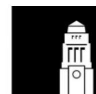


UNIVERSITY OF LEEDS

This is a repository copy of *Modulation of cellular glucose metabolism in human HepG2 cells by combinations of structurally related flavonoids*.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/85477/

Version: Accepted Version

Article:

Kerimi, A, Jailani, F and Williamson, G (2015) Modulation of cellular glucose metabolism in human HepG2 cells by combinations of structurally related flavonoids. Molecular Nutrition and Food Research, 59 (5). 894 - 906. ISSN 1613-4125

https://doi.org/10.1002/mnfr.201400850

Reuse
Unless indicated otherwise, fulltext items are protected by copyright with all rights reserved. The copyright exception in section 29 of the Copyright, Designs and Patents Act 1988 allows the making of a single copy solely for the purpose of non-commercial research or private study within the limits of fair dealing. The publisher or other rights-holder may allow further reproduction and re-use of this version - refer to the White Rose Research Online record for this item. Where records identify the publisher as the copyright holder, users can verify any specific terms of use on the publisher's website.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

Modulation of cellular glucose metabolism in human HepG2 cells by combinations of structurally-related flavonoids

Asimina Kerimi[1], Fadhilah Jailani[1,2], Gary Williamson[1]

[1]School of Food Science and Nutrition
Faculty of Mathematics and Physical Sciences
University of Leeds, UK
UK
Phone +44 113 343 8380
Fax: +44 113 343 2982
Email: a.kerimi@leeds.ac.uk
g.williamson@leeds.ac.uk

[1,2]Food Technology Programme
Faculty of Applied Sciences
Universiti Teknologi MARA
40450 Shah Alam
Selangor, Malaysia
fadhi478@salam.uitm.edu.my

Correspondence to: Prof Gary Williamson, School of Food Science and Nutrition, Faculty of Mathematics and Physical Sciences, University of Leeds, Leeds, LS2 9JT, UK

Contributions: All authors were involved in planning the research. AK carried out the in vitro experiments, and FJ carried out the human study. AK and GW wrote the paper.

Abstract

Scope: Insulin-regulated glucose metabolism in cells is critical for proper metabolic functioning, and insulin resistance leads to type 2 diabetes. We performed a human study to assess the availability of structurally related dietary flavonols and tested their ability to affect cellular glucose uptake, metabolism and glucose transporter gene expression in a liver HepG2 cell model.

Methods/results: Eight healthy volunteers consumed a meal containing galangin, kaempferol, quercetin and myricetin. In plasma, myricetin was absent, but the others were present, mostly as conjugates. In HepG2 cells, a combination of galangin, kaempferol and quercetin (5 μM each) for 12 h increased the acute uptake of [U-^{14}C]-glucose and 2-[U-^{14}C]-deoxyglucose by almost 100 and ~10% respectively. All of the combinations increased glucose metabolism, but the effect on transport was less pronounced and mixed. A mixture of all flavonols significantly increased mRNA expression of the main glucose transporter Glut1 in HepG2 cells.

Conclusions: These results for the first time show the presence of galangin conjugates in human plasma, and allow direct comparison between absorption of flavonols. A combination of flavonols has the potential to modulate sugar metabolism, both uptake into cells as evident from effects on deoxyglucose, and also further cellular glucose metabolism.

Abbreviations:

C_{max}: maximum concentration in the blood

DAD: diode array detector

DMSO: dimethylsulfoxide

DPM: disintegrations per minute

EDTA: ethylenediaminetetraacetic acid

[U-^{14}C]-G: radiolabelled glucose

2-[U-^{14}C]-DG: radiolabelled deoxy-glucose

GLUT1: Solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1)

GLUT2: Solute carrier family 2 (facilitated glucose transporter), member 2 (SLC2A2)

GLUT3: Solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)

GLUT4: Solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4)

GLUT9: Solute carrier family 2 (facilitated glucose transporter), member 9 (SLC2A9)

GLUT10 Solute carrier family 2 (facilitated glucose transporter), member 10 (SLC2A10)

HPLC: High performance liquid chromatography

IS: internal standard

MRM: multiple reaction monitoring

MS: mass spectrometry

PTFE: polytetrafluoroethylene

RPMI: Roswell Park Memorial Institute medium

T_{max}: time that C_{max} is reached

Introduction

Regulation of glucose uptake from the blood and metabolism in peripheral tissues is one of the key steps in maintaining a healthy metabolic phenotype. Glucose uptake into cells is facilitated and tightly controlled by glucose transporters that show a diverse expression between different tissues. Only specific isoforms (Glut2, Glut4) are activated in response to insulin. Postprandially blood insulin levels rise, leading to an increase of intracellular glucose in some tissues that is used to generate energy through glycolysis and the TCA cycle. These finely-tuned processes are disrupted in patients with metabolic syndrome or diabetes. Many bioactive compounds have been reported to inhibit the uptake of glucose into cells in vitro, including the flavonoid quercetin. However, less is known about the ability of mixtures of these compounds to modulate glucose responses.

Dietary flavonoids are small molecules present in many plant-based foods and supplements and are proposed to reduce the risk of diabetes and heart disease according to population studies [1-4]. Flavonols are a biologically active sub-group consisting of galangin, kaempferol, quercetin and myricetin. These have identical chemical structures except for substitution of the B ring, where the number of hydroxyl groups increases from 0 to 3 for galangin, kaempferol, quercetin and myricetin respectively, and this provides a convenient platform for performing structure-function studies. Quercetin constitutes the most abundant of these compounds and is found widely in the diet in plant-based foods and beverages, such

as onions, apples and tea. Kaempferol content is high in many Brassica vegetables, salads and herbs, myricetin exists at fairly high levels in walnuts, fennel, blackcurrants, blackberries and many other berries, and galangin is contained mainly in flavourings such as oregano [5]. The biological functions of flavonols in vivo and in vitro broadly support their protective effects against cardiovascular disease and diabetes. Most studies have been performed with quercetin [1;6], while the other flavonols have also been reported to exert some beneficial effects. Galangin reduced inflammation in fructose-fed rat liver [7], and inhibited increased body weight, energy intake and PAT weight induced by a "cafeteria diet" in rats [8]. Myricetin was reported to affect glycogen metabolism in diabetic rats [9]. In diets, however, these compounds are not consumed in isolation but together with other polyphenols, nutrients and vitamins. Despite this, very few studies have considered the effect of combinations of flavonols on any biological activity.

Since these compounds have been shown to potentiate multiple diverse effects, we took a first step towards a mechanistic understanding of their combined action. We endeavoured to test the potential of flavonols to affect cellular glucose uptake and metabolism in a mixture compared to each one alone, and further investigated any transcriptional changes of glucose transporters. However, although quercetin absorption and bioavailability is well studied [1], there is much less information on kaempferol [10] and very little on myricetin and galangin in humans. In order for the flavonols to modulate energy metabolism in cells, we considered it necessary to investigate plasma appearance before conducting in vitro experiments on activity.

To achieve this, we performed a human intervention study to evidence bioavailability, and assessed biological activity of the flavonols using the human HepG2 cell model. This is suitable for studying certain functions of human hepatocytes [11] including secretion of plasma proteins characteristic for hepatocytes [12]. HepG2 cells show a blunted response to insulin, i.e. a lower glycerol uptake and conversion to glucose-3-phosphate, while ketone body production and peroxisomal β-oxidation is comparably lower but still functional compared to human hepatocytes [13]. They also retain acyl-CoA oxidase and very long chain fatty acyl-CoA synthase activities and this alteration may be attributed to

the high lipogenic character of HepG2 cells as they primarily metabolise glucose to lactate instead of CO_2 which leads to a lower oxidative capacity [14], a generalised common phenomenon in cell culture models. Glucose uptake is facilitated and regulated by glucose transporters, and HepG2 cells express GLUT1> GLUT3 > GLUT2 > GLUT9 > GLUT10 [15]. These factors make the well understood HepG2 model suitable for carrying out experiments to assess glucose uptake and metabolism [16].

Experimental

Materials

Pickled capers, low fat mayonnaise, tuna chunks in brine, white bread and granulated sugar were obtained from a local supermarket (Morrisons, Leeds). Propolis tablets were purchased online (Amazon.co.uk,Natural Plus Ltd) and freeze-dried blackcurrant powder was from by Healthy Supplies (Brighton, UK). Equipment for blood sampling was from BD Medical (Oxford, UK) and from Bunzl Healthcare (London, UK). K_3EDTA tubes and adapters with holders were from Greiner Bio-One Ltd (Gloucestershire, UK) and cryotubes for plasma storage were from Nunc Thermoscientific (UK). Milli-Q water nuclease free water (≥ 18.2 MΩ) was used (Milli-Q Advantage A10, Millipore UK Ltd, Hertfordshire, UK).

Authentic standards of myricetin, quercetin, kaempferol, kaempferol-3-O-glucuronide, isorhamnetin, galangin and daidzein were from Extrasynthese (Genay, France). Quercetin-3'-O-sulphate, quercetin-7-O-glucuronide, quercetin-3-O-glucuronide, quercetin-3'-O-glucuronide and isorhamnetin-3-O-glucuronide were enzymatically synthesised and purified by Dr D. Wong, University of Leeds as described previously [17]. Sinapic acid, ascorbic acid, formic acid, DMSO, sodium azide, ethanol, and deoxy-D-glucose were from Sigma-Aldrich (Dorset, UK). Hexane, D-glucose and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). 2-[U-^{14}C] deoxy-D-glucose and [U-^{14}C] D-glucose were from Perkin Elmer (Life Analytical Sciences, Beaconsfield, UK).

Selection of food samples and flavonol content analysis

Blackcurrant, broad bean, red and yellow onions, pickled capers, curly endive, and dried oregano were analysed for their content of myricetin, quercetin, kaempferol and galangin. The selection was based on flavonol content from the PhenolExplorer database [5], and from the literature for galangal (Alpina officinarum) and propolis [18;19]. All fresh samples were frozen at -80°C prior to lyophilisation and ground to a fine powder. Propolis supplements in tablet form were crushed and ground using a mortar and pestle. For each food, at least three replicates of 0.2 g dried samples were extracted. Samples were homogenised in 5 ml of 70% methanol containing daidzein as internal standard and ascorbic acid to deter flavonol oxidation, with a final concentration of 20 and 100 μM respectively. After centrifugation (3000 x g, 4°C, 10 min), the supernatant was collected. The extraction was repeated twice and all supernatants were combined and evaporated to dryness (Genevac EZ-2 Plus, Suffolk, UK).

To achieve identification and quantification of flavonols, the food extracts were subjected to hydrolysis with cellulase and hesperidinase. The dried food extracts were reconstituted in acetate buffer (pH 5) containing 20 units/ml of cellulase from Aspergillus niger and the solution was incubated at 37°C for 18 h. The reaction was stopped by the addition of ethyl acetate (1:1) and aglycones were extracted. The solutions were vortexed, centrifuged (3000 x g/ 4°C/ 10 min) and aliquots of the supernatant were used. The extraction procedure was repeated twice. Three supernatants were pooled, reduced to dryness and reconstituted in 50% aqueous ethanol (50:50, v/v). Aliquots of the supernatant were removed and filtered through a 0.2 μm PTFE membrane filter and analysed by HPLC. Enzyme hydrolysis was repeated with hesperinidase in acetate buffer (pH 3.8, 20 units/ml) and solutions incubated for 18 h at 40°C. To account for flavonol aglycones losses due to oxidation throughout the enzyme hydrolysis protocol, a known concentration of aglycone standards containing either cellulase or hesperidinase enzyme was used for comparison. Recovery was calculated as the amount of flavonols remaining after the incubation and ethyl acetate extraction.

Study design and experimental meal

The protocol of the study was approved by the MEEC Faculty Research Ethics Committee at the University of Leeds (MEEC 10-010). A subset of 8 volunteers in good health as assessed by self-reported medical history donated blood samples. Ages ranged from 21 to 36 years, body mass index from 19.1–27.1 kg/m^2, non-smokers and not on prescription of any medication. The protocol was explained to volunteers and written informed consent was obtained.Volunteers were requested to follow a low-flavonol diet by refraining from foods with high levels of flavonols (mostly fruits and vegetables especially onions, leeks, chives, tomato, kale, endive, spinach, apples, wild berries and drinking any alcoholic beverages or tea) for two days prior to (washout period), and during the course of the study period (24 h post the experimental meal). A list of prohibited foods and suggestions of meals during the low-flavonol diet was provided.

On the day of intervention, volunteers arrived between 8:00 and 9:00 am after an overnight fast. An intravenous catheter (cannula) was placed and a baseline blood sample was taken as control (time zero) prior to consuming the experimental meal. During the intervention, volunteers were asked to fill in a 24 h food record to assess their compliance with the recommended low-flavonol diet. Meals were prepared on the day of the experiment, comprising 50 g pickled capers as sources of quercetin and kaempferol, 65 g tuna in brine and 15 g low fat mayonnaise. 50 g of freeze-dried blackcurrants was used to prepare the jam which provided a source of myricetin. Sugar was added to taste (25 g). The jam was served with 90 g of white bread (2-3 slices). Galangin was supplied from two tablets of a commercially available propolis supplement, and meals were consumed within an average time window of 20 min. A low-polyphenol lunch and snack were provided during the day of intervention. The timing of the study began after consumption of the experimental meal, and venous blood samples (12 ml) were obtained at 0.5, 1, 2, 3, 4, 5, 6, 8 and 24 h post-ingestion. Samples were collected in EDTA tubes and immediately centrifuged (3000 x g/ 4°C/ 10 min) to separate the plasma from the red blood cells. Aliquots of 500 µl plasma were acidified with 15 µl of 50% formic acid and the oxidation of flavonoids was prevented by addition of ascorbic acid (final concentration 1 mM) [20]. Samples were frozen at -80°C until further analysis.

Plasma extraction protocol

Flavonols were extracted from plasma based on the method described previously [21] with slight modifications. The protocol for plasma extraction was validated for extraction efficiency, and intraday/interday variability. Blank plasma was spiked with a known amount of flavonol standards containing myricetin, quercetin, isorhamnetin, kaempferol, galangin, quercetin-7-O-glucuronide, quercetin-3-O-glucuronide, quercetin-3′-O-glucuronide, quercetin-3′-O-sulphate, kaempferol-3-O-glucuronide, isorhamnetin-3-O-glucuronide and daidzein (IS) at the very beginning of the extraction procedure. Four concentrations were spiked in three independent replicates of blank plasma (plasma collected at 0 h). The recovery value is expressed as % of the initial spiked amount (Table 1, supplementary information).

HPLC-DAD-MS-MS analysis

Identification and quantification of flavonols and conjugates was performed on an Agilent 1200 LC system coupled to a diode array detector and triple quadrupole mass spectrometer according to the method described previously [22].

Data analysis of the human study

The results were expressed as mean ± SE unless stated otherwise. Distribution of the data was evaluated using normal probability plots and by performing the Shapiro-Wilk test. The intra-individual variation of the flavonol concentration was expressed as coefficient variation (CV) from the mean. A p-value of ≤ 0.05 was considered statistically significant. All of the statistical analyses were conducted using SPSS for Windows, software version 22.

Cell culture

The human hepatocellular carcinoma cell line, HepG2 (HB-8065), was obtained from the American Type Culture Collection (LGC Promochem, Teddington, UK) at passage 74 and was used for experiments between passages 78 and 91. Cells were routinely cultured in 75 cm^2 cell culture flasks (Corning, 430641, Sigma-Aldrich, Poole, Dorset, UK) at 37 °C in a humidified atmosphere of 5 % CO_2/95% O_2. They

were seeded at a density of approximately 10^5 cells/ cm^2 and allowed to grow in Eagle's minimal essential medium (LGC Promochem, Teddington, UK) supplemented with 10% heat inactivated FBS (v/v) (Sigma Aldrich, Poole, Dorset, UK) and 100 U/ml penicillin–streptomycin (Sigma Aldrich, Poole, Dorset, UK), until they reached 80-90% confluence, as judged by observation under a light microscope. Medium was changed twice weekly and cells were subdivided usually on day 4 from seeding (96 h, 80-90% confluence) at a ratio of 1:4 or less, according to yields. Assessment of yield at 80-90% confluency was carried out by counting of intact cells on a haemocytometer after mixing cells with trypan blue (Sigma Aldrich, Poole, Dorset, UK). Cells were detached from the flask by addition of 0.25% trypsin-EDTA and centrifugation at 1170 g. The resultant pellet was reconstituted in fresh Eagle's minimal essential medium and cells were counted. Cells were fed with fresh medium without FBS for 10 h before any experimental treatment with the flavonols. Flavonols were added to cells from a DMSO stock solution (final concentration 0.08%) of 30 mM either separately (20 µM) or in a mixture (5 µM each) for 12 h before assays.

[U-^{14}C]-glucose and 2-[U-^{14}C]-deoxy-glucose assay

For glucose and deoxy-glucose uptake assays, cells were seeded in 6 well plates at a seeding density of 0.6 x 10^6 cells/ well. At the end of the experimental treatment, cells were washed twice with 37º C PBS buffer (pH 7.4) and transferred to assay medium without glucose for 10 min (HEPES, 20 mM; NaCl, 137 mM; KCl, 4,7 mM; MgSO4, 1.2 mM; CaCl2; 1.8 mM) followed by the addition of [U-^{14}C]-glucose or 2-[U-^{14}C]-deoxy-glucose (0.1 µCi/ well) in 0.55 mM glucose or deoxy-glucose in RPMI medium pH corrected to 7.4, for an additional 15 min. The assay was terminated by withdrawal of the medium and subsequent washing of the cells with ice-cold phosphate-buffered saline on ice. Cells were lysed and scraped in 0.5 mM NaOH, equal amounts of 0.5 mM HCl added and radioactivity evaluated in the cell extract (n=6). For scintillation counting samples were mixed with 10 ml of Ecoscint XR scintillation fluid (National Diagnostics, UK) and counted over 10 min on a Tri-Carb 1600TR (Packard Instruments, UK). Efficiency of the scintillation counter was evaluated in every experiment with the use of a radiolabelled ^{14}C standard (Perkin Elmer Life Analytical Sciences, Beaconsfield, UK).

RNA isolation and quantitative real time RT-PCR

For gene expression assays, cells were seeded in 12 well plates at 0.4 x 10^6 cells/ well. Three biological replicates were pooled from three individual experiments and analysed four times (n=4). RNA isolation from harvested HepG2 cells in cell lysis buffer was carried out using an RNAqueous-4PCR kit (AM1912, Ambion, Applied Biosystems, Warrington, UK). Assessment of purity and concentration of RNA was carried out with a Nanodrop 1000ND spectrophotometer (Thermo Scientific, Labtech, East Sussex, UK) and 1 µg of RNA was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA master mix kit (Applied Biosystems, Warrington, UK).

Quantitative RT-PCR was performed using TaqMan universal PCR master mix. RT-PCR was performed using the Biosystems ABI PRISM HT Fast Real-Time PCR sequence detection system (Applied Biosystems, Foster City, CA, USA) while for reverse transcription a StepOne Real-Time PCR (Applied Biosystems, Warrington, UK) system was used. Target-specific probes (FAM labelled) were purchased from Applied Biosystems (SLC2A1 (GLUT1) solute carrier subfamily 2A member 1 (Hs00892681_m1, NM_006516.2); SLC2A9 (GLUT9) solute carrier subfamily 2A member 9 (Hs00252242_m1, NM_020041.2). Primers were multiplexed with GAPDH (VIC labelled_PL) for normalisation and the relative quantity was obtained using the comparative threshold method. In the ΔΔCt method, cells treated with DMSO were used as the reference sample.

Determination of protein concentration of cell samples

A Coomassie protein assay kit (Pierce Scientific, Thermo Scientific, Leicestershire, UK) was performed according to the manufacturer’s protocol.Cell extracts were appropriately diluted with either NaOH 0.5 mM or PBS.

Statistical analysis

Results shown for [U-^{14}C]-glucose and 2-[U-^{14}C]-deoxy-glucose assays (n=6) and for mRNA expression (n=9) represent mean values and error bars SD. A One-Way-ANOVA was used to compare between different treatments and a p-value of ≤ 0.05 was considered statistically significant. Levenes' test was employed to check for homogeneity of means, and where the equality criterion was met, the Tukey HSD test was used for post-hoc comparisons and where not true the Dunnet C test was used instead.

Results

Flavonol content in the experimental meal

Foods were analysed for their content of flavonols before and after enzymic hydrolysis (Table 1). Pickled capers and propolis tablets contained considerable amounts of quercetin, kaempferol and galangin in the free aglycone form constituting ~ 46%, 59% and 40% of the total amount, respectively. The main conjugated forms of flavonols in pickled capers are kaempferol-3-O-rutinoside, quercetin-3-O-rutinoside and trace amounts of kaempferol-3-O-rhamnosyl-rutinoside [23]. In blackcurrants, myricetin is conjugated with various glycosides such as rutinoside, glucuronide and glucoside groups and small amounts of quercetin were also conjugated as rutinosides and glucosides [24]. The serving size of 50 g of pickled capers and 50 g of blackcurrant jam provided an estimate of 91 µmol quercetin, 76 µmol myricetin and 72 µmol kaempferol while two tablets of propolis supplement amounted to 50 µmol galangin (Table 1).

Identification of flavonols in plasma

Plasma samples collected after the consumption of the experimental meal were analysed using HPLC-MS-MS, and a typical MRM trace chromatogram is shown in Figure 1. No aglycone, sulphated or glucuronidated forms of myricetin were detected in the plasma samples collected at any time point after consumption of the experimental meal. Among seven quercetin metabolites detected by LC-MS-MS, only two of them, quercetin-3'-O-sulphate and isorhamnetin-3-O-glucuronide, could be quantified in the plasma of every volunteer. C_{max} and T_{max} values for quercetin

are detailed in Table 2. The T_{max} of isorhamnetin-3-O-glucuronide in plasma was somewhat later than quercetin-3'-O-sulphate, and the former was still present at ~ 8 h post-ingestion at a level of ~ 60% of the C_{max}. Figure 2 shows the mean circulating concentrations of kaempferol found as kaempferol-7-O-sulphate and kaempferol-3-O-glucuronide, and the resulting C_{max} and T_{max} values are included in Table 2.

The appearance of galangin-7-O-sulphate in plasma occurred between 4 to 6 h after the intake of the experimental meal. It becomes evident from Figure 3 that there is a large inter-individual variability in the appearance of galangin-7-O-sulphate. The majority of subjects exhibited maximum concentration in plasma at the 8 h time point. Figure 4 illustrates the individual profiles of circulating galangin-3-O-glucuronide in plasma of eight volunteers. Similar to galangin-7-O-sulphate, high variability was observed between the volunteers. The level of galangin-3-O-glucuronide was ~ 30% of the C_{max} value at 24 h post-ingestion. Moreover, there were two volunteers that exhibited their apparent maximum absorption of both galangin-7-O-sulphate and galangin-3-O-glucuronide at the 24 h time point. The average time to reach the maximum plasma concentration of galangin-7-O-sulphate and galangin-3-O-glucuronide was estimated to be at least 10 h post consumption.

Effect of flavonols on uptake of glucose by HepG2 cells

Treatment of HepG2 cells with [U-^{14}C]-glucose ([U-^{14}C]-G) resulted in rapid uptake of the radiolabel, which was increased signficantly by ~20% in the presence of 100 nM insulin (Figure 5b). The effect of flavonols on uptake of both [U-^{14}C]-G and 2-[U-^{14}C]-deoxy-glucose (2-[U-^{14}C]-DG) (Figure 5a) was tested, by pre-incubating the cells with flavonol(s) for 12 h and then assessing the uptake of radiolabelled sugars in the presence of freshly added flavonol(s). Cell viability in all cases was >90%. Treatment with quercetin alone decreased significantly the uptake of both [U-^{14}C]-G and 2-[U-^{14}C]-DG by ~10 and ~20% respectively. In contrast, treatment with kaempferol or galangin enhanced the uptake of [U-^{14}C]-G by ~30 and 70% respectively, while similarly decreasing 2-[U-^{14}C]-DG uptake by ~15%. Myricetin was the only flavonol found to increase both [U-^{14}C]-G and 2-[U-^{14}C]-DG by ~30 and ~10% respectively. Because of the contrasting effects of the flavonols alone at higher concentrations, we also tested their combinations at lower individual

concentrations. Quercetin and kaempferol together had very little effect effect on either [U-^{14}C]-G or 2-[U-^{14}C]-DG uptake, just a ~10% increase in the former. The addition of myricetin to the mixture did not affect the result on [U-^{14}C]-G but enhanced 2-[U-^{14}C]-DG uptake to similar levels as that of myricetin alone (~10%). Quercetin and galangin together produced a modest (~30%) increase in the uptake of [U-^{14}C]-G and a ~15% decrease in the uptake of 2-[U-^{14}C]-DG similar to that observed of galangin alone. Finally, a combination of quercetin, kaempferol and galangin led to a large almost 2-fold increase in [U-^{14}C]-G uptake and a ~10% increase in 2-[U-^{14}C]-DG uptake.

Effect of flavonols on transcription of glucose transporters in HepG2 cells

The effect of the different flavonols on the mRNA levels of Glut1 and Glut9 was explored individually at a 20 µM concentration or in a mixture of 5 µM each after 12 h treatments in medium without FBS (Figure 6). Quercetin and galangin significantly increased Glut1 mRNA expression to similar levels and comparable to the mixture of the four flavonols together (~2 fold) while kaempferol further enhanced Glut1 transcription (~2.7 fold). Myricetin had no effect on Glut1 expression levels but moderately decresed Glut9 (~0.8 fold) similar to galangin and kaempferol. Quercetin treatment was the only flavonol found to induce Glut9 mRNA expression (~1.3 fold) while the flavonol mixture treatment led to a significant downregulation of mRNA expression of Glut9 (~0.6 fold).

Discussion

Blood glucose levels are raised as part of the metabolic syndrome and in diabetes, and polyphenols may attenuate glucose levels by interacting with key metabolic enzymes and transporters. The overall effect is to modify glycemic responses and glucose distribution which could reduce the risk of developing diabetes [25]. It is important to link any biological effects to polyphenol bioavailability as far as possible. As a first step to dissecting the mechanism by which one of the classes of polyphenols, the flavonols, can modulate glucose metabolism, we conducted firstly a human intervention study. This showed, for the first time, that galangin is present in plasma after consumption of a flavonol-rich meal. We also confirmed the absence of myricetin and the presence of kaempferol and quercetin, and our study also allowed, for the first time, direct comparison between the different compounds in the same individuals. Although the extraction efficiency of synthesised myricetin aglycone and conjugates from plasma indicated good recovery, neither myricetin aglycone nor conjugates could be detected in plasma after consumption of the flavonol-rich experimental meal. The absence of myricetin in plasma is consistent with previous studies on healthy volunteers who consumed 1800 ml cranberry juice and detection carried out by GC-MS [26], or a polyphenol-rich juice drink [27]. The dose of myricetin used in our study was >4 times higher than in previous reports, however myricetin was still undetectable; we can therefore conclude that intact myricetin or glucuronide/sulphate conjugates are not present in the plasma when consumed in normal doses by humans, although conjugates of the methylated forms could still be present. Other forms of myricetin, such as microbiota-dehydroxylated forms, could be present, but these were not specifically looked for. This dehydroxylation could also convert myricetin to quercetin, which would then be "lost" in the quercetin pool. Kaempferol in pickled capers is present in the aglycone form as well as rutinoside and rhamnosyl-rutinoside conjugates [23]. In a study which provided ~ 27 mg kaempferol, mainly as the 3-glucuronide form from endive [12], kaempferol 3-O-glucuronide was detected in plasma. In agreement, we also found kaempferol-3-O-glucuronide as the predominant conjugate resulting from consumption of kaempferol, indicating that the circulating form is independent of the nature of the kaempferol glycoside found in the original matrix. There is very limited information on the absorption and metabolism of galangin in humans.

Although some galangin was present in plasma after consumption of propolis, no galangin conjugates were reported since the plasma samples were enzymatically deconjugated prior to detection [28]. Both galangin metabolites in our study showed a marked delay in absorption when compared to quercetin and kaempferol. Since the estimated T_{max} was much greater than the other flavonols, we have not attempted to do a formal pharmacokinetic calculation for the compounds. A further study on galangin should follow to fully estimate the pharmacokinetic parameters. No pharmacokinetic parameters could be calculated for myricetin for obvious reasons, while many reports on quercetin pharmacokinetics exist in literature. As a matter of fact, the steady state concentration of flavonols in the blood could exceed the single dose values reported here if the food or supplement is consumed at regular intervals. Using pharmacokinetic modelling, we have found that the concentration of one of the major plasma metabolites from chlorogenic acid was several-fold higher after 4 or 5 cups of coffee throughout the day than after a single dose (unpublished data).

The data from the human intervention study was used as guide for the selection of flavonols to use in the in vitro studies. It was not our intention at this stage toattempt to fully replicate the in vivo situation, since most of the compounds are present in vivo as conjugates, the timing of their appearance is somewhat different, and the concentrations are less than 1 μM. Further, the concentration of many flavonoids inside cells is higher than the cell culture medium owing to either active uptake of the conjugates [17] or passive diffusion followed by conjugation [22]. The goal at present was to initially test the concept that flavonols could influence glucose uptake and metabolism when present in mixtures in comparison to single compounds. In order to achieve measurable effects within a short time frame, we used 5 μM of each of the compounds as mixtures, although this strictly exceeds physiological levels. The effect of quercetin, kaempferol and galangin together (5 μM each) increased 2-fold the metabolism of glucose. In vivo, physiological concentrations reached could translate to a modest 10% change in glucose metabolism which, however, could be physiologically significant over a long period of time. The effect of mixtures of flavonols on glucose metabolism has not been reported before, and the experiments open the way for future studies to look at both the mechanisms involved and further effects of flavonol conjugates and mixtures. The in vitro studies were designed as a

structure-function relationship between the flavonols, and we therefore tested all four compounds. However, as we confirmed thatmyricetin is not absorbed, this compound is relevant only for structure-function information. Taking into account that monitoring the intracellular [^{14}C]-label after [U-^{14}C]-G glucose uptake represents both the transport into cells and also the rate of its subsequent metabolism we assessed both the effect of the flavonols on the uptake of [U-^{14}C]-G and 2-[U-^{14}C]-DG into HepG2 cells. Deoxyglucose is transported into cells, but does not undergo further metabolism such as glycolysis, and hence deoxyglucose uptake is directly indicative of the rate and extent of transport of glucose into cells. Glucose is transported into cells and then undergoes further metabolism especially to pyruvate, from where it can be either converted to lactate or can enter the TCA cycle. The effects on glucose uptake can be due to several factors including (1) competitive interactions at the active site of the sugar transporters, (2) short term modification of the transporter or function through indirect binding, phosphorylation, etc., (3) longer term effects resulting from changes in gene expression or (4) consumption of glucose for glycolysis and subsequent pathways.

Kinetically, quercetin is already known to inhibit GLUT1 in human erythrocytes with $IC_{50} < 10$ μM [29] and GLUT2 expressed in Xenopus oocytes with $IC_{50} \sim 20$-40 μM [30]. Reports from experiments with erythrocytes which abundantly express GLUT1 and also some other GLUTs have supported an inhibitory role for quercetin [31] on the transport of deoxy-glucose, linked to tyrosine kinase-inhibition. Flavonols could interact with the ATP binding sites of GLUT1 and competitively inhibit the transport activity of GLUT1 with or without any other involvement of cellular phosphorylation-dephosphorylation events. The transporter is thought to be sensitive to intracellular ATP levels, either indirectly via transporter-nucleotide dependent interactions with accessory proteins which require ATP, or via direct ATP-GLUT1 interactions. For other flavonoids and GLUT1-mediated glucose flux in human erythrocytes, it was shown that an increasing inhibiting potency was associated with the number of hydroxyl groups of the test compounds [32]. The OH-groups of the glucose molecule interact via hydrogen bonds with the side chains of polar amino acids (so called QLS sites) lining the wall of the aqueous channel of the facilitative GLUTs, and it has been proposed that flavonoids with OH-groups may

mimic the feature of glucose. In a different system, in isolated rat adipocytes, quercetin, myricetin and catechin-gallate were proposed to interfere with GLUT4 through a direct interaction with the QLS sites based on in silico data [33].

Flavonols have also been reported to produce some changes in cell signalling, in phosphorylation and gene expression in HepG2 and other cells in vitro, but for many experiments the concentration of flavonol used was much higher than described here. With the mixture of flavonols at 5 μM each, we obtained a very substantial 2-fold change in the rate of glucose metabolism. Many other studies have used 10-fold or higher concentrations of test compound: galangin induced autophagy in HepG2 cells, but 130 μM was required [34], and quercetin activated p38 and stimulated Nrf2 but only at 50 μM [35] and above.

The model chosen for the in vitro experiments is well established and very well characterised for glucose and energy metabolic properties. HepG2 cells have been shown to possess an intracellular storage pool for GLUT2, but lack the insulin-responsive glucose transporter translocation mechanism [36]. The fact that we could still evidence a small insulin driven increase in glucose transport (22%) in the HepG2 cells may be explained by the fact that GLUT1 can also undergo some insulin-dependent trafficking to the cell surface, a 2-fold compared to a 20-fold increase with GLUT4 [37].

We further investigated whether the acute treatment of HepG2 cells with the flavonols and the changes in glucose uptake and metabolism affected the transcription of GLUT1 and GLUT9 at the mRNA level after 12 h. This choice was made because GLUT1 was found to be the main isoform responsible for glucose entry into HepG2 cells and GLUT9, although expressed at low levels, still contributes to the flux [38]. Interestingly, GLUT9 is expressed exclusively in gluconeogenic tissues such as kidney and liver, expression is low in the small intestine while a GLUT9-splice variant is upregulated in diabetes. GLUT9 pancreatic expression is regulated by glucose but the overall physiological functions of this facilitative transporter as well as its transport properties remain unclear.

We found that 20 μM of galangin and quercetin produced the same upregulation on GLUT1 while kaempferol had an even greater effect leading to a 2.5 fold induction, while 5 μM of each of them in a mixture had no additive nor synergistic effect. GLUT9 gene expression was mildly downregulated by myricetin and kaempferol and further by the flavonol mixture. These changes only partially account for results found with the radiolabelled glucose assay, indicating that flavonol-induced changes in glucose metabolism are important and do not just stem from changes in transport. With the flavonol treatments, more glucose was found to be retained in the cells. Given the fact that this radiolabelled substrate may be further metabolised, and that with the assay we account only quantitatively for total radioactivity, we may assume that less glucose oxidation occured. This retardation of glucose breakdown could hypothetically lead to an increase of the oxidative capacity of the cells. Our results agree with [39] where it was reported that quercetin did not significantly affect glucose transport but decreased [5-^{3}H] removal by 33%, reflecting the unidirectional flow of free glucose into glycolysis. In those experiments, quercetin was infused in the range of 50-500 μM over 30 min in livers from fed rats, a state at which respiration takes place at the expense of endogenous fatty acids and glycogen catabolism is manifested by glucose release and production of pyruvate and lactate.

Quercetin has also been shown to inhibit glucose-6-phosphatase and increase glucose-6-phosphate levels intracellularly. Glucose, after entering into the cells, is converted to glucose-6-phosphate by hexokinase II which is highly expressed in HepG2 cells, and followed by subsequent catabolism in the glycolytic pathway [40]. In HepG2 cells the glycolytic pathway is commonly used for energy and precursor metabolite production for biosynthesis of nucleic acids, fatty acids and other macromolecules [38]. Under both high and low concentrations of glucose, glycolysis is one of the main sources of energy while glutaminolysis replenishes tricarboxylic acid cycle metabolites, and oxidative phosphorylation is low compared to primary hepatocytes. This alteration may be attributed to the high lipogenic character of HepG2 cells as they primarily metabolise glucose to lactate instead of CO_2, and this leads to a lower oxidative capacity. Clearly the flavonols could affect these processes, with galangin overall having the most significant effect on glucose metabolism and kaempferol on Glut1 mRNA induction.

In summary, we have shown that the flavonols galangin, kaempferol and quercetin, but not myricetin, are absorbed from food and appear in plasma mostly as conjugates rather than aglycones in healthy volunteers. A mixture of galangin, kaempferol and quercetin potently enhanced glucose metabolism in HepG2 cells. If this effect occurs in vivo, flavonols may stimulate glucose uptake into the liver where it is metabolised, consequently lowering the glucose concentration in the blood. We suggest as the next step to determine the effect and the mechanism of quercetin, galangin and kaempferol conjugates on glucose uptake and metabolism, but using a cellular system where the conjugate uptake transporters are expressed, to mimic the in vivo situation in tissues. These uptake transporters are known for quercetin conjugates [17] but remain to be discovered for galangin and kaempferol, and in addition, the galangin conjugates must first be synthesised as this is the first time reported in humans.

Acknowledgements

We gratefully acknowledge financial support received from the Ministry of Higher Education of Malaysia and Universiti Teknologi MARA for a PhD studentship to FJ, from Nestle Research Center, Lausanne for a PhD studentship to AK, and to the European Research Council for an Advanced Grant (POLYTRUE? 322467).

Reference List

1. Perez-Vizcaino, F., Duarte, J., Flavonols and cardiovascular disease. Mol. Aspects Med. 2010, 31, 478-494.
2. Suksomboon, N., Poolsup, N., Boonkaew, S., Suthisisang, C. C., Meta-analysis of the effect of herbal supplement on glycemic control in type 2 diabetes. J. Ethnopharmacol. 2011, 137, 1328-1333.
3. Hodgson, J. M., Croft, K. D., Tea flavonoids and cardiovascular health. Mol. Aspects Med., 2010, 31, 495-502.
4. Kay, C. D., Hooper, L., Kroon, P. A., Rimm, E. B., Cassidy, A., Relative impact of flavonoid composition, dose and structure on vascular function: A systematic review of randomised controlled trials of flavonoid-rich food products. Mol. Nutr. Food Res., 2012, 56, 1605-1616.
5. Neveu, V., Perez-Jimenez, J., Vos, F., Crespy, V. et al., Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. Database (Oxford), 2010, bap024.
6. Kobori, M., Masumoto, S., Akimoto, Y., Takahashi, Y., Dietary quercetin alleviates diabetic symptoms and reduces streptozotocin-induced

disturbance of hepatic gene expression in mice. Mol.Nutr.Food Res., 2009, 53, 859-868.

7. Sivakumar, A. S., Anuradha, C. V., Effect of galangin supplementation on oxidative damage and inflammatory changes in fructose-fed rat liver. Chem. Biol. Interact., 2011, 193, 141-148.

8. Kumar, S., Alagawadi, K. R., Anti-obesity effects of galangin, a pancreatic lipase inhibitor in cafeteria diet fed female rats. Pharm. Biol., 2013, 51, 607-613.

9. Ong, K. C., Khoo, H. E., Effects of myricetin on glycemia and glycogen metabolism in diabetic rats. Life Sci., 2000, 67, 1695-1705.

10. DuPont, M. S., Day, A. J., Bennett, R. N., Mellon, F. A., Kroon, P. A., Absorption of kaempferol from endive, a source of kaempferol-3-glucuronide, in humans. Eur. J. Clin. Nutr., 2004, 58, 947-954.

11. Wilkening, S., Stahl, F., Bader, A., Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metab. Dispos., 2003, 31, 1035-1042.

12. Slany, A., Haudek, V. J., Zwickl, H., Gundacker, N. C., et al., Cell characterization by proteome profiling applied to primary hepatocytes and hepatocyte cell lines Hep-G2 and Hep-3B. J. Proteome. Res., 2010, 9, 6-21.

13. Watkins, P.A., Ferrell, E.V. Jr, Pedersen, J.I., Hoefler, G. (1991). Peroxisomal fatty acid beta-oxidation in HepG2 cells Arch. Biochem. Biophys., 289, 2, 329-336.

14. Iyer, V.V., Yang, H., Ierapetritou, M.G., Roth, C.M. (2010). Effects of glucose and insulin on HepG2-C3A cell metabolism. Biotechnol. Bioeng. 107, 2, 347-356.

15. Takanaga, H., Chaudhuri, B., Frommer, W. B., GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. Biochim. Biophys. Acta, 2008, 1778, 1091-1099.

16. Nakajima, K., Yamauchi, K., Shigematsu, S., Ikeo, S., et al., Selective attenuation of metabolic branch of insulin receptor down-signaling by high glucose in a hepatoma cell line, HepG2 cells. J. Biol. Chem., 2000, 275, 20880-20886.

17. Wong, C. C., Akiyama,Y., Abe, T., Lippiat, J. D., et al., Carrier-mediated transport of quercetin conjugates: Involvement of organic anion transporters and organic anion transporting polypeptides. Biochem. Pharmacol., 2012, 84, 564-570.

18. Tao, L., Wang, Z. T., Zhu, E. Y., Lu, Y. H., Wei, D. Z. HPLC analysis of bioactive flavonoids from the rhizome of Alpinia officinarum. S. African J. Bot., 2006, 72, 163-166.

19. Gardana, C., Scaglianti, M., Pietta, P., Simonetti, P., Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography-tandem mass spectrometry. J. Pharm. Biomed. Anal., 2007, 45, 390-399.

20. Mullen, W., Edwards, C. A., Crozier, A., Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. Br. J. Nutr., 2006, 96, 107-116.

21. Guy, P. A., Renouf, M., Barron, D., Cavin, C., et al., Quantitative analysis of plasma caffeic and ferulic acid equivalents by liquid chromatography tandem mass spectrometry. J. Chromatogr. B, 2009, 877, 3965-3974.

22. Jailani, F., Williamson, G., Effect of edible oils on quercetin, kaempferol and galangin transport and conjugation in the intestinal Caco-2/HT29-MTX co-culture model. Food Funct., 2014, 5, 653-662.
23. Inocencio, C., Rivera, D., Alcaraz, F., Tomas-Barberan, F. A., Flavonoid content of commercial capers (Capparis spinosa, C-sicula and C-orientalis) produced in mediterranean countries. Eur. Food Res. Technol., 2000, 212, 70-74.
24. Borges, G., Degeneve, A., Mullen, W., Crozier, A., Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. J. Agric. Food Chem., 2010, 58, 3901-3909.
25. Williamson, G., Possible effects of dietary polyphenols on sugar absorption and digestion. Mol. Nutr. Food Res., 2013, 57, 48-57.
26. Zhang, K. Zuo, Y., GC-MS determination of flavonoids and phenolic and benzoic acids in human plasma after consumption of cranberry juice. J. Agric. Food Chem., 2004, 52, 222-227.
27. Borges, G., Mullen, W., Mullan, A., Lean, M. E., et al., Bioavailability of multiple components following acute ingestion of a polyphenol-rich juice drink. Mol. Nutr. Food Res., 2010, 54, S268-S277.
28. Gardana, C., Simonetti, P., Berti, C., and Pietta, P., Evaluation of propolis polyphenols absorption in humans by liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom., 2007, 21, 3849-3854.
29. Cunningham, P., Afzal-Ahmed,I., Naftalin, R. J., Docking studies show that D-glucose and quercetin slide through the transporter GLUT1. J. Biol. Chem., 2006, 281, 5797-5803.
30. Kwon, O., Eck, P., Chen, S., Corpe, C. P., et al., Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. FASEB J., 2007, 21, 366-377.
31. Vera, J. C., Reyes, A. M., Velasquez, F. V., Rivas, C. I., et al., Direct inhibition of the hexose transporter GLUT1 by tyrosine kinase inhibitors. Biochemistry, 2001, 40, 777-790.
32. Martin, H. J., Kornmann, F., Fuhrmann, G. F., The inhibitory effects of flavonoids and antiestrogens on the Glut1 glucose transporter in human erythrocytes. Chem. Biol. Interact., 2003, 146, 225-235.
33. Strobel, P., Allard, C., Perez-Acle, T., Calderon, R., et al., Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes. Biochem. J., 2005, 386, 471-478.
34. Wen, M., Wu, J., Luo, H., Zhang, H., Galangin induces autophagy through upregulation of p53 in HepG2 cells. Pharmacol., 2012, 89, 247-255.
35. Granado-Serrano, A. B., Martin, M. A., Bravo, L., Goya, L., Ramos, S., Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38. Chem. Biol. Interact., 2012, 195, 154-164.
36. Hah, J., Jo, I., Chakrabarti, R., Jung, C. Y., Demonstration of an insulin-insensitive storage pool of glucose transporters in rat hepatocytes and HepG2 cells. J. Cell Physiol., 1992, 152, 56-63.
37. Claussnitzer, M., Skurk, T., Hauner, H., Daniel, H., Rist, M. J. (2011) Effect of flavonoids on basal and insulin-stimulated 2-deoxyglucose uptake in adipocytes. Mol. Nutr. Food Res., 55, S26-S34.
38. Mathupala, S. P., Colen, C. B., Parajuli, P., Sloan, A. E., Lactate and malignant tumors: a therapeutic target at the end stage of glycolysis. J. Bioenerg. Biomembr., 2007, 39, 73-77.

39. Gasparin, F. R., Spitzner, F. L., Ishii-Iwamoto, E. L., Bracht, A., Constantin, J., Actions of quercetin on gluconeogenesis and glycolysis in rat liver. Xenobiotica, 2003, 33, 903-911.
40. Mathupala, S. P., Ko, Y. H., Pedersen, P. L., Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene, 2006, 25, 4777-4786.

Figure legends

Figure 1: HLPC-MS-MS chromatograms of plasma.

Chromatograms obtained by MRM of flavonol metabolites identified in the plasma of volunteer 2, collected at the times indicated, after flavonol-rich meal consumption, are shown. IS1: internal standard 1 (m/z 223 = sinapic acid), IS2: internal standard 2 (m/z 253 = daidzein), m/z 349 for galangin sulphate, m/z 365 for kaempferol sulphate, m/z 381 for quercetin-3'-O-sulphate, m/z 445 for galangin-3-O-glucuronide, m/z 461 for kaempferol-3-O-glucuronide, m/z 477 for quercetin-3-O-glucuronide and m/z 491 for isorhamnetin-3-O-glucuronide.

Figure 2: Kaemperfol conjugates in plasma.

Plasma mean kaempferol-7-O-sulphate (A) and kaempferol-3-O-glucuronide (B) concentrations after consumption of the flavonol-rich meal. Error bars are SE, n = 8.

Figure 3: Galangin sulphate conjugates in plasma.

Plasma galangin-7-O-sulphate concentration profile of individual subjects (A) and average (B) (error bars are SE, n = 8) after consumption of flavonol-rich meal.

Figure 4: Galangin glucuronide conjugates in plasma.

Plasma galangin-3-O-glucuronide concentration profile of individual subjects (A) and average (B) (error bars are SE, n = 8) after consumption of flavonol-rich meal.

Figure 5: Uptake of 2-[U-^{14}C]-DG (A) and [U-^{14}C]-G (B) into HepG2 cells.

Cells were treated as indicated for 12 h. Fresh assay medium was added together with the [^{14}C]-labelled sugar and indicated compound, and incubated for 15 min. Concentrations of flavonols were 20 µM when assessed individually, while in mixtures it was 5 µM of each; insulin when added was 100 nM. Values shown are means of six biological replicates per treatment carried out on the same day and expressed as percent of control (CTRL) which was DMSO alone. CTRL represents average of DMSO from five experiments with six replicates per experiment (n=30). For deoxy-glucose uptake, CTRL was 0.25± 0.07 nmol deoxy-glucose/ mg of protein/ min and for glucose uptake 0.08± 0.02 glucose/ mg of protein/ min. Error

bars represent SD (n=6) and different symbols represent statistically significant different groups ($p < 0.05$). Q quercetin, K kaempferol, G galangin, M myricetin

Figure 6. mRNA levels of Glut1(A) and Glut9 (B) in HepG2 cells.

Cells were treated as indicated for 12 h with individual flavonols (20 µM) or a combination of all 4 flavonols at 5 µM each. Values shown are means of three biological replicates per treatment pooled and analysed by RT-qPCR (n=4). Error bars represent SD.

Table 1: Flavonol content of foods used in the intervention study

Food/extracts	Flavonol	Enzyme hydrolysed	Non-hydrolysed	Total content per portion
		mg aglycone equivalents/100 g		mg
Blackcurrant	Myricetin	48.3 ± 6.8	3.0 ± 1.2	24.1
	Quercetin	13.1 ± 1.6	1.1 ± 0.3	6.5
Pickled capers	Quercetin	42.1 ± 2.4	19.2 ± 5.3	21.0
	Kaempferol	41.3 ± 3.7	24.3 ± 4.1	20.6
Propolis	Galangin	671.0 ± 5.4	268.9 ± 3.1	13.5

Samples were incubated in acetate buffer containing cellulase (blackcurrant for myricetin and propolis) at 37°C pH 5, or hesperidinase (all others) at 40°C pH 3.8 for 18 h. Sample analysis was performed by HPLC-DAD, with compounds confirmed by retention time and UV spectra compared to aglycones. Values are mean ± SD (n=4). A portion comprised 50 g pickled capers and 50 g of freeze-dried blackcurrants with two tablets of a commercially available propolis supplement.

Table 2: Pharmacokinetic parameters of flavonols metabolite in the plasma of healthy volunteers after ingestion of the flavonol-rich meal

Flavonol metabolites	C_{max}	T_{max}
	(nM)	(h)
Quercetin-3'-O-sulphate	62 ± 16	1.9 ± 0.4
Isorhamnetin-3-O-glucuronide	28 ± 10	4.3 ± 0.7
Kaempferol-7-O -sulphate	20 ± 7	2.3 ± 0.3
Kaempferol-3-O-glucuronide	360 ± 83	3.0 ± 0.5
Galangin-7-O sulphate	120 ± 42[a]	>8
Galangin-3-O-glucuronide	119 ± 25 [a]	>8

Values are mean ± SE, n = 8.

[a]Estimated as the maximum was not reached for all volunteers.

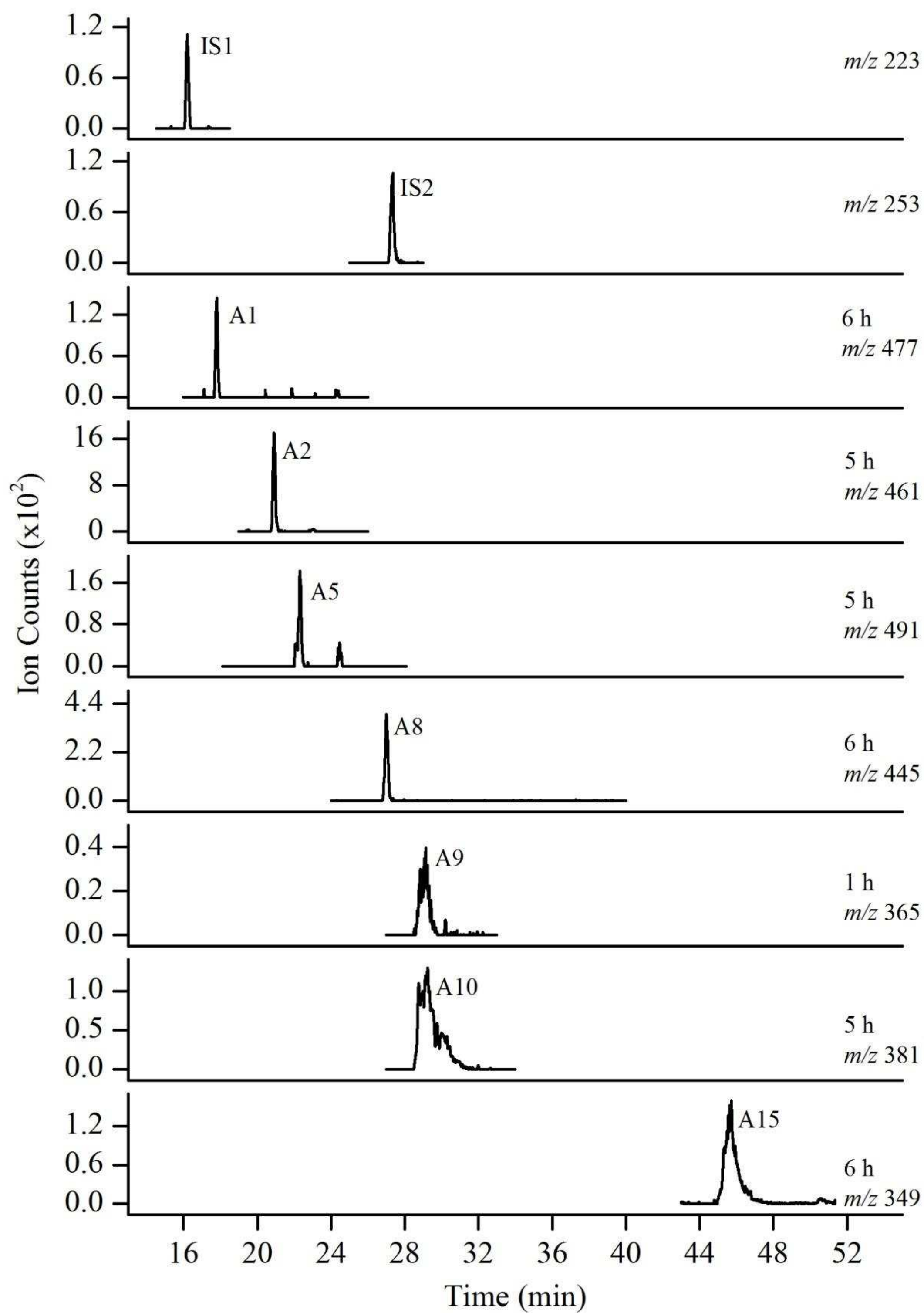


Figure 1

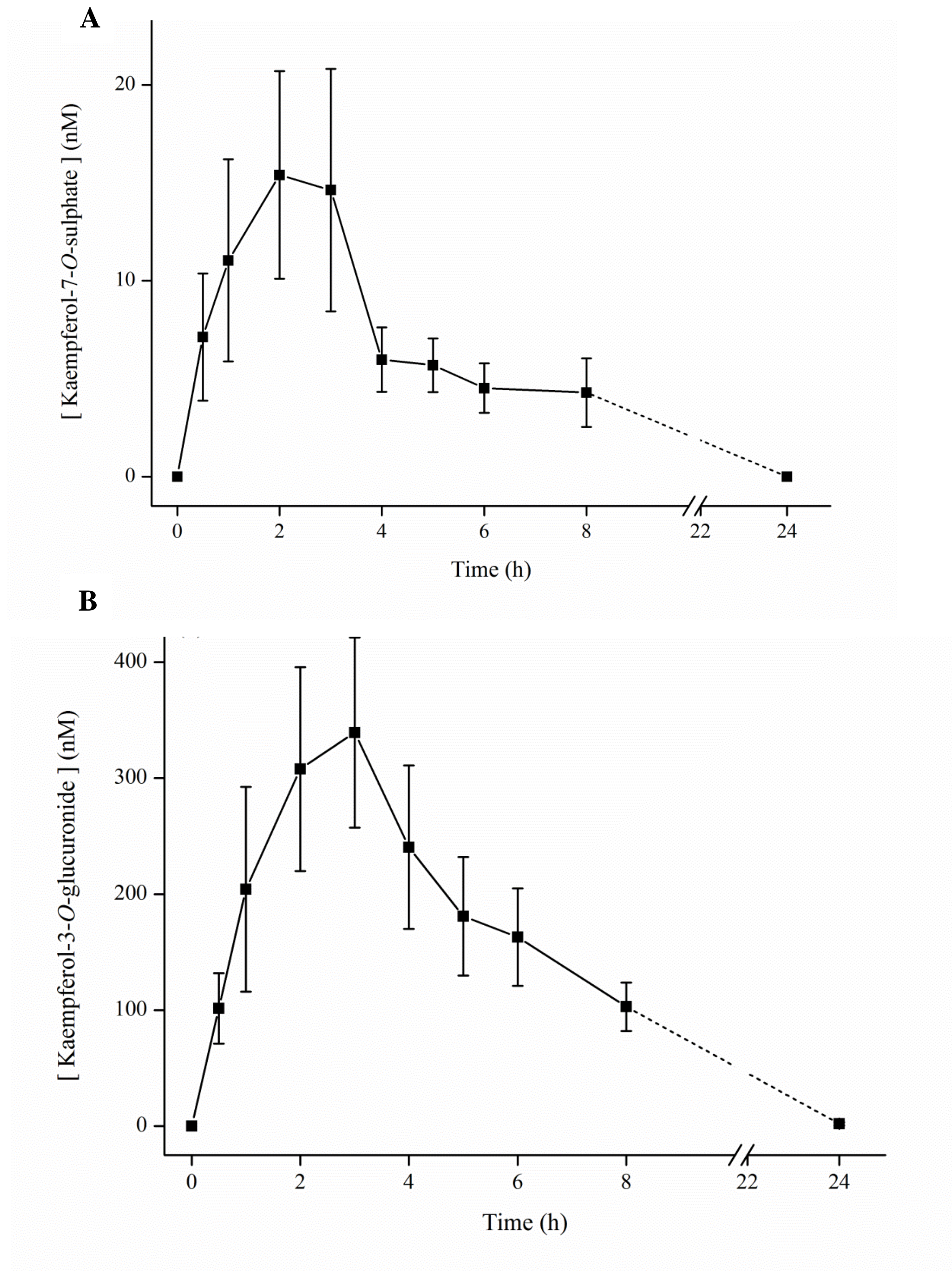


Figure 2

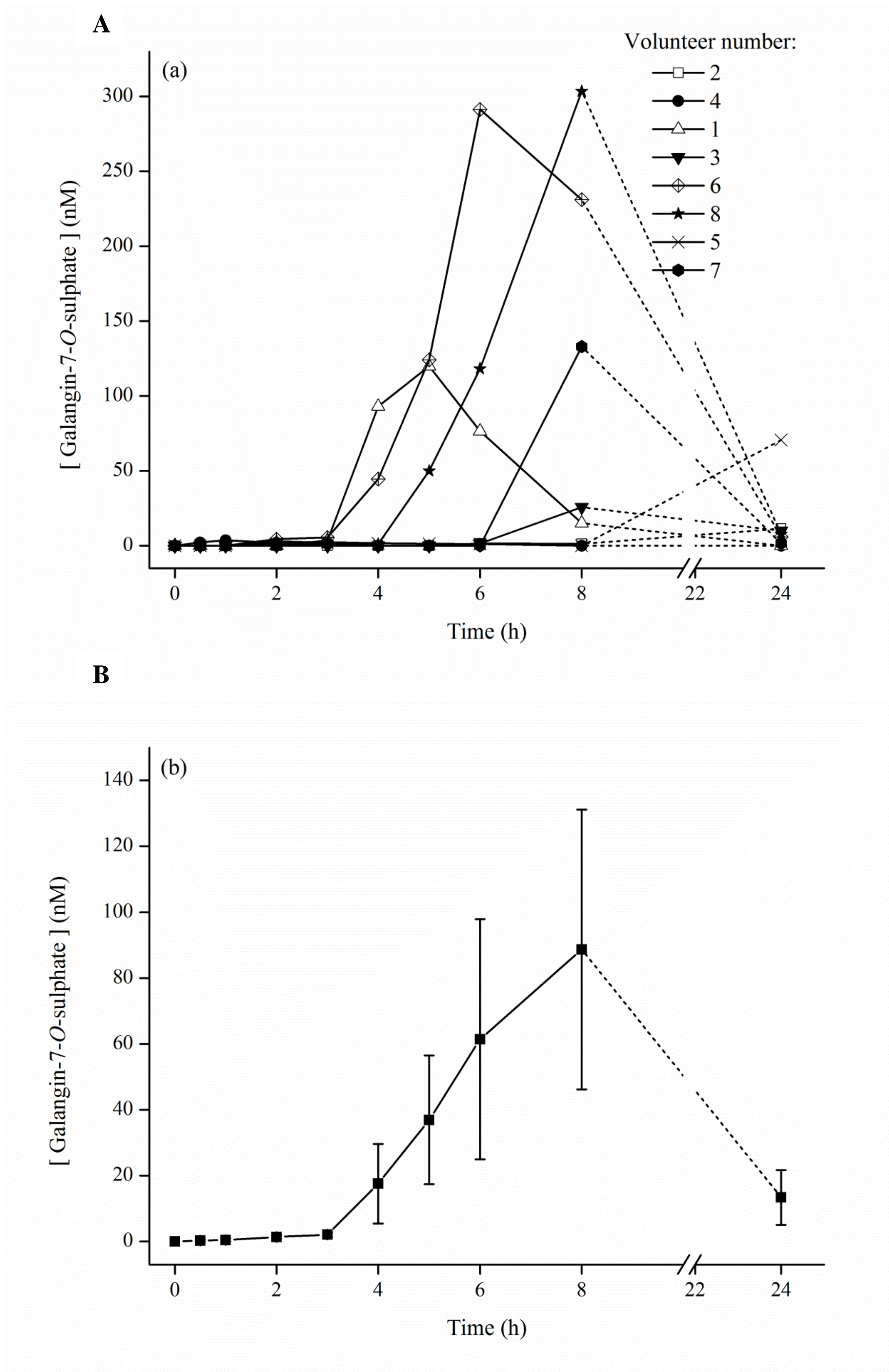


Figure 3

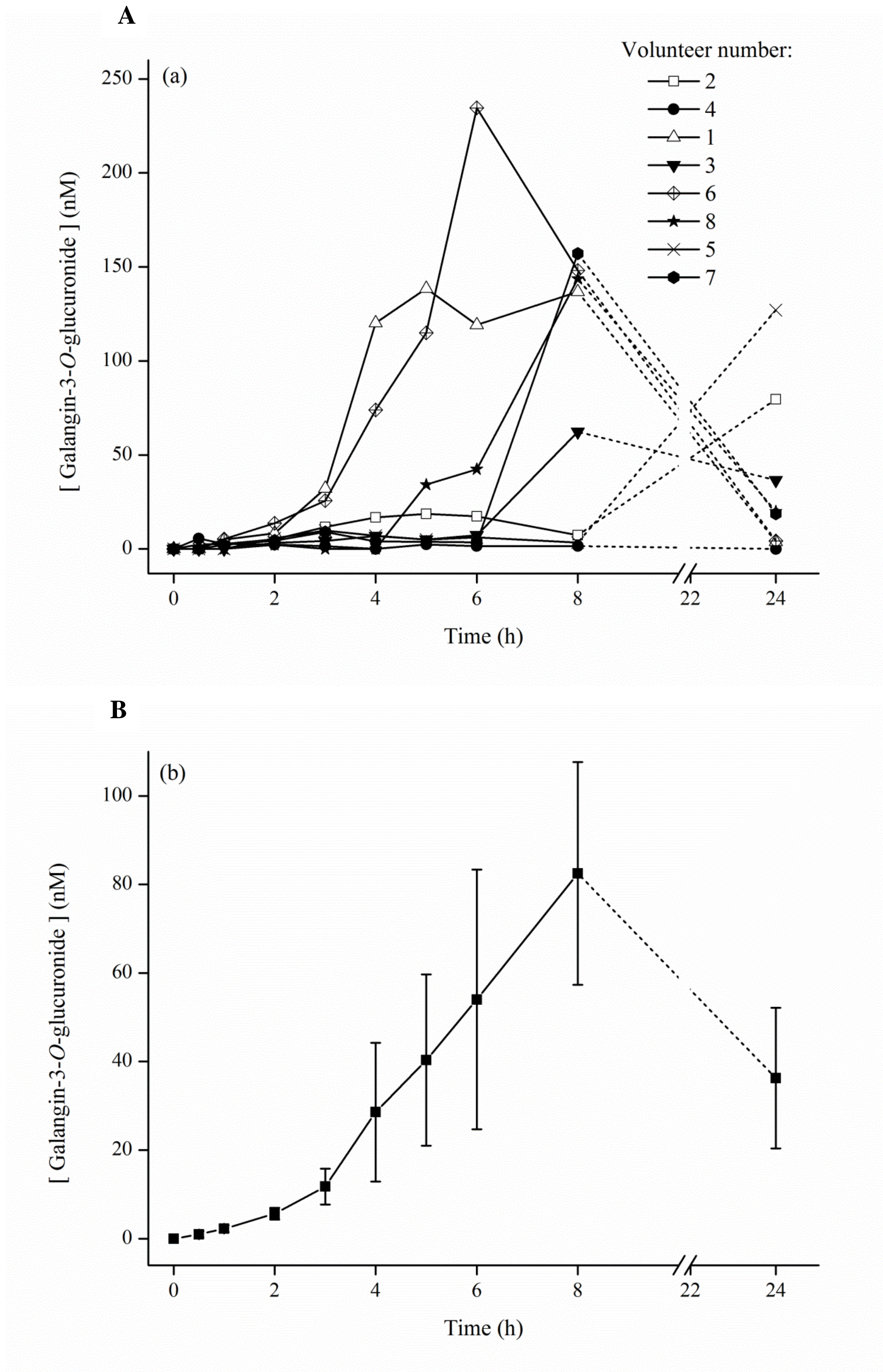
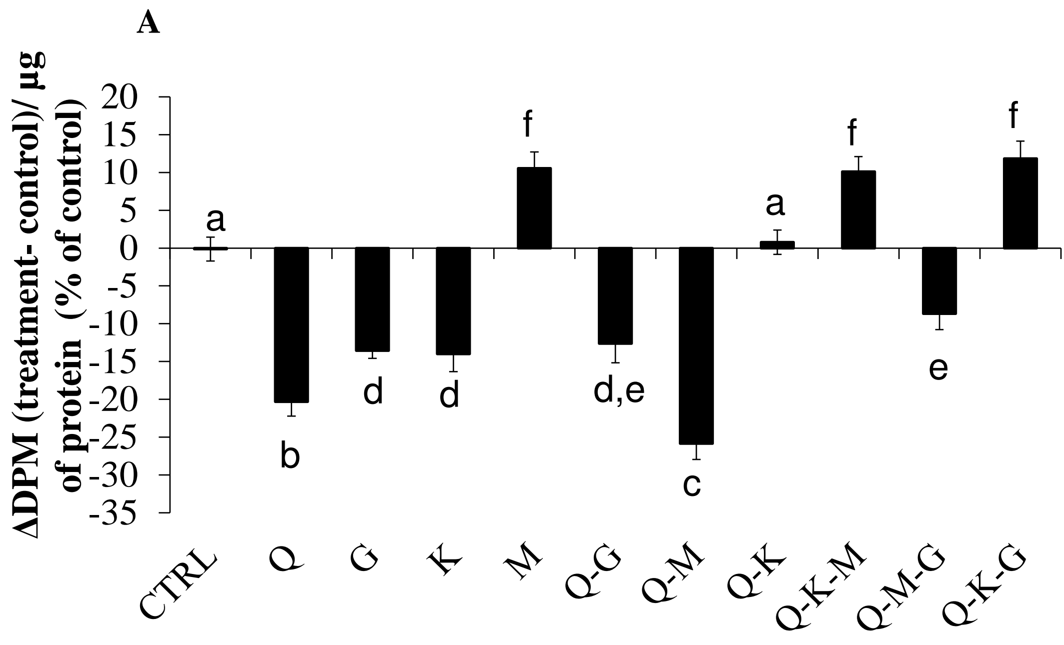


Figure 4



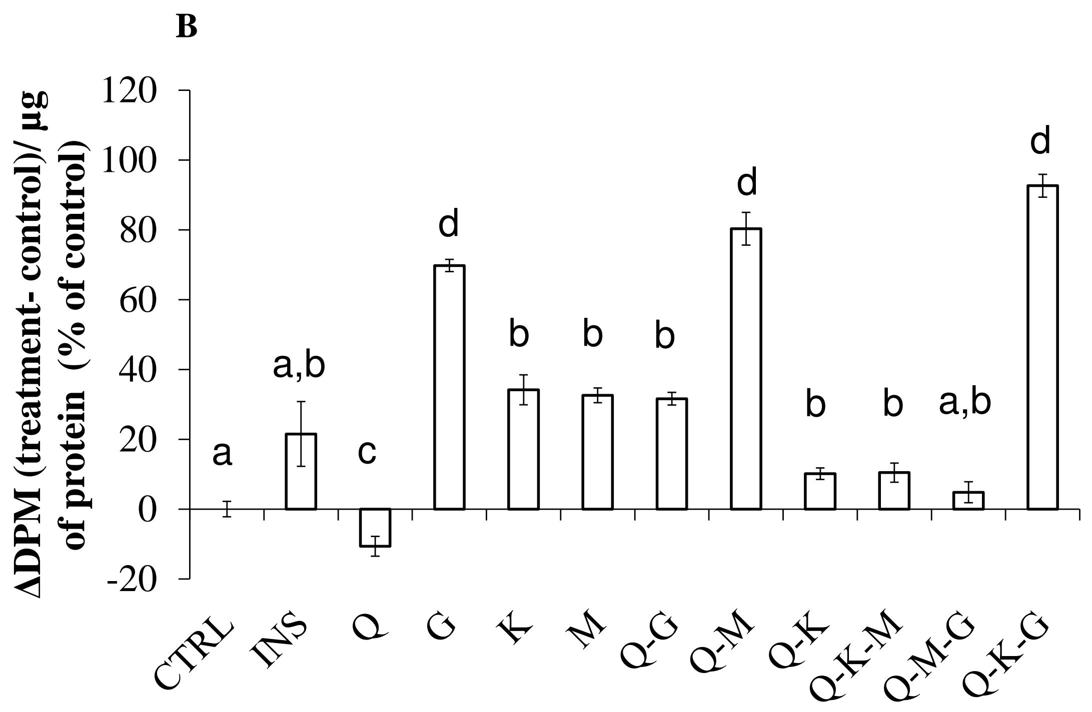


Figure 5

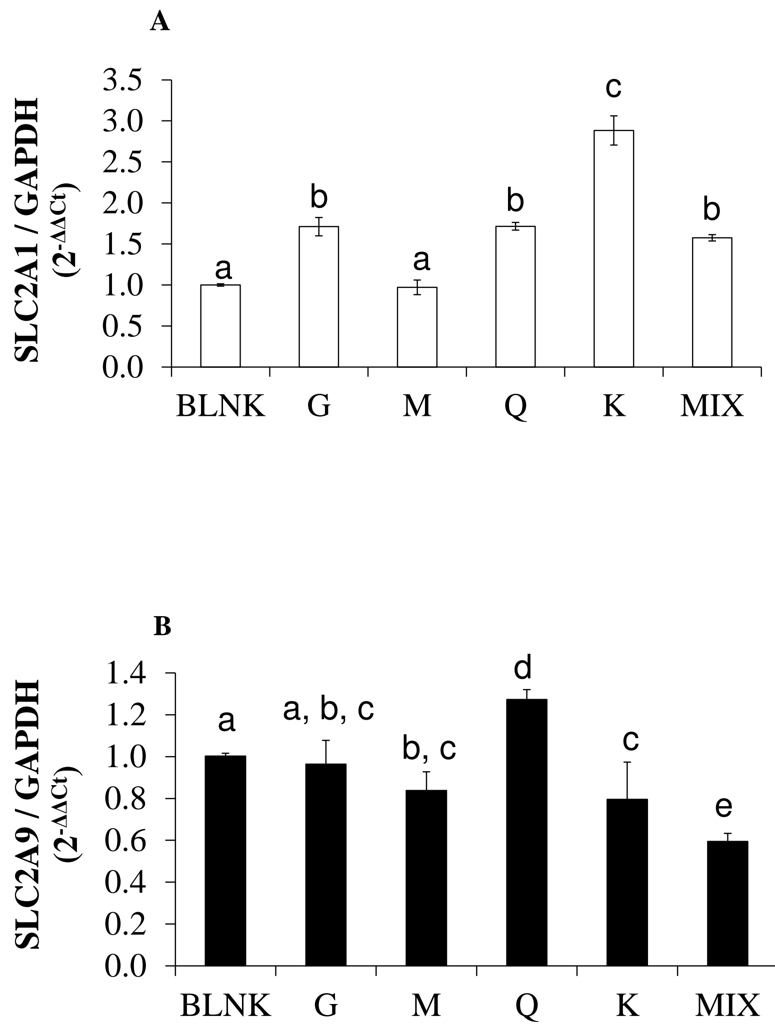


Figure 6

Table 1, supplementary information: Recovery values after spiking flavonol metabolites into blank plasma samples

Flavonol metabolites	Spiking concentration			
	50 nM	100 nM	500 nM	2500 nM
	% Recovery			
Myricetin	n.d.	115 ± 18	109 ± 19	108 ± 19
Quercetin	n.d.	109 ± 7	99 ± 5	98 ± 4
Quercetin-7-O-glucuronide	93 ± 12	79 ± 11	86 ± 14	90 ± 15
Quercetin-3-O-glucuronide	82 ± 7	85 ± 9	91 ± 13	90 ± 8
Quercetin-3'-O-glucuronide	81 ± 9	79 ± 8	74 ± 7	76 ± 4
Quercetin-3'-O-sulphate	85 ± 12	88 ± 11	83 ± 11	88 ± 5
Isorhamnetin	n.d.	108 ± 2	100 ± 4	100 ± 2
Isorhamnetin-3-O-glucuronide	82 ± 13	85 ± 14	78 ± 9	79 ± 4
Kaempferol	n.d.	104 ± 16	94 ± 9	99 ± 10
Kaempferol-3-O-glucuronide	79 ± 5	74 ± 11	74 ± 10	75 ± 7
Galangin	n.d.	74 ± 11	77 ± 4	80 ± 11
Daidzein (IS)	99 ± 3	101 ± 5	110 ± 6	107 ± 5

Concentration of the internal standard (IS) was 2 μM for all spiking samples. Values are mean ± SD (n=3). n.d. not determined