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HUMAN BIOAVAILABILITY OF QUERCETIN

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Abstract

The present study investigated the bioavailability of quercetin, epicatechin, catechin and caftaric acid in humans after oral ingestion of raisins. A sensitive and specific method was developed and validated for the quantitation of quercetin in human urine. Interest in dietary consumption of grapes and its products has increased because of their possible health benefits in preventing cardiovascular disease and certain types of cancer. These diseases and cancer types caused from polyphenolic compounds that are present in grape and its products. Raisins are derived from grapes and contain some polyphenols and some of them are present at a higher level in raisins compared to grapes. Also raisins have high amount of quercetin (quercetin-3-*O*-rutinoside). This research study summarizes that urinary excretion of quercetin after oral consumption of 100 g Sun-Maid raisins to 18 healthy volunteers demonstrated that the amount of quercetin in human urine.

1. Introduction

In recent years, flavonoids, especially derivatives of quercetin have gained quite a lot of importance as dietary constituents (Boots *et al*, 2008). Most of the recent studies reveal that these molecules prevent the onset of cardiovascular diseases and cancer (Boots *et al,* 2008). Murota & Terao (2003) pointed out that this health-promoting activity has strong linkage to the antioxidant (free-radical scavenging) activity of flavonoids (Murota & Terao, 2003). Flavonoids are classified into groups such as the flavonols, flavanols, flavanones, flavones, anthocyanidins and isoflavones that are found in plants (Wach *et al*, 2007).

Current studies have supported the health benefits of polyphenols especially their importance in the prevention of cardiovascular disease, cancer and osteoporosis. These studies indicate the role of polyphenols in the prevention of neurodegenerative diseases, diabetes and inflammatory disorders (Carughi, 2008). According to Kaliora *et al*. (2009), polyphenols are potent antioxidants they support the biological resistance to free radicals and decreases the risk of degenerative disease such as cardiovascular disease (Kaliora *et al*., 2009). Plant foods, especially fruits and vegetables such as apple, onion, grapes, raisins etc have high amounts of these polyphenolic components. Many researchers have investigated the phenolic composition of grapes, grape juices and wines but there are only plenty of reports about the polyphenols composition of raisins (Karadeniz *et al*., 2000; Parker *et al*., 2007; Williamson and Carughi unpublished).

Raisins are an excellent source of polyphenols (Carughi, 2008) and they are considered to be a desirable source of dietary fibre, with polymerized phenolics contributing to that fibre (Karadeniz *et al*., 2000). Raisins are derived from grapes in two different ways. One involves 15-20 s exposure to hot water (87- 93 °C) followed by dehydration tunnel at 71 °C for 20-

24 h. And the second one involves sun-drying for 2 to 3 weeks (Williamson and Carughi, unpublished). The

results of these reports look similar quercetin glycosides present at the highest among flavonols followed by kaempferol. According to Williamson and Carughi's study some compounds such as caffeoyl tartaric acid and some quercetin and kaempferol derivatives are present at higher level in raisins compare to grapes (Williamson and Carughi unpublished). Karadeniz *et al* (2000) reported that compared to fresh grapes a 60% loss of flavonols was observed. They find out a loss in phenolic acids of up to 90% while Parker's results show lower losses. Also according to Karadeniz report, procyanidins and flavonols are completely degraded during raisin production. The differences in polyphenolic composition of raisins are affected by oxidation and non-enzymatic browning reactions during dehydration of grapes (Karadeniz *et a*l., 2000).

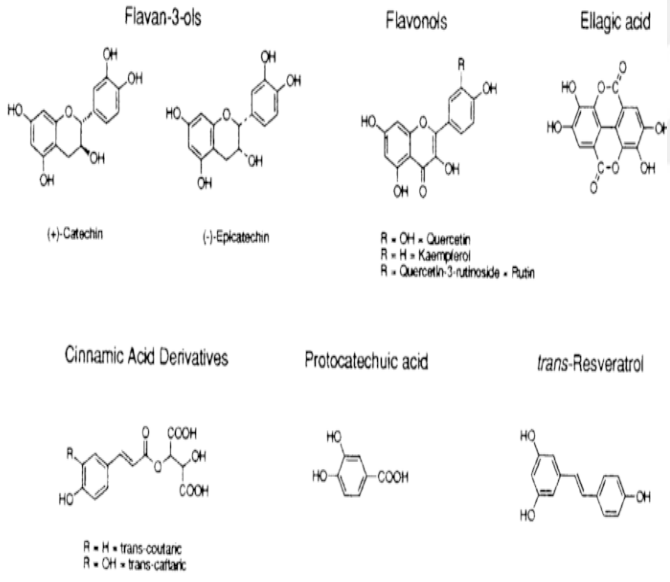

Figure 1 Structures of polyphenolics featured of raisins (Karadeniz et al., 2000). (Quercetin glycosides are the highest among flavonols in raisins). Quercetin (Fig. 1) represents one of the most abundant flavonoid in food and present in vegetables and fruits such as apple skins, raisins, onions, tea (green and black tea), citrus fruits, red wine, leafy green vegetables, berries, beans, parsley, olive oil, grapes, kale, broccoli, cherries (Karadeniz *et al*., 2000). Its antioxidant effect has importance for human health. As a molecule *"Quercetin is a yellow powdered crystalline compound, synthesized or occurring as a glycoside in the rind and bark of numerous plants. Quercetin is considered a phytoestrogen. Phytoestrogen is a plant substance with similar functions as that of estrogen. Quercetin is categorised in the class of flavonoids called flavonols. It is widely distributed in the plant kingdom in rinds and barks. In the body, quercetin has potent antioxidant and anti-inflammatory activity, where it can protect cellular structures and blood vessels from the damaging effects of free radicals. These features of the quercetin make this substance to be studied much* (http://www.vitamins-supplements.org/quercetin.php)."

One specific kind of quercetin, 3,30,40,5,7-pentahydroxylflavone, is one of the most abundant flavonoids could be found in vegetables and fruits (Wach *et al*., 2007). Vegetables, fruits and beverages are the main sources with their high content of quercetin. As Wach points out, regarding his survey, onion (*Allium cepa* L.) is so rich in quercetin among 28 vegetables and 9 fruits (Wach *et al*., 2007). Glucose is the most common sugar, with galactose and rhamnose frequently found in composition with flavonoids. In general, quercetin glycosides contain a sugar group at the 3-position.

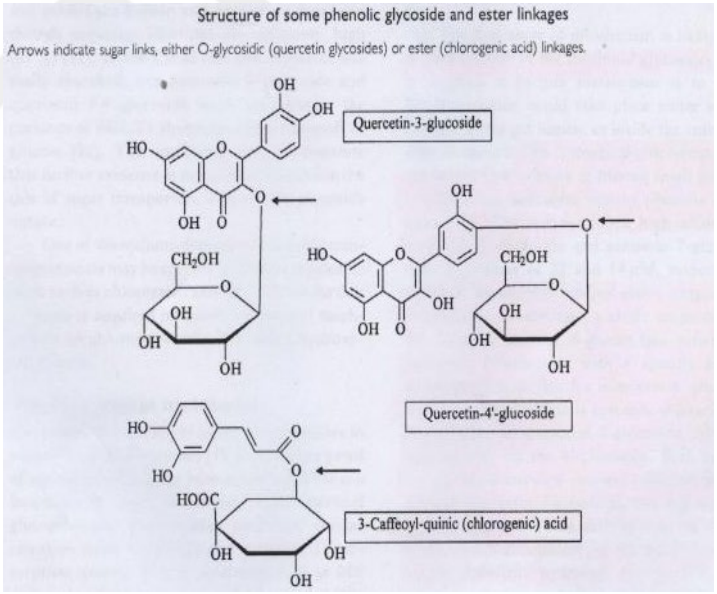

Figure 2 Structure of some phenolic glycoside and ester linkages (Williamson *et al*., 2000)

When sugar or organic acid are linked to phenolic, the water solubility increases than this situation impedes the passive diffusion. Polyphenol absorption in the small intestine is affected by linkage type and position in foods. For instance, the onion glycosides were absorbed better than tea flavonol glycosides or apple flavonol glycosides

(Williamson *et a*l., 2000). Daily intake of quercetin glycosides has been estimated by Manach *et al*., 2000 to between 20 and 30 mg/d.

Quercetin has antioxidant, anti-inflammatory, antiviral, immunomodulatory, anticancer and gastro-protective activities. *"Quercetin blocks an enzyme that leads to accumulation of sorbitol, which has been linked to nerve, eye, and kidney damage in those with diabetes (Kaliora et al., 2009)".* They may prevent the inflammation emerged from fever, allergies, bursitis, gout, arthritis, and asthma. Accordingly, it may also decrease other asthma symptoms (Karadeniz et al., 2000).

In this regard Kaliora and colleagues (2009) deduced that *"quercetin possesses potent antioxidant properties. It protects LDL cholesterol from becoming damaged. Quercetin prevents damage to blood vessels by certain forms of cholesterol and other chemicals produced by the body. LDL cholesterol is an underlying cause of heart disease. Quercetin also works as an antioxidant by scavenging damaging particles in the body known as free radicals. People with diabetes are at higher risk of blood vessel damage from free radicals".* Quercetin may reduce the risks of development of certain cancers (Kaliora *et al.,* 2009). Animal studies and in vitro studies suggest that dietary flavonols could inhibit cancer in humans (Hollman, 1999). In recent years, research on quercetin has ranged from it is suggested carcinogenic to its promise as an anti-cancer agent (Lamson & Brignall 2000). According to Lamson & Brignall (2000), the quercetin can be utilized safely in the cancer cure. *In vitro* studies show that quercetin has several separate and independent mechanisms of anti-tumor action and *in vivo* studies confirmed that quercetin may be used for therapeutic activity for cure in some cancer situations (Lamson & Brignall 2000). However, there is still needed further clarification on its possible benefits is required.

2. Absorption and Metabolism of Quercetin

Flavonoids are present in foods bound to sugars as β-glycosides (with the exception of catechins) that make hard to absorption of flavonoids from the diet. Only aglycones which has not sugar molecule, were considered to be transferred through the gut wall, and no enzymes that can split these predominantly β - glycosidic bonds are secreted into the gut or present in the intestinal wall. During degradation of dietary flavonoids hydrolysis starts to occur in the colon by microorganisms. Thus, only a marginal absorption of dietary flavonoids is to be expected. However, research on the mechanisms for aglycone transfer across the gut wall is lacking. Hollman et al. (1995) studied onion absorption in humans and found that in a human study with ileostomists, the absorption of orally taken quercetin aglycone was 24% and the absorption amount of quercetin glycosides from onions was 52%, and 17% for pure quercetin rutinoside, a common glycoside in foods. This research provided information about humans can absorb appreciable amounts of quercetin which is occurring in the small intestine (Crepsy et al., 2001). Thus absorption from small intestine can be more decisive than absorption from colon. The appearance of flavonols and flavones are less than 7 h after their absorption from ingested food.

Generally the diffusion of phenolic aglycones occurs passively through biological membranes. However linkage of a phenolic to a sugar or organic acid increases the water solubility and severely limits passive diffusion. Recent studies indicate that flavonol glucosides are absorbed in the gut selectively. Hollman (1999) deduced that attached sugar influence the rate of absorption.

The initial step in the absorption process for glycosylated flavonoids is deglycosylation which is essential if further metabolism is to occur (Williamson et al., 2000) and it provides conjugation from intestinal enzymes and transport to the serosal or mucosal sides (Williamson, 2004). "Deglycosylation can potentially occur at several sites in the duodenum and jejunum:

(1) within the intestinal lumen;

(2) brush border hydrolases; or
(3) intracellular hydrolases after transport of the flavonoid into the enterocyte (Williamson, 2004)."

There are 2 pathways of absorption for flavonoids. Both way give rise to intracellular aglycone, and in fact transient intracellular free aglycone (Williamson, 2004). The first step is an enzyme lactase phlorizin hydrolase (LPH) that found in the brush border of the small intestine provide deglycosylation of glycosylated flavonoids. LPH is an enzyme that is located in the brush border of the small intestine and is responsible for lactose hydrolysis (Williamson, 2004). The deglycosylation reaction produces a free aglycone which can then diffuse into epithelial cells either passively or by facilitated diffusion. The enzyme provides deglycosylation in the lumen such as acting like outside the epithelial without first having to traverse the enterocyte membrane (Williamson, 2004).

The second pathway of absorption involves transport of the flavonoid glycoside into the enterocyte in an intact form via the function of a sugar transporter such as SGLT1. After transporting of flavonoid glycoside into the cell deglycosylation is started by cytosolic β-glucosidase (Williamson, 2004). Quercetin-4'-glucoside is a good substrate for the cytosolic-glucosidase and the research on rat everted intestine was shown that the sugar transporter/cytosolic β -glucosidase pathway accounted for 20% of the absorbed quercetin while LPH accounted for the remaining 80%. However the LPH pathway for quercetin-3-glucoside accounted for 100% of the absorbed quercetin although "it is not a substrate for cytosolic β-glucosidase, (Williamson, 2004)."

The initial conjugation of flavonoids occurs in the small intestine. The intestine's conjugating capacity has glucuronosyl transferases (UGTs) and glutathione transferases. These enzymes catalyze the conjugation of flavonoids in the human small intestine. Using models of the small intestine studies showed that the transfers of flavonoids from the mucosal (gut) compartment to the serosal (blood) compartment have found quercetin, predominantly in the glucuronidated form (Williamson, 2004). Sometimes glucuronide residues are removed and replaced with a sulphate during glucuronidation reactions then a mixture of glucuronide and sulfated flavonoid conjugates can be present in peripheral blood. The sulfation reaction is thought to occur predominantly in the liver (Williamson, 2004).

The liver receives flavonoids from the blood, including blood from the small intestine. Hepatic cells provide transportation of quercetin glucuronides from the small intestine to liver although quercetin-3-glucuronide and quercetin-7-glucuronide appear to be taken up by a different mechanism. After this transport the glucuronides are deglucuronidated inside the cell by β-glucuronidase and then sulfated or methylated. MRP2 export the conjugates of flavonoids into the bile and back to the small intestine. Flavonoids are delivered to tissues throughout the body by the blood. While aglycones could enter peripheral tissues by passive or facilitated diffusion, glucuronide conjugates need to be transported into peripheral tissues, because they are relatively hydrophilic and diffuse through membranes only very slowly (Williamson, 2004). The reason is that not only sulphate conjugates may be relatively hydrophobic. The cells which have β-glucuronidase activity are present in the lysosomal fraction and in the endoplasmic reticulum; in liver cells. This enzyme provides deconjugation in tissues and it is also active on quercetin glucuronides (Williamson, 2004). According do Williamson the yield of quercetin in the urine less than 1.5% the amount of flavonoids in the urine dependent on the flavonoid and also the urinary content of flavonoids cannot be used as a biomarker of bioavailability or dietary intake. In the small intestine quercetin is much less efficiently absorbed than in the colon probably because quercetin is more readily broken down into low molecular weight phenolics by colonic microflora, and the aglycone of quercetin is unstable (Williamson, 2004).

The aim of the experiments was to find out the amount of rutin in the raisins and then to evaluate how much

was recovered in urine after 24 hours of consumption in 18 individuals.

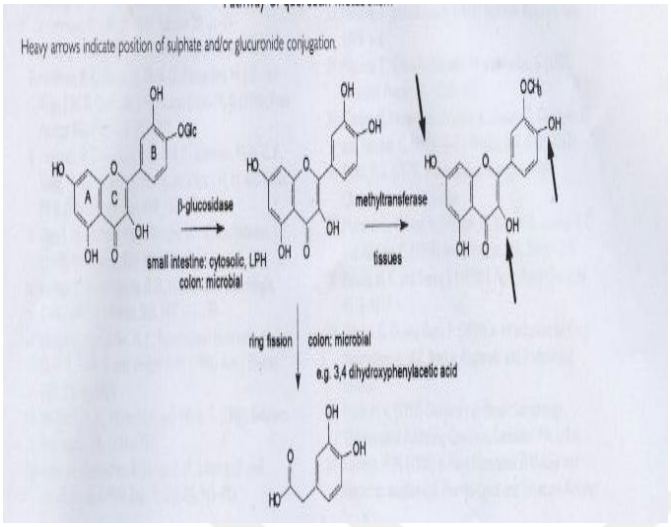

Figure 3 Pathway of quercetin metabolism (Williamson *et al.,* 2000)

3. Materials and Methods

For determination of the total amount of the quercetin concentration, there are several analytical methods in biological samples which are used to obtain after the enzymatic hydrolysis of conjugated quercetin metabolites, and for the analysis of quercetin metabolites. These analytical methods have included LC/MS, LC/MS/MS, HPLC with UV detection, HPLC with fluorescent detection, HPLC with electrochemical detection and HPLC-radiocounting and tandem mass spectrometry. LC/MS method allows the analysis of low concentrations of parent quercetin in urine with good reproducibility (Ishii *et al.,* 2003).

3.1. Chemical and reagents

Quercetin and rutin were purchased from Sigma-Aldrich Inc (St Louis, MO). Stock solutions of quercetin and rutin were prepared by dissolving these compounds in ethanol followed by dilution with water (50% EtOH solution). Taxifolin was dissolved by using distilled water. β-Glucuronidase was purchased from Sigma-Aldrich Inc (St Louis, MO) of type IX-A extract from *E.Coli*. Sulfatase was purchased from Sigma-Aldrich Inc (St Louis, MO) of type VI extract from *Aerobacter aerogenes*. All other chemicals and solvents were used without further purification. Methanol, ethanol, acetonitrile and sodium azide were all HPLC grade and purchased from Fisher.

3.2. Preparation of standard solutions for urine samples

A stock solution of 1 mg/ml quercetin was prepared in 50% ethanol solution. Dilution of the stock solution with 20% acetonitrile solution yielded working stock solutions at concentrations of 1, 20, 40, 60, 80, 100 µg/ml. A stock solution of the internal standard taxifolin was prepared in distilled water at a concentration of 1 mg/ml. A 100 µl aliquot of quercetin stock solution, 100 µl taxifolin stock solution and 100 µl of epicatechin, catechin, caftaric, ferulic acid and caffeic acid were added to 2 ml eppendorf tube and vortexed for 1 min prior and then transfer into vial insert for analysis by LC-MS.

3.3. Participants

In our study 18 healthy people with no history of major disease were recruited. A brief health questionnaire, description of the study, liability waiver, and right to opt out of the study were provided for each participant. The study was approved by and performed under the University of Leeds. During the washing period (3 days) detailed dietary recording forms were requested from participant. Also, during the diet, volunteers were requested to avoid certain types of foods containing the quercetin for two days before the consumption and on the third day morning before consumption of raisins their control urine was collected. After that, they were fed with 1 slice of bread, butter, banana and 100 g Sun-Maid raisins. After consumption, they were requested to collect 24h urine. Subjects were coded and data was recorded in a form which cannot be traced back to the name of the volunteer and then the addition of raisins; thus, we rely on the research to determine and quantify which components of foods are absorbed and appear in

the urine within a period of 24 hours. This allows us to estimate the bioavailability of quercetin and other compounds in raisins, and to see how much and in what form the body takes up and excretes the compounds. (Appendix form)

3.4. Raisins Extraction

Extraction was done according to Zhao and Hall's method. To increase the efficiency of the raisins extractions, 3 g well-chopped raisins were extracted with 15 ml solvent (EtOH, MeOH and ACN). The solvents were combined with distilled water to make solvents containing 0, 25, 50, 75 and 95% water. Then the raisin extracts were vortexed for 2 minutes. Following vortex they were homogenized for 5 minutes from low speed to higher speed to reduce the particle size. After that, this mixture was placed into centrifuge (25 min 20 °C 3000 rpm) then the pellet was re-extracted by adding 15ml of same solvent before putting the re-extract into centrifuge in the same protocol (A). The solvents from the repeat extractions were combined and stored in the freezer (-20°C). Each extraction process was done in triplicate. The aqueous extract was then filtered through 0.2 µm polytetrafluoroethylene filter and injected into HPLC column for the determination of polyphenols that they are investigated. Ethanol had better extraction compare to methanol and acetonitrile. According to Williamson and Carughi, the effectiveness of extraction is affected by solvent, extraction method, pH, and temperature.

Apart from Zhao and Halls method we used 2 different methods. However, the results from this extraction were not as good as Zhao and Halls method. One of these methods called protocol 2 was done according to following protocol; 3 g well-chopped raisins were extracted with 15 ml water after which it was homogenised and sonicated for 10 minutes. From this, aliquot 5 ml took out and centrifuge (25 min 20 °C 3000 rpm) then the pellet called A and supernatant filtered through 0.2µm polytetrafluoroethylene filter and injected onto HPLC column. From the left mixture 5 ml took out and re-extracted with 15ml EtOH after that sonicated of mixture for 10 minutes was placed in centrifuge (25 min 20 °C 3000rpm) then this pellet called pellet B and supernatant was filtered through 0.2µm polytetrafluoroethylene filter and injected onto HPLC column. Last 5ml of mixture was put into freezer at -20°C. The pellets that were obtained from the mixture pellet A and pellet B extracted with 5ml EtOH/H_2O (70%/30%) after that they were vortexed and sonicated for 10 minutes. Last mixtures were put into centrifuge (25 min 20 °C 3000 rpm) then the pellet from pellet A was called C and from B called as pellet D. Their supernatants filtered through 0.2µm polytetrafluoroethylene filter and injected onto HPLC column. Into pellet C and D 5ml Acetone/H_2O (70:30) was added and they were vortexed and sonicated for 10 minutes than were put into the centrifuge (25 min 20 °C 3000 rpm) then the pellets put into freezer. Their supernatants were filtered through 0.2µm polytetrafluoroethylene filter and injected onto HPLC column. Second method called protocol 3 was done by increasing the raisins amount. 10 g well-chopped raisins were extracted with 10 ml water after that it was homogenised and 10ml MeOH was added into mixture and homogenisation repeated second time. The reason why homogenisation was done twice was to increase the solubility of compounds in solvent. Using a measuring cylinder, 2 ml of mixture was placed into centrifuge (25 min 20 °C 3000rpm). The aqueous extract filtered through 0.2µm polytetrafluoroethylene filter and injected onto HPLC column for the determination of polyphenols that they are investigated.

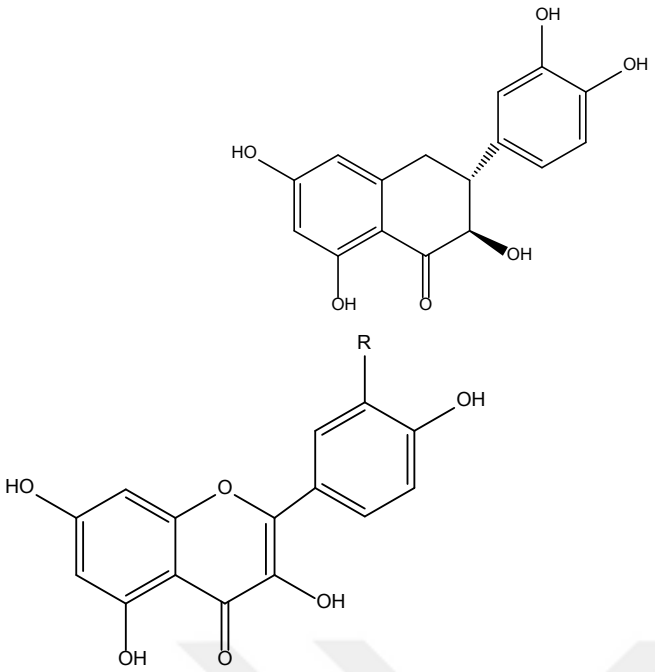

Figure 4 Taxifolin structure, (2) R = OH...QUERCETIN, R = H... KAEMPFEROL, R = QUERCETIN-3-RUTINOSIDE...RUTIN

3.4.1. HPLC analysis

The HPLC system consisted of an Agilent Eclipse XDB-C18 RRHT threaded column with Merc Hitachi interface D-7000 Lachrom, Merc Hitachi autosampler L-7200, Merc Hitachi column oven L-7300, Merc Hitachi diode array detector L-7450 and Merc Hitachi pump L-7100. Solvent A 95% acetonitrile, 5% distilled water and 0.1% formic acid, solvent B 95% distilled water, 5% acetonitrile and 0.1% formic acid. The elution program with a flow rate of 1.0 ml/min followed by a linear gradient from 100% to 10% A and from 0% to 90% B. The pressure limit was between 0 to 400 bar and the temperature was 35 °C. Simultaneous detection was at 260, 280, 310 and 370 nm. Total quercetin glycosides were quantitated as rutin and it can be estimated from absorbance measures at 370 nm. All peaks eluted within 26.7 min.

3.5. Urine Sampling and analysis (Analytical Methods of Assay)

We have 4 steps for preparing urine samples which are given below (Laboratory instructions).

1) Collection
2) Preparation for storage
3) Enzymatic hydrolysis
4) Reconstitution and filtration

Twenty-four hour collected urine samples were gathered on the third day in plastic 3000 ml containers containing 3 g ascorbic acid. Urine sample volume was measured. After that 10 x 10 ml portions of urine were measured into 15 ml falcon tubes. These falcon tubes also had 1 ml sodium-azide (0.1% concentration) that used as a biocide and prevents deterioration of samples during storage. They were stored in -20°C until analysis. The following flavonoids were quantified in the urine samples by LC-MS quercetin, catechin, epicatechin, ferulic acid, caffeic and caftaric acid. In brief, 100µl of 0.01% Taxifolin was added to 1ml of urine sample as internal standard. One control and 24 h urine from same volunteer were spiked with 100 µl/ml quercetin and also from the same volunteer 100 µl of taxifolin and 100 µl of quercetin was added to 1ml of urine sample. The majority of flavonoids in urine will exist as conjugates (sulphates or glucuronides) rather than as aglycones. Therefore enzyme hydrolysis was performed to liberate aglycones. To each 1 ml aliquots of urine were hydrolyzed by enzyme-enriched sodium phosphate buffer (0.2 M, pH 7). An enzyme solution from *E.coli* is contained 50 activity units of β-glucuronidase and from *Aerobacter aerogenes* 0.3 activity units of sulfatase in 0.2M sodium phosphate buffer solution (pH 7). The reaction mixture was incubated at 37 °C for 2 hours with continuous shaking (100 rpm). After hydrolysis, 275 µl of 2% HCL was added to the mixture to stop the enzymatic activity and decrease pH to 3 to provide non-polar analytes removing through ethyl acetate part during washing procedure. Then 3 series of 1500 µl ethyl acetate washes provided selective extraction of non-polar analytes. The resulting supernatant was evaporated by using centrifugal evaporator at 40°C for 6-7 hours. After drying up samples, they were stored at -20°C until

reconstitution. Before analysis, the residue was dissolved in 50 µl ACN and 200 µl 0.125% ascorbic acid solution. Until getting the perfect dissolving they were sonicated. After all residues were re-dissolved properly the samples were centrifuged at 1700 rpm for 10 minutes. Then, they were placed into vials for LC/MS analysis. Also same protocol applied for control urine. Each urine samples was done in duplicate.

3.6. LC/MS analyses

The dried sample was re-constituted in 50 µl acetonitrile and 200 µl 0.125% ascorbic acid. After they were vortexed they were centrifuged at 1700 rpm for 10 minutes in IEC Microcl 17 centrifuge. After they were filtered through 0.2 µm polytetrafluoroethylene filter, they were placed into LC/MS. Each sample was extracted and analyzed by LC/MS in duplicate. LC/MS Agilent Technologies 6410 Triple Quad LC/MS was used for the analysis. Chromatographic separation of the analytes of interest was achieved on a C18 (particle size 3.1micron, 150 mm×2.1 mm) column (Phenomenx Kinetix) and the mobile phase consisted of acetonitrile/water with 0.1% formic acid. The injection volume was 5 µl Solvent A had 0.1% formic acid in water and solvent B 0.1% formic acid in acetonitrile. Wang *et al*. (2005) reported that an acidic mobile phase using formic acid provided optimal separation and quantification of quercetin. By the method of comparison of retention and relative retention time the aromatic and phenolic compounds in the samples were identified.

3.7. Statistical Analysis

Statistical software was used to analyse the significance of the data and results indicated that there were significant levels of quercetin in the urine after 24 hours because Pr was less than 0.005.

4. Results

General

In this study we investigated the rutin (quercetin glycosides), catechin, epicatechin and caftaric acid content in the raisins. 18 participants were fed with 100 g of Sun Maid raisins. Before consumption of raisins they had 2 days washing period the third day they were fed with 100 g raisins. After consumption, they were requested to collect 24 h urine.

Results showed that the yields of raisin extract were affected by solvent type and extraction method and each participant has different amount of quercetin absorption depending on their metabolism and diet.

4.1. Phenolic Content

4.1.1. Raisins

Three different methods were done however Zhao and Halls (2008) method had the best efficiency compared with the other two. The 100% and 50% EtOH solvents produced extracts with the lowest rutin content. The extracts obtained from 25% ethanol had significantly higher yield than the other treatments that is shown in table 1. Acetonitrile and methanol produced extracts with significantly lower levels of rutin than the EtOH and MeOH solvent showed better extraction than acetonitrile. The 5 and 100% MeOH solvents produced extracts with no detectable levels of rutin. Furthermore the 5% MeOH had significantly lower rutin compared with concentrations observed for the 5% EtOH. Generally for ACN the main differences for rutin was lower than the comparable alcohol solvents as it seen in table 1. From this information it is clear that extraction method (pH, solvent, temperature (Williamson and Carughi unpublished) affects the apparent composition of polyphenols.

Concentration of solvent (%)	Ethanol (EtOH)	Methanol (MeOH)	Acetonitrile (ACN)
5	63.2	None	None
25	82.2	62.2	61.8

50	None	64.6	None
75	62.2	62.7	60.6
100	None	None	None

Table 1 The rutin content (µg/g) in raisins obtained from ethanol extraction.

Zhao and Halls (2008) reported that the best solvents for total phenolic content are MeOH and EtOH. Apart from this report, it is shown that 60% and 100% EtOH solvent extraction had better extraction in raisins extract and Williamson and Carughi reported that nearly 40mg/100g wet weight quercetin 3-*O*-rutinoside in raisin extract. According to Williamson and Carughi's study they found that 12 mg/100g quercetin 3-*O*-glycoside and 3 mg/100g quercetin 3-*O*-rutinoside were found in raisins making up a total of 15mg/100g quercetin present. Total losses during extraction are figured out by using taxifolin as an internal standard. Before adding solvent into raisins, 100µg of taxifolin added that helped to provide information about losses during extraction and identification of interested compound's peak. Figure 5 showed taxifolin (1 mg/ml) and rutin standard (50 µg/ml) chromatogram besides rutin and taxifolin content from raisin extraction. According to these results loss of taxifolin amount was figured out as 13.4%. Compared to this result, total amount of rutin content was found as 95 µg/g in raisins. Participants were fed with 100 g raisins in this experiment. Williamson, (2004) reported that the yield of flavonoids in the urine depends on flavonoid type. According to Scalbert and Williamson (2000), excretion of quercetin in urine is less than 1.5% such as 1.39% for onion and 0.44% for apple.

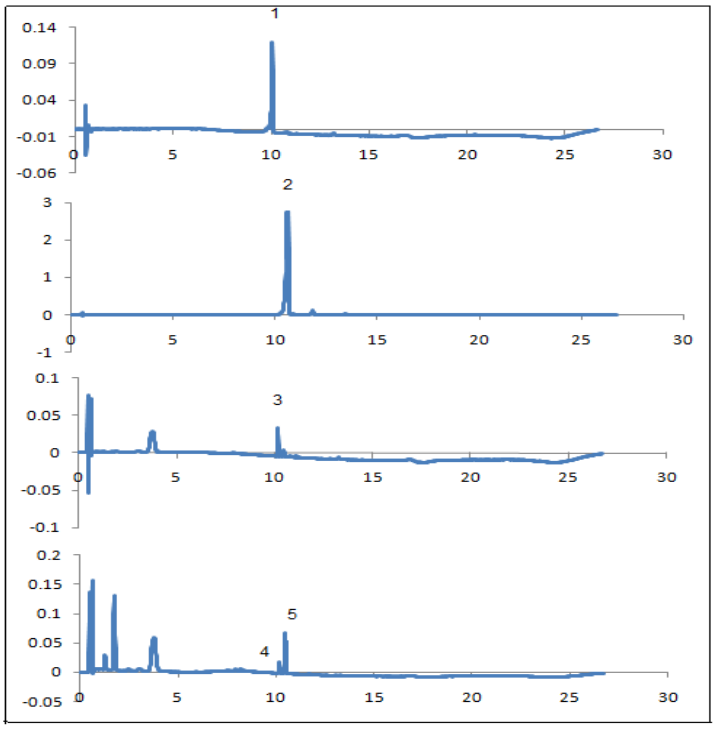

Figure 5 HPLC chromatogram of Sun-Maid Raisins rutin content, internal and rutin standard. (1) Rutin standard (370 nm), (2) taxifolin (310 nm), (3) rutin content in raisin (370 nm), (4) rutin content in 310 nm, (5) taxifolin content in raisin (310 nm).

4.2. Urine excretion

Phenolic compounds were detected in the participants' 24-h urine samples. The urinary excretion of phenolic compounds increased after feeding with raisins in 24-h urine. The absorption of quercetin was investigated by measuring the total quercetin concentrations present in the control urine and 24-hour urine. The differences between control urine and 24-hour urine give the total quercetin concentration in urine. The total amounts of quercetin excreted in urine during the 24 h period were also significantly increased in all participants after feeding. The participants in our study consumed a flavonoid-restricted diet (avoiding fruits, vegetables, and beverages rich in flavonoids) for 2 days before feeding with raisin. According to Hollman *et al*. (1997) elimination half life of quercetin is nearly 24 h therefore 48 h washing period should be enough to clear quercetin that is present in the urine. However, the amount of quercetin present in control urine has shown some variations between 1.5 µg/ml and 18.9 µg/ml. In figure 6 it is shown that LC/MS chromatogram of

extracts of 24-hour urine (UC) and control urine (UB) for one of the volunteer with code 113 and retention time for quercetin is for urine consumption 16.242 while for control urine 16.250 there was shift in the retention time to left.

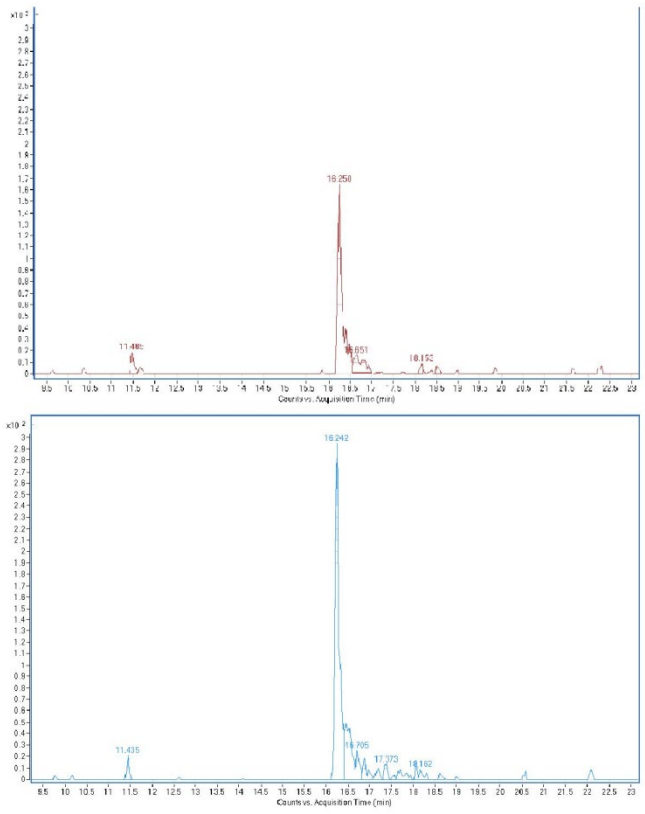

Figure 6 LC/MS chromatogram of extracts of 24-hour urine (UC) and control urine (UB) for one of the volunteer with code 113 for quercetin.

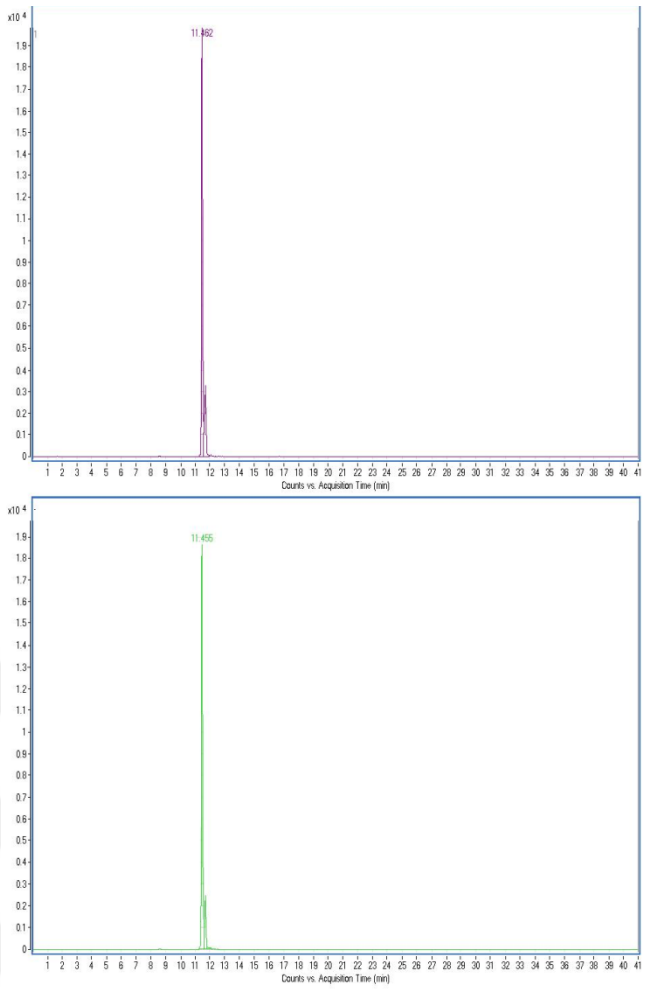

Figure 7 LC/MS chromatogram of extracts of 24-hour urine (UC) and control urine (UB) for one of the volunteer with code 113 for taxifolin as internal standard.

Retention time for taxifolin also shifts left as it is shown in figure 7 and the relative retention time confirms that it is quercetin in control urine and 24-hour urine. The participants in the study consumed a flavonoid-restricted diet (avoiding fruits, vegetables, and beverages rich in flavonoids) for 2 days before consuming raisins. According to Hollman *et al*. (1997) elimination half life of quercetin is nearly 24 h therefore 48 h washing period should be enough to clear quercetin that is present in the urine. However, the amount of quercetin present in control urine has shown some variations between 1.5 µg/ml and 18.9 µg/ml. As it is seen in figure 4, control urine has some quercetin and the amount of it is 1.5 µg/ml. The reason for that might come from different metabolism and some other factors such as the possibility that the subject

consumed foods or beverage with ingredients rich in quercetin. According to raisins extraction, each participant was fed with 9500 µg/100g rutin, therefore if the excretion of quercetin in urine is accepted less than 1.5% than the amount of total quercetin in urine should be less than 142.5 µg. Participants were fed with raisins in the same condition because it was important to provide same conditions for all of them. The reason for that reported by Hollman *et al*. (1995); absorption of quercetin is affected by the diet. Also they discovered that quercetin conjugation with sugar provide increase in the absorption of quercetin (Hollman *et al*., 1995). Firstly, all volunteers were fed with bread, butter, banana and water after which they were requested to eat 100 g of raisins. Each participant's 24h urine had different amounts of quercetin that is, between 21.8 µg/ml and 238.8 µg/ml. For the final results of total quercetin in urine different values between 16.2 µg/ml and 229.3 µg/ml were shown giving the total urinary excretion of quercetin between 2.4% to 0.17%. According to Hollman *et al*. (1995), absorption of quercetin-glucosides from onion was 52% while rutin from onion renders absorption only 15%.

5. Discussion

Three different extraction methods were done with different solvents during the experiment. The best efficiency was provided in the Zhao and Halls (2008) method. According to Zhao and Halls (2008), a method with three solvents was used for extraction and instead of using raisin extraction well-chopped raisins used. These solvents were ethanol, methanol and acetonitrile. EtOH extraction solvent had significantly higher yield than the other treatments. Acetonitrile and methanol solvents produced extracts with significantly less rutin content than ethanol solvent and MeOH solvent showed better extraction than acetonitrile. Acetonitrile (CH_3CN) produced substantially lower yield than yields obtained from the comparable alcohol solvents. Alcohols have an important role in the extraction efficiency (Zhao and Halls, 2008). Other two extraction method had lower yields. Maximum extraction result for protocol 2 (64.4 µg/g) and the extracts obtain from protocol 3 was 75.6 µg/g. All the three extractions gave better extraction yields with ethanol. For quercetin and caftaric acid, 25% EtOH had better extraction compared to other concentrations and catechin and epicatechin showed higher yields with 5% EtOH. A similar trend was reported by Zhao and Halls (2008) and they reported that EtOH and MeOH showed higher extraction than acetone. The presence and identity of quercetin in human urine samples and raisin extraction were verified by two criteria which are a) spiking urine samples and raisin extracts increased the expected peak heights; b) adding taxifolin as an internal standard into urine samples and raisin extracts to determine relative retention time. Beside losses from the process was identified by calculating the lost taxifolin during process. The retention time for taxifolin was 10.44 and the concentration was 0.01 µg/ml and the taxifolin solution was made with 20% ACN. Total loss of taxifolin amount was figured out as 13.4% during process. This loss might come from homogenization process.

Subject code	103	105	107	111	113	116	118
UC	238.87	113.76	216.18	29.79	110.60	21.80	83.44
UB	9.55	17.34	12.13	2.28	1.56	5.58	3.73
TOTAL	229.31	96.41	204.04	27.51	109.04	16.21	79.71

Table 2 The amount of total excreted quercetin $\mu g/ml$.

The accuracy of measurements was wanted to determine in duplicate by adding 100µg of quercetin to 1.0ml aliquots from subject 101. However during re-constitution of this spiked urine some amount of quercetin precipitated. Therefore the accuracy of measurement could not be done by using spiked urine. Total amount of rutin content was found in raisins 95 µg/g. Participants fed with 100 g raisins in this experiment. In that case, all participants consumed the

amount of 9500 µg/100g rutin and Scalbert and Williamson (2000) reported that excretion of quercetin in urine is less than 1.5% such as for onion 1.39% and for apple 0.44%. Hydrolysation of rutin can be done by intestinal microflora with α-rhamnosidase and β-glucosidase to quercetin and then quercetin is absorbed and the absorbed quercetin excreted into bile and urine (Shimoi, 2003). Scalbert and Williamson (2000) reported that the amount of quercetin present in the urine should be lower than 142.5 µg/ml. The variation of quercetin excretion is from the concentration 16.2 µg/ml to 229.3 µg/ml as it is shown in table 2. The participant that has the highest excretion ate chocolate in third day during dinner time. The lowest excretion should base on participant absorption metabolism because 24 h urine sample of this participant (subject code: 116) also has lower amount (21.8 µg/ml) although the consumption amount of raisin was same for all participants. During exclusion diet participant with the code 105 consumed dried plums and pineapple therefore control urine has high concentration of quercetin. For three days 107 ate mayonnaise that is a stable emulsion of olive oil, egg yolk and either vinegar or lemon juice with other herbs and spices. Except egg yolk other ingredients of mayonnaise include polyphenols. Therefore excretion of quercetin is higher for both baseline and 24-hour urine for this participant. The participants the codes are 111 and 113 have the best exclusion diet compare to other 16 participants. The amount of quercetin in control urine is very little. After consumption with raisin as it is seen in table 2 113 has more quercetin compare to 111 in 24 h urine. The reason might be related with different metabolism therefore it can be said that 111 has better absorption than 113. Catechin, epicatechin, caffeic acid, caftaric acid and ferulic acid excreted lower than quercetin. Catechin and epicatechin was shown the lower excretion. The reason why quercetin has less absorption compared to other polyphenols was reported by several researchers. In foods, quercetin exists in the form of glycosides and rutin is one of the most commonly occurring glycoside of quercetin (Gross *et al.,* 1996) and first hydrolysed by the microflora before being absorbed (Ishii *et al.,* 2003). Day *et al.,* (2000) reported that naturally flavonols are present as glycosylated forms in foods therefore absorption of quercetin is dependent on the nature of glycoside and metabolism of these compounds hydroxyl groups conjugated with sulphate, glucuronic acid and limited methylation of the catechol functional group (Day *et al*., 2000). Therefore removal of sugar from flavonols should be provided by enzymes. Williamson (2004) reported that absorption of flavonols is started with deglycosylation by LPH in the lumen. Also quercetin hydrolysis occurs with β-glucosidase in the small intestine but it cannot hydrolyse in the liver (Scalbert & Williamson, 2000). However flavonols (especially epicatechin, catechin) can pass through biological membranes and absorbed because there is no needs for hydrolysis or deconjugation (Scalbert & Williamson, 2000). Phenolic acid esters (especially ferulic acid, caffeic acid) are esterified to organic acids, sugars and lipids. However, there are no esterase enzymes in humans to metabolise it (Scalbert & Williamson, 2000). According to all these papers it is clear that quercetin cannot metabolise well for some participants and also high amount of present quercetin in control urine can be thought there is some interference present in urine which has the same retention time with quercetin. Therefore it might cause increase the amount of present quercetin in urine baseline.

Bioactivities of polyphenols in raisins provide health benefits. Flavanols, flavonols, and hydroxycinnamics are the major functional components that are responsible for most of the biological activities of raisins. During the recent years, the importance of flavonoids which is generally present in fruit and vegetables has become popular due to their effects on human health. Derivatives of quercetin have gained importance as dietary constituents that are present in high amount in raisins. The amount of quercetin in raisins is relevant to choose this food for feeding volunteers to see the absorption of quercetin. Raisins are a good dietary

source of flavonol glycosides and phenolic acids and are considered to be a desirable source of dietary fibre, with polymerized phenolics contributing to that fibre. The antioxidant activity of flavonoids has strong linkage to the antioxidant activity of flavonoids.

REFERENCES

BOOTS, A. W., WILMS, L. C., SWENNEN, E. L. R., KLEINJANS, J. C. S., BAST, A. & HAENEN, G. R. M. M. (2008). In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. *Nutrition,* 24**,** 703-710.

CARUGHI, A. (2008). health benefits of sun-dried raisins. California-Kingsburg: Health Research & Studies Center.

CRESPY, V., MORAND, C., BESSON, C., MANACH, C., DEMIGNE, C. & REMESY, C. (2001). Comparison of the intestinal absorption of quercetin, phloretin and their glucosides in rats. *Journal of Nutrition,* 131**,** 2109-14.

CRESPY, V., MORAND, C., MANACH, C., BESSON, C., DEMIGNE, C. & REMESY, C. (1999). Part of

quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal lumen. *American Journal of Physiological Gastrointestinal Liver Physiology,* 277**,** 120-126.

DAY, A. J., BAO, Y., MORGAN, M. R. A. & WILLIAMSON, G.(2000). Conjuction position of quercetin glucuronides and effect on biological activity. *Free Radical Biology & Medicine,* 29**,** 1234-1243.

GROSS, M., PFEIFFER, M., M. MARTINI, CAMPBELL, D., SLAVIN, J. & POTTER, J. (1996a). The Quantitation of Metabolites of Quercetin Flavonols in Human Urine[1-2]. *Cancer Epidemiology, Biomarkers & Prevention,* 5**,** 711-720.

HOLLMAN, P. C. H., DE VRIES, J. H. M., VAN LEEUWEN, S. D., MENGELERS, M. J. B. & KATAN, M. B. (1995). Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers[1-3]. *American Journal of Clinical Nutritional,* 62**,** 1276-82.

HOLLMAN, P. C. H. & KATAN, M. B. (1999). Dietary flavonoids: intake, health effects and bioavailability. *Food and Chemical Toxicology,* 37**,** 937-42.

Http://www.vitamins-supplements.org/quercetin.php [Accessed 2010, May].

ISHII, K., FURUTA, T. & KASUYA, Y. (2003). H igh-performance liquid chromatographic determination of quercetin in human plasma and urine utilizing solid-phase extraction and ultraviolet detection. *Journal of Chromatogrophy B,* 794**,** 49-56.

KALIORA, A. C., KOUNTOURI, A. M. & KARATHANOS, V. T. (2009). Antioxidant properties of raisins. *Journal of Medical Food,* 12 1302-09.

KARADENIZ, F., DURST, R. W. & WROLSTAD, R. E. (2000). Polyphenolic composition of raisins. *Journal of Agricultural Food Chemistry,* 2000**,** 5343-50.

LAMSON, D. W. & BRIGNALL, M. S. (2000). Antioxidants and cancer III: Quercetin. *Alternative Medicine Review* 5**,** 196-208.

MUROTA, K. & TERAO, J. (2003). Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. *Archives of Biochemistry and Biophysics,* 417**,** 12–17.

PARKER, T. L., WANG, X., PAZMINO, J. & ENGESETH, N. J. (2007). Antioxidant capacity and phenolic content of grapes, sun-dried raisins, and golden raisins and their effect on ex vivo serum antioxidant capacity. *Journal of Agricultural and Food Chemistry,* 55**,** 8472-8477.

SCALBERT, A., WILLIAMSON, G. (2000). Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition, 130, 2073-2085.*

SHIMOI, K., YOSHIZUMI, K., KIDO, T., USUI, Y. & YUMOTO, T. (2003). Absorption and urinary excretion of quercetin, rutin, and αG-Rutin, a water soluable flavonoids, in rats. *Journal of Agricultural and Food Chemistry,* 51**,** 2785-2789.

WACHA, A., PYRZYŃSKAA, K. & BIESAGA, M. (2007). Quercetin content in some food and herbal samples. *Food Chemistry,* 100**,** 699-704.

WILLIAMSON, G. & CARUGHI, A. unpublished. polyphenol content and health benefits of raisins.

WILLIAMSON, G. (ed.) (2004). *Common features in the pathways of absorption and metabolism of flavonoids* New York: CRC Press.

WILLIAMSON, G., DAY, A. J., PLUMB, G. W. & COUTEAU, D. (2000). Human metabolic pathways of dietary flavonoids and cinnamates. *Biochemical Society Transactions,* 28**,** 16-22.

ZHAO, B. & HALL III, C. A. (2008). Composition and antioxidant activity of raisin extracts obtained from various solvents. *Food Chemistry,* 108**,** 511-518.