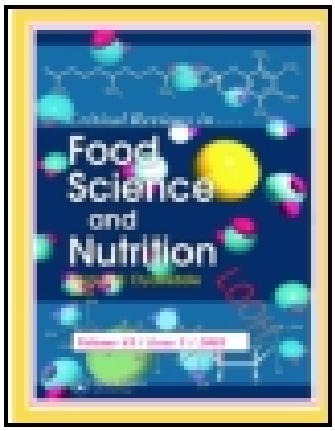

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The occurrence, fate and biological activities of C-glycosyl flavonoids in the human diet.

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Title: The occurrence, fate and biological activities of *C*-glycosyl flavonoids in the human diet.

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Abbreviations:

2-AAF, 2-acetamido-fluorene; AFB_1, aflatoxin B_1; Ara, arabinose; COMT, catechol-*O*-methyltransferase; Fer, ferulic acid; Gal, galactose; Glc, glucose; HUVEC, human umbilical vein endothelial cell; ICAM-1, inter-cellular adhesion molecule 1; Mal, malic acid; Rut, rutinose; Syn, synapic acid; TNF-α, tumour necrosis factor-α; Xyl, xylose.

Abstract

The human diet contains a wide variety of plant-derived flavonoids, many of which are glycosylated via an *O*- or less commonly a *C*-glycosidic linkage. The distribution, quantity and biological effects of *C*-glycosyl flavonoids in the human diet have received little attention in the literature in comparison to their *O*-linked counterparts however, despite being present in many common foodstuffs. The structural nature, nomenclature and distribution of *C*-glycosyl flavonoids in the human diet is therefore reviewed. Forty-three dietary flavonoids are revealed to be *C*-glycosylated, arising from the dihydrochalcone, flavone and flavan-3-ol backbones, and distributed amongst edible fruits, cereals, leaves and stems. *C*-linked sugar groups are shown to include arabinose, galactose, glucose, rutinose and xylose, often being present more than once on a single flavonoid backbone and occasionally in tandem with *O*-linked glucose or rutinose groups. The pharmacokinetic fate of these compounds is discussed with particular reference to their apparent lack of interaction with hydrolytic mechanisms known to influence the fate of *O*-glycosylated dietary flavonoids, explaining the unusual but potentially important appearance of intact *C*-glycosylated flavonoid metabolites in human urine following oral administration. Finally, the potential biological significance of these compounds is reviewed, describing mechanisms of anti-diabetic, anti-inflammatory, anxiolytic, anti-spasmodic, and hepatoprotective effects.

Keywords:

C-glycosylation, polyphenol, dihydrochalcone, flavone, flavan-3-ol, pharmacokinetics.

1. Introduction: *C*-glycosylation of flavonoids

The flavonoids are a highly diverse class of polyphenolic organic compounds formed principally as secondary plant metabolites with broad functional roles *in planta,* acting as siderophores, colourants, antioxidants and attractants, whilst providing protection against UV-radiation, insects, fungi and bacteria (Franz and Grun, 1983). Their wide distribution throughout the plant kingdom results in their inevitable presence in diets rich in plant-derived foods or beverages, where they appear to contribute to the overall health benefits of consuming such a diet. The epidemiological link of flavonoid intake to prevention of diabetes appears to be the strongest thus far (Nicolle *et al*., 2011), however specific links of flavonoid intake to cardiovascular mortality, chronic inflammation, cancer and other conditions are also evident (Arab and Liebeskind, 2010; Hirvonen *et al*., 2000; Manthey, 2000; Mink *et al*., 2007; Steinmetz and Potter, 1996).

The flavonoid family tree begins with seven heterocyclic backbone families differentiated by fundamental structural features usually related to the C-ring, such as its presence, carbon saturation and position of B-ring bonding (Tomás-Barberán and Clifford, 2000). These backbones are the flavan-3-ols, flavonols, flavones, flavanones, isoflavones, anthocyanidins and chalcones (Scalbert and Williamson, 2000), which are subsequently differentiated by the presence of functional moieties such as hydroxyl groups at multiple positions about the carbon backbone, allowing for a diverse array of possible structures. Whilst additional substitution, including *O*- and *C*- methylation (Erlund, 2004), prenylation (Stevens and Page, 2004), gallation (Singh *et al*., 2011), glucuronidation (Hegnauer and Gpayer-Barkmeijer, 1993) and polymerisation (Manach *et al*., 2004) at various positions about the backbone structure all also further expand the diversity of the flavonoid family, glycosylation is arguably the most significant single native structural feature in determining the

pharmacokinetics of any flavonoid in the human diet. The addition of a sugar moiety is capable of fundamentally altering parent compound bioavailability following oral consumption by modulating critical physicochemical parameters such as structural polarity (Day *et al*., 2000). Flavonoid glycosylation usually proceeds by the attachment of a sugar substituent to a hydroxyl group during *in planta* flavonoid synthesis, thus conferring *O*-glycosylation via preservation of the hydroxyl oxygen in the glycosidic linkage. Such a biochemically demanding reaction is assumed to be required in order to stabilise the receiving flavonoid, which subsequently exhibits the function of both the donor and receptor molecules *in planta* (Hultin, 2005). This synthetic *O*-glycosylation reaction is catalysed by a myriad of family-1 *O*-glycosyltransferases (OGTs) in the presence of nucleotide-diphospho-sugars (UDP-sugars) as substituent group donors participating in a final step during flavonoid synthesis (Lairson *et al*., 2008). It is common for both the glycosylated and aglycone forms of the same parent flavonoid to be present in the same food crop, as in the example of the onion (*Allium cepa*). The edible onion bulb contains both the flavonol aglycone quercetin and its four *O*-glycosylated derivatives: rutin, quercetin 3-*O*-glucoside, 4′-*O*-glucoside, and 3,4′-*O*-diglucoside (Marotti and Piccaglia, 2002). *O*-glycosylation most usually occurs on the aglycone flavonoid hydroxyl group at the 7 position of the flavonoid backbone, at the 3 position of flavonol structures, at the 4 position, and less frequently at other positions of hydroxylation (Ferreres *et al*., 2007). The general system of flavonoid ring numbering is shown in figure 1.

C-glycosyl flavonoids, the subject of this review, are a less well understood sub-class of secondary plant metabolites in comparison to their more common and structurally diverse *O*-glycosyl cousins. The defining feature and divergent characteristic of their formation and subsequent nature is a *C*-*C* covalent bond between the aglycone flavonoid backbone and a sugar moiety: generally monomer glucose or galactose. This linkage is found in dietary

examples of *C*-glycosyl flavonoids at either the 6 or 8 positions about the carbon backbone A-ring, and differs from *O*-glycosyl examples in the limited number of glycosylation positions, the form and range of attached saccharide polymerisation, and critically in the lack of an oxygen intermediate required for glycosidic linkage synthesis (Franz and Grun, 1983). Another less common sub-class are the *O*-glycosyl-*C*-glycosyl flavones, characterised by *O*-glycosylation at the same positions as described for mono-*O*-glycosyl flavonoids in addition to *C*-glycosylation elsewhere on the flavone backbone, or *O*-glycosylation at one of the hydroxyl groups of a *C*-linked sugar moiety. The latter form of *O*-glycosylation occurs most usually at the 2 or less frequently at the 6 carbon of a *C*-linked hexose sugar (Ferreres *et al*., 2007).

It is likely that *C*-glycosylation usually occurs as an integral part of *in planta* flavonoid biosynthesis rather than as a final stabilising step. In this regard, the lack of requirement for positional hydroxylation and the involvement of a separate synthetic pathway show that the biosynthetic process is distinct from that of *O*-glycosylation. This pathway has been investigated most thoroughly in wheat (*Triticum aestivum*), rice (*Oryza sativa*) and buckwheat (*Fagopyrum esculentum*). The principal reaction is catalysed in these plants by the *C*-glycosyltransferase (CGT) family of enzymes, the best characterised of which is OsCGT: a 49 kDa enzyme related strongly in amino acid sequence to the known *O*-glycosyltransferases (Brazier-Hicks et al., 2009). More recently, aglycone flavan-3-ol structures such as epicatechin have been shown to be non-enzymically mono- or di- *C*-glycosylated with glucose or galactose during post-harvest processing, although as with OsCGT-catalysed glycoconjugation, positions of substitution are limited to the hydroxyl groups at 6 and 8 positions (Stark and Hofmann, 2006).

The significance of the *C*-glycosidic linkage to the overall flavonoid structure is greater than might at first be imagined in view of the seemingly relatively minor chemical divergence

from the *O*-glycosidic linkage. The *C*-glycosidic bond between a saccharide moiety and flavonoid carbon skeleton largely protects the flavonoid glycoside from the hydrolytic effect of both acidic and enzymic treatments known to readily cleave *O*-glycosidic linkages, leading to fundamental differences in the analysis, degradation, pharmacokinetics and bioactivity of those flavonoids possessing a *C*-glycosyl group (Harborne, 1965). Whilst *C*-glycosylation of flavonoid compounds *in planta* is not widespread, especially in those plants traditionally used as food crops, plants capable of *C*-glycosylation may produce *C*-glycosyl flavonoids in greater amounts than *O*-glycosyl flavonoids by weight, such as in the examples of the *C*-glycosyl flavones in wheat (*Triticum aestivum*) and *C*-glycosyl dihydrochalcones in rooibos tea leaves and stems (*Aspalathus linearis*) (Bramati *et al*., 2002; Brazier-Hicks *et al*., 2009). As such, it is reasonable to suggest that these compounds deserve greater interest than has previously has been afforded. Various plants produce a wide variety of *C*-glycosyl flavonoids in the leaves, however the parts of such plants traditionally present in the human diet appear to contain a narrower range. In durum wheat (*Triticum durum*) for example, 29 *C*-glycosyl derivatives of apigenin, luteolin and chrysoeriol, including some di-*C*-glycosides, have been identified in the leaves (Cavaliere *et al*., 2005). In contrast, wheat grain is known to contain only 5 such compounds, 4 of which are derivatives of the flavone luteolin. Biosynthetic pathways *in planta* have been well characterised in certain species not traditionally included in the human diet, initially being studied in duckweed (*Spirodela polyrhiza*) (Wallace and Alston, 1966). Even prior to this publication, other workers had reported the presence of *C*-glycosylated flavonoids in over 40 plant species. Harborne had also determined the hydrolytic fragility of over 100 flavonoid glycosides and glucuronides by both chemical (strongly acidic) and enzymic means, providing a comprehensive assessment of the conditions that could be easily employed to distinguish *C*- from *O*-glycosyl flavonoids (Harborne, 1965). It was concluded that β-D-glucosidase glucohydrolase (β-glucosidase), an

endogenous mammalian enzyme typically employed to non-specifically deglycosylate *O*-glycosylated flavonoids *in vitro*, had no specificity to the *C*-glycosyl linkage. Hydrolytic attempts in acidic ethanol/water mixtures at 100 °C for 24 hr proved similarly ineffective. Thus a simple, specific and robust analytical means of distinguishing this critical structural divergence in glycosyl flavonoids extracted from plant or crop materials was provided. Known flavonoids found exhibiting native *C*-glycosylation in the human diet are limited to the flavones and dihydrochalcones, whilst *C*-glycosyl flavan-3-ols are known to be formed during food processing. A notable disparity exists between the structural nomenclature of the three-ringed flavones and flavan-3-ols and the two-ringed dihydrochalcones insofar as A- and B-ring carbon numerical priming is reversed (the flavone and flavan-3-ol B-ring is primed, whilst the dihydrochalcone A-ring is primed). The lack of a C-ring also dictates that dihydrochalcone A-ring numbering is offset by 3 fewer carbons about the phenolic ring in comparison to the flavone and flavan-3-ol A-rings, as shown in figures 1 - 4. As with many *O*-glycosyl flavonoids, all known dietary *C*-glycosyl flavonoids possess a hydroxyl group at the 7 position on the flavonoid backbone A-ring (or the equivalent 4′ position on the dihydrochalcone structure). This group is often additionally *O*-glycosylated *in planta* however, presumably as a downstream step in the flavonoid biosynthetic pathway. It seems likely that, whilst unglycosylated, this group confers substrate specificity to the CGTs, with equal potential of glycosylation at the 6 or 8 position (3′ and 5′ respectively for dihydrochalcones) through spontaneous rotation of the A-ring whilst in the open-ringed 2-hydroxyflavanone form during biosynthesis. These exist in equilibrium between open-chain and closed-chain (C-ringed) forms. Closed-chain forms are subsequently enzymically dehydrated, fixing the position of *C*-glycosylation in either the 6 or 8 A-ring positions of the flavone backbone (Brazier-Hicks *et al*., 2009). In many examples, *C*-glycosylation in one of

these positions does not appear to prevent *C*-glycosylation of the other, nor does it prevent *O*-glycosylation at the 7 (dihydrochalcone 5′) position.

2. Nature and distribution of *C*-glycosyl flavonoids in the human diet

The most common sources of *C*-glycosylated flavonoids in the human diet are tomatoes, dates, lemons, limes, wheat, oats, maize, rice, buckwheat, rooibos tea, processed cacao, and Swiss chard, as summarised in tables 1 - 4. Whilst literature describing the distribution of these compounds in specific organs of the plant typically consumed as part of the human diet is limited in comparison to characterisation in other parts (and in particular the leaves), even less data is available on the abundance or variation of these compounds in unprocessed crops, or indeed quantities of individual structures in the diet. Nevertheless, indications are provided where available.

2.i. Fruit

The flavonoids of tomato (*Lycopersicon esculentum*) fruits are generally confined to the skin, with little or no presence in the central flesh. In one study, chalconaringenin dominated the composition in the skins of 9 cultivars quantified by HPLC-DAD (35 – 71%), however the *C*-glycosyl dihydrochalcone phloretin 3′,5′-di-*C*-β-D-glucoside was found to contribute 5 - 14% of the total flavonoid content and was present in all cultivars tested (Slimestad *et al*., 2008). This is currently the only *C*-glycosyl flavonoid identified in tomatoes, and indeed is the first dihydrochalcone identified in genus *Lycopersicon* and family Solanaceae. It is unusual in the presence of two *C*-linked glucose substituent groups, both present on the A-ring (3′ and 5′ positions) of the dihydrochalcone backbone. It is possible however that the biosynthetic pathway of phloretin 3′,5′-di-*C*-β-D-glucoside is a diversion from the formation of chalconaringenin, differing only in a single glycosyl group and the saturation of the α and

β carbons (i.e. in the difference between chalcone and dihydrochalcone). Flavonoid *C*-glycosylation occurs in nature almost exclusively at either the 6 or 8 positions on the flavonoid A-ring. As such, phloretin 3′,5′-di-*C*-β-glucopyranoside appears unusual in structure simply as a result of dihydrochalcone nomenclature (as previously discussed) rather than disposition. A di-*C*-glycosyl flavonoid is also present in the edible fruit of the Deglet Noor cultivar of the date palm (*Phoenix dactylifera*). The flavone apigenin 6,8-di-*C*-β-D-glucoside (vicenin-2) was identified by LC-MS/MS as the only *C*-glycosylated flavonoid alongside a variety of *O*-glycosyl flavonoids (Yun *et al*., 2006).

2.ii. Citrus fruit

Vicenin-2 is also present in the lemon fruit (*Citrus limon*) (Gil-Izquierdo *et al*., 2003), accompanied by three further dietary di-*C*-glycosylated flavonoid examples. The flavones diosmetin 6,8-di-*C*-β-D-glucoside (Miyake *et al*., 1997), luteolin 6,8-di-*C*-β-D-glucoside (lucenin-2) (Baldi *et al*., 1995) and chrysoeriol 6,8-di-*C*-β-D-glucoside (stellarin-2) (Garg *et al*., 2001) are present alongside two mono-*C*-glycosylated analogues, diosmetin 6-*C*-β-D-glucoside (Miyake *et al*., 1997), and diosmetin 8-*C*-β-D-glucoside (Abad-Garcia *et al*., 2008). Of all of these forms, only lucenin-2 in lemon fruit is partnered by its respective aglycone, luteolin (González-Molina *et al*., 2010). This again serves to support the assertion that unlike *O*-glycosylated flavonoids, where aglycone and glycoside often coexist in the same source, *C*-glycosylation does not occur as the final reaction in the biosynthetic pathway. Lime (*Citrus aurantifolia*) fruits contain both diosmetin 6- and 6,8-di-*C*-β-D-glucoside, although these compounds are not found in other members of the *citrus* family (Caristi *et al*., 2006). Vicenin-2 has also been shown to be present in freshly squeezed orange (*Citrus sinensis*) juice at 19.6 mg L^{-1}, and interestingly at the greater concentration of 26.3 mg L^{-1} in a commercially pasteurised juice (Gil-Izquierdo *et al*., 2001). It is now clear that this

phenomenon may be due to post-harvest *C*-glycosylation of apigenin in the presence of D-glucose and catalysed by the heat of pasteurisation, furthermore suggesting that mono-*C*-glucosyl apigenin derivatives such as vitexin may also be present.

2.iii. Cereal grains

The flavones of wheat (*Triticum aestivum*) grain were first discovered by King in 1962, who observed the presence of two flavones comprising 0.2 – 0.3% w/w dry-matter in commercial wheat germ samples (King, 1962). UV spectral analysis after partial purification revealed these compounds to be 8-*C*-glycosyl apigenin derivatives, one of which was acylated with syringic acid. Characterisation of the glycosidic substituent was reported to not be possible for these compounds, however acylation of *C*-glycosyl apigenin derivatives with hydroxycinnamic acids has since been shown to be common in cereal grains, and can be circumvented during routine analysis by alkaline hydrolysis of the substituent moiety to release the glycosyl flavone from the phenolic acid ester (Ferreres *et al*., 2007). The advent of tandem mass spectrometry has provided a useful tool in the detection and structural elucidation of the *C*-glycosyl flavonoids (Kazuno *et al*., 2005), especially those found natively esterified or acylated. LC-MS/MS analyses of wheat and rye (*Secale cereal*) grains have shown the presence of vicenin-2, apigenin 6-*C*-β-D-glucoside-8-*C*-arabinoside (schaftoside) and apigenin 6-*C*-arabinoside-8-*C*-β-D-glucoside (isoschaftoside) in both free and acylated forms, both being esterified with ferulic or synapic acid in the 6 position of the glucose moiety (Gallardo *et al*., 2006; Wagner *et al*., 1980). Both schaftoside and its reverse-glycosylated isomer are the predominant flavonoids in hard red spring wheat bran (Feng *et al*., 2008; Feng and McDonald, 1987; Feng *et al*., 1988). Buckwheat (*Fagopyrum esculentum*) hulls contain the mono-*C*-glycosyl flavones orientin, isoorientin (luteolin 8- and 6-*C*-β-D-glucoside respectively), vitexin and isovitexin (apigenin 8- and 6-*C*-β-D-glucoside

respectively), whereas the groats (the commonly consumed fraction of the seed) contain only isovitexin (Dietrych-Szostak and Oleszek, 1999; Watanabe, 1998). Significant quantities of vitexin have also been found in the flour of millet seeds (*Pennisetum americanum*), with reported amounts ranging between 77 and 275 mg g^{-1} (Akinbala, 1991), whilst isovitexin is also present in rice hulls (Ramarathnam *et al*., 1989). Barley grains contain nine *O*-glycosylated *C*-glycosyl flavonoids, in which both pathways of glycosylation are followed by the same compound during *in planta* biosynthesis, resulting in at least one of each *O*- and *C*-glycosyl substituent groups being present at separate positions on the aglycone flavonoid backbone (Ferreres *et al*., 2009). In barley grain, these are isoorientin-7-*O*-β-D-glucoside (lutonarin), isovitexin-7-*O*-β-D-glucoside (saponarin), isoorientin-7-*O*-β-D-[6-feruoyl]-glucosyl-4′-*O*-β-D-glucoside, apigenin-6-*C*-arabinosyl-8-*C*-β-D-glucoside, isovitexin-7-*O*-rutinoside, chrysoeriol-6-*C*-β-D-glucosyl-7-*O*-β-D-glucoside (isoscoparin-7-*O*-glucoside), apigenin-6-*C*-β-D-glucosyl-8-*C*-arabinoside, isovitexin-7-*O*-β-D-[6-sinapoyl]-glucosyl-4′-*O*-β-D-glucoside and isoscoparin-7-*O*-rutinoside (Ferreres *et al*., 2009). It is worth noting that this complex form of glycosylation differs to those equally complex but structurally divergent compounds containing an *O*-glycosidic linkage between the two hexose sugars of a *C*-linked disaccharide flavonoid sub-species that are present in the inedible leaves of plants such as barley (Ferreres *et al*., 2007). Isoorientin is the only additionally identified mono-*C*-glycosyl flavonoid in barley seed (Ferreres *et al*., 2009).

2.iv. Edible leaves and stems

Chard (*Beta vulgaris*) leaves, commonly known as Swiss Chard, contain vitexin 2′-*C*-xyloside and vitexin 2′-*C*-[6′-malonyl]-xyloside: the only example of a dietary *C*-glycosyl flavonoid acylated with a dicarboxylic acid (Gil *et al*., 1998). These compounds were extracted with methanol from the leaf material to yield 1.39 and 0.34 mg g^{-1} un-dried leaf

material respectively. In a separate study, the edible stems of rhubarb (*Rheum rhabarbarum*), a relative of chard, were shown to contain isovitexin, vicenin-2, schaftoside and isoschaftoside (Krafczyk *et al*., 2008).

Rooibos tea, a tisane produced from the leaves and thin stems of the rooibos plant (*Aspalathus linearis*), contains significant amounts of two examples of dietary *C*-glycosyl dihydrochalcones (Koeppen and Roux, 1966; Schmandke, 2005). These unusual compounds have received a significant deal of attention, not least because of their abundance in this tisane and the importance of this principal South African export to its home economy. Indeed, the routine quantification of both aspalathin (2′,3,4,4′,6′-pentahydroxy-3-*C*-β-D-glucopyranosyldihydrochalcone) and the 2′,4,4′,6′ tetrahydroxylated analogue, nothofagin, has become an accepted measure of rooibos quality, with several publications detailing methodologies for analysis and abundance in material from various processing yards and following several processing protocols (Bramati *et al*., 2003; Bramati *et al*., 2002; Joubert, 1996; Joubert and deVilliers, 1997; Joubert *et al*., 2008; Krafczyk and Glomb, 2008; Manley *et al*., 2006; Pengilly *et al*., 2008; Rabe *et al*., 1994; Schulz *et al*., 2003; Standley *et al*., 2001; vonGadow *et al*., 1997). The predominant factor determining the concentration of these two *C*-glycosyl dihydrochalcones in rooibos tea is the ‘fermentation’ process. This comprises a heating and / or sun-drying step (usually between 35 - 45°C) that catalyses the oxidation of phenolic compounds in the dried leaf and stem material (Joubert, 1996). This process is responsible for the distinct red-colour of both the tea material and resulting aqueous extract, and is best understood in the case of oxidative ring-cyclisation of aspalathin in the presence of heat or UV radiation and oxygen, a reaction that forms two additional closed-ring *C*-glycosylated eriodictyol products that, whilst not native to the rooibos plant, can be considered to be part of the fermented rooibos tisane (Marais *et al*., 2000). A-ring cyclisation prior to closure and formation of a third C-ring dictates the formation of two isomeric *C*-

glycosylated forms, eriodictyol-6-*C*-β-D-glucoside and eriodictyol-8-*C*-β-D-glucoside, from the 8 *C*-glycosylated precursor aspalathin. Unfermented, freeze-dried rooibos tea therefore contains ~ 14 times the total quantity of aspalathin (~ 15 g kg^{-1}) and presumably due to a similar oxidation reaction in production of the fermented product, 12 times the quantity of nothofagin (4.31 g kg^{-1}) when compared to oxidised rooibos tea (1.02 and 0.35 g kg^{-1} respectively) (Joubert, 1996). This is an important factor in considering the dietary burden of the rooibos *C*-glycosyl dihydrochalcones, as both unfermented ('green') and fermented (often known as 'redbush') rooibos tea is consumed. Rooibos also contains the flavones orientin and isoorientin, both native to the plant and derived from the pathway of aspalathin oxidation during fermentation via the two aforementioned eriodictyol-*C*-glucoside isomers (Marais *et al*., 2000; Rabe *et al*., 1994). These isomeric compounds are reported to be interconvertable via a Wessely-Moser rearrangement of the flavone structure under heating in an aqueous solution: conditions similar to those that occur during tisane extraction (Koeppen and Roux, 1965). They are extractable in hot aqueous media from commercial fermented rooibos tea at 1.00 and 0.83 g kg^{-1} respectively; comparable to the aspalathin extracted in the same manner (1.23 g kg^{-1}) (Bramati *et al*., 2002). The flavones vitexin and isovitexin are also present in rooibos tea, however are significantly less abundant than the aforementioned compounds (0.33 and 0.27 g kg^{-1} respectively).

Extraction of sugar from the stems of the sugarcane plant (*Saccharum officinarum*) is the first step in an extended process of refinement, the products of which are various forms of sugar-based products. During processing, a surprising array of flavonoids is found in the extracted juice and bagasse (the lignin-based material left after juice extraction), many of which are *C*-glycosylated. These include the dimethylated flavone 4′,5′-dimethyl luteolin-8-*C*-β-D-glucoside, luteolin-8-*C*-rutinoside, vitexin, orientin, schaftoside, isoschaftoside, and diosmetin-8-*C*-β-D-glucoside (Colombo *et al*., 2008; Colombo *et al*., 2006). Whilst these

compounds are easily detectable during processing, no data is currently available detailing flavonoid survival through further refinement to consumed products such as molasses or refined sucrose.

2.v. Synthesis during cocoa processing

The pH and temperature conditions often used in the post-harvest processing of foods have been shown to catalyse the *C*-glycosylation of aglycone flavonoids. The only dietary example to date is the non-enzymatic formation of flavan-3-ol *C*-glycosides during cocoa tree (*Theobroma cacao*) seed processing during the production of chocolate, however other theoretical structures have been synthesised in the laboratory and the chemistry of their production elucidated. (-)-Epicatechin-8-*C*-β-D-galactopyranoside was first evidenced in processed cocoa (Hatano *et al*., 2002), followed by the 6- and 8-*C*- β-D-glucopyranosyl, 6,8-*C*-β-D-diglucopyranosyl and 6,8-*C*-β-D-digalactopyranosyl derivatives of (-)-epicatechin, summarised in table 3. (-)-Catechin-*C*-glycopyranosides have also been identified in cocoa, produced both as a product of the *C*-glycosylation reaction and in increasing amounts following alkalinisation, presumably as a result of (-)-epicatechin-*C*-glycoside epimerisation under basic conditions. These are the corresponding (-)-catechin epimers of the cocoa (-)-epicatechin *C*-glycosides alongside (-)-catechin-6-*C*-β-D-galactopyranoside (Stark and Hofmann, 2006; Stark *et al*., 2007). Table 4 outlines these structures. In view of the universal commercial and domestic use of high temperature in the sterilisation and preparation of food products, is highly likely that many untold *C*-glycosylated flavonoid species are formed as a result, and are therefore present in the human diet. Further research is required to elucidate the extent of their formation.

3. The biological fate of *C*-glycosyl flavonoids in the human diet

Deglycosylation has often been referred to as a critical step in the 'activation' of flavonoids following human consumption (Hollman *et al*., 1999; Walle *et al*., 2005). This appears to be generally true for *O*-glycosylated compounds, as buccal, intestinal or colonic enzymic hydrolysis results in improved bioavailability of the resulting aglycone (Day *et al*., 1998; Hollman and Katan, 1997; Walle *et al*., 2005), however greater resistance of the *C*-glycosidic linkage to hydrolysis dictates that the bioavailability of *C*-linked flavonoid glycosides is somewhat different. Increasing evidence suggests that deglycosylation is not a prerequisite for *C*-glycosyl flavonoid absorption in the small intestine, resulting in the presence of the intact glycosyl flavonoid in the urine of humans following oral consumption. This phenomenon has been best characterised for the dietary *C*-glycosyl dihydrochalcone, aspalathin, intact urinary methyl metabolites of which were quantified in the human subjects of two separate studies following single-dose oral administration of similar aspalathin-rich rooibos tea beverages, and also in pigs fed high chronic doses of rooibos tea extract (Courts and Williamson, 2009; Kreuz *et al*., 2008; Stalmach *et al*., 2009). Interestingly however, intact aspalathin metabolites were not detected in either human or porcine plasma in two of these studies, despite sensitive mass-spectrometric analysis; findings that are at odds with the common observation of circulating aglycone flavonoid metabolites in human plasma following ingestion of *O*-glycosyl flavonoids (Mullen *et al*., 2004). The presence of intact aspalathin in urine suggests that the efficacy of existing plasma extraction protocols may not be suitable for the analysis of circulating *C*-glycosyl flavonoids, possibly due to the intact glucosylated structure having greater affinity for plasma carrier proteins such as serum albumin. This assertion is supported by the identification of intact puerarin, a non-dietary *C*-glucosyl isoflavone from kudzu root (*Pueraria lobata*), in the plasma of humans, rats and

dogs (Ma *et al*., 2005; Ren *et al*., 2006; Shen *et al*., 2007). Despite these findings, the authors of a similar study reported the absence of *C*-glycosyl flavonoids in the collected urine or plasma of Sprague Dawley rats (n = 4) at any point in the 72 hr following a high, single-dose oral administration of 1 g kg^{-1} bamboo leaf extract in water (100 mg ml^{-1}) containing the four flavone *C*-glycosides orientin, isoorientin, vitexin and isovitexin. Similarly, none of these compounds were detected in the excised liver, brain, thigh muscle and kidney tissue of these animals, prompting the authors to conclude that *C*-glycosyl flavones are not readily absorbed in the small intestine (Zhang *et al*., 2007). In this case, it is highly likely that the HPLC-DAD methodology used for the analysis of these samples did not provide sufficient sensitivity in the detection of these compounds however, nor was provision made for the hydrolysis of metabolic conjugates prior to analysis of parent structures. Evidence from studies on the bioavailability of aspalathin, measured by the appearance of glucuronide and sulphate conjugates, suggests that like their aglycones, flavonoid *C*-glycosides are readily conjugated *in vivo* (Courts and Williamson, 2009; Kreuz *et al*., 2008; Stalmach *et al*., 2009). Furthermore, we have characterised the kinetics of catechol-*O*-methyltransferase (COMT)-catalysed aspalathin *O*-methylation, showing that *C*-glycosyl flavonoids bearing a catechol moiety may also participate in this reaction. This was confirmed by the presence of 3-*O*-methylaspalathin in human urine following consumption of an aspalathin-rich beverage (Courts and Williamson, 2009).

Unpublished data from our laboratories suggests that aspalathin is capable of passive diffusion across the intestinal epithelium in transport studies using confluent filter-grown monolayers of the intestinal cell-line, caco-2. These monolayers were also found to be incapable of deglycosylating aspalathin during transepithelial flux, supporting data from human bioavailability studies. Moreover, enterocyte uptake of the intact *C*-glycoside in a similar model was negligible compared to the structurally similar dihydrochalcone *O*-

glycoside, phlorizin, suggesting that bioavailability of the intact *C*-glycoside in humans is limited by non-specificity to enterocyte glucose carrier proteins known to transport flavonoid *O*-glycosides, such as sodium-dependant glucose transporter 1 (SGLT-1) (Walgren *et al*., 2000). Position of glycosylation and other structural features are known to affect substrate affinity of *O*-glycosyl flavonoids to these transporters however, providing a likely explanation for the lack of facilitated aspalathin diffusion, rather than non-specificity of *C*-glycosidic linkages to glucose transporters.

Generally, *O*-glycosylated structures are not detected *in vivo* due to first-pass intestinal and hepatic hydrolysis, presumably catalysed by broad-specificity β-glucosidases (Day *et al*., 2000; Day *et al*., 1998; Hays *et al*., 1996). *C*-Glycosyl flavonoids survive hepatic hydrolysis for reasons outlined previously, explaining their seemingly unusual presence in human urine following oral consumption (Courts and Williamson, 2009; Hasslauer *et al*., 2010). As such, it is very likely that *C*-glycosylation does not confer diffusive flavonoid absorption *per se*; rather that the *C*-glycosyl bond of these compounds has greater stability *in vivo*.

Whilst the *C*-glycosidic linkage appears to remain intact in the upper- and mid-gastrointestinal tract, and no known mammalian *C*-deglycosylating enzyme with specificity towards flavonoid structures exists, increasing evidence suggests that bacteria capable of cleaving the *C*-glycosidic linkage (putatively via expression of *C*-deglycosylating enzymes) are present in the colon, as outlined in figure 5. Hasslauer *et al*. (2010) examined the respective difference in the *ex vivo* stability of (-)-epicatechin-6-*C*-glucoside in buffered human ileostomy and colostomy fluids at 37°C for 10 hr (pH 6.3 and 7.4 respectively). No significant degradation occurred in ileostomy fluid, however complete degradation occurred following incubation for between 2 and 4 hr in colostomy fluid. Also reporting excellent stability in saliva and simulated gastric juice, the authors conclude that *C*-glycosyl flavan-3-ols undergo rapid degradation via microbial metabolism only in the human colon.

C-Deglycosylating enzymes from plants have already been identified, such as an example purified from safflour (*Carthamus tinctorius*) which was demonstrated to cleave the *C*-glycosyl linkage of orientin to yield its respective aglycone precursor, luteolin (Saito, 1990). No such enzyme has yet been isolated from bacteria, however several studies have provided detailed information on the mechanism by which the colonic microflora may metabolise *C*-glycosyl flavones present in the lumenal milieu. Zhang *et al.* (2007) observed a decline in total *C*-glycosyl flavone presence in the total gastrointestinal contents of rats from 83 ± 1 % at 30 min to 52 ± 1 % at 12 hr following oral administration of a bamboo leaf extract containing orientin, isoorientin, vitexin and isovitexin. This decline was not accompanied by the presence of these compounds in the urine or plasma of these animals, indicating that absorption was not wholly responsible for this effect, but was allied to increased concentrations of small aromatic products of microbial flavonoid metabolism. The four *C*-glycosyl flavones were shown to undergo significant deglycosylation in both dehydrogenated and native forms, with the former product being further metabolised by C-ring fissure to form small aromatic metabolites such as phloroglucinol, hydrocaffeic acid and phloretic acid in the gastrointestinal tract (Zhang *et al*., 2007). Similarly, human faecal preparations have been shown to catalyse the *C*-deglycosylation of flavonoids. Braune and Blaut (2011) recently identified a strain of the colonic bacterium *Lachnospiraceae* (CG19-1) from human faecal suspensions capable of deglycosylating the dietary *C*-glycosyl flavones vitexin and isoorientin as well as mangiferin, a *C*-glycosyl xanthanoid from mango (*Mangifera indica*), and puerarin (daidzein 8-*C*-β-D-glucoside). This work confirmed and furthered the earlier findings of a number of authors, including Jin *et al.* (2008), who isolated an uncharacterised strain of intestinal bacteria from human faecal cultures capable of deglycosylating puerarin by reductive cleavage. Unlike *Lachnospiraceae* CG19-1 however, this unidentified strain demonstrated high specificity towards puerarin, and was unable to deglycosylate a number of

other natural cyclic *C*-glycosylated structures, including mangiferin. A *Bacteroides* species (sp. MANG) isolated from human faecal cultures has also been shown to deglycosylate mangiferin, producing norathyriol. Interestingly, the *C*-deglycosylating activity was induced by the presence of this xanthanoid (yet native *O*-deglycosylating activity was unaffected) and was not prevented by glucosidase inhibitors such as gluconolactone. The authors cite these findings as evidence that the enzyme or enzyme system responsible for *C*-deglycosylation in the colon expressed by this bacterium may differ significantly from those capable of *O*-deglycosylation (Sanugul *et al*., 2005).

4. Known biological activities of dietary *C*-glycosyl flavonoids

The majority of studies concerning *C*-glycosyl flavonoid bioactivity are principally derived from ethnopharmacological investigations detailing biological modulation and protective effects of plant extracts in cell and animal models. This approach traditionally focuses on the activity of broad plant-derived chemical mixtures, some of which are rich in *C*-glycosyl flavonoids. Whilst it is tempting to include these examples in a review of the activities of such compounds, it is neither useful nor efficient to do so where no attempt has been made to ascribe the defined effect to a particular compound, as structure-function correlations are broadly masked by confounding unknowns in these extracts. As such, this review will principally focus on literature describing the defined bioactivities of enriched or purified *C*-glycosyl flavonoid fractions only. Additionally, direct antioxidant effects described *in vitro* will be omitted due to the lack of significant correlation between *in vitro* antioxidant capacity and *in vivo* plasma antioxidant capacity for some tested *C*-glycosyl flavonoids (Breiter *et al*., 2011; Hollman *et al*., 2011; Mladenka *et al*., 2010).

4.i. Anti-diabetic activity

Interest surrounding the ability of *C*-glycosyl flavonoids to modulate glucose tolerance presumably stems from the hypothesised preservation of the glycosyl moiety *in vivo*, due to its aforementioned stable carbon-carbon glycosyl linkage (Harborne, 1965). Studies concerning the anti-diabetic properties of these compounds have focussed primarily on the modulation of glucose uptake by the key glycogen storage organs (i.e. muscle and liver tissue) and the independent effects on insulin secretion by pancreatic β-cells. These effects were individually characterised in cell cultures *in vitro* by Kawano *et al*. (2008). RIN-5F cells were used as a model of pancreatic β-cells, incubated with or without the *C*-glycosyl

dihydrochalcone aspalathin at 100 μM for 3 hr. Insulin secretion was found to have increased by 30% over this time with no increase in cytotoxicity, however in view of the low bioavailability of aspalathin, the concentration of aspalathin required to elicit this effect appears biologically irrelevant (Courts and Williamson, 2009; Kreuz *et al*., 2008; Stalmach *et al*., 2009). L6 rat myotubes were also cultured to determine the modulation of glucose uptake in muscle cells in the presence of aspalathin. Uptake of glucose (11 mM) present in the culture media was determined by the rate of myocyte-mediated glucose depletion over a 4 hr time-course. In this experiment, the aspalathin concentration required to produce an effect was significantly lower than in the insulin secretion study. Glucose uptake was increased by 24% at 1 μM, and 64% at 10 μM aspalathin ($n = 6$, $p \leq 0.05$).

The combined significance of these findings can be validated in animal models through an oral glucose tolerance test, useful in determining how a fixed dietary glucose load is tolerated by an animal through the measurement of plasma glucose in response to the loading. Chronic or acute doses of a proposed modulating substance are added to the system in experimental groups to observe the change in tolerance of the animal to the acute glucose dose. Two recent studies have defined the specific effects of orally administered dietary *C*-glycosyl flavonoids in rodent models of diabetes. Folador *et al*. (2010) showed the improvement in glucose tolerance following single, acute doses of enriched fractions and isolated compounds from the roots of Taiuiá (*Wilbrandia ebracteata*), a non-dietary Brazilian-native crop that contains swertisin, isoswertisin, vitexin, isovitexin, spinosin, orientin and isoorientin. A butanolic fraction possessed the greatest activity in minimising blood glucose elevation in a Wistar rat model of hyperglycaemia generated through the administration of 4 g kg^{-1} oral glucose to fasted animals via gavage, and compared to a fasted euglycaemic control (nil oral glucose) cohort. Both isovitexin and swertisin were partially purified from the butanolic fraction and identified as the two principal compounds involved in this effect. Both *C*-glycosyl flavones

were active, significantly reducing hyperglycaemia by 18% at a dose of 15 mg kg^{-1} after 30 min ($n = 6$, $p \leq 0.05$). The relevance of this result to the human diet is again limited however due to the high dose used in this study, and therefore may in turn explain a lack of evidence for such an effect in humans.

Isovitexin was also shown to be active in improving the insulin response after glucose administration, although no isovitexin control in the euglycaemic group was monitored. As such, it is uncertain whether effect of isovitexin in this study is simply to non-selectively enhance insulin secretion independently of blood glucose concentrations, or whether it is capable of enhancing glucose sensitivity, improving the overall insulinemic response. Nevertheless, the response in isovitexin-treated animals occurred after 60 min time-course of the experiment, thus improving serum insulin concentrations by 58% ($n = 6$, $p \leq 0.05$). In agreement with this finding, the glycogen content of the soleus muscle in the same hyperglycaemic animals treated with isovitexin was 27% greater than the hyperglycaemic control ($n = 6$, $p \leq 0.05$) (Folador *et al*., 2010).

Kawano *et al*. (2009) demonstrated the effects of chronic oral aspalathin intake on a bd/bd male mouse model of progressive hyperglycaemia. Glucose was administered to the fasted animals by oral gavage, and serum glucose concentrations measured after 2 hr. Six control animals at four weeks old were provided a standard diet containing no aspalathin as a model of type-2 diabetes, whilst a matched test cohort ($n = 4$) was provided an identical diet with the addition of 0.2% w/w aspalathin. No difference was observed in food intake. An almost linear serum glucose concentration increase was shown in control db/db animals over the 5 week duration of the experiment, whilst a significantly lower increase was observed in the aspalathin-fed animals ($n = 4$, $p \leq 0.05$). Prior to the experiment, both groups had blood glucose concentrations of 1.4 g L^{-1}, however aspalathin-fed animals only increased to 4.25 g L^{-1}; 70% of the control increase to 5.5 g L^{-1} glucose (Kawano *et al*., 2009).

It is possible that the mechanism by which these effects are potentiated is via modulation of one or all of the hexokinase isoforms present in the pancreatic β-cells. The low expression of these enzymes in this cell-type allows (under normal conditions) for sensitive detection of blood glucose, as high expression of the glucose transporter GLUT-2 allows for extracellular glucose to closely relate to intracellular concentrations. The rate of hexokinase-catalysed glucose phosphorylation is therefore closely related to the rate of insulin release (Schuit *et al*., 2001). Provided the proposed flavonoid was bioavailable, it is possible that the intact glucose moiety may interact in this pathway, acting as a glucose-type ligand, up-regulating the glycaemic response.

4.ii. Anti-inflammatory activity

Literature detailing the anti-inflammatory effects of *C*-glycosyl flavonoids has only recently emerged, primarily focusing on vicenin-2. Dos Santos *et al*. (2010) used the U-937 macrophage model to study the anti-inflammatory effects of the medicinal plant *Lychnophora ericoides*. Macrophage stimulation with lipopolysaccharide (LPS) was used to generate a controlled immune response, measured by the production of the two inflammatory biomarkers, tumour necrosis factor (TNF) -α and prostaglandin (PG) E-2. Vicenin-2 derived from *Lychnophora ericoides* had a dose-dependent effect on PG E-2 without variation in the expression of cyclooxygenase (COX) -2, the enzyme responsible for prostaglandin synthesis. TNF-α production was unchanged by vicenin-2 in this study, however the work of Shie *et al*. (2010) showed this *C*-glycosyl apigenin-derivative amongst other synthetic examples to inhibit TNF-α expression in LPS-activated Raw264.7 mouse monocyte macrophages (IC_{50} 6.8 ± 2.5 μM). Nitric oxide release as part of the macrophage respiratory burst was also inhibited by viceinin-2 with an IC_{50} of 3.9 ± 0.9 μM.

Vicenin-2 was also recently evaluated alongside vitexin for its role in the anti-nociceptive effects of the medicinal plant, *Urtica circularis*, in inflammation. Intraperitoneal injection of 10 mg kg^{-1} body-weight vitexin produced a 91% reduction in nociception induced by intraperitoneal injection of 1% acetic acid at 10 µL g^{-1} body weight in mice versus an untreated control, as measured by the number of abdominal writhes over the duration of the study (n = 5). The effect of this *C*-glycosyl flavonoid was in excess of the anti-inflammatory pharmaceutical indomethecin, a positive control administered at the same concentration as the flavonoid candidates that non-selectively inhibits the COX family of enzymes (62%). Vicenin-2 also produced anti-nociceptive effects, the number of writhes being 41% lower than the control, supporting the COX-2 inhibitory data of Dos Santos *et al*. (2010) (Gorzalczany *et al*., 2011). De Melo *et al*. (2005) have also shown the activity of both schaftoside and vitexin towards preventing neutrophil influx in a mouse model of lung inflammation induced by inhalation of aerosolised LPS at interperitoneally injected amounts of 400 µg kg^{-1} body-weight, with inhibition of 62% and 80% of control influx respectively. Neutrophil release of hyperchlorous acid ($HOCl_2$) and hydrogen peroxide (H_2O_2) during the inflammatory 'respiratory burst' response to an alien agonist occurs through myeloperoxidase (MPO) activity. Quilez *et al*. (2010) showed that an ethanolic sub-fraction of *Piper carpunya* containing the *C*-glycosyl flavones vitexin, isovitexin, rhamnopyranosylvitexin and isoembigenin significantly reduced MPO activity to 48.2% of a control in an *in vitro* co-suspension of polymorphic nuclear leukocytes (PMNs) and mononuclear cells isolated from male Wistar rats at 50 µg mL^{-1} ($P \leq 0.001$, n = 3). Whilst activity was measured by the oxidation rate of *O*-dianisidine in the flavonoid-containing media over time and therefore may be due to the direct radical-scavenging activity of the flavonoid structure in this *in vitro* system, Zucolotto *et al*. (2009) also showed the MPO inhibitory activity of 25 mL kg^{-1} body

weight interperitoneally injected isoorientin *in vivo* in a carrageenan-induced mouse model of the lung disorder, pleurisy ($P \leq 0.05$, $n = 3$).

4.iii. Anxiolytic effects

Passiflora is a large genus of tropical flowering plants that, whilst not typical components of the human diet themselves (although are used for juice production in Brazil), contain many of the *C*-glycosyl flavones found in dietary sources. Sena *et al*. (2009) uniquely attempted to ascribe some of the reported anxiolytic effects of various extracts of this genus to these flavones. Extracts were administered by intragastric cannula and effects on the performance of 3 month-old Swiss mice in a light-dark transition (LDT) were observed. A butanolic extract containing a high concentration of isoorientin, vicenin-2, 6,8-di-*C*-glycosyl chrysin and spinosin administered at 25, 50 and 100 mg kg^{-1} body weight all produced a significant decrease in baseline anxiety as measured by an increase in time spent in the light compartment of the LDT test ($P \leq 0.01$), mimicking the effects of the positive control, diazepam. This extract also significantly increased the number of transitions between the light and dark compartments of the test apparatus ($P \leq 0.01$). Interestingly, an aqueous extract containing only isoorientin did not produce such effects versus the untreated controls. Isoorientin alone therefore had no anxiolytic effects in this experiment, however both extracts also containing vicenin-2, 6,8-di-*C*-glycosyl chrysin and spinosin appeared to improve baseline anxiety in the mouse model.

This data is supported by the earlier work of Dhawan *et al*. (2001), eliminating chrysin, apigenin, quercetin, hesperidin and orientin as candidate mediators of this effect by adopting a progressive fractionation of *Passiflora incarnata* to isolate a fraction found to elicit the greatest anxiolytic activity using an elevated plus-maze model of anxiety in Swiss mice. The

final fraction contained β-sitosterol and a single unidentified flavone (Dhawan *et al*., 2001) which may therefore be either vicenin-2 or spinosin.

4.iv. Antispasmodic effects

Two studies have demonstrated the antispasmodic effects of *C*-glycosyl flavonoids from non-dietary herbal extracts on tissues from rodents. Both studies used similar force transducer apparatus to monitor the responses of isolated segments of various tissues after treatment with isolated compounds *ex vivo*, leading to the attribution of effect to a *C*-glycosyl flavonoid in both cases. Afifi *et al*. (1999) treated isolated rat aorta, ileum, trachea, uterus and guinea-pig uterus with orientin purified from *Arum palaestinum*. Despite previous work by Abdalla *et al*. (1994) showing the antispasmodic effect of the orientin aglycone, luteolin, on spontaneously contracting illeal tissue, and epinephrine or carbachol-induced aortic and tracheal tissue contraction, addition of the 8-*C*-glycosyl group appeared to abolish the activity of this flavone towards these tissues in this study. However, the amplitude of spontaneous contraction of both rat and guinea-pig uterus tissue was diminished through treatment with a range of orientin concentrations between 100 nM and 600 µM, giving IC_{50} values of 205 ± 99 µM in rat tissue (n = 7) and 57 ± 20 µM in guinea-pig tissue (n = 8). The frequency of muscular contraction was also attenuated in a dose-dependent manner (Afifi *et al*., 1999). Ragone *et al*. (2007) provided greater mechanistic evidence for effect of the dietary *C*-glycosyl flavone vitexin, but not its isomer isovitexin, isolated from the herbal plant *Aloysia citriodora* on attenuating induced rat duodenum contraction. Both acetylcholine and Ca^{2+} dose-response curves were employed to potentiate contraction of the smooth muscle within this tissue. Increasing extracellular Ca^{2+} concentrations caused a dose-dependent extracellular release of acetylcholine, which has the downstream effect of polarising the muscle fibre and eventually causing a contractile response. Whilst both agonists play different but dose-

dependent roles in the same pathway of contraction, the use of a dose-response curve to both of these reagents allows mechanistic elucidation of flavonoid effect mediation. Vitexin non-competitively inhibited the acetylcholine dose-response to give a maximal inhibition of 69.4 ± 0.9 µM at 48.6 ± 12.0% of the E_{max} of acetylcholine (n = 5). However isovitexin, the 6-*C*-glycosylated isomer of vitexin, had no significant activity on the effect of acetylcholine at 69 µM (81.4 ± 9.5% of E_{max} of acetylcholine,) indicating that the position of *C*-glycosylation may be crucial in producing this effect. Interestingly, despite the crude extract of *Aloysia citriodora* from which these compounds were isolated being capable of so doing, neither compound had a significant effect on the tissue response to increasing extracellular Ca^{2+} concentrations at 46.3 µM (Ragone *et al*., 2007). Vitexin therefore has been shown to non-competitively inhibit the activity of acetylcholine in potentiating smooth muscle contraction in rat duodenum, thus demonstrating its capability to produce a significant antispasmodic effect in the gastrointestinal tract at a high but lumenally relevant concentration (69.4 µM).

4.v. Hepatoprotection

Despite intriguing evidence of efficacy, the hepatoprotective effects of specific *C*-glycosyl flavonoids have been little studied. Hoffmannh-Bohm *et al*. (1992) first discussed the important correlation between the position of flavonoid *C*-glycosylation and hepatoprotective effects *in vitro*. Extracts of *Allophyllus edulis*, a Paraguayan herb traditionally used in the treatment of jaundice, was studied in a carbon tetrachloride (CCl_4) model of hepatic damage using cultured primary rat hepatocytes. Isolated 8-*C*-glycosylated flavones (vitexin and orientin) from these extracts showed hepatotoxic effects, whilst the 6-*C*-glycosylated flavone isovitexin protected the hepatocyte culture following CCl_4 treatment. Further investigation revealed that addition of a rhamnose group to the existing *C*-glycosidic moiety reversed the

effect of the 8-*C*-glycosides. The effect of isovitexin was also enhanced by substitution at the free 7 hydroxyl group, in this case by an *O*-methyl or *O*-glucosyl moiety.

This structure-function information is in agreement with the *in vitro* study of Orhan *et al*. (2003), investigating the effects of phenolic compounds from the Turkish folk remedial herb *Gentiana olivieri*, including the 6-*C*-glycosyl flavone isoorientin, on single dose CCl_4-induced liver damage in Sprague-Dawley rats. Sub-acute oral administration of 15 mg kg^{-1} body-weight isoorientin in 0.5% aqueous carboxymethyl cellulose (CMC) occurred daily for 5 days prior to a single dose of 50% (v/v) CCl_4 in liquid paraffin at 2.5 mL kg^{-1} body-weight to induce liver damage, and samples were harvested for analysis 60 min following CCl_4 administration. Isoorientin appeared to normalise liver tissue malondialdehyde (MDA) compared to a negative control group that did not undergo CCl_4 treatment but was fed only blank CMC (control, 341.9 ± 13.6 nmol g^{-1}; isoorientin treated, 340.4 ± 17.5 nmol g^{-1}). MDA concentrations were found to be significantly improved in both of these groups in comparison to a positive control group that underwent CCl_4 treatment and was fed blank CMC (578.0 ± 39.5 nmol g^{-1}, $p \leq 0.01$, $n = 6$). Accordingly, plasma MDA concentrations were also significantly decreased in the isoorientin-treated cohort versus the positive control, although modulation did not reach the control baseline in this case ($p \leq 0.01$, $n = 6$). Hepatic glutathione GSH concentrations also remained at the level of the control after isoorientin treatment (control, 15.3 ± 1.3 µmol g^{-1}; isoorientin treated, 17.1 ± 1.2 µmol g^{-1}), again giving significance versus the positive control (6.2 ± 1.2 µmol g^{-1}, $p \leq 0.001$, $n = 6$). Transaminase markers of liver function were also improved to –75% and –80% of the positive control respectively ($p \leq 0.001$, $n = 6$). Taken together, these data indicate a significant decrease in oxidative stress *in vivo*, possibly via reduction in trichloromethyl radical generation, the major mechanism of hepatic damage following CCl_4 administration. Hepatic histological examination revealed that many parameters were unchanged between the control and

isoorientin-treated animals, whereas severe or moderate damage was observed in the positive control. Indeed, central vein and sinusoid congestion were both improved by isoorientin-treated animals versus the untreated control group. Interestingly however, isoorientin treatment did not decrease the mild lymphocyte infiltration or Kupffer cell proliferation observed in the positive control group.

Despite the conclusion of these authors to the contrary, the remarkably diverse hepatic effects of structurally similar *C*-glycosyl flavones suggest that radical scavenging is not a primary mechanism of activity in the two studies outlined here. This is an assertion that is supported by the poor predicted bioavailability of these compounds, discussed in section 3.

4.vi. Other biological effects

Snijman *et al*. (2007) demonstrated the moderate antimutagenic effects of isolated *C*-glycosyl flavonoids from rooibos tea. Two model systems were employed to study the inhibition or stimulation of mutagenicity in metabolically activated tester strains *Salmonella typhimurium* TA98 and TA100 induced by 2-acetamido-fluorene (2-AAF) and aflatoxin B_1 (AFB_1) respectively. The *C*-glycosylated flavonoids tested were aspalathin, nothofagin, orientin, isoorientin, vitexin and isovitexin. With the exceptions of vitexin and isovitexin, all compounds exhibited antimutagenic effects in both systems. Isovitexin (0.8 mM) showed a significant 17 ± 10% increase in the mutagenic response of T98 to 2-AAF compared to an untreated control, as measured by the number of His^+ revertants (i.e. the number of mutants reverting back to the previous genotype) ($p \leq 0.05$, $n = 5$). This was in stark contrast to the antimutagenic effect of the same compound in the T100 tester strain following AFB_1 treatment. A significant dose-dependent response was recorded, with a 0.8 mM concentration giving a 62 ± 5% relative reduction in revertant numbers, falling to 50 ± 3% at 0.4 mM and 37 ± 4% at 0.08 mM ($p \leq 0.05$, $n = 5$). Nothofagin presented a similar dose-dependent profile

of antimutagenicity towards AFB_1 in this assay (59 ± 3% at 0.8 mM), and whilst a shallower curve of effect was observed, the nothofagin analogue aspalathin gave a similar low-concentration effect at 0.08 mM (aspalathin, 33 ± 3%; nothofagin, 33 ± 8%, N.S.). The antimutagenic effects of these *C*-glycosyl dihydrochalcones also were demonstrated in the T98 : 2-AAF model, however as with orientin and isoorientin, a lack of dose-response was observed. These two isomeric flavones also exhibited a similar pattern of antimutagenicity in the T100 : AFB_1 assay to the vitexin isomers (orientin, 59 ± 3%; vitexin, 51 ± 4%; isoorientin, 66 ± 10%; isovitexin 62 ± 5% at 0.8 mM).

A human umbilical vein endothelial cell (HUVEC) line was cultured by Miyake *et al*. (2007) to study the effects of two di-*C*-glycosyl flavones, diosmetin 6,8-di-*C*-glucoside and vicenin-2, isolated from citrus fruit, on inter-cellular adhesion molecule-1 (ICAM-1) expression after stimulation by TNF-α (10 ng mL^{-1}). ICAM-1 is a cell adhesion protein expressed on the surface of endothelial and immune system cells, allowing for the adhesion of these two cell types, a process that precedes migration of the immune cell (i.e. leukocyte) into the endothelium. Overexpression of this protein has, however, been associated with atherosclerotic lesion and plaque formation. Whilst 10 μM diosmetin 6,8-di-*C*-glucoside had no significant effect on the expression of ICAM-1, vicenin-2 demonstrated a significant reduction at this concentration (85 ± 3% activity versus control, $p \leq 0.05$, $n = 4$), although a lower concentration (1 μM) had no significant effect.

5. Conclusions

The *C*-glycosyl flavonoids have received little concerted attention in the literature with regard to human nutrition, as the majority of data relating to biological activity arises from ethnopharmacological investigations of various traditional medicinal plants. This lack of consideration from a nutritional standpoint does not appear to reflect the potential of this interesting class of compounds from the diet to confer novel biological effects, as the hydrolysis-resistant *C*-glycosidic linkage appears to uniquely result in the circulation of intact glycosyl flavonoids *in vivo*. The influence of an intact glycosyl moiety on the bioactivity of a given polyphenolic structure *in vivo* therefore requires much further consideration. No attempt has yet been made to separate highly-diverse known biological effects of aglycone flavonoids from those exhibiting *C*-glycosylation, however in view of the biological significance of hexose sugars, the potential for intact glycosylated flavonoids to confer biological effects via this putative pharmacophore appears substantial. The true significance may only be truly elucidated by employing matched aglycone and *C*-glycosylated standards in all investigations where practicable. This is becoming an increasingly possibility with the commercial availability of an ever-expanding range of diverse phytochemicals and the publication of a relatively simple chemical means by which aglycone polyphenols may be efficiently *C*-glycosylated (Hasslauer *et al*., 2010; Stark *et al*., 2007). Further pharmacokinetic studies are also required for these compounds, as limited data suggests low bioavailability, likely by virtue of the intact glycosyl group.

6. References

Abad-Garcia, B., Garmon-Lobato, S., Berrueta, L. A., Gallo, B., and Vicente, F. (2008). New features on the fragmentation and differentiation of *C*-glycosidic flavone isomers by positive electrospray ionization and triple quadrupole mass spectrometry. *Rapid Communications in Mass Spectrometry*. **22**(12):1834-1842.

Abdalla, S., Zarga, M. A., and Sabri, S. (1994). Effects of the flavone luteolin, isolated from *Colchicum richii*, on guinea-pig isolated smooth muscle and heart and on blood pressure and blood flow. *Phytotherapy Research*. **8**(5):265-270.

Afifi, F. U., Khalil, E., and Abdalla, S. (1999). Effect of isoorientin isolated from *Arum palaestinum* on uterine smooth muscle of rats and guinea pigs. *Journal of Ethnopharmacology*. **65**(2):173-177.

Akinbala, J. O. (1991). Effect of processing on flavonoids in millet (*Pennisetum americanum*) flour. *Cereal Chemistry*. **68**(2):189-183.

Arab, L., and Liebeskind, D. S. (2010). Tea, flavonoids and stroke in man and mouse. *Archives of Biochemistry and Biophysics*. **501**(1):31-36.

Baldi, A., Rosen, R. T., Fukuda, E. K., and Ho, C.-T. (1995). Identification of nonvolatile components in lemon peel by high-performance liquid chromatography with confirmation by mass spectrometry and diode-array detection. *Journal of Chromatography A*. **718**(1):89-97.

Bramati, L., Aquilano, F., and Pietta, P. (2003). Unfermented rooibos tea: quantitative characterization of flavonoids by HPLC-UV and determination of the total antioxidant activity. *Journal of Agricultural and Food Chemistry*. **51**(5):7472-7474.

Bramati, L., Minoggio, M., Gardana, C., Simonetti, P., Mauri, P., and Pietta, P. (2002). Quantitative characterization of flavonoid compounds in Rooibos tea (*Aspalathus linearis*) by LC-UV/DAD. *Journal of Agricultural and Food Chemistry*. **50**(20):5513-5519.

Braune, A., and Blaut, M. (2011). Deglycosylation of puerarin and other aromatic *C*-glucosides by a newly isolated human intestinal bacterium. *Environmental Microbiology*. **13**(2):482-494.

Brazier-Hicks, M., Evans, K. M., Gershater, M. C., Puschmann, H., Steel, P. G., and Edwards, R. (2009). The *C*-glycosylation of flavonoids in cereals. *Journal of Biological Chemistry*. **284**:17926-17934.

Breiter, T., Laue, C., Kressel, G., Gröll, S., Engelhardt, U. H., and Hahn, A. (2011). Bioavailability and antioxidant potential of rooibos flavonoids in humans following the consumption of different rooibos formulations. *Food Chemistry*. **128**(2):338-347.

Caristi, C., Bellocco, E., Gargiulli, C., Toscano, G., and Leuzzi, U. (2006). Flavone-di-*C*-glycosides in citrus juices from Southern Italy. *Food Chemistry*. **95**(3):431-437.

Cavaliere, C., Foglia, P., Pastorini, E., Samperi, R., and Laganà, A. (2005). Identification and mass spectrometric characterization of glycosylated flavonoids in *Triticum durum* plants by

high-performance liquid chromatography with tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*. **19**(21):3143-3158.

Colombo, R., Yariwake, J. H., and McCullagh, M. (2008). Study of *C*- and *O*-glycosylflavones in sugarcane extracts using liquid chromatography - Exact mass measurement mass spectrometry. *Journal of the Brazilian Chemical Society*. **19**(3):483-490.

Colombo, R., Yariwake, J. H., Queiroz, E. F., Ndjoko, K., and Hostettmann, K. (2006). On-line identification of further flavone *C*- and *O*-glycosides from sugarcane (*Saccharum officinarum* L. Gramineae) by HPLC-UV-MS. *Phytochemical Analysis*. **17**(5):337-343.

Courts, F. L., and Williamson, G. (2009). The C-glycosyl flavonoid, aspalathin, is absorbed, methylated and glucuronidated intact in humans. *Molecular Nutrition & Food Research*. **53**(9):1104-1111.

Day, A. J., Cañada, F. J., Diaz, J. C., Kroon, P. A., McLauchlan, R., Faulds, C. B., Plumb, G. W., Morgan, M. R., and Williamson, G. (2000). Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Letters*. **468**(2-3):166-170.

Day, A. J., DuPont, M. S., Ridley, S., Rhodes, M., Rhodes, M. J. C., Morgan, M. R. A., and Williamson, G. (1998). Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS Letters*. **436**(1):71-75.

De Melo, G. O., Muzitano, M. F., Legora-Machado, A., Almeida, T. A., De Oliveira, D. B., Kaiser, C. R., Koatz, V. L. G., and Costa, S. n. S. (2005). *C*-Glycosylflavones from the aerial parts of *Eleusine indica* inhibit LPS-induced mouse lung inflammation. *Planta Medica*. **71**(4):362-363.

Dhawan, K., Kumar, S., and Sharma, A. (2001). Anti-anxiety studies on extracts of *Passiflora incarnata* Linneaus. *Journal of Ethnopharmacology*. **78**(2-3):165-170.

Dietrych-Szostak, D., and Oleszek, W. (1999). Effect of processing on the flavonoid content in buckwheat (*Fagopyrum esculentum* Möench) grain. *Journal of Agricultural and Food Chemistry*. **47**(10):4384-4387.

dos Santos, M. D., Chen, G. J., Almeida, M. C., Soares, D. M., de Souza, G. E. P., Lopes, N. P., and Lantz, R. C. (2010). Effects of caffeoylquinic acid derivatives and *C*-flavonoid from *Lychnophora ericoides* on *in vitro* inflammatory mediator production. *Natural Product Communications*. **5**(5):733-740.

Erlund, I. (2004). Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research*. **24**(10):851-874.

Feng, X., Jiang, D., Shan, Y., Dai, T. B., Dong, Y. F., and Cao, W. X. (2008). New flavonoid-*C*-glycosides from *Triticum aestivum*. *Chemistry of Natural Compounds*. **44**(2):171-173.

Feng, Y., and McDonald, C. E. (1987). *C*-Glycosyl flavonoid isolated from wheat bran. *Cereal Foods World*. **32**:652.

Feng, Y., McDonald, C. E., and Vick, B. A. (1988). *C*-Glycosylflavones from hard red spring wheat bran. *Cereal Chemistry*. **65**:452-456.

Ferreres, F., Gil-Izquierdo, A., Andrade, P. B., Valentao, P., and Tomas-Barberan, F. A. (2007). Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*. **1161**(1-2):214-223.

Ferreres, F., Krskova, Z., Goncalves, R. F., Valentao, P., Pereira, J. A., Dusek, J., Martin, J., and Andrade, P. B. (2009). Free water-soluble phenolics profiling in barley (*Hordeum vulgare* L.). *Journal of Agricultural and Food Chemistry*. **57**(6):2405-2409.

Folador, P., Cazarolli, L. H., Gazola, A. C., Reginatto, F. H., Schenkel, E. P., and Silva, F. R. M. B. (2010). Potential insulin secretagogue effects of isovitexin and swertisin isolated from *Wilbrandia ebracteata* roots in non-diabetic rats. *Fitoterapia*. **81**(8):1180-1187.

Franz, G., and Grun, M. (1983). Chemistry, occurrence and biosynthesis of *C*-glycosyl compounds in plants. *Planta Medica*. **47**(3):131-140.

Gallardo, C., Jiménez, L., and García-Conesa, M. T. (2006). Hydroxycinnamic acid composition and *in vitro* antioxidant activity of selected grain fractions. *Food Chemistry*. **99**(3):455-463.

Garg, A., Garg, S., Zaneveld, L. J. D., and Singla, A. K. (2001). Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytotherapy Research*. **15**(8):655-669.

Gil-Izquierdo, A., Gil, M. I., Ferreres, F., and Tomas-Barberan, F. A. (2001). *In vitro* availability of flavonoids and other phenolics in orange juice. *Journal of Agricultural and Food Chemistry*. **49**(2):1035-1041.

Gil-Izquierdo, A., Riquelme, M. a. T., Porras, I., and Ferreres, F. (2003). Effect of the rootstock and interstock grafted in lemon tree (*Citrus limon* (L.) Burm.) on the flavonoid content of lemon juice. *Journal of Agricultural and Food Chemistry*. **52**(2):324-331.

Gil, M. I., Ferreres, F., and Tomas-Barberan, F. A. (1998). Effect of modified atmosphere packaging on the flavonoids and vitamin C content of minimally processed Swiss chard (*Beta vulgaris* subspecies cycla). *Journal of Agricultural and Food Chemistry*. **46**(5):2007-2012.

González-Molina, E., Domínguez-Perles, R., Moreno, D. A., and García-Viguera, C. (2010). Natural bioactive compounds of *Citrus limon* for food and health. *Journal of Pharmaceutical and Biomedical Analysis*. **51**(10):327-345.

Gorzalczany, S., Marrassini, C., Miño, J., Acevedo, C., and Ferraro, G. (2011). Antinociceptive activity of ethanolic extract and isolated compounds of *Urtica circularis*. *Journal of Ethnopharmacology*. **134**(3):733-738.

Harborne, J. B. (1965). Plant polyphenols-XIV. : Characterization of flavonoid glycosides by acidic and enzymic hydrolyses. *Phytochemistry*. **4**(1):107-120.

Hasslauer, I., Oehme, A., Locher, S., Valotis, A., van't Slot, G., Humpf, H.-U., and Schreier, P. (2010). Flavan-3-ol *C*-glycosides – Preparation and model experiments mimicking their human intestinal transit. *Molecular Nutrition & Food Research*. **54**(11):1546-1555.

Hatano, T., Miyatake, H., Natsume, M., Osakabe, N., Takizawa, T., Ito, H., and Yoshida, T. (2002). Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. *Phytochemistry*. **59**(7):749-758.

Hays, W. S., Jenison, S. A., Yamada, T., Pastuszyn, A., and Glew, R. H. (1996). Primary structure of the cytosolic β-glucosidase of guinea pig liver. *Biochemical Journal*. **319**(3):829-837.

Hegnauer, R., and J. Gpayer-Barkmeijer, R. (1993). Relevance of seed polysaccharides and flavonoids for the classification of the *leguminosae*: A chemotaxonomic approach. *Phytochemistry*. **34**(3):3-16.

Hirvonen, T., Virtamo, J., Korhonen, P., Albanes, D., and Pietinen, P. (2000). Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers. *Stroke*. **31**(10):2301-2306.

Hoffmann-Bohm, K., Lotter, H., Seligmann, O., and Wagner, H. (1992). Antihepatotoxic C-glycosylflavones from the leaves of *Allophyllus edulis* var. edulis and gracilis. *Planta Medica*. **58**(6):544-548.

Hollman, P. C. H., Bijsman, M. N. C. P., van Gameren, Y., Cnossen, E. P. J., de Vries, J. H. M., and Katan, M. B. (1999). The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Research*. **31**(6):569-573.

Hollman, P. C. H., Cassidy, A., Comte, B., Heinonen, M., Richelle, M., Richling, E., Serafini, M., Scalbert, A., Sies, H., and Vidry, S. p. (2011). The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. *The Journal of Nutrition*. **141**(5):989S-1009S.

Hollman, P. C. H., and Katan, M. B. (1997). Absorption, metabolism and health effects of dietary flavonoids in man. *Biomedicine & Pharmacotherapy*. **51**(8):305-310.

Hultin, P. G. (2005). Bioactive *C*-glycosides from bacterial secondary metabolism. *Current Topics in Medicinal Chemistry*. **5**(14):1299-1331.

Jin, J.-S., Nishihata, T., Kakiuchi, N., and Hattori, M. (2008). Biotransformation of *C*-glucosylisoflavone puerarin to estrogenic (3S)-equol in co-culture of two human intestinal bacteria. *Biological & Pharmaceutical Bulletin*. **31**(8):1621-1625.

Joubert, E. (1996). HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing. *Food Chemistry*. **55**(4):403-411.

Joubert, E., and deVilliers, O. T. (1997). Effect of fermentation and drying conditions on the quality of rooibos tea. *International Journal of Food Science and Technology*. **32**(2):127-134.

Joubert, E., Richards, E. S., Merwe, J. D., De Beer, D., Manley, M., and Gelderblom, W. C. (2008). Effect of species variation and processing on phenolic composition and *in vitro* antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). *Journal of Agricultural and Food Chemistry.* **56**(3):954-963.

Kawano, A., Nakamura, H., Hata, S., Minakawa, M., Miura, Y., and Yagasaki, K. (2009). Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. *Phytomedicine*. **16**(5):437-443.

Kazuno, S., Yanagida, M., Shindo, N., and Murayama, K. (2005). Mass spectrometric identification and quantification of glycosyl flavonoids, including dihydrochalcones with neutral loss scan mode. *Analytical Biochemistry*. **347**(2):182-192.

King, H. G. C. (1962). Phenolic compounds of commercial wheat germ. *Journal of Food Science*. **27**(5):446-454.

Koeppen, B. H., and Roux, D. G. (1965). *C*-Glycosylflavonoids – chemistry of orientin and iso-orientin. *Biochemical Journal*. **97**(2):444-448.

Koeppen, B. H., and Roux, D. G. (1966). *C*-Glycosylflavonoids. The chemistry of aspalathin. *Biochemical Journal.* **99**(3):604-609.

Krafczyk, N., and Glomb, M. A. (2008). Characterization of phenolic compounds in rooibos tea. *Journal of Agricultural and Food Chemistry*. **56**(9):3368-3376.

Krafczyk, N., Kotke, M., Lehnert, N., and Glomb, M. (2008). Phenolic composition of rhubarb. *European Food Research and Technology*. **228**(2):187-196.

Kreuz, S., Joubert, E., Waldmann, K.-H., and Ternes, W. (2008). Aspalathin, a flavonoid in *Aspalathus linearis* (rooibos), is absorbed by pig intestine as a *C*-glycoside. *Nutrition Research*. **28**(10):690-701.

Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008). Glycosyltransferases: structures, functions, and mechanisms. *Annual Review of Biochemistry*. **77**:521-555.

Ma, Z., Wu, Q., Lee, D. Y. W., Tracy, M., and Lukas, S. E. (2005). Determination of puerarin in human plasma by high performance liquid chromatography. *Journal of Chromatography B*. **823**(2):108-114.

Manach, C., Scalbert, A., Morand, C., Rãmãsy, C., and Jimãnez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*. **79**(5):727-747.

Manley, M., Joubert, E., and Botha, M. (2006). Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus linearis*) by means of near infrared spectroscopy. *Journal of near Infrared Spectroscopy*. **14**(4):213-222.

Manthey, J. A. (2000). Biological properties of flavonoids pertaining to inflammation. *Microcirculation*. **7**(6):S29-S34.

Marais, C., van Rensburg, W. J., Ferreira, D., and Steenkamp, J. A. (2000). (S)- and (R)-eriodictyol-6-*C*-beta-D-glucopyranoside, novel keys to the fermentation of rooibos (*Aspalathus linearis*). *Phytochemistry*. **55**(1):43-49.

Marotti, M., and Piccaglia, R. (2002). Characterization of flavonoids in different cultivars of onion (*Allium cepa* L.). *Journal of Food Science*. **67**(3):1229-1232.

Mink, P. J., Scrafford, C. G., Barraj, L. M., Harnack, L., Hong, C. P., Nettleton, J. A., and Jacobs, D. R., Jr. (2007). Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *American Journal of Clinical Nutrition*. **85**(3):895-909.

Miyake, Y., Yamamoto, K., Morimitsu, Y., and Osawa, T. (1997). Isolation of *C*-glucosylflavone from lemon peel and antioxidative activity of flavonoid compounds in lemon fruit. *Journal of Agricultural and Food Chemistry*. **45**(3):4619-4623.

Mladenka, P., Zatloukalová, L., Filipský, T., and Hrdina, R. (2010). Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radical Biology and Medicine*. **49**(6):963-975.

Mullen, W., Boitier, A., Stewart, A. J., and Crozier, A. (2004). Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by liquid chromatography with photodiode array and full scan tandem mass spectrometric detection. *Journal of Chromatography A*. **1058**(1-2):163-168.

Nicolle, E., Souard, F., Faure, P., and Boumendjel, A. (2011). Flavonoids as promising lead compounds in type 2 diabetes mellitus: molecules of interest and structure-activity relationship. *Current Medicinal Chemistry*. **18**(17):2661-2672.

Orhan, D. D., Aslan, M., Aktay, G. k., Ergun, E., Yesilada, E., and Ergun, F. (2003). Evaluation of hepatoprotective effect of *Gentiana olivieri* herbs on subacute administration and isolation of active principle. *Life Sciences*. **72**(20):2273-2283.

Pengilly, M., Joubert, E., van Zyl, W. H., Botha, A., and Bloom, M. (2008). Enhancement of rooibos (*Aspalathus linearis*) aqueous extract and antioxidant yield with fungal enzymes. *Journal of Agricultural and Food Chemistry*. **56**(11):4047-4053.

Quílez, A., Berenguer, B., Gilardoni, G., Souccar, C., de Mendonça, S., Oliveira, L. F. S., Martín-Calero, M. J., and Vidari, G. (2010). Anti-secretory, anti-inflammatory and anti-*Helicobacter pylori* activities of several fractions isolated from *Piper carpunya* Ruiz & Pav. *Journal of Ethnopharmacology*. **128**(3):583-589.

Rabe, C., Steenkamp, J. A., Joubert, E., Burger, J. F. W., and Ferreira, D. (1994). Phenolic metabolites from rooibos tea (*Aspalathus-linearis*). *Phytochemistry*. **35**(6):1559-1565.

Ragone, M. I., Sella, M., Conforti, P., Volonté, M. G., and Consolini, A. E. (2007). The spasmolytic effect of *Aloysia citriodora*, Palau (South American cedrón) is partially due to its vitexin but not isovitexin on rat duodenums. *Journal of Ethnopharmacology*. **113**(2):258-266.

Ramarathnam, N., Osawa, T., Namiki, M., and Kawakishi, S. (1989). Chemical studies on novel rice hull antioxidants. 2. Identification of isovitexin, a *C*-glycosyl flavonoid. *Journal of Agricultural and Food Chemistry*. **37**(2):316-319.

Ren, F., Jing, Q., Shen, Y., Ma, H., and Cui, J. (2006). Quantitative determination of puerarin in dog plasma by HPLC and study on the relative bioavailability of sustained release tablets. *Journal of Pharmaceutical and Biomedical Analysis*. **41**(2):549-553.

Saito, K. (1990). Enzyme-catalyzed cleavage of the *C*-glycosidic linakage to the aromatic ring-A of a 3',4',5'7-tetrahydroxyflavone 8-*C*-glycoside. *Biochimica et Biophysica Acta*. **1035**(3):340-347.

Sanugul, K., Akao, T., Li, Y., Kakiuchi, N., Nakamura, N., and Hattori, M. (2005). Isolation of a human intestinal bacterium that transforms mangiferin to norathyriol and inducibility of the enzyme that cleaves a *C*-glucosyl bond. *Biological & Pharmaceutical Bulletin*. **28(9)**:1672-1678.

Scalbert, A., and Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition*. **130**(8S):2073S-2085S.

Schmandke, H. (2005). Antioxidants in rooibos tea: dihydrochalcones and flavonoids. *Ernahrungs-Umschau*. **52**(1):18-23.

Schuit, F. C., Huypens, P., Heimberg, H., and Pipeleers, D. G. (2001). Glucose sensing in pancreatic β-cells. *Diabetes*. **50**(1):1-11.

Schulz, H., Joubert, E., and Schutze, W. (2003). Quantification of quality parameters for reliable evaluation of green rooibos (Aspalathus linearis). *European Food Research and Technology*. **216**(6):539-543.

Sena, L. M., Zucolotto, S. M., Reginatto, F. H., Schenkel, E. P., and De Lima, T. C. M. (2009). Neuropharmacological activity of the pericarp of *Passiflora edulis* flavicarpa degener: putative involvement of *C*-glycosylflavonoids. *Experimental Biology and Medicine.* **234(8)**:967-975.

Shen, J., Meng, Z., Su, H., Xing, D., Ding, Y., and Du, L. (2007). Different kinetics of puerarin in plasma of normal and depressed rats after oral administration of Chinese medicine TZ18. *Tsinghua Science & Technology*. **12**(4):394-399.

Shie, J. J., Chen, C. A., Lin, C. C., Ku, A. F., Cheng, T. J. R., Fang, J. M., and Wong, C. H. (2010). Regioselective synthesis of di-*C*-glycosylflavones possessing anti-inflammation activities. *Organic & Biomolecular Chemistry*. **8**(19):4451-4462.

Singh, R., Akhtar, N., and Haqqi, T. M. (2011). Green tea polyphenol epigallocatechin-3-gallate: inflammation and arthritis. *Life Science*. **86**(26):907-918.

Slimestad, R., Fossen, T., and Verheul, M. J. (2008). The flavonoids of tomatoes. *Journal of Agricultural and Food Chemistry*. **56**(7):2436-2441.

Snijman, P. W., Swanevelder, S., Joubert, E., Green, I. R., and Gelderblom, W. C. A. (2007). The antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*): some dose-response effects on mutagen activation-flavonoid interactions. *Mutation Research / Genetic Toxicology and Environmental Mutagenesis*. **631**(2):111-123.

Stalmach, A., Mullen, W., Pecorari, M., Serafini, M., and Crozier, A. (2009). Bioavailability of *C*-linked dihydrochalcone and flavanone glucosides in humans following ingestion of unfermented and fermented rooibos teas. *Journal of Agricultural and Food Chemistry*. **57**(15):7104-7111.

Standley, L., Winterton, P., Marnewick, J. L., Gelderblom, W. C., Joubert, E., and Britz, T. J. (2001). Influence of processing stages on antimutagenic and antioxidant potentials of rooibos tea. *Journal of Agricultural and Food Chemistry*. **49**(1):114-117.

Stark, T., and Hofmann, T. (2006). Application of a molecular sensory science approach to alkalized cocoa (*Theobroma cacao*): structure determination and sensory activity of nonenzymatically *C*-glycosylated flavan-3-ols. *Journal of Agricultural and Food Chemistry*. **54**(25):9510-9521.

Stark, T., Keller, D., Wenker, K., Hillmann, H., and Hofmann, T. (2007). Nonenzymatic *C*-glycosylation of flavan-3-ols by oligo- and polysaccharides. *Journal of Agricultural and Food Chemistry*. **55**(23):9685-9697.

Steinmetz, K. A., and Potter, J. D. (1996). Vegetables, fruit, and cancer prevention: a review. *Journal of the American Dietetic Association*. **96**(10):1027-1039.

Stevens, J. F., and Page, J. E. (2004). Xanthohumol and related prenylflavonoids from hops and beer: to your good health! *Phytochemistry*. **65**(10):1317-1330.

Tomás-Barberán, F. A., and Clifford, M. N. (2000). Flavanones, chalcones and dihydrochalcones – nature, occurrence and dietary burden. **80**(7):1073-1080.

vonGadow, A., Joubert, E., and Hansmann, C. F. (1997). Effect of extraction time and additional heating on the antioxidant activity of rooibos tea (*Aspalathus linearis*) extracts. *Journal of Agricultural and Food Chemistry*. **45**(4):1370-1374.

Wagner, H., Obermeier, G., Chari, V. M., and Galle, K. (1980). Flavonoid-*C*-glycosides from *Triticum aestivum* L. *Journal of Natural Products*. **43**(5):583-587.

Walgren, R. A., Lin, J.-T., Kinne, R. K.-H., and Walle, T. (2000). Cellular uptake of dietary flavonoid quercetin β-glucoside by sodium-dependent glucose transporter SGLT1. *Journal of Pharmacology and Experimental Therapeutics*. **294**(3):837-843.

Wallace, J. W., and Alston, R. E. (1966). *C*-Glycosylation of flavonoids. *Plant and Cell Physiology*. **7**(4):699-700.

Walle, T., Browning, A. M., Steed, L. L., Reed, S. G., and Walle, U. K. (2005). Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans. *The Journal of Nutrition*. **135**(1):48-52.

Watanabe, M. (1998). Catechins as antioxidants from buckwheat (*Fagopyrum esculentum* Möench) groats. *Journal of Agricultural and Food Chemistry*. **46**(3):839-845.

Yun, J. H., Tomas-Barberan, F. A., Kader, A. A., and Mitchell, A. E. (2006). The flavonoid glycosides and procyanidin composition of deglet noor dates (*Phoenix dactylifera*). *Journal of Agricultural and Food Chemistry*. **54**(6):2405-2411.

Zhang, Y., Tie, X., Bao, B., Wu, X., and Zhang, Y. (2007). Metabolism of flavone *C*-glucosides and p-coumaric acid from antioxidant of bamboo leaves (AOB) in rats. *British Journal of Nutrition*. **97**(3):484-494.

Zucolotto, S. M., Goulart, S., Montanher, A. B., Reginatto, F. v. H., Schenkel, E. P., and Frãde, T. n. S. (2009). Bioassay-guided isolation of anti-inflammatory *C*-glucosylflavones from *Passiflora edulis*. *Planta Medica*. **75**:1221-1226.

Name / nomenclature	Dietary sources	2′	3′	4′	5′	6	7	8	Glc-6
4′,5′-dimethyl luteolin-8-*C*-glucoside	Sugarcane	H	OH	O-CH_3	O-CH_3	H	OH	Glc	-
Apigenin 6-*C*-[6-feruoyl]-glucoside-8-*C*-arabinoside	Wheat and rye grains	H	H	OH	H	Glc	OH	Glc	Fer
Apigenin 6-*C*-[6-sinapoyl]-glucoside-8-*C*-arabinoside	Wheat and rye grains	H	H	OH	H	Glc	OH	Glc	Syn
Apigenin 6-*C*-arabinoside-8-*C*-[6-feruoyl]-glucoside	Wheat and rye grains	H	H	OH	H	Ara	OH	Glc	Fer
Apigenin 6-*C*-arabinoside-8-*C*-[6-sinapoyl]-glucoside	Wheat and rye grains	H	H	OH	H	Ara	OH	Glc	Syn
Apigenin-6-*C*-arabinosyl-8-*C*-glucoside	Barley grains	H	H	OH	H	Ara	OH	Glc	-
Apigenin-6-*C*-glucosyl-8-*C*-arabinoside	Barley grains	H	H	OH	H	Glc	OH	Ara	-
Diosmetin 6,8-di-*C*-glucoside	Lemon and lime fruits	H	OH	O-CH_3	H	Glc	OH	Glc	-
Diosmetin 6-*C*-glucoside	Lemon and lime fruits	H	OH	O-CH_3	H	Glc	OH	H	-
Diosmetin 8-*C*-glucoside	Lemon fruit, sugarcane	H	OH	O-CH_3	H	H	OH	Glc	-
Isoorientin	Buckwheat hulls, rooibos tea	H	OH	OH	H	Glc	OH	H	-
Isoorientin-7-*O*-[6-feruoyl]-glucosyl-4′-*O*-glucoside	Barley grains	H	OH	O-Glc	H	Glc	O-Glc	H	Fer
Isoschaftoside	Wheat and rye grains, rhubarb, sugarcane	H	H	OH	H	Ara	OH	Glc	-
Isoscoparin-7-*O*-glucoside	Barley grains	H	O-CH_3	OH	H	Glc	O-Glc	H	-
Isoscoparin-7-*O*-rutinoside	Barley grains	H	O-CH_3	OH	H	Glc	O-Rut	H	-
Isovitexin	Buckwheat hulls and groats, rice hulls, rhubarb, rooibos tea	H	H	OH	H	Glc	OH	H	-
Isovitexin-7-*O*-[6-sinapoyl]-glucosyl-4′-*O*-glucoside	Barley grains	H	H	O-Glc	H	Glc	O-Glc	H	Syn
Isovitexin-7-*O*-rutinoside	Barley grains	H	H	OH	H	Glc	O-Rut	H	-
Lucenin-2	Lemon fruit	H	OH	OH	H	Glc	OH	Glc	-
Luteolin-8-*C*-rutinoside	Sugarcane	H	OH	OH	H	H	OH	Rut	-
Lutonarin	Barley grains	H	OH	OH	H	Glc	O-Glc	H	-
Orientin	Buckwheat hulls, rooibos tea, sugarcane	H	OH	OH	H	H	OH	Glc	-
Saponarin	Barley grains	H	H	OH	H	Glc	O-Glc	H	-
Schaftoside	Wheat and rye grains, rhubarb, sugarcane	H	H	OH	H	Glc	OH	Ara	-
Stellarin-2	Lemon fruit	H	O-CH_3	OH	H	Glc	OH	Glc	-
Vicenin-2	Lemon fruit, orange juice, wheat and rye grains, rhubarb, dates	H	H	OH	H	Glc	OH	Glc	-
Vitexin	Buckwheat hulls, millet flour, rooibos tea, sugarcane	H	H	OH	H	H	OH	Glc	-
Vitexin 2′-*C*-[6-malonyl]-xyloside	Chard leaves	Xyl	H	OH	H	H	OH	Glc	Mal
Vitexin 2′-*C*-xyloside	Chard leaves	Xyl	H	OH	H	H	OH	Glc	-

Table 1. Summary of the chemical structures, nomenclature and sources of known dietary *C*-glycosyl flavones. The flavone backbone structure is defined in fig. 1. Ara, arabinose; Glc, glucose; Rut, rutinose; Xyl, xylose; Fer, ferulic acid; Mal, malic acid; Syn, synapic acid.

Name / nomenclature	Dietary source	3	5′
Aspalathin	Rooibos tea	OH	H
Nothofagin	Rooibos tea	H	H
Phloretin 3′,5′ di-*C*-glucoside	Tomato skin	H	Glc

Table 2. Summary of the chemical structures, nomenclature and sources of known dietary *C*-glycosyl dihydrochalcones. The dihydrochalcone structure is defined in fig. 2. Glc, glucose.

Nomenclature	6	8
(-)-Epicatechin-6-*C*-glucoside	Glc	H
(-)-Epicatechin-8-*C*-glucoside	H	Glc
(-)-Epicatechin-8-*C*-galactoside	H	Gal
(-)-Epicatechin-6,8-di-*C*-glucoside	Glc	Glc
(-)-Epicatechin-6,8-di-*C*-galactoside	Gal	Gal

Table 3. Summary of the chemical structures and nomenclature of *C*-glycosylated (-)-epicatechin derivatives from processed cocoa. The (-)-epicatechin structure is defined in fig. 3. Glc, glucose; Gal, galactose.

Nomenclature	6	8
(-)-Catechin-6-*C*-glucoside	Glc	H
(-)-Catechin-8-*C*-glucoside	H	Glc
(-)-Catechin-6-*C*-galactoside	Gal	H
(-)-Catechin-8-*C*-galactoside	H	Gal
(-)-Catechin-6,8-di-*C*-glucoside	Glc	Glc
(-)-Catechin-6,8-di-*C*-galactoside	Gal	Gal

Table 4. Summary of the chemical structures and nomenclature of *C*-glycosylated (-)-catechin derivatives from processed cocoa. The (-)-catechin structure is defined in fig. 4. Glc, glucose; Gal, galactose.

Figure captions (figures attached separately as required):

Figure 1. Flavone backbone structure.

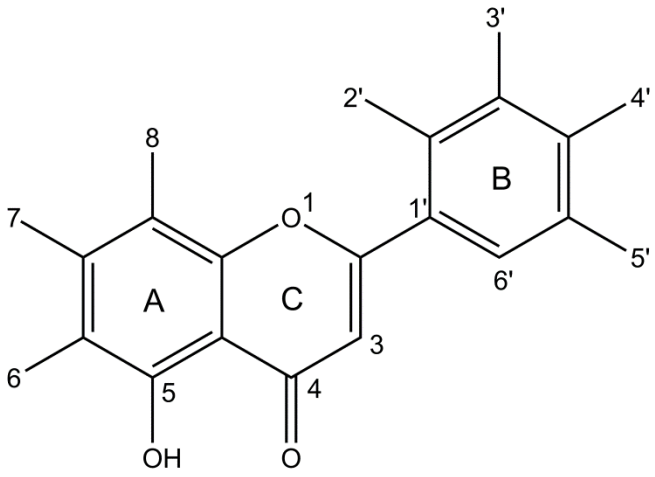

Figure 2. 3′-*C*-Glycosyl dihydrochalcone backbone structure.

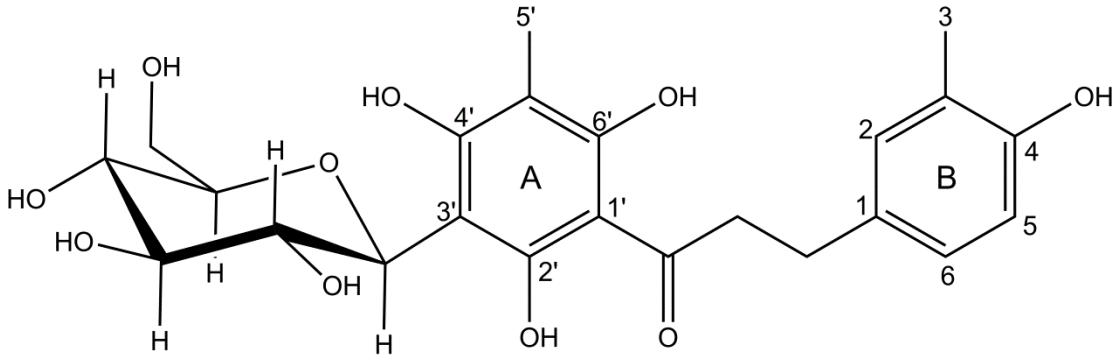

Figure 3. (-)-Epicatechin backbone structure.

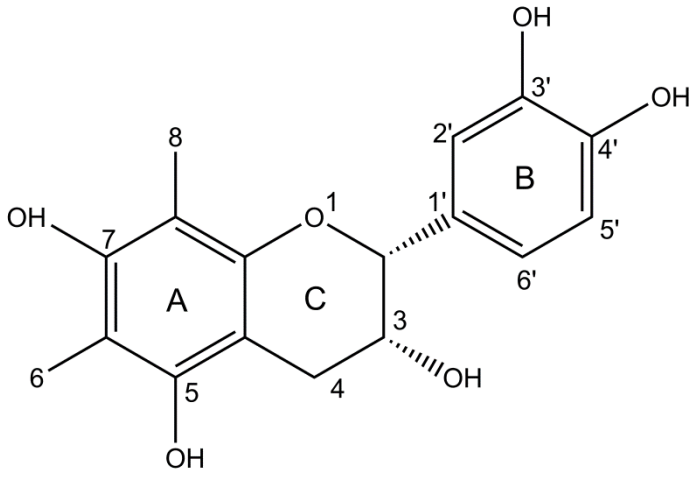

Figure 4. (-)-Catechin backbone structure.

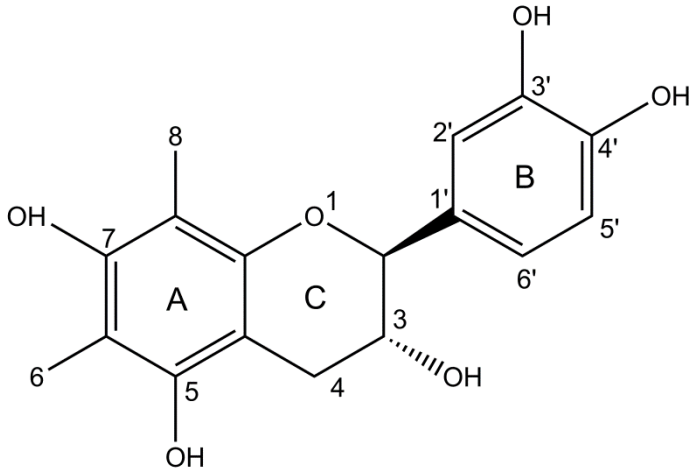

Figure 5. Comparative diagrammatical representation of flavonoid *C*- and *O*-glycoside pharmacokinetics *in vivo*. Unlike *O*-glycosyl flavonoids, resistance of the *C*-glycosidic linkage to hydrolytic mechanisms in the upper- and mid- gastrointestinal tract and during hepatic processing results in the presence of intact *C*-glycosyl flavonoids in human urine.