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Liquid chromatographic method for the separation and quantification of prominent flavonoid aglycones

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Abstract

Many beneficial health effects have been attributed to flavonoids, which are prevalent in plant-based foods. The literature is replete with chromatographic systems which are capable of measuring flavonoid content across one, two, and even three of the five common subclasses of flavonoids found in foods. However many foods and mixed diets, in particular, contain members of all five subclasses of flavonoids. We have developed an HPLC system for the separation and quantification of seventeen flavonoids, as their aglycones, which represent all five subclasses and are expected to be prominent in commonly consumed foods. Representative foods with significant concentrations of flavonoids from each of these subclasses were analyzed employing the new system. © 2000 Published by Elsevier Science B.V.

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1. Introduction

Flavonoids are widespread throughout the plant kingdom and as a result may be important in human diets [1] Several milligrams to as much as 1 g of mixed flavonoids are consumed per day in the western diet [2]. They have several benefits to human health, including antioxidant activities, metal chelation [3,4], and antiproliferative [2], anticarcinogenic, antibacterial, anti-inflammatory, antiallergic, and antiviral effects [4]. They also have shown the ability to stimulate the immune system [4] and to prevent nitration of tyrosine [5]. Detailed knowledge of the flavonoid content of foods and the development of analytical databases of values is essential in understanding the relationship of these dietary constituents and human health [6].

Flavonoids have a diphenylpropane skeleton [1]. The monomeric flavonoids common in food can be divided into five subclasses: anthocyanidins, catechins (flavan-3-ols), flavanones, flavones, and flavonols (Fig. 1) [7]. Glycosylation is often on C3 and less often on C5, C7 [4], and C4′ [8]. The most common sugar is glucose, but other sugars are found, including rhamnose, galactose, xylose [4], rutinose, and neohesperidose [9].

Harborne and colleagues have edited several volumes on the science of the flavonoids, the most recent of which is a 15-chapter work [10]. We recently reviewed the analysis of flavonoids in foods by HPLC ([11]and refs. therein). Most of the analytical procedures that have been developed for

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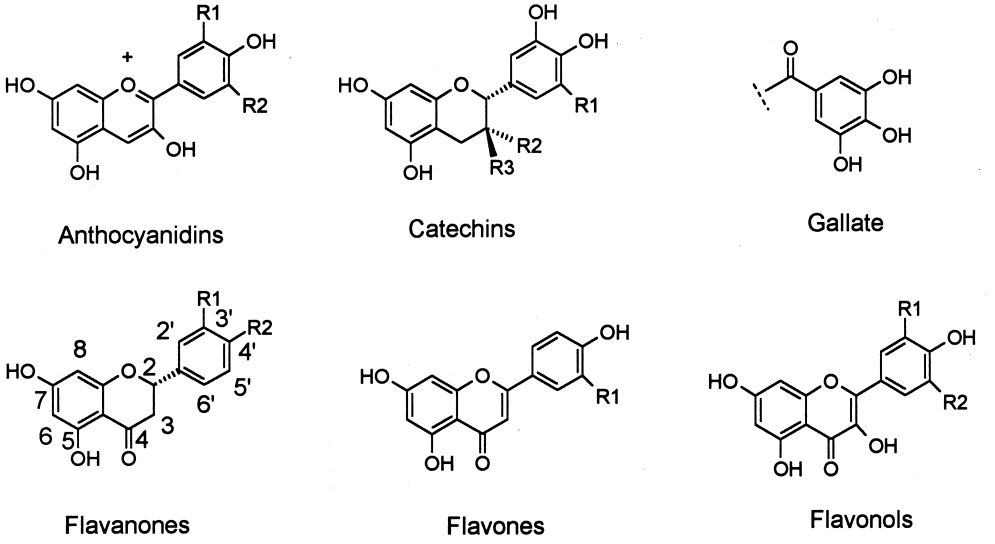

Fig. 1. Aglycones of flavonoids common in foods, with the numbering system shown on the flavanones [11]. Anthocyanidins: cyanidin, R1=OH, R2=H; delphinidin, R1=R2=OH; malvidin, R1=R2=OMe; pelargonidin: R1=R2=H; peonidin: R1=OMe, R2=H. Catechins: (+)-catechin [(+)-Cat], R1=R2=H; R3=OH; (−)-epicatechin [(−)-EC], R1=R3=H, R2=OH; (−)-epicatechin gallate [(−)-ECG], R1=R3=H, R2=OGallate; (−)-epigallocatechin [(−)-EGC], R1=R2=OH, R3=H; (−)-epigallocatechin gallate [(−)-EGCG], R1=OH, R2=OGallate, R3=H. Flavanones: hesperetin, R1=OH, R2=OMe; naringenin, R1=H, R2=OH. Flavones: apigenin, R1=H; luteolin, R1=OH. Flavonols: kaempferol, R1=R2=H; myricetin, R1=R2=OH; quercetin, R1=OH, R2=H.

flavonoids in foods, measure flavonoids in their native form which is usually as glycosylates. Over fifty flavonoid glycosides are common in foods [1]. The development of a useful and functional database of monomeric flavonoid values for foods requires knowledge of the total aglycone content for each flavonoid, because that is the form in which it is probably absorbed. In addition, it would be convenient to have a single HPLC procedure which could be employed to simultaneously measure all of the prominent food flavonoids as their aglycones. Hertog and colleagues [1] developed HPLC conditions for the separation and quantification of five prominent food flavonoids as their aglycones from three of the five subclasses, flavanones, flavones and flavonols. However, anthocyanidins and catechins were not measured and may constitute a substantial proportion of total dietary flavonoid intake.

In this report, we describe an HPLC system for the separation and quantification of seventeen prominent monomeric flavonoid aglycones which represent all five of the common subclasses. The applicability of the newly developed system was demonstrated in the measurement of flavonoid aglycones in extracts from typical foods.

2. Experimental

2.1. Chemicals

Gallic acid, myricetin, and spectrophotometric-grade trifluoroacetic acid (TFA) were purchased from Aldrich Chemical (Milwaukee, WI). *tert.*-Butylhydroquinone was purchased from Eastman Chemical Products, Inc. (Kingsport, TN). Kaempferol was purchased from Fluka Chemie AG (Switzerland). Apigenin, cyanidin chloride, delphinidin chloride, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, kaempferol, luteolin, malvidin chloride, pelargonidin chloride, and peonidin chloride were purchased from Indofine Chemical Company (Somerville, NJ). Petunidin chloride was purchased from Polyphenols AS (Sandnes, Norway). (+)-Catechin hydrate, (−)-

gallocatechin gallate, hesperetin, naringenin, and quercetin were purchased from Sigma (St. Louis, MO). Hydrochloric acid and HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Chemical (Fair Lawn, NJ). HPLC-grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA).

2.2. Preparation of standards

Solutions were made by dissolving a 1–6 mg accurately weighed portion of standard in 10 ml methanol. Each solution was sonicated 5 min. Aliquots of 3.0, 2.0, 1.0, and 0.5 ml were put in 5.0-ml volumetric flasks, and made to volume with MeOH. These five solutions bracketed the expected concentrations found in foods. Mixtures of standards used in HPLC method development were made by mixing certain standard solutions.

2.3. LC instrumentation, columns, and conditions

The LC system was a Hewlett-Packard (HP) Series 1100 system (Hewlett-Packard, Wilmington, DE), consisting of a liquid chromatograph connected to a variable-wavelength diode array detector (DAD) and controlled by an HP HPLC 3D ChemStation software. A Zorbax Eclipse XDB-C18 column (Hewlett-Packard) (250×4.6 mm, 5 μm) was preceded by a guard column (12.5×4.6 mm) of the same stationary phase. The column and guard column were thermostatically controlled at 30°C, and the flow rate was 1.0 ml/min. Injections were 5 μl. Detection was monitored at 210, 260, 278, 370, and 520 nm. The solvents consisted of water (A), methanol (MeOH) (B), and acetonitrile (ACN) (C), each containing 0.05% (w/w) trifluoroacetic acid (TFA).

2.4. Calibration

Calibration lines of peak area versus analyte concentration were plotted from varying concentrations of commerically available standards. Calibrations were made for all compounds at 210 nm, then at whichever of the four wavelengths (260, 278, 370, 520 nm) showed the highest absorbance. The high concentration of methanol in the last half of the run caused the baseline at 210 nm to rise. This was corrected by subtraction of a blank run. Slope, intercept, and the other statistics of calibration lines were calculated with linear regression programs supplied as part of Microsoft Excel Version 7.0.

Limits of detection were determined by running a dilute standard ten times, multiplying the standard deviation of the peak area by three, and converting from area to mass and concentration of flavonoid [12,13]. This was done for EGC at 210 nm and luteolin at 260 nm, which elute at 11.5 and 44.3 min respectively, and bracket most of the flavonoids in a chromatographic run. The limits are based on ten 5-μl injections containing 416 ng EGC and 154 ng luteolin.

2.5. Foods and extractions

Foods were purchased from local supermarkets, except for Dragon Well green tea which was a gift from a colleague in China.

The food extractions were modified, following a method reported by Hertog [1] and used by several other groups. Freeze-dried food samples were ground in a Thomas-Wiley Intermediate Mill (Arthur H. Thomas Co., Philadelphia, PA, USA) through 20 mesh screen. Parsley was dehydrated flaked parsley leaf, ground in a Wiley mill. Samples (0.5 or 5.0 g) were refluxed 2 h in a solution of 40 ml 62.5% aqueous methanol in which 0.5 g/l *tert.*-butylhydroquinone (TBHQ) was dissolved, and 10 ml aqueous 6N HCl was added. This resulted in 50 ml of 50% aqueous methanol which was 1.2 N in HCl and contained 0.4 g/l TBHQ. After cooling, the solution was sonicated 5 min, made to a known volume, and filtered through a 0.2 μm Anotop syringe filter (Whatman) for HPLC analysis. Foods rich in anthocyanins were preferentially sampled at 5.0 g, due to the low extinction coefficients of anthocyanidins.

Dragon Well green tea leaves were stirred and refluxed in deionized water for 20 min and filtered, prior to HPLC analysis [14]. This minimized hydrolysis of the gallate esters.

Table 1
Solvent parameters for the separation of flavonoid aglycones[a,b]

Time (min)	Water (%)	MeOH (%)	ACN (%)
0	90	6	4
5	85	9	6
30	71	17.4	11.6
60	0	85	15
61[c]	90	6	4
66	90	6	4

[a] All solvents include 0.05% (w/w) TFA.
[b] All gradients are linear.
[c] For equilibration.

3. Results and discussion

3.1. Development of the chromatographic system

Dalluge and colleagues [15] recognized the problem of the irreproducibility of retention times for catechins while trying to reproduce work in the literature. Using the Zorbax Eclipse XDB-C18 column which worked best for Dalluge, we modified one of their two solvent systems, giving us the system that yielded the best resolution for seventeen flavonoid aglycones (Table 1).

3.2. Chromatography of solutions of standards

Fig. 2 shows the separation of a solution of seventeen common food flavonoid standards at 210 nm. The anthocyanidins are seen at 520 nm, where none of the other standards absorb (Fig. 3).

These separations were conducted on a reversed-phase column, therefore as expected the most polar flavonoids (catechin and epicatechins) eluted early. In general, the anthocyanidins as a class eluted next, followed by members of the flavonols, flavones and flavanones. The polarity of the basic ring structure of the later three subclasses is quite similar, so the addition of hydroxyl group(s) to the B-ring results in substantial alterations in polarity of the molecule. Thus myricetin, a flavonol (2,3-dehydro, 3-hydroxy, 4-keto), which has three hydroxyl groups on the B-ring elutes among the anthocyanidins, whereas kaempferol and apigenin which have identical structures in their B-rings and considerable electronic resonance among all three rings, elute last suggesting they are the least polar of the common flavonoids tested.

Most flavonoids had peak-to-peak separation of 0.5 min or greater (Table 2). However, two pairs of compounds, myricetin (peak 8) and pelargonidin (peak 9) as well as kaempferol (peak 16) and

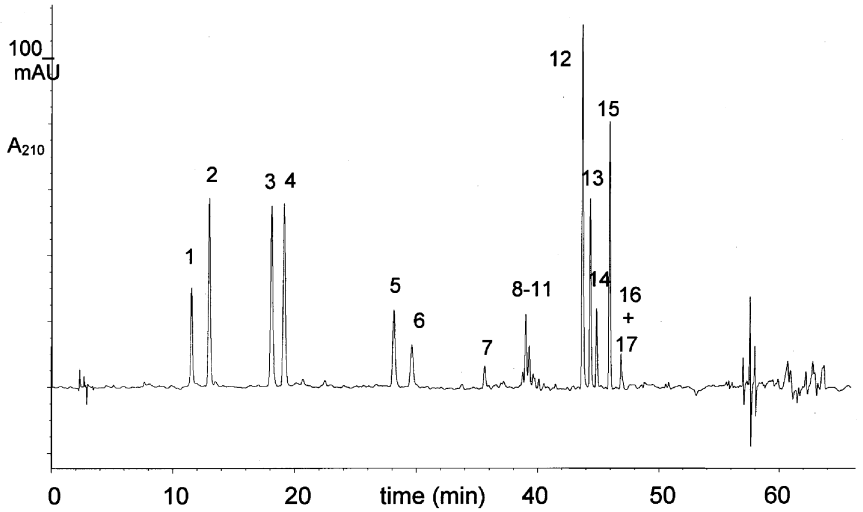

Fig. 2. Chromatogram of a solution of 17 flavonoid standards monitored at 210 nm. Chromatographic conditions are described in text. See Table 2 for peak identification.

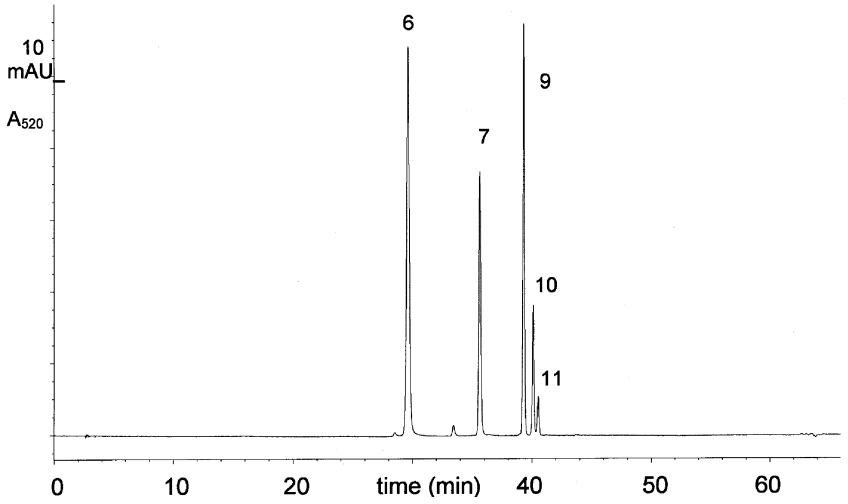

Fig. 3. Chromatogram of a solution of 17 standards monitored at 520 nm. Anthocyanidins are the only flavonoids tested which absorb at 520 nm. Chromatographic conditions are described in text. See Table 2 for peak identification.

apigenin (peak 17) were only partially resolved with the current chromatographic system. It is doubtful these combinations of flavonoids will be observed in the same foods. However if they are, pelargonidin can be quantified at 520 nm where myricetin has baseline absorbance and myricetin can be measured at 350 nm where pelargonidin has minimal absorbance. The estimation of values for apigenin and kaempferol, in the presence of each other, requires use of their isobestic absorption at 354 nm and peak absorbance values at 338 nm and 368 nm, respectively.

Table 2
Retention times of the flavonoids

Peak	Standard	t_R (min)
1	(−)-EGC	11.5
2	(+)-Catechin	13.0
3	(−)-EGCG	18.0
4	(−)-EC	19.1
5	(−)-ECG	28.1
6	Delphinidin	29.8
7	Cyanidin	35.8
8	Myricetin	39.1
9	Pelargonidin	39.5
10	Peonidin	40.3
11	Malvidin	40.7
12	Quercetin	43.7
13	Luteolin	44.3
14	Naringenin	44.9
15	Hesperetin	45.9
16	Kaempferol	46.8
17	Apigenin	47.0

3.3. Calibration

Table 2 shows the seventeen flavonoids in order of their retention times. All flavonoids were calibrated at 210 nm, and whichever of the other four wavelengths, 260, 278, 370, and 520 nm, showed the highest absorbance. Linearity was found over all the calibrations reported. All intercepts were not significantly different from the origin, and all correlation coefficients were 0.995 or greater.

Limits of detection were determined to be 9.3 ng column load EGC at 210 nm (t_R=11.5 min), and 45 ng luteolin at 260 nm (t_R=44.3 min).

3.4. Food analysis

Flavonoids are present in foods primarily as glycosides [11]; catechins are an exception in that they occur only as aglycones and gallate esters. To hydrolyze all flavonoids to aglycones, representative samples of typical foods were freeze dried, ground, extracted under refluxing acid conditions and the extracts analyzed with the current HPLC system [1].

Tea was extracted with boiling water, filtered and an aliquot subsequently analyzed.

Four anthocyanidins, delphinidin, cyanidin, petunidin and malvidin, were identified in the extract of blueberries (Fig. 4). We and others [16] have recently discovered that anthocyanidins and catechins are not stable in the acid-reflux conditions employed in these food extractions. Therefore, anthocyanidin concentrations were not calculated for blueberries. We are currently developing hydrolysis conditions for the quantitative recovery of these two subclasses of flavonoids from foods employing a single extraction procedure.

Tea is one of a few foods known to contain large amounts of catechins [14]. By simply boiling tea in water, gallate esters (ECG, EGCG) are not hydrolyzed, and quantification of catechins could be successfully accomplished. The chromatogram of an extract of Dragon Well green tea is shown in Fig. 5. Several compounds, other than flavonoids, known to be present in tea were identified in this extract. The calculated concentrations in g/kg dry tea are: catechin, 2.1; EC, 4.5; ECG, 15; EGC, 15; EGCG, 3.4. These values are within the ranges reported by Lin et al. [17] and Kuhr and Engelhardt [18] for commercial Chinese green tea products. No myricetin or quercetin was detected in this sample, although they have been found in other teas when extracted with 85% aqueous ethanol [19].

Orange juice concentrate was analyzed, showing hesperetin at 0.84 g/kg dry concentrate. Literature values for hesperetin 7-β-rutinoside, or hesperidin, a flavonoid glycoside of significant concentration in orange juice, range from 0.05 to 3.5 g/kg of dry orange juice concentrate [20]. Analysis of parsley flakes showed apigenin was present at 13 g/kg dried parsley. Analysis of onion was processed and showed quercetin at 3.1 g/kg, compared to a literature value of 5.1 g/kg [1].

Analysis of extracts of these few foods demonstrates that the newly developed analytical system for flavonoid aglycones functions successfully. The high resolving power of the Zorbax Eclipse XDB-C18 column in combination with the ternary mobile phase permits flavonoids as well as additional compounds to be separated and quantified in foods (tea as an example) that may be related to health.

4. Conclusions

We have developed an HPLC system for the

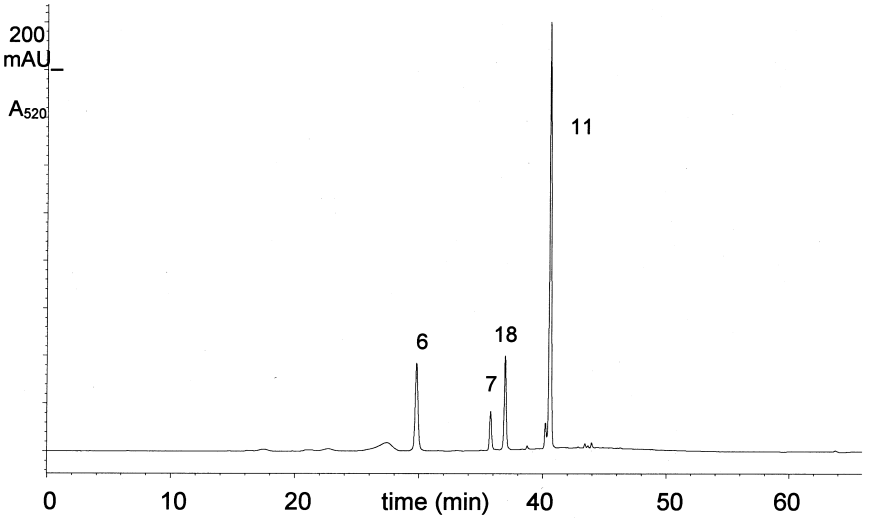

Fig. 4. Chromatogram of extract of blueberries at 520 nm. Chromatographic conditions are described in text. Peak identification for peaks 6, 7, and 11 as in Table 2; peak 18 is petunidin.

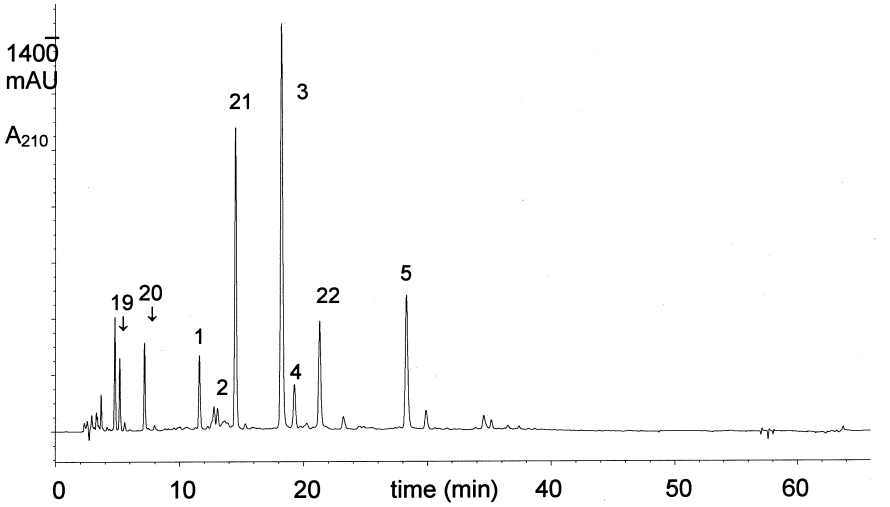

Fig. 5. Chromatogram of extract of Dragon Well Green Tea at 210 nm. Chromatographic conditions are described in text. Peak identification for peaks 1, 2, 3, 4, and 5 as in Table 2. Peak 19 is gallic acid, peak 20 is theobromine, peak 21 is caffeine, and peak 22 is gallocatechin gallate (GCG).

separation and quantification of seventeen flavonoids, as their aglycones, expected to be prominent in commonly consumed foods. The system is based on a highly endcapped, reversed-phase column (Zorbax Eclipse XDB-C18) and a gradient, ternary mobile phase consisting of water, methanol and acetonitrile. All solvents contain 0.05% (w/w) trifluoroacetic acid to reduce peak tailing. Responses of standards were linear over a 6-fold concentration range at 210 nm and selected other wavelengths characteristic of the respective flavonoids. Limits of detection were estimated at 9.3 and 44.8 ng column load based on the variance of repeated analysis of a low concentration of two flavonoids. The utility of the system was demonstrated by analyzing extracts of typical foods which contain representative flavonoids.

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