	O	O	O	O
Donut, Sweet Rolls, Danish, Pop-Tart	1 (60g)	O	O	O	O	O	O	O	O	O
Pudding, Custard, Jello	1/2 Cup	O	O	O	O	O	O	O	O	O
Fresh Fruit	1/2 Cup or 1 Small Whole Fruit	O	O	O	O	O	O	O	O	O
Sweet Muffin, Dessert Bread	1 Muffin/Slice of Bread	O	O	O	O	O	O	O	O	O
Condiments (Ketchup, Sweet and Sour Sauce, etc.)	1 Tbsp	O	O	O	O	O	O	O	O	O