FEBS Letters 401 (1997) 78–82 FEBS 18048

The identification of flavonoids as glycosides in human plasma

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Received 29 November 1996

Abstract This study describes evidence for the absorption of flavonoids and their presence in human plasma in the glycosylated form by HPLC analysis with photodiode array detection. Rutin and other quercetin glycosides, phloridzin, as well as an anthocyanin are detected simultaneously. In addition, a compound eluting with the spectral properties of the aurone family is identified. The results reveal that phloretin and quercetin are absorbed from the diet as glycosides. The polyphenols are detected in plasma from non-supplemented humans at individual levels in the range 0.5–1.6 μM.

Key words: Flavonoid; Rutin; Phloridzin; Quercetin; Glycoside; Flavonoid uptake; Anthocyanin

1. Introduction

The protective effects of diets rich in fruit and vegetables against cardiovascular disease and certain cancers [1–3] have been attributed partly to the antioxidant vitamins and carotenoids contained therein [3]. Recent work is beginning to highlight the potential role of the phenolic components of fruit, vegetables, beverages and grains, the flavonoids, phenylpropanoids and phenolic acids which may act as antioxidants, or indeed, in combination with other constituents such as glucosinolates, indoles, dithiolthiones, isothiocyanates, etc, as agents of alternative mechanisms that may contribute to the anticarcinogenic or cardioprotective actions.

Many in vitro studies demonstrate the free radical scavenging properties of dietary phenolic phytochemicals [4–9]. Indeed, the antioxidant activities of the flavonoids are, in many cases, higher than those of vitamins C and E on a molar basis [6,7]. This is attributed to the redox properties of the phenolic hydroxyl groups, their number and chemical relationships [4]. Many flavonoids occur in foods in the glycosidic form and while substitution of phenolic groups decreases the antioxidant activity, in many cases it is still elevated in comparison with vitamin C.

There is little information concerning the extent to which the polyphenolics are absorbed in humans, their metabolism, pharmaco-kinetics and bioavailability. Although a small number of investigations have provided evidence for uptake of specific flavonoids in rats [10–15], few studies have demonstrated flavonoid uptake in humans in vivo. Furthermore, it is not clear whether the aglycone or the glycoside is absorbed, nor has a method been applied which can identify more than one family simultaneously in any particular plasma sample.

The study presented here describes a method for identifying and quantifying flavonoids in plasma from human subjects applying HPLC analysis with photodiode array detection. The preparative approach to the plasma extraction, the solvent system, column and detection conditions described allows detection of glycosides of flavonol, flavone and dihydrochalcone families. Members of the aurone and anthocyanidin families are also detected but not characterised. The system provides evidence for the absorption of the polyphenols as individual glycosides at levels of detection down to approx. 400–600 nM in human plasma.

2. Materials and methods

2.1. Chemicals

Methanol and acetonitrile, all HPLC grade, were obtained from Rathburn Limited, Walkerburn, UK. All phenolics were obtained from Extrasynthese, ZI Lyon Nord, Genay, France. Elgastat UHP double-distilled water (18+Ω grade) was used in all experiments.

2.2. Preparation of plasma extract

Fresh venous blood was taken by venipuncture, from normal individuals on non-supplemented diets, into acid-citrate-dextrose as anticoagulant at a 1:5 dilution. The blood was immediately centrifuged at 800×*g* (and 4°C) and the plasma separated from the packed erythrocytes and buffy coat. Plasma (1 ml volume) was transferred to a tube containing salicylic acid as internal standard (at final diluted concentration of 10 μg/ml) and the sample was deproteinised by adding 4 vols. of methanol. After standing for 5–10 min, the tubes were vortexed and centrifuged at 800×*g* (at 4°C). The supernatant was collected and the methanol removed by rotary evaporation under vacuum at 40°C. The resultant aqueous fraction was filtered using a Flowpore 0.22 μm sterile non-pyrogenic membrane filter directly into an HPLC vial and analysed directly.

2.3. Determination of polyphenols in plasma

The HPLC system used consisted of an autosampler with Peltier temperature controller, a Photodiode Array Detector and the Software system which controlled all the equipment and carried out data processing. A Nova-Pak C^{18} column (4.6×250 mm) with a 4 μm particle size was used and the temperature maintained by the column oven set at 30°C. Injection was by means of an autosampler, with a 100 μl fixed loop and the volume injected was 30 μl. Elution (0.5 ml/min) was performed using a solvent system comprising solvent A (20% methanol in 0.1% hydrochloric acid) and acetonitrile mixed using a linear gradient held at 95% solvent A for 10 min and then decreasing linearly to 50% solvent A at 50 min, back to 95% solvent A at 55 min and held at these conditions for a further 5 min. There was a 10 min delay before the next injection to ensure re-equilibration of the column. The chromatograms were obtained according to the retention time of each fraction with detection at both 280 and 350 nm. Peak identification of each component was effected post-run using spectroscopic analysis by photodiode array detection from 200 to 600 nm.

2.4. Preparation of standards

Stock solutions of a large range of standards were prepared by dissolving 0.6–1.2 mg of sample into 1 ml of methanol. Solutions were stored at 0–4°C and used within 4 weeks from the date of preparation, after spectroscopic monitoring, except for the anthocyanidins which were prepared freshly.

Stock solutions were diluted 1:10 in methanol, followed by dilution of aliquots of 10, 15 and 20 μl into 1 ml of water. This was taken through the same extraction procedure as the plasma samples after incorporation of 10 μg internal standard. The standard curves for the rutin (quercetin 3-rutinoside), quercetin 3-glucoside, quercetin 4′-glucoside and phloridzin (phloretin 2′-glucoside) are shown in Fig. 1.

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PII S0014-5793(96)01442-1

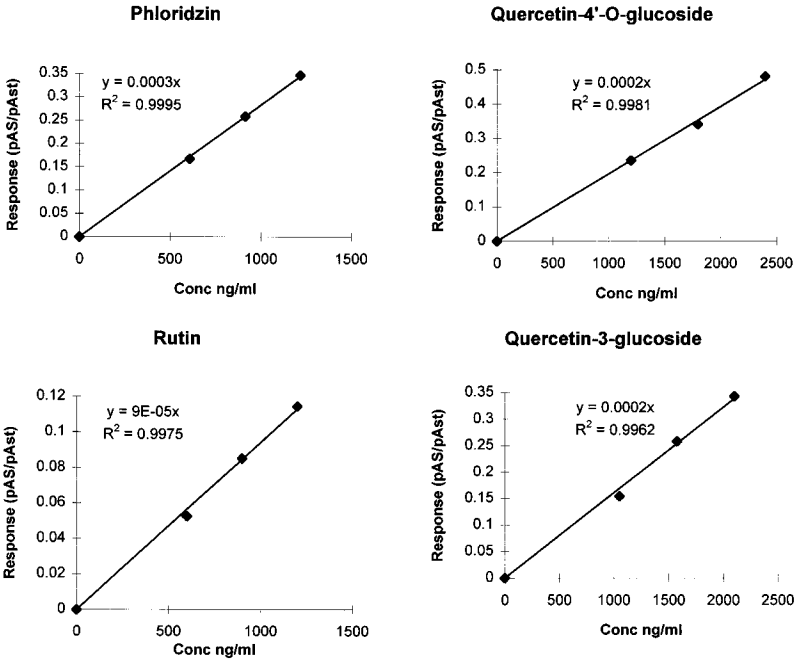

Fig. 1. Standard curves for phloridzin, rutin, quercetin 4′-*O*-glucoside, quercetin 3-glucoside. The response unit on the *y*-axis represents the ratio of peak area of sample to peak area of internal standard.

2.5. The recovery of flavonol glucosides from plasma

For recovery experiments, rutin, quercetin 3-glucoside, quercetin 4′-*O*-glucoside and phloridzin were diluted into 1 ml of water (control) or plasma, with salicylic acid at final concentrations of 0.6, 1.05, 1.2, 0.61 and 10 μg/ml, respectively. The samples were deproteinised and prepared for HPLC analysis as described above. Recoveries were in the range 90–100%.

3. Results

Fig. 2 shows the chromatograms of flavonoids from representative plasmas from normal healthy individuals (VLM/24 and LCB/1), with UV detection at 280 and 350 nm, respectively. The polyphenols are identified from their retention times relative to the internal standard. The glycosides have shorter retention times than their corresponding aglycones due to their higher polarities, and thus, in comparison with the range of standards applied, it is possible to distinguish them. Linking together the retention times with the spectral profiles of the individual peaks from the chromatograms (Fig. 3), the specific individual polyphenols identified are rutin, phloridzin and other quercetin glycosides with retention times and concentrations in plasma as shown in Table 1. Spiking plasma with individual compounds further confirmed the identification of phloridzin and rutin but the quercetin glucosides were not the 3- or 4′-derivative. The typical profile of an aurone (specific characteristic, the peak in the 421 nm region), and an anthocyanin (main visible peak at 526 nm) were also detected but not yet identified.

4. Discussion

This study describes an HPLC method for photodiode array detection of several specific families of dietary flavonoids in human plasma in one run including flavonol or flavone glycosides, dihydrochalcone, aurone and anthocyanidin families. The identification and quantification as phloridzin and rutin indicates their absorption as glycosides. Few previous studies have achieved such detection. Little is known about the bioavailability, absorption and metabolism of polyphenols in humans and it is likely that different groups of flavonoids have different pharmacokinetic properties. Absorption of dietary quercetin in healthy ileostomy volunteers has been concluded from studies defining absorption of quercetin as oral intake minus ileostomy excretion [16]. Three volunteers received quercetin-glucoside-rich fried onions equivalent to 89 mg aglycone, pure rutin equivalent to 100 mg aglycone, or 100 mg pure quercetin, from which absorption according to their above definition, was defined as 52, 17 and 24%, respectively. The same group also studied the time course of plasma quercetin concentration in two subjects after ingestion of fried onions containing quercetin glycosides to the level of quercetin aglycone equivalent to 64 mg [17]. Quercetin was determined after extraction with simultaneous hydrolysis so the question of absorption as the glycoside or aglycone was not addressed. Fluorescence detection at 485 nm emission revealed quercetin as an aluminium-quercetin complex after post-column derivatisation with aluminium nitrate. Peak plasma levels of 196 ng/ml total quercetin were detected. Manach et al. [18] have studied the absorption of quercetin in rats. Quercetin conjugated with isorhamnetin was recovered in the plasma of rats fed a quercetin diet, but curiously the conjugated complex was reported to be bound to albumin.

The pharmacokinetics and metabolism of a single oral supplement of diosmin (the 7-rhamnoglucoside of diometin, 5,7,3′-trihydroxy-4′-methoxy flavone) in human volunteers were studied by Cova et al. [19] arising from its attributed properties of vascular protection [20]. Diosmetin was detected by liquid chromatography-mass spectrometry at peak levels of 400 ng/ml in plasma after 1 h, decreasing to 50 ng/ml at 48 h. Evidence exists that catechin is absorbed by the human gut [21] and other investigations [22] involving oral administration of 3-*O*-methyl-(+)-[U-^{14}C]catechin (three volunteers) detected

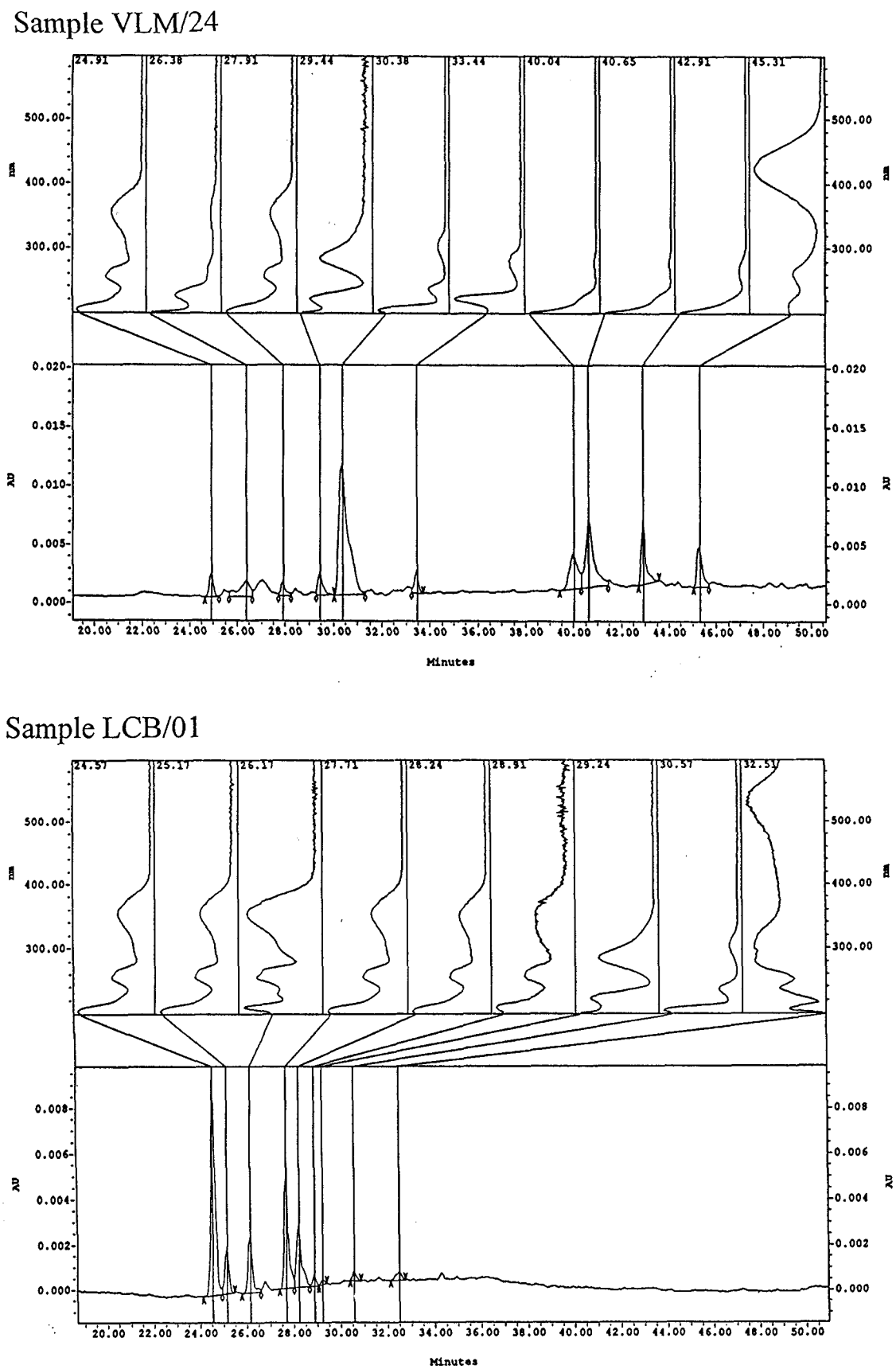

Fig. 2. HPLC chromatograms of two representative plasma samples (volunteers VLM/24 and LCB/1) depicting the retention times on UV detection at 280 and 350 μm, respectively, and absorption intensities (conditions described in Section 2).

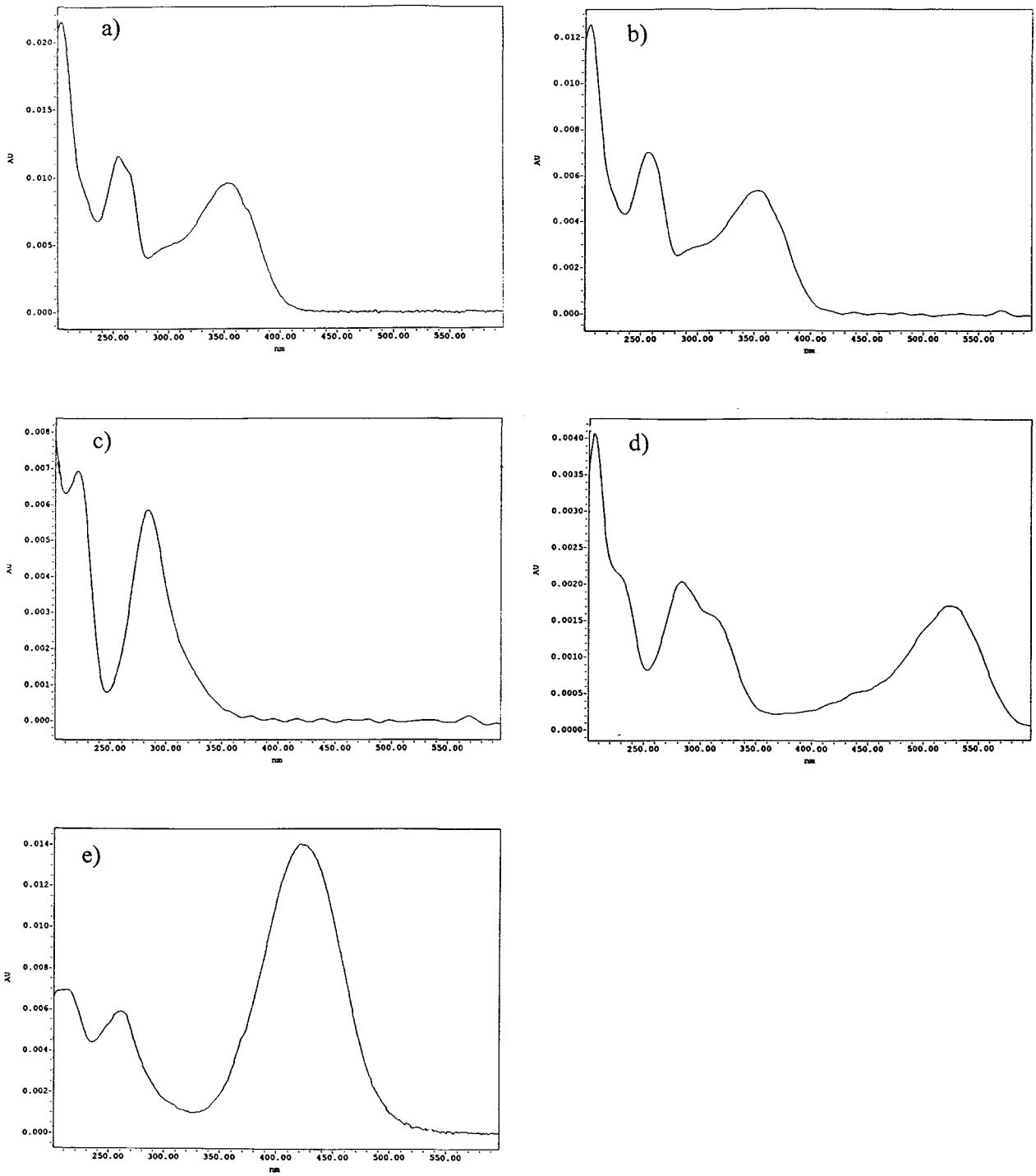

Fig. 3. Spectral profiles of selected individual polyphenolic components as they elute from the column: (a) rutin, (b) quercetin glucoside, (c) phloridzin, (d) anthocyanidin, (e) aurone.

and measured the supplement in the plasma by HPLC with UV detection at 280 nm. Peak levels of 11–18 μg/ml were found within 2 h of administration. Radioactivity also peaked with the same time scale at concentrations between 48 and 60 μg equivalents/ml plasma.

The study reported here describes a simple method for the simultaneous detection in one run of several of families of flavonoids in plasma of non-supplemented normal healthy human subjects. Individual levels down to approx. 0.6 μM are detected by HPLC with photodiode array detection. The uptake of flavonoids in the glycosylated form is demonstrated. In addition, constituents identified, from their spectroscopic properties [23] and their range of retention times, as the anthocyanidin, aurone and other members of the flavone glycoside families are indicated, although precise identification and quantification remain to be resolved.

Table 1
Retention times of flavonoid peaks and concentrations (μM) of identified substances in plasma sample VLM/24 with detection at 280 nm and plasma sample LCB/1 with detection at 350 nm

Retention time	Family	Component identification	Concentration (μM)
Sample VLM/24 (280 nm detection)			
24.91	flavonol	rutin	0.72
26.38	NI	NI	NQ
27.91	flavonol	quercetin glycoside (not rutinoside)	0.60[a]
29.44	dihydrochalcone	phloridzin	0.66
30.38	internal standard (IS)	salicylic acid	
33.44–42.91	4 peaks		
45.31	aurones	NI	NQ
Sample LCB/1 (350 nm detection)			
24.57	flavonol	rutin	0.76
25.17	flavonol	quercetin glycoside (not rutinoside)	0.40[a]
26.17	flavonol	quercetin glycoside (not rutinoside)	0.81[a]
27.71	flavonol	quercetin glycoside (not rutinoside)	1.26[a]
28.24	flavonol	quercetin glycoside (not rutinoside)	1.34[a]
29.24	dihydrochalcone	phloridzin	1.64
30.57	internal standard (IS)	salicylic acid	
32.51	anthocyanin	NI	NQ

NI, not identified; NQ, not quantified. HPLC conditions in Section 2.
[a]Indicates concentration calculated with reference to the standard to quercetin 3-glucoside.

Acknowledgements: Financial support from the Biotechnology and Biological Sciences Research Council is gratefully acknowledged.

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