Effects of Quercetin on Uric Acid Metabolism

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Chapter 3 contains work which has also been used in the publication

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Chapter 4 contains work which has also been used in the publication

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Chapter 5 contains work which has also been used in the publication

Shi, Y., Fisher, J. and Williamson, G., Supplementation of quercetin showed distinct metabolic profile of plasma in healthy males as assessed by a ^{1}H NMR-based metabolomic approach: a randomised, double-blinded, placebo-controlled, cross-over trial (manuscript in preparation)

Chapter 6 contains work which has also been used in the publication

Shi, Y. and Williamson, G., Inhibition of enzymes involved in uric acid production/purine degradation by quercetin and its metabolites (manuscript in preparation)

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Abstract

Background and Objective: High blood uric acid (hyperuricemia) is a common phenomenon in populations with hypertension, hyperglycemia, obesity and/or dyslipidemia. This study was to investigate the effects of quercetin supplementation on blood uric acid level and the biochemical mechanism behind it.

Methods: A pilot trial confirmed the delivery of quercetin from a supplement tablet in healthy males (n=6). Randomised, double-blind, cross-over, placebo-controlled 4-week dietary intervention trial with the same supplement tablet containing 500 mg quercetin d^{-1} was conducted in selected healthy males (n=22, with higher blood uric acid but within normal range). Changes of uric acid and glucose were analysed in fasting blood plasma at 0, 2 and 4 weeks. Plasma metabolomics were profiled by 1H-NMR. Where quercetin and its metabolites may affect in the pathway of uric acid metabolism was investigated *in vitro* and *ex vivo*.

Results: At the end of the 4-week trial, plasma uric acid levels were significantly reduced (mean change -26.5 µM, 95% CI -7.6 to -45.5, P = 0.008, n=22), as were diastolic blood pressures in normotensive subjects (-3.1 mm Hg, -0.5 to -5.8, P = 0.048, n=10). Paired plasma ^{1}H-NMR spectrum showed lowered glutamine (P = 0.008), acetoacetate (P = 0.005) and lactate (P = 0.03) after quercetin treatment. A dose-dependent inhibition of quercetin, quercetin-3'-*O*-sulfate and 3,4-dihydroxyphenylacetic acid on xanthine oxidase *in vitro* and a mild inhibitory effect of quercetin on plasma adenosine deaminase was found.

Conclusions: Quercetin supplementation can maintain blood uric acid level and blood pressure within a low-risk range. It is probably a result of regulated purine metabolism by quercetin, its microbial derivatives and their metabolites.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

%	Percent
$^{\bullet}NO$	Nitric oxide
$^{\bullet}O_2^-$	Superoxide
^{1}H-NMR	Proton nuclear magnetic resonance
ADA	Adenosine deaminase
AUC	Area under curve
BMI	Body mass index
℃	Degree celsius
CI	Confidence interval
Da	Dalton
DAD	Diode array detector
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FID	Free induction decay
FRAP	Ferric reducing ability of plasma
g	Gram
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
kg	Kilogram
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ratio
mAU	Milli absorbance unit
M	Mol/L

mg	Milligram
mL	Millilitre
MS	Mass spectrometry
MWCO	Molecular weight cut off
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PCA	Principle component analysis
PLS, OPLS	Partial least-squares, orthogonal PLS
PNP	Purine nucleoside phosphorylase
RNA, mRNA	Ribonucleic acid, messenger rna
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of mean
SIMCA	Soft independent modeling of class analogy
TAG	Triacylglycerol
UV	Ultra violet
v/v	Volume/volume
XD	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase
µL	Microlitre
µM	Micromolar
µmol	Micromol

Chapter 1. Introduction

1.1 Hyperuricemia and metabolic syndromes

1.1.1 Epidemiology of hyperuricemia

Abnormally high blood uric acid (hyperuricemia) is the most determinant risk factor for gout, an inflammatory arthritis caused by uric acid crystals (1). Patients who develop diabetes (2), obesity (3), hyperglycemia (4, 5), hypertension (6), stroke (7), heart disease (8, 9), kidney dysfunction (10, 11) and cancer (12), often display hyperuricemia, although this often is not noticed until their first, if any, gout attack. Gout prevalence increased from ~0.5 to ~3% between 1960 and 2010 in the US (13) and other areas (14) accompanied by a parallel increase in the number of individuals with hyperuricemia (15, 16). The prevalence of gout is much higher in men than in women and rises with age (17). Data show a rise in the prevalence of gout that is potentially attributable to shifts in diet and lifestyle, improved medical care, and increased longevity (18). Interestingly, allopurinol, a uric acid lowering agent in gout therapy, also has a protective effect on hypertension (19), which suggests that excess uric acid synthesis is associated with hypertension (20, 21). The fact that 25-34 is the age group with highest blood uric acid level (22) may suggest that hyperuricemia precedes the development of metabolic syndromes (23).

1.1.2 Epidemiology of metabolic syndromes

Metabolic syndrome consists of dyslipidemia (i.e., high triglycerides and lipoproteins containing apolipoprotein B and low high-density lipoproteins), hypertension,

hyperglycemia, hyperinsulinemia and obesity (24). The prevalence of metabolic syndrome in patients with gout has been reported to be as high as 62% (25). Results of animal studies suggest that hyperuricemia might play a part in development of metabolic syndrome (26). Hyperuricemia often precedes development of diabetes (27), obesity (28) hyperinsulinemia (20). Elevated blood uric acid has been shown to be an independent predictor of mortality in high-risk groups including populations with hypertension (29, 30) and coronary heart disease (31), stroke (32), diabetes (33), heart failure (34) and renal hypertension (35). A meta-analysis review showed that high blood uric acid levels increase all-cause mortality in patients with heart failure, and that this increase in risk seems to start at an uric acid level of 420 μM (36).

Growing number of experimental and clinical evidences proposed uric acid as having a causal role in hypertension (37). Hypertension is commonly associated with hyperuricemia (38). For example, hyperuricemia has been reported in nearly 90% of children with newly diagnosed untreated hypertension, and uric acid concentrations were directly related to systolic and diastolic blood pressure in these patients (39). Normotensive men with hyperuricemia but not metabolic syndrome showed a higher risk of hypertension than those with a uric acid concentration within the normal range (23). Men with gout have a two-fold higher risk of kidney stones than do patients without gout (40). Cardiovascular diseases are the greatest threat for patients with gout. Evidence from prospective and interventional studies suggests that hyperuricemia is an independent risk factor for cardiovascular diseases (41). However, an elevation of uric acid in hypertension could be a consequence of reduced renal function, the use of diuretics, the presence of hyperinsulinemia and oxidative stress, or elevated renal vascular resistance, which are commonly present in

this condition (42). As such, hyperuricemia is not considered a true metabolic syndrome.

1.1.3 Pathophysiology of uric acid metabolism

The human diet contains little uric acid. It is produced in the liver and to a lesser extent in the small intestine from the metabolism of purines. Purines come exogenously from specific foods and endogenously from cellular metabolism. Uric acid is a weak acid with pKa of 5.75. At a physiological pH of 7.4 in the extracellular compartment, 98% of uric acid is present as monosodium urate, with a low solubility limit of about 380 µM (43). Humans and higher primates do not have the enzyme uricase that degrades uric acid to the highly soluble allantoin. Consequently, the physiological concentration of urate in blood is close to its limit of solubility. When urate concentration exceed 380 µM, risk of monosodium urate crystal formation and precipitation increase (1, 44). In urine, which is acidified along the renal tubule, urinary urate is converted to low solubility uric acid. Therefore, humans are particularly susceptible to changes induced by diet which parallels hyperuricemia, causing gout, arthritis, and/or kidney stone (45). Uric acid level in blood depends on the balance between purine ingestion, synthesis in cells recycling and the degradation function of xanthine oxidase at the end of the purine pathway (Figure 1-1).

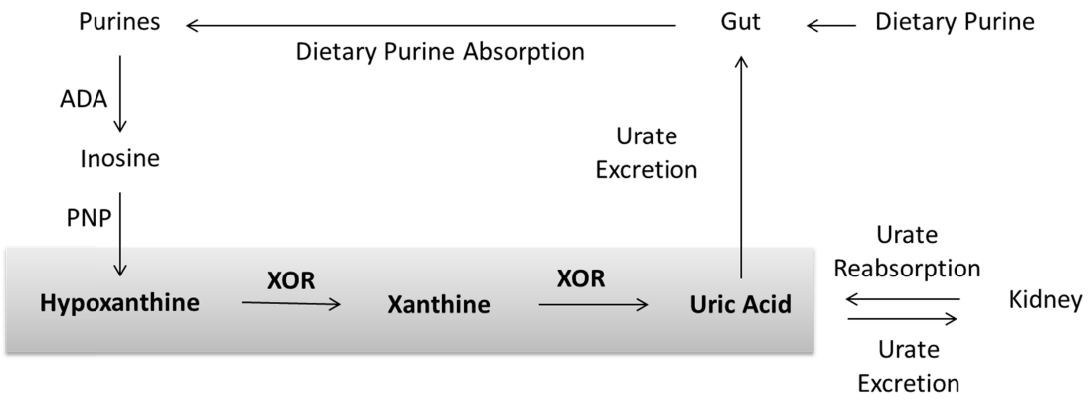


Figure 1-1 Metabolism pathway and excretion of uric acid.

Purines are absorbed from the diet through the gut, synthesised in the body and derived from the degradation of endogenous DNA and RNA. They are further oxidised by xanthine oxidoreductase (XOR). Uric acid is the major end product of purine metabolism in humans. The majority of uric acid is excreted by the kidney and the rest in feces, but a substantial amount of uric acid is reabsorbed at the proximal tubule of the kidney. Figure is adapted from (46).

The major pathways of purine catabolism in humans are outlined in Figure 1-2. The various nucleotides are first converted to nucleosides by intracellular nucleotidases. These nucleotidases are under strict metabolic regulation so that their substrates, which act as intermediates in many vital processes, are not depleted below critical levels. Nucleosides are then degraded by the enzyme purine nucleoside phosphorylase (PNP) to release the purine base and α-D-deoxyribose-1-phosphate. Note that adenosine is not a substrate for PNP. Instead, it is first converted to inosine by ADA (47). The PNP products are merged into xanthine by guanine deaminase and XOR, and xanthine is then oxidised to uric acid by this latter enzyme.

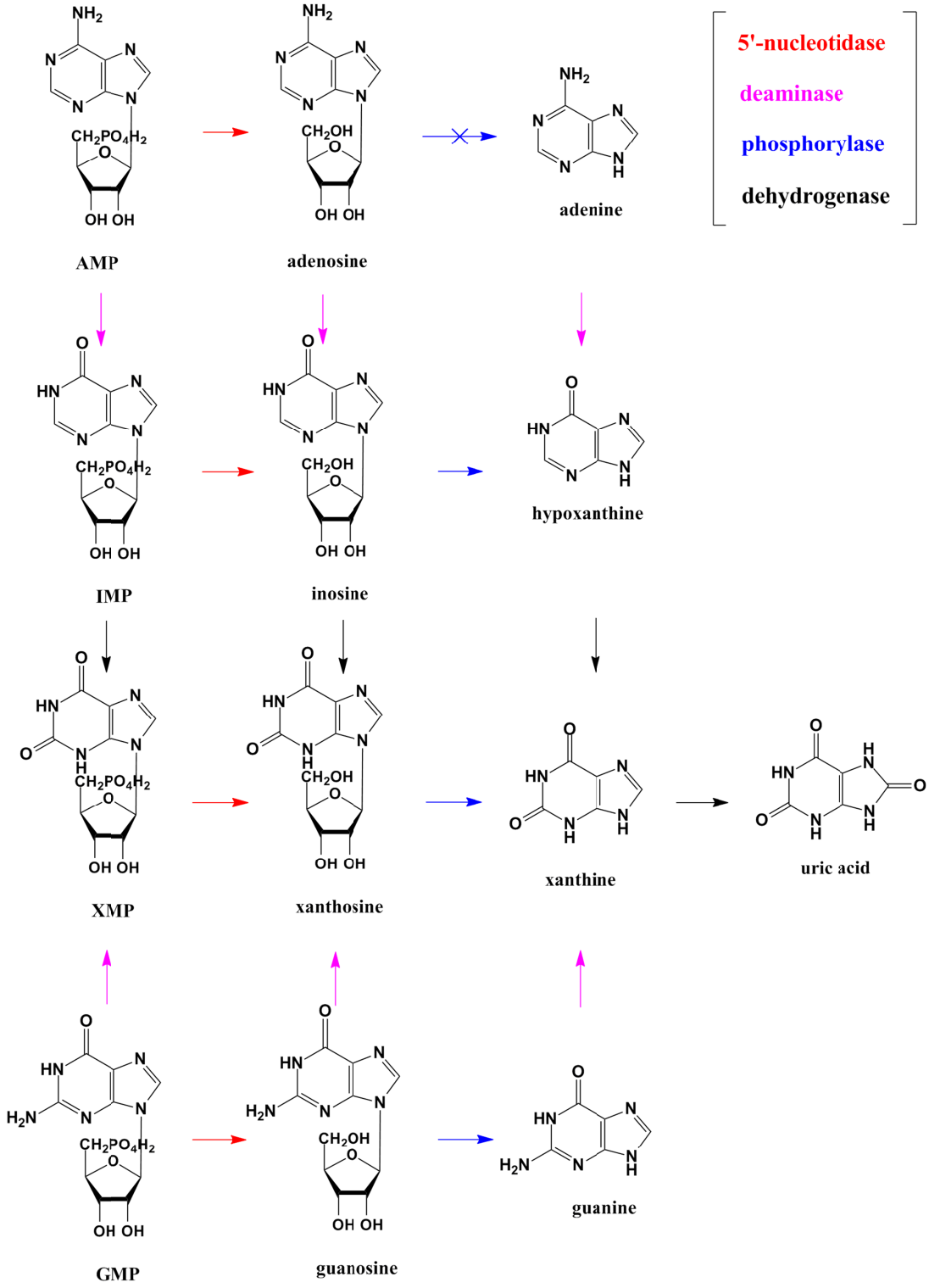


Figure 1-2 The major pathways of purine catabolism that lead to uric acid.

Adenosine monophosphate (AMP), inosine monophosphate (IMP), xanthine monophosphate (XMP) and guanosine monophosphate (GMP) are catabolised by 5′-nucleodases, deaminases (e.g. adenosine deaminase, ADA), phosphorylase (e.g.

purine nucleoside phosphorylase, PNP) and dehydrogenase (xanthine oxidoreductase, XOR or IMP dehydrogenase), end up to uric acid. Figure is personally drawn by Yuanlu Shi.

Some dietary factors, including purines, alcohol and fructose, were critical in elevating blood uric acid level (48-51). Persons with gout may benefit from limiting dietary purines. Serum uric acid levels are increased with increasing total meat or seafood intake and decrease with increasing dairy intake. In an observational study of 47 150 men, the risk for gout increased by 21% for each additional portion of meat (such as beef, pork, lamb, broth, gravy, sweetbreads, liver and kidney) per day and by 7% for each additional portion of seafood (such as shrimp, anchovies, mussels, scallops, sardines, herring, fish roe, canned tuna, shrimp and lobster) per week (17). It is important to note that moderate consumption of purine-rich food, such as mushrooms, was not associated with an increased risk for gout (52). Furthermore, the incidence of gout reversely associates to intake of dairy products. Protein from dairy products does not seem to carry the same risk as protein from meat or fish sources (52). In comparison to men who did not drink alcohol, the multivariate relative risk of gout increased from 1.25 (95% CI, 0.95 to 1.64) for alcohol consumption of 5 to 9.9 g per day to 2.53 (95% CI, 1.73 to 3.70) for 50 g per day or more ($P<0.001$). This risk varied substantially according to the type of alcoholic beverage: beer conferred a larger risk than liquor, whereas moderate wine drinking did not increase the risk (53).

Chronic exposure to fructose can lead to development of hyperuricemia (54). Fructose phosphorylation by fructokinase causes intracellular phosphate depletion leading to the activation of AMP deaminase (AMPD1), which converts the adenosine monophosphate to inosine monophosphate. As illustrated by Figure 1-3, fructose

enters hepatocytes and other cells (including tubular cells, adipocytes, and intestinal epithelial cells), where it is completely metabolised by fructokinase with the consumption of ATP; unlike in glucose metabolism, there is no negative regulatory mechanism to prevent the depletion of ATP. Rapid conversion of fructose to fructose 1-phosphate catalysed by fructokinase results in decreased ATP and inorganic phosphate (Pi) levels. Intracellular Pi has a regulatory effect on urate production in the liver by inhibiting AMP deaminase. In addition, ATP levels have a regulatory effect on 5′-nucleotidase. Thus, fructose induces acute depletion of ATP and Pi and causes increased activity of the enzymes involved in the degradation of purine nucleotides to urate. This leads to the activation of AMP deaminase, which converts the AMP to IMP, inosine and eventually uric acid (55).

As a consequence, uric acid is generated in the process, and uric acid concentrations may rise by 60-240 μM after the ingestion of a large fructose-based meal (56). Chronic hyperuricemia may reversely up-regulate fructokinase expression thus leading to the amplification of the lipogenic effects of fructose in human hepatocytes (55). Cohort studies found that fructose-sweetened beverages have a notable effect on increased levels of BMI and serum uric acid level in adolescents (49) and adults (50). Results from intervention studies are in accordance with this finding. An acute hyperuricemic response was observed ($P<0.01$) after a 64 g fructose load in healthy humans (48). It was widely believed that intake of polyphenol rich beverages can increase antioxidant capacity in plasma (57, 58), but was later proven to be the effect of fructose on plasma uric acid production (59, 60) since uric acid behaves like antioxidant in these assays. The concentration of plasma uric acid at the end of a 1 week treatment of ~213 g d^{-1} (26% energy in take) of fructose is significantly higher than that of control group in healthy young males ($P<0.05$) (61). However this effect

can only be confirmed in long-term, high-dose fructose treated intervention trials (62) while others fail to prove the relationship between fructose and gout (63).

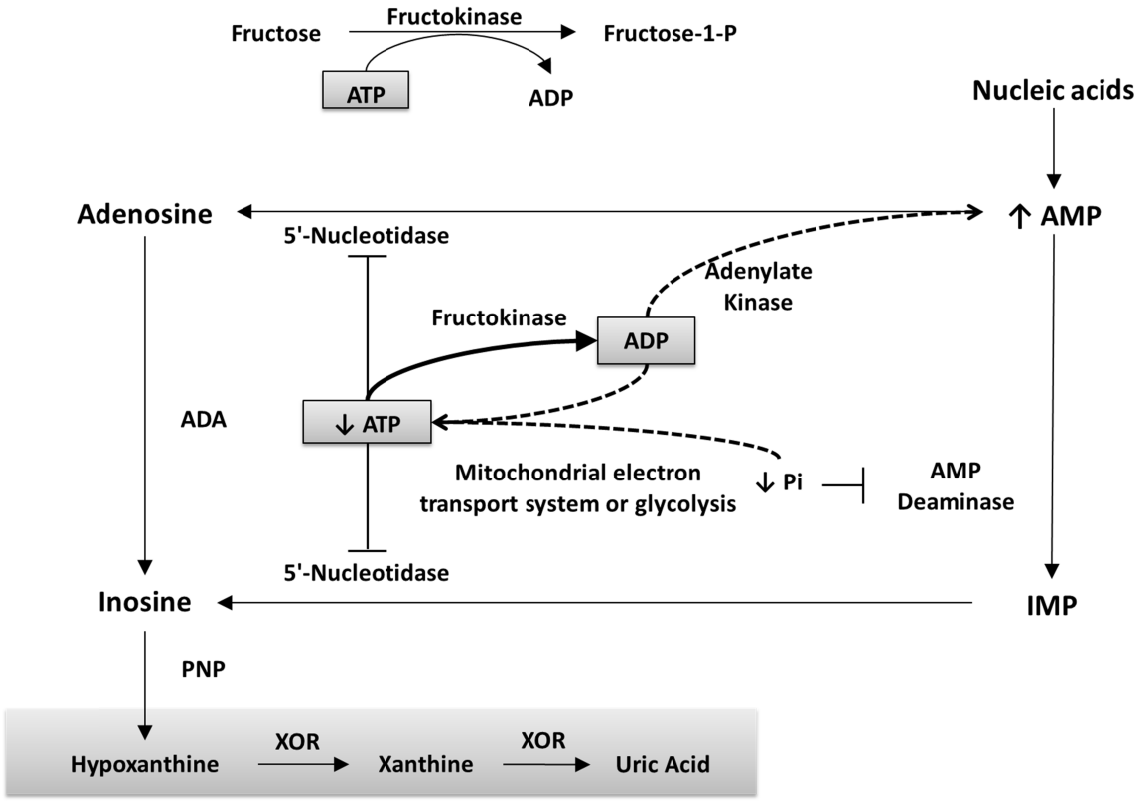


Figure 1-3 Mechanism of fructose-induced purine nucleotide degradation.

In liver, the phosphorylation of fructose to fructose-l-phosphate causes ATP to be degraded to ADP. To convert ADP back to ATP, the mitochondrial electron transport system or glycolysis use inorganic phosphate, and adenylate kinase build up intracellular AMP. The elevated AMP concentrations also lead to increased IMP. Intracellular Pi has a regulatory effect on urate production in the liver by inhibiting AMP deaminase, the enzyme controlling purine breakdown. In addition, ATP levels have a regulatory effect on 5′-nucleotidase. Thus, fructose induces acute depletion of ATP and Pi and causes increased activity of the enzymes involved in the degradation of purine nucleotides to inosine. AMP, adenosine monophosphate; IMP, inosine monophosphate; PNP, purine nucleoside phosphorylase. Figure is partially adapted from (64)

1.1.4 Hyperuricemia and metabolic syndromes

A proposed causal relationship between hyperuricemia and metabolic syndromes is shown in Figure 1-4. Insulin resistance seems to be the core reason for the association of hyperuricemia and metabolic syndromes (65). Studies in healthy humans (66, 67) and animal models (68) substantiate an explicable role of increased insulin resistance to hyperuricemia, and *vice versa*. Over production of uric acid could be a sign of over production of free radicals which induce oxidative metabolism (69, 70), and uric acid itself may also inhibit nitric oxide production (71). The combined result would be insulin resistance and platelet aggregation (72) and pro-inflammatory activity (73). Then hypertension is caused via pathways that involved a reduction in nitric oxide synthase in the macula densa of the kidney, stimulation of renin-angiotensin system and reduction of renal perfusion (73). Importantly, each of these effects was ameliorated by uric acid lowering drugs (74).

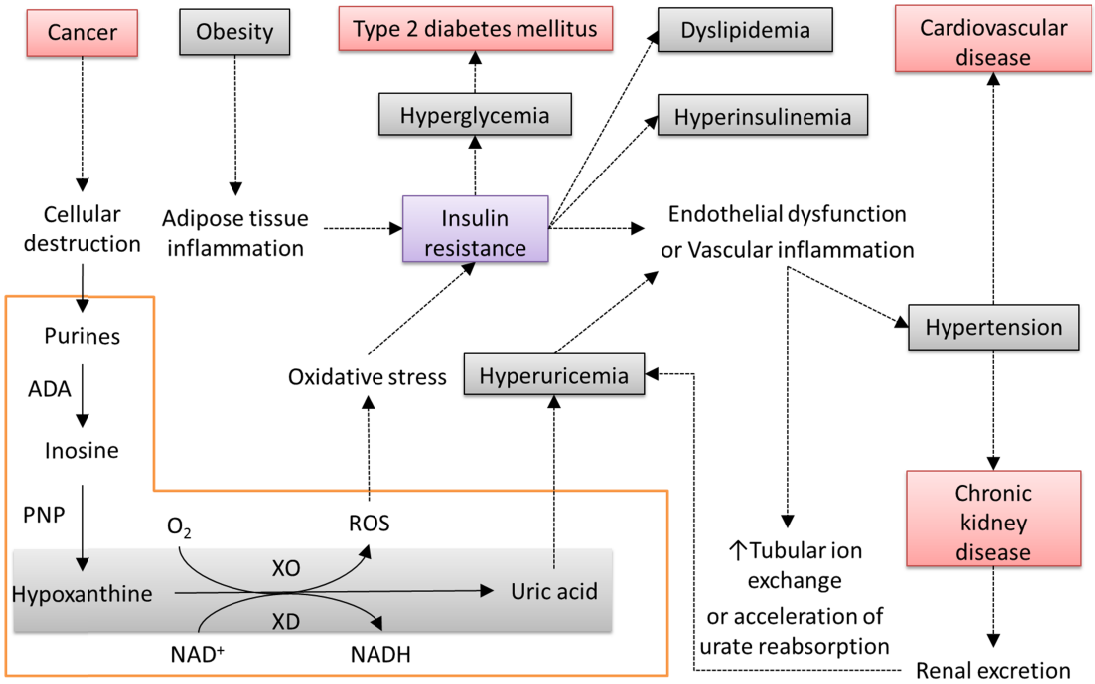


Figure 1-4 Proposed causal relationship between hyperuricemia, metabolic syndromes and disease.

Solid lines indicate chemical reaction in cells and dotted lines show causal relationship. Figure is personally drawn by Yuanlu Shi and partially adapted from (16, 65).

1.2 Quercetin and metabolic syndromes

Diabetes and diabetic complications: Higher dietary flavonol intake is associated with lower incidence of type 2 diabetes (75). A human intervention study using quercetin was conducted in 34 patients with type 1 or 2 diabetes and diabetic neuropathy, a reduction in the severity of numbness, jolting pain, and irritation was reported following four weeks of treatment topically with a mixture that contained quercetin, ascorbyl palmitate and vitamin D_3 (76). When regularly administered a mix of chlorogenic acid, myricetin, quercetin and metformin for 1 week, fasting

blood glucose, 2 h postprandial, actual peak glucose and AUC (post-50 g) were decreased in 40 diabetic patients (77).

Hypertension: Quercetin also appears to have effects on lowering blood pressure in overweight or obese (78, 79) subjects, smokers (80) and hypertensive (81) but not normotensive (healthy) (82, 83) individuals. Evidence from human intervention studies suggests that the antihypertensive effect of quercetin might involve improved endothelial function, since a single 200 mg dose of quercetin augmented nitric oxide status and reduced endothelin-1 concentrations (84).

Metabolic syndrome traits and obesity: In animal studies, quercetin tends to improve overall status of diet-induced problematic animals. For example, to the diet of obese Zucker rats, quercetin was added at a dose of 2 or 10 mg kg^{-1} body weight for 10 weeks, and this improved total cholesterol, triglycerides and insulin levels and decreased inflammatory molecules produced by visceral adipose tissue (85). In a 8-week study, quercetin (1.2% of the diet) was not effective in preventing hepatic insulin resistance caused by a high-fat diet in mice (86). However another 20-week study reports that mice fed by a diet high in fat, cholesterol and sugar induced increases in visceral and hepatic fat, cholesterol, triglycerides, blood glucose, insulin and inflammatory adipokines but their changes were partially prevented if quercetin was administered (0.5% of the diet) (87). Quercetin was reported to be effective in reducing high-fat induced elevations in cholesterol and triglyceride levels and associated with a reduced formation of atherosclerotic plaques in the aorta and carotid artery in rabbits over 12 weeks (88).

Mixed results have also been reported in human studies. Two weeks of supplementation with doses of quercetin ranging from 50-150 mg d^{-1} had no effect

on serum uric acid, lipids/lipoproteins, body composition or resting energy expenditure (89). A supplement containing 100 mg d^{-1} quercetin along with 128 mg of other mixed flavonoids (composition not specified) or placebo was given to male smokers for 10 weeks significantly decreased total cholesterol, LDL and serum glucose, as well as increases in HDL compared to placebo group, triglycerides, body mass index and waist circumference did not change (80). Quercetin supplementation (150 mg d^{-1}) decreased blood pressure and HDL-cholesterol concentrations while having no effect on total cholesterol or triglycerides in overweight and obese subjects (78). The effect on aspects of metabolic syndrome might be influenced by apolipoprotein E (apoE) genotype. A dose of 150 mg d^{-1} for 6 weeks decreased systolic blood pressure by 3.4 mm Hg in the apoE3 group, but had no effect in the apoE4 group. Quercetin decreased HDL and apoA1 and increased the LDL:HDL cholesterol ratio in the apoE4 subgroup, but the apoE3 subgroup experienced no significant changes in these variables (79). In a study with 1 002 subjects receiving either 500 mg quercetin or 125 mg vitamin C with 5 mg niacin, this efficiently led to a small decrease in blood pressure, and the group treated with double of that dose also had a small decrease in HDL levels (90). Healthy men and women received either 1 000 mg d^{-1} quercetin or placebo for 28 days but quercetin did not modify total cholesterol, LDL, HDL, triglyceride levels, blood pressure, resting heart rate or thrombogenic risk factors (platelet aggregation and platelet thromboxane B_2 production) (83).

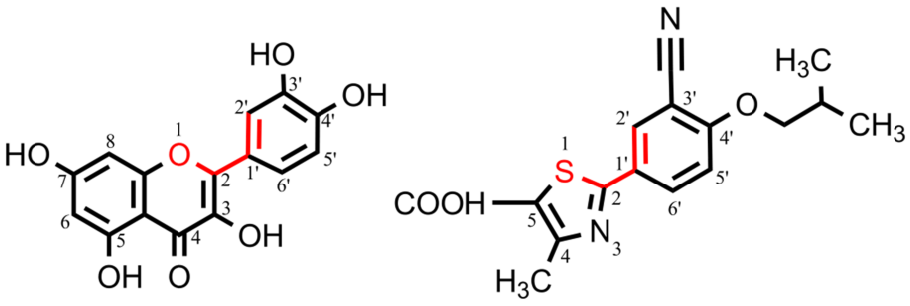
Hyperuricemia: An extensive amount of *in vivo* human and animal researches have focused on the uric acid lowering effect of quercetin. An inverse association of blood uric acid level with vitamin C supplementation (91), orange juice intake (92) or coffee intake (93) was confirmed. However mixed results have been reported from

epidemiology studies and intervention trials with quercetin and effect on blood uric acid level. For example, among 50 healthy Japanese females (aged 53 ± 9.7 years), flavonoids were found not to be associated with blood uric acid level (94). 6 cups of black or green tea daily during a 4-week period had no effect on plasma uric acid on healthy smokers (95). 400 g onion with 6 cups of tea daily providing 76-110 mg of flavonols (mostly quercetin) for 2-week had no effect on plasma uric acid level in 10 stable type 2 diabetic patients (96). 2-week of consumption of red grape juice did not affect concentrations of uric acid in both haemodialysis patients and healthy subjects (97). 4-week of 15 mg d^{-1} quercetin glucoside from blueberry-apple juice gave no significant difference of the uric acid levels between before and after supplementation in 4 females and 3 males (98). 2-week of quercetin supplementation in capsule with doses up to 150 mg d^{-1} had no effect on serum uric acid in human (89). However, animal evidence suggests the uric acid lowering effects of quercetin affords protection for both healthy and induced-hyperuricemia cases. 2-week of quercetin treatment (5 mg kg^{-1}) significantly reduced serum uric acid levels in normal and induced-hyperuricemia rats (99). Oral administration of quercetin, morin, myricetin, kaempferol, apigenin and puerarin at 50 and 100 mg kg^{-1} for 3 d was able to elicit hypouricemic actions in hyperuricemic mice induced by potassium oxonate (100). A crude apple polyphenol extract and low-viscosity apple fibres isolated from cider apples were administered separately or in association with the diet of apolipoprotein E deficient mice. After 4 months of supplementation, uric acid concentrations and antioxidant capacity (Ferric reducing ability of plasma, FRAP) in plasma were reduced in all groups supplemented with polyphenols or fibre (101). 3-week supplementation of procyanidins from grape had no significant effect on plasma uric acid level in both young and aged rats (102).

1.3 Quercetin

Flavonoids, including quercetin, are molecules that have been largely studied for their numerous biochemical and physiological activities (103, 104). Apparently, a habitual diet approach allowing maintenance of health has been of interest to researchers for a long time, since quercetin has an *in vivo* effect on biomarkers and other metabolic syndromes that have been proven to associate with hyperuricemia such as hypertension, diabetes and limited evidence on oxidised LDL. Quercetin is the main flavonol in the human diet, present in many fruits, vegetables and beverages. It is particularly abundant in onions and black tea (105). Quercetin usually occurs as *O*-glycosides, with D-glucose as the most frequent sugar residue.

Quercetin is particularly interesting since it was proven to inhibit xanthine oxidase (106). Quercetin can also inhibit enzymes responsible for superoxide anion production, such as NADPH oxidase (107), superoxide dismutase (108) and lipoxygenase (109). As shown in Figure 1-5, chemical structure of quercetin is compared to that of fabuxostat, an sythesised inhibitor of xanthine oxidase. It works by non-competitively blocking the molybdenum pterin center which is the active site on xanthine oxidase. In particular, the dihedral angle O1C2C1′C2′ of quercetin, is in good agreement with the previous calculated values for distinguishing the important aspects in the inhibition of xanthine oxidase: attraction inside the cavity and anchorage (110).



Quercetin Fabuxostat

Figure 1-5 Chemical structures of quercetin and fabuxostat.

Quercetin and fabuxostat are novel non-purine selective inhibitors of xanthine oxidase, one is naturally occurred and another synthesised, their structures share a similarity in dihedral angle (red). The various rings-numbering schemes for the heterocycles are also indicated. Systematic name of quercetin is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one.and of fabuxostat is 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-1,3-thiazole-5-carboxylic acid (according to International Union of Pure and Applied Chemistry, IUPAC).

The conjugation position of quercetin glucuronides also affect the biological activity followed the order 4′, 3′, 7 and 3 (111). As (112) has reported, the conjugation position of quercetin metabolites in human plasma and urine could be diverse (Table 1-1) as the proposed structures are shown in Figure 1-6 and Figure 1-7. They cannot be identified due to the lack of standards, however these are most reasonable conjugates positions according to the identifiable structures.

Table 1-1 HPLC–tandem MS (MS^2) identification of quercetin metabolites detected in plasma and urine (112).

Peak	Rt (min)	Skeleton	Conjugation position			$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)	Location
			3	4'	3'			
01	43·2	Quercetin	OH	OH	OH	301	179, 151	Plasma
02	47·9	Quercetin	OH	OH	SO_3	381	301 ($[M-H]^-$-SO_3)	Urine, plasma
03*	48·3	Quercetin	OH	SO_3	OH	381	301 ($[M-H]^-$-SO_3)	Plasma
04	28·4	Quercetin	Glc	OH	OH	463	301 ($[M-H]^-$-Glc)	Plasma
05	28·4	Quercetin	GlcUA	OH	OH	477	301 ($[M-H]^-$-GlcUA)	Urine, plasma
06	33·2	Isorhamnetin	Glc	OH	methyl	477	315 ($[M-H]^-$-Glc)	Plasma
07	34·4	Quercetin	OH	GlcUA	OH	477	301 ($[M-H]^-$-GlcUA)	Urine
08	36·3	Quercetin	OH	OH	GlcUA	477	301 ($[M-H]^-$-GlcUA)	Urine, plasma
09	34·1	Isorhamnetin	GlcUA	OH	methyl	491	315 ($[M-H]^-$-GlcUA)	Urine, plasma
10	37·2	Isorhamnetin	OH	GlcUA	methyl	491	315 ($[M-H]^-$-GlcUA)	Urine, plasma
11*	29·6	Quercetin	OH	Glc	SO_3	543	463 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-Glc), 301 ($[M-H]^-$-SO_3-Glc)	Urine
12*	30·6	Quercetin	Glc	OH	SO_3	543	463 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-Glc), 301 ($[M-H]^-$-SO_3-Glc)	Urine
13*	30·1	Quercetin	GlcUA	OH	SO_3	557	477 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-SO_3-GlcUA)	Urine
14*	30·3	Quercetin	OH	GlcUA	SO_3	557	477 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-GlcAU), 301 ($[M-H]^-$-SO_3-GlcUA)	Urine, plasma
15	22·8	Quercetin	Glc	Glc	OH	625	463 ($[M-H]^-$-Glc), 301 ($[M-H]^-$-Glc-Glc)	Plasma
16*	21·5	Quercetin	Glc	GlcUA	OH	639	477 ($[M-H]^-$-Glc), 463 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-Glc)	Urine
17*	26·2	Quercetin	Glc	OH	GlcUA	639	477 ($[M-H]^-$-Glc), 463 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-Glc)	Urine
18*	27·0	Quercetin	GlcUA	Glc	OH	639	477 ($[M-H]^-$-Glc), 463 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-Glc-GlcUA)	Urine
19*	15·6	Quercetin	GlcUA	GlcUA	OH	653	477 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-GlcUA)	Urine
20*	24·8	Quercetin	GlcUA	OH	GlcUA	653	477 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-GlcUA)	Urine
21*	27·4	Quercetin	OH	GlcUA	GlcUA	653	477 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-GlcUA)	Urine, plasma
22*	20·4	Methylquercetin	GlcUA	methyl	GlcUA	667	491 ($[M-H]^-$-GlcUA), 315 ($[M-H]^-$-GlcUA-GlcUA)	Urine
23*	22·7	Methylquercetin	GlcUA	GlcUA	methyl	667	491 ($[M-H]^-$-GlcUA), 315 ($[M-H]^-$-GlcUA-GlcUA)	Urine

Note: Retention time is not necessarily related to indicated group position and structures with peak number with *are proposed structures.

Abreviations: Glc, glucosyl unit; GlcUA, glucuronyl unit; SO_3, sulfate; $[M-H]^-$, negatively charged molecular ion; Rt, retention time.

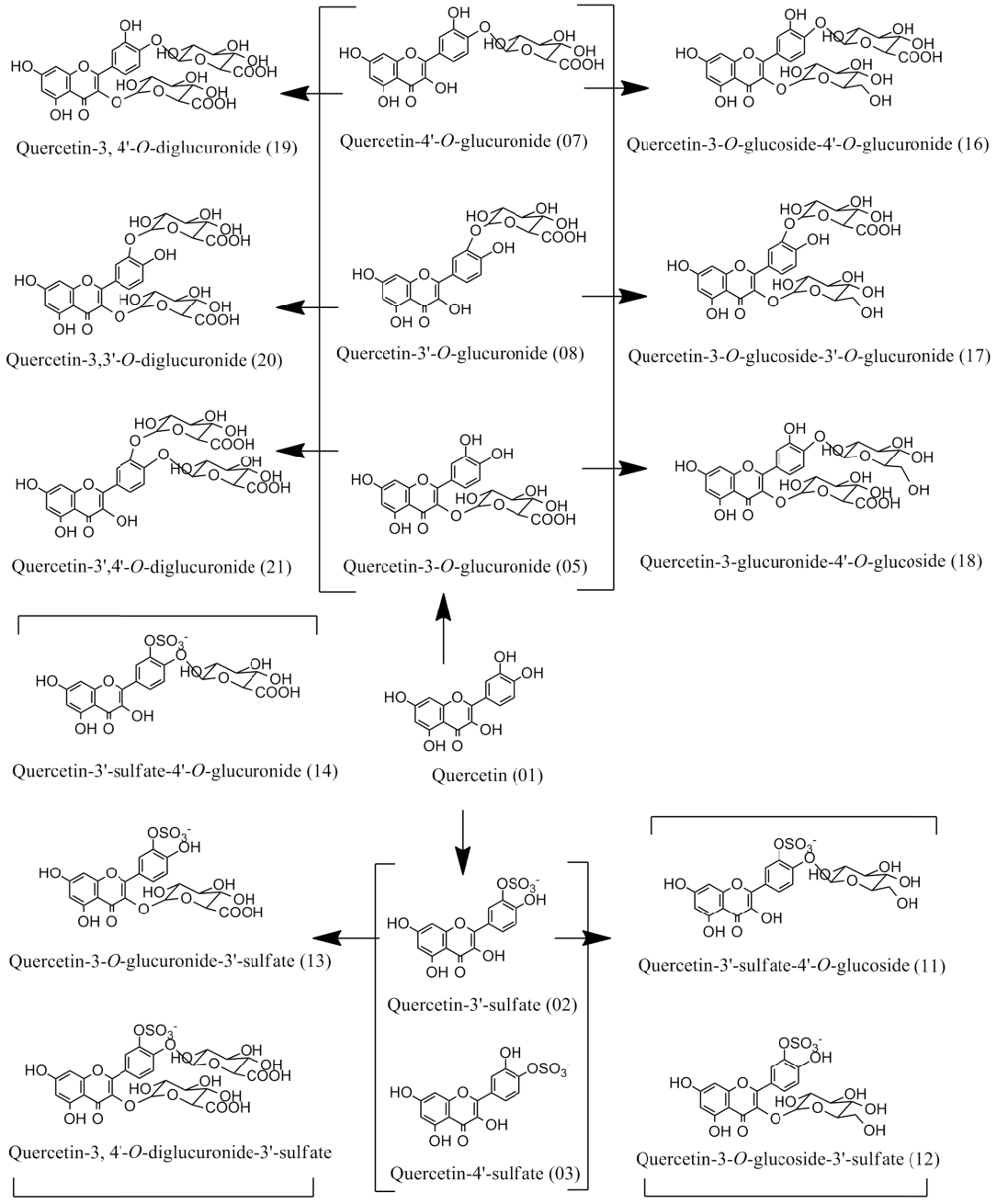


Figure 1-6 Structures of quercetin 3, 3′,4′ metabolites (A).

Enterocytes convert quercetin into glucuronide by uridine 5′-diphospho-glucuronosyltransferase (UGT) and sulfated derivatives by sulphotransferase (SULT) and then into glycoside by glucosyltransferase (GT) in kidney. Structures followed by numbered bracket are found in human plasma and urine after consuming onions (112).

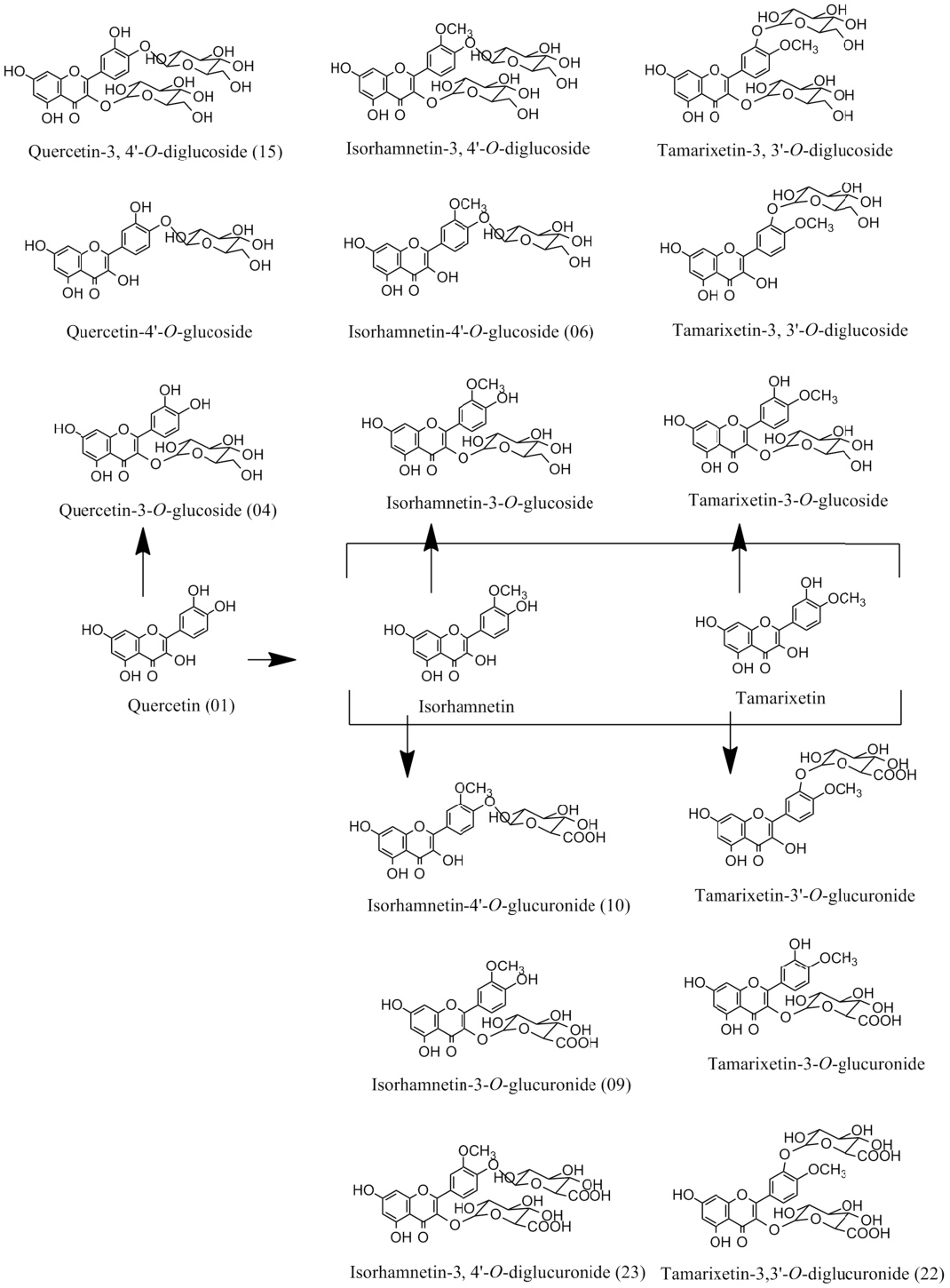


Figure 1-7 Structures of quercetin 3, 3′,4′ metabolites (B).

Enterocytes convert quercetin into methylated forms by catechol-*O*-methyl-transferase (COMT), into glucuronide by uridine 5′-diphospho-glucuronosyltransferase (UGT) and them into glycoside by glucosyltransferase (GT) in kidney. Structures followed by numbered bracket are found in human plasma and urine after consuming onions (112).

1.3.1 Absorption, bioavailability and biotransformation

Quercetin bioavailability may vary depending on the food or the conjugation and may vary among people due to the diverse amount of transporters. The maximum concentration in plasma can reach up to 1 to 2 μM and the excretion in urine may typically range from 0.5 to 1.4 % as portion of intake (113). Figure 1-8 summarises the proposed metabolic fate of quercetin once is ingested. To date, no enzymes involved in the biotransformation of flavonoid glycosides were identified in the stomach, however 50% of quercetin was hydrolysed there (114). Once it enters the small intestine, quercetin may undergo deglycosylation by β -glucosidase present on the outer surface of the intestinal brush border or microbial enzymes in colon (115). The quercetin-glucoside can alternatively be absorbed intact by passive diffusion and then the glycosylated quercetin is hydrolysed in enterocytes by cytosolic β-glucosidases (116). Based on the fact that glycosylated quercetin is more bioavailable than quercetin, it was suggested that a glucose transport system may be involved in the absorption of quercetin-3-*O*-glucoside (117). The liberated quercetin is then glucuronidated, sulfated and/or methylated by UDP-glucuronyltransferases (UGTs), sulfotransferases (SULTs) and catechol-*O*-methyl transferases (COMT), all of which are found in human intestine. A significant amount of conjugated flavonoids are re-secreted by enterocytes via multidrug resistance-associated protein 2 (MRP2) or breast cancer resistance protein (BCRP) into the intestinal lumen (118).

The large intestine is by far the most colonised region of the digestive tract, with a total population of 10^{11}-10^{12} CFU mL^{-1} of contents with more than 500 bacterial species, of which over 99% are anaerobic (119). Intestinal bacteria also play an important role in quercetin metabolism. Quercitin-3-*O*-glucoseide can be hydrolysed

by *Enterococcus casseliflavus* and the released aglycone can be degraded to 3,4-dihydroxyphenylacetic acid and phloroglucinol (120). However, flavonoids in general are further degraded into numerous phenolic and carboxylic acid products (121), as well as to carbon dioxide (122). As shown in Figure 1-9, the proposed pathway for colonic bacterium-mediated catabolism of quercetin in the human large intestine results in the production of 3,4-dihydroxyphenylacetic acid (DOPAC) and phloroglucinol (123), smaller quantities of 3,4-dihydroxybenzoic acid, 4-hydroxyphenylacetic acid (124) and 3-hydroxyphenylacetic acid. Like intact quercetin, those microbial-derived phenolic metabolites are readily conjugated by glucuronidation, sulftation, and methylation in the small intestine, liver and other organs (Figure 1-6, Figure 1-7). The polyphenol molecule provides multiple potential sites for these reactions and the extent of these reactions depends on substrate specificity of the enzyme that determines the reactivity and the position of the conjugation and the availability of the conjugation enzymes and their respective cofactors that are affected by the proteome and metabolome.

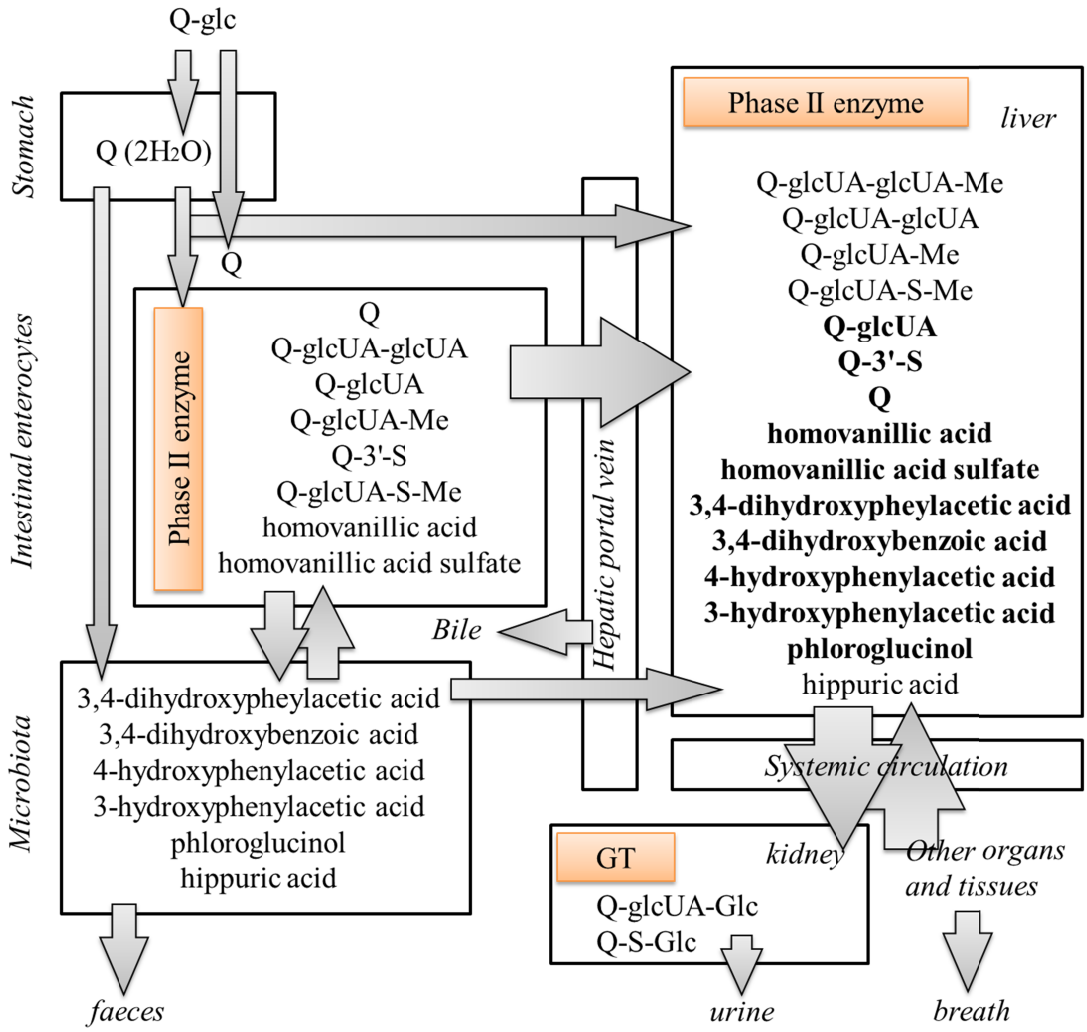


Figure 1-8 Schematic diagram of mammalian organs and enzymes involved in the biotransformation of quercetin.

After ingestion, while in the small intestine, Phase II enzymes in enterocytes convert quercetin into glucuronide, methylated and sulfated derivatives. On entering the cecum and the colon, the flavonol metabolites are converted to smaller phenolic acids by microbial metabolism. These metabolites are transported from the small intestine to the liver where they are further metabolised before returning to the bloodstream and being excreted in urine via the kidneys. Metabolites in liver (in bold) were selectively of interest in this work. Phase II enzymes include catechol-*O*-methyl-transferase (COMT), sulphotransferase (SULT), uridine 5′-diphospho-glucuronosyltransferase (UGT) and β-glucosidase. Glc, glucoside; glcUA, glucuronide; Q, quercetin; S, sulfate; Me, methyl; GT, glucosyltransferase. Figure is personally drawn by Yuanlu Shi with reference to (112, 114, 125).

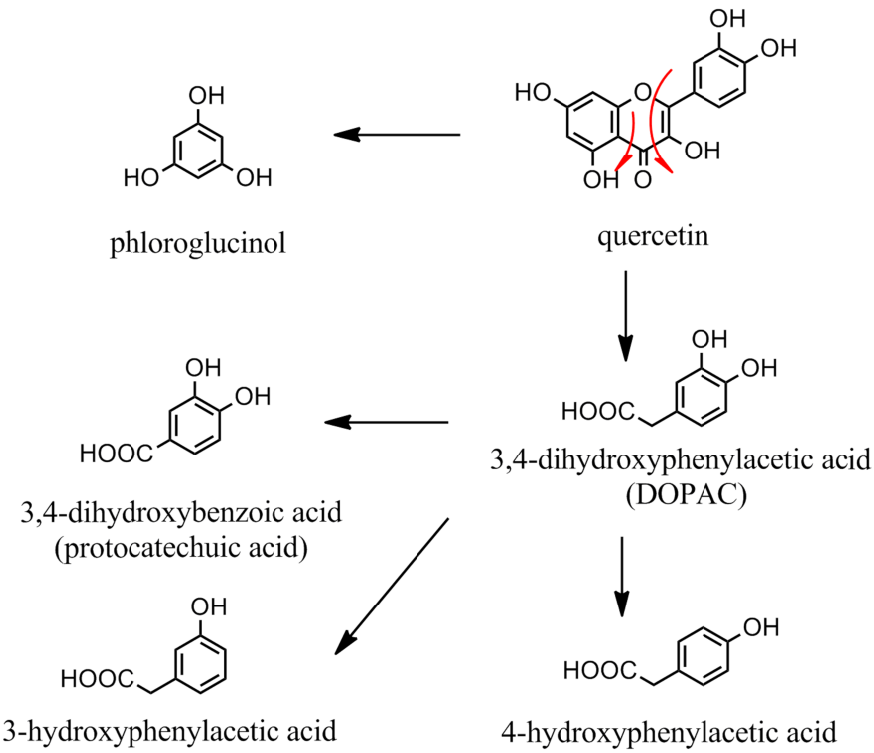


Figure 1-9 Schematic representation of colonic bacterium-mediated catabolism of quercetin in human large intestine. They are phloroglucinol, 3,4-dihydroxyphenylacetic acid (DOPAC) (123), 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxyphenylacetic acid (124) and 3-hydroxyphenylacetic acid (126, 127)

1.4 Objectives and methodology

Quercetin intake has been shown to affect various biomarkers of metabolic syndromes in human. A few studies have reported the inhibition effect of quercetin and related metabolites on xanthine oxidoreductase, the main enzyme on uric acid production, *in vitro*. Quercetin treatment has significantly improved blood uric acid level in induced-hyperuricemic mice, but it has not been established so far in humans (see Section 1.2: Hyperuricemia). Because of the relatively lower bioavailability of quercetin, the dosage in those human studies may not be able to deliver a decent amount of quercetin into target organs. For the same reason, the treatment period of 2-weeks may not be long enough to allow a consistent amount of quercetin being accumulated in blood. Therefore, a human study with a higher dosage and a longer treatment period is necessary.

The aim of the current study is to test the hypothesis that chronic oral supplementation of quercetin can modify the blood uric acid level in healthy men (Figure 1-10).

Experimental Hypotheses:

1) Quercetin or its metabolites inhibit the enzymes that are involved in uric acid metabolic pathway. They are adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP) and xanthine oxidoreductase (XO).
2) Quercetin reduce blood uric acid in human volunteers.
3) Quercetin affects fasting glucose and/or blood pressure.
4) Quercetin reduces blood uric acid by inhibiting uric acid production and/or other mechanism, such as increasing kidney excretion.

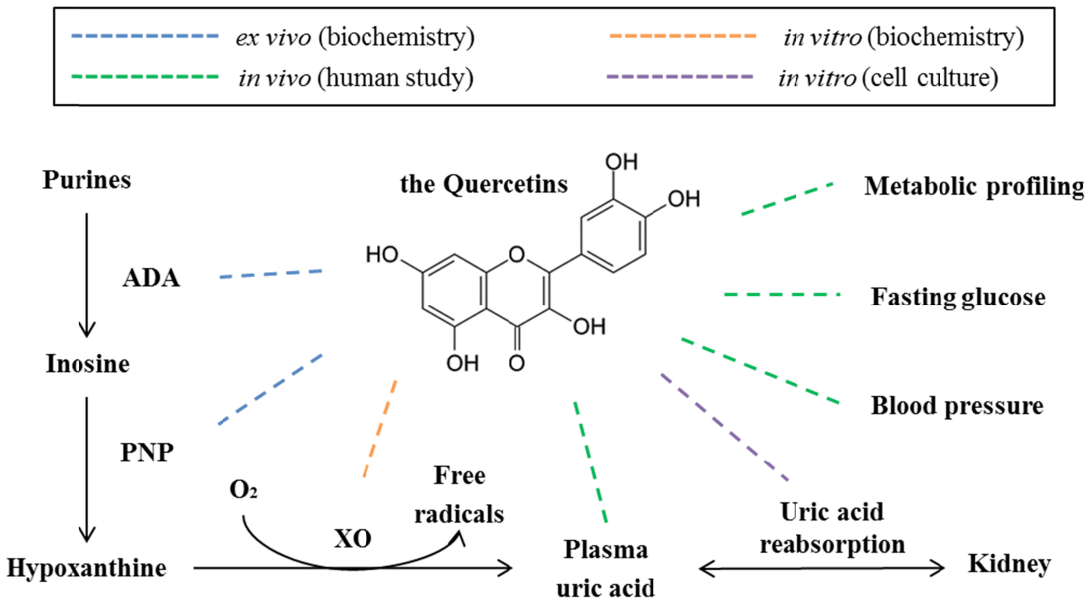


Figure 1-10 Potential interaction/mechanisms of quercetin on the pathway of uric acid metabolism.

The aims were achieved by the following objectives:

1) Randomised clinical trial to evaluate the delivery efficiency of quercetin supplements.
2) Randomised clinical trial to investigate the chronic effect of quercetin on metabolic biomarkers, including blood uric acid level as primary outcome.
3) Plasma profiling by ^{1}H-NMR and chemometric analysis aiming to explore other possible changes caused by quercetin supplementation.
4) *In vitro* inhibition of enzyme activity on the pathway of uric acid metabolism by quercetin metabolites.

and the following methodology was developed:

1) Development of an analytical HPLC-ESI/MS method that efficiently identifies both free-form and conjugated forms of quercetin in urine aiming to check quercetin deliverance.

2) Development of an enzymatic assay to quantify uric acid level in biological samples (urine, plasma, cell tissue extract or cell culture medium) aiming to detect plasma uric acid changes.
3) Development of HPLC-DAD method that accurately quantify purine derivatives in biological samples (plasma, cell tissue extract or cell culture medium) aiming to evaluation the validity of *in vitro* enzyme kinetic assay and the effects of quercetin and its metabolites on uric acid production.

Chapter 2. Materials and Methods

2.1 Uric acid assay

Uric acid in plasma samples and urine samples was determined by a specific coupled enzyme reaction, followed by a colorimetric determination at 520 nm (128). The protocol was modified for use in a 96-well plate reader for high-throughput and improved accuracy. Within-run variation was 1.99 ± 1.20%, and between-run variation was 2.17 ± 0.52%. Recovery was 92.8 ± 1.6% for plasma and 80.4 ± 3.8% for 10-fold diluted urine. Calibration curve was prepared for each plate, with a regression curve of 0.550 ± 0.003 mM^{-1} uric acid and a maximum concentration of 1.0 mM with $R^2 \geq 0.999$.

2.1.1 Introduction

In this assay, uric acid concentration is determined selectively by a coupled enzyme reaction, which results in a red dye (520 nm). 8 μL of sample (serum/plasma/10-fold diluted urine/working standard) is needed per test. The reaction is carried at room temperature (25℃) for 15 min in 96 well plate reader. Protocol is modified according to Fossati *et al.* (128).

Figure 2-1 shows the enzymatic reaction sequence of this assay. In brief, the amount of H_2O_2 produced by uricase selectively from uric acid would be detected by a colorimetric quantification of the product of peroxidase which is proportional to the uric acid concentration. Modifications from the original protocol include standard curve being made by at least 5 points (including blank), instead of 2 points (including

blank). 3,5-dichloro-2-hydroxybenzenesulfonic acid being replaced by sodium 3,5-dichloro-2-hydroxybenzenesulfonate from Sigma-Aldrich (USA). The assay being read on a 96 well plate and plate reader instead of cuvette and spectrophotometer ensures the accuracy and increases throughput.

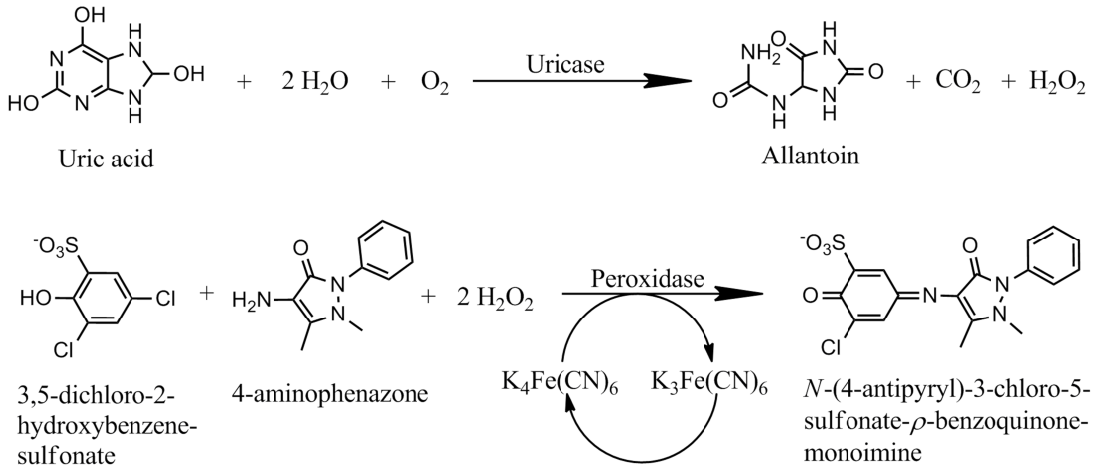


Figure 2-1 Reaction sequence of uric acid enzymatic assay.

2.1.2 Chemicals and apparatus

Potassium monohydrogen phosphate, potassium dihydrogen phosphate, potassium hexacyanoferrate (II) trihydrate, 4-aminoantipyrine, peroxidase from *horseradish*, ascorbate oxidase from *cucurbita sp.*, sodium 3,5-dichloro-2-hydroxybenzenesulfonate, lithium carbonate, uricase from *candida sp.* and uric acid were purchased from Sigma-Aldrich (USA); Triton X-100 was from Fisher Scientific (UK). A plate reader modelled PHERAstar FS (BMGlabtech, Germany) with 96 well micro-plates (COSTAR or Greiner bio-one) was used.

2.1.3 Reagents and standards

Buffer/enzymes/4-aminoantipyrine reagent Phosphate buffer, 200 mM, pH 7.0; horseradish peroxidase, > 0.20 kU L^{-1}; ascorbate oxidase, > 0.20 kU L^{-1}; 4-aminophenazone, 0.33 mM; and potassium hexacyanoferrate (II) trihydrate, 40 µM. This reagent is stable for six weeks at 2-8℃

DHBS Sodium 3,5-dichloro-2-hydroxybenzenesulfonate 8 mM and Triton X-100, 5 g L^{-1}. This reagent is stable for months at room temperature if protected from direct light.

Uricase reagent Uricase, 6 kU L^{-1}, in doubly-distilled water. This is stable for months at room temperature, barring bacterial contamination.

Working reagent Mix the buffer/enzymes/4-aminoantipyrine, DHBS and uricase reagents in the ratio 1.5:0.5:0.02; store in an amber glass bottle. The mixture was used during one working day.

Standards 0.168 g of uric acid and 0.102 g of lithium carbonate were dissolved in 100 mL of Milli-Q water. To achieve this, add 30 mL of Milli-Q water and keep in 60℃ water bath for 20 minutes till totally dissolve and then bring the volume to 100 mL after cooling to room temperature. Aliquots of this stock were protect from light, stored in -20℃ and used within one month.

2.1.4 Procedure

For 100 assays (96-well plate), prepare working reagent by mixing 24 mL of Buffer/enzymes/4-aminophenazone, 8 mL of DHBS, and 320 µL of uricase reagents in an amber glass bottle. Quickly mix 8 µL of sample or working standards and 320

μL working reagent to each well. Multi-dispenser pipette is an ideal alternative to multi-channel pipette since reverse pipetting is recommended for avoiding bubbles. Shortly shake the plate before incubating and let stand in dark without lid for 15 min at 25℃. Shortly shake the plate before reading the absorbance at 520 nm. When preparing samples into well plates, substitute H_2O for urine or plasma in the first and last sample in each plate to produce blank samples. This provides a check for carry-over between samples.

2.1.5 Linearity and sensitivity

A moderate increase in the reagent′s pink colour does not influence the results. The background for the assays is the value obtained for the uric acid standard 0 (blank). Correct for the background by subtracting the standard 0 (blank) value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate uric acid standards to plot a standard curve.

Calibration curve (Figure 2-2) gives a conversion k value for each plate and it is 0.550 ± 0.001 mM^{-1} uric acid (mean ± SEM, n=10). Recovery was calculated from pooled plasma spike with 300 μM standards of each plate and it is 92.8 ± 0.5% (mean ± SEM, n=10). Concentration was corrected by the corresponding recovery value.

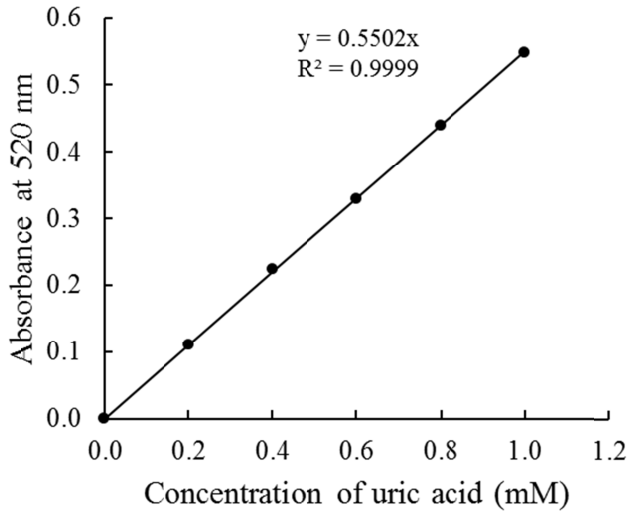


Figure 2-2 Calibration curve of uric acid at concentrations up to 1.0 mM

2.1.6 Reliability and reproducibility

Residual standard deviation of the linear regression is 0.002 mM^{-1} among 10 plates. Mean ± SD of Slope of 10 sets of calibration curve is 0.550 ± 0.003 mM^{-1}. This method can be applied to plasma, serum or 10-fold diluted urine or tissue samples. The limit of detection is 12.4 μM and limit of quantification is 41.3 μM. The normal range of baseline plasma uric acid in men of previous report is 360 ± 75 μM (129).

2.2 Measurement of blood glucose

Plasma glucose was measured with a commercial hexokinase assay kit for D-glucose (Sigma-Aldrich, St. Louis, USA) (130). The protocol was modified for use in a 96-well plate reader. Within-run variation was 4.29 ± 2.21% and between-run variation was 3.33 ± 2.51%. Recovery was 104 ± 8%. Calibration curve was prepared for each plate, with a regression curve of 0.923 ± 0.006 per mg mL^{-1} D-glucose with R^2 > 0.998 and a maximum concentration of 1.5 mg mL^{-1}.

2.2.1 Introduction

This method for measurement of D-glucose in plasma is based on the detection of absorbance at 340 nm of NADH produced using the hexokinase assay from Sigma-Aldrich (USA) with modification in use of 96-well micro-plate reading which increases the amount of sample analysed in time, as well as reduces the amount of plasma required, from 1 mL to as little as 10 μL.

The first reaction is catalysed by the hexokinase, where glucose is phosphorylated by adenosine triphosphate (ATP):

$$\text{Glucose} + \text{ATP} \rightarrow \text{G-6-P} + \text{ADP (non-selectively)}$$

The glucose-6-phosphate (G-6-P) formed is then oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NAD^+). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH):

$$\text{G-6-P} + NAD^+ \rightarrow \text{6-PG} + \text{NADH (selectively)}$$

During this oxidation, an equimolar amount of NAD^+ is reduced to NADH. The consequent increase in NADH concentration is directly proportional to the glucose concentration and can be measured at 340 nm.

2.2.2 Chemicals and equipment

Glucose (hexokinase) assay kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). The working reagent contains the following lyophilised compounds: 1.5 mM of NAD^+, 1.0 mM of ATP, 1.0 unit mL^{-1} of hexokinase (1.0 unit is defined to phosphorylate 1.0 μmol of D-glucose per min at pH 7.6 at 25℃; rate of phosphorylation: K_m, 0.12 mM, pH 7.5, 30℃), and 1.0 unit mL^{-1} of glucose-6-phosphate dehydrogenase (1.0 unit is defined to convert 1.0 μmol of glucose-6-phosphate to 6-phospho-D-gluconate per min in the presence of NADP at pH 7.4 at 25℃) with sodium benzoate and potassium sorbate as preservatives. D-glucose was from Fisher Scientific (UK) and 96-well plate compatible for UV reading was from Greiner bio-one (Austria). A micro-plate reader modelled PHERAstar FS (BMGlabtech, Germany) was used for UV absorbance reading.

2.2.3 Procedure

A series of concentrations of standard solutions at 1.5, 1.0, 0.5, 0.25, 0.025 and 0 mg mL^{-1} (0 mg mL^{-1} was used to create reagent blank) of D-glucose were prepared freshly for each set of experiment. Mix 10 μL of standard or sample with 250 μL of working reagent or Milli-Q water (as blank). Incubate the 96-well micro-plate for 15 min in a 30℃ incubator and read the absorbance at 340 nm.

2.2.4 Linearity and sensitivity

Background values can be significant and were subtracted from all readings. The background for the assays is the value obtained from the reagent blank, and for each plasma sample is the value obtained from the corresponding plasma blank. Correct

for the background by subtracting the reagent blank value and their sample blank values from all readings. Use the values obtained from the appropriate D-glucose standards to plot a standard curve. D-glucose standards ranging from 0.025 mg mL^{-1} to 1.5 mg mL^{-1} were prepared freshly before each time assay was performed. standard curve was leaner as shown in Figure 2-3.

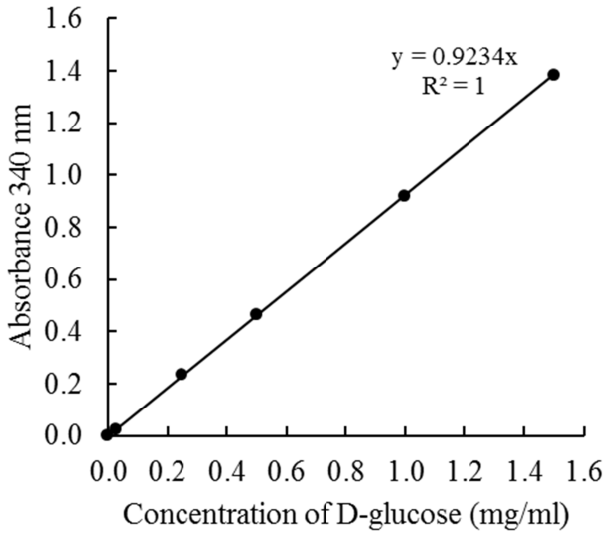


Figure 2-3 Calibration curve of D-glucose. To convert mg mL^{-1} concentrations into mM, multiply by 5.55.

Recovery of the plasma glucose was tested by calibration curve prepared within plasma pool and it is 104 ± 8%. Each biological sample was duplicated when carry on the assay and analyses were repeated on a different day. Within run variation of absorbance at 340 nm of plasma blank is 2.62 ± 3.09% and of plasma test is 4.29 ± 2.21%. Between run variation of glucose concentration of biological samples is 3.33 ± 2.51% (n=122).

2.3 Quantification of quercetin content in food

This method was modified and shared between group members with purpose driven modification specified to various food type. Modification for onion extraction was also contributed by Dr. Patthamawadee Charoensuk.

2.3.1 Sample preparation

The quercetin content of the red onion soup and of the supplement tablet was determined by HPLC-diode-array analysis. To 5 g of frozen red onion, 5 mL of absolute methanol was added and to 0.4 g soup powder, 5 mL of 70% methanol was added. Extraction was performed using ultra sonication and vortex. The samples were centrifuged (3 000 g, 4℃, 10 min) and the supernatant was collected. The extraction was repeated twice with 5 mL of 70% aqueous methanol (containing 0.1 mM ascorbic acid, pH 5.08). 1 mL of the combined extracts was fully dried in a centrifugal evaporator (Genevac Ltd, Ipswich, UK), and then reconstituted with 1 mL of 50% aqueous ethanol containing 100 µM daidzein as internal standard. Before HPLC analysis, the samples were filtered through polytetrafluoroethylene (PTFE) membrane syringe filter (pore size of 0.2 µm). Extraction was performed in duplicate for each food sample.

2.3.2 HPLC analysis of quercetin and its conjugates

The reconstituted samples were analysed on an Agilent HPLC 1200 instrument (Agilent Technologies, Waldbronn, Germany) equipped with C18 column (ZORBAX Eclipse XDB-C18, 4.6×50 mm, 1.8 µm particle size, rapid resolution

high throughput, 600 bar column, Agilent, USA) and a pre-column (Eclipse XDB-C18, 4.6×12.5 mm, 5 µm, analytical guard cartridge, Agilent, USA).

A modified version of the analytical HPLC method from (131) and (132), was used. Solvents A (water with 0.1% of formic acid, v/v) and B (acetonitrile with 0.1% of formic acid, v/v) were run at a flow rate of 0.5 mL min^{-1}. The chromatographic conditions of elution were as follows: 0-2 min, 15% solvent B; 2-22 min, increase solvent B from 15% to 40%; 22-24 min, isocratic for 2 min. A post-run column clean up procedure was applied by increasing B to 90% in 1 min, isocratic for 3 min and finally rapidly returning to initial conditions with re-equilibration at 29 min for 5 min of 15% B. Each sample (10 µL) was injected and analysed twice. A column clean-up stage maintained B at 90% (30 min) which was followed by a re-equilibration at 15% B (30 min) to initiate each new batch of analysis. Diode array detection monitored the eluent at 255 nm and 370 nm. A standard curve ranging from 15.6 to 1 000 pmol quercetin equivalents was produced using standard solutions of quercetin 3,4′-*O*-diglucosides (AUC_{370nm} of 0.736 $pmol^{-1}$), quercetin 4′-*O*-glucoside (AUC_{370nm} of 1.49 $pmol^{-1}$), daidzein (AUC_{255nm} of 1.68 ± 0.01 $pmol^{-1}$), and quercetin (AUC_{370nm} of 1.26 $pmol^{-1}$), with retention times of 3.20, 9.44, 12.6 and 14.3 min, respectively. HPLC chromatograms of standard mix, supplement extract and red onion extract are shown in Figure 2-4.

After HPLC analysis to confirm that the supplement contained pure quercetin (Figure 2-4), the quantification was performed by spectrophotometry using the extinction coefficient (ε) at λ_{max} (quercetin) 19.95 mM^{-1} cm^{-1} (257 nm) and 21.88 mM^{-1} cm^{-1} (376 nm) against 95% aqueous ethanol (133). In brief, 5 tablets were finely ground in an electric coffee grinder and about 2 mg of the powder was accurately weighed and

fully dissolved in 95% ethanol. Absorbance spectra were compared with quercetin standards prepared in 95% ethanol.

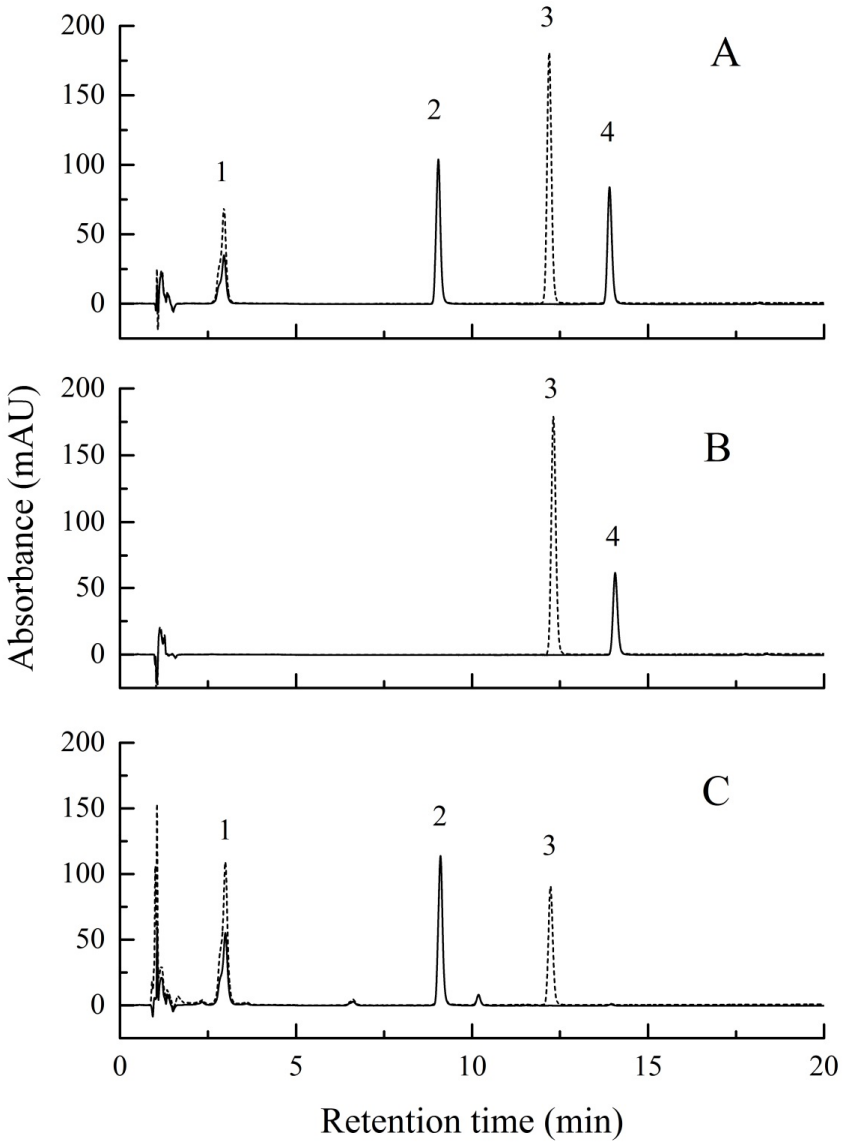


Figure 2-4 HPLC chromatograms of (A) quercetin standards (B) supplement extracts and (C) onion extracts at 255 nm (dash line) and 370 nm (solid line): (1) quercetin 3,4′-*O*-diglucoside; (2) quercetin 4′-*O*-glucoside; (3) daidzein (added as internal standard); (4) quercetin.

2.4 HPLC-ESI/MS analysis of metabolite

2.4.1 Chemicals and enzymes

Absolute methanol, ethanol, acetonitrile (LC-MS grade) and ethyl acetate were from VWR international, France; ascorbic acid was from MP Biomedicals, LLC, France; formic acid, sodium acetate trihydrate, acetic acid, hydrochloric acid, β-glucuronidase from *Helix pomatia*, and sulfatase from *Helix pomatia*, were purchased from Sigma-Aldrich, USA. Standards of quercetin dihydrate, quercetin-4′-*O*-glucoside (spiraeoside), quercetin-3,4′-*O*-diglucoside, isorhamnetin (3-*O*-methylquercetin), tamarixetin (4′-*O*-methyquercetin), daidzein and taxifolin, are all HPLC grade and were purchased from Extrasynthese, France.

2.4.2 Processing of urine samples and analysis of quercetin

24-h urine was collected into a 3 L sterile urine storage container with 3 g of ascorbic acid added. Once the sample arrived at the laboratory, the weight was measured and two 45 mL aliquots were taken into 50 mL falcon tubes, then centrifuged at 2 000 g at 4℃ for 10 min. The supernatant was stored at -20℃ until analysis.

2.4.2.1 Enzyme hydrolysis of quercetin conjugates and liquid phase extraction

Metabolites of methyl-, glucuronyl-, glucosyl- and sulfo-conjugates of quercetin in human urine were hydrolysed to quercetin and the monomethylated derivatives isorhamnetin (3-*O*-methylquercetin) and tamarixetin (4′-*O*-methylquercetin) using β-glucuronidase and sulfatase (112). To 200 µL of urine, 20 µL of 0.2 M sodium acetate - acetic acid buffer, pH 5.0 containing 200 units β-glucuronidase and 5 units

of sulfatase were added; 2 μL of 100 μM taxifolin was added as internal standard, then incubated in a shaking water bath at 37℃, 100 rpm for 1 h. The completion of hydrolysis of all quercetin conjugates was assured by parallel experiments running from 1 h every 0.5 h up to 3 h (Figure 2-5). Results showed that hydrolysis was complete within 1 h. The pH of the hydrolysis mixture was adjusted to 2.0 by addition of 30 μL of 0.1 M HCl. To the hydrolysis mixture (about 250 μL), 500 μL of ice-cold ethyl acetate was added, mixed vigorously by vortex for 2 min, followed by standing on ice for 2 min and centrifugation at room temperature at 17 000 g for 2 min. The procedure was repeated twice and 3 supernatants pooled. Extracts were fully dried by nitrogen gas, then reconstituted with 150 μL of 50% ethanol and filtered through 0.2 μm PTFE filters before analysis. An enzyme unit was defined at 37℃ at pH 5.0 according to the manufacturer: one unit of β-glucuronidase liberates 1.0 μg of phenolphthalein from phenolphthalein glucuronide per h; one unit of sulfatase hydrolyses 1.0 μmol 4-nitrocatechol sulfate per h. Extraction was performed in duplicate for each biological sample.

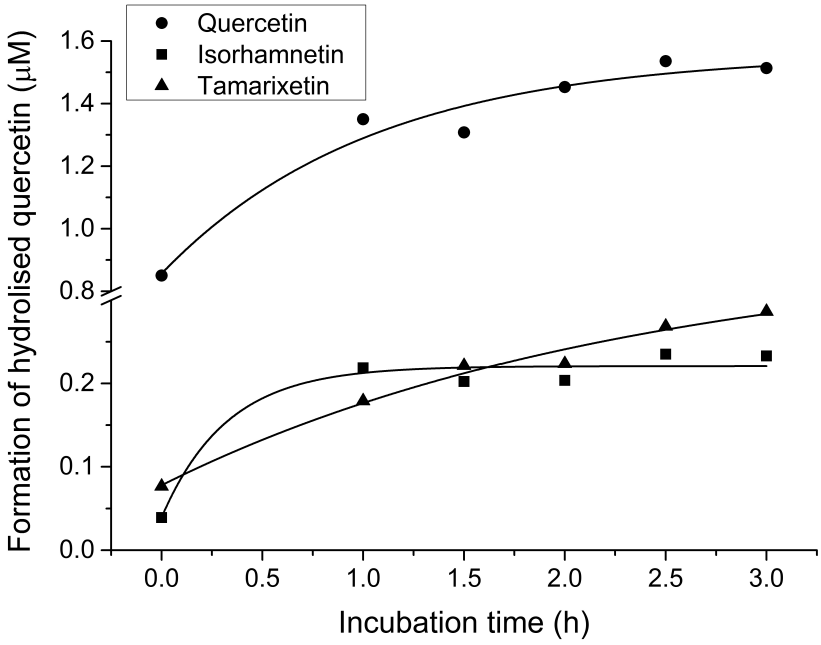


Figure 2-5 Enzymatic hydrolysis of urinary quercetin conjugates against incubation time.

2.4.2.2 HPLC-ESI/MS

Analysis of urine concentrations of quercetin and of the monomethylated derivatives: isorhamnetin (3-*O*-methylquercetin) and tamarixetin (4′-*O*-methylquercetin) was performed by HPLC (detailed in Section 2.3.2) with mass spectrometry using a Shimadzu LC-2010C HT with single ion monitoring (Shimadzu, Tokyo, Japan) operated in negative electrospray ionization (-ESI) mode. Nitrogen was used both as drying and nebulising gas at a flow rate of 15.0 L h^{-1} and 1.5 L h^{-1}. The DL temperature was maintained at 250℃ with detector voltage set at 1.80 kV and interface voltage at -3.5 kV. The standard curve was 0.05 - 2.00 µmol, within-run variance was 6.8 ± 5.6% and between-run variance was 14.5 ± 8.2%. The recovery of quercetin extraction from urine was calculated using the yield of taxifolin (internal standard, 111 ± 14.3%, n=92). All chromatograms in the same batch were processed automatically by software (Labsolutions, ver. 5, Shimadzu, Tokyo, Japan) using the same processing parameters, such as integration, peak-to-peak amplitude, and peak detection. Manual integration was performed only rarely when necessary.

Figure 2-6 shows a typical LC-MS Chromatogram of quercetin and conjugates after enzymatic hydrolysis of urine. The retention times of quercetin (*m/z* 301), isorhamnetin (*m/z* 315), tamarixetin (*m/z* 315) and taxifolin (*m/z* 303) are 16.1 min, 20.4 min, 20.6 min and 8.8 min, respectively.

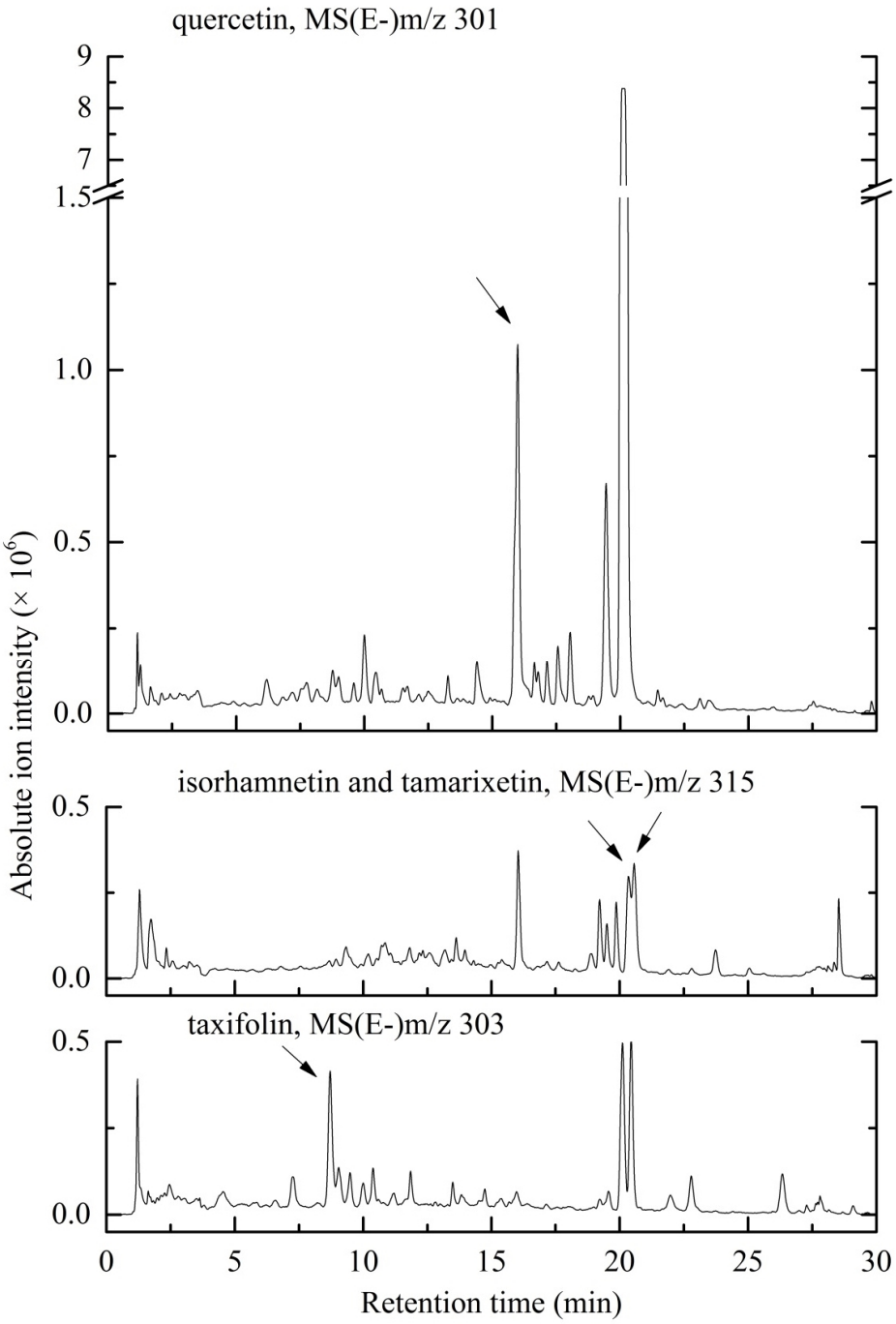


Figure 2-6 LC-MS chromatogram of quercetin and methylquercetin after β-glucuronidase and sulfatase hydrolysis of urine.

2.5 Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy

Method is detailed in Section 5.2.

2.6 Development of HPLC method of analysing adenosine, inosine, hypoxanthine, xanthine, uric acid and adenine simultaneously

A method that can accurately measure the product of these enzymes: inosine (of ADA), hypoxanthine (of PNP) and uric acid (of XOR) without interference of their enzymatic substrate adenosine (of ADA), inosine (of PNP) and hypoxanthine (of XOR), of tested compounds (in this case, quercetin and its metabolites) or of plasma contents was built. This method can be used for investigating interaction of interested compounds on these enzymes by which the uric acid was produced. The HPLC reverse phase separation was achieved on a C18 column, using a mobile phase consisting of water containing 3- 20% methanol, at a flow rate of 0.6 mL min^{-1}. The column effluent was monitored by UV detection referencing to 450 nm. A linear response was achieved over the concentration range 50 - 500 pmol. The analytical method LOQ was below 50 pmol. This method is standardised for enzyme activity study both of purified (or) recombinant enzymes and of delicate samples, such as plasma or cell extract.

2.6.1 Introduction

Uric acid production results from endogenous purine degradation in hepatocyte, by adenosine deaminase (ADA; E.C.3.5.4.4), purine nucleoside phosphorylase (PNP; E.C.2.4.2.1) and xanthine oxidoreductase (XOR): xanthine oxidase (XO,

E.C.1.17.3.2) or xanthine dehydrogenase (XD, E.C. 1.17.1.4). It is of our interest to know whether quercetin and its derivatives have effects on human enzymes and furthermore, on plasma enzymes. A method that can separate and accurately measure inosine, hypoxanthine, and uric acid (Structures are shown in Figure 2-7) without interference of their enzymatic substrates, of the tested compounds and of other chemicals in plasma is necessary in experiment investigating interaction of quercetin and its metabolites on plasma enzymes by which the uric acid was produced.

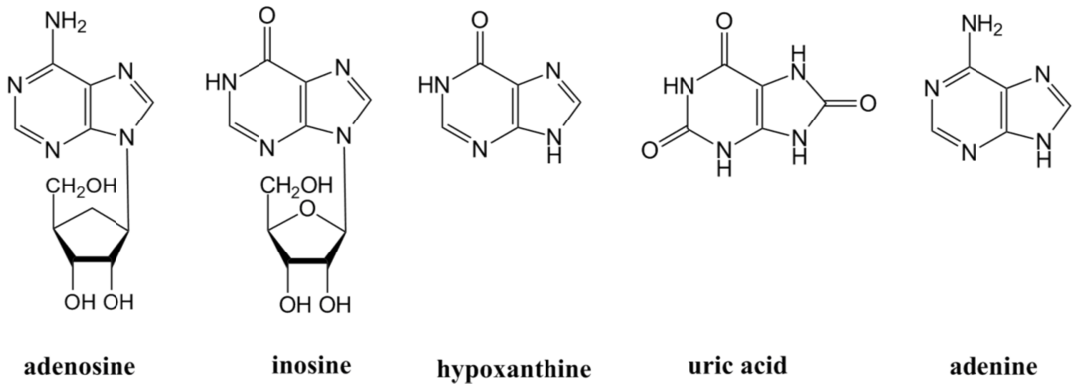


Figure 2-7 Structures of adenosine, inosine, hypoxanthine, uric acid and adenine.

For example, xanthine oxidase activity has usually been determined by the rate of uric acid formation from xanthine spectrophotometrically at 295 nm (134). Xanthine itself has UV absorption at around 260 nm, and some flavonoids also have strong absorption from the UV to visible region. Such absorption not only enhances the background absorption but also sometimes disturbs an accurate spectrophotometric determination of uric acid formation. We determined xanthine oxidase activity by analysing the amount of uric acid separation on a HPLC column. This method could eliminate the disturbance by other UV-absorbing compounds present in the reaction medium. This method is therefore useful to evaluate the inhibitory effect on XO activity of various compounds in various biological samples.

The traditional way of separating purine nucleosides is with HPLC on a C18 column in 8% aqueous methanol containing 30 mM of phosphate, pH 5.5 (135). Mei *et al.* have reported an ideal HPLC method (136) that is satisfactory for our purpose of separating inosine from adenosine, hypoxanthine from inosine, uric acid from hypoxanthine and xanthine, and adenine (added as internal standard). Current method was built by employing a combination of HPLC method using methanol (7% - 30%) to separate inosine and adenosine (137) and one to separate inosine and hypoxanthine (138). In our present method, a C18 column 150 mm × 4.6 mm is equipped with a gradient combination of solvent A: 0.1% formic acid in deionised water at pH 2.2 and solvent B: absolute methanol with reverse-phase gradient from 3% to 20% methanol. A 95% methanol cleaning step is adopted to application on plasma samples.

2.6.2 Chemicals and methods

With adenine added as internal standard, the metabolites of adenosine, inosine, hypoxanthine, xanthine and uric acid were analysed by HPLC-diode-array 1200 Series (Agilent Technologies) equipped with a C18 column (150 × 4.6 mm 125 Å,3 µm, Phenomenex Aqua). Solvents A (water with 0.1% v/v of formic acid) and B (methanol) were run at a flow rate of 0.6 mL min^{-1}. The chromatographic conditions of elution were as follows: 0 - 2 min, 3% solvent B; 2 - 14 min, increase solvent B from 3% to 20%. A post-run column clean up procedure was applied by increasing B to 95% in 1 min, isocratic for 2 min and finally rapidly returning to initial conditions with re-equilibration at 18 min for 2 min of 3% B. Each sample (10 µL) was injected and analysed twice. A column clean-up stage maintained B at 95% (30 min) which was followed by a re-equilibration at 3% B (30 min) to initiate each new batch of

analysis. A photo diode array detector (DAD) was used to simultaneously record chromatograms at 260 nm (adenosine and adenine), 250 nm (inosine and hypoxanthine), 270 nm (xanthine) and 292 nm (uric acid) with a reference wavelength of 450 nm.

2.6.3 Result and discussion

2.6.3.1 HPLC chromatogram

HPLC chromatogram of standards was as shown in Figure 2-8. Adenine (4.50 min), adenosine (8.52 min), inosine (9.84 min), hypoxanthine (6.37 min) and uric acid (6.27 min) were well separated. The retention times of hypoxanthine and uric acid are similar therefore the two peaks overlap at 250 nm at low concentration (Figure 2-8 **B**). However the uric acid can be identified at 292 nm exclusively (Figure 2-8 **A, C**) from the rest metabolites which compromised to the interference from hypoxanthine (Figure 2-9). Hypoxanthine cannot be quantified for this reason but as substrate, it is not important in quantifying reaction progress as long as it is certain that it does not interfere with product (uric acid) quantification.

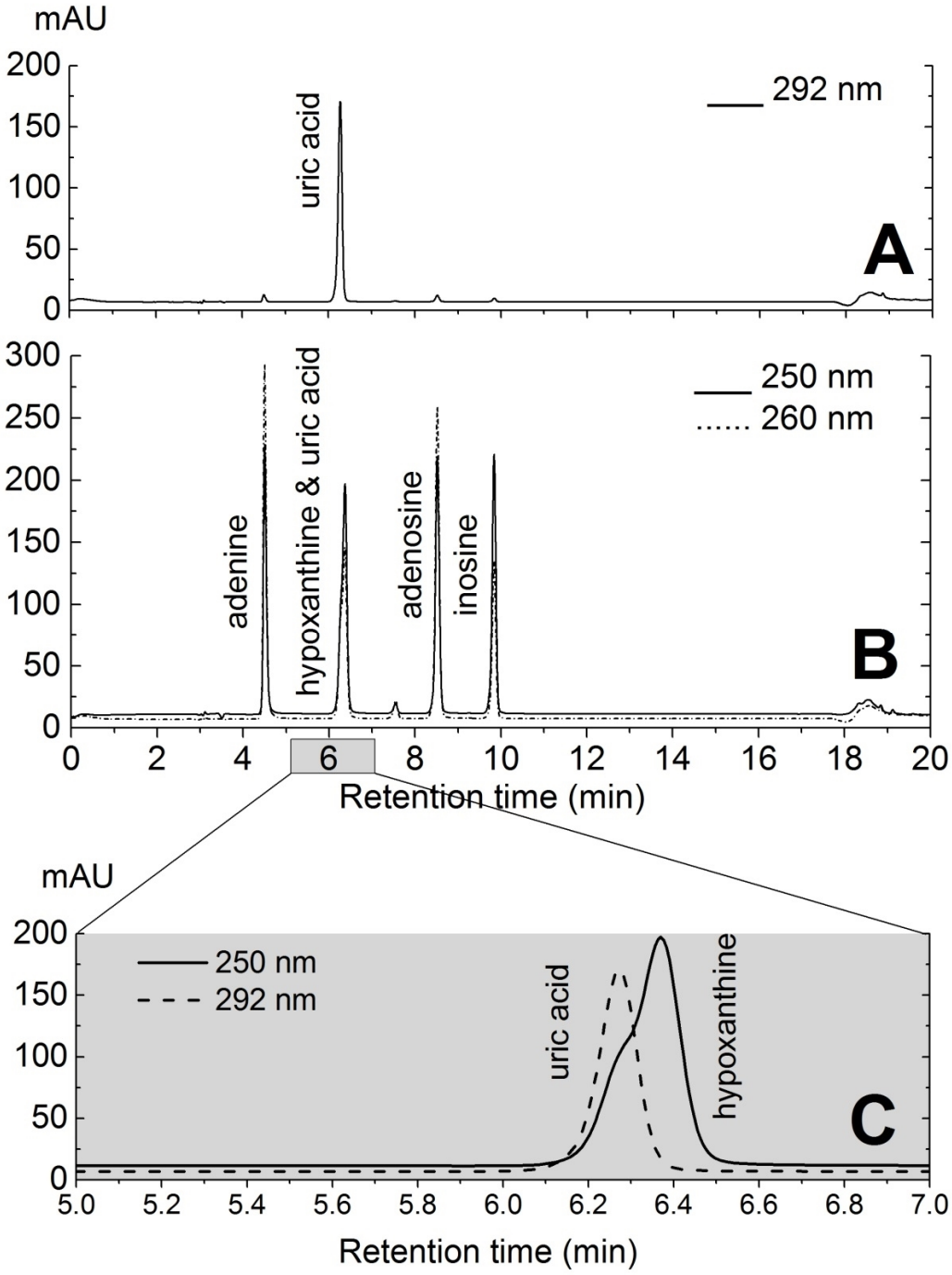


Figure 2-8 HPLC chromatogram of standards of (A) uric acid (6.27 min) at 292 nm, of (B) adenine (4.50 min), adenosine (8.52 min), inosine (9.84 min) and hypoxanthine (6.37 min) at 250 or 260 nm, (C) Peak identification of uric acid by zoom in from 5 min to 7 min. Overlap of the peaks of uric acid and hypoxanthine does not interfere the quantification of uric acid due to the difference of wavelength of maximum absorbance

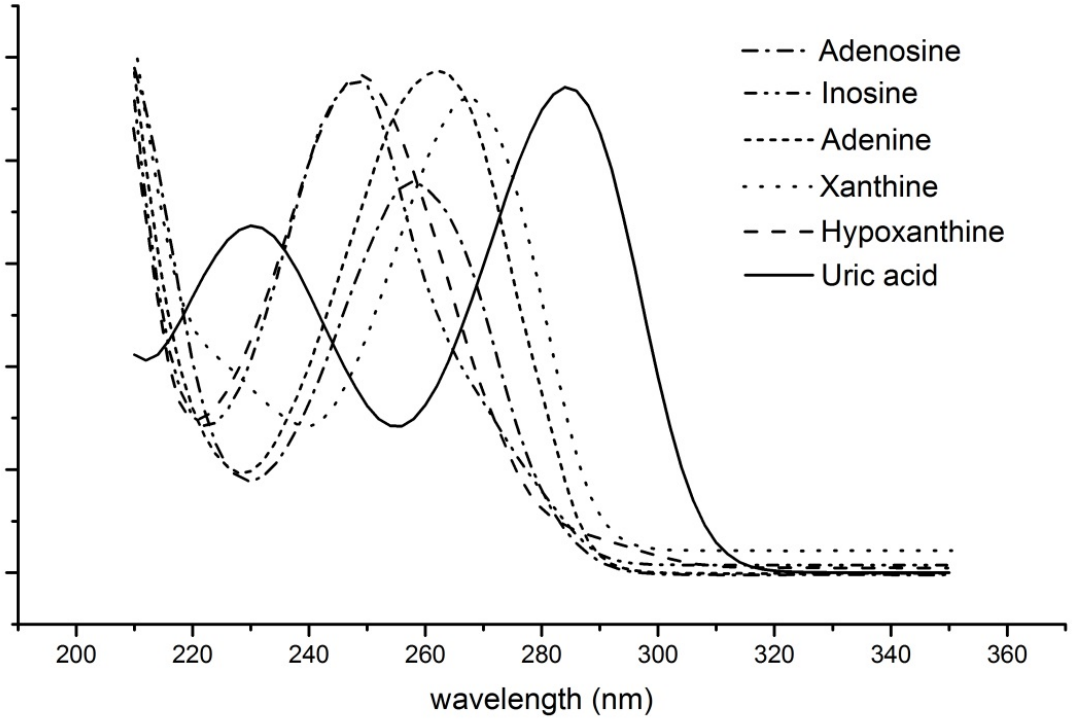


Figure 2-9 Overlay of UV spectra of adenosine, inosine, adenine, xanthine, hypoxanthine and uric acid. At 292 nm, only uric acid absorb extensively.

2.6.3.2 Linearity and sensitivity

Linearity of reagent responses was assessed by use of aqueous solutions of adenosine, inosine, hypoxanthine, xanthine and uric acid at concentration up to 500 pmol, or to 4 000 pmol. The responses are linear within this range, with a linear regression equation presented in Figure 2-10.

2.6.3.3 Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the equations below

$LOD=3S_a/b$,

$LOQ=10S_a/b$,

where S_a is the residual standard deviation of the linear regression of the response and b is the slope of the calibration curve (AUC $pmol^{-1}$).

The calculation of LOD and LOQ are presented in Table 2-1. Data suggests that the accuracy of detection is excellent for detecting small difference of product when conduct enzyme kinetic experiment.

Table 2-1 Limit of detection and limit of quantification of compounds with this HPLC method.

Compound	b ($pmol^{-1}$)	S_a	range (pmol)	LOD (pmol)	LOQ (pmol)
Adenosine	0.829	33.6	500-5000	122	405
Inosine	0.672	2.86	50-500	12.8	42.5
Adenine	0.632	6.38	1000	30.3	101
Xanthine	0.581	45.6	500-5000	236	785
Hypoxanthine	0.617	1.26	50-500	6.13	20.4
Uric acid	0.631	4.93	50-4000	23.4	78.2

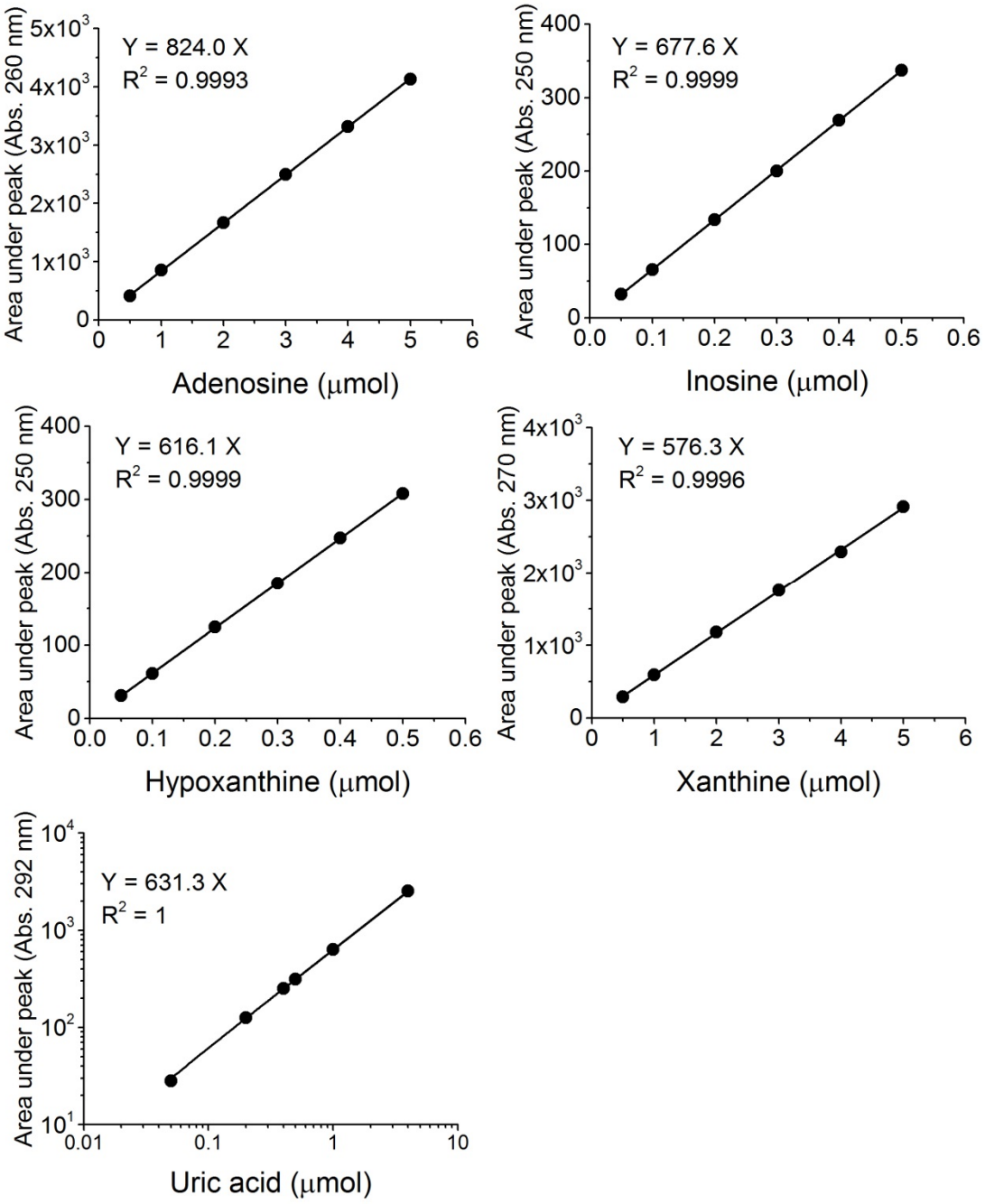


Figure 2-10 Calibration curve of purine derivatives quantified by HPLC-DAD at 260 nm (adenosine and adenine), 250 nm (inosine and hypoxanthine), 270 nm (xanthine) and 292 nm (uric acid).

2.6.4 Conclusion

We have developed a new HPLC method for quantitatively studying the kinetics and inhibition of human (mammalian) enzymes using body fluids (and pure enzyme system or using cell extracts). The same method can also be extended for screening a large number of compounds for the discovery of enzyme inhibitors.

2.7 Statistical analysis

All statistical analyses were performed using the SPSS statistics software (version 21; International Business Machines Corp., New York, USA). Normality of data distribution was checked with the Shapiro-Wilk test and data are normally distributed; independent samples t test was used to compare means between treatments. Data that were not normally distributed were compared using the Wilcoxon signed-rank tests. Relationships between variables were evaluated using Pearson′s correlation coefficient. All calculations were carried out with CI 95%, and differences were considered significant at $P<0.05$. Correlation coefficient r was calculated by the Pearson test. Unless otherwise indicated, the results were reported as mean values with their standard deviations.

Chapter 3. Comparison of the Urinary Excretion of Quercetin Glycosides from Red Onion and Aglycone from Dietary Supplements in Healthy Subjects a Randomised, Single-blinded, Cross-over Study

Abstract

Some intervention studies have shown that quercetin supplementation can regulate certain biomarkers, but it is not clear how the doses given relate to dietary quercetin (e.g. from onion). We conducted a two-period, two-sequence cross-over study to compare the bioavailability of quercetin when administered in the form of fresh red onion meal (naturally glycosylated quercetin) or dietary supplement (aglycone quercetin) under fasting conditions. Six healthy, non-smoking, adult males with BMI 22.7 ± 4.0 kg m^{-2} and age 35.3 ± 12.3 y were grouped to take the two study meals in random order. In each of the 2 study periods, one serving of onion soup (made from 100 g fresh red onion, providing 156.3 ± 3.4 μmol quercetin) or a single dose of a quercetin dihydrate tablet (1800 ± 150 μmol of quercetin) were administered following 3 d washout. Urine samples were collected up to 24 h, and after enzyme deconjugation, quercetin was quantified by LC-MS. The 24-h urinary excretion of quercetin (1.69 ± 0.79 μmol) for 100 g of red onion in soup was not significantly different to that (1.17 ± 0.44 μmol) for 500 mg quercetin supplement tablet (P = 0.065, paired t-test). This means that in practice, 500 mg of quercetin supplement can provide bioavailable quercetin comparable to ~100 g fresh red onion. These data allow intervention studies on quercetin giving either food or supplements to be more effectively compared.

3.1 Introduction

Quercetin is a flavonoid (class: flavonol) that is present at high levels in onions, apples and tea, in the form of a 3-*O*-glucoside, 4′-*O*-glucoside or 3,4′-*O*-diglucoside. Intervention studies using those foods to examine long term effects are rare, not only because of the extensive food preparation required with consistent composition, but also that volunteers grow tired of the same food for months which limits compliance.

Many studies using quercetin supplements (aglycone) in humans indicate effects on antioxidant status, oxidised LDL, inflammation and metabolism (summarised in Table 3-1). 500 mg quercetin supplementation twice per day improved the NIH (National Institution of Health) prostatitis symptom score after 30 d in 30 men with chronic pelvic pain syndrome (139) and improved cystitis symptoms after 28 d in 22 interstitial cystitis patients (140). 150 mg of quercetin significantly affected expression of key genes, glycolipid catabolism, cell proliferation and apoptosis after 42 d intake in 20 subjects with a cardiovascular risk phenotype (141), and decreased systolic blood pressure, serum HDL-cholesterol, and plasma concentrations of atherogenic oxidised LDL in 96 healthy subjects (78). Daily consumption of 100 mg quercetin for 70 d reduced serum total and LDL/HDL cholesterol, glucose and systolic and diastolic blood pressure in 49 health subjects (80). 14 d of daily dose of 30 mg quercetin improved the oxidative resistance of LDL (109) and significantly decreased tissue inhibitor of metallopeptidase-1 (TIMP-1) in plasma and lymphocyte mRNA (142) in healthy subjects.

Whether dietary quercetin could achieve the same effects remains unknown since the bioavailability of quercetin aglycone in supplements is much lower than quercetin glucoside (143) and this makes interpretation and comparison of studies using

supplements or foods difficult. Although studies have delaminated the correlation between quercetin intake and urinary excretion (112, 116, 127, 143-150), it was never done with quercetin dihydrate supplements at dosage up to 500 mg or in a crossover trial in comparison to onion meal. Previous data suggests that a 10-fold dosage difference might yield similar urinary excretion and thus present study adopted 500 mg quercetin tablet and 47 mg quercetin from 100 g of fresh red onion. Although limited, clinical data relating to the safety of quercetin were available from studies in which quercetin or plant extracts containing quercetin glycosides were provided to study subjects for oral ingestion for periods of up to 12 weeks at dose levels ranging between 3 and 1000 mg quercetin d^{-1} (139, 151-153). None of them reported significant adverse health effects following oral administration of quercetin to humans at doses up to 1000 mg d^{-1} for up to 12 weeks (154). This randomised, single-blind, two period, two sequence, cross-over intervention study, conducted under fasting conditions with a 3 d washout period, compared different dosages of quercetin from dietary supplements (aglycone) and fresh red onion (naturally conjugated as glucosides). This comparison allows to calculate the dosage of different quercetin sources needed to achieve similar effective absorption in healthy subjects to aid in the design of meaningful intervention studies.

Table 3-1 Human intervention studies on quercetin supplementation [a]

dose per day [b]	days	No. of subjects per group [c]	Biomarkers significantly affected	Biomarkers not significantly affected	Ref
500 mg x 2	30	30 men with chronic pelvic pain syndrome	Improvement in NIH prostatitis symptom score		(139)
500 mg x 2	28	22 interstitial cystitis patients	Improvement in cystitis symptoms	No side effects or adverse reactions	(140)
250 mg x 4	21	63		Blood antioxidant capacity or plasma lipid during ultramarathon	(141)
150 mg	14	12		Serum uric acid, plasma α- and γ -tocopherols, oxidised LDL, tumour necrosis factor-α, serum lipids and lipoproteins, plasma antioxidant capacity, body composition, or resting energy expenditure supplementation	(89)
150 mg	42	42 with cardiovascular risk phenotype	Gene expression of *C1GALT1*, O-glycan biosynthesis; *GM2A*, glycolipid catabolism; *HDGF*, cell proliferation; *SERPINB9*, apoptosis	Gene expression of the other target genes	(155)
150 mg	42	93 overweight or obese	Decrease of systolic blood pressure, serum HDL, plasma concentrations of atherogenic oxidised LDL	Total cholesterol, TAG, LDL/HDL, TAG/HDL, TNF-α, C-reactive protein, nutritional status, blood parameters of liver and kidney function, haematology or serum electrolytes	(78)
150 mg	56	49 men	Decrease of waist circumference, postprandial systolic blood pressure and postprandial triacylglycerol concentrations; increase of HDL-cholesterol and TNFα	Endothelial function	(156)
100 mg	70	49	Increase of HDL; decrease of serum total cholesterol and LDL; decrease of systolic and diastolic blood pressure, blood glucose	Inflammatory IL-6, sVCAM-1	(80)

(continued from Table 3-1)

dose per day [b]	days	No. of subjects per group [c]	Biomarkers significantly affected	Biomarkers not significantly affected	Ref
30 mg	14	10	Improved oxidative resistance of LDL	Plasma triglycerides, HDL or LDL	(109)
30 mg	14	4	Decrease in TIMP-1 plasma protein and lymphocyte mRNA	TIMP-2 and matrix metalloprotein-2 lymphocyte mRNA or plasma protein	(142)
500 mg quercetin-3-*O*-glucoside	7	15		Repeated-sprint performance, percent fatigue decrement, blood XO activity, IL-6 or uric acid	(157)
250 mg quercetin and other polyphenols	28	27		HDL/LDL cholesterol, platelet aggregation, plasma thromboxane B2, blood pressure, resting heart rate	(83)
100 mg of quercetin with 50 mg of each [resveratrol,pterostilbene, δ-tocotrienol, nicotinic acid]	28	81 Elderly with normal total serum cholesterol	decreased serum nitric oxide, C-reactive protein, uric acid levels, and γ-glutamyltransferase activity		(158)
100 mg [curcumin, 400 mg]	1	15 renal transplant recipients	Improved renal function only in patients with elevated serum creatinine, improved urine output and lowered isoprostanes in patients with delayed graft function	Blood pressure, calcineurin levels	(159)

[a] Some of the entries were derived from (160)

[b] Quercetin aglycone, unless otherwise stated.

[c] Healthy subjects, unless otherwise stated.

Abbreviation: NIH, national institution of health; C1GALT1, Core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase; GM2A, ganglioside monosialic 2 activator; HDGF, hepatoma-derived growth factor; SERPINB9, Serpin B9; IL-6, Interleukin 6; sVCAM-1, soluble vascular cell adhesion molecule 1; TIMP-1, tissue inhibitor of metallopeptidase -1; TIMP-2, tissue inhibitor of metallopeptidase-2.

3.2 Subjects and methods

3.2.1 Subjects

Six healthy male volunteers participated in the present study. They were non-smokers, not on any medication, aged 35.3 ± 12.3 y (range 20.0 - 48.9) and had a BMI of 22.7 ± 4.0 kg m^{-2} (range 18.5 - 29.9). Exclusion criteria were metabolic and endocrine diseases, malabsorption syndromes, alcohol abuse, use of dietary supplements or any form of regular medication. All subjects were asked to maintain their normal lifestyle and usual extent of physical activities throughout the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the MaPS and Engineering joint Faculty Research Ethics Committee (MEEC 12-019), University of Leeds, UK. Written informed consent was obtained from all subjects.

3.2.2 Study design

The study was conducted with a single-blinded (researcher blind), diet-controlled, cross-over design. Subjects were required to avoid flavonols in the diet for 3 d washout prior to the breakfast and for 1 d during 24-h urine collection. For this purpose, a list of food items rich in flavonols was given to each participant as a guideline. This diet excluded vegetables like onion, spring onion, shallots, leeks, chives, spinach, kale, endive, lettuce, broccoli, asparagus, tomato, olive, pepper, courgette, green beans, broad bean, common bean and galangal; all types of berries and currants, apple, apricot, grape and plum; all types of alcoholic beverages and tea;

and propolis supplements. On the morning of the study, baseline urine was collected immediately before breakfast and 24-h urine was collected following the breakfast. The six participants were randomly assigned to treatment group A or B (n=5 and 1). Group A ingested one quercetin supplement (1800 ± 150 µmol quercetin equivalents) with a standard breakfast; after another 3 d washout, they ingested onion-enriched soup (156.3 ± 3.4 µmol quercetin equivalents). Group B had treatments in reverse order to Group A. The baseline urine was used as compliance control and no apparent deviation from the low-quercetin diet was observed. Accordingly, the concentrations of quercetin were very low (0.095 ± 0.037 µM, SEM) in baseline urine.

3.2.3 Preparation of standard breakfasts

Red Onion Soup Fresh local red onions were washed, skinned and sliced after removing the top and bottom of the bulb. The slices were frozen at -20℃ for 1 h and quickly minced with a kitchen electronic blender while still frozen. 100 g of the onion mince was stored individually at -20℃ until the day of the human study. A breakfast was freshly made consisting of one portion of instant tomato soup mix 52 g (Slim a Soup, Batchelorsrange, UK) and 100 g of frozen onion by adding hot water and stirring into a soup-paste after heating in a 800 W microwave for 1 min. The standard meal was served with buttered white bread. The soup powder did not contain any quercetin.

Supplement Quercetin dihydrate tablets (500 mg) were Purchased from Nature′s Best (Kent, UK) without further processing. One tablet was consumed with buttered white bread and instant tomato soup as above.

3.2.4 Laboratory analysis

Method of quantification of quercetin in study food was detailed in Section 2.3. Protocol for processing of urine samples and analysis of quercetin was as described in Section 2.4.

3.2.5 Statistical analysis

All statistical analyses were performed using the SPSS statistics software (version 21; International Business Machines Corp., New York, USA). Normality of data distribution was checked with the Shapiro–Wilk test and data are normally distributed; independent samples t test was used to compare means between treatments. All calculations were carried out with CI 95%, and differences were considered significant at $P < 0.05$. Unless otherwise indicated, the results were reported as mean values with their standard deviations.

3.3 Results

3.3.1 Control variables and intervention compliance

The baseline urine was used as compliance control and no deviation from the low-quercetin diet was observed. Accordingly, the concentration of quercetin was very low 0.095 ± 0.037 μM (SEM) in baseline urine.

3.3.2 Quercetin content of the study meals

Based on individual analysis of compounds, red onion soup contained 156.3 ± 3.4 µmol quercetin equivalents per portion made from 100 g fresh red onion (quercetin 3, 4′-*O*-diglucoside 59.3% and quercetin 4′-*O*-glucoside 40.7%, substance equivalents). Quercetin dihydrate tablets contained 1800 ± 150 µmol of quercetin (100% quercetin aglycone).

3.3.3 Urinary excretion of quercetin

The LC-MS chromatogram of quercetin and methylquercetin after β-glucuronidase and fulfatase hydrolysis of human urine can be found in Section 2.4.2.2, Figure Figure 2-6. The 24-h urinary excretion of quercetin for each individual after consuming a meal of 100 g red onion or a single study tablet is shown in Table 3-2 and Figure 3-1. 24-h urinary excretion of quercetin after consuming red onion soup, made from 100 g fresh red onion, was 1.69 ± 0.79 µmol (of which 72.9 ± 6.0% of quercetin, 7.70 ± 5.92% of isorhamnetin and 19.4 ± 5.95% of tamarixetin), and that from the 500 mg quercetin supplement was 1.17 ± 0.44 µmol (71.4 ± 11.1%, 7.54 ± 6.38% and 21.0 ± 11.7%). No significant difference in quercetin excretion was observed within subject ($P = 0.065$, paired t test) or among populations ($P = 0.189$, independent t test, n=6) for the total quercetin.

Table 3-2 Total urinary excretion of quercetin and methyl quercetin (mean ± SEM) and the proportion of each metabolites (%)

Subject	Treatment	Total quercetin, (μmol)	24-hour urinary excretion,%		
			Quercetin	Isorhamnetin	Tamarixetin
369	Supplement	1.43 ± 0.11	74.6 ± 6.9	4.69 ± 2.77	20.7 ± 2.43
	Onion soup	1.73 ± 0.12	82.5 ± 6.3	3.22 ± 2.01	14.2 ± 1.91
434	Supplement	1.07 ± 0.03	83.3 ± 1.8	12.1 ± 2.53	4.58 ± 2.89
	Onion soup	0.87 ± 0.06	71.1 ± 6.9	13.0 ± 7.54	15.9 ± 9.20
442	Supplement	0.82 ± 0.05	77.4 ± 6.3	7.77 ± 2.73	14.9 ± 2.81
	Onion soup	1.72 ± 0.08	71.8 ± 3.5	8.57 ± 0.38	19.6 ± 1.58
569	Supplement	1.85 ± 0.18	54.8 ± 5.3	17.5 ± 0.85	27.8 ± 3.74
	Onion soup	3.17 ± 0.33	65.8 ± 9.3	15.6 ± 0.92	18.6 ± 2.11
723	Supplement	1.17 ± 0.13	60.9 ± 9.6	0.00 ± 0.00	39.1 ± 1.65
	Onion soup	1.37 ± 0.12	69.1 ± 7.8	0.00 ± 0.00	30.9 ± 1.32
959	Supplement	0.66 ± 0.06	77.7 ± 8.0	3.18 ± 1.94	19.1 ± 5.31
	Onion soup	1.26 ± 0.06	76.9 ± 3.9	5.74 ± 0.24	17.3 ± 0.72
Mean ± SD	Supplement	1.17 ± 0.44	71.4 ± 11.1	7.54 ± 6.38	21.0 ± 11.7
	Onion soup	1.69 ± 0.79	72.9 ± 6.0	7.70 ± 5.92	19.4 ± 5.95

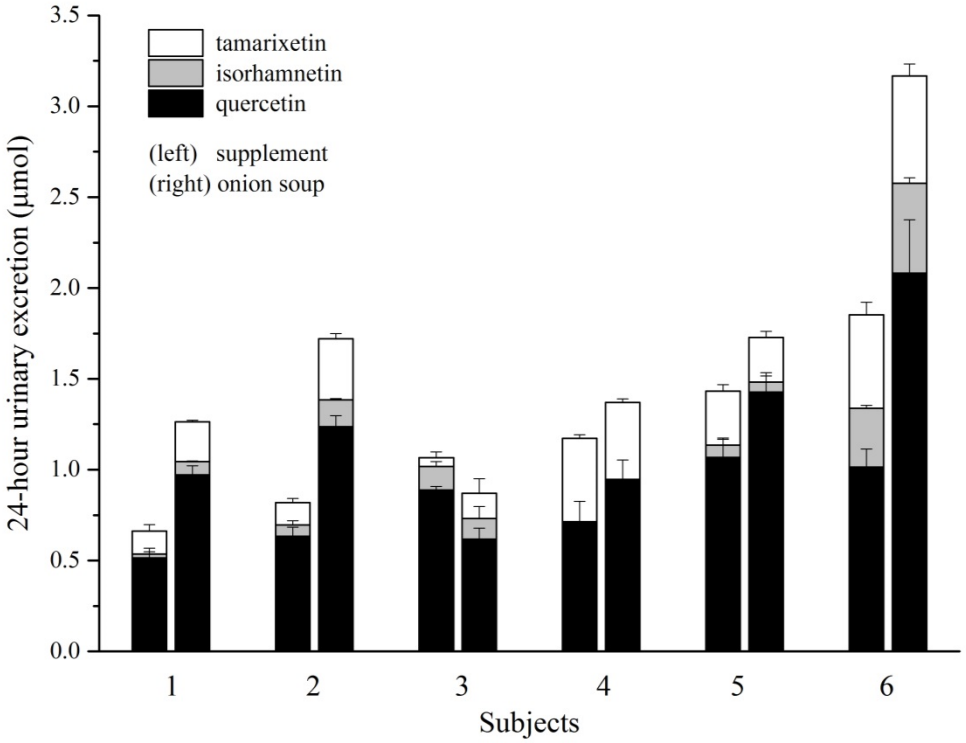


Figure 3-1 Urinary excretion of quercetin and methyl quercetin (mean ± SEM). 1800 ± 150 µmol quercetin from supplements or 156.3 ± 3.4 µmol quercetin from red onion soup was provided to each individual on separate occasions. Metabolites of methyl-, glucuronyl-, glucosyl- and sulfo-conjugates of quercetin in human urine were hydrolysed to quercetin and the monomethylated derivatives isorhamnetin (3′-*O*-methylquercetin) and tamarixetin (4′-*O*-methylquercetin) using β-glucuronidase and sulfatase (detailed in Section 2.4.2).

3.4 Discussion

The aim of the present randomised, single-blind, two-period, two-sequence, cross-over intervention study, conducted under fasting conditions with a 3 d washout period, was to compare the absorption of quercetin from fresh red onion (156.3 ± 3.4 µmol, naturally conjugated) and dietary supplements (1800 ± 150 µmol, aglycone) in healthy subjects. This resulted in similar amounts of quercetin being absorbed as assessed by quantifying 24-h urinary excretion of quercetin.

Quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans (89), and incorporation of the washout period was designed to diminish the impact of carry-over effects. According to other reports, the plasma concentrations after quercetin-4′-*O*-glucoside supplementation (equivalent to 100 mg quercetin) reached a peak after 0.7 ± 0.3 h and the apparent elimination half-life was about 11 h (149). Quercetin accumulated in plasma after repeated intake of onion (elimination half-life of 28 h), apples (elimination half-life of 23 h) and tea (143), but a steady state concentration in plasma was reached after about 4 d (147) and so plasma concentrations would reflect the intake of only the previous 3 d. For this reason, the length of the washout period was designed to be 3 d.

24-h urinary excretion of quercetin after consumption of red onion (mainly glucoside conjugated quercetin) and supplement (quercetin aglycone) was significantly different when compared by percentage dose ($P<0.0001$, paired t test, 1.08 ± 0.51% and 0.065 ± 0.024%). These values are consistent with other human studies. For example, 24-h urinary excretion of quercetin as a proportion of intake after consumption of conjugated quercetin from fried onion was 0.8 ± 0.4% (116) and 1.1 ± 0.5% (145). 13-h urinary excretion of quercetin as a proportion of intake from

onion was 0.31 ± 0.14% and that from 100 mg quercetin aglycone was 0.12 ± 0.08% (148). A systematic review confirmed that the correlation between the dose of quercetin ingested and its recovery in 24-h urine samples in humans is on average 1.5 % but with recovery ranging from 0.07 to 8.4% (161). Table 3-3 summarises the maximum plasma concentration and the urinary excretion of quercetin after consuming quercetin rich food, quercetin-3-O-rutinoside supplement or quercetin supplement and Figure 3-2 shows the relevance between urinary excretion and quercetin intake, within category of each form. It should be noted that the amount in urine reflects the minimum amount of quercetin absorbed, and other experiments such as intestinal perfusion show that the actual amount absorbed is considerably higher (162). Nevertheless, the amount in urine is a suitable biomarker for some polyphenols since it allows comparisons between different foods or supplements, and between individuals for the same compound (143, 163). The low amount of compounds such as quercetin in the urine means that the remainder of the dose is either excreted in the bile, in the feces or may end up as chemically-altered microbial metabolites, which can then be absorbed in the colon (164). Typical microbial metabolites of quercetin are 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid and 3-hydroxyphenylacetic acid (114). After absorption, these compounds participate in metabolism and so may ultimately contribute to the physiological effects of quercetin (122). Even though the amount of intact quercetin in urine after these dosages of supplementation and onion intake were similar, it is likely that the supplement could deliver higher concentrations of microbial metabolites to the blood.

Supplements have consistent quality and a relatively long shelf life, and are preferred in many intervention studies since they remove the complication of the activity of

other components in the food, and are well tolerated long-term by volunteers. However, it is important to know the bio-equivalence of quercetin-containing foods and supplements, to allow for future design and to compare existing studies. According to the result of this study in practical terms, 100 g of onion gives a comparable amount of quercetin in the urine to a 500 mg quercetin aglycone supplement. Based on this data, we can compare reported intervention studies on quercetin from onions and from supplements (Table 3-4), which lists the human intervention studies using dietary sources of quercetin. The obvious difference between the dose ranges between Table 3-1 and Table 3-4 may explain, for example, why plasma LDL/HDL reduction after 14 d administration was observed by Kim *et al*. (165) but not by Egert *et al*. (89) or Chopra *et al*. (109). This pilot study provides a guideline for design of future human studies when using supplements and foods, and also facilitates comparison of studies in existing literature.

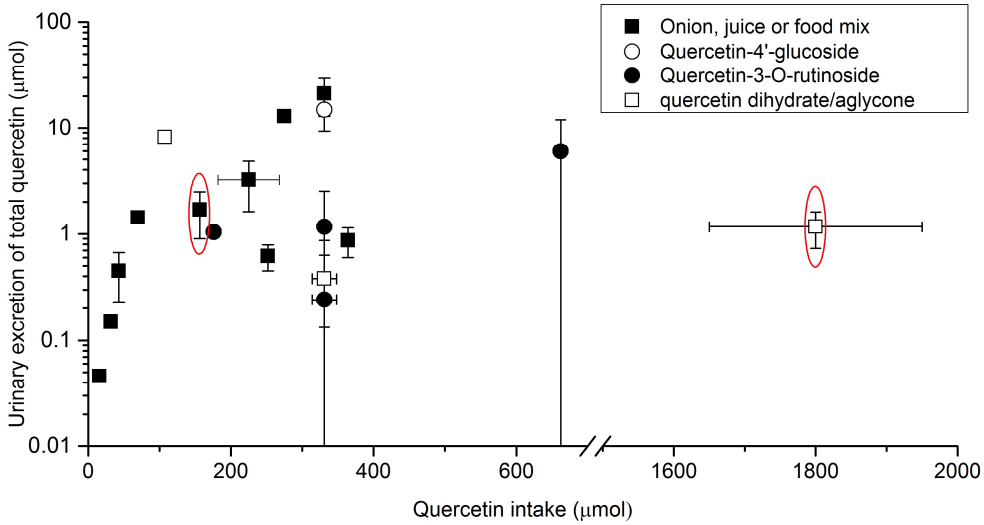


Figure 3-2 Comparison of bioavailability among human trials. 24-h (or 13-h) urinary excretion of quercetin from onion or supplement. Data were extracted from (112, 116, 127, 143-150) and present study (red circled).

Table 3-3 Bioavailability of quercetin from onion or supplement.

	Quercetin intake (μmol)	24 h Urinary excretion (μmol)	Urinary excretion as portion of intake (%)	ref
Juice	15.9	0.046	0.29	(144)
Juice	31.8	0.149	0.47	(144)
Onion	43	0.45 ± 0.22	1.1 ± 0.5	(145)
food mix	69.5	1.43	2.06	(146)
Onion	156.3 ± 3.4	1.69 ± 0.79	1.08 ± 0.51	Present study
Onion	225 ± 43	3.22 ± 1.6	1.39 ± 0.49	(143)
Onion	252	0.62 ± 0.17	0.24 ± 0.07	(147)
Onion	275 ± 8.8	12.9	4.7	(112)
Onion (13h)	295 ± 46	0.911 ± 0.427	0.31 ± 0.14	(148)
Onion	331	21.2 ± 8.3	6.4 ± 2.5	(149)
Quercetin-4′-glucoside	331	14.9 ± 5.6	4.5 ± 1.7	(149)
Onion	364	0.87 ± 0.27	0.24 ± 0.07	(147)
Onion	411 ± 74	3.29 ± 0.59	0.8 ± 0.4	(116)
Quercetin-3-*O*-rutinoside	176	1.04 ± 0.04	0.59 ± 0.02	(127)
Quercetin-3-*O*-rutinoside	331 ± 7	1.17 ± 1.34	0.35 ± 0.41	(143)
Quercetin-3-*O*-rutinoside (13h)	331 ± 17	0.241 ± 0.629	0.07 ± 0.19	(148)
Quercetin-3-*O*-rutinoside	662	5.96 ± 5.96	0.9 ± 0.9	(149)
Quercetin aglycone	107.6	8.18	7.6	(150)
Quercetin aglycone (13h)	331 ± 17	0.381 ± 0.248	0.12 ± 0.08	(148)
Quercetin dihydrate	1800 ± 150	1.17 ± 0.44	0.065 ± 0.024	Present study

Note: All data are presented by mean ± SD.

Table 3-4 Human intervention studies on dietary quercetin [a]

Dose per day [b]	Supplement equivalent [c]	Days	No. of subjects per group [d]	Biomarkers significantly affected	Biomarkers not significantly affected	Ref
76-110 mg quercetin and other flavonols from 400 g onion (with tomato sauce) + 6 cups of tea	1200-1800 mg with other	14	10 type 2 diabetic patients	Decrease oxidative damage to lymphocyte DNA	Fasting plasma glucose, fructoseamine, vitamin C, carotenoids, α-tocopherol, urate, albumin and bilirubin	(96)
200 g onion	1500 mg	1	6 female	Increase resistance of lymphocyte DNA to strand breakage, decrease in urinary 8-hydroxy-2′-deoxyguanosine	Urinary malondialdehyde	(166)
21 mg dietary quercetin, 9 mg dietary kaempferol	350 mg with other	1	19 female	Increase in erythrocyte superoxide dismutase activity, decrease in lymphocyte DNA damage (tail moment)	Plasma α-tocopherol or β-carotene	(146)
51 mg quercetin from 4.3 g onion extract	850 mg	30	23 male with oral maltose load induced postprandial endothelial dysfunction	Increase postprandial flow-mediated vasodilation (FMD) responses	Fasting FMD systemic or forearm hemodynamic	(167)
100 mg quercetin + 128 mg other flavonoids, onion peel extract	1660 mg with other	14	12 female	Decrease total cholesterol level, LDL cholesterol and atherogenic index	Erythrocyte antioxidant enzymes, lipid peroxidation markers, plasma antioxidant vitamin (retinol, tocopherol, carotenoids, coenzyme Q10), *ex vivo* H_2O_2-provoked oxidative DNA damage	(165)

[a] Some of the entries were derived from (160)

[b] Quercetin aglycone, unless otherwise stated.

[c] Calculation is based on 16.6-fold since 100 mg quercetin aglycone equivalents from onions would be comparable to about 1660 mg quercetin aglycones from supplements, according to this study.

[d] Healthy subjects, unless otherwise stated.

Chapter 4. Effect of Quercetin Supplements on Healthy Males: a Four-Week Randomised Cross-Over Trial

Abstract

Hypertension, diabetes and obesity are often accompanied by hyperuricemia, which, if left unattended, may lead to gout. Allopurinol is usually administered to mediate hyperuricemia during gout treatment but is associated with side effects including joint problems and rashes. We explored non-drug strategies to reduce plasma uric acid using the naturally occurring biologically active molecule, quercetin, which inhibits XO, a key enzyme in the production of uric acid. To determine effects of one month oral supplementation of quercetin on plasma uric acid level, on blood pressure and on fasting glucose, a randomised, double-blinded, placebo-controlled, cross-over trial (between February 2013 to April 2014) involving 22 healthy males aged 19 to 60 years with baseline plasma uric acid concentration in the higher range (but still considered healthy; 339 ± 51 μM) was conducted with one quercetin tablet containing 500 mg quercetin daily for 4 weeks, compared to placebo (one lactose tablet), once daily for 4 weeks, with a 4-week washout period between treatments. The order of the treatments was randomised. At the end of 4 weeks of daily supplementation of quercetin, plasma uric acid levels were significantly lowered by -26.5 μM (95% confidence interval [CI], -7.6 to -45.5 μM; P=0.008), without affecting fasting glucose, urinary excretion of uric acid or blood pressure. In conclusion, daily supplementation of 500 mg quercetin for 4 weeks significantly reduces plasma uric acid concentrations in healthy males.

4.1 Introduction

Hyperuricemia is the strongest determinant risk factor for gout, an inflammatory arthritis caused by uric acid crystals (1). Hyperuricemia is common in patients who develop diabetes (2), obesity (3), hyperglycemia (4, 5), hypertension (6), stroke (7), heart disease (8, 9), kidney dysfunction (10, 11) and cancer (12), although it is often unattended until their first, if any, gout attack. Gout prevalence increased from ~0.5 to ~3% between 1960 and 2010 in the US (13) and other areas (14) accompanied by a parallel increase in the number of individuals with hyperuricemia (15, 16). The fact that 25-34 is the age group with highest blood uric acid level (22) may suggest that hyperuricemia precedes the development of metabolic syndromes (23). Interestingly, allopurinol, a uric acid lowering agent in gout therapy, has a protective effect on hypertension, which suggests that excess uric acid synthesis is a casual factor in developing hypertension (20).

Some dietary factors, including purines, alcohol and fructose, also elevate blood uric acid (48-51). For example, chronic exposure to fructose can lead to development of hyperuricemia (54). Fructose phosphorylation by fructokinase causes intracellular phosphate depletion leading to the activation of deaminase, which converts the adenosine monophosphate to inosine monophosphate, inosine, and hypoxanthine, xanthine and ultimately uric acid through XOR (Figure 1-3). Chronic hyperuricemia may also up-regulate fructokinase expression therefore leading to the amplification of the lipogenic effects of fructose in human hepatocytes (55). Apparently, fructose induced insulin resistance occurs as a result of fructose-induced hyperuricemia (168, 169). Studies in healthy humans (66, 67) and in animal models (68) substantiate the importance of increased insulin resistance to hyperuricemia, and *vice versa*.

Recently, quercetin was identified to be able to induce insulin secretion (170), protect INS-1 pancreatic β-cells against oxidative damage (171) and reduce insulin resistance in adipose tissue and skeletal muscle (172).

Apparently, XOR, as the major enzyme in uric acid production, became an extensively pharmacologically targeted enzyme in extensive exploration of inhibition strategies, including allopurinol (173). The plant-derived flavonoid, quercetin, and its metabolites inhibit XO *in vitro* (111) and regulate blood uric acid level *in vivo* in animal studies (99-101), yet whether uric acid metabolism could be similarly affected in humans is still highly debatable (89, 94-98).

Therefore, we performed this randomised, double-blindeded, placebo-controlled, cross-over trial to test the hypothesis that habitual 500 mg d^{-1} of oral quercetin supplementation might result in a long-term reduction in plasma uric acid in subjects with non-optimal blood uric acid.

4.2 Materials and methods

4.2.1 Sample size calculation

A sample size of 17 was estimated to be required to detect a 10% difference for the primary efficacy variable, plasma concentration of uric acid, and to achieve 80% power to meet the two-tailed equality criteria between quercetin and placebo. A significance level of 0.05 from paired 2-sample t test was set for this two-sequence, two period cross-over design (174). Coefficient of variation of blood uric acid level among population was about 20% according to previous cohort reports (78, 175, 176) and 10% of coefficient of variation among study population was estimated since

we pre-screened and selected the upper 50% of the volunteers in plasma uric acid concentration. About 50 ± 10 volunteers shall be screened in the first stage. The volunteers with plasma uric acid level exceeding 300 μM would be selected.

4.2.2 Subjects

22 healthy males were eligibly assigned and successfully compliant to the complete study. Selection criteria included being apparently healthy, age between 19 to 65, BMI between 18.5 and 29.9 kg m^{-2}, non-smoking and not a heavy drinker (less than 3 units of alcohol regularly per day). Volunteers with diagnosed gout and/or kidney stone, who were experiencing intestinal disorders, or whose plasma uric acid concentration was lower than 300 μM, were excluded. The study was conducted according to the guidelines laid down in the declaration of Helsinki of 1975 as revised in 1983 and all procedures involving human subjects were in accordance with the ethical standards of the University of Leeds, MaPS and Engineering joint Faculty Research Ethics Committee (MEEC12-019), UK. Written informed consent was obtained from each of the subjects before commencement of the study. All data were collected and analysed in the School of Food Science and Nutrition in the University of Leeds, UK.

4.2.3 Study design

The main goal of the present study was to examine the chronic effect of quercetin on plasma uric acid concentration, as well as on biochemical markers associated with chronic pre-diabetes and hypertension. For this purpose, the study was a randomised, double-blinded, placebo controlled, cross-over, 4-week intervention trial with 2 treatment groups, with daily consumption of either quercetin dihydrate tablet (500

mg stated, actual measured 544 mg, purchased from Nature′s Best, Kent, UK) (177) or placebo (the placebo formulation is a white oval tablet and contain lactose monohydrate, magnesium stearate and cellulose, purchased from Fagron, Barsbuttel, Germany). There was a 4-week washout period between each treatment. Blood and urine samples were taken before, during and the end of each study phase (Figure 4-1). Each participant was independently and randomly assigned in one of two groups, receiving both treatments in one order or another.

During the protocol, volunteers made 6 visits to the research unit at day 0, 14 and 28 of each experimental period for measurement and sample collection. In practice, with 24-hour urine collected at home during the day and night before the visit, overnight-fasted subject arrived at the research unit between 7-10 am. A fasting blood sample was collected, followed by questionnaires and measurements of weight, height and blood pressure. Subjects received a light meal and the study tablets before leaving the research unit. All participants were assured not to exceed the dosage described in this study. Subjects were asked to maintain their lifestyle and their normal dietary habits from 4 weeks before the first visit till the end of the entire study. Compliance was assessed at the end of each 4-week period by call back questionnaires recording date of missing dose, changes of physical activity and intensity, use of exotic diet or non-routine medications, and the occurrence of any side effects. Subjects were also asked to return the unconsumed tablets at each follow-up visit.

Intervention was randomised independently by a coin toss for each volunteer who received a random 3 digital code. A decode list (participant identification and subject code) was kept by a third person in order to blind the researcher assessing outcomes.

The size and shape of study tablets were the same and of different colour, and participants were not aware of the identification of the two types of study tablets.

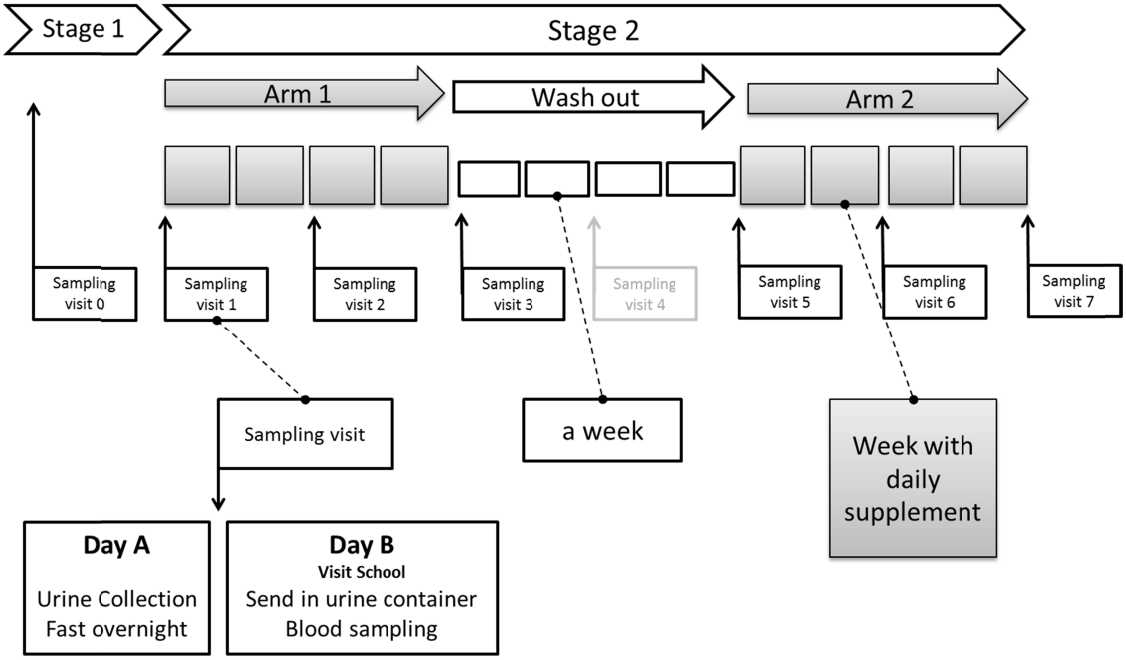


Figure 4-1 Diagram of research program as appears on volunteer information sheet.

4.2.4 Sample collection

Blood pressure was measured on the left upper arm in a quiet room at normal temperature 25±3℃, with use of a cuff-less upper arm blood pressure monitor (Panasonic Co., Tokyo, Japan). Before blood pressure recording were made, participants rested 15 min in a seated position. At each assessment, 3 consecutive blood pressure readings were recorded at 5 min intervals. The average of these measures was used for analysis.

Venous blood was collected following a standard venepuncture protocol into a sodium fluoride/potassium oxalate blood collection tube (Greiner-Bio-One, Austria). Blood samples were immediately centrifuged at 3 000 g, 4℃ for 10 min and aliquots

were stored at -80℃ until analysis. 24-hour urine samples were collected by volunteers in 3 L sterile urine container (Simport, Beloeil, Canada) which contained 3 g of L-ascorbic acid (MP Biomedicals, France). The urine samples were weighed before centrifugation at 2 000 g, 4℃ for 10 min before storage at -20℃. Urine samples for uric acid assay was 10-fold diluted before storage at -80℃. Urinary quercetin was quantified by HPLC-ESI/MS as previously described in Section 2.4 (177).

4.2.5 Uric acid assay

Assessment of uric acid in plasma and urine samples was by a specific coupled enzyme reaction, followed by a colorimetric determination at 520 nm (128). The protocol was modified for use in a 96-well plate reader (BMGlabtech, Germany) for high-throughput and improved accuracy. Within-run variation was 1.99 ± 1.20%, and between-run variation was 2.17 ± 0.52%. Recovery was 92.8 ± 1.6% for plasma and 80.4 ± 3.8% for 10-fold diluted urine. Calibration curve was prepared for each plate, with a regression curve of 0.550 ± 0.003 mM^{-1} uric acid with $R^2 > 0.999$ and a maximum concentration of 1.0 mM. The protocol is detailed in Section 2.1.

4.2.6 Measurement of blood glucose

Plasma glucose was measured with a commercial hexokinase assay kit for D-glucose (Sigma-Aldrich, St. Louis, USA). The protocol was modified for use in a 96-well plate reader. Within-run variation was 4.29 ± 2.21% and between-run variation was 3.33 ± 2.51%. Recovery was 104 ± 8%. Calibration curve was prepared for each plate, with a regression curve of 0.923 ± 0.006 per mg mL^{-1}, D-glucose with $R^2 \geq$

0.999 and a maximum concentration of 1.5 mg mL^{-1}. The protocol is detailed in Section 2.2.

4.2.7 Statistical analysis

Normality of data distribution was tested by Shapiro-Wilk tests. The paired 2-sample t test was used for comparison of normally distributed data. Data that were not normally distributed were compared using the Wilcoxon signed-rank tests. Relationships between variables were evaluated using Pearson′s correlation coefficient. In all cases, a value for $P \leq 0.05$ (2-tailed) was taken to indicate a significant effect. Unless otherwise indicated, results are expressed as mean values and standard deviations (SD). All statistical analyses were performed using the SPSS statistics software (version 21; International Business Machines Corp., New York, USA).

4.3 Results

4.3.1 Subjects

During the recruitment, among the potential volunteers who expressed their interest in participation, 54 provided contact information, measurements of height and weight, age, history of disease, and written consent. 2 failed to donate blood at this stage because of fear of needles or difficulty in locating vein, so they were no longer continuing in the following study. 52 of them successfully donate a single fasting blood sample for uric acid assay. They aged 28.6 ± 11.6 years (ranging 19-60 years), with a body mass index of 24.2 ± 3.36 kg m^{-2} and plasma uric acid at 316 ± 56.5 μM

(ranging 194-472 µM, n=52) (Table 4-1). 23 subjects were selected and 22 of them completed the study (Figure 4-2).

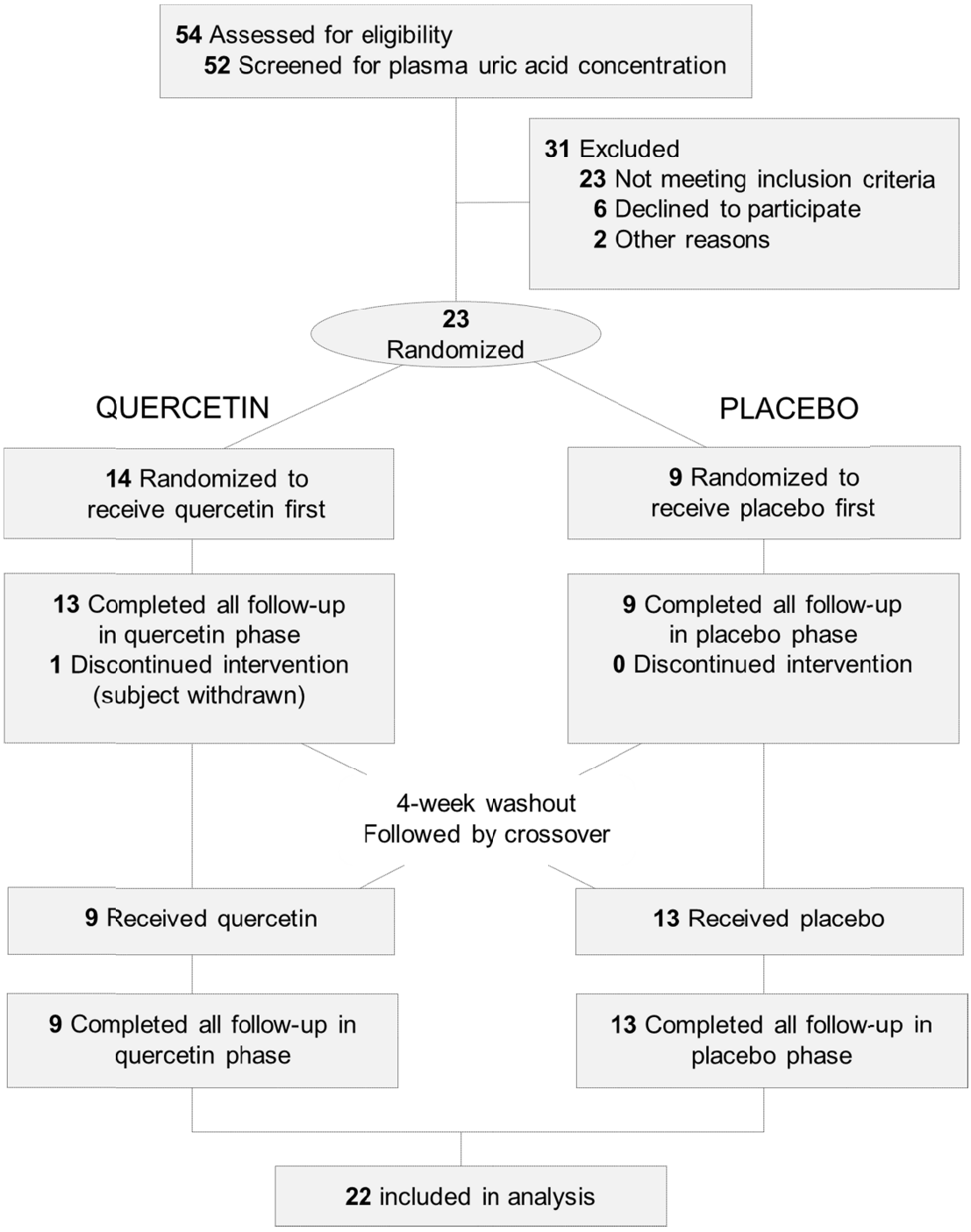


Figure 4-2 Flow diagram for current randomised cross-over clinical trials according to the CONSORT statement. (Consolidated standards of reporting trials (178))

Table 4-1 Physical characteristics of volunteer participants at screen visit, n=52

Variable	Mean	SEM	SD	Min	Max
Age (years)	28.6	1.62	11.6	19.0	60.0
Body mass index (kg m^{-2})	24.2	0.43	3.36	18.5	32.7
Uric acid (μM)	316	7.92	56.5	194	472

The study recruitment attracted mostly adults in their early 20s (Figure 4-3) since the advertisement was posted around the campus of the University of Leeds. However, among the volunteers who made contact (n=54) and donated blood sample (n=52), a strong correlation was found between age and BMI (Figure 4-3), while no correlation was found between blood uric acid level and BMI or between that and age (Figure 4-4). This finding confirmed the necessity of screening subject by blood analysis.

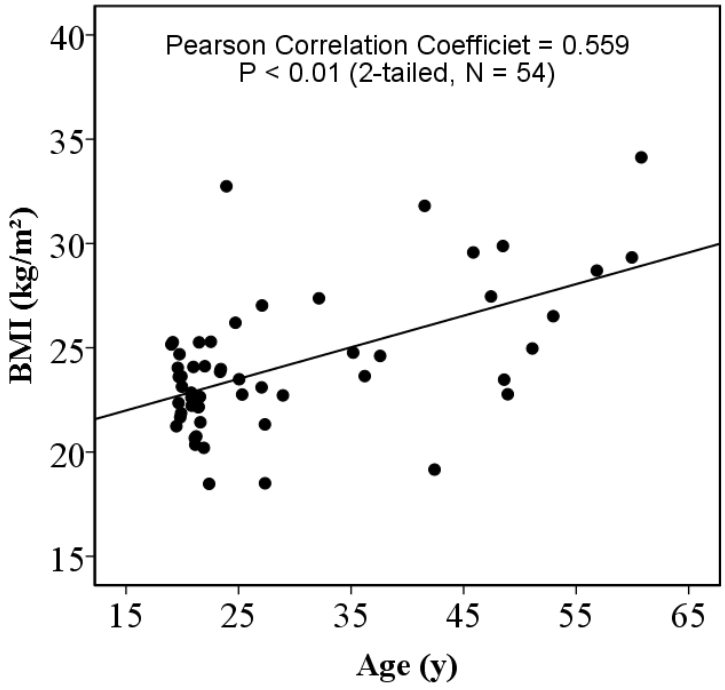


Figure 4-3 Correlation between age and BMI.

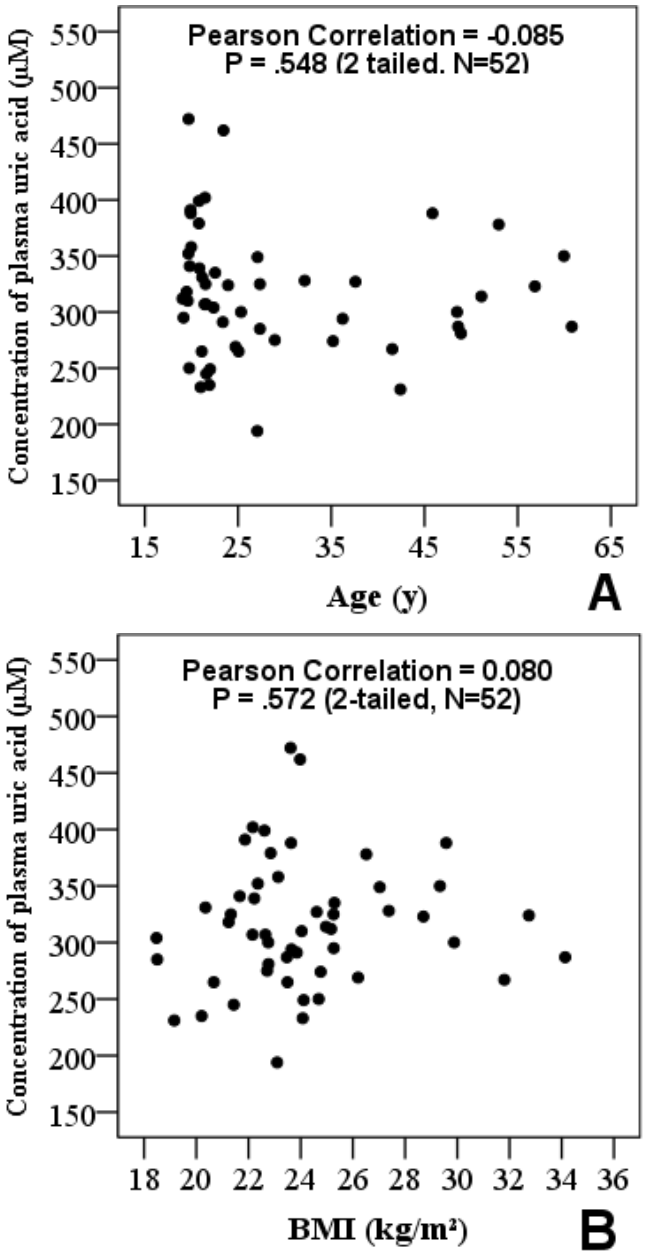


Figure 4-4 No correlation can be found between concentration of plasma uric acid with either age (A) or BMI (B) (n=52).

The study population (n=22) selectively contains volunteers with a relatively high uric acid level after exclusion. Comparison of the histogram between populations included and excluded shows a clear shift of the main range (Figure 4-5).

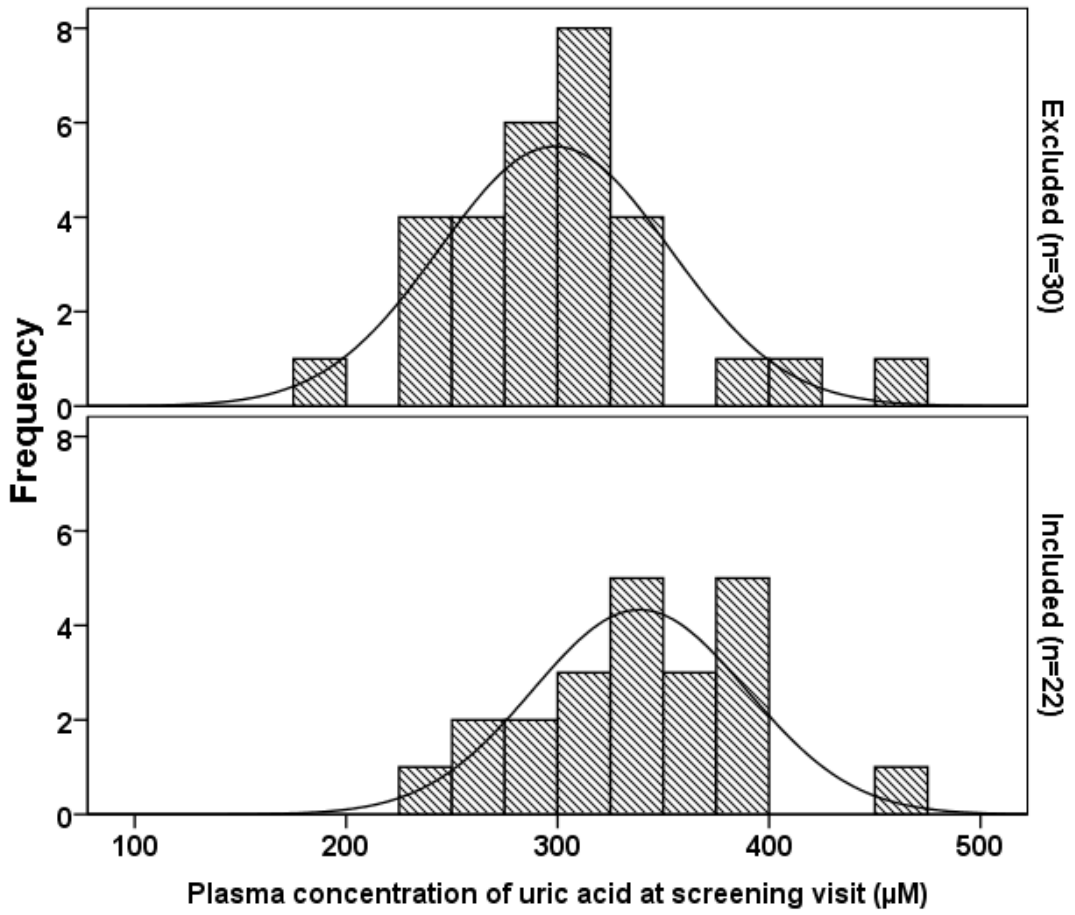


Figure 4-5 Histogram of plasma uric acid concentration at screening visit.

The distribution of volunteers who were excluded because of either low plasma uric acid level or other personal reasons. Figure at bottom shows the plasma uric acid concentration distribution of the 22 volunteers who successfully complete the study. The upper shift of normal curve suggested that selection in this stage is efficient.

They were healthy adult males aged 29.9 ± 12.9 y with BMI of 24.8 ± 3.0 kg m^{-2}, blood pressure of normal to (pre-) hypertensive (Systolic 123 ± 8 mm Hg, Diastolic 74.3 ± 9.0 mm Hg), and fasting blood glucose of normal to impaired fasting glycemia of 5.04 ± 0.56 mM and plasma uric acid of 339 ± 51 μM (Table 4-2). The BMI of the study subject are distributed across normal weight and overweight. The age of the study subjects is mainly young adults and ranges up to 60 year however an age gap between younger and elder was observed (Figure 4-6).

Table 4-2 Physical characteristics of volunteer participants who complete the study, n=22

	Mean ± SD	Range
Age (y)	29.9 ± 12.9	19.2 - 60.0
BMI (kg m^{-2})	24.8 ± 3.0	21.3 - 32.7
Heart rate (beats min^{-1})	66.5 ± 11.6	42 - 85
Systolic blood pressure (mm Hg)	123 ± 8	105 - 138
Diastolic blood pressure (mm Hg)	74.3 ± 9.0	65 - 100
Plasma uric acid (μM)	339 ± 51	245 - 462
Fasting glucose (mM)	5.04 ± 0.56	4.33 - 6.71

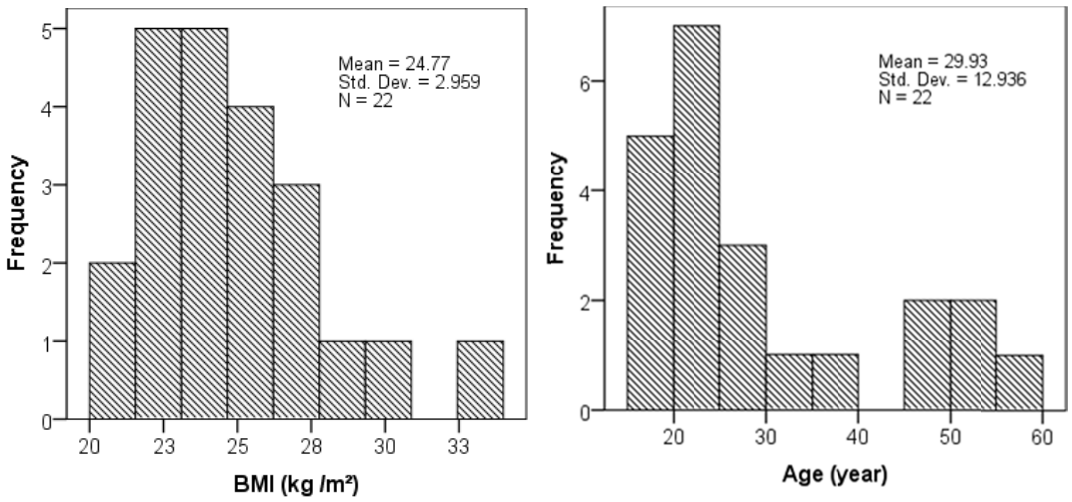


Figure 4-6 Histogram showing distribution of age and BMI of the subjects entering final analysis, n=22

4.3.2 Correlations between baseline biomarkers

Correlations (two-tailed) were calculated for each pair of the following biomarkers in baseline blood sample and measurement on baseline visit (Table 4-3): fasting

glucose, uric acid, age, BMI, DBP and SBP. Significant correlations were found between age and fasting glucose (Pearson correlation = 0.666, $P<0.01$), age and DBP (Pearson correlation = 0.611, $P<0.01$), age and BMI (Pearson correlation = 0.559, $P<0.01$); and between DBP and BMI (Pearson correlation = 0.470, $P<0.05$), DBP and fasting glucose (Pearson correlation = 0.811, $P<0.01$), DBP and SBP (Pearson correlation = 0.548, $P<0.01$). No other significant correlation was found. No significant correlation between plasma uric acid with any other factors were found, although this contradicts a previous publication (179).

It is important to notice the correlation between DBP and fasting glucose at baseline visit (Pearson correlation = 0.811, $P<0.01$), as shown in Figure 4-7 E and another three following visits (Figure 4-8). This may be an observation confirming the correlation between pre-hypertension and pre-diabetes (impaired glucose tolerance).

Table 4-3 Correlations of baseline biomarkers, n=22.

	Glucose (mM)	Uric acid (μM)	Age (year)	BMI (kg m^{-2})	SBP (mm Hg)
Uric acid (μM)	.285 (*.198*)	--	--	--	--
Age (year)	.666 (*.001*) **	-.038 (*.866*)	--	--	--
BMI (kg m^{-2})	.315 (*.153*)	.080 (*.572*)	.456 (*.033*) *	--	--
SBP (mm Hg)	.202 (*.366*)	.056 (*.806*)	.119 (*.596*)	.326 (*.139*)	--
DBP (mm Hg)	.811 (*<.001*) **	.184 (*.412*)	.611 (*.003*) **	.470 (*.027*) *	.548 (*.008*) **

Note: Data presented in Pearson Correlation (P value, 2-tailed). * indicates a trend (P<0.1) and ** indicates P<0.05.

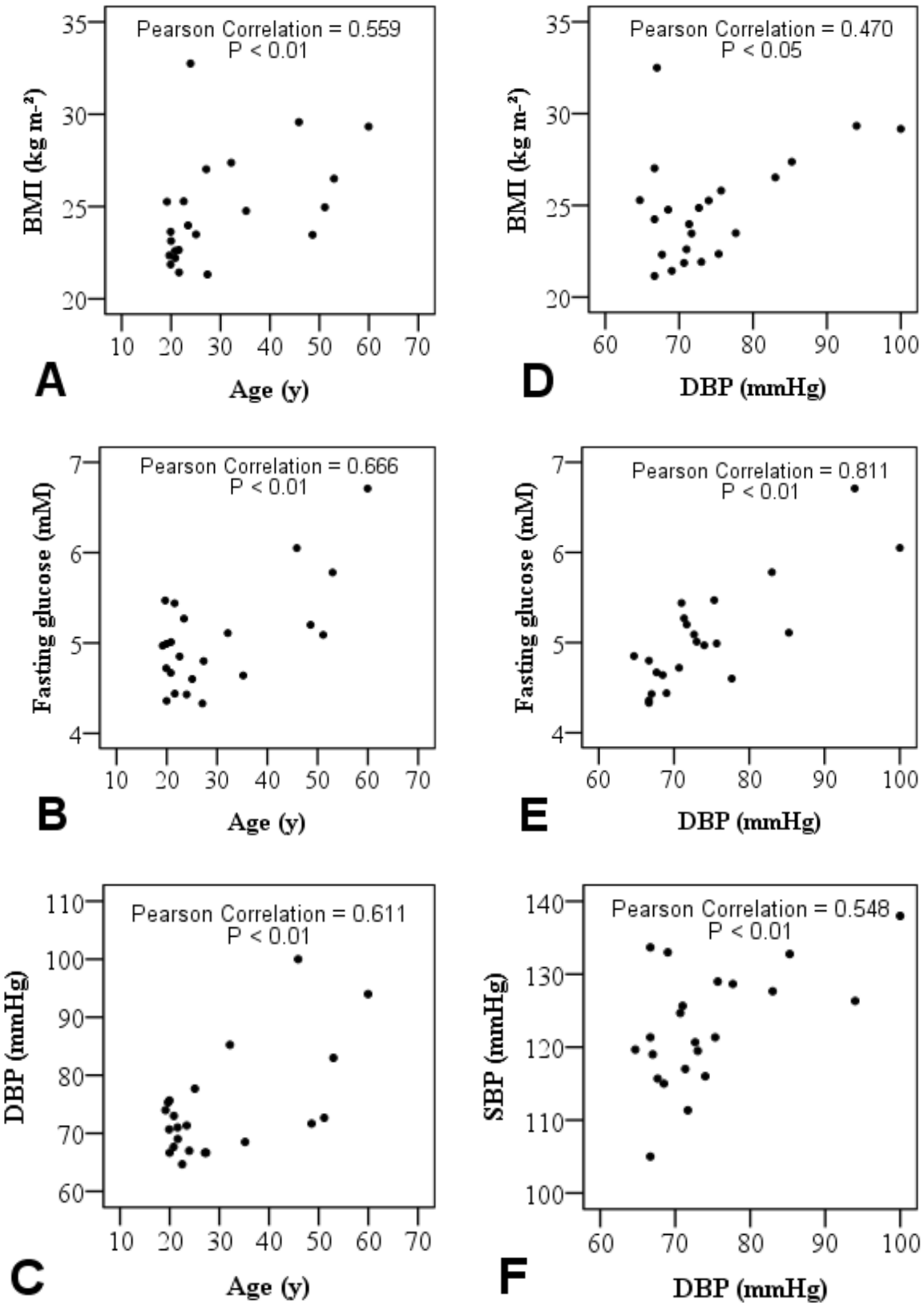


Figure 4-7 Correlations among age, BMI, fasting glucose, DBP and SBP in all subjects (n=22) at baseline visit.

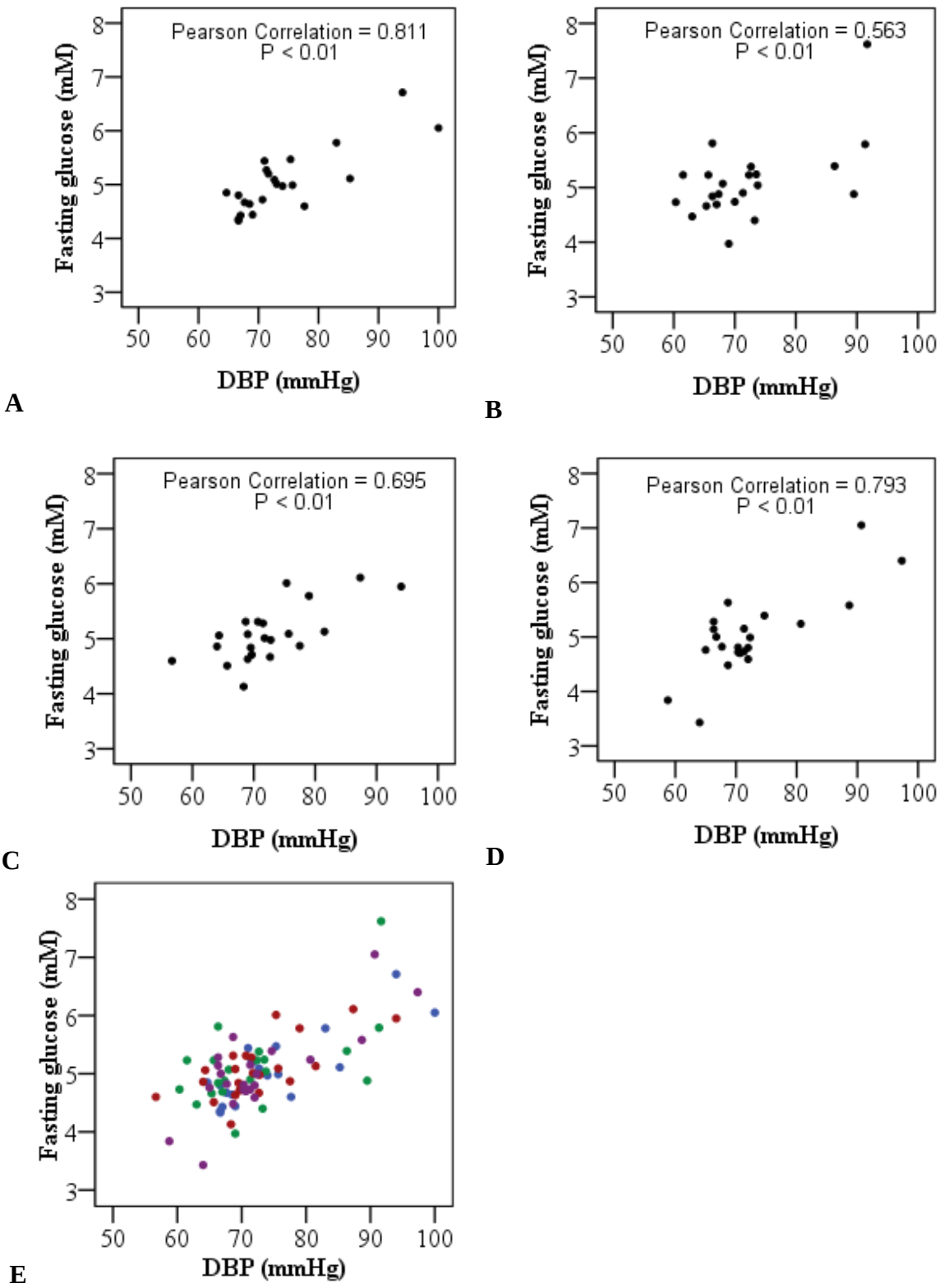


Figure 4-8 Strong correlation between DBP and fasting glucose was confirmed repeatedly in all 4 different visits with 4 weeks interval (panel A, B, C, D) of 22 subjects. Overlay of 4 measurements is shown in panel E.

4.3.3 Intervention compliance

The study population was homogenous because strict eligibility criteria and participant surveillance were used to prevent confounding influences of gender, medications, diet, or other lifestyle factors. The BMI of each subject was consistent compared to previous visits and this suggests the consistency of physiological status during the whole study as the subjects were required to avoid vigorous change of physical activity or dietary habits (Table 4-4). According to participant′s reports, no significant change of lifestyle or medication occurred during the study on lifestyle maintenance questionnaire and no adverse event for receiving quercetin or placebo was reported (Table 4-5). 24-h urinary excretion of quercetin was 0.810 ± 0.704 μmol during quercetin treatment and 0.200 ± 0.366 μmol during placebo treatment (Figure 4-9, Figure 4-10). According to the returned unconsumed tablets, participant self-reports and significant difference of the uptake of quercetin during interventions, none of the participants was classified as non-compliant.

Table 4-4 BMI change during the study (n=22)

		BMI (kg m^{-2})	
	Time	Mean	SEM
1^{st} arm	Baseline	24.9	0.6
	Endpoint	25.1	0.6
2^{nd} arm	Baseline	25.1	0.6
	Endpoint	25.1	0.6

Table 4-5 Summary of questionnaire of lifestyle maintenance, number of Yes (parts out of 22)

	1st arm		2nd arm	
	Baseline	Endpoint	Baseline	Endpoint
Change of physical activity	3 (13.6%)	2 (9.09%)	3 (13.6%)	0
Change of dietary habits	0	1 (4.55%)	2 (9.09%)	0
Non-routine medication	4 (18.2%)	3 (13.6%)	2 (9.09%)	1 (4.55%)
Problem following the diet	0	0	0	1 (4.55%)
Vomiting or diarrhea	0	0	0	0
More than 2 changes	0	0	0	1 (4.55%)

Note: The specific details of answers of Yes were recorded and exanimated. No event can be classified as having a significant impact on study progress.

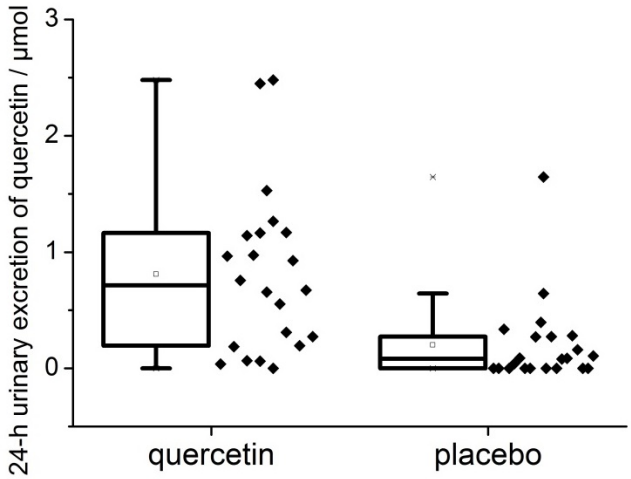


Figure 4-9 Box plot showing the variety of 24-h urinary excretion of intact quercetin among study population and compared between that affected by treatment.

24-h urinary excretion of intact quercetin was 0.810 ± 0.150 µmol and 0.200 ± 0.078 µmol for quercetin and placebo respectively (mean ± SEM, n=22). Quercetin supplementation is significantly improving quercetin intake in 22 free living subjects.

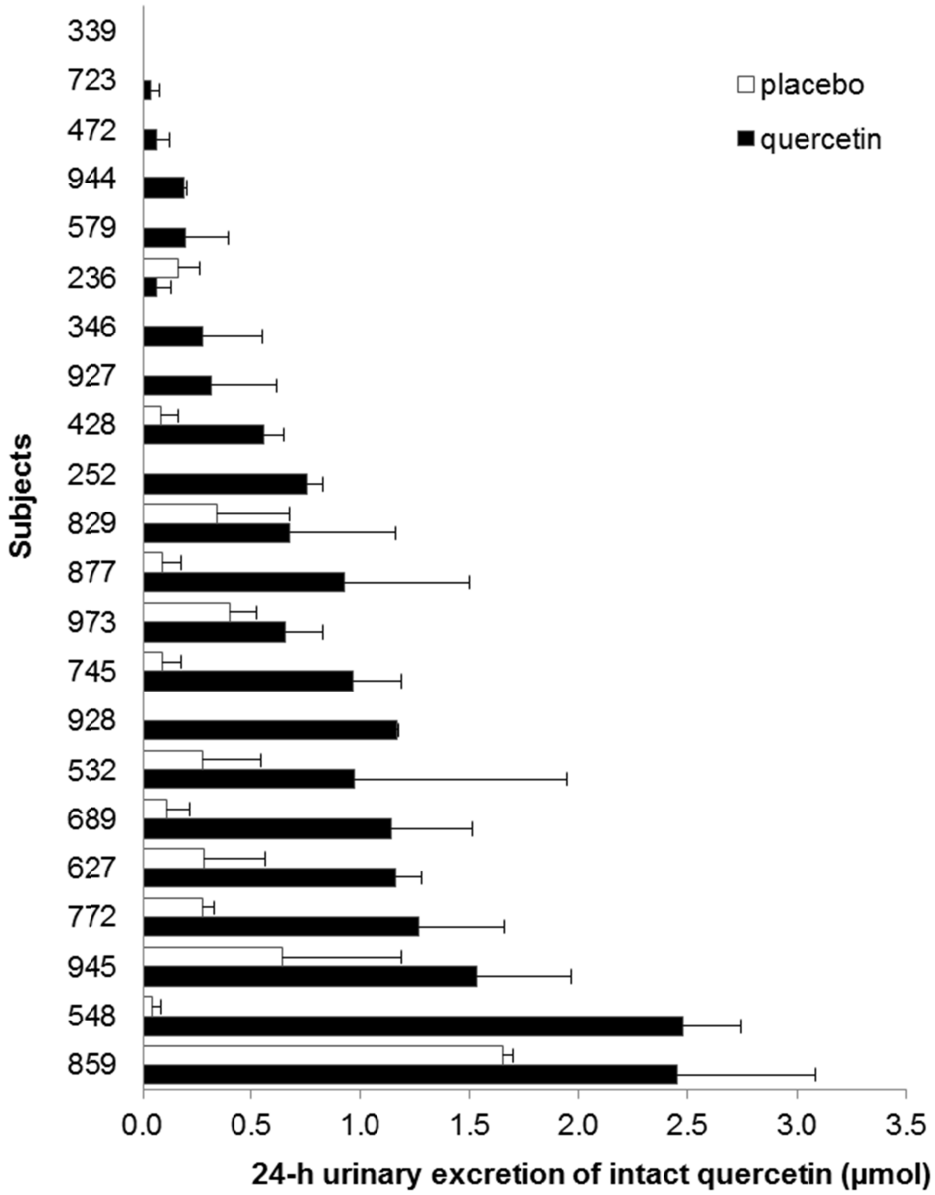


Figure 4-10 24-h urinary excretion of intact quercetin in each subjects. Compliance is confirmed if that of placebo is not significantly higher than that of quercetin.

4.3.4 Uric acid level in plasma

A two-way repeated measures ANOVA was run to determine the effect of different treatments over time on plasma uric acid concentration. Analysis of the studentized residuals showed that there was normality, as assessed by the Shapiro-Wilk test of normality and no outliers, as assessed by no studentized residuals greater than ± 3 standard deviations. There was sphericity for the interaction term, as assessed by Mauchly's test of sphericity ($P > 0.05$). There was a statistically significant interaction between treatment and time on plasma uric acid concentration, $F (2, 42) = 3.387$, $P = .043$, partial $\eta^2 = .139$. Therefore, simple main effects were run for treatments. Plasma uric acid concentration was not statistically significantly different in the control trial (315 ± 45 µM) compared to the intervention trial (330 ± 56 µM) at the baseline of the trials, $F (1, 21) = 1.709$, $P = .205$, partial $\eta^2 = .075$. Plasma uric acid concentration was not statistically significantly different in the control trial (325 ± 52 µM) compared to the intervention trial (314 ± 55 µM) at 2-week of the trials, $F (1, 21) = 1.315$, $P = .264$, partial $\eta^2 = .059$. However, plasma uric acid concentration was statistically significantly different in the control trial (320 ± 47 µM) compared to the intervention trial (304 ± 48 µM) at the 4-week of the trials, $F (1, 21) = 4.894$, $P = .038$, partial $\eta^2 = .189$, with a mean difference of 16.5 (95% CI, 1.0 to 32) µM. Also, simple main effects were run for time. There was no statistically significant effect of time on plasma uric acid concentration in the control trial, $F (2, 42) = 0.589$, $P = .59$, partial $\eta^2 = .027$. Plasma uric acid concentration was statistically significantly reduced along time during quercetin treatment, $F (2, 42) = 6.166$, $P = .004$, partial $\eta^2 = .227$.

Table 4-6 Effect on plasma uric acid (μM) of quercetin and placebo during intervention (n=22)

Treatment	Study Period			ANOVA *P* (effect of time)	2-Week		4-Week	
	Baseline	2-Week	4-Week		Change (95% CI)	*P*	Change (95% CI)	*P*
Quercetin	330 ± 56	314 ± 55	304 ± 48	.004 **	-15.9 (0.9, -32.8)	.063	-26.5 (-7.6, -45.5)	.008 **
Placebo	315 ± 45	325 ± 52	320 ± 47	.559	10.6 (-8.9, 30.0)	.273	5.2 (-15.1, 25.5)	.598
ANOVA *P* (effect of treatments)	.205	.264	.038 *	.043 *				

Note: All values are presented as mean ± SD. Data were tested by paried sample t test since the data is normally distributed * indicates $0.01<P<0.05$ and ** indicates $P<0.01$.

Plasma uric acid improved progressively over time among participants during 4 weeks of quercetin supplementation. From baseline to 2 weeks, plasma uric acid showed a downward trend by -15.9 μM (95% CI, 0.9 to -32.8; $P = 0.063$). From baseline to 4 weeks, plasma uric acid was decreased significantly by mean ± SEM from 330 ± 12 μM to 304 ± 10 μM (95% CI, -7.6 to -45.5; $P = 0.008$). Plasma uric acid by mean ± SEM remained unchanged throughout the treatment period when participants received placebo from baseline of 315 ± 10 μM to 325 ± 11 μM (mean change of 10.6 μM; 95% CI, -8.9 to 30.0; $P = 0.273$) at 2-week interval and to 320 ± 10 μM by mean change of 5.2 μM (95% CI, -15.1 to 25.5; $P = 0.598$) at 4-week interval. No difference was observed between the baselines of each arm ($P = 0.205$). (Table 4-6, Figure 4-11). The magnitude of plasma uric acid reduction was higher in individuals with higher baseline plasma uric acid in both treatments. Plasma uric acid in the majority of subjects declined after 4 weeks in treatment by quercetin (17/22) but not by placebo (10/22) (Figure 4-12).

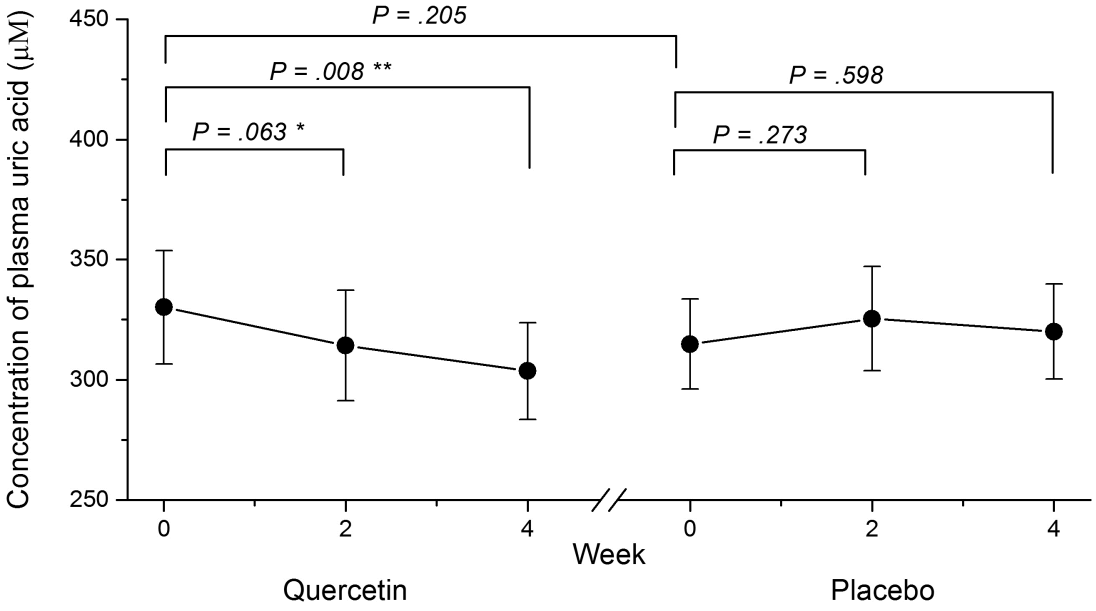


Figure 4-11 Distribution of plasma uric acid concentration (μM) in 22 healthy man before, during and after the 4-week supplementation or placebo. Data are presented by mean and 95% confidence interval.

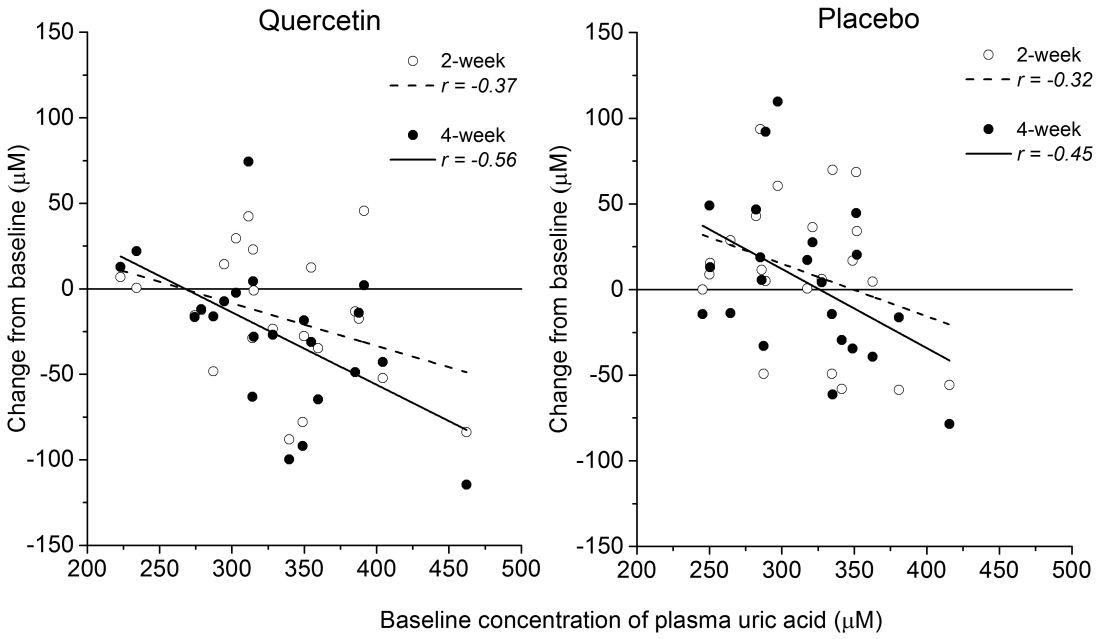


Figure 4-12 Regressions of changes of plasma uric acid after treatment of quercetin or placebo within subject compared to baseline reading.

Compared to the placebo which does not affect the plasma uric acid in population (n=22), supplementation have reduced the plasma uric acid level in majority of the subjects (n=17 of 22). Correlation coefficient *r* was calculated by the Pearson test.

Due to the huge between-subjects variation of quercetin absorption and renal excretion, compliance cannot be check by urinary excretion alone. Therefore, as shown in Figure 4-13, each subject was reviewed by comparing the change of concentration of plasma uric acid from baseline to the 24 h urinary excretion of total quercetin during each arm. This plot helped to explain some of the outliers and unexpected effects of the placebo.

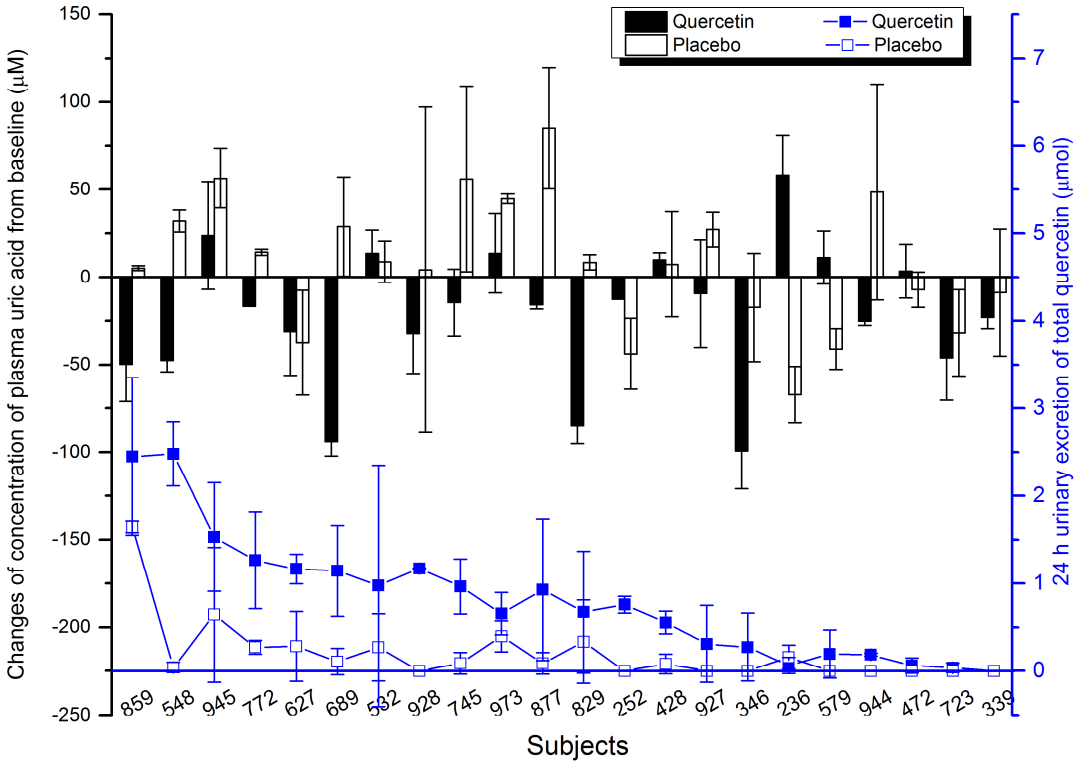


Figure 4-13 Relevance between blood uric acid reduction and quercetin supplementation is presented for each individual. The mean value ± SD were calculated by averaging the data from 2-week and 4-week samples.

The optimised coherence of efficient sample size and efficient inclusion criteria. In consider of the U-shape association between plasma uric acid levels and mortality risk (180) indicating that lower uric acid level subjects should be carefully excluded because treatment might cause hypoururicemia in them.

4.3.5 Resting blood pressures

Blood pressure measures are presented in Table 4-7. Subjects were grouped according to guidelines of World Health Organization-International Society of Hypertension (WHO-ISH). Subjects with SBP lower than 120/80 mm Hg (n=10) were considered as normotensive, between 120/80 and 140/90 mm Hg as pre-

hypertensive (n=10), higher than 140/90 as hypertensive (n=2). The DBP in the normotensive group (n=10) were significantly reduced after quercetin supplementation (P<0.05) but not in the placebo group (Table 4-7). SBP was not changed significantly in either group (Figure 4-14 and Figure 4-15).

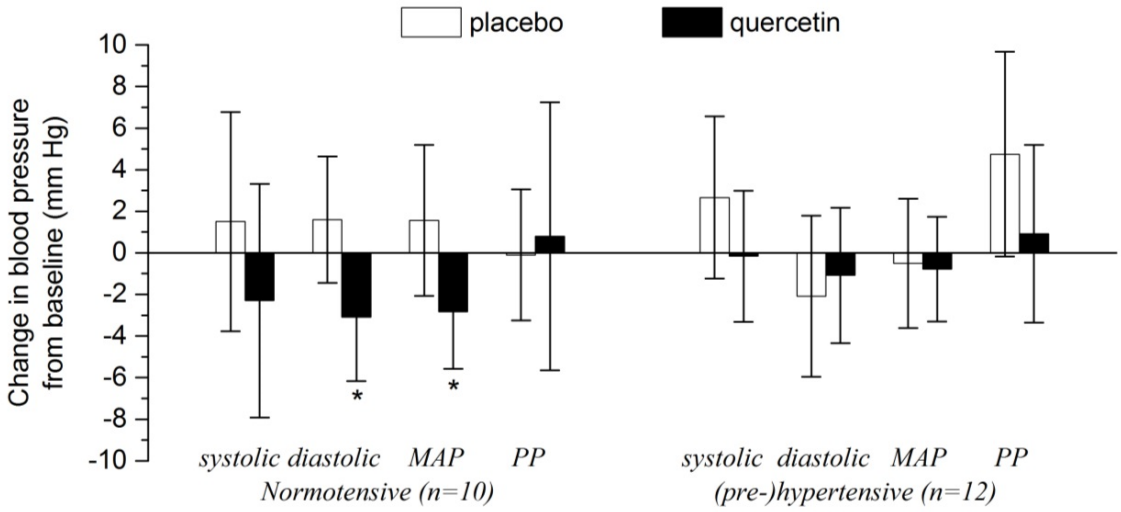


Figure 4-14 Comparison of changes in blood pressure from baseline (mm Hg) in subgroups.

Comparison to baseline observation after a double-blinded, placebo-controlled trial compared 4 week of 500 mg d^{-1} quercetin versus placebo in 22 healthy males. MAP, mean arterial pressure; PP. pulse pressure. Error bar indicates 95% CI. *Difference from baseline; P<0.05.

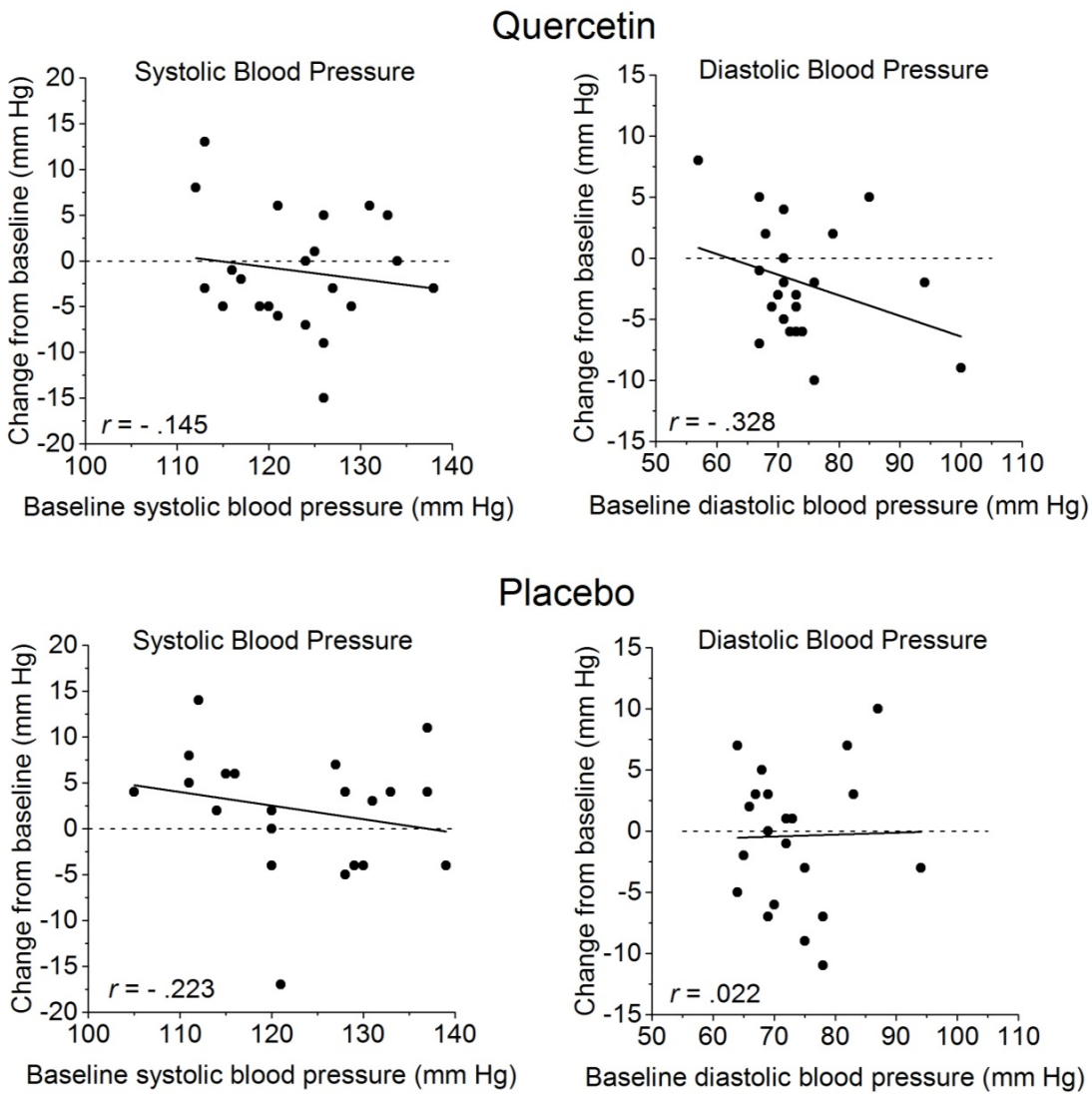


Figure 4-15 Regressions of changes of blood pressures compared to baseline reading after 4 week of quercetin or placebo. Correlation coefficient *r* was calculated by the Pearson test.

Table 4-7 Resting systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse rate in male subjects before and after 4-week supplementation with quercetin (500 mg d^{-1}) or placebo

		Study period		Change[a]	*P* value
		Baseline	4-week	(95% CI)	
Systolic blood pressure, SBP (mm Hg)					
Total (n=22)	Quercetin	123.2 ± 1.5	122.0 ± 1.9	-1.1 (-4.0, 1.7)	.412
	Placebo	122.5 ± 2.1	124.6 ± 2.3	2.1 (-0.8, 5.1)	.143
Normotensive (n=10) [b]	Quercetin	117.6 ± 1.5	115.3 ± 1.5	-2.3 (-7.9, 3.3)	.378
	Placebo	114.4 ± 1.6	115.9 ± 1.8	1.5 (-3.8, 6.8)	.536
(Pre-) Hypertensive (n=12) [c]	Quercetin	127.8 ± 1.6	127.7 ± 2.2	-0.2 (-3.3, 3.0)	.910
	Placebo	129.3 ± 2.2	131.9 ± 2.3	2.7 (-1.2, 6.6)	.161
Diastolic blood pressure, DBP (mm Hg)					
Total (n=22) [d]	Quercetin	73.8 ± 2.0	71.8 ± 1.9	-2.0 (-4.1, 0.1)	.067
	Placebo	73.1 ± 1.7	72.7 ± 2.1	-0.4 (-2.9, 2.0)	.794
Normotensive (n=10) [b]	Quercetin	71.4 ± 0.9	68.3 ± 1.0	-3.1 (-6.2, 0.0)	.048 *
	Placebo	69.2 ± 1.1	70.8 ± 0.8	1.6 (-1.4, 4.6)	.264
(Pre-) Hypertensive (n=12) [c]	Quercetin	75.8 ± 3.5	74.8 ± 3.2	-1.1 (-4.3, 2.2)	.479
	Placebo	76.4 ± 2.6	74.3 ± 3.7	-2.1 (-6.0, 1.8)	.261
Mean arterial pressure, MAP (mm Hg)					
Total (n=22)	Quercetin	90.3 ± 1.6	88.6 ± 1.7	-1.7 (-3.5, 0.0)	.055
	Placebo	89.6 ± 1.6	90.0 ± 1.8	0.4 (-1.8, 2.6)	.683
Normotensive (n=10) [b]	Quercetin	86.8 ± 0.8	84.0 ± 0.9	-2.8 (-5.6, -0.1)	.044 *
	Placebo	84.3 ± 1.1	85.8 ± 1.0	1.6 (-2.1, 5.2)	.355
(Pre-) Hypertensive (n=12) [c]	Quercetin	93.2 ± 2.6	92.4 ± 2.5	-0.8 (-3.3, 1.7)	.511
	Placebo	94.0 ± 2.2	93.5 ± 3.0	-0.5 (-3.6, 2.6)	.730
Pulse pressure, PP (mm Hg)					
Total (n=22)	Quercetin	49.4 ± 1.9	50.2 ± 2.0	0.9 (-2.4, 4.3)	.602
	Placebo	49.4 ± 1.7	51.9 ± 2.3	2.5 (-0.5, 5.6)	.093
Normotensive (n=10) [b]	Quercetin	46.2 ± 1.7	47.0 ± 1.8	0.8 (-5.6, 7.2)	.785
	Placebo	45.2 ± 1.3	45.1 ± 1.4	-0.1 (-3.1, 3.1)	.944
(Pre-) Hypertensive (n=12) [c]	Quercetin	52.0 ± 3.1	52.9 ± 3.1	0.9 (-3.4, 5.2)	.646
	Placebo	52.8 ± 2.5	57.6 ± 3.1	4.8 (-0.2, 9.7)	.058

Abbreviation: MAP, mean arterial pressure, MAP ≈ (2DBP+SBP) /3; PP, pulse pressure, PP = SBP - DBP

[a]. mean ± SEM and mean change (95% CI). Paired *t*-test, 2 tailed, if not stated otherwise.

[b]. Subjects with blood pressure lower than 120/80 mm Hg (n=10) according to guidelines of World Health Organization-International Society of Hypertension (WHO-ISH).

[c]. Subjects with blood pressure between 120/80 and 140/90 mm Hg are defended as pre-hypertensive (n=10), higher than 140/90 as hypertensive (n=2).

[d]. Wilcoxon signed-rank test was used as the data is not normally distributed.

* indicates $P<0.05$ and ** indicates $P<0.01$ when compare to baseline by paired t test.

4.3.6 Fasting glucose

The baseline concentration of fasting plasma glucose of the 22 healthy subjects was 5.04 ± 0.58 mM for 1st arm and 5.09 ± 0.51 mM for 2nd arm. Consistency of subjects is good and carry-over effect was not observed. Figure 4-16 shows the effect of quercetin supplementation on plasma glucose compared to control group, no significant difference were observed in either group (Table 4-8). Two-way ANOVA showed there was not a significant interaction between treatment and time (P=0.297), maineffect of treatment was not significant (P=0.607) nor time (P=0.997).

Comparison of the effect on fasting plasma glucose of quercetin and placebo were carried within each subject, as Figure 4-17 shows. Apparently, the quercetin intake had increased the status of fasting plasma glucose level for some individuals but not for some of the others. The number of each were very balanced, same was in placebo treatment.

Table 4-8 Concentration of fasting plasma glucose in male subjects before, during and after 4-week supplementation with quercetin (500 mg d^{-1}) or placebo.

	C. glucose (mM), Mean ± SEM	
	Quercetin (P value, 2-tailed)	Placebo (P value, 2-tailed)
Total (n=22)	(P=.350) [a]	
Baseline	5.04 ± 0.13	5.09 ± 0.11
2-week	5.01 ± 0.14 (.729)	5.13 ± 0.12 (.648)
4-week	5.10 ± 0.15 (.480)	5.02 ± 0.16 (.567)

Note: Mean value was compared with that at baseline (paired t test). [a] Change during quercetin treatment was compared with that during placebo treatment (95% CI distribution is normal, paired t test), [b] normal blood glucose level 3.9-5.9 mM, [c] pre-diabetes 5.9-7.0 mM

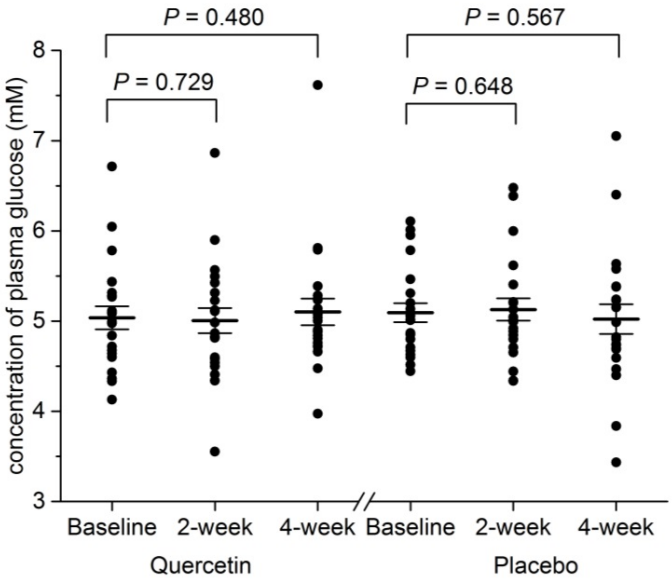


Figure 4-16 Fasting plasma glucose concentration by mean ± SEM and individual observations in 22 healthy man before, during and after the 4-week supplementation placebo.

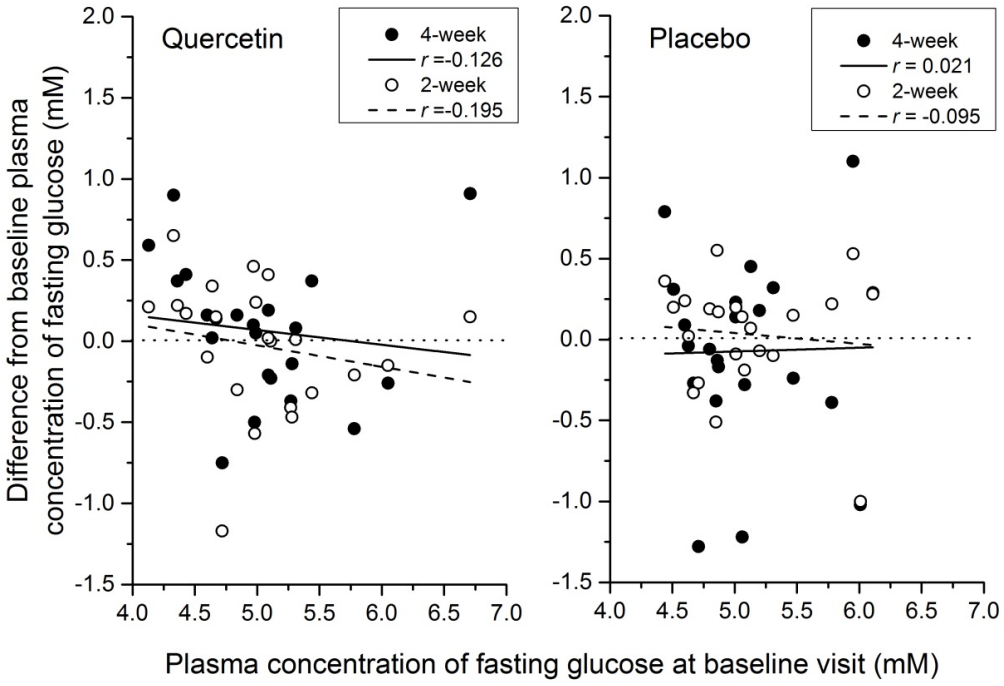


Figure 4-17 Linear regressions of changes of fasting plasma glucose after treatment of quercetin or placebo within subject compared to baseline reading. Correlation coefficient *r* was calculated by the Pearson test.

4.3.7 Renal clearance of uric acid

The concentration of uric acid presented in urine was trendily reduced. 2-week of quercetin supplementation reduced the urine uric acid concentration by mean ± SEM from 1.23 ± 0.21 to 0.94 ± 0.18 mM (P<0.1, two tailed) but no change was observed during placebo treatment (from 0.80 ± 0.12 to 0.84 ± 0.13 mM, SEM). Renal excretion of uric acid was exanimated by total 24-h urinary uric acid level. It did not significantly vary among the two time points for either treatment (Table 4-9).

Table 4-9 Urinary uric acid level of each visit (n=22) [a]

		Day 14	Day 28	*Asymp. P* [b]
Concentration, mM	Quercetin	1.23 ± 0.21	0.94 ± 0.18	0.082 *
	Placebo	0.80 ± 0.12	0.84 ± 0.13	0.673
24 h excretion, mmol	Quercetin	2.15 ± 0.38	1.61 ± 0.33	0.105
	Placebo	1.41 ± 0.28	1.64 ± 0.31	0.348

[a] Data is presented by mean ± SEM *indicates 0.05<P<0.1

[b] *Asymp. P* is calculated by Wilcoxon signed-rank test, 2 tailed.

Renal excretion of uric acid was exanimated by total 24-h urinary uric acid level. This data alone is not sufficient to draw any conclusion on whether kidney function was affected, since this is usually indicated by glomerular filtration rate (GFR, shown in Figure 4-18, 1). After all uric acid is mainly reabsorbed once was filtrated (Figure 4-18, 2) while what we have detected is overall excretion (Figure 4-18, 4). It is possible that quercetin can have an effect on kidney function (Table 4-9), but this requires a biomarker that can accurately describe kidney function *in vivo*. Apparently uric acid is not an ideal biomarker for such purpose.

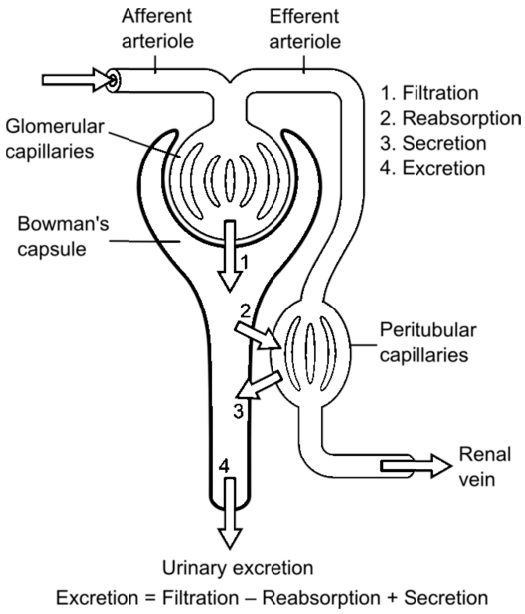


Figure 4-18 Diagram showing the basic physiologic mechanisms of the kidney.

Apparently, plasma uric acid level was significantly reduced after 4-week treatment of quercetin at 500 mg d^{-1} while the amount of uric acid excreted through the kidney was maintained at the same level during the last 2 weeks (Table 4-9). In other words, daily supplementation of quercetin can significantly reduce plasma concentration but does not affect renal excretion of uric acid. This may suggest that liver, where uric acid was mainly produced, was affected by quercetin which inhibits the production of uric acid.

The consistency of the result of urinary uric acid level indicates that the four sampling point were consistent as the volunteers were required to maintain their normal lifestyle and diet habit while none of the sampling points show any abnormality. This validates the whole study and results obtained through this study.

4.4 Discussion

4.4.1 Novelty

Previous nutritional intervention studies have not been designed for blood uric acid level as their primary outcome, therefore the power estimation is not specific for uric acid variation. Another overlooked issue of previous studies, is the method for uric acid quantification may not be validated precisely engough to detect a difference between samples. Besides, the U-shape association of uric acid level and mortality requires a careful study design by conducting trial in subjects with blood uric acid in the upper range. For this reason the human study has selected (screen) male volunteers with uric acid level higher than average. For accurate measurements we have modified an enzymatic spectrophotometric detection for uric acid assay (Chapter 2).

4.4.2 Primary outcome

In this randomised controlled trial, supplementation with quercetin at 500 mg d^{-1} for 4 weeks progressively reduced plasma concentration of uric acid without inducing changes in BMI, in fasting blood glucose or showing any adverse effects.

At the interval of 2 weeks, a mean change of -15.9 µM (95% CI, 0.9 to -32.8, P = 0.063) showed a trend towards decreasing. A significant decrease in plasma uric acid level was observed at the end of 4-week treatment with mean change of -26.5 µM (95% CI, -7.6 to -45.5, P = .008). On the basis of previous reports of lack of effect of quercetin on blood uric acid after 2 weeks of dosage at 150 mg d^{-1} of quercetin supplement in healthy subjects (89), 76-110 mg of flavonols (mostly quercetin

glucosides) from 400 g onion with 6 cups of tea daily in type 2 diabetic patients (96), or 15 mg d^{-1} quercetin glucoside from blueberry-apple juice for 4 weeks in healthy subjects (98), the present study was purposely designed for a population with higher uric acid level, with an apperently higher dosage and longer intervention period.

In vitro tests on *bovine* XOR showed that the inhibition constant for allopurinol is 0.34 ± 0.22 μM while that of quercetin is 1.40 ± 0.78 μM (181), indicating that intact quercetin could be comparable to allopurinol *in vivo* based on this data, and depending on the amount absorbed. Furthermore, quercetin in physiological fluids is present in methyl-, glucuronosyl-, and sulfo- conjugated forms (see Figure 1-6, Figure 1-7), some of which retain the inhibitory effects of quercetin (see Table 6-5) (106, 111).

Besides the apparent inhibition of XOR, additional mechanisms are also possible, including activated renal excretion of uric acid, which could be as a result of an increased glomerular filtration of uric acid. Some drugs such as Losartan inhibit directly URAT1, involved in uric acid reabsorption, and thereby decrease plasma uric acid (182), whereas some treatments down regulate mURAT1 and mGLUT9 in mice (183). Up-regulation of transporters (mOAT1 (183), rOAT1 (184) and hOAT1 (185)) which increase kidney urate secretion in the proximal tubules of the renal cortex is also possible. Other additional mechanisms could involve an indirect antioxidant effect that reduces microvascular ischemia in glomeruli and leads to increased blood flow at the site, dilation of afferent arterioles, and competition for reabsorption with ions such as sodium and potassium that exert osmotic effects (186).

It is worthy to notice in our study that the hypouricemic effect of quercetin is more significant in subjects with higher uric acid level (Figure 4-12), which is in accordance with animal tests (183). This suggests safety of quercetin compared to allopurinol (154, 187), because quercetin may exhibit fewer side effects than allopurinol in the amelioration of hyperuricemia. Alongside to the drugs used in gout therapy, febuxostat and allopurinol, the uric acid lowering effect of 500 mg quercetin (8.0 % reduction in mean plasma uric acid) during 14 d are not comparable to 10 mg of febuxostat (26 % reduction in mean serum uric acid) (188). In comparison to daily intake of 80 mg of febuxostat (253 µmol), which could decrease serum uric acid by 51 ± 14 % in healthy subjects by 6 days and reach the primary end point in 50~80% of gout patients eventually, 300 mg d^{-1} of allopurinol (2204 µmol) cab only cure 20~40 % of the patients (Table 4-10). Apparently a 26.5 µM reduction (95% CI, -7.6 to -45.5) is not medically significant for gout patients. Therefore these findings are especially beneficial: **1)** to maintain a healthy population, i.e. preventing formation of uric acid crystals (gouty arthritis) by preventing hyperuricemia defined as serum uric acid concentration above its solubility limit 6.8 mg dL^{-1} (to convert milligram per decilitre to micromoles per litre, multiply by 59.485) (189). Although hyperuricemia alone is not sufficient to cause gout, a dose-response relationship between serum uric acid and the risk of developing gout is well documented (190). **2)** For recovering gout patients: The primary treatment is to achieve an end point of serum uric acid levels less than 6.0 mg dL^{-1} for a period of three months (189); this includes the use of allopurinol to inhibit XO and uric acid production, or the use of uricosuric drugs which increase renal excretion of uric acid. However for patients also presenting kidney disease, liver disease, diabetes, congestive heart failure, hypertension, etc., the dosage of allopurinol has to be adjusted in this stage (173).

Once restored, patients are often advised on comprehensive dietary modifications for prevention against recurrent gout attacks. In the above situations, adoption of one quercetin tablet that has proven efficacy to reduce blood uric acid in the habitual diet is easy to adhere to compared to a comprehensive dietary modifications. Therefore it may be a promising behavioural approach to lower uric acid in individuals with above-optimal blood uric acid either for those at high risk who have not yet developed any disease, or for patients recovering after therapy.

Table 4-10 Summary of drug (febuxostat and allopurinol) usage and their effect on blood uric acid level.

Subjects, n	Design	Duration	Daily treatment	Decrease in mean serum urate, %	Primary result, %	Reference
10 healthy subjects	DR, PC	14 d	febuxostat 10 mg	26		Becker, 2004 (188)
10 healthy subjects	DR, PC	14 d	febuxostat 70 mg	50		Becker, 2004 (188)
23 healthy subjects	CO, DR	6 d	febuxostat 80 mg	51 ± 14		Khosravan, 2008 (191)
37 gout patients	R, DB, DR, PC	28 d	febuxostat 80 mg		76	Becker, 2005 (192)
267 gout patients	R	28 wk	febuxostat 80 mg		48	Schumacher, 2008 (193)
598 gout patients	R, DR	28 wk	febuxostat 80 mg		67	Becker, 2010 (194)
255 gout patients	R, DB	52 wk	febuxostat 80 mg		81	Becker, 2005 (195)
268 gout patients	R	28 wk	Allopurinol 300 mg		22	Schumacher, 2008 (193)
620 gout patients	R, DR	28 wk	Allopurinol 300 mg		42	Becker, 2010 (194)
251 gout patients	R, DB	52 wk	Allopurinol 300 mg		39	Becker, 2005 (195)

DB, double blind; DR, dose response; PC, placebo-controlled; CO, cross-over; R, randomized. Gout patients are with gout and with serum uric acid concentrations of at least 8.0 mg dL^{-1} (480 µM); the primary end point was a serum uric acid concentration of less than 6.0 mg dL^{-1} (360 µM) at the last three monthly measurements. Molecular weight of allopurinol is 136.112 g mol^{-1} and of febuxostat is 316.374 g mol^{-1}.

4.4.3 Cardiovascular protective effects

A trend for reduction of diastolic blood pressure (P = 0.067) after quercetin supplementation lends support to this hypothesis. The -2.00 mm Hg (95% CI, 0.11 to -4.11) reduction is noteworthy, since this decrease has been calculated to result in a 17% decrease in the prevalence of hypertension in population studies (196, 197). Uric acid-induced aging and death of human endothelial cells are mediated by local activation of oxidative stress and the renin-angiotensin system, which provides a novel mechanism of uric acid-induced endothelial dysfunction (198). It is alarming that a recent estimates that ~25% of the US population has prehypertension, which is defined as untreated blood pressure of 120-139 mm Hg systolic or 80-89 mm Hg diastolic (199). Although hypertension could frequently exist with other cardiovascular disease (CVD) risk factors such as metabolic syndrome, it is usually asymptomatic. Importantly, there is a positive and direct correlation between hypertension and the risk of other CVDs such as cardiac arrhythmia, coronary artery disease, cardiac hypertrophy, myocardial infarction, and heart failure (200).

Significant reduction of -3.1 mm Hg (95% CI, 0.0 to -6.2) in DBP was observed in normotensive group (n=10, P = 0.048) but not in (pre-) hypertensive group (n=12), but no difference was found in SBP in either group. Although the magnitude of the DBP reduction was small, the effects are clinically noteworthy, Cook *et al*. reported that a 2-mm Hg reduction in the population average of DBP for white US residents aged between 35 to 64 years would result in a 17% decrease in the prevalence of hypertension, a 15% reduction in the risk of stroke and transient ischemic attacks, and a 6% reduction in the risk of coronary heart disease (196, 197). Prolonged decrease in usual DBP of 5-6 mm Hg is associated with about 35-40% less stroke

and 20-25% less CHD (201). This is not consistent to previous findings. 150 mg of daily quercetin supplementation decrease the systolic blood pressure significantly after 6-week treatment in contrast to placebo (78). 730 mg of quercetin supplementation for 4 weeks significantly reduced both SBP by -7 ± 2 mm Hg and DBP by -5 ± 2 mm Hg ($P<0.01$) in stage 1 hypertensive patients but not in pre-hypertensive subjects (81). The following limitation of blood pressure measurement should be noted. Although blood pressure measurements were carefully taken, we acknowledge that 24-h ambulatory blood pressure monitoring would have been more accurate. The statistical calculation for subgroups as well as total might be underpowered since subjects were not selected according to blood pressure, neither was sample size estimated on it.

4.4.4 Blood glucose

Fasting glucose or BMI were not affected by either of 2 or 4-week quercetin or placebo supplementation. This may be explained by the normal healthy status of volunteers. Although many researches have confirmed the protective effects of quercetin and other polyphenols in animal models of diabetes mellitus (202, 203), this clinical trial still cannot prove the reduction of risk of diabetes effect of chronic polyphenol supplementation. Volunteers in this study were recommended to take the quercetin tablet (or placebo) with a meal. It might be able to lower the uptake of glucose from a variety types of food by inhibiting the glucose transporters in small intestine however simply relying on one dose of quercetin tablet per day, apparently, may not be more helpful than a balanced diet rich of polyphenols and fibre.

Concentration of glucose estimated by this external means will serve as internal standard to obtain the absolute concentration of interested analytes for NMR metabolic profiling of plasma, which is detailed in Section 5.2.7

4.4.5 Correlation between diastolic blood pressure and fasting glucose

We have found a moderate correlation between age and BMI (Figure 4-3) but no correlation between concentration of plasma uric acid with either age or BMI (Figure 4-4) in our early stage. Previous study among Chinese ethnic reports that chance for hyperuricemia of people aged 18-24 years is 2.01 fold (95% CI, 1.52 to 2.64) and people aged 45-64 years is 0.56 fold (95% CI, 0.45 to 0.71) compared to age group 25-44 years and shows that age group 25-34 years has the highest blood uric acid level. BMI became a risk factor for hyperuricemia only if above 30 (22).

The mean arterial pressure correlates with plasma glucose, insulin, and body weight in type 2 diabetic mice (204). Purnamasari, D., *et al*. reported the most weighed metabolic component contributing to the prevalence of insulin resistance among siblings of T2DM patients is central obesity (56.7%), hypertension (46.7%), decreased HDL cholesterol level (26.6%), hyper-triglyceridemia (26.6%) and hyperglycemia (20%) (205). It was commonly believed that DBP and fasting glucose in non-hypertensive, non-diabetic population was not related. This is the first time bringing up interest in testing this hypothesis since a moderate correlation between DBP and fasting glucose (Pearson Correlation =0.811, $P<0.01$, n=22) was observed among males whose fasting glucose level were mostly below 6.0 mM while their DBP were mostly below 90 mm Hg (Figure 4-8). However the sample size (n=22) is small and can hardly draw any conclusion while a biological reason of this correlation was not clear yet. This hypothesis cannot be tested within this study since

the population was selective (males with higher blood uric acid level). Cohort studies with representative population and larger sample size are required to test this hypothesis. Apparently, the presumption of “non-hypertensive non-diabetic population” has limited the test to be conducted in this subgroup. Another possible explanation is that an unidentified confounding factor was affecting both DBP and fasting blood glucose. Again, this requires a carefully designed cohort study. This confounding factor could be the key to understanding diabetes and heart disease risk and relevant metabolic syndromes.

4.4.6 Subjects compliance

24-h urinary excretion of intact quercetin was 0.81 ± 0.03 μmol (SEM, n=22) in present study (Chapter 4) and was 1.17 ± 0.18 μmol (SEM, n=6) in previous study (Chapter 3) after taking the same amount of quercetin dihydrate supplement tablet. They are significantly different (P=0.04 by two-sample t test with non-equal variance). Firstly, the purpose of the studies are different therefore the means of sample collection were different. Our previous study (Chapter 3 or (177)) indirectly detect the bioavailability of quercetin by quantifying 24-h urinary excretion of intact quercetin in urine immediately started from quercetin intake. In contrast, present study detected the 24-h urinary excretion of intact quercetin to reflect the habitual intake of quercetin during the past several days. Besides, only one volunteer participated in both studies, therefore, a difference in population may also contribute to this difference. Even though Egert, S., et al. showed that habitual quercetin intake can accumulate plasma quercetin, our result does not support or disagree that finding (89).

As shown in Figure 4-10, a high variance between subjects of 24 hour urinary excretion of quercetin with mean of 0.810 ± 0.704 µmol during quercetin treatment and 0.200 ± 0.366 µmol during placebo suggests that the variability in response to quercetin is most likely associated with differences in absorption and tolerance, also individuals varying in their dietary or exercise might modify the effect. Because of the small size of this study, we were unable to identify the reason of the variable response. Future studies may face the same situation and it is suggested to involve a food frequency questionnaire (FFQ) as compliance check to make sure their own diet does not contain significantly higher amount of quercetin in placcebo period. However, the study population was very homogenous because strict eligibility criteria and participant surveillance were used to prevent confounding influences of gender, medications, diet, or other lifestyle factors. This was further confirmed by plasma profiling (^{1}H-NMR) in Chapter 5. Hence, our result may be valid only for male individuals who are mildly hyperuricemic but otherwise healthy and we do not know if the findings will extend to populations that include lower plasma uric acid level, females, hypertensive, older or younger population or different ethnic or geographic mixture. More clinical trials are needed to determine the re-productivity of the data and whether it can be generalised to the larger population.

4.4.7 Application

The role of habitual diet should also be considered. Similar effects might be achieved by consuming quercetin-rich foods, which may provide more bioavailable quercetin. We (Chapter 3) recently showed that, for example, quercetin (as glycoside conjugates) in 100 g fresh red onion provides a similar amount of bioavailable quercetin to the tablet used here (500 mg of pure quercetin aglycone) (177). This preliminary study provides proof of principle and only one dosage was tested. Further clinical research should determine the minimal and maximal dosage of quercetin to establish a new therapy by evaluation of clinical efficacy and safety. However, the present study adopted 500 mg d^{-1} of quercetin supplement, may provide much more microbial metabolites to be absorbed into the blood stream and have a physiological effect. This study did not evaluate the concentration of plasma phenols thus did not identify the relationship to the observed effect. The lack of adverse events for participants receiving quercetin in this study is promising, but the absence of side effects cannot be extrapolated to higher doses or longer times. However, quercetin is part of the normal diet and is consumed in very different amounts according to the individual′s dietary pattern.

In summary, quercetin seems to be an attractive alternative to available anti-hyperuricemia medications. Adoption of one querctin tablet that has proven efficacy to reduce blood uric acid in the habitual diet is easy to adhere to compared to a comprehensive dietary modifications. Therefore it may be a promising behavioural approach to lower uric acid in individual with above-optimal blood uric acid. Establishment of effective amount of quercetin in diet may lead to recommendation for serving of vegetables and fruits on a daily basis to take advantage of this benefit.

Chapter 5. Metabolic Profiling of Plasma by ^{1}H NMR Spectroscopy from Randomised Controlled Trial

Abstract

The consumption of quercetin supplement at dosage of 500 mg d^{-1} has been proven effective in lowering blood uric acid concentration and diastolic blood pressure in pre-hyperuricemic males. The objective in this chapter is to investigate changes in the plasma metabolite profile caused by 4-week intervention of 500 mg quercetin d^{-1}, using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy-based metabolomics. In this double-blinded randomised placebo-controlled cross-over study, 22 healthy males received 500 mg quercetin d^{-1} for 4 weeks. Blood plasma samples were collected at the baseline (day 0), 2-week (day 14) and 4-week (day 28) of the treatments. The metabolic profiles were compared across samples using PCA, PLS-DA and OPLS-DA to analyse the multivariate spectral data. Absolute concentrations of corresponding signals (matabolites) were interpreted by external measurement of glucose for each sample. Lipoproteins, amino acids and carbohydrate profiles that related to metabolic pathways responsible for energy metabolism can be detected. PLS-DA revealed a significant difference in plasma profiles after administrations of quercetin and placebo. Glutamine and acetoacetate were significantly reduced after quercetin supplementation compared to placebo treatment. Valine, alanine, β-hydroxybutyrate and lactate levels were not affected by either treatment. 4-week intervention of 500 mg quercetin d^{-1} in healthy males significantly alters the metabolite composition of plasma therefore has an effect on metabolic progress.

5.1 Introduction

As the metabolite composition within living tissue and its waste management reflects the physiological or pathophysiological state of the total organism, a fast and reliable analysis of metabolites within a specimen or the intact living system will supplement medical diagnosis as well as basic research in various biochemical aspects. Metabolic profiling, metabolomic and metabonomic studies encompass the comprehensive and simultaneous systematic profiling of multiple metabolite concentrations and their cellular and systemic fluctuations in response to drugs, diet, lifestyle, environment, stimuli and genetic modulations, in order to characterise the beneficial and adverse effects of such interactions (206). ^{1}H-NMR (proton nuclear magnetic resonance) spectroscopy provides structural and conformational information on multiple metabolite classes without prior isolation of individual chemical compounds. The obtained spectra of biofluids such as plasma and urine, or of tissues and tissue extracts, provide metabolic patterns corresponding to the metabolic status of the organism as a function of genetic, environmental or toxicological influence (Figure 5-1).

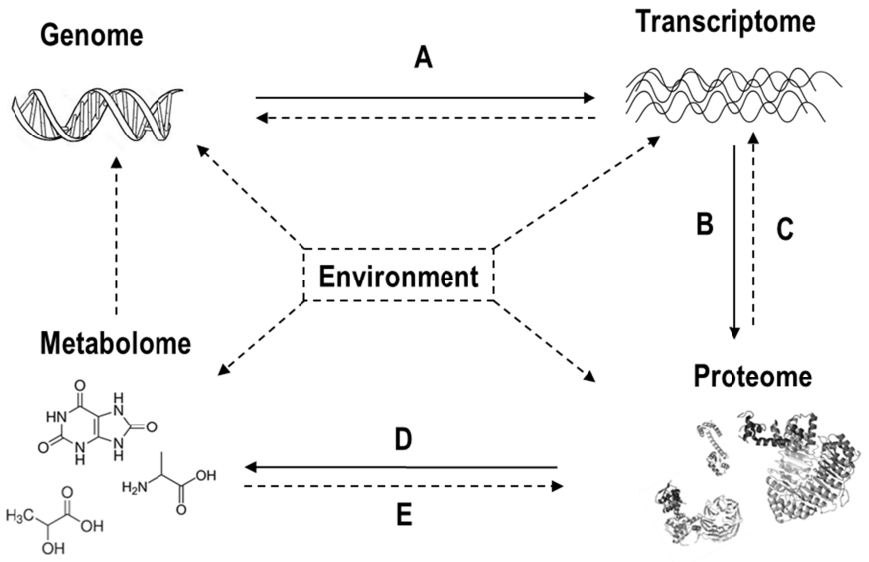


Figure 5-1 Flow of biological information.

Genomic information is transcribed into RNAs **(A)**, which thereafter are translated into proteins **(B)**. Proteins act in the regulation of transcription (e.g. as transcription factors) (C) or directly on metabolite levels as enzymes or transporters **(D)**. Metabolites, in turn, can regulate the activity of proteins for instance as ligands or via protein modifications **(E)**. All organizational levels are affected by environmental factors like diet, lifestyle or mutagenic exposure. Figure is adapted from (207).

Metabolomics involves maximum data capture from biofluid or tissue analysis through the use of technologies such as NMR spectroscopy or mass spectrometry, resulting in complex multivariate data sets that require pattern recognition statistics using chemometric and bioinformatics methods for interpretation (also called multivariate data analysis). Multivariate statistical and pattern recognition methods have been developed to extract sample classification and associated biomarker information from NMR spectroscopic data because of the high complexity of bio-fluids containing potentially thousands of different metabolites. The aim of these procedures is to produce biochemically based fingerprints that are of diagnostic or

other classification value (Figure 5-2). A second stage, crucial in such studies, is to identify the substances causing the diagnosis or classification, as these become the potentially complex set of biomarkers that define the biological or clinical context and help explain the mechanisms related to tissue damage or disease (Figure 5-3).

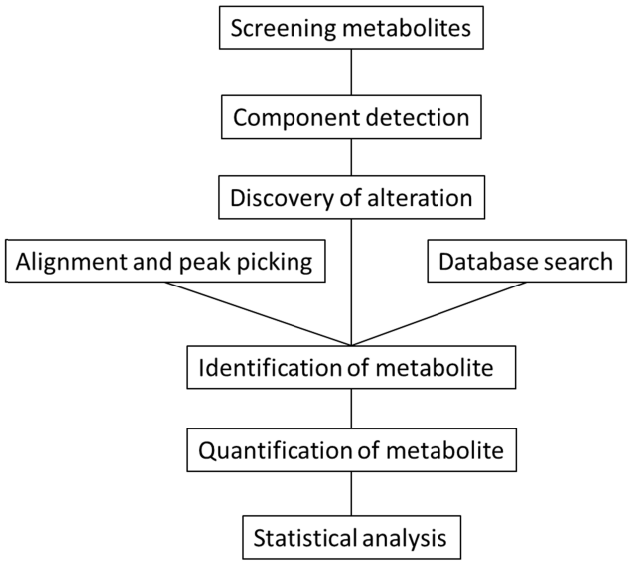


Figure 5-2 Untargeted metabolomics. An untargeted approach allows the identification of alterations in metabolic profiles induced by a nutritional intervention (or a disease). Figure is personally drawn by Yuanlu Shi.

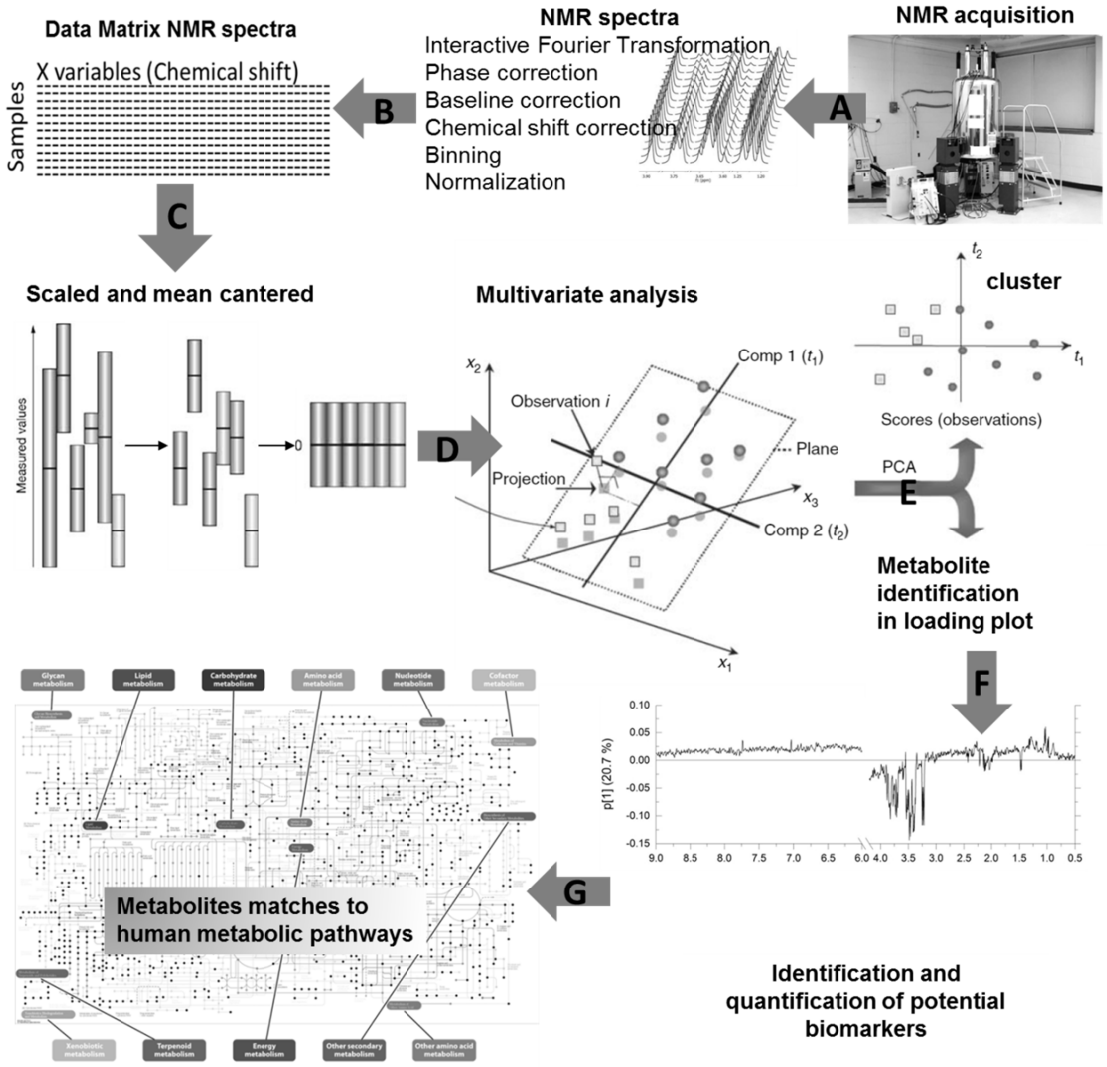


Figure 5-3 Schematic illustration of the chemometric approach to metabolomics.

Multiple blood samples are analysed via NMR (A) and then the spectra are processed (B, C) using principal component analysis (D, E). After identifying significant differences (F), the most informative peaks in the spectra are identified using a variety of methods. Later quantitative approach to metabolomics will be performed if necessary. In this example the biofluid spectrum is annotated and most (all) of the compounds in the sample are identified and quantified. Metabolite differences between groups can be analysed using informatics solutions, which provide multivariate statictical analysis tools and database searches functionalities (e.g. METLIN, Human Metabolome Database and LipidMaps for the identification

of the metabolites and pathways (G). Figure is personally drawn by Yuanlu Shi with part adapted from (208, 209).

This technique permits a non-targeted comparison of physiological variation in experimental animals/humans, such as male/female differences, healthy/patients classification, age-related changes, dietary modulation, diurnal/nocturnal effects and phenotyping of mutant and transgenic animals. Development of pharmaceutical applications in which metabonomic biofluid profiles were used to identify specific biomarkers of organ toxicity, an important economic factor of attrition in the preclinical pharmaceutical discovery process has led to a quick diagnose at early stage of the likelihood of developing disease (210, 211). Metabonomics and metabolomics have found application not only in the study of diseases like cardiovascular disease (212), multiple sclerosis (213), hypertension (214), breast cancer (215), diabetes (216) and epithelial ovarian cancer (217), but also of personalised medicine (218) and nutritional factors on gut microflora (219), in animals fed by different grains (220), in humans given soy isoflavones (221), soya milk (222), tea (223), grape products (224, 225), proteins (226), vitamins (227) or fatty acids (228). The application of pattern-recognition techniques have been used in nutrition and metabolomics research and challenges have been discussed extensively (229-232).

Knowing the effect of lowering plasma uric acid, proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy-based metabolite profiling was adopted to investigate changes in plasma metabolomics caused by daily supplementation of 500 mg quercetin for 4 weeks. The aim of this study is to investigate the possibility of predicting postdose quercetin effects from predose metabolic profiles thus identify potential biomarkers or toxicity through this pharmaco-metabonomics approach. It is

particularly of interest in identifying other potential metabolites that may be associated with uric acid reduction or to quercetin supplementation.

5.2 Materials and methods

5.2.1 Subjects and study design

22 healthy males were eligibly assigned and successfully compliant for the complete study. They were healthy adult males aged 29.9 ± 12.9 y with BMI of 24.8 ± 3.0 kg m^{-2}, blood pressure of normal to (pre-) hypertensive (Systolic 123 ± 8 mm Hg, diastolic 74.3 ± 9.0 mm Hg), and fasting blood glucose of normal to impaired fasting glycemia of 5.04 ± 0.56 mM and plasma uric acid of 339 ± 51 μM (Table 4-2). They were non-smokers and not a heavy drinker (less than 3 units of alcohol regularly per day). The study was conducted according to the guidelines laid down in the declaration of Helsinki of 1975 as revised in 1983 and all procedures involving human subjects were in accordance with the ethical standards of the University of Leeds, MaPS and Engineering joint Faculty Research Ethics Committee (MEEC12-019), UK. Written informed consent was obtained from each of the subjects before commencement of the study. All data were collected and analysed in the University of Leeds, UK.

The study was a randomised, double-blinded, placebo-controlled, cross-over, 4-week intervention trial with 2 treatment groups, with daily consumption of either quercetin dehydrate tablet (500 mg stated, actual measured 544 mg, purchased from Nature′s Best, Kent, UK) (177) or placebo (the placebo formulation is a white oval tablet and contain lactose monohydrate, magnesium stearate and cellulose, purchased from

Fagron, Barsbuttel, Germany). There was a 4-week washout period between each treatment. Blood and urine samples were taken before, during and the end of each study phase. Each participant was independently and randomly assigned in one of two groups, receiving both treatments in one order or another.

5.2.2 NMR sample preparation

Fasting plasma were collected at day 0, 14 and 28 of each arm and were stored at -80℃ until analysis. 300 µL of plasma was mixed with 350 µL of deuterium oxide (D_2O, Sigma-Aldrich, USA), flicked to remove bubbles and then vortexed at level 5 (Stuart vortex mixer, Bibby Scientific Limited, Staffordshire, UK). The mixture was then centrifuged at 12 000 g (equivalent to 11 300 rpm) (MIKRO 120, Andreas Hettich GmbH & Co.KG. Tuttlingen, Germany) for 5 min at room temperature. 600 µL of supernatant was transferred into a 5 mm, 7" select series NMR tube (S-5-500-7; Norell. Inc).

5.2.3 ^{1}H NMR spectra acquisition

^{1}H-NMR spectra were acquired on a Varian Unity Inova 500 spectrometer operating at 499.97 MHz proton frequency at 20℃. A standard NMR pulse sequence, Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence, was used to selectively highlight the signals from the small molecule metabolites where the observed peak intensities are edited on NMR T_2 relaxation times. The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [RD - 90°- $(\tau$ - 180° - $\tau)_n$ - acq] was used, with a relaxation delay (RD) of 2 seconds, during which the water resonance was selectively irradiated with a τ of 1.5 ms and an n of 150. For each FID (free induction decay), 256 transients were collected into 16 384 data points with a spectral width of 6 000.2 Hz.

5.2.4 Data binning/spectra fitting

Spectral data were processed with ACD/Spectrus Processor 2014 (Advanced Chemistry Development Labs software 14, Toronto, Canada). An exponential line broadening of 1 Hz was applied to the FIDs before zero filling with a transform size of 32 768 (32 K) data points. The resulting spectra were phased, baseline corrected, and referenced to the α-glucose signal at 5.23 ppm. The spectra sections were then divided into bins (frequency windows) of 0.005 ppm over the range 0.5-9.0 ppm, and the integrals (areas; directly relating to concentration) of the signals within each bin calculated. This serves to stabilise effects of physicochemical environment differences (pH, ionic concentration, etc.) that may cause peak position variation and allow a smaller, more manageable, number of variables for statistical processing. The chemical shift region 4.2-6.0 ppm was set to zero to eliminate the effects of varying water resonance suppression efficiency. Prior to principal components analysis (PCA), the integrals were normalised to the total spectral area (hence total concentration of all metabolites) and was converted into a numerical format, consisting of observations (the actual samples) and variables (the individual intensity and chemical shift pairs symbolising individual chromatographic peaks).

5.2.5 Chemometric analysis of NMR data

Analysis was then carried out using chemometric tools, such as principal component analysis (PCA) and projection to latent structure also called partial least squares data analysis (PLS-DA), to discover the variables describing the metabolic variation involved in the particular study or to allow categorization of the samples.

5.2.5.1 Multivariate analysis (Ordinary PCA)

The NMR data were *Pareto*-scaled and mean cantered before PCA was carried out with the use of SIMCA-P+ 13.0.0.0 (Umetrics,Umea, Sweden). PCA reduces multivariate data into a set of variables (principal components, PCs) which each describe the variability in the data under the constraints that each PC is orthogonal to the last and each accounts for the greatest source of variation remaining after previous PCs have been established. In brief, multivariate data was plotted in multi dimensions where the position of the data points represents the numerical intensity of each variable. Having plotted out all the data points, a structure is visible and a new latent variable, principal component (PC) 1, can be drawn through the centre of the data swarm. However, there is still significant variance across the data points and a second PC is generated at 90° to the first PC. In this manner three variables was reduced down to 2 new PCs. PCA typically reduces datasets of thousands of variables down to 10-11 PCs.

Two plots are produced in PCA: first, a scores plot, which displays how individual samples relate to one another as a point on the plot (score, t) and allows intrinsic clustering among individuals to be examined; and second, a loadings plot which describes which variables are most influential in producing the scores plot (loadings, p). The parts of the spectra corresponding to the most discriminatory variables are displayed in order to allow identification of the varying metabolites or biomarkers for a particular condition. For ^{1}H-NMR spectral data, the variables are the chemical shifts corresponding to the centres of the bins containing metabolite signals. The quality of the model can be assessed based on the goodness of fit (R2X value, where X refers to the original variable matrix) and the model′s ability to predict the class

membership of new samples (Q2 value). PCA only investigates the general variance structure of the blood plasma spectral data.

5.2.5.2 Multilevel data analysis (MLPCA)

A further data mining approach, multilevel PCA (233) was used to analyse individual contrast between quercetin intervention and placebo treatment. Let for example:

> $\mathbf{x}_{iQ\text{-}w0}$, $\mathbf{x}_{iQ\text{-}w2}$ and $\mathbf{x}_{iQ\text{-}w4}$ be samples from subject i at week 0 (baseline), 2-week, and 4 week of quercetin (Q) intervention. Further, let $\mathbf{x}_{iP\text{-}w0,}$ $\mathbf{x}_{iP\text{-}w2}$ and $\mathbf{x}_{iP\text{-}w4}$ be the corresponding samples during placebo (P) intervention.

The purpose is to investigate between the supplementary response, defined as the difference between plasma composition after intervention and at baseline ($\mathbf{dx}_{iQ\text{-}w4\text{-}w0} = \mathbf{x}_{iQ\text{-}w4} - \mathbf{x}_{iQ\text{-}w0,}$ $\mathbf{dx}_{iQ\text{-}w2\text{-}w0} = \mathbf{x}_{iQ\text{-}w2} - \mathbf{x}_{iQ\text{-}w0}$) for quercetin and the same for placebo, as illustrated in Figure 5-4 of the baseline adjustment scheme. This corresponds to comparing $\mathbf{dx}_{iQ\text{-}w4\text{-}w0}$ and $\mathbf{dx}_{iQ\text{-}w2\text{-}w0}$ with $\mathbf{dx}_{iP\text{-}w4\text{-}w0}$ and $\mathbf{dx}_{iP\text{-}w2\text{-}w0}$ across all individuals (i=1, 2, 3, …, 22). Following analysis was carried on with normal PCA.

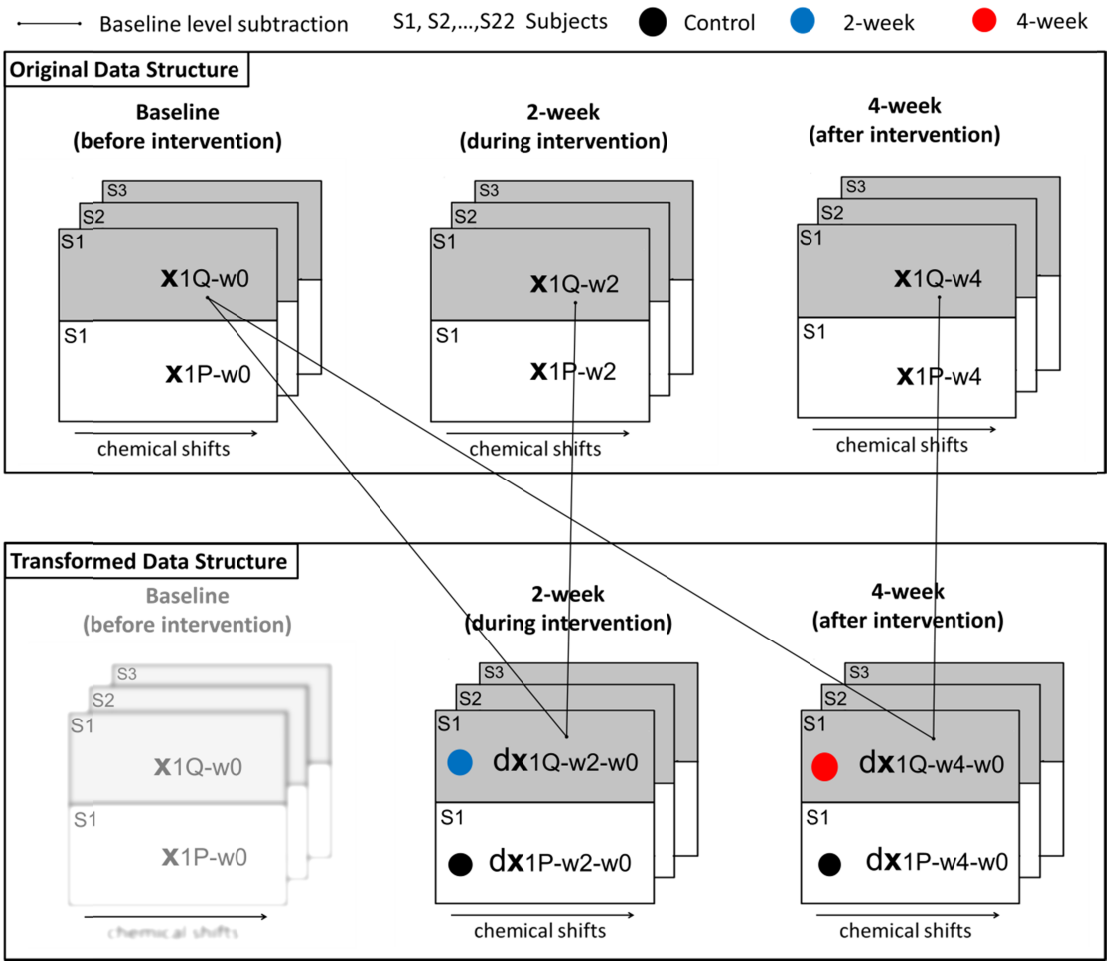


Figure 5-4 Data structure and arrangement scheme. Baseline subtraction concatenation of sampling points. Figure is personally drawn by Yuanlu Shi.

5.2.6 OPLS-DA

Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to confirm any class separations identified in the PCA. OPLS-DA is a supervised technique, which means a model is built with a priori knowledge of the class membership of a set of samples and so acts to try to find the variation in the data that best describes the difference between the classes. Because it is supervised, OPLS-DA models require validation, usually by testing the model to see if it can correctly predict the class membership of a new set of samples that were not used to build the

original model. In the present study, it was not possible to obtain any more samples and there were not enough samples to leave out a set for prediction. Therefore, the OPLS-DA models were validated with the use of “leave-one-out” cross-validation, where one third of the samples were excluded from the model and the OPLS-DA model built from the remaining two third of the samples was used to predict the class membership of the excluded samples. This step was repeated until all samples had been excluded. Furthermore, OPLS-DA models were validated using permutation testing. This involved randomly permuting the class memberships of the samples, and remodelling the data to see whether the resulting models were of similar quality to the original model (in terms of R2Y and Q2, where Y refers to the classification). If permuted models are of similar or better quality than the original model, then the original model could be spurious.

5.2.7 Quantification of metabolites

In order to quantify the absolute concentration of interesting metabolites, all NMR spectra data were normalised to the whole spectrum integral. With α-glucose referred to δ 5.23 ppm, the baseline of regions of δ 5.20-5.25, 0.80-4.66 were phased to remove the interference of signal of water.

Then the spectra were binned into windows of 0.01 ppm width. The area of peaks of metabolites of interest were checked to make sure that all bins that were covered in the signal region were combined (Table 5-1). For example, peaks at 5.23 d α-glucose covers from 5.217-5.236 ppm with width of 0.019 ppm and the two bins cross 5.220-5.240 ppm were combined for α-glucose integral.

Table 5-1 ^{1}H-NMR resonance assignments with chemical shifts for signals identified in human plasma

Metabolite	δ (ppm) multiplicity	Peak region (ppm)	Bin width (ppm)	Selected and combined bins (ppm)
α-glucose	5.23 d	5.217 - 5.236	0.019	5.220 - 5.240
β-glucose	4.65 d	4.626 - 4.653	0.027	4.620 - 4.660
Valine	0.99 d	0.968 - 0.991	0.023	0.970 - 0.990
Valine	1.04 d	1.020 - 1.045	0.025	1.020 - 1.050
Lactate	4.11 q	4.091 - 4.117	0.026	4.090 - 4.120
Lactate And threonine	1.334 d 1.332 d	1.303 - 1.338	0.035	1.300 - 1.340
Glutamine	2.13 q	2.090 - 2.147	0.057	2.090 - 2.150
Glutamine	2.46 m	2.415 - 2.466	0.051	2.410 - 2.470
Acetoacetate	2.27 s	2.265 - 2.275	0.010	2.260 - 2.280
Alanine	1.48 d	1.457 - 1.480	0.023	1.450 - 1.480
β-hydroxybutyrate	1.20 d	1.179 - 1.205	0.026	1.180 -1.210

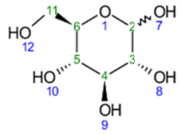
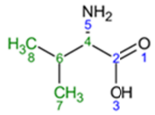
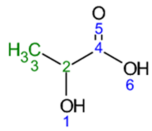
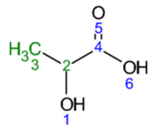
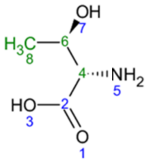
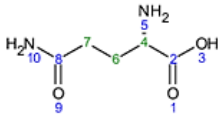
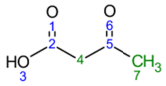
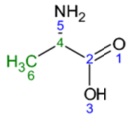
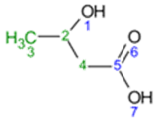
Note: s: singlet; d: doublet; q: quadruplet; m: multiplet.

For each sample, integrals of α-glucose and β-glucose were combined and this was referred to glucose concentration (mM) that was measured by external mean (detailed in Section 2.2 and Section 0). The conversion factor **(F)** was used to predict the concentration of other metabolites. In brief, the combined peak area of the combined resonance at δ 4.65 ppm and δ 5.23 ppm ($A_{glucose}$) is used to normalise the raw NMR signals integrals (X) in each spectrum.

$$F = C._{glucose}/A_{glucose} \text{ /number of } ^{1}H.$$

e.g. $C._{valine} = F \times A_{valine} / 3$ (since doublet signal at 1.04 ppm was contributed by the protons of 3 hydrogen of $-CH_3$ of valine). A list of metabolites and their structure was used for this calculation (Table 5-2).

Table 5-2 Metabolite chemical information

Metabolite	Chemical shift (ppm)	Structure	Identical proton	Number of ^{1}H
α, β-glucose	4.65 d, 5.23 d		(2)-CH-	1
Valine	1.04 d		(7)-CH_3	3
Lactate	4.11 q		(2)-CH-	1
Lactate	1.334 d		(3)-CH_3	3
Threonine	1.332 d		(8)-CH_3	3
Glutamine	2.46 m		(7)-CH_2-	2
Acetoacetate	2.27 s		(7)-CH_3	3
Alanine	1.48 d		(6)-CH_3	3
β-hydroxybutyrate	1.20 d		(3)-CH_3	3

5.2.8 Univariate analysis

PCA and OPLS-DA statistics were performed in SIMCA and other data comparison were performed in SPSS. After tests of normality, the paired Student t test or the Wilcoxon signed-rank test was performed to compare the mean values of the integrals from influential regions of the NMR spectra, using SPSS.

5.3 Results

5.3.1 ^{1}H NMR spectral analysis of plasma profiles

Lipoproteins, amino acid and carbohydrate profiles that relate to metabolic pathways responsible for energy metabolism can be detected. Figure 5-5 shows a 500 MHz ^{1}H-NMR spectrum of plasma sample from an apparently heathy man. The reference table from (234) and the Human Metabolome Database (235) were used for peak assignment for resonances in the spectrum. Among them, the resonances of broad lipid signals around 0.9 ppm (-CH_3) and 1.3 ppm (-CH_2-) stemming from the lipoprotein contents of triglycerides, cholesterol compounds and phospholipids can be assigned (Table 5-1). Spikes at δ 1.18 ppm of β-hydroxybutyrate (BHB), methyl group (δ 1.33 ppm, e.g. lactate or threonine; δ 1.48 ppm, e.g. alanine; δ 2.22 ppm, e.g. acetone, propionate) can be assigned and δ 2.46 ppm is glutamine, the most abundant free amino acid in human blood (236).

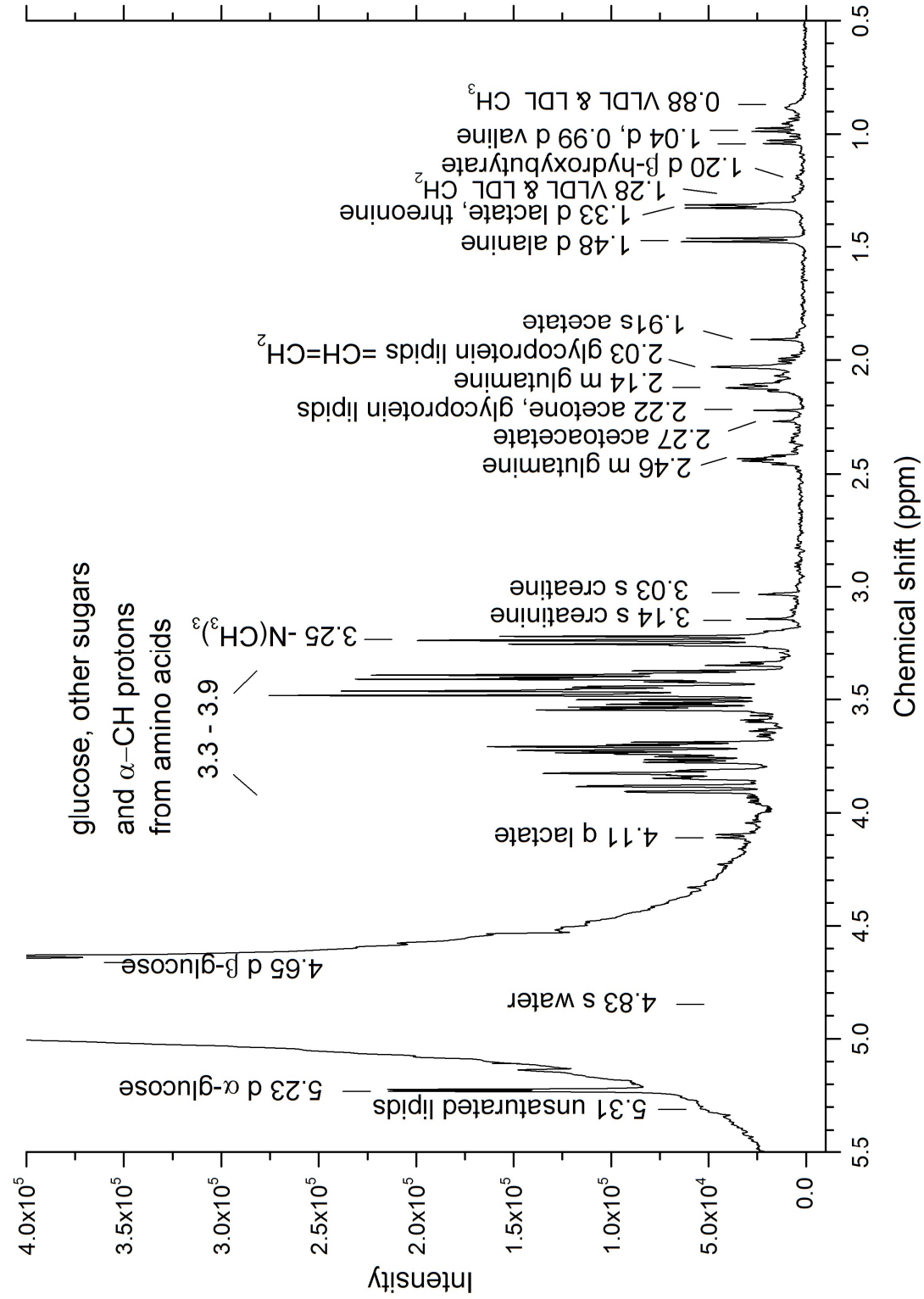


Figure 5-5 500 MHz ^{1}H-NMR Carr-Purcell Meiboom-Gill (CPMG) spectrum (δ 0-6 ppm) of plasma from a healthy volunteer (samplecode 5792) with spectral region δ 3-6 ppm dominated by sugars and δ 0-3 ppm dominated by lipids. Assignment of the most prominent peaks are included (234).

In the spectral region δ 0.5-2.5 ppm, the biological variations between the data-pairs are illustrated (Figure 5-6). $\mathbf{dx}_{iQ\text{-}w4\text{-}w0}$ and $\mathbf{dx}_{iP\text{-}w4\text{-}w0}$ were calculated for each subject and a mean and standard deviation for the NMR spectra of aliphatic region (δ 0.5 - 4.5 ppm) is shown in Figure 5-6. A comparison of the changes brought about by interventions on each region of chemical shift shows clearly the changes of concentration of correspondent chemicals and a summary is listed in Table 5-3.

From visual inspection of the ^{1}H NMR spectra of plasma samples, a high degree of inter-subject and temporal variability which obscured the more subtle metabolic changes related to dietary intervention was observed (Table 5-3). To be able to detect more subtle treatment and related metabolic differences, pattern recognition techniques were applied. Chemometric analysis was used to aid interpretation and to detect latent variables associated with quercetin intervention.

Table 5-3 Visual inspection of comparison of $dx_{iP\text{-}w4\text{-}w0}$ and $dx_{iQ\text{-}w4\text{-}w0}$.

	Chemical shift (ppm)	Quercetin	Placebo	Comparison
VLDL, LDL	0.90	↑	↑↑	↓
Leucine	0.98	↓	↓↓	↑
Valine	1.03	↓	↓↓	↑
β- hydroxybutyrate	1.18	↓	o	↓
Lactate	1.31	↓	↑	↓
Alanine	1.48	↑	↑↑	↓
Acetate	1.90	o	o	o
Acetoneacetate	2.22	o	↓	↑
Glutamine	2.46	o	↓↓	↑
Carbohydrate, sugar	3.4-3.7	↑	o	↑
Glycerol of lipid	3.7-3.9	o	↓	↑

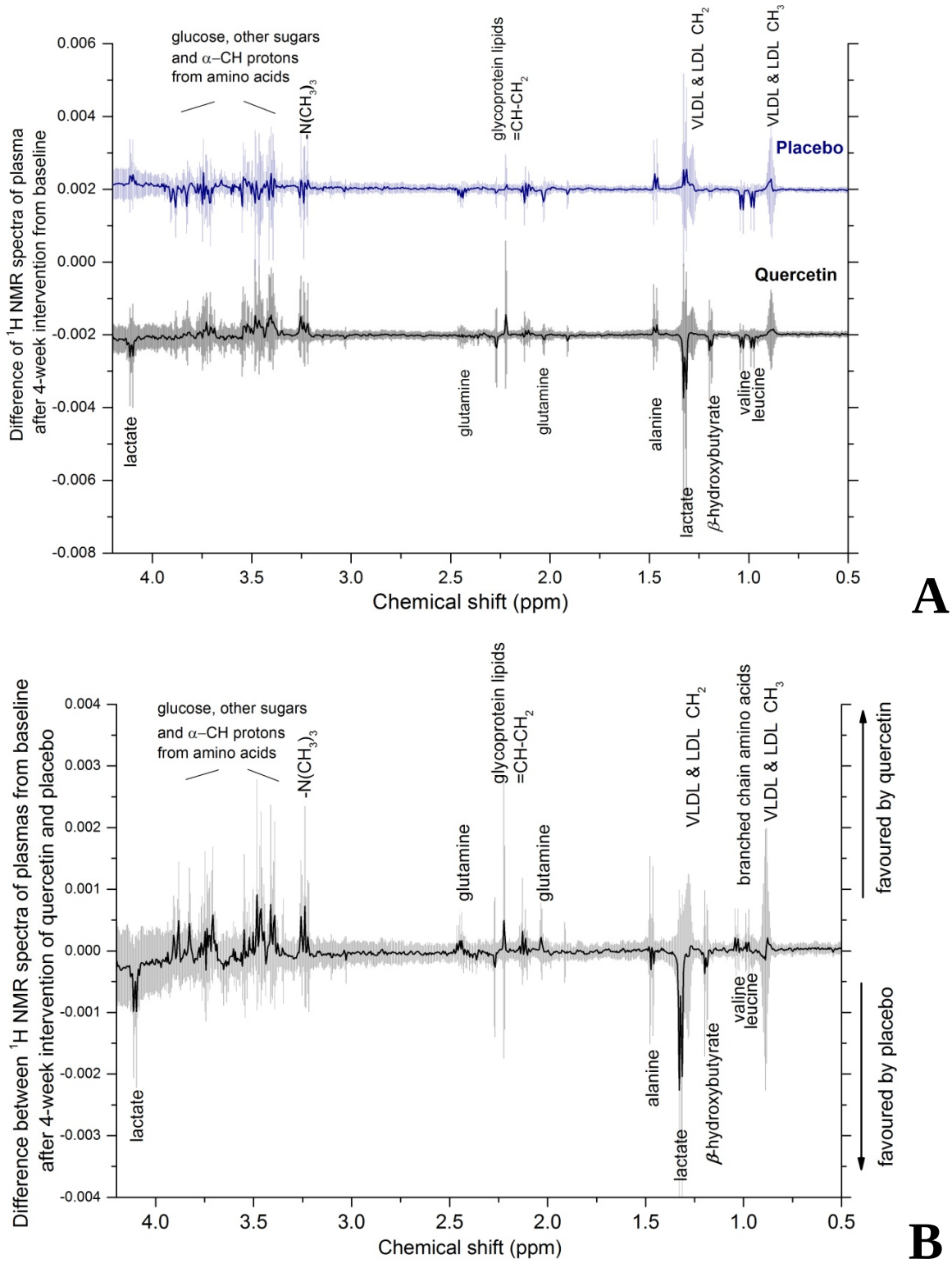


Figure 5-6 Spectra of $dx_{iP\text{-}w4\text{-}w0}$ and $dx_{iQ\text{-}w4\text{-}w0}$ (A) and mean difference between spectra of $dx_{iP\text{-}w4\text{-}w0}$ and $dx_{iQ\text{-}w4\text{-}w0}$ (B) for 22 subjects in the region of δ 0.5 - 4.6 ppm. Line and shadow show mean ± SD. Lactate was reduced significantly (paired t test $P<0.05$) after quercetin treatment compared to placebo.

5.3.2 PCA and unsupervised classification

PCA was performed on all plasma metabolite profiles. Two plasma samples were recognised as outliers and were removed from the analysis because of the presence of a substantial signal of ethanol. The PCA score plot (Figure 5-7) of the plasma profiles did not display intrinsic clustering related to supplementations in the 1st and 2nd PCs. Initial PCA does not take the experimental design into account, therefore the power of this multivariate method is not fully employed. However, this demonstrates that the study population was in healthy condition and was homogenous.

Apparently huge variance between subjects made it impossible to compare within subject (Figure 5-8) with ordinary PCA that overwhelm the variance with subject even though we have accounted for the BMI and gender, two of the most important factors on plasma metabolic variations (237). Figure 5-8 presents that the variation within subject is much smaller than that between subjects making it difficult to identify the changes within subject. In conclusion, multilevel data analysis is necessary in cooperating interventional cross-over study in order to draw any meaningful conclusion.

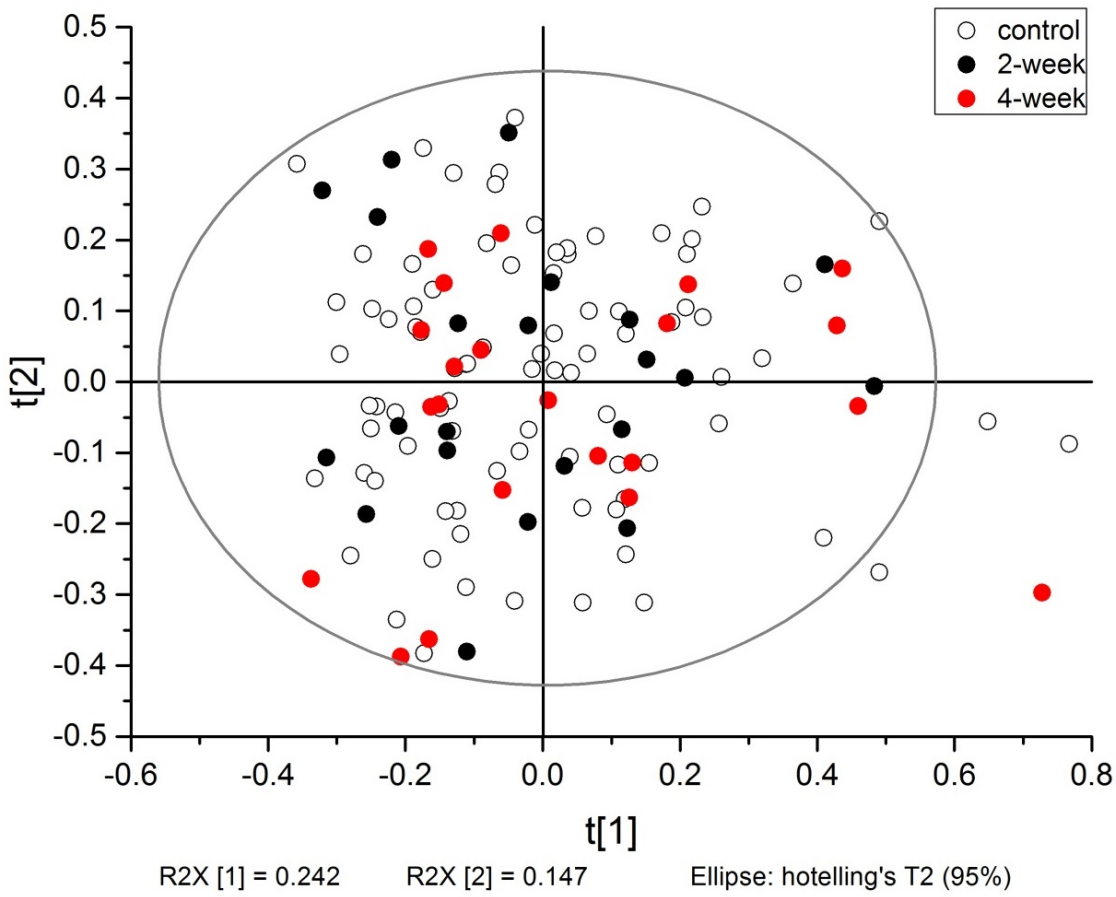


Figure 5-7 PCA score plot of human plasma ^{1}H-NMR spectra.

The presence of outliers was investigated using PCA-Hotelling T^2 Ellopse (95% CI) and the normality of multivariate data using the normal probability plot of PCA model. Samples were classified into three groups. They are control (open circle) contains samples of $\mathbf{x}_{iP\text{-}w0,}$ $\mathbf{x}_{iP\text{-}w2,}$ $\mathbf{x}_{iP\text{-}w4}$ and $\mathbf{x}_{iQ\text{-}w0}$; 2-week (black filled circle) of $\mathbf{x}_{iQ\text{-}w2}$ and 4-week (red filled circle) of $\mathbf{x}_{iQ\text{-}w4}$ (i=1, 2, 3, …, 22). This plot shows 38.9% of the total difference. Component 1 represents 24.2% of the variance. The 4 observations representing samples from each laboratory visit for subject 339 who has the highest fasting glucose level among all subjects fell outside the Hotelling′s T^2 Ellopse (95% CI). Hotelling′s T^2 is a multivariate t-test to assign a probability that the predicted score value of any given observation is different from the mean of the distribution (238).

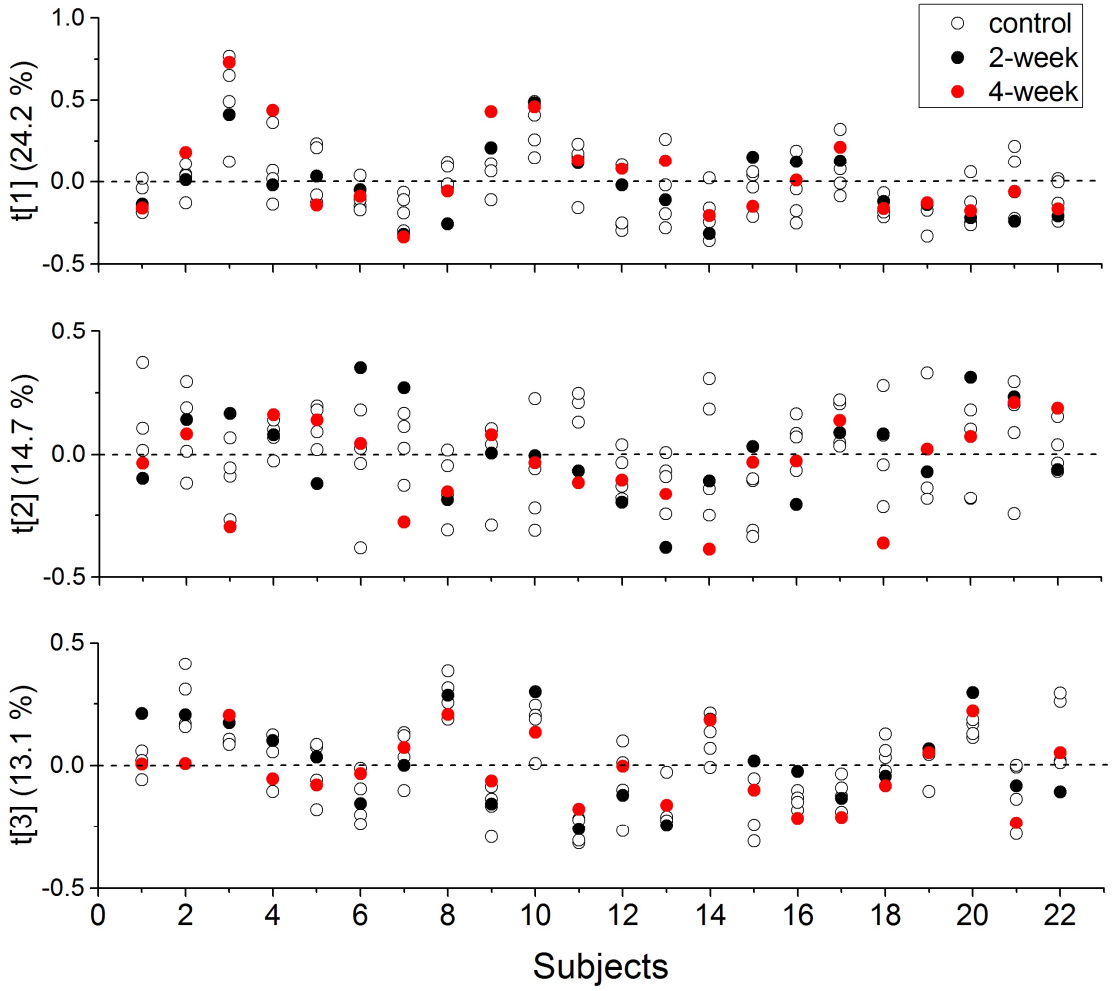


Figure 5-8 Scoring plot of each subject of first three major components. Note how much less variable the human plasma is when is compared with other individuals.

5.3.3 Chemometric techniques with multilevel data analysis

After sample transformation detailed in section 5.2.5.2, examination of the scores and score plots for PC1 vs. PC2 (Figure 5-9). The PCA model discriminating between groups was not significant (α=0.05, Figure 5-9 A). Apparently blood sugars (δ 3-4 ppm) contribute to the major variance between samples. Interestingly we have not discovered any significant change of blood glucose level by external measurement. However, some visual clustering in the second comment (PC2:R^2X=19.6%) suggests an association between quercetin intake and lower level of plasma lactate (1.31 ppm) and LDL (0.90 ppm) and higher levels of glutamine (2.46 ppm) and acetoneacetate (2.22 ppm) (Figure 5-9 B). The scores of PC2 were significantly different between control and 4-week for quercetin treatment (*P* = 0.021, Figure 5-10)

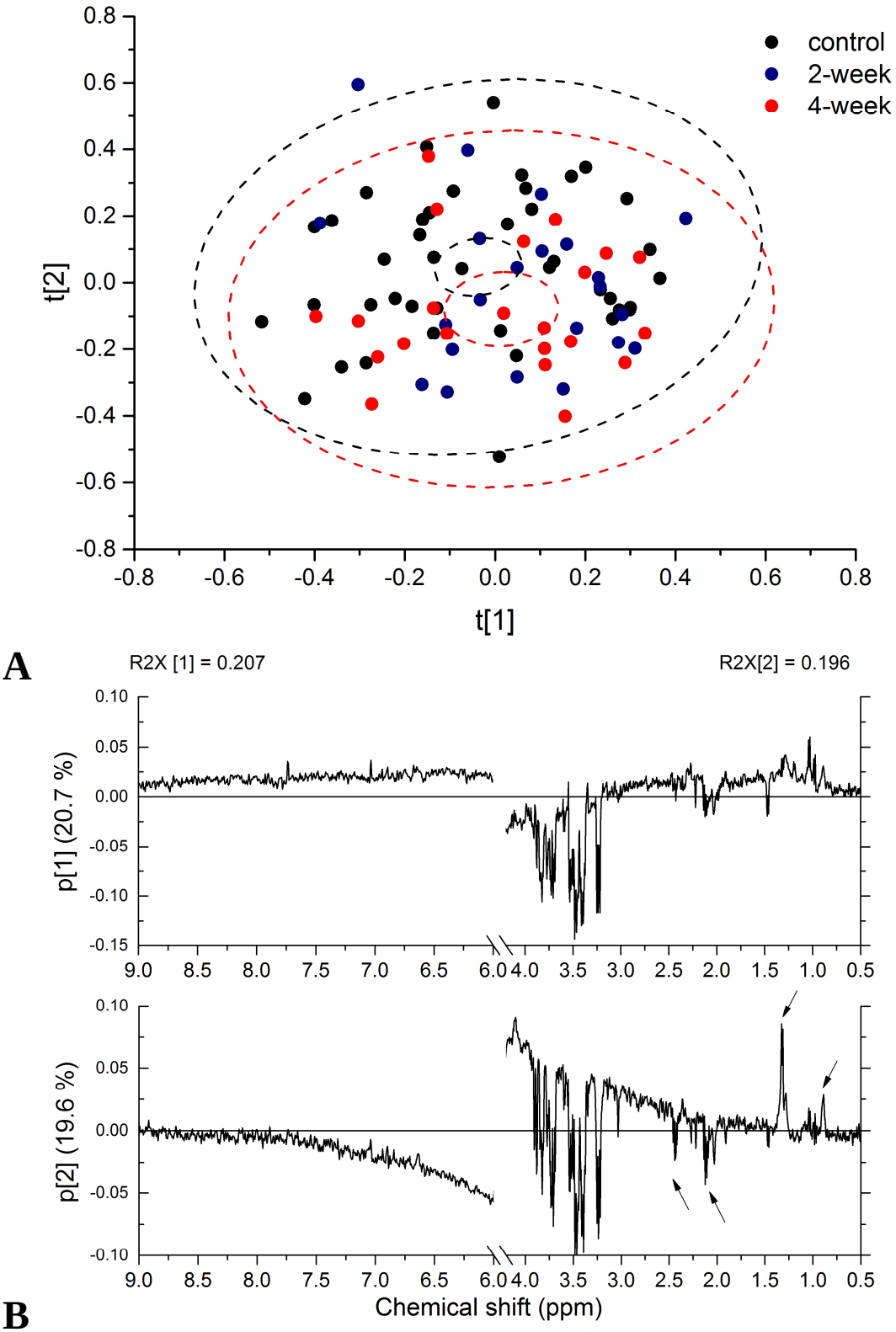


Figure 5-9 **(A)** ^{1}H-NMR score plot of subtracted data. Inner circle indicates mean 95% CI ellipse and out circle is predicted 95% CI. **(B)** Loadings plot of component 1 (top, 20.7%) and component 2 (bottom, 19.6%) across δ 0.5 to 4.2 ppm and 6.0 to 9.0 ppm.

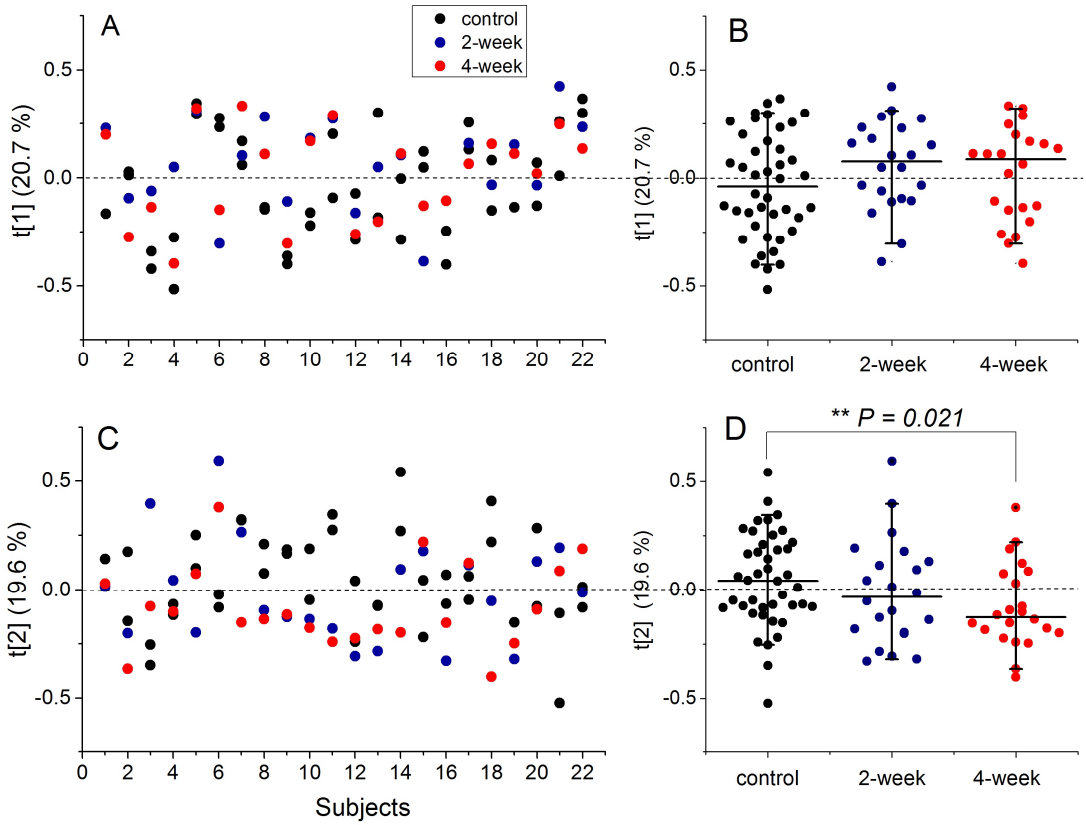


Figure 5-10 Subtracted scoring plot of each subject of first two major components.

Statistic was conducted by two sample non-equal variance t test. Panel A, C shows the within-subject variance and between subject variance of scores of component 1 and component 2. Panel B, D shows the box plot of scores of component 1 and component 2, showing the difference between interventions. Bar with whisker represent median and 5-95%.

5.3.4 OPLS-DA and supervised classification

The OPLS-DA analysis revealed a model for comparison of changes of quercetin and placebo (Figure 5-11). Validation procedures indicate that for a two-component OPLS-DA model the sample classification could be predicted accurately in 59.5 ± 6.3% (mean ± SEM) of the cases, indicating that the observed quercetin-induced plasma changes were robust. The OPLS-DA loading plot (Figure 5-12) highlighted the metabolites that weighs most in describing the difference between classes. They are valine, alanine, glutamine, acetoacetate, β-hydroxybutyrate and lactate.

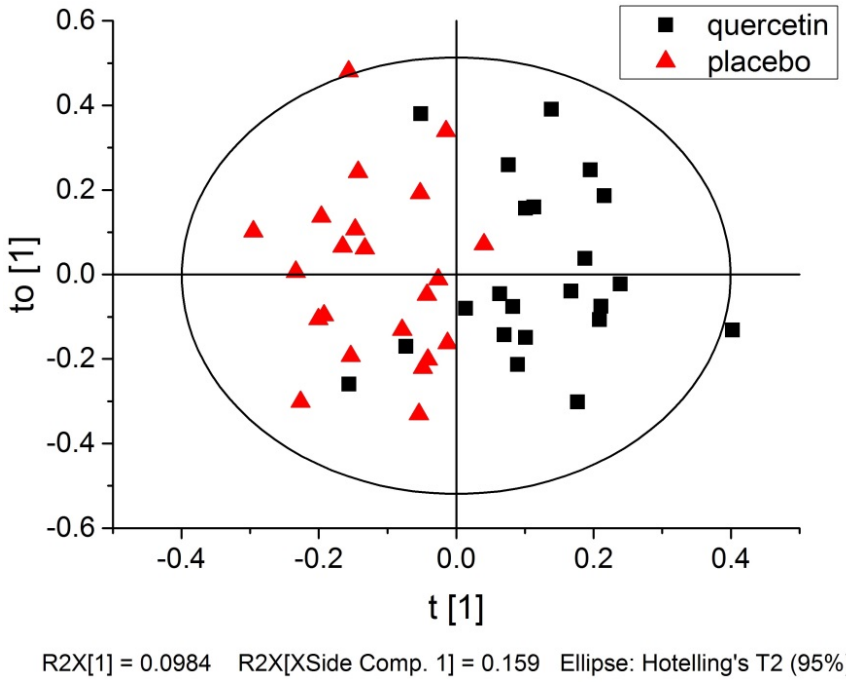


Figure 5-11 OPLS-DA score plot of human plasma 1H-NMR spectra substracted from baseline spectra: $dx_{iP\text{-}w4\text{-}w0}$ (placebo) and $dx_{iQ\text{-}w4\text{-}w0}$ (quercetin).

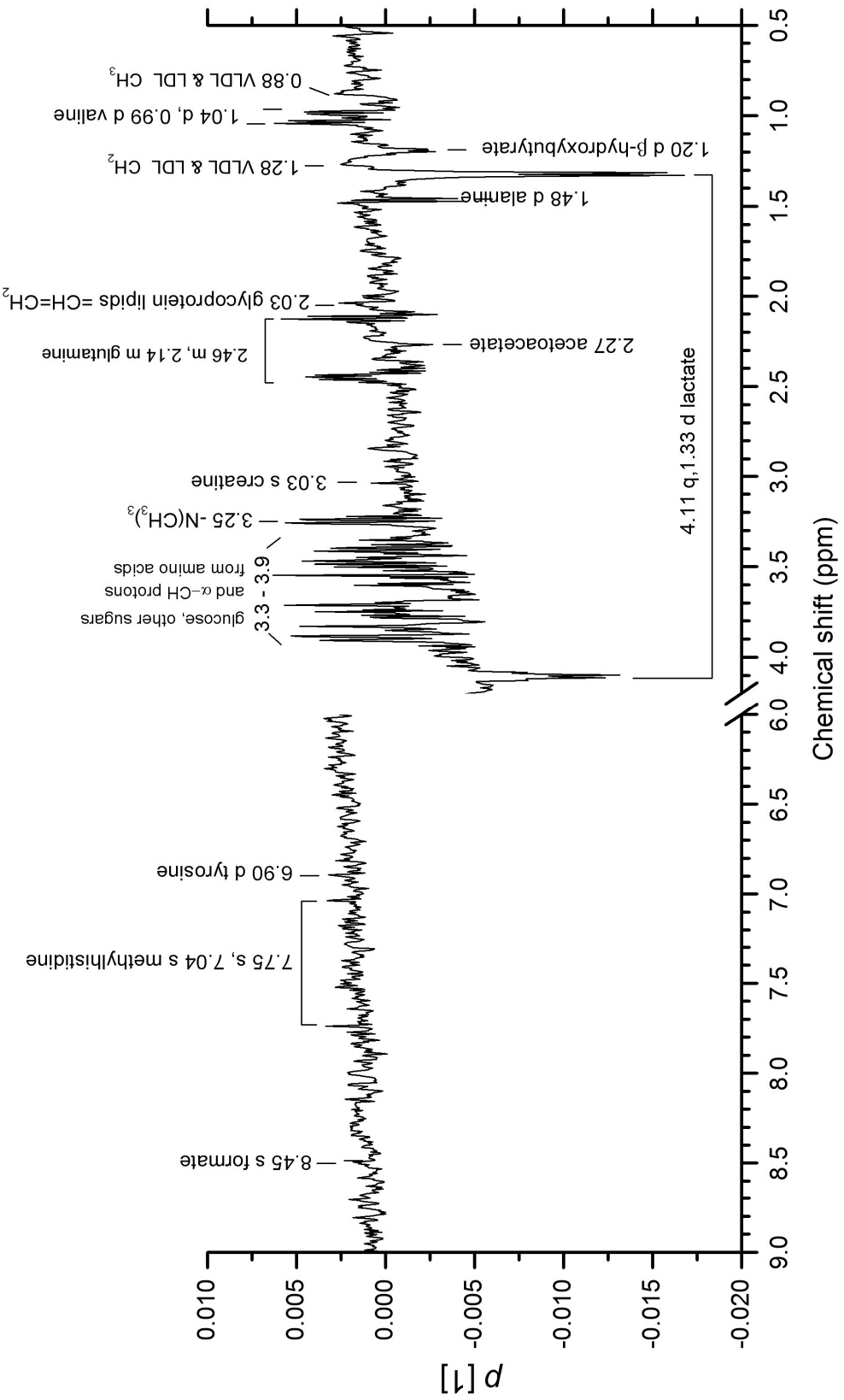


Figure 5-12 OPLS-DA loading plot of component 1 (9.8 %) across δ 0.5 to 4.2 ppm and 6.0 to 9.0 ppm.

5.3.5 Changes in metabolites

Metabolites that were caught in chemometric analysis were quantified by the proton numbers comparing to concentration of glucose as indicated in Table 5-2 and result is shown in Table 5-4 and Figure 5-13. Valine, glutamine, acetoacetate and lactate were significantly reduced after quercetin supplementation compared to placebo treatment. Alanine and β-hydroxybutyrate were not affected by either treatment (Table 5-5, Figure 5-14).

Table 5-4 NMR quantification of these metabolites (n = 6*12-2 = 130).

	Plasma concentration (μM)			
	Mean ± SD	Median (95% CI)	Range	Ref. Mean ± SD
Valine	207 ± 90	179 (121-412)	37-579	212 ± 63
Acetoacetate	62.2 ± 42.7	55.0 (26.6-105.8)	12-350	40.6 ± 36.5
Alanine	389 ± 115	373 (248-603)	99-911	427 ± 84
β-hydroxybutyrate	52.1 ± 64.6	42.3 (12.1-106.4)	6.4-559	76.9 ± 66.3
Glutamine	607 ± 171	581 (421-957)	136-1 280	510 ± 118
Lactate	943 ± 449	837 (392-1886)	101- 2 896	1 490 ± 371

Note: The predicted concentration of those metabolites were consistent with previous report (239).

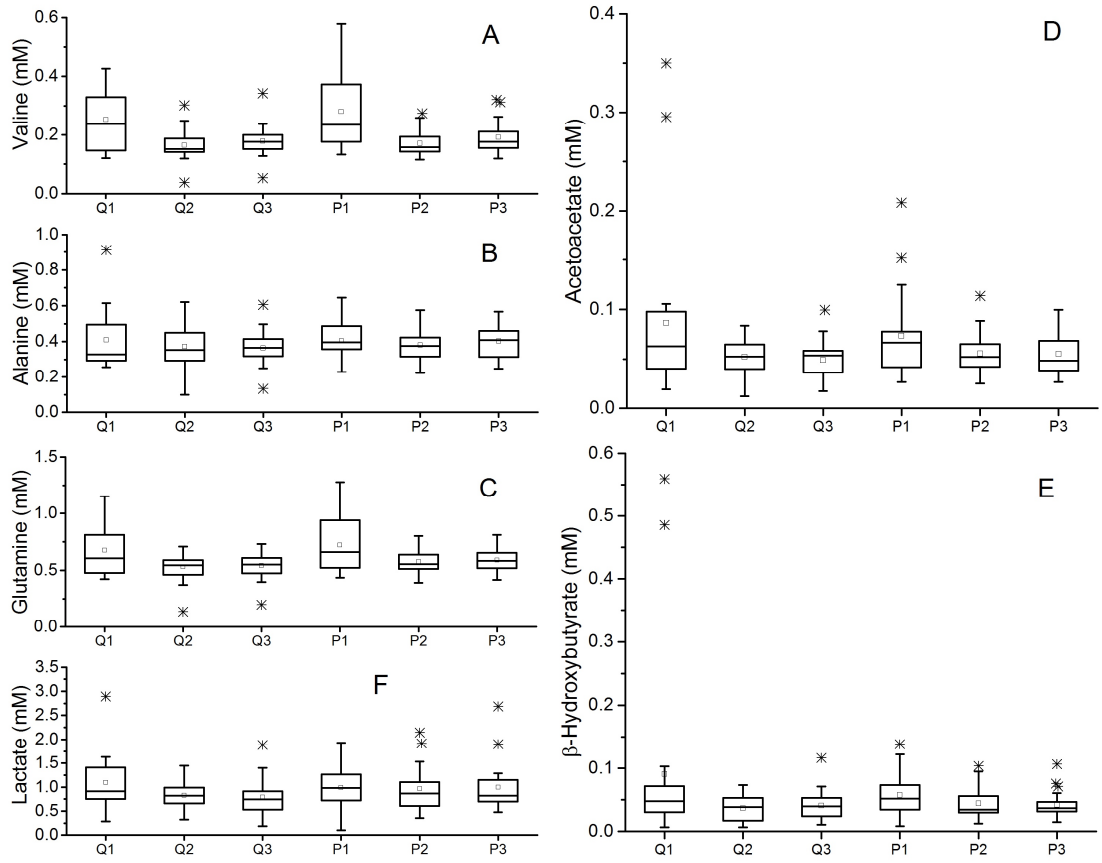


Figure 5-13 Data distribution of each metabolites shown in boxplots. **(A)** Valine, **(B)** Alanine, **(C)** Glutamine, **(D)** Acetoacetate, **(E)** β-hydroxybutyrate and **(F)** Lactate. Open square shows mean value and box represents upper quintile, median and lower quintile with whiskers showing 1.5 × interquartile range (IQR). Q1, Q2, Q3, P1, P2 and P3 represents baseline, 2-week and 4-week of quercetin arm and baseline, 2-week and 4-week of placebo arm, respectively.

Table 5-5 Metabolites quantified by NMR to glucose reference (n=22 or 20).

		Quercetin		Placebo		*P* by one way ANOVA main effects	
		Measures	*P*	Measures	*P*	of time	of treatment
Glucose, mM		Between baseline *P* (.35)				.99	.61
	Baseline	5.04±0.60		5.09±0.49			
	2-week	5.01±0.65	.73	5.13±0.58	.65		
	4-week	5.10±0.69	.48	5.02±0.77	.57		
Valine, µM		Between baseline *P* (.08 *)				.006 *	.10
	Baseline	253 ± 23		281 ± 27			
	2-week	165 ± 11	.01 *	170 ± 10	.02 *		
	4-week	178 ± 11	.02 *	191 ± 12	.07		
Alanine, µM		Between baseline *P* (.83)				.42	.33
	Baseline	410 ± 35		403 ± 21			
	2-week	372 ± 27	.28	381 ± 20	.45		
	4-week	365 ± 20	.25	402 ± 21	.96		
Glutamine, µM		Between baseline *P* (.13)				.01 *	.02 *
	Baseline	676 ± 45		723 ± 49			
	2-week	532 ± 27	.008 **	577 ± 26	.03 *		
	4-week	542 ± 25	.008 **	588 ± 22	.02 *		
Acetoacetate, µM		Between baseline *P* (.71)				.02 *	.51
	Baseline	86.6 ± 17.4		73.5 ± 9.1			
	2-week	52.5 ± 3.9	.02 *	55.8 ± 4.5	.06		
	4-week	49.0 ± 4.4	.006 **	55.2 ± 4.8	.08		
β-hydroxybutyrate, µM		Between baseline *P* (.71)				.05	.76
	Baseline	90.7 ± 30.4		57.4 ± 7.5			
	2-week	36.9 ± 4.0	.001 **	44.2 ± 5.9	.12		
	4-week	40.6 ± 5.0	.13	42.1 ± 4.7	.13		
Lactate, µM		Between baseline *P* (.37)				.44	.54
	Baseline	1093 ± 118		989 ± 98			
	2-week	827 ± 59	.08	965 ± 107	.53		
	4-week	792 ± 76	.03 *	993 ± 105	.94		

Note: All data are presented as mean ± SEM. There was not a statistically significant interaction between treatment and time on any metabolites which was determined by two-way ANOVA. Alanine and glutamine were tested by paried sample t test since the data is normally distributed and others were tested by paired sample Wilcoxon signed-rank test since the data were not normally distributed. * indicates $0.01<P<0.05$ and ** indicates $P<0.01$.

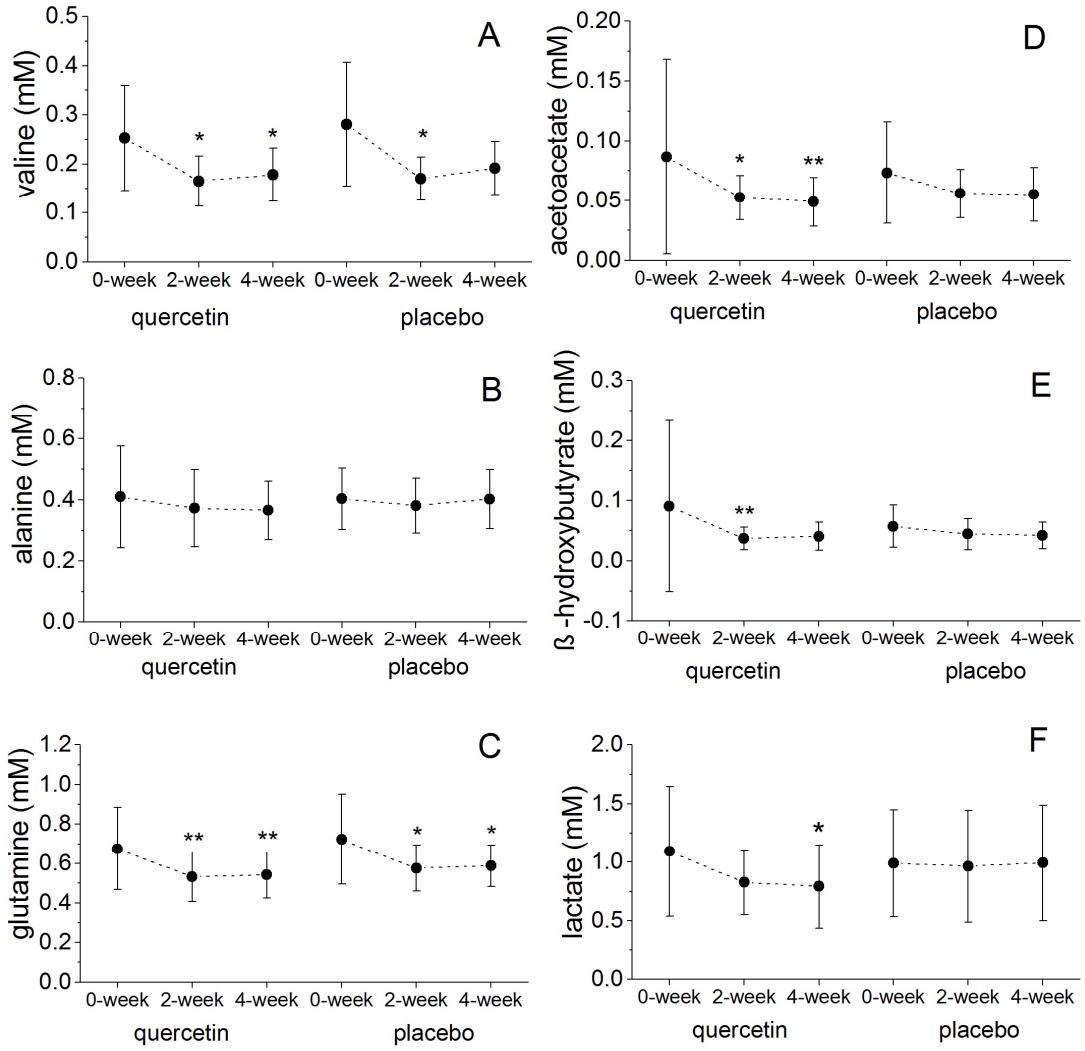


Figure 5-14 Concentration of metabolites at each visit by mean ± SD.

(A) Valine, **(B)** Alanine, **(C)** Glutamine, **(D)** Acetoacetate, **(E)** β-hydroxybutyrate and **(F)** Lactate. * indicates $P<0.05$ and ** indicates $P<0.01$. **(B)** Alanine and **(C)** Glutamine were tested by paired sample t test since the data is normally distributed and others were tested by paired sample Wilcoxon signed-rank test since the data were not normally distributed.

5.4 Discussion

The principle objective of the present study was to assess the effect of long term intake of quercetin supplement on the plasma uric acid and one of the secondary outcomes was plasma metabolic profiles.

Among the 132 analysed plasmas, two samples displayed an unexpected biochemical profile (2362 and 9282). When their spectra were examined, triplet and quadruplet resonances at 1.18 and 3.65 ppm, for the methyl and methylene groups of ethanol were unexpectedly detected. Contamination during handling was ruled out as a potential source of ethanol. Endogenous ethanol is known to be produced in small quantity in mammalian tissue via the reduction of acetaldehyde, itself derived from the decarboxylation of pyruvate (240), as an intermediate step for the elimination of excess energy-releasing substrates from mitochondria (241) during intense physical effort. But cases in this study may be caused by alcohol intake before donating their fasting blood because results of other visits of these two volunteers do not show such abundant ethanol loads. For these assumptions, those two samples were excluded from analysis because this may show the most negative scores (outlier of the score plot).

The peak integrals relate directly to the number of protons giving rise to the peak and hence to the relative concentrations of the substances in the sample. Absolute concentration was obtained for glucose quantified by a conventional biochemical assay (assay protocol is detailed in Section 2.2 and result is in 4.3.6). Trimethylsilyl propionic acid (TSP) was not used as reference standard in plasma samples, since the compound binds to high molecular weight proteins (primarily albumin) resulting in a much reduced signal with a very broad line width.

Generally, NMR-based metabolomics studies of biofluids have shown a high reproducibility (206), therefore it is sufficient to have one sample per time point. We also tested two or three aliquots of three representative plasma samples across the whole run and this serves as a quality control measure. As each plasma sample was prepared between each test individually, carry-over was avoided. Metabolites like uric acid is NMR invisible. This is a result of a very rapid exchange of protons on the metabolite with matrix protons (242).

Apparently we have exposed two challenges in nutritional metabolomics studies. These metabolic responses are often small and subtle since the volunteers are generally healthy and in metabolic homeostasis. Moreover, the effect of the nutritional treatment tend to be much smaller than the biological variation that exists between the individuals. Suhre *et al*. (243) and Gieger *et al*. (244) showed that changes in the concentration levels of biochemically related metabolite pairs are often highly correlated with genetic variance in the general population. Humans are extremely diverse beings and it is not surprising that most human metabolomics studies have found that spectral outputs are strongly influenced by inter- and intra-individual variation. For example, dietary intervention with isoflavone (245) or with barley (237). Some other have been undertaken involved clinical cases and controls (246) which is probably making it more difficult to detect the changes in metabolic profiles resulting from dietary intervention as the contrast between the metabolic profiles variances produced from individuals with a clinical disease compared with the healthy controls.

Ongoing analytical developments include development of cryogenic probes and increases in magnetic field strength and resolution for NMR. The enhanced

sensitivity and resolution gained by these developments increases the capability for biomarker detection, at the expense of increased spectral complexity. Spectral complexity was traditionally addressed by peak picking or by binning signals across the spectra in order to generate a smaller or more manageable data set (247). In this study, 1340 buckets of 0.005 ppm resolution was generated and the analysis was performed using the full data set rather than a peak-picked selection, which increase the information recovered, for example potential biomarker, from the statistical modelling.

Soft Independent Modelling of Class Analogy (SIMCA) (248) is an established method for multivariate classification. Principle Component Analysis (PCA) (249) models are fitted for each class, and model residents are utilised to classify unknown observations to no class, one class or several classes (250). However, each disjoint PCA model is generated based on the direction in the data demonstrating the higher variation, which might be distinctly different from the direction separating the classes. Consequently, maximum class separation is not explicitly the objective function of the method.

A new method for identifying multiple NMR peaks from the same molecule in a complex mixture, based on the concept of Statistical Total Correlation Spectroscopy (STOCSY) takes advantage of the multicolinearity of the intensity variables in a set of spectra to display the correlation among the intensities of the various peaks across the whole spectra (251). However this technique would require the biological samples to be carefully prepared to prevent the disturbance of pH or temperature that causes variation on chemical shift of metabolites. For current study, binning spectra has avoided this problem caused by unadjusted pH of plasma samples.

The particular strength of the cross-over design is that interventions are evaluated on the same subjects, allowing comparison at the individual level rather than on the group level. Taking advantage of cross-over design and the underlying data structure can be useful to uncover minor treatment effects. Unfortunately, most traditional multivariate data analysis techniques such as Principle Component Analysis (PCA), Partial Least Squares (PLS) and Partial Least Square - Discriminant Analysis (PLS-DA) do not optimally exploit the paired data structure in cross-over design. A specific limitation of using PCA and PLS-DA in cross-over designed experiments is that the net treatment effect is not separated from the biological variation between the subjects. As a result, subtle treatment effects within the subjects are often largely overwhelmed by the strong biological variation between subjects (Figure 5-8). Figure 5-15 summarises the extrinsic and intrinsic factors affecting to metabolome. With a cross-over study design, the extrinsic factors can be simplified down to treatment and the intrinsic variation such as resting metabolite rate can be accounted when compare results. Therefore, a hypothesis of a metabolic shift was proclaimed from our finding that quercetin varies the balance between substrates metabolised, both acutely and chronically, according to plasma substrate concentrations, including fatty acids, glucose, ketone bodies, lactate and amino acids hormones, oxygen availability, workload and disease difference in plasma.

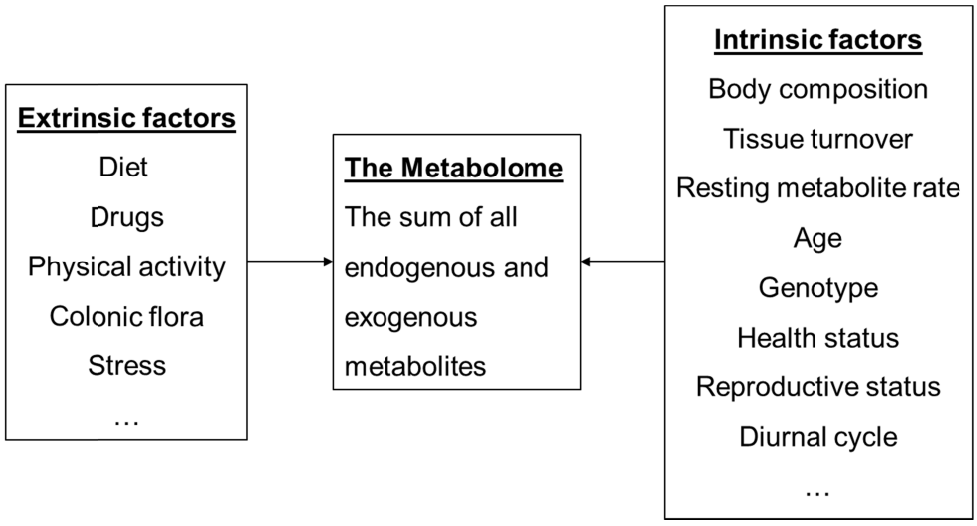


Figure 5-15 Exogenous and endogenous factors likely to influence the human nutritional metabolome (230).

Blood lactate is a measure of not only energy imbalance but also oxidative capacity. Prior work suggested that lactate is elevated among obese, insulin-resistant subjects (252) and decreases with weight loss (253). One prospective study further suggested that serum lactate may be an independent risk factor for the development of type 2 diabetes (254). Quercetin may also potentially play a part altering carbohydrate metabolism in mitochondrial oxidative metabolism and thus diminish the production of lactate. In fact, lactate is more than merely a by-product of glycolysis: it can be used as a metabolic fuel by oxidative cancer cells (Figure 5-16). This phenomenon resembles processes that have been characterised in tumour cells (255) and non-tumour tissues (256) including skeletal muscle, brain and liver that involve what are known as intracellular lactate shuttles (257). Lactate shuttles are dependent on the lactate dehydrogenase (LDH) that convert lactate into pyruvate (and backwards) and on the specific monocarboxylate transporters (MCTs) of lactate into and out of cells (258). Quercetin inhibits glucose uptake by breast cancer cells independently from oestrogen signalling (259) and also inhibits lactate transport of HeLa cells (260).

Under normal biochemical circumstances, lactate is shuttled into gluconeogenesis and thus is not maintained at high concentration in the plasma. Since, as shown by our data, plasma concentration of lactic acid was found to be lowered by quercetin supplementation, we postulate that an activation in the gluconeogenic pathway caused by quercetin activated the consumption of lactic acid by liver cells. The observed change in plasma concentrations of the glucogenic amino acids like glutamine would also support this hypothesis. In humans, the main gluconeogenic precursors are lactate, alanine, glycerol and glutamine, accounting for over 90% of the overall gluconeogenesis (221) and their blood levels were all reduced along quercetin treatment.

In conclusion, the study population was, as expected, homogenous. Some changes were observed through plasma profiling if by ^{1}H NMR when analysed after subtraction from baseline without being confounded by the other variation source (mainly between subject variation). 4-week intervention of 500 mg quercetin d^{-1} in healthy males significantly altered the metabolomics of plasma therefore has a significant effect on metabolism.

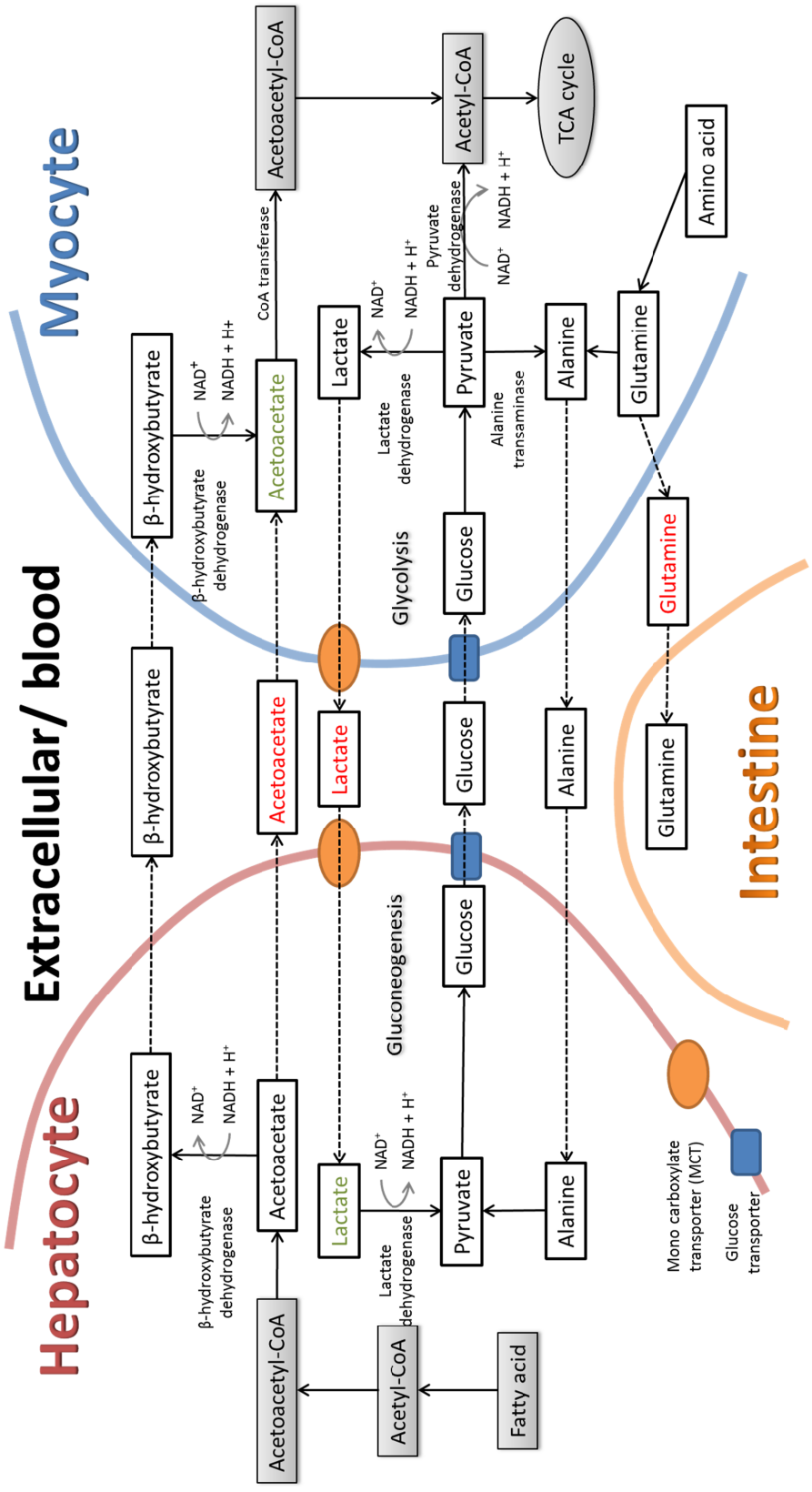


Figure 5-16 Schematic chart of metabolites that may relate in glucose regulation.

Figure is personally drawn by Yuanlu Shi.

Chapter 6. Kinetics of Inhibition of Enzymes Involved in Uric Acid Metabolism by Quercetin and its Metabolites

Abstract

Various dietary flavonoids have been evaluated *in vitro* for their inhibitory effect on xanthine oxidase (XO), the final step in uric acid metabolism. This study investigated the inhibition of enzymes involved in uric acid metabolism by quercetin and its metabolites: quercetin-3-*O*-glucuronide, quercetin-3′-sulfate, quercetin, 3,4-dihydroxyphenylacetic acid (DOPAC), phloroglucinol, 3-hydroxyphenylacetic acid, 3-methyoxy-4-hydroxyphenylacetic acid (homovanillic acid), homovanillic acid sulfate, 3,4-dihydroxybenzoic acid and 4-hydroxyphenylacetic acid; as well as the mechanisms involved based on studies *ex vivo* and *in vitro*. Enzyme activity was determined by direct measurement of product formation by HPLC. The result shows that healthy human plasma contains the activities of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP), but not xanthine oxidoreductase (XOR) activity. There was a dose-dependent inhibitory effect of quercetin (IC_{50}, 23.6 ± 8.1 μM), quercetin-3′-*O*-sulfate (IC_{50}, 2.4 ± 1.1 μM) and 3,4-dihydroxyphenylacetic acid (IC_{50}, 124 ± 16 μM) on *bovine* XO and an inhibition of quercetin on plasma ADA. None showed interaction with plasma PNP activity. These results suggest that quercetin and its microbial-derived metabolites may suppress the formation of uric acid *in vivo*.

6.1 Introduction

XOR catalyses the oxidation of hypoxanthine to xanthine and of xanthine to uric acid in the metabolic pathway of purine degradation in humans. It appears in two inter-convertible forms of the same gene product: xanthine oxidase (XO; E.C.1.17.3.2) using oxygen as the electron acceptor, and xanthine dehydrogenase (XD; E.C.1.17.1.4) which predominates *in vivo* in non-pathological conditions, using FAD as a flavin cofactor and NAD^+ as the preferred electron acceptor (110). Under physiological conditions XOR exists in the form XD, but can convert to XO by pathological events such as ischemia *in vivo*. Because XO produce the cytotoxic intermediates, active oxygen species like superoxide anion radicals and hydrogen peroxide, the irreversible conversion from XD to XO (D-to-O conversion) has been implicated in ischemic injury to intestine, heart, kidney, skin, pancreas, liver, and brain, as well as in the pathogenesis of inflammatory and virus-induced tissue injury. It has, moreover, been suggested that these active oxygen species interact with nitric oxide synthesised by the endothelia. XOR also catalyses the metabolism of some other naturally occurring heterocyclic compounds (261).

Excess activity of XOR in XD form produces excess cytosolic concentration of NADH which may leads to metabolic disorders such as fatty liver, similar to alcoholism (262) and, in XO form, produces ROS (reactive oxygen species). Inhibition of XOR is a primary objective in treating hyperuricemia, for example using allopurinol or febuxostat. However, as shown in Figure 1-2, which summarises the pathway of uric acid formation from purine degradation and the enzymes involved with their inhibitors Figure 6-1, these enzyme were also of interest. Adenosine deaminase (ADA, E.C.3.5.4.4) catalyses the deamination of adenosine

and to inosine (263). A positive inhibitor of ADA is erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, CAS#59262-86-1) (264). Phosphorolysis of inosine generates α-D-deoxyribose-1-phosphate and hypoxanthine by purine nucleoside phosphorylase (PNP, E.C.2.4.2.1) which could be inhibited by 9-benzylguanine. Hypoxanthine would be oxidised to uric acid by xanthine oxidoreductase (XOR, in the form of either XO, E.C.1.17.3.2 or XD, E.C.1.17.1.4) and allopurinol serves a positive control of the inhibition of this reaction.

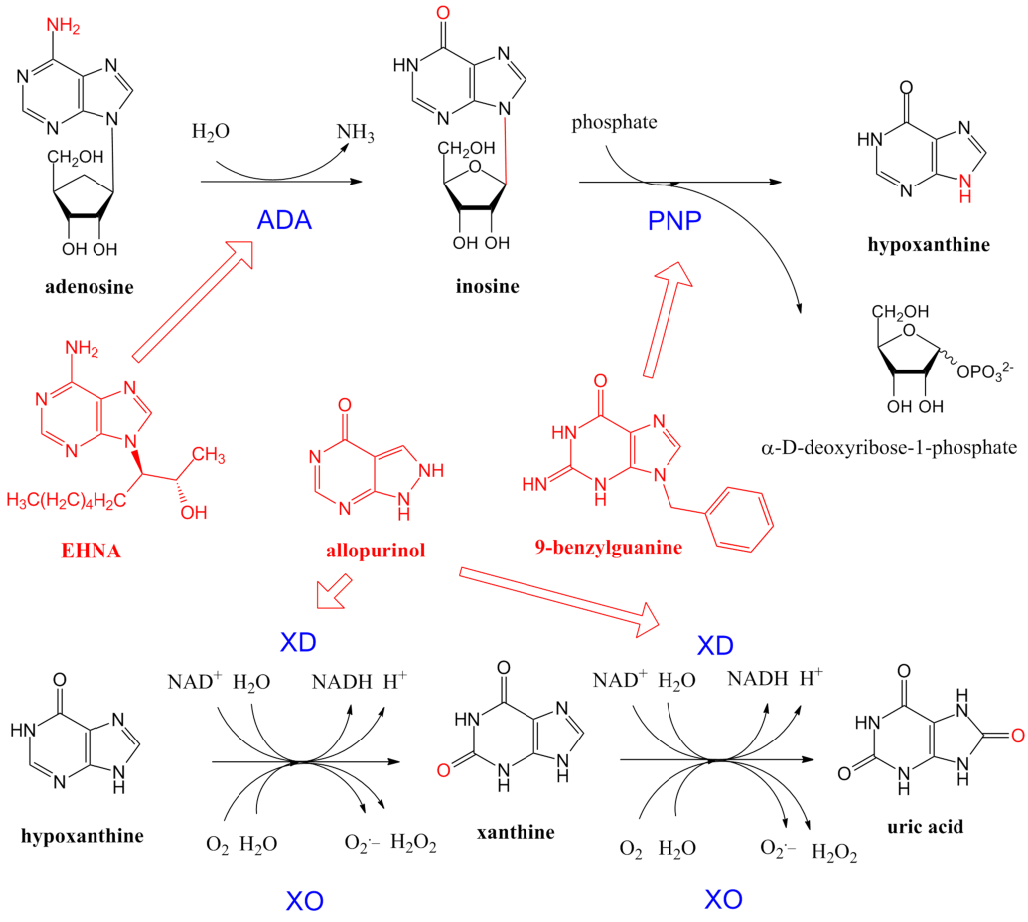


Figure 6-1 Enzymatic reaction scheme of the formation of uric acid and where the positive inhibitors interfere. EHNA is a positive inhibitor of ADA, 9-benzylguanine is of PNP and allopurinol is of XOR.

Quercetin and many other polyphenols and food extracts were tested for their inhibitory effect on xanthine oxidase. However, none of them have detected those compounds (Figure 6-2). When considering the effect of oral intake of quercetin, or of other polyphenols, studies have mainly focused on intact polyphenol present in blood or in urine, for example, the aglycone or their conjugates. The colonic microflora may transform a polyphenol to a more potent biologically active compound (265), and its effect may be crucial. The potential biological activity of quercetin *in vivo* is dependent on the intestinal absorption and subsequent interaction with target tissues, and the absorption, metabolism, distribution, and excretion of quercetin have been extensively studied. To fully assess the health benefits of dietary quercetin, the biological properties of both the aglycone, conjugates and microbial metabolites was investigated. As shown in Figure 6-2, the proposed pathway for colonic bacterium-mediated catabolism of quercetin in the human large intestine results in the production of 3,4-dihydroxyphenylacetic acid (DOPAC) and phloroglucinol (123), smaller quantities of 3,4-dihydroxybenzoic acid, 4-hydroxyphenylacetic acid (124), 3-hydroxyphenylacetic acid, with subsequent hepatic conversion of 3,4-dihydroxyphenylacetic acid to 3-methyoxy-4-hydroxyphenylacetic acid (homovanillic acid) (126, 127, 266) and further to homovallinic acid sulfate. The tested compounds include the two initial quercetin metabolites found in human hepatic portal vein: quercetin-3-*O*-glucuronide and quercetin-3′-sulfate (112). Those compounds (include but not limited to) can be found in human organ after ingesting quercetin. They all may play a crucial part in interfering uric acid metabolites in liver (Figure 1-8) and this study tested this hypothesis.

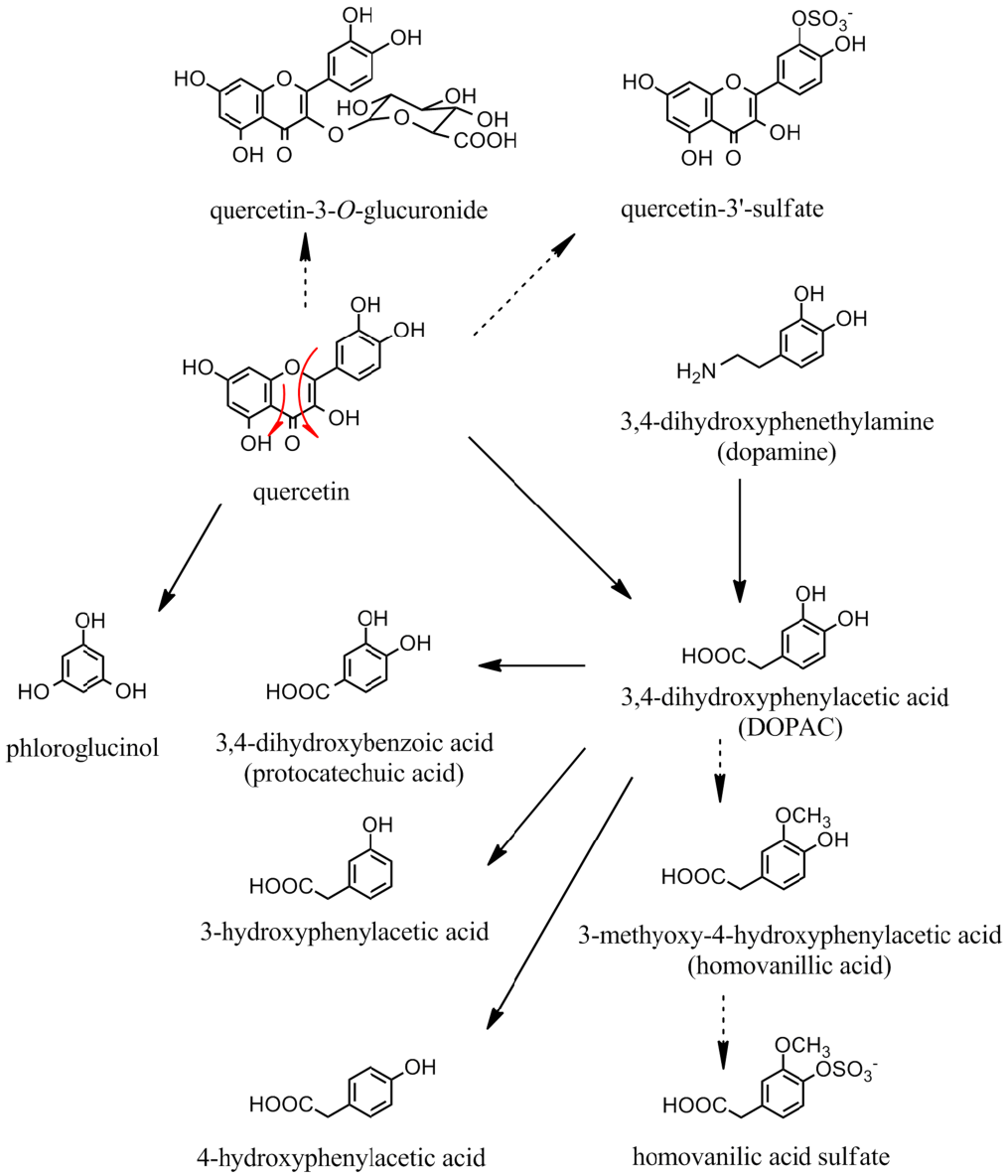


Figure 6-2 Interested compounds for inhibition testing are listed.

quercetin; *schematic representation of colonic bacterium-mediated catabolism of quercetin in human large intestine (solid arrow):* phloroglucinol, 3,4-dihydroxyphenylacetic acid (DOPAC) (123), 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxyphenylacetic acid (124) and 3-hydroxyphenylacetic acid (126, 127); *metabolites in human liver (dash arrow):* quercetin-3-*O*-glucuronide, quercetin-3′-sulfate (112), 3-methyoxy-4-hydroxyphenylacetic acid (homovanillic acid) and homovanillic acid sulfate (126, 127). The precursor of DOPAC, 3,4-dihydroxyphenethylamine (dopamin) was also tested.

We have previously reported that daily supplementation of 500 mg quercetin for 4 weeks significantly reduces plasma uric acid concentrations in healthy males (Chapter 4). This could be a result of intact quercetin circulating in blood that inhibits XOR. Hypothetically, this may also be caused by the large amount of penolic acids released by microbial from unabsorbed quercetin. Besides XO, present study also tested the interaction of these compounds with human ADA and PNP.

6.2 Materials and methods

6.2.1 Chemicals and equipment

Substrates and inhibitors were dissolved in PBS pH 7.4. Substrates: adenosine, inosine, hypoxanthine, xanthine and uric acid; co-enzymes: NAD^+, FAD (Flavin adenine dinucleotide disodium salt hydrate) are all from Sigma-Aldrich (St. Louis, MO, USA). Internal standard adenine was from Alfa Aesear (Ward Hill, MA, USA); inhibitors: EHNA hydrochloride was from Calbiochem (Merk KGaA, Darmstadt, Germany); 9-benzylguanine was from AldrichCPR and allopurinol from Sigma (Sigma-Aldrich).

Potential inhibitors were dissolved in DMSO at a stock concentration of 20 mM and stored at -20℃. Quercetin-3-*O*-glucoronide and quercetin-3′-*O*-sulfate (70% purity) were sythesised in our lab and the following were purchased: quercetin (Extrasynthese, France), 3,4-dihydroxyphenylacetic acid (Alfa Aesar), 3-hydroxyphenylacetic acid (Alfa Aesar), 4-hydroxyphenylacetic acid (Alfa Aesar), homovanillic acid (Alfa Aesar), phloroglucinol (ACROS organic, Fisher Scientific, UK), 3,4-dihydroxybenzoic acid (Sigma-Aldrich), 3,4-dihydroxyphenethylamine

(Sigma-Aldrich), homovanillic acid sulfate (Santa Cruz Biotechnology, Dallas, TX, USA). Experiment was carried on a digital incubator (Dry Block Heater, AccuBlock^{TM} Digital Dry bath, Labnet) at 37℃.

6.2.2 Enzyme preparation

Venous blood was obtained in EDTA K3 tube form a healthy subject with written consent. Whole blood was 1:1 (vol:vol) diluted with PBS buffer pH 7.4 before centrifugation at 3 000 g, 4℃ for 10 min. The supernatant plasma was stored at -80℃ till analysis. About 2 mL of plasma was dialysed in PBS pH 7.4 buffer with a slide-A-Lyzer dialysis Cassettes, 20 K molecular weight cut off (MWCO) (Thermo Fisher Scientific, Waltham, MA, USA) to purify the plasma enzymes from abundant small molecules such as uric acid. 20 K MWCO was chosen because molecular weight of ADA is about 42 kDa (267); PNP is 87-92 kDa (268) or 32-33kDa (269); XO is 300 kDa (270) and they will be caged in the dialysis membrane with 20 K MWCO.

Xanthine oxidase from *bovine* milk and human adenosine deaminase and purine nucleoside phosphorylase (recombinant and expressed in *E.coli*) were from Sigma-Aldrich

6.2.3 Kinetic analyses and determination of the inhibitory effect

A protocol for the experiment of enzyme inhibitory analysis is displayed in Figure 6-3. In brief, a reaction mixture consisted of 200 µL of processed plasma and 100 µL of testing compound at certain concentration were pre-incubated at 37℃ for 5 min. An enzyme reaction was initiated by adding 200 µL of 37℃ pre-incubated 1 mM substrate. At 15 and 30 min after the enzyme reaction was initiated, 200 µL of

reaction mixture was taken and mixed to another test tubes containing 40 µL of 5 M HCl (ice-cold) and was then vigorously shaken by vortex. 10 µL of 1 mM of adenine was then added as internal standard (10 nmol). The resulting solution was centrifuged at 17 000 g at 4℃ for 10 min. The supernatant solution was transferred to another clean test tube and was frozen at -20℃. On the day of analysis, samples were thawed and centrifuged at 17 000 g at 4℃ for 15 min to remove the precipitated lipids-protein sediment. Supernatant was injected to HPLC without further processing. The freeze-thaw cycle was essential for samples containing plasma otherwise the sample become cloudy after 24 h at 4℃. Determination of the inhibitory effect against XO in *bovine* milk, against human plasma purine nucleoside phosphorylase (PNP) and against human plasma adenosine deaminase (ADA) activity were performed using this protocol with their correspondent substrates, inhibitors and tested compounds.

Inhibition of each enzyme is measured by decreased product production. Quercetin and its metabolites (Figure 6-2) were initially tested, and from those, only the compounds which expressed inhibitory capacity were explored further.

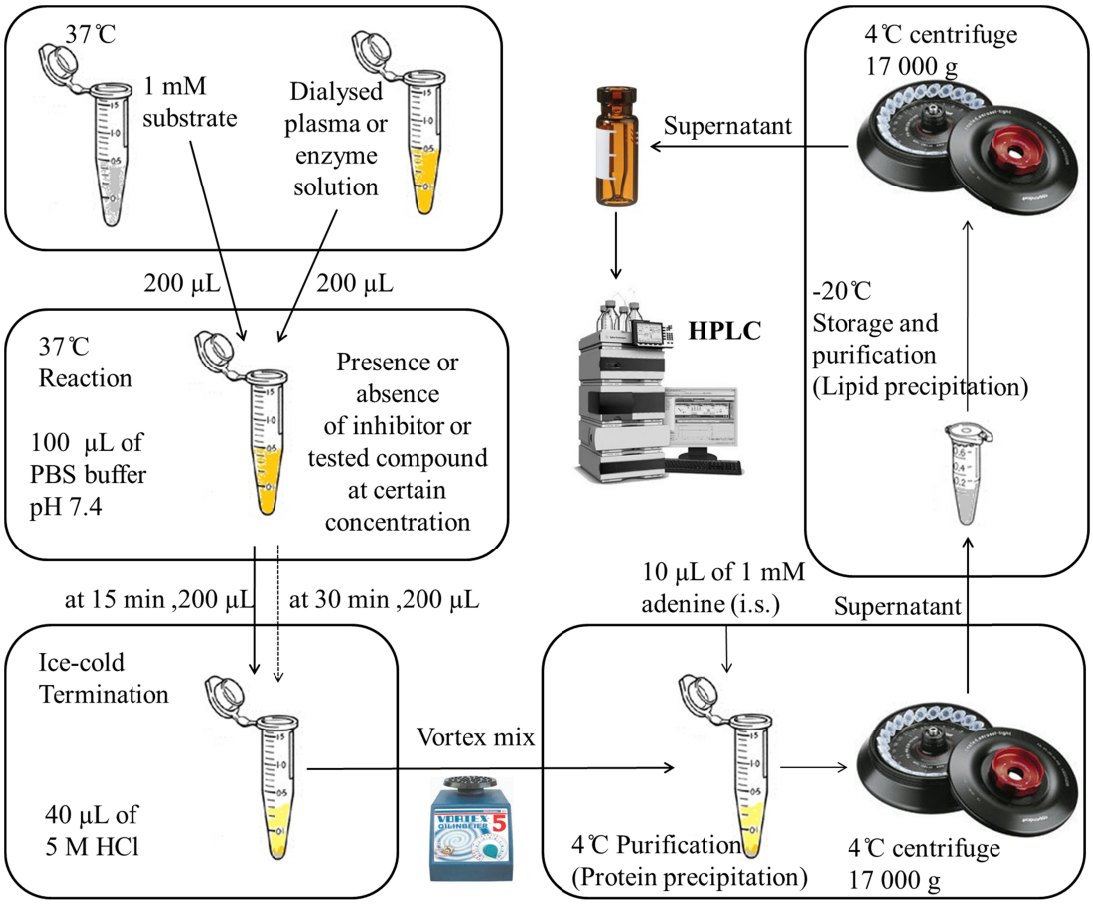


Figure 6-3 Protocol of enzyme kinetic experiments for ADA, PNP and XO. Enzyme of XO is not from plasma, it is purified from *bovine* milk.

The human plasma ADA and PNP activity were analysed in the plasma matrix instead of purified form. Because the enzyme products were diverse (Data not shown), indicating that other enzyme activities were present in bacterial recombinant enzymes when same assays were conducted with them. It is possible that the tested compounds prefer to bind to other proteins in plasma thus the actual IC_{50} could be lower than the concentration estimated. Compared to intact poplyphenols, higher amounts of phenolic acids are found in plasma up to 200 nM at 1.5 h (271) and 34.2 μg mL^{-1} at 8-10 h (272) following flavonoids intake. The actual concentration in the hepatic portal vein might be higher than the rest of the circulation and it is vital to understand whether the plasma enzyme could be highly active and affected. Therefore, using human plasma enzyme *ex vivo* is an advantage compared to purified/recombinant enzymes.

XO activity is normally low in healthy human plasma and we confirmed this in study plasma sample. Therefore the inhibitory effect of testing compound on XO was determined using XO purified from *Bovine* milk. 300 μL enriched buffer containing 5 μL of 1 mM FAD, 10 μL of 5 mM EDTA, 40 μL of 10 mM NAD^+, 120 μL of air saturated PBS buffer, pH 7.4 and 0.01 U XO were pre-incubated at 37℃ with or without test compound or inhibitor (in this case, allopurinol). To initialise the reaction, 200 μL of 37℃ pre-incubated hypoxanthine was added to the mix. The XO activity was also studied in the absence of NAD^+, FAD or EDTA and was compared to that at optimal condition. The inhibition of XO by tested compounds is expressed as the concentration that results in half-maximal enzyme velocity (IC_{50}).

6.2.4 HPLC reverse phase separation of nucleosides

The corresponding products were analysed using HPLC method as detailed in Section 2.6. Uric acid production represented XO activity from hypoxanthine; hypoxanthine production represented plasma PNP activity from inosine (bacause of the lack of XO activity in plasma, hypoxanthine was not further converted to xanthine or uric acid); ADA activity was defined by the total production of inosine and hypoxanthine since PNP activity was also presented in human plasma.

6.2.5 Purity of quercetin-3-*O*-glucoronide and quercetin-3′-*O*-sulfate

Quercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate were synthesed in the lab. According to the product specification record of quercetin-3′-*O*-sulfate, the purity is 70% and this was corrected. Quercetin-3-*O*-glucoronide and quercetin-3′-*O*-sulfate stock solutions were diluted in 50% ethanol and was identified by RP-HPLC. The HPLC method is detailed in Section 2.3. As shown in Figure 6-4, both standard samples were free of quercetin and they are identical according to the overlay UV spectra (Figure 6-5).

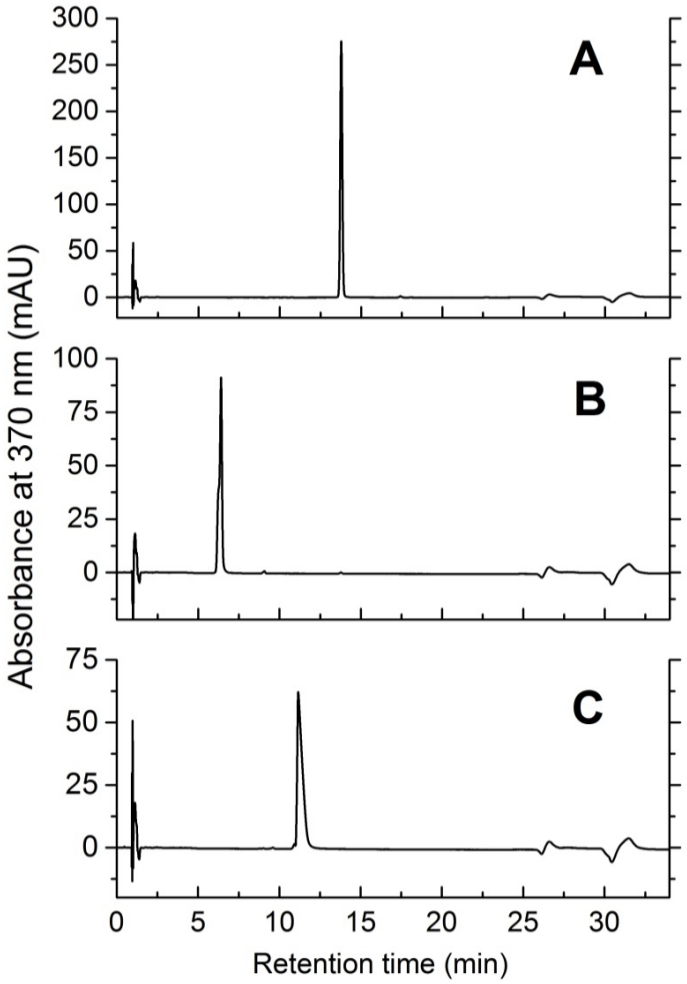


Figure 6-4 HPLC chromatogram of individual standard (1 nmol) of quercetin (A, Rt, 13.8 min), quercetin-3-*O*-glucoronide (B, Rt, 6.44 min) and quercetin-3′-*O*-sulfate (C, Rt, 11.5 min).

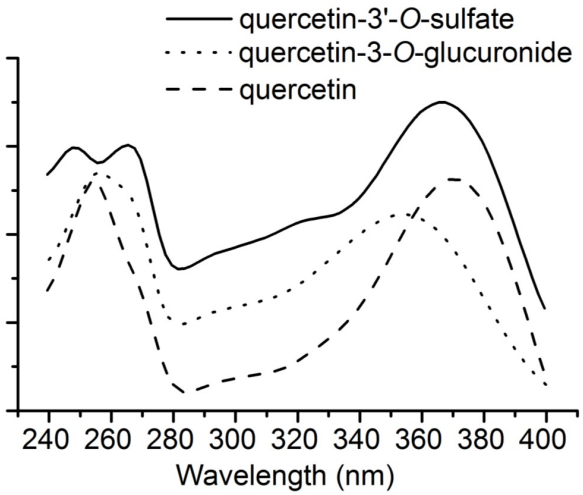


Figure 6-5 Overlay of UV spectra of quercetin, quercetin-3-*O*-glucoronide and quercetin-3′-*O*-sulfate standard from the chromatogram.

6.2.6 Statistics

Statistical calculation in this Chapter was carried in Excel 2010 (Microsoft, USA) by independent t test (2 tailed, non-equal variance). Comparison was made between 100% enzyme activity (control) and percentage of inhibition/activation. Replicate number was 4 for tested compounds and 8 for known inhibitors. The Curve fitting in achieving IC_{50} value was gained in using OriginPro 9.1 (OriginLab, USA).

6.3 Results and discussion

6.3.1 Experimental concentrations

To achieve the same amount of intact quercetin in urine to fresh red onion meal, quercetin dehydrate supplement tablet was adopted as study meal. However, a dosage of 544 ± 45 mg d^{-1} (500 mg d^{-1} stated) of quercetin is much higher than usual intake (3.3 ± 2.4 mg d^{-1}) (273). Even though, the maximum concentration of intact

quercetin in circulatory blood from majority studies cannot exceed 2 μM (Table 6-3, Figure 6-6). It is possible that the extensive quercetin was metabolised into smaller phenolic acids by colon bacteria and the plasma peak concentrations of those phenolic acid may reach higher than intact quercetin after quercetin intake (Table 6-2 in comparison to Table 3-3), thus may play a more important role in physiological system. However, the concentration of subsequent compounds might be higher in hepatic portal vein and/or, most importantly, in hepatocytes.

Table 6-1 Plasma C_{max} of total quercetin after quercetin intake.

	Quercetin intake (μmol)	Plasma C_{max} (μM)	Ref
Onion	225 ± 43	0.74 ± 0.15	(143)
Onion	331	7.65 ± 4.83	(149)
Quercetin-4′-glucoside	331	7.02 ± 5.40	(149)
Onion	411 ± 74	1.34 ± 0.30	(116)
Quercetin-3-*O*-rutinoside	26.2	0.078	(274)
Quercetin-3-*O*-rutinoside	65.6	0.158	(274)
Quercetin-3-*O*-rutinoside	164	0.298	(274)
Quercetin-3-*O*-rutinoside	331 ± 7	0.30 ± 0.30	(143)
Quercetin-3-*O*-rutinoside	662	1.06 ± 1.13	(149)
Quercetin aglycone	26.5	0.137	(274)
Quercetin aglycone	66.2	0.219	(274)
Quercetin aglycone	166	0.285	(274)

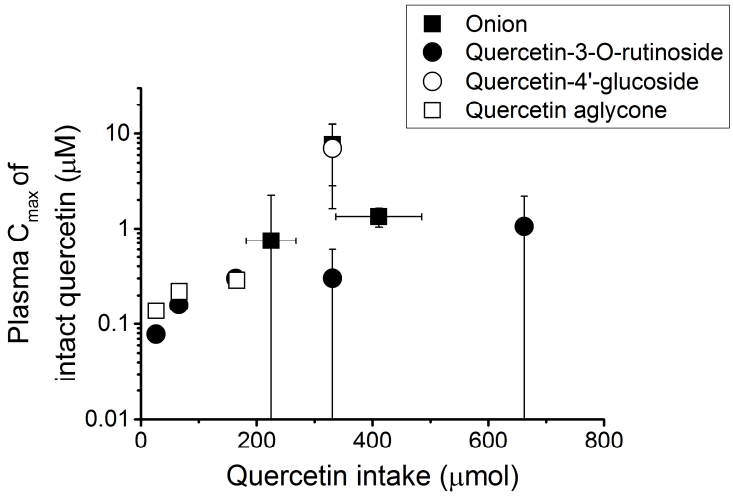


Figure 6-6 Plasma C_{max} of intact quercetin against quercetin intake by food form. Data are extracted from Table 6-1.

Table 6-2 Excretion of phenolic acids in the urine (μmol) of human subjects 24 h after consumption by mean ± SD.

Phenolic acids	Quercetin-3-rutinoside supplemented tomato juice (per 176 μmol) by mean ± SD (127)	Quercetin-3-rutinoside Supplement (per 100 μmol) by mean ± 95% CI (275)
3,4-Dihydroxyphenylacetic acid	16 ± 3	5.0 ± 1.9
3-Methoxy-4-hydroxyphenylacetic acid	19 ± 6	7.8 ± 4.2
3-Hydroxyphenylacetic acid	4.4 ± 1.1	36.1 ± 16.1

6.3.2 Inhibition of human plasma adenosine deaminase

Plasma ADA activity was quantified by the total production of inosine and hypoxanthine after a certain duration of time incubating with adenosine. The plasma used in all enzyme assays was taken from one healthy male subject at one time point. The plasma ADA activity was 5.14 ± 0.76 U L^{-1} (mean ± SD, n=12) and this is used as the control value. 1 unit of ADA produces 1 μmol of inosine and hypoxanthine per min at 37℃ pH 7.4.

The total amounts of inosine and hypoxanthine produced in assays with 50 μM of the interested compounds were compared to that of absence (control) and the result is shown as a ratio (Figure 6-7). EHNA, as positive inhibitor of ADA, efficiently inhibits the production of inosin at the rate of 90.6 ± 3.9% ($P<0.001$). At 50 μM, quercetin (inhibition of 6.54 ± 3.17%, SEM, $P = 0.022$) shows significant inhibition of plasma ADA activity. 3,4-dihydroxyphenylacetic acid (inhibition of 4.62 ± 3.95%, SEM) and homovanillic sulfate (inhibition of 3.44 ± 2.90%, SEM) shows a mild potential inhibition. Quercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate at 50 μM have activated human plasma ADA by 2.32 ± 1.34% (SEM) and 2.46 ± 1.94% (SEM), respectively. However, none of these were significant. 9-benzylguanine, as PNP inhibitor and allopurinol, as an XO inhibitor, failed to inhibit plasma ADA.

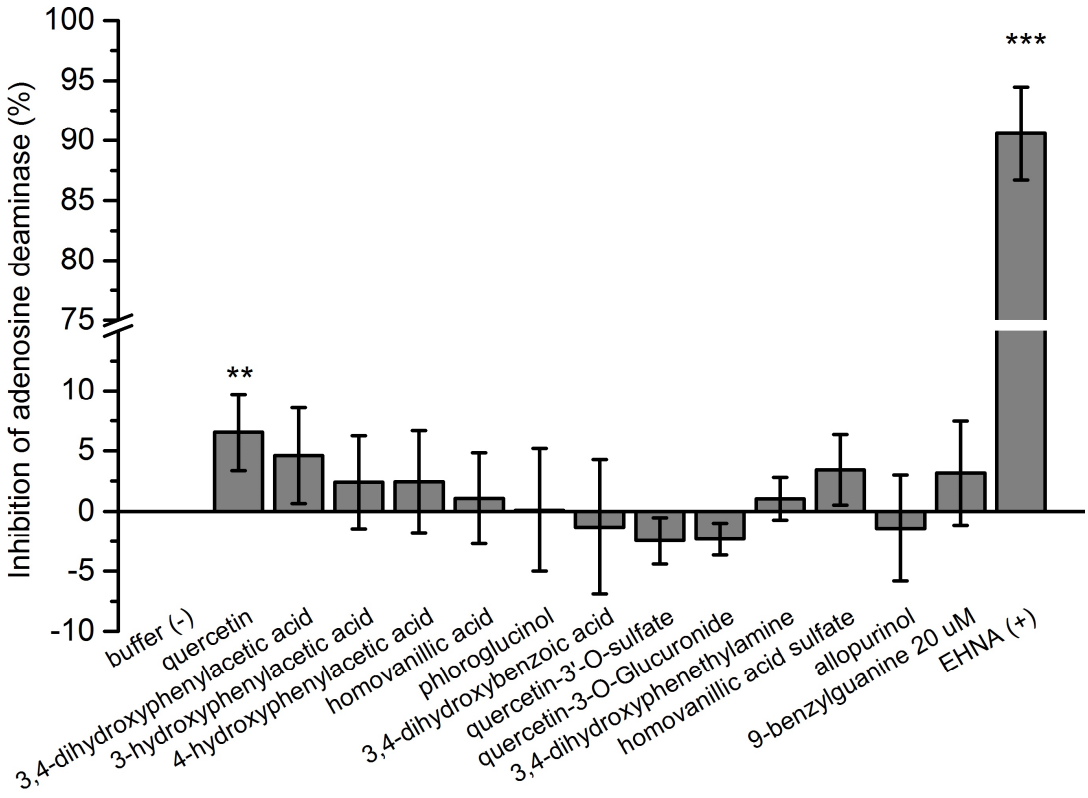


Figure 6-7 Inhibition of human plasma ADA of tested compounds at concentration of 50 µM. Data are presented by mean ± SEM, n=4. ** indicates $P < 0.05$ and *** indicates $P < 0.01$.

To evaluate the dose dependency of the observed inhibitory effect, a higher concentration of quercetin and of 3,4-dihydroxyphenylacetic acid was tested at 200 µM and homovanillic acid sulfate at a series of concentrations at 5, 50, 500 µM. Result is as shown in Figure 6-8. A dose dependent inhibition of quercetin on human plasma ADA was proven for the first time (Figure 6-9). 3,4-dihydroxyphenylacetic acid was an activator of plasma ADA activity at high concentration (200 µM) while had no effect at low concentration (50 µM). Homovanillic acid sulfate does not inhibit or activate plasma ADA activity.

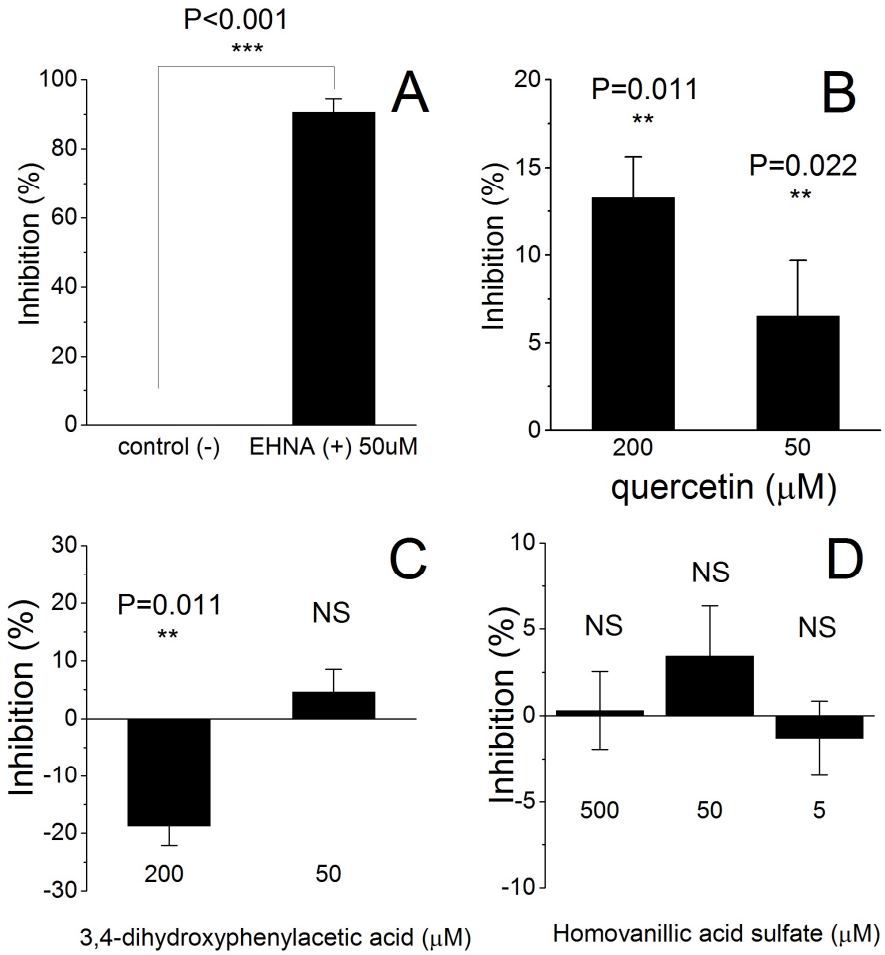


Figure 6-8 Inhibition of human plasma ADA. Data are presented as mean ± SEM.

(A) EHNA, as positive control, efficiently inhibites the production of inosine at the rate of 90.6 ± 3.9%. **(B)** 200 μM of quercetin (inhibition of 13.3 ± 2.4%) and 50 μM of quercetin (inhibition of 6.54 ± 3.17%) **(C)** 200 μM of 3,4-dihydroxyphenylacetic acid (inhibition of -18.8 ± 3.4%) and 50 μM of 3,4-dihydroxyphenylacetic acid (inhibition of 4.62 ± 3.95%). **(D)** the inhibition of homovanillic acid sulfate were 0.30 ± 2.24%, 3.44 ± 2.90% and -1.30 ± 2.14% of the concentration at 500 μM, 50 μM, and 5 μM. ** indicates $P< 0.05$, *** indicates $P< 0.01$ and NS indicates not significant.

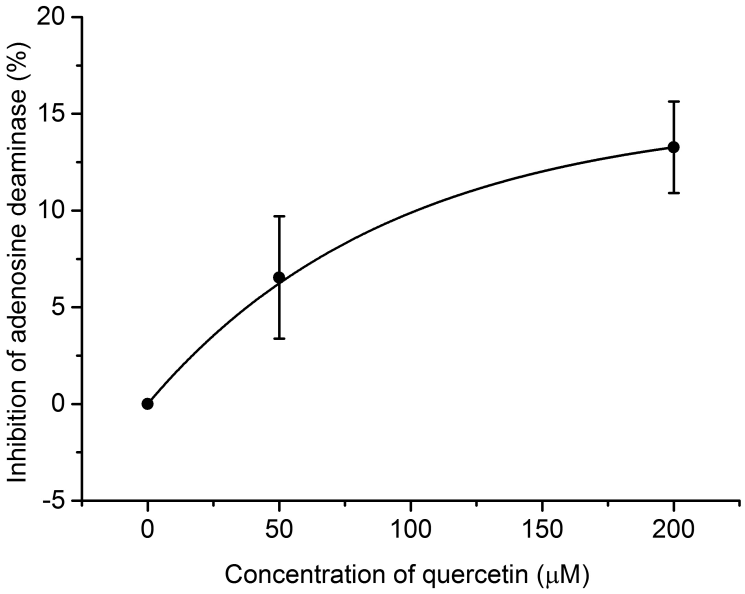


Figure 6-9 Dose dependent inhibition of quercetin on plasma adenosine deaminase. Data are presented by mean ± SEM.

Adenosine deaminase (ADA) is an enzyme present in cells of the immune system, and ADA activity is considered an indicator of cellular inflammation (276, 277). Plasma ADA activity is mainly contributed by ADA 2, the major isoenzyme in the serum of normal individuals. Normal serum ADA activity varies among studies, ranging from 2.38 ± 1.43 to 17.05 ± 3.75 U L^{-1} (278) and 10.11 ± 2.01 U L^{-1} (279). The mean ADA activity in fasting plasma was higher in T2DM patients (23.1 ± 0.6 U L^{-1} SE) compared with that in non-diabetic control subjects (18.6 ± 0.8 U L^{-1} SE; $P<0.05$) (280), higher in pulmonary tuberculosis patients (35.5 ± 6.93 U L^{-1}) compared to 16.20 ± 2.85 U L^{-1} in healthy subjects (281) and higher in patients with systemic vasculitis (20.6 ± 6.3 U L^{-1}) than age-matched and gender-matched healthy subjects (12.8 ± 1.8 U L^{-1}; $P<0.001$) (282). A positive correlation between plasma uric acid and plasma ADA activity was found in women with preeclampsia ($p <0.01$) with a significant higher ADA activity compared to normotensive pregnant women or non-pregnant women (283). The levels of ADA and uric acid were simultaneously

found to be significantly higher in patients of head and neck cancers as compared to the levels in controls ($P<0.001$) and a positive correlation was also observed between ADA and uric acid ($P<0.001$) (284).

6.3.3 Interaction of plasma purine nucleoside phosphorylase (PNP)

Plasma activity was quantified by the production of hypoxanthine after incubation with inosine. The plasma used in all enzyme assays was taken from one healthy subject with plasma PNP activity of 21.4 ± 3.1 U L^{-1} (n=6). 1 unit of PNP produces 1 μmol of hypoxanthine per min at 37℃ pH 7.4. The total amounts of hypoxanthine produced in assays in the presence of 50 μM of the test compounds were compared to control and the result is shown as a ratio (Figure 6-10). 20 μM 9-benzylguanine, as positive control, efficiently inhibited the production of inosine at a rate of 26.5 ± 1.9% ($P<0.001$). At 50 μM, none of the tested compounds showed inhibition of human plasma PNP activity ($P>0.05$). However, a higher concentration was tested of 3,4-dihydroxyphenylacetic acid at 200 μM and homovanillic acid sulfate at 500 μM and 5 μM were tested. The result is shown in Figure 6-11. Neither compounds showed any significant effect on plasma PNP activity.

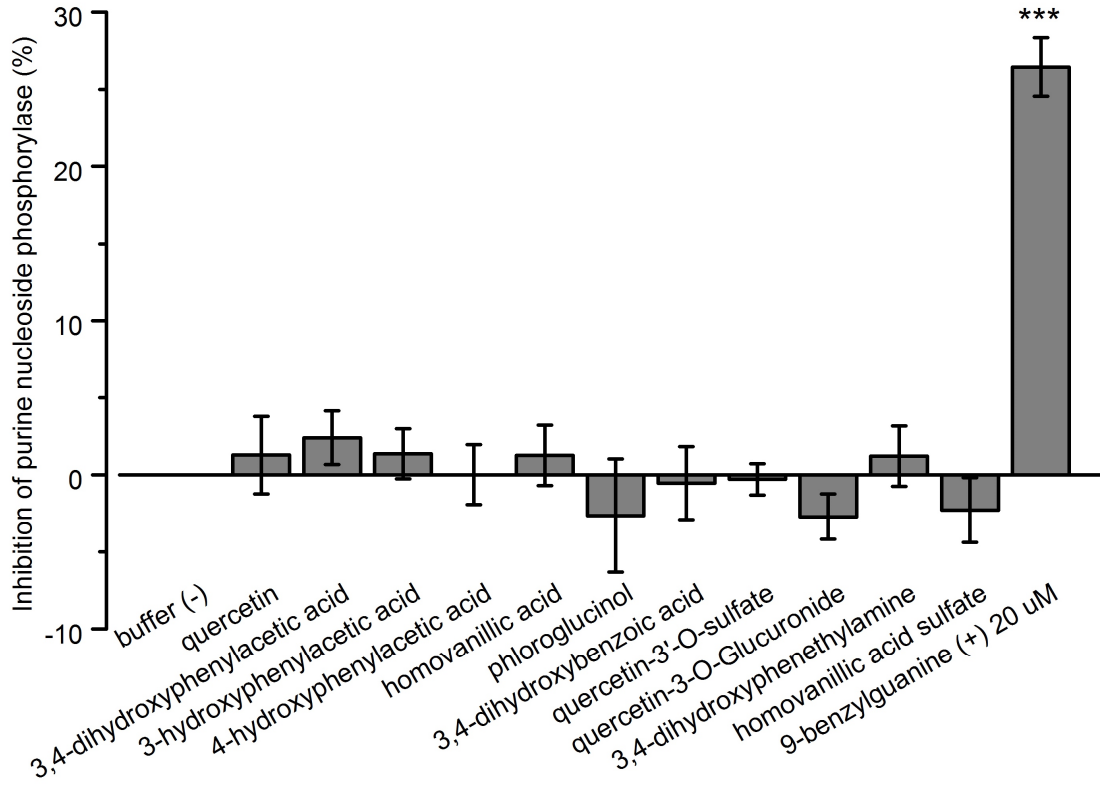


Figure 6-10 Inhibition of purine nucleoside phosphorylase by 50 µM of indicated compound. Data are presented by mean ± SEM. 3,4-dihydroxyphenylacetic acid inhibits by 2.40 ± 1.74% (NS) and homovanillic acid sulfate activate 2.30 ± 2.10% (NS) and quercetin-3-*O*-glucuronide activated 2.7 ± 1.5% (NS) of plasma PNP activity. *** $P<0.01$.

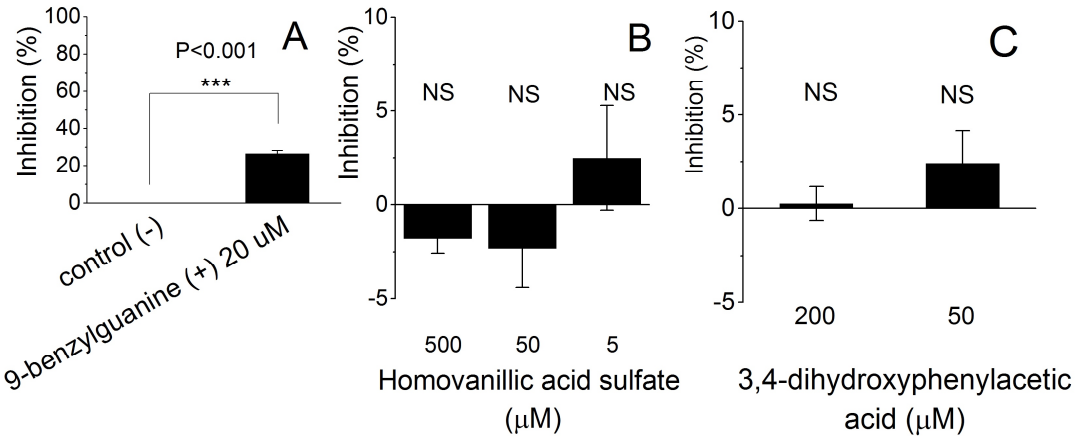


Figure 6-11 Inhibition of plasma purine nucleoside phosphorylase of 3,4-dihydroxyphenylacetic acid and homovanillic acid sulfate.

Inhibition is not dose dependent even though at high concentration. Data are presented by mean ± SEM. **(A)** 20 μM of 9-benzylguanine, as positive control, efficiently inhibited the production of hypoxanthine by 26.5 ± 1.9%. **(B)** The inhibitory effect of homovanillic acid sulfate was -1.79 ± 0.79%, -2.30 ± 2.10% and 2.49 ± 2.79% of the concentration at 500 μM, 50 μM, and 5 μM. **(C)** The inhibitory effect of 3,4-dihydroxyphenylacetic acid was 0.25 ± 0.90% at 200 μM and 2.40 ± 1.74%) at 50 μM. *** indicates $P < 0.01$ and NS indicates not significant.

It was proven that none of the testing compound can effectively inhibit plasma PNP activity, therefore do not contribute to the prevention of uric acid production. However, PNP activity is 3.26 ± 1.63 μmol min^{-1} L^{-1} in gout patients, 3.91 ± 2.15 μmol min^{-1} L^{-1} in asthma which were all lower than 6.69 ± 3.92 μmol min^{-1} L^{-1} in healthy subjects (285).

Inhibitors of PNP have been used for the treatment of T-cell cancers and autoimmune disorders where T-cell clones are misdirected against self-antigens causing disorders, including psoriasis, rheumatoid arthritis, and multiple sclerosis. A rare genetic deficiency of PNP reveals that the enzyme is essential for recycling d-guanosine and

formation of free purines leading to uric acid synthesis (286). This finding is showing for the first time the enhancing function of quercetin metabolites on plasma PNP.

6.3.4 Inhibition of xanthine oxidase

6.3.4.1 Assay parameters

This experiment was conducted with enzyme purified from *bovine* milk (XO from *B. taurus*) instead of from human biological samples. Experiments were conducted with hypoxanthine as substrate in either human plasma (dialysed) or red blood cells (induced hemolysis) from several other subjects but failed to produce uric acid or xanthine. This indicates that plasma XD activity cannot be found in this blood sample (Figure 6-12).

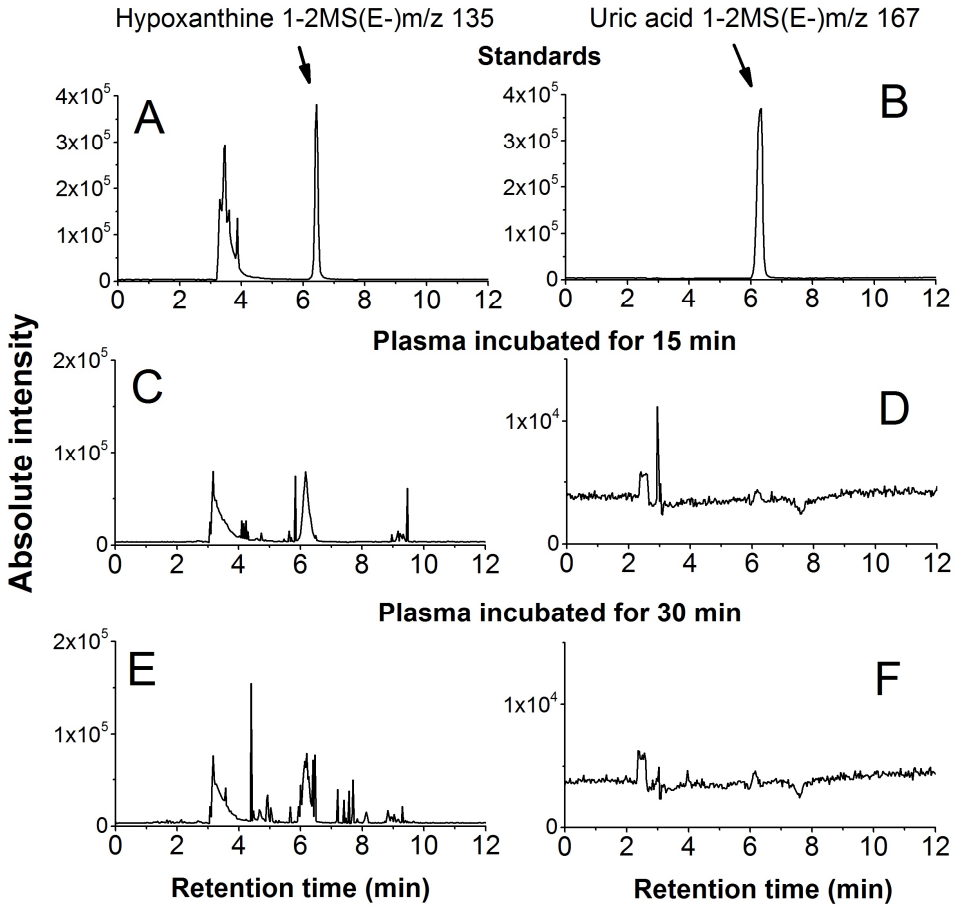


Figure 6-12 LC-MS detection of uric acid production from hypoxanthine by *ex vivo* plasma and none was found within 15 min (D) or 30 min (F) of incubation.

Panels on left show chromatograms of 1-2MS (E-) 135 *m/z* with arrow indicates hypoxanthine and panels on right show chromatograms of 1-2MS (E-) 167 *m/z* with arrow indicates uric acid. MS chromatograms of standards sample **(A, B)**, plasma incubated with hypoxanthine for 15 min **(C, D)** and plasma incubated with hypoxanthine for 30 min **(E, F)** are shown.

The contribution of co-factors, EDTA, FAD and NAD^+, was investigated by excluding them in assay system (Figure 6-13). Result shows that neither EDTA or FAD affects the overall performance of XO. NAD^+ inhibited XO activity astonishingly and FAD is not essential whenever NAD^+ is present or not.

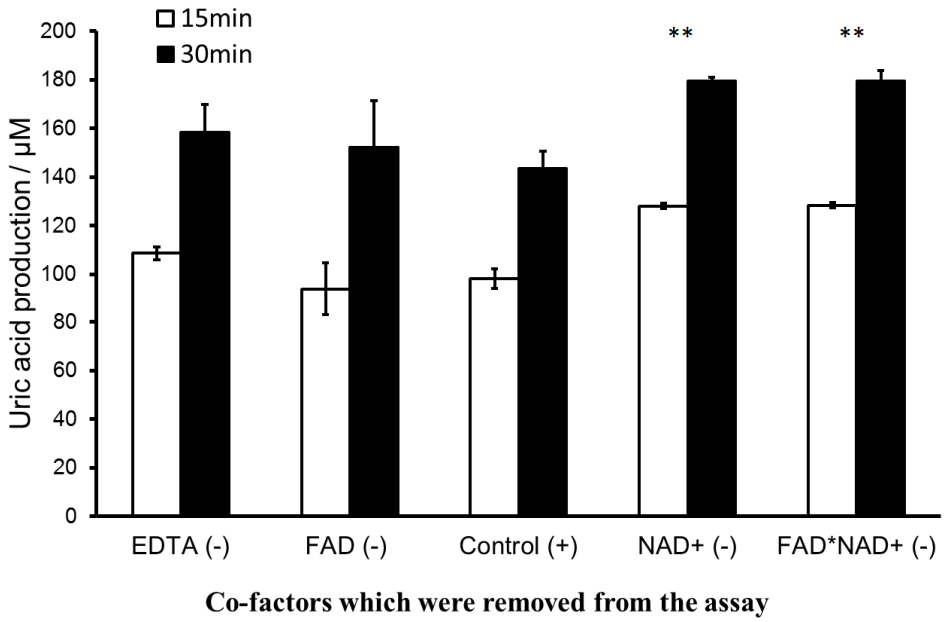


Figure 6-13 Comparison of uric acid production through XO when co-factor was absent. Error bar indicates SEM.**$P < 0.05$.

EDTA was administered during blood sampling as an anticoagulant agent so it is normally present. By adopting a commercial enzyme instead of a human biological sample, it is considered for examination of XO activity to scavenging metal ions so to deactivate metal-dependent enzymes (287). However, it is not essential in contributing to the enzyme activity (Figure 6-13).

FAD and NAD^+ were involved in this experiment for the purpose of generating comparable results to conditions in liver *in vivo* or in liver extract (*ex vivo*) where XD takes the main role of producing uric acid (288) and was found to be more sensitive to flavonoid treatment (289). XO and XD are composed of two identical

subunits of approximately 145 kDa. Each subunit contains one molybdenum centre, one flavin (FAD), and two non-identical [2Fe-2S] iron-sulfur centres. The oxidative hydroxylation takes place at the molybdenum centre with a concomitant reduction of NAD^+ for XD at the flavin centre (290). The absence of NAD^+ significantly ($P<0.01$) enhanced the enzyme activity by ~1.23 fold and 1.20 fold at 15 min and 30 min, respectively. These evidences all suggest that the nature of *bovine* milk XOR is mainly in the form of XO, either of a result of readily conversion from XD to XO either irreversibly by proteolysis or reversibly by oxidation of Cys residues to form disulfide bridges (291) or of the nature of enzyme processing. The enzyme could be purified in its XD form from species such as chicken, whereas it was always isolated in its XO form from mammalian sources such as *bovine* milk (292).

However, results of present inhibition assay were determined with all co-factors. The 100% enzyme activity in this assay setting can convert 400 μM of hypoxanthine to 101 ± 3 μM (mean ± SEM) of uric acid in 15 min and 153 ± 4 μM (mean ± SEM) in 30 min. Inhibition was defiended as the proportion (%) of less uric acid produced with presence of each testing compound at certain concentrations compared at the same condition except without presence of testing compound.

6.3.4.2 Inhibition of *bovine* milk xanthine oxidase

The XO activity was reflected by the formation of uric acid when hypoxanthine serves as substrate, since we have not detected stable accumulation of xanthine during incubation. Results are as shown in Figure 6-14. Allopurinol, as a positive control, inhibits the XO activity by 97.6 ± 0.3% (mean ± SEM). Among the tested compounds, quercetin (91.6 ± 1.2%, mean ± SEM, P<0.001), 3,4-dihydroxyphenylacetic acid (24.3 ± 5.1%, mean ± SEM, $P = 0.017$) and quercetin-3′-

O-sulfate (93.4 ± 0.4%, mean ± SEM, P<0.001) were found to significantly inhibit XO activity.

Inhibition was further determined for quercetin, 3,4-dihydroxyphenylacetic acid, quercetin-3′-*O*-sulfate, phloroglucinol and homovanillic acid sulfate at other concentrations (Figure 6-15). 3,4-dihydroxyphenylacetic acid dose dependently inhibited XO but at a relatively high concentration (Figure 6-15 B). Quercetin and quercetin-3′-*O*-sulfate were found to be inhibitors showing activating effects at low concentration (Figure 6-15 C, D, Table 6-3) while phloroglucinol at high concentration (Figure 6-15 F). Homovanillic acid sulfate did not affect XO activity either at high or low concentration (Figure 6-15 E).

Table 6-3 Dose dependent inhibition of xanthine oxidase by quercetin and quercetin-3′-*O*-sulfate (mean ± SEM)

Concentration (μM)	Quercetin		Quercetin-3′-*O*-sulfate	
	Inhibition (%)	*P* value	Inhibition (%)	*P* value
0.08	-9.87 ± 3.78	0.080	-3.15 ± 6.33	0.652
0.4	-9.63 ± 4.72	0.134	-12.78 ± 2.96	0.050
2	-2.90 ± 1.34	0.118	36.25 ± 3.35	0.008
10	20.66 ± 7.57	0.072	72.14 ± 5.16	0.001
50	91.62 ± 5.08	<0.001	93.44 ± 0.36	<0.001

Note: P value was calculated by t test (2-tailed, two sample non-equal variance)

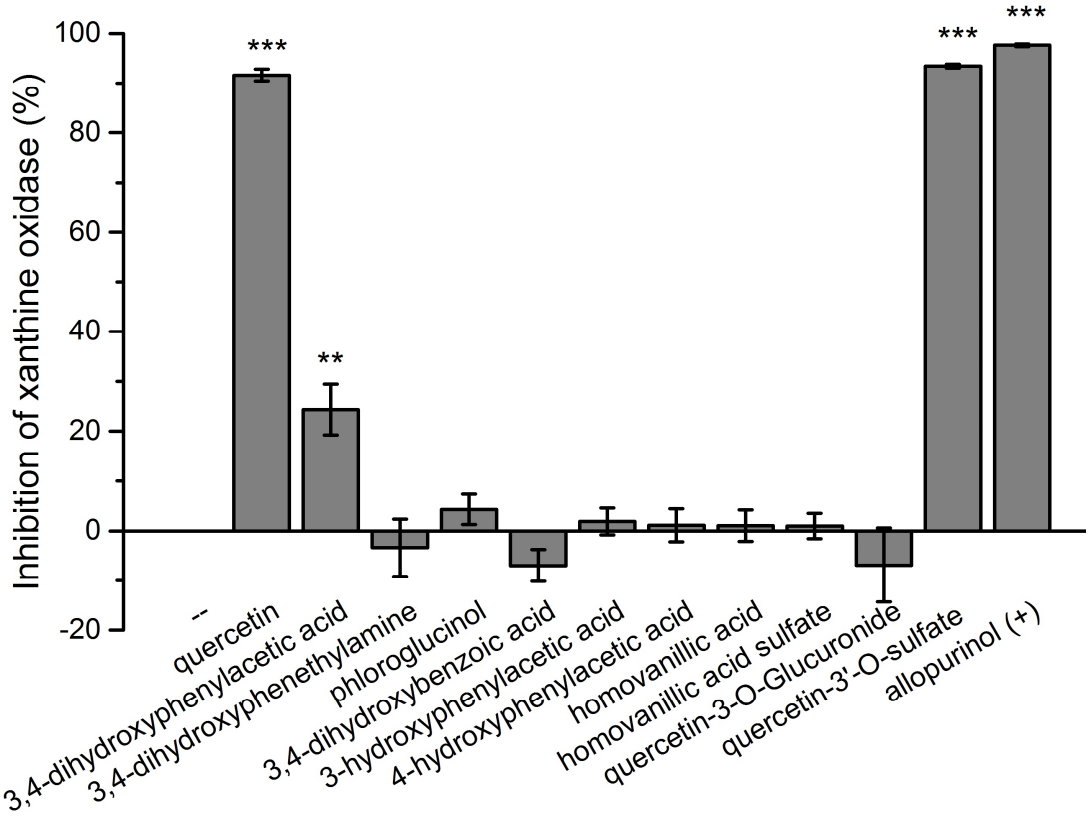


Figure 6-14 Inhibition of xanthine oxidase by 50 μM of each compound against 400 μM of hypoxanthine. Data are presented as mean ± SEM.

Quercetin, 3,4-dihydroxyphenylacetic acid, quercetin-3′-*O*-sulfate and phloroglucinol were found to significantly inhibit XO activity. 3,4-dihydroxybenzoic acid were found to be a activator at a mild promotion level. ** indicates $P < 0.05$ and *** indicates $P < 0.01$.

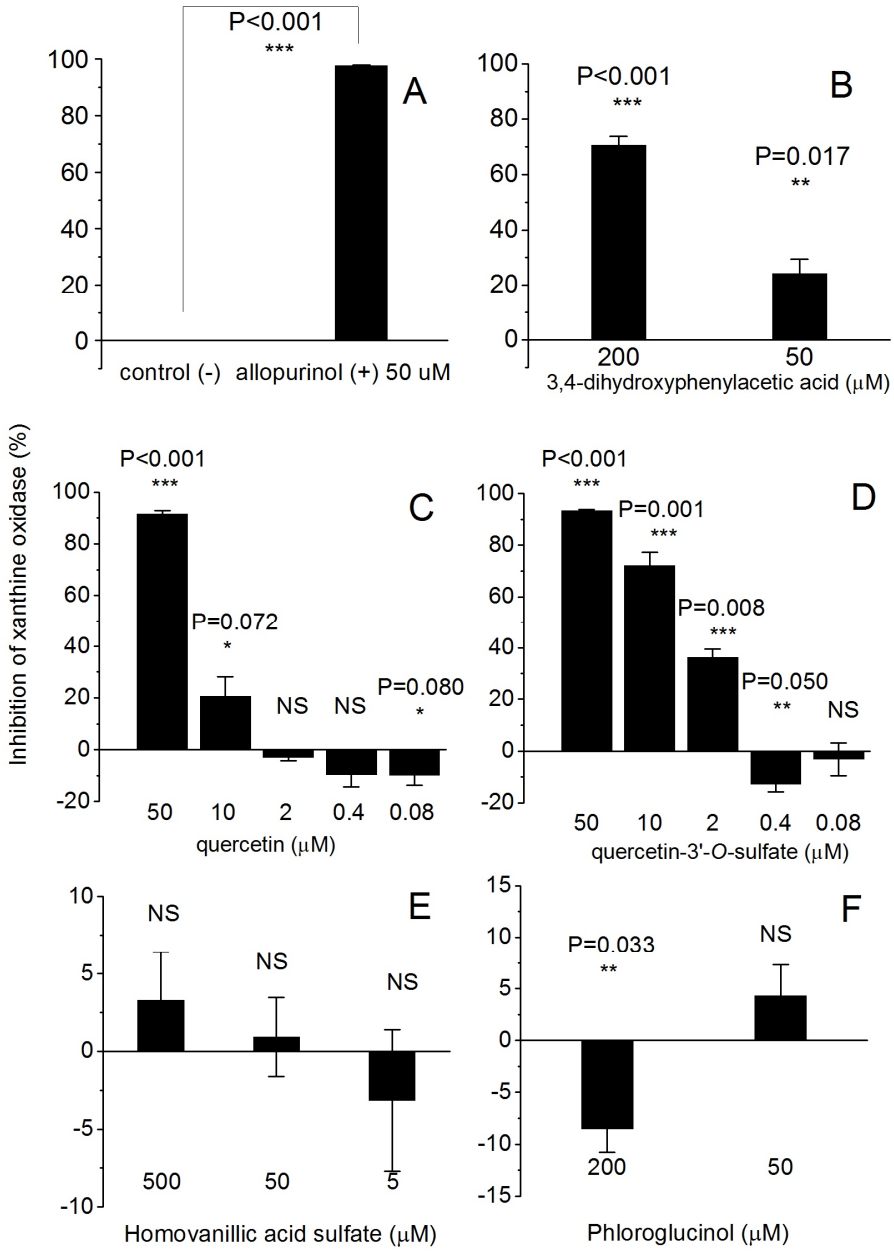


Figure 6-15 Comparison of inhibition of xanthine oxidase at series of concentrations. Data are presented by mean ± SEM.

(A) 50 μM of allopurinol by 97.65 ± 0.28%; **(B)** 200 μM and 50 μM of 3,4-dihydroxyphenylacetic acid by 70.72 ± 3.11% and 24.34 ± 5.08%, respectively; **(C)** quercetin ranging from 0.08 μM to 50 μM; **(D)** quercetin-3′-*O*-sulfate ranging from 0.08 μM to 50 μM; **(E)** homovanillic acid of 5 μM, 50 μM and 500 μM; **(F)** phloroglucinol of 200 μM and 50 μM by -8.51 ± 2.25% and 4.30 ± 3.04%, respectively. * indicates $0.05 < P < 0.1$, ** indicates $P < 0.05$, *** indicates $P < 0.01$ and NS indicates not significant.

6.3.4.3 IC_{50}

Half maximal inhibitory concentration (IC_{50}), IC_{10} (if apply) and IC_{90} (if apply) of XO were determined from plots of dose dependent inhibitors: quercetin (Figure 6-16), quercetin-3′-*O*-sulfate (Figure 6-17) and 3,4-dihydroxyphenylacetic acid (Figure 6-18) and are summarised in Table 6-4.

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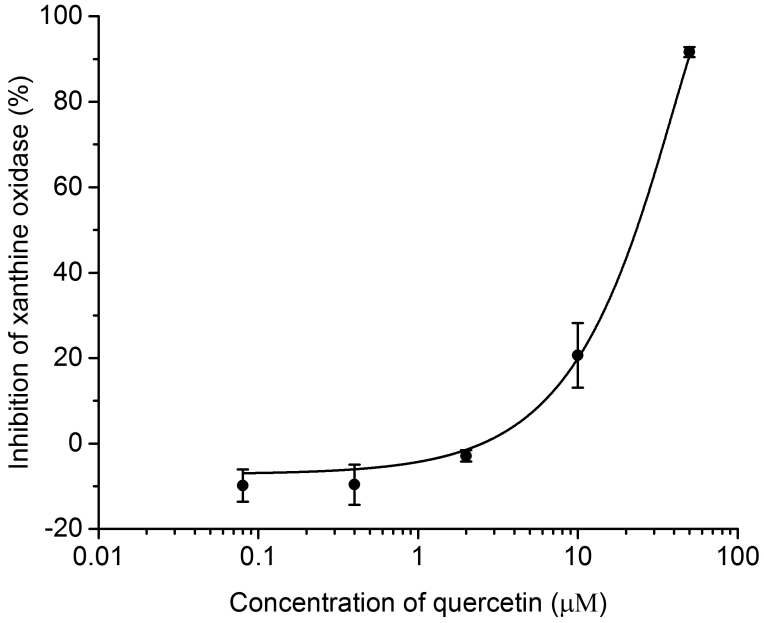

Figure 6-16 Dose dependent inhibition of quercetin on xanthine oxidase. Data are presented by mean ± SEM. Parabola polynomial fitting was generated in OriginPro 9.1, $R^2 > 0.99$

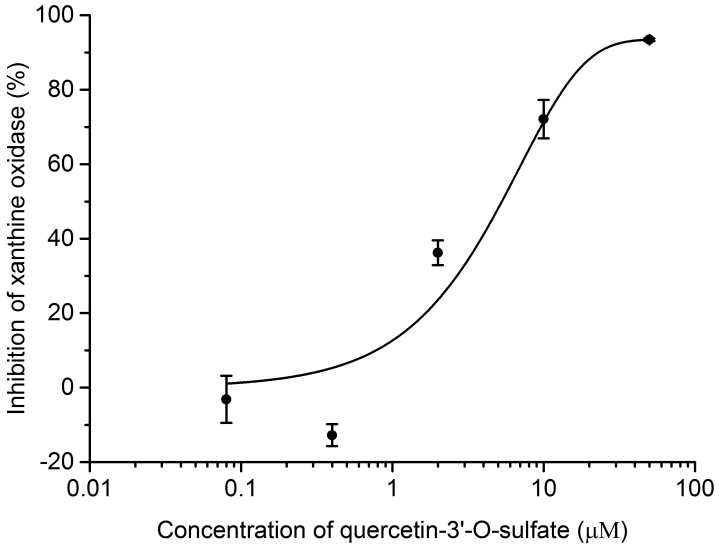

Figure 6-17 Dose dependent inhibition of quercetin-3′-*O*-sulfate on xanthine oxidase. Experimental polynomial fitting with instrumental weight was generated in OriginPro 9.1, Adjusted R^2=0.95.

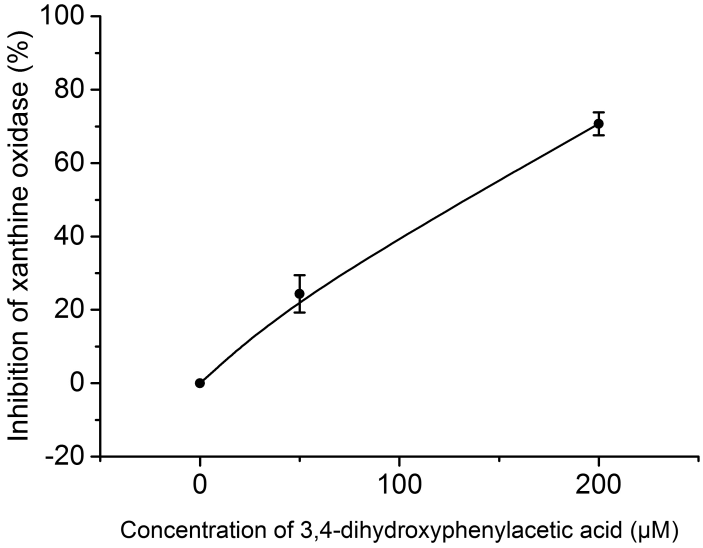

Figure 6-18 Dose dependent inhibition of 3,4-dihydroxyphenylacetic acid on xanthine oxidase. Data are presented by mean ± SEM. Experimental polynomial fitting was generated in OriginPro 9.1. Adjusted R^2 is not applicable for this plot due to the small number of data.

Table 6-4 IC_{10}, IC_{50}, IC_{90} of quercetin, quercetin-3′-*O*-sulfate, and 3,4-dihydroxyphenylacetic acid on xanthine oxidase from *bovine* milk

Compound	IC_{10} (μM)	IC_{50} (μM)	IC_{90} (μM)
Quercetin	6.06 ± 2.08	23.6 ± 8.1	48.9 ± 16.7
Quercetin-3′-*O*-sulfate	--	2.4 ± 1.1	40.1 ± 19.3
3,4-dihydroxyphenylacetic acid	18.2 ± 2.3	124 ± 16	--

Note: when [E] is producing 5.91 ± 0.25 μmol min^{-1} L^{-1} at 37℃ from hypoxanthine to uric acid and initial [S] is 400 μM. All data are presented by mean ± SEM

It is worthy to mention that quercetin-3′-*O*-sulfate inhibits XO by 50 % at the concentration of 2.4 ± 1.1 μM which, theoretically, can be easily achieved in circulation blood by quercetin intake, for example, plasma peak concentration of quercetin-3′-*O*-sulfate can be 0.665 ± 0.082 μM (SEM) (112). As an inhibitor, quercetin-3′-*O*-sulfate activates *bovine* milk XO by 12.8 ± 3.0 % at concentration of 0.4 μM and similar behaviour was also observed for theaflavin-3-gallate and epicatechin gallate (134). Therefore, without knowing the physiological activity of XD/XO in healthy living liver cells, the physiological concentration of quercetin-3′-*O*-sulfate in liver cells and the comparability between human XD in living hepatic cells and bovine milk purified XO, it is difficult to link this result to human trials.

Table 6-5 summarises various studies and their findings on the Ki and IC_{50} of quercetin or quercetin conjugates on *bovine* milk xanthine oxidase. This incosistancy could be caused by the way they quangtify uric acid All of them were using abosrbance 295 nm in detecting uric acid production, as mentioned before in Section 2.6.1, this may be interfered by many other compounds especially polyphenols. The studies also varies in reaction temperature and pH. Present study was conducted at 37 ℃, pH 7.4, whilst the maximum activity of *bovine* milk xanthine oxidase is 25 ℃,

pH 7.5. Also it was possible that the manufactured xanthine oxidase contains (usually <0.1%) of uricase activity at 25 ℃, pH 8.5. Those are the possible reasons that Ki value cannot be consistantly measured.

In Table 6-5, it is worthy to mention that quercetin-3′-*O*-sulfate and quercetin-3′-*O*-glucuronide are better inhibitors of *bovine* milk XO than quercetin and other quercetin 3-*O*-conjugates. As shown in Figure 6-19 C, The 3′-sulfo-conjugates of quercetin rotate the B ring towards A/C rings thus forms a dihedral angle which is similar to that of febuxostat and luteolin.

Table 6-5 Ki and IC_{50} of quercetin and quercetin metabolites on xanthine oxidase from *bovine* milk *in vitro*.

	Ki (μM)	IC_{50} (μM)	Ref
Allopurinol	0.34 ± 0.22		(181)
Quercetin	1.40 ± 0.78		(181)
Quercetin		2.08	(293)
Quercetin		1.5	(294)
Quercetin	1.2 ± 0.7		(295)
Quercetin	0.28	0.44	(106)
Isorhamnetin/ tamarixetin	0.17	0.40	(106)
Quercetin	0.2		(111)
Isorhamnetin	0.25		(111)
Quercetin-4′-*O*-glucuronide	0.25		(111)
Quercetin-3′-*O*-glucuronide	1.4		(111)
Quercetin-3-*O*-sulfate	78		(111)
Quercetin		23.6 ± 8.1	Present study
Quercetin-3′-*O*-sulfate		2.4 ± 1.1	Present study
3,4-dihydroxyphenylacetic acid		124 ± 16	Present study

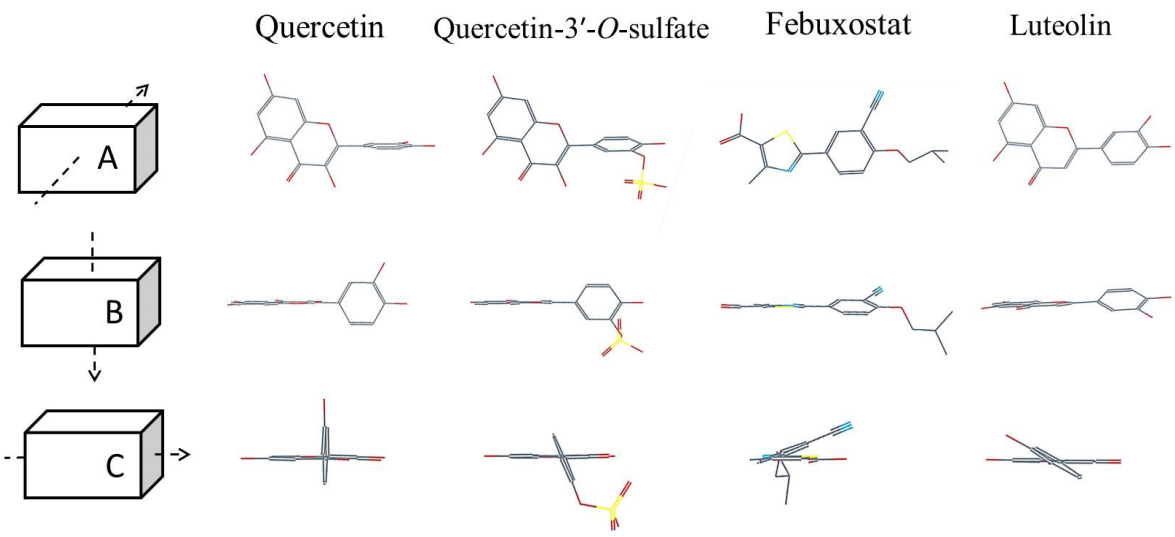

Figure 6-19 Three-dimensional (3D) structures of quercetin, quercetin-3′-O-sulfate, febuxostat and luteolin are found in PubChem Substance and PubChem Compound Database (296). (A) shows the full view of A/C rings; (B) shows the full view of B ring and (C) shows the dihedral angle between A/C ring and B ring formed by O1C2C1′C2′ (Figure 1-5).

Xanthine oxidoreductase (XD, not XO) is also known as a crucial upstream regulator of activity of PPAR-γ, a master-regulator of adipogenesis, expression of adiponectin, and an anti-inflammatory factor in adipocytes. Adipose tissue could produce and secrete uric acid through XOR and the production was enhanced in obesity (297). $XOR^{-/-}$ mice showed 50% reduction of adipose mass compared to wild type whilst obsess mice demonstrated higher level of XOR mRNA and uric acid in adipose tissue (298).

XO, which has been reported to increase its activity during oxidative stress (299, 300), produces uric acid and superoxide (Figure 6-1). In healthy individuals, XO is present in appreciable amounts only in the liver and jejunum; only in various liver disorders the enzyme is released into circulation (300). Plasma XO activity were

reported to be 2.1 ± 0.3 μmol min^{-1} L^{-1} in infant (301), 2.1 ± 0.8 μmol min^{-1} L^{-1} (302), 0.3 ± 0.2 μmol min^{-1} L^{-1} (303) and 0.32 ± 0.09 μmol min^{-1} L^{-1} (304) in healthy subjects and 0.36 ± 0.05 μmol min^{-1} L^{-1} in in healthy males (304). According to Yamamoto *et al*., there was no significant difference between healthy subjects and gout patients of plasma XO activity (305). Jawed *et al*. firstly purified human XOR from plasma using protein A beads coated with rabbit antihuman XOR polyclonal antibody. They found 75% of the plasma NADH oxidase activity was contributed by XOR, and it is significantly higher in rheumatoid arthritis patients (306). Our study has not found plasma XOR activity, since the plasma was collected from an apparently healthy subject. The produced uric acid, in reflecting of plasma enzyme activity, could not reach the limit of quantification (detailed in section 2.6.3.3). Brief calculation, plasma XO activity was reported, at highest, to be 2.1 ± 0.8 μmol min^{-1} L^{-1} (302) in healthy subjects. In each assay, 200 μL diluted plasma [2×] was added to 500 μL reaction system.

$$2.1\ \mu mol\ min^{-1}\ L^{-1} \times 200\ \mu L / 2 = 210\ pmol\ min^{-1}$$

Plasma provides 210 pmol min^{-1} enzyme activity. By the end of reaction, 200 μL of mixture was taken then be terminated by adding 50 μL of acid and methanol to 250 μL. If allow reacting for 30 min, the 250 μL of sample would contain:

$$210\ pmol\ min^{-1} \times 200\ \mu L \times 30\ min / 500\ \mu L = 2.520\ nmol$$

10 μL of sample was injected to HPLC. So the amount of uric acid in 10 μL sample would be:

$$2.520\ nmol / 250\ \mu L \times 10\ \mu L = 100.8\ pmol$$

And 100.8 pmol is close to the limit of quantification (78.2 pmol) of uric acid.

Healthy human erythrocytes XD activity is reported by μmol min^{-1} g^{-1} of haemoglobin is abundant than that of plasma (307) and is 0.61 ± 0.15 μmol min^{-1} g^{-1} of haemoglobin (308). We have also used healthy human erythrocytes for the experiment. Results show that healthy human erythrocytes show XO activity however the activity varies from sample to sample. Therefore the plan was terminated. It might because of a limit of current treatment which cannot validate the reproducibility. The treatment was, in brief, that erythrocytes were obtained from the blood sample of the human study MEEC 12-019 with mononuclear leukocytes isolated by Ficoll centrifugation (GE Healthcare, USA) and destroyed with detergent (DECON 90, Decon Laboratories Limited, UK) according to (308). The remain erythrocytes were harvested and washed in PBS, pH 7.4 (Sigma, USA) three times and aliquots were stored at -80℃ till use.

IC_{50} of quercetin on milk XO was reported to be 0.44 μM (106), 1.5 μM (294) and 2.08 μM (293). Ki of quercetin on milk XO varies from 1.40 ± 0.78 μM (181), 1.2 ± 0.7 μM (295), 0.28 μM (106) to 0.2 μM (111). By which, a comparison of the inhibitory effect between isorhamnetin and quercetin can be made. K_i of isorhamnetin is about 0.6 fold (0.17 μM (106)) and 1.25 fold (0.25 μM (111)) of K_i of quercetin (Table 6-5). Results from the present study provide comparisons of IC_{50} between quercetin-3′-*O*-sulfate and quercetin and 3,4-dihydroxyphenylacetic acid and quercetin. At inhibiting XO activity, quercetin-3′-*O*-sulfate is almost 10-fold more efficient than quercetin and 3,4-dihydroxyphenylacetic acid is about 0.2-fold. Quercetin-3′-*O*-sulfate was found to be an effective inhibitor of XOR, like isorhamnetin and quercetin-4′-*O*-glucuronide (111) whilst quercetin-3-*O*-glucuronide was found not able to inhibit, like quercetin-3-*O*-sulfate (111). This finding agreed with previous findings that the position of conjugation is much more determinant on

the function than the conjugated groups because of the exposure of the crucial structure O1C2C1′C2′ angle (Figure 1-5). It can be concluded that the intact quercetin is more likely to inhibit XO than derived phenolics. This is probably due to the loss of O1C2C1′C2′ structure where XOR favours (110).

Some parameters of this assay brought limits. Besides the presence of NAD^+ exhausted the enzyme, the adoption of hypoxanthine but not of xanthine as substrate (as high concentration of xanthine is not stable in reaction buffer) introduced various factors that may interfere with the observation since xanthine and uric acid inhibits enzyme activity as well (309). Therefore K_m and K_i of assay was not determined.

In conclusion, quercetin and its microbial-derived metabolites may suppress *in vivo* the formation of uric acid by inhibiting XO and ADA. This finding may also provide hypothesis of the relationship between quercetin supplement and other diseases related to ADA activity.

Chapter 7. Summary and Future Perspectives

7.1 Purpose of investigation and novelty

The primary hypothesis is that blood uric acid concentration can be modulated by quercetin supplementation, especially in pre-hyperuricemic subjects. We investigated the effect of oral supplementation of quercetin at 500 mg d^{-1} on blood uric acid level by a randomised, double-blinded, cross-over human study. The result shows that the blood uric acid levels were lowered after 4 weeks of quercetin supplementation without affecting BMI, systolic blood pressure or fasting blood glucose level. The lack of association of blood uric acid with other biomarkers during recruitment and the lack of effect of quercetin treatment on BMI, systolic blood pressure or fasting blood glucose suggest that hyperuricemia advances or at least is independent from metabolic syndromes (Chapter 4). But this does not propose that metabolic syndromes are independent from hyperuricemia.

The uric acid lowering effect was chronic (effect did not show significance until 4 weeks) so it might be caused by a more complex mechanism other than a simple direct enzyme inhibition (Chapter 6). In Chapter 6, *ex vivo* characterisation of the interaction of quercetin and its microbial and mammalian metabolites with enzymes that are involved in uric acid metabolism, ADA, PNP and XOR was discussed to advance previous knowledge that quercetin and some of its metabolites efficiently inhibited XO *in vitro* (Chapter 6). Results showed that XO activity *in vitro* was inhibited by not only quercetin, but also by quercetin-3′-*O*-sulfate and 3,4-dihydroxyphenylacetic acid, dose-dependently. Also quercetin inhibits human plasma ADA activity, dose-dependently, Therefore, this justifies the mechanism of

the uric acid lowering effect of quercetin and its metabolites by direct inhibition of the production of uric acid and its precursors.

Since blood uric acid is independent from other targeted biomarker levels (all within healthy range) and it does not respond to quercetin immediately, as mentioned above, we therefore explored the potential changes in all detectable plasma metabolites using ^{1}H-NMR spectroscopy and pattern-recognition techniques (Chapter 5). This methodology was adjusted specifically for the study design and successfully recognised several carbohydrate metabolites responding to quercetin treatment. For example, lactate was significantly reduced after 4 weeks of quercetin treatment, but not of placebo. The untargeted scanning of the differences in ^{1}H-NMR spectra was powered by the cross-over design of this study thus providing two study groups that were matching in phenotype yet controlled by placebo. This randomised trial with sufficient sampling points provided a valuable data set to explore potential biomarkers that may have been affected by quercetin supplementation.

However, the effects of functional foods are not always as significant as pharmaceutical strategies and usually they do not affect a healthy system as much as an unhealthy system. Therefore, one of the advantages of the current study is that a careful selected population were involved in this randomised controlled trial. It was designated to use a male pre-hyperuricemic population to investigate the uric acid lowering effect of quercetin. Similar to what we might anticipate, the effect was found to be more remarkable in subjects with a higher initial uric acid level. Also this lack of influence can be confirmed in other outcomes respectively, such as BMI, fasting glucose and blood pressure since none of those were correlated to plasma uric acid level and were normally distributed in normal range. Therefore the lack of effect

on these biomarkers could be partially due to the normal healthy status. After all the function of functional foods may be to dramatically protect against stress. The development of disease is usually a result of years of accumulation of stress caused by either an unhealthy lifestyle or lifelong susceptibility due to typical genetic predisposition. Therefore a reasonably long exposure to the beneficial treatment must be safe. This elongated treatment period is another advantage of present study allowing a repeated supplementation chronically simulates diet.

A dosage 500 mg d^{-1} quercetin aglycone might be difficult to be efficiently delivered, since quercetin aglycone yields less quercetin in urine as portion of intake than quercetin glucosides from food. A pilot human study compared the renal excretion of intact quercetin from aglycone supplement and quercetin glucoses from food. We have found out that one 500 mg quercetin aglycone tablet is comparable to 100 g fresh onion (Chapter 3). This result provided the confidence of the delivery efficiency of supplement tablet for human study and provided a constant to compare the previous results from humans studies conducted with different form of quercetin.

7.2 Discussion of outcomes

The key findings in this study is a favourable reduction in blood uric acid and blood lactate after the habitual consumption of quercetin supplements, in healthy male subjects with higher range of blood uric acid level, without affecting fasting glucose blood pressure or BMI. This is probably not a result of reduced production of uric acid through the inhibition of quercetin and its metabolites on XOR activity and ADA, two important enzymes in purine metabolic pathways. Although we have found the inhibitory effect of quercetin on both enzymes, the effective concentration

(IC_{50}) are apparently higher than the physiological concentration (see Table 6-1 and Table 6-2). In another word, further investigation on mechanism are needed to comprehend with this observation. Present study has tested the hypotheses and possible interactions proposed (Figure 7-1).

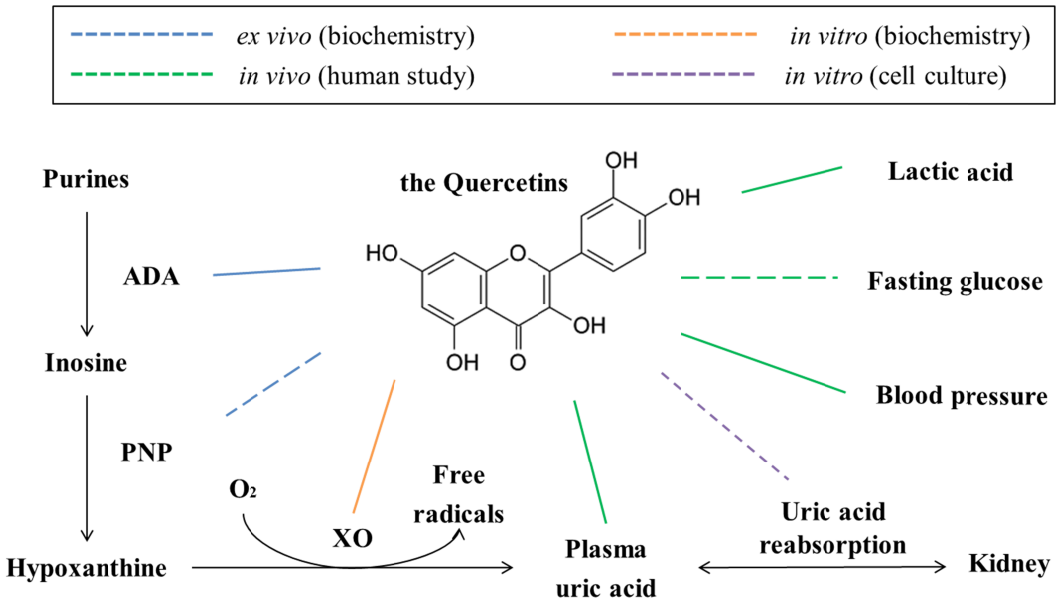

Figure 7-1 Summary of the findings in this work in understanding the effect of oral intake quercetin on metabolism and general health. Solid lines indicate significant effects, dash lines indicate no effect and short dash line indicates effect which remains unknown. Figure is personally drawn by Yuanlu Shi.

Hyperuricemia is often accompanied by higher oxidative stress (310). The link between hyperuricemia and metabolic syndromes seems to be oxidative stress as previously proposed in Chapter 1 (Figure 1-4). However, there is an ongoing debate as to whether uric acid itself or free oxygen radicals produced while generating uric acid, both or neither are/is the main culprits responsible for these effects.

A strong *in vitro* antioxidant does not necessarily show the same beneficial effect *in vivo*. Uric acid is a well-known antioxidant that contributes significantly to the

plasma antioxidant capacity (311, 312). This is not surprising given the high concentration of urate in human plasma (160–450 μM) and its powerful reducing and free radical scavenging activities (313). In the extracellular environment, urate can scavenge hydroxyl radical, singlet oxygen, and peroxynitrite, especially when combined with ascorbic acid or thiols (314-316). However available data suggest that uric acid is not essentially a biological antioxidant and, depending on the chemical milieu, may become a prooxidant. Uric acid loses its antioxidant ability in the hydrophobic environment (317) and even becomes a strong prooxidant in the presence of lipid peroxides (318).

The products of XO, superoxide ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2) would surely cause oxidative stress. In healthy human liver and jejunum, only 21% of XOR activity was XO (319). However an irreversible conversion from XD into XO happens in cell death in isolated hepatocytes subjected to ischemia (320) and in acute minimal liver damage when XOR leaks into the blood. Like other cytoplasmic and mitochondrial enzymes (321), the dehydrogenase form of XOR could be rapidly converted into the oxidase form which may then produce extracellular oxygen radicals (322). As XO produces $^{\bullet}O_2^-$ and H_2O_2, the conversion from the XD to the XO is hypothetically proposed to be responsible for causing oxidative stress. If this is true, inhibition of XOR by allopurinol would not reduce oxidative stress when the enzyme was mainly dehydrogenase since allopurinol/oxypurinol blocks the production of uric acid and of $^{\bullet}O_2^-$ and H_2O_2 by shutting down the enzymatic system (Figure 7-2) (323). Allopurinol is an irreversible mechanism-based inhibitor of the enzyme XOR in both forms (Figure 7-2). The enzyme commits suicide by initially activating allopurinol into a transition state analogue, oxypurinol, that bind very tightly to the molybdenum-sulfide (Mo-S) complex at the active site (324). It is

worth mentioning that higher doses of allopurinol profoundly reduced vascular oxidative stress (as well as uric acid, of course) in healthy humans (325) but does not modify kinetics of O_2 consumption or reactive oxygen species overproduction after severe exercise (326). All of this evidence suggests that there are other mechanisms that are responsible for causing oxidative stress where allopurinol may also interfere.

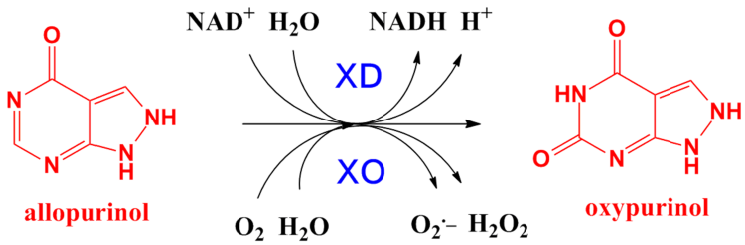

Figure 7-2 Oxidation of allopurinol. Allopurinol is metabolised irreversibly to oxypurinol. Oxypurinol, a non-competitive XOR inhibitor, binds tightly to the enzyme, therefore blocks uric acid production.

In the current study, diastolic blood pressure was reduced by quercetin intake, consistent with previous evidence (327). This probably is a result of reduction of intracellular uric acid, in parallel with reduced blood uric acid (Figure 7-3). These mechanisms could be an interference with the renin-angiotensin-aldosterone system and/or improving vascular function in an endothelium-dependent or -independent manner (200).

Uric acid, as a metabolic waste product, can stimulate ROS synthesis through pathways such as NADPH oxidase, although activation of NADPH oxidase by uric acid occurs via unknown mechanism (328). Uric acid can enter adipocytes through uric acid transporters such as GLUT9, URAT1 and OATs. ROS generated from superoxide produced by NADPH oxidase is followed by ROS-dependent activation of the pro-inflammatory signalling. An activation of this mechanism in response to

uric acid is followed by an increase in the production of chemokines. In animal tests, accumulation of uric acid in blood induced by means other than XOR inhibition cause acute inflammation (by injection of uric acid solution) (329) and insulin resistance (by inhibition of uricase) (68). An example for the mechanism is the effect of hyperuricemia might be partially responsible for the low-grade inflammation and insulin resistance in the adipose tissue and for increased risk of cardiovascular disease induced by obesity.

Development of renal disease, endothelial dysfunction, and activation of the renin-angiotensin system might also be the underlying cause of hyperuricemia-induced hypertension. Since uric acid inhibits the expression of renin (330) and stimulates renin release via a macula densa dependent mechanism (331) causing decreased internal renin. This suggests a promising protective function for the endothelial cells and smooth muscle cells by quercetin intake. Quercetin interrupts the renin-angiotensin system signalling pathway by inhibiting the angiotensin-converting enzyme activity and decreasing its mRNA production (332). This direct inhibition of quercetin on the renin-angiotensin system may explain the antihypertensive effects of quercetin.

Despite a long standing association between increased XOR activity and negative clinical outcomes, recent report described a paradigm shift where XOR mediates beneficial actions by catalysing the reduction of NO_2^- to $^{\bullet}NO$ in both forms (Figure 7-4) (333).

$$R\text{-}C\text{-}H + 2NO\mathbf{O}^- + H^+ \rightarrow R\text{-}C\text{-}\mathbf{O}^- + 2^{\bullet}NO + H_2\mathbf{O}$$ (Figure 7-4 C)

$$NADH + 2NO\mathbf{O}^- + 3H^+ \rightarrow NAD^+ + 2^{\bullet}NO + 2H_2\mathbf{O}$$ (Figure 7-4 D)

The alteration of micro-environmental factors, like acidic pH, elevation of NADH levels oxidation of biopterin and diminution of O_2 tension, that coalesce to facilitate the conversion from NO_2^- to $^{\bullet}NO$ by XOR, diminish the capacity of eNOS to catalyse $^{\bullet}NO$ formation and enhance its potential to uncouple and produce $^{\bullet}O_2^-$ (334).

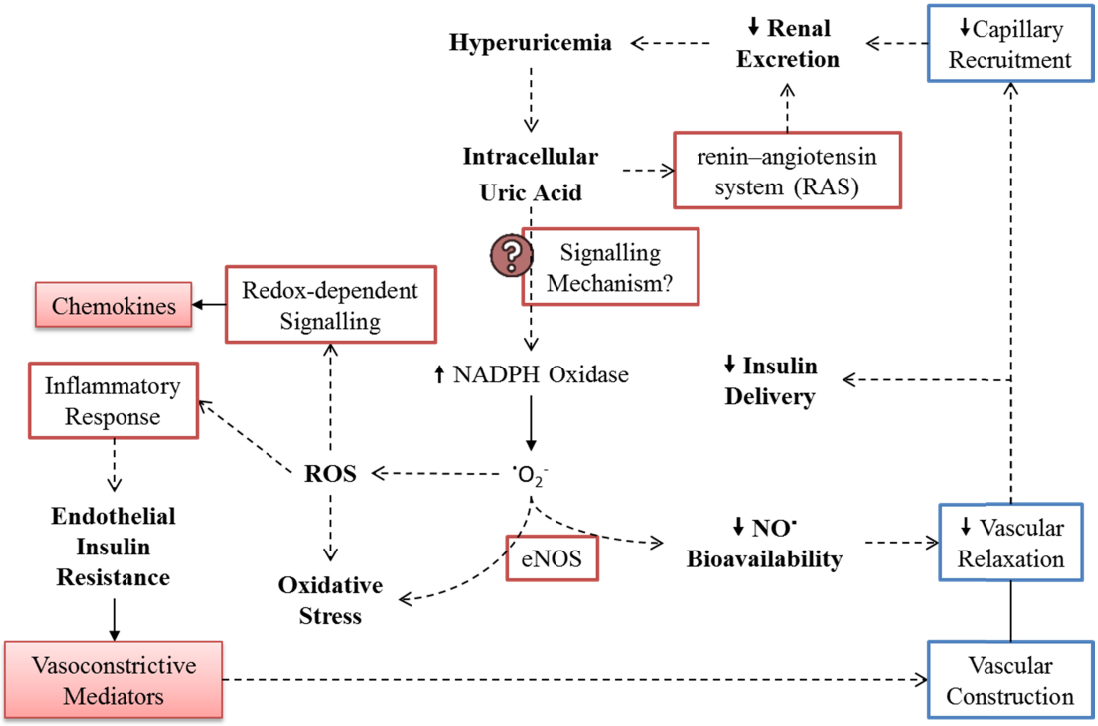

Figure 7-3 Model for the effect of hyperuricemia on the endocrine balance.

Solid lines indicate chemical reaction in cells and dotted lines show causal relationship. This model summarises the results of the current and the previous studies (328). eNOS, endothelial nitric oxide synthase; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; NO; nitric oxide; XOR, xanthine oxidoreductase . Figure is personally drawn by Yuanlu Shi and partially adapted from (335-338).

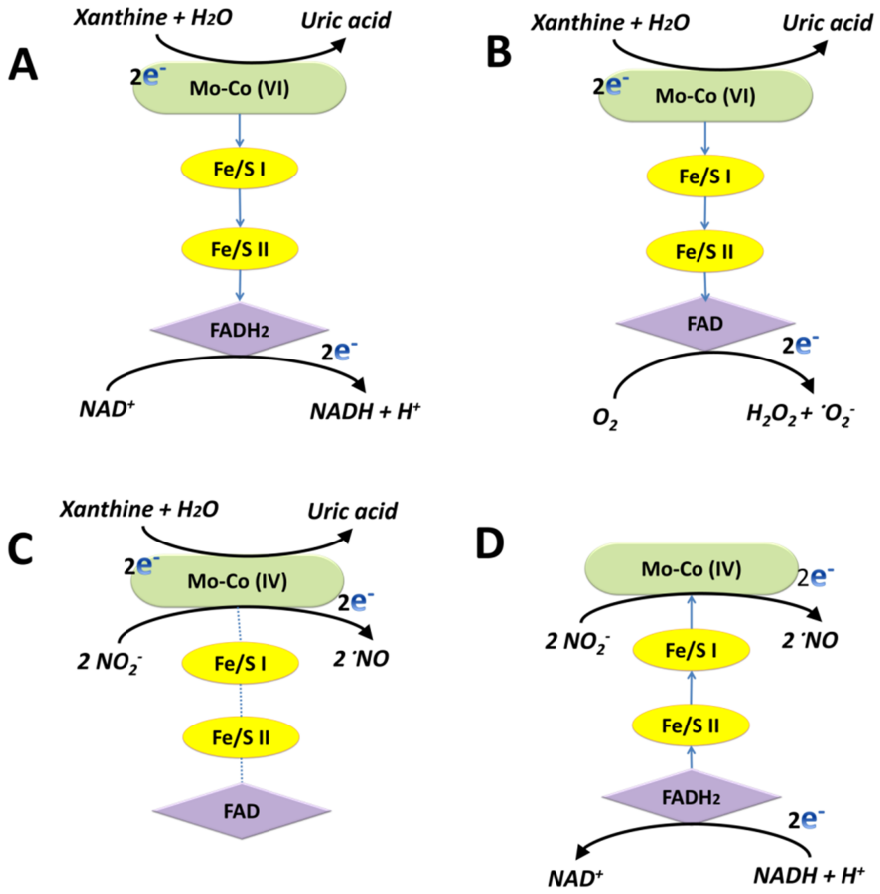

Figure 7-4 Reduction of nitrite to nitric oxide catalysed by XOR.

(A) For XD, xanthine is oxidised to uric acid and electrons transferred via 2 Fe/S centers to the FAD where NAD^+ is reduced to NADH. **(B)** For XO, xanthine is oxidised to uric acid and electrons are transferred to the FAD where O_2 is reduced to $^{\bullet}O_2^-$ and H_2O_2. Under normal O_2 tension and pH the Mo-co would reside more often in the oxidised +6 (VI) valence as electrons are rapidly transferred to O_2 at the FAD. **(C)** Nitrite (NO_2^-) undergoes a 1 electron reduction to $^{\bullet}NO$ at the Mo-cofactor of XOR (electrons are donated directly to Mo by xanthine). **(D)** NO_2^- is reduced to $^{\bullet}NO$ at the Mo-cofactor of XO (electrons are supplied by NADH and transferred retrograde reducing the Mo). Under low O_2 tensions and pH the Mo-co would reside more often in the reduced +4 (IV) valence as electrons are more slowly transferred to O_2. This decrease in electron flux from the Mo-co to the FAD is depicted in **(C)** as diminished arrows whereas in **(D)** NADH-mediated electron donation at the FAD is out-competing O_2-mediated electron withdrawal and thus the arrows are reversed indicating flux from the FAD to the Mo-co. Mo-co, molybdenum-cofactor; Fe/S, iron-sulfur centre; FAD, flavin adenine dinucleotide. Figure is adapted from (333, 339, 340).

The synchronised decrease of blood urate and lactate after chronic oral consumption of quercetin may indicate a relationship of their metabolism or a multi-functional intervention by quercetin. Uric acid and lactic acid share the renal transport system of organic acids (341) so that increased blood level of one may lead to accumulation of another in blood because of the competition on transporters in renal excretion. This hypothesis is also consistent with the known association of fructose induced hyperuricemia and simultaneous generation of lactic acid (56) and the association of hyperuricemia with other conditions of excessive generation of acid in the liver, like diabetic acidosis (342) and alcoholism (343). Judging by the fact that allopurinol treatment does not prevent lactic acid elevation caused by exercise in rats (344) or in humans (345), uric acid formation is not likely to affect physiological lactic acid balance. Therefore, hypothetically, the increase in serum concentration of uric acid is due to the decreased urinary excretion of uric acid. It was demonstrated that ethanol induced excessive production of lactic acid and NADH was accompanied by decreased urinary excretion of uric acid, as well as the increase of serum uric acid (346) especially when NADH inhibits production of uric acid (347). This may indicate that uric acid excretion may be dependent on blood lactic acid. It is possible that quercetin improved the efficiency of renal anion transporters however this needs to be further investigated.

To summarise, as a key factor for gout, blood uric acid levels were significantly reduced by oral supplementation of quercetin. The observed effect might be a result of inhibition of uric acid production, or indirectly activated renal excretion of uric acid through the reduction of lactate production or increase of lactate usage. The downstream effects, as hypothesis, could involve a reduction of oxidative stress in adipose, endothelial cells, etc., and a mediation on insulin delivery.

7.3 Overall conclusion and future perspectives

Among healthy male subjects with a higher range of blood uric acid level, we observed favourable reduction in blood uric acid and blood lactate after the habitual consumption of quercetin supplements, without affecting fasting glucose or BMI. This is probably a result of the inhibitory effect on XO/XD or other purine metabolic enzymes.

We did not identify the role of the phenolic breakdown products of quercetin and their metabolites in lowering blood uric acid. However bearing this goal in mind, a series of quercetin metabolites, both microbial and mammalian, were tested on the enzymes that were involved in uric acid metabolism, both *ex vivo* and *in vitro*. Unfortunately we could not detect *ex vivo* human plasma XOR activity so the relevant knowledge about XOR cannot advance beyond *in vitro*. The gap between requires several more specific experiments to be performed.

Three hypotheses were proposed through the results of this thesis.

1) Quercetin may affect energy metabolism that activates gluconeogenesis in liver or glycolysis in muscles by reduced oxidative stress and insulin tolerance;
2) Quercetin may inhibit MCTs (monocarboxylate transporters) thus reduced blood lactate and improved renal excretion of uric acid.;
3) Quercetin may inhibit renal transporters in reabsorption of uric acid and consequently cause a depletion of blood uric acid.

In conclusion, this research has exposed many potential functions of polyphenols *in vivo*, for example, on energy metabolism, on cancer prevention, on kidney function and on general healthy well-being.

On the basis of these results, it is highly recommended that plasma/serum uric acid level should be considered as an outcome or a risk factor in future nutrition cohort study in public health research. Basic science research is still required in confirming the causal relationships between metabolic syndromes and development of diseases. To our hesitation, investigation of quercetin on kidney function is not included in this study, as multiple lines of evidence suggest that kidney dysfunction is the fundamental cause of hyperuricemia (348). A study looking into the interaction of quercetin with human URAT1 (SLC22A12) and GLUT9 (SLC2A9) would be helpful to better understand the *in vivo* effect of quercetin and may provide a new approach in preventing gout and other metabolic disease.

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