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Short communication

Quantitative analysis of flavonoids by reversed-phase high-performance liquid chromatography

Alan Crozier[a,*], Einar Jensen[b], Michael E.J. Lean[c], Morag S. McDonald[a]

[a]*Plant Molecular Science Group, Bower Building, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK*

[b]*Department of Pharmacology, University of Tromsø, Science Park, N-9037 Tromsø, Norway*

[c]*Department of Human Nutrition, University of Glasgow, Queen Elizabeth Building, Royal Infirmary, Glasgow G31 2ER, UK*

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Abstract

Large variations were found in the effectiveness of five reversed-phase high-performance liquid chromatography columns used for the analysis of free and conjugated flavonoids. The best results were obtained with C_{18} Nova-Pak, C_{18} Symmetry and C_{18} Genesis columns but substantial band broadening and peak asymmetry were observed when free flavonoids were chromatographed on ODS-Hypersil and LiChrospher RP-18 supports. The Symmetry and Genesis columns provided gradient elution separations of rutin, quercetin-3-glucoside, quercitrin, myricetin, luteolin, quercetin, apigenin, kaempferol and isorhamnetin. This procedure was used for the quantitative analysis of endogenous flavones and flavonols in acid hydrolysed extracts from onions and celery.

Keywords: Plant extracts; Flavonoids

1. Introduction

Flavonoids are a large family of secondary plant metabolites, comprising anthocyanins, flavonols, flavones, catechins and flavonones, and many are present in plant tissues in relatively high concentrations as sugar conjugates [1]. There is increasing interest in flavonoids because it is becoming apparent that they are an important group of compounds in both plants and animals. In plants their production is induced by and they provide protection against UV-B radiation (see Ref. [2]). Flavonoids are also involved in the regulation of pollen tube growth in the stigma [3,4] and they act as regulatory signals in the transcription of nodulation genes in *Rhizobium* cells as the first step towards legume root nodule formation and symbiotic nitrogen formation [5,6]. Flavonols and flavones are of particular importance in the human diet as there is evidence that they act as antioxidants [7,8], and epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer [9–12] and cardiovascular disease [13–15]. The main dietary sources of the flavonols, primarily as conjugates of quercetin (**I**) and kaempferol (**II**), and the corresponding flavones, luteolin (**III**) and apigenin (**IV**), are vegetables, fruit and beverages (Scheme 1) [16,17].

Among the more ubiquitous flavonoids over 50

*Corresponding author.

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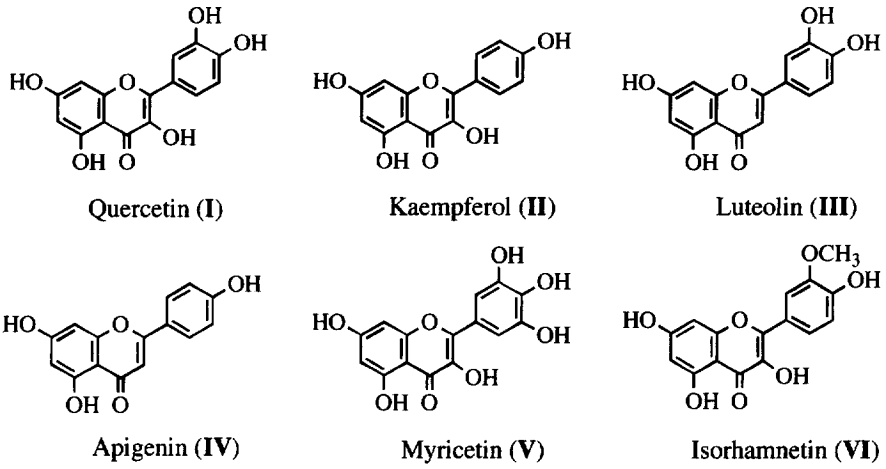

Scheme 1. Flavone and flavonol structures.

different glycosides have been identified [18,19]. As few reference compounds of these glycosides are available commercially, direct quantitative analysis of flavonoid conjugates is rarely a practical proposition. As a consequence, when investigating the levels of the more common flavonoids, it has become accepted practice to acid hydrolyse the glycosides [20] and to identify and quantify the released aglycones. A recent study by Hertog et al. [21] assessed the effectiveness of acid hydrolysis conditions for the release of free flavonoids, such as quercetin, kaempferol, myricetin (**V**), luteolin and apigenin, from conjugated forms in plant extracts. These investigators also developed improved reversed-phase high-performance liquid chromatography (HPLC) procedures for the quantitative analysis of flavonoid aglycones [21].

This paper reports on an evaluation of the performance of five reversed-phase supports for HPLC of flavonoids. The data obtained enabled refinements in HPLC procedures to be made, providing enhanced resolution of free and conjugated flavonoids by robust, highly reproducible analytical methodology. Examples of the use of these procedures are demonstrated by the quantitative analysis of flavonoids in onion and celery extracts in which use was made of an internal standard and samples were analysed both before and after acid hydroysis.

2. Experimental

2.1. High-performance liquid chromatography

Samples were analysed using a Waters (Milford, MA, USA) automated liquid chromatograph comprising a Millennium 2010 chromatography manager, a 717 plus autoinjector, a 616 LC system and a 990 photodiode array detector. Reversed-phase separations were carried out at 30°C using the following columns: (1) 250×4.6 mm I.D., 5 μm ODS-Hypersil column (Shandon, Runcorn, UK); (2) 250×4.6 mm I.D., 5 μm RP-18 LiChrospher column (Merck, Darmstadt, Germany); (3) 150×3.9 mm I.D., 4 μm C_{18} Nova-Pak column (Waters); (4) 150×3.9 mm I.D., 5 μm C_{18} Symmetry column (Waters); (5) 150×4.6 mm I.D., 4 μm C_{18} Genesis column (Jones Chromatography, Mid-Glamorgan, UK). The columns were eluted at flow-rates ranging from 0.6–1 ml min^{-1} in isocratic and gradient modes, with mixtures of either (i) acetonitrile in water or (ii) either acetonitrile or methanol in water adjusted to pH 2.5 with trifluoroacetic acid (TFA). In tests of column performance parameters with benzophenone, the HPLC eluate was monitored at 254 nm. For the analysis of flavonoids, HPLC traces were plotted at an absorbance of 365 nm. At this wavelength the limit of detection was <5 ng and a 5–250 ng linear

calibration curve was obtained for each flavonoid aglycone standard.

2.2. Reference compounds

Benzophenone, which was used to test HPLC columns, and the flavonoids, apigenin, kaempferol, myricetin, quercetin, quercitrin (quercetin-3-L-rhamnoside) and rutin (quercetin-3β-D-rhamnoside) were purchased from Sigma (Poole, UK). Isorhamnetin (**VI**), luteolin and quercetin-3-glucoside were obtained from Apin Chemicals (Abingdon, UK).

2.3. Plant material

White onions (*Allium cepa* L.) (UK), and white celery stalks (*Apium graveolens* L.) (UK) were purchased from Safeway Stores, Glasgow, UK in May 1995. On the day of purchase, and after cleaning the celery and removing the outer dry skin from the onions, the plant material was finely sliced and frozen in liquid nitrogen prior to lyophilisation in an Edwards (Welynn Garden City, UK) Modulyo freeze-drier. When the tissue was freeze-dried it was powdered in a pestle and mortar and stored at −20°C. Fresh and dry mass measurements were obtained for all plant material.

2.4. Extraction and hydrolysis conditions

Optimisation of acidic conditions for the hydrolysis of flavonoid conjugates in a range of fruit and vegetables have been described by Hertog et al. [21], following an earlier detailed study by Harborne [20] on the release of free flavonoids by acid and enzymic hydrolyses. In the present study, preliminary screenings were carried out to determine the most effective acid hydrolysis conditions for onion and celery. The data obtained were in agreement with the findings of Hertog et al. [16,21]. Thus, 0.25 g aliquots of lyophilised onion were extracted with 20 ml, 60% aqueous methanol containing 125 μg kaempferol as an internal standard and 20 m*M* sodium diethyldithiocarbamate as an antioxidant. Five ml 6 *M* HCl was added to each extract to give a 25 ml solution of 1.2 *M* HCl in 50% aqueous methanol. Extracts were refluxed at 90°C for 2 h. Extract aliquots of 500 μl, taken both before and after hydrolysis, were filtered through a 0.45 μm filter after which 50 μl samples were made up to 250 μl with distilled water adjusted to pH 2.5 with TFA, prior to analysis of 200 μl volumes (1/1250 aliquot of total sample) by gradient, reversed-phase HPLC on a 150×3.9 mm C_{18} Symmetry column. Identical procedures were used with celery except that 125 μg isorhamnetin was used as an internal standard and samples were refluxed in 2 *M* HCl for 4 h at 90°C.

With the onion and celery extracts, the homogeneity of the endogenous flavonoid and internal standard peaks in acid hydrolysed samples was confirmed by both co-chromatography and a comparison of their 300–450 nm absorbance spectra with those of reference compounds.

3. Results and discussion

3.1. Assessment of reversed-phase HPLC columns for flavonoid analysis

Our preliminary studies indicated that a solvent comprising mixtures of acetonitrile and water adjusted to pH 2.5 with TFA provided more efficient reversed-phase HPLC separations of flavonoids than the more traditional mobile phases in which the aqueous solvent is acidified with either phosphoric acid or acetic acid. In order to assess and optimise HPLC conditions, the performances of ODS-Hypersil, LiChrospher RP-18, C_{18} Nova-Pak, C_{18} Symmetry and C_{18} Genesis columns were investigated using benzophenone, which is used by manufacturers to test column efficiency, and the flavonoids rutin and its aglycone, quercetin. The column performance parameters that were monitored were theoretical plates cm^{-1} ($1/H$) and peak tailing (T) which was calculated using the USP method [22]. The data obtained are presented in Table 1. As anticipated, when used to analyse benzophenone, all five columns exhibited high efficiencies, with figures for $1/H$ ranging from 505 to 959 cm^{-1} with T values of 1.17 to 1.32. With rutin, both the ODS-Hypersil and LiChrospher RP-18 columns exhibited a slight decline in efficiency and an increase in T which was more noticeable with LiChrospher. The fall off in

Table 1
Performance of five reversed-phase HPLC columns when tested with benzophenone, a flavonol glycoside conjugate, rutin, and a free flavonol, quercetin

Column	Performance parameter	Test compound		
		Benzophenone	Rutin	Quercetin
ODS-Hypersil	$1/H$	625	459	80
	T	1.27	1.51	9.63
LiChrospher RP-18	$1/H$	505	373	3
	T	1.32	2.21	10.52
C_{18} Nova-Pak	$1/H$	959	823	935
	T	1.22	1.22	1.44
C_{18} Symmetry	$1/H$	959	801	840
	T	1.22	1.24	1.29
C_{18} Genesis	$1/H$	850	769	874
	T	1.17	1.18	1.30

Mobile phase: water–acetonitrile for benzophenone; water adjusted to pH 2.5 with TFA–acetonitrile for rutin and quercetin; ratios of the two solvents adjusted for each column to provide a k' value of 4–5 for each analyte. Performance parameters calculated by Millenium 2010 software: number of theoretical plates cm^{-1} ($1/H$); USP tailing factor (T).

performance was much less marked when rutin was analysed with the Nova-Pak, Symmetry and Genesis columns. Analysis of quercetin revealed marked differences in the performance of the five columns. Broad, low efficiency peaks with excessive tailing were obtained with ODS-Hypersil and to an even greater extent with the LiChrospher RP-18 support. In contrast, no deterioration of $1/H$ figures were observed and there was minimal effect on T values when quercetin was chromatographed on the Nova-Pak, Symmetry and Genesis columns.

The data presented in Table 1 demonstrate that ODS-Hypersil and LiChrospher RP-18 columns can provide acceptable reversed-phase analyses of flavonoid conjugates, as indicated by the separations reported by Bailey et al. [23], Finger et al. [24] and Van Sumere et al. [25]. However, they are clearly not suitable for use with free flavonoids because of unacceptably high band broadening and excessive peak tailing. In contrast, high efficiency analyses of both free and conjugated quercetin were obtained with the Nova-Pak, Symmetry and Genesis columns. The quercetin $1/H$ value obtained with the Nova-Pak column represents a 2.5-fold improvement on the figures achieved by Hertog et al. [21] with the same reversed-phase support.

The C_{18} Symmetry and C_{18} Genesis columns were used for gradient elution analysis of a range of free and conjugated flavonoid standards and the traces obtained are presented in Fig. 1. The chromatograms obtained with the two columns were broadly similar with only a marginal separation of luteolin and quercetin in the acetonitrile-based solvent. However, as indicated by Hertog et al. [21], these compounds be can resolved by isocratic analysis when methanol is used as the organic component in the mobile phase.

3.2. Analysis of endogenous flavonoids in plant extracts

A kaempferol internal standard was added to extracts of lyophilised white onions prior to hydrolysis of flavonoid conjugates by refluxing with 1.2 *M* HCl in 50% aqueous methanol for 2 h. Preliminary studies had ascertained that the hydrolysed onion extracts did not contain detectable quantities of kaempferol. Aliquots of pre- and post-hydrolysis samples were analysed by gradient elution, reversed-phase HPLC using a C_{18} Symmetry column. The data obtained are presented in Fig. 2. In the unhydrolysed sample, two peaks are present in addition to the internal standard. Although neither peak co-chromatographed with either rutin, quercetin-3-glucoside or quercitrin, they can be considered to be putative quercetin glycosides because when the extract was acid hydrolysed they disappeared and were replaced by a major peak at

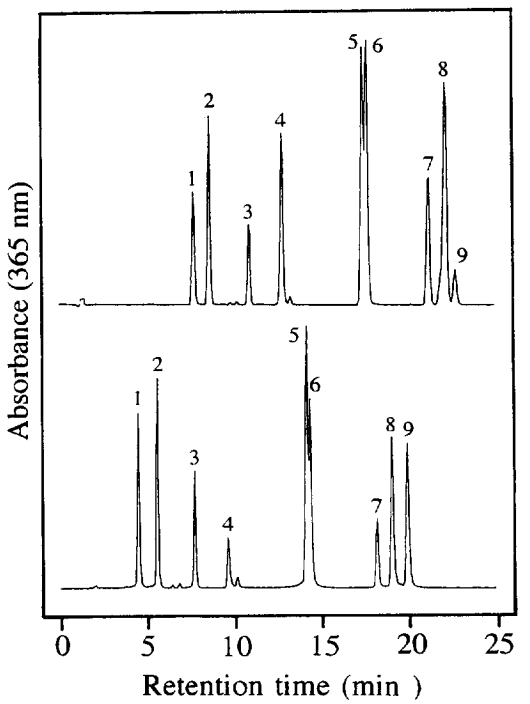

Fig. 1. Gradient reversed-phase HPLC analysis of free and conjugated flavonoids. Upper trace: 150×3.9 mm I.D. 5 μm C_{18} Symmetry column eluted with a 20 min gradient of 15–35% acetonitrile in water adjusted to pH 2.5 with TFA. Lower trace: 150×4.6 mm I.D. 4 μm C_{18} Genesis column eluted with a 20 min gradient of 20–40% acetonitrile in water adjusted to pH 2.5 with TFA. Flow-rate: 1 ml min^{-1}. Detector: absorbance monitor operating at 365 nm. Sample: (1) rutin, (2) quercetin-3-glucoside, (3) quercitrin, (4) myricetin, (5) luteolin, (6) quercetin, (7) apigenin, (8) kaempferol and (9) isorhamnetin.

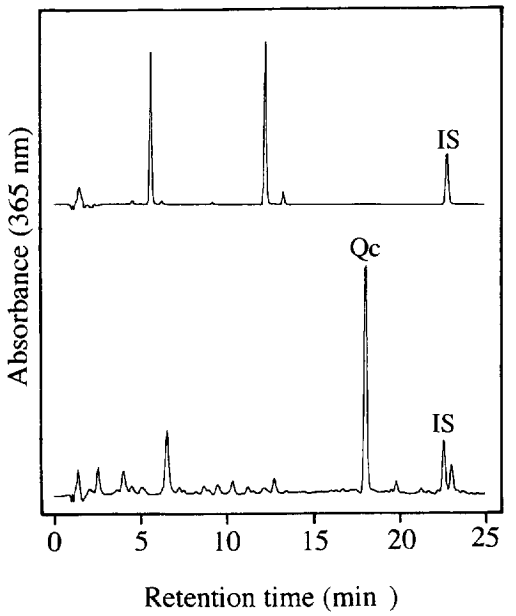

Fig. 2. Gradient reversed-phase HPLC analysis of flavonoids in white onions. Column: 150×3.9 mm 5 μm C_{18} Symmetry. Mobile phase: 20 min gradient of 15–35% acetonitrile in water adjusted to pH 2.5 with TFA. Flow-rate: 1 ml min^{-1}. Samples: extract aliquots before acid hydrolysis at 90°C in 1.2 *M* HCl for 2 h (upper trace) and after acid hydrolysis (lower trace). Detector: absorbance monitor operating at 365 nm. Qc: quercetin, I.S.: kaempferol internal standard.

18.0 min, which co-chromatographed with quercetin and had a 300–450 nm absorbance spectrum which very closely matched that of a quercetin standard. In addition to the main quercetin peak, the acid hydrolysed extract also contained a relatively polar peak at t_R 6.1 min and a number of minor constituents. Four replicate samples of onions were analysed similarly and found to contain 227±3.4 μg quercetin g^{-1} fresh mass. This represents a relative standard deviation of 1.5%.

In keeping with the reports of Hertog et al. [16,21], preliminary studies indicated that acid hydrolysed extracts of celery stalks contained significant quantities of apigenin and luteolin and that quercetin and kaempferol were not present in detectable amounts. In view of the proximity of kaempferol to apigenin in the reversed-phase HPLC system (Fig. 1), isorhamnetin was used as the internal standard when investigating the flavonoid content of celery. The HPLC traces obtained with the celery extracts before and after hydrolysis with 2 *M* HCl for 4 h are illustrated in Fig. 3. The unhydrolysed extract contained a number of peaks which disappeared during acid hydrolysis and were replaced by apigenin, luteolin and an unknown component, that chromatographed between luteolin and the internal standard. Based on the analysis of four replicate samples, it was estimated that celery contained 35±1.5 μg luteolin and 191±4.0 μg apigenin g^{-1} fresh mass.

The data obtained in these studies demonstrates that the analytical methodology employed provides acceptable levels of precision when used to quantify flavonoids in plant extracts. The limit of detection for endogenous quercetin and the other flavonoid aglycones was ca. 3 μg g^{-1} fresh mass. However, if

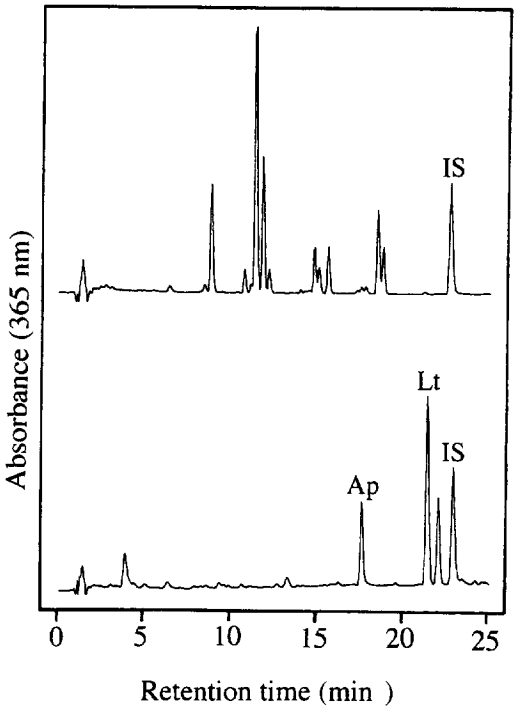

Fig. 3. Gradient reversed-phase HPLC analysis of flavonoids in celery. Column: 150×3.9 mm 5 μm C_{18} Symmetry. Mobile phase: 20 min gradient of 15–35% acetonitrile in water adjusted to pH 2.5 with TFA. Flow-rate: 1 ml min^{-1}. Samples: extract aliquots before acid hydrolysis at 90 °C in 2 *M* HCl for 4 h (upper trace) and after acid hydrolysis (lower trace). Detector: absorbance monitor operating at 365 nm. Ap: apigenin, Lt: luteolin, I.S.: isorhamnetin internal standard.

necessary, this figure can be lowered substantially as larger sample aliquots can be analysed by HPLC without impurities interfering with the analysis. In practice, the exact limit of detection is going to vary, being dependent upon the spectrum of impurities and the flavonoid levels present in extracts from different plant materials.

4. Conclusions

The data presented in this report indicate that there are wide variations in the effectiveness of different reversed-phase HPLC columns when used to analyse free and conjugated flavonoids. High efficiency separations were obtained with C_{18} Nova-Pak, C_{18} Symmetry and C_{18} Genesis columns with $1/H$ values markedly better than those obtained in previous studies. Gradient elution of the Symmetry and Genesis columns with acetonitrile in water adjusted to pH 2.5 in TFA facilitated the separation of three quercetin conjugates and a number of flavone and flavonol aglycones. This procedure was used for the quantitative analysis of quercetin in acid hydrolysed onion extracts and luteolin and apigenin in hydrolysed celery samples. The estimates obtained were precise and the limit of detection was ca. 3.0 μg g^{-1} fresh mass. The identity and homogeneity of the flavonoid peaks was confirmed by co-chromatography and a comparison of the 300–450 nm absorbance spectra with those of standards.

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