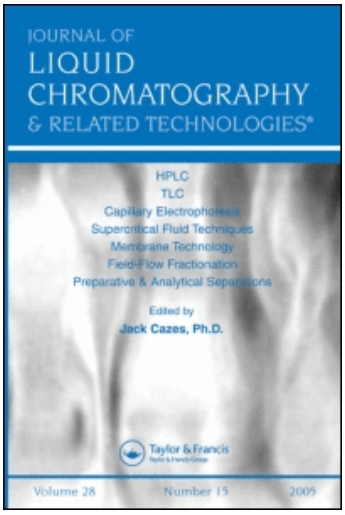

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Analysis of Flavonoids by HPLC: An Update

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ANALYSIS OF FLAVONOIDS BY HPLC: AN UPDATE

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INTRODUCTION

From 1973 to 1982 there was a dramatic increase of interest in the application of HPLC to the study of flavonoids. In 1983, a general review of the use of HPLC in flavonoid analysis by Daigle and Conkerton was published in this journal [1]. This paper extends that review from 1982 to the present.

Several other review papers have been published since 1983 but they are not general applications of HPLC to flavonoid analysis. Nagel [119] described the applications of HPLC to analysis of flavonoids in wine and beer; Harborne [118, 120] compared HPLC to other methods of separation and identification of flavonoids; Hostettmann and Marston [121, 122] discussed a recently developed technique for preparative-scale separation of flavonoids and Strange [123] reviewed the application of HPLC to the analysis of phytoalexins.

In our original review, methoxylated flavone references were listed in a separate section; however, in this review, these compounds are included with the flavones. In addition, at the end of the last two sections of the table, cross-references to citations noted earlier, which include flavones or flavonols, are indicated.

COLUMNS

The initial choice of a column still involves matching the class of flavonoid to be separated to the column capable of providing good retentivity and selectivity characteristics. Although particle size should make a difference, size popularity remains: 10 μm > 5μm > 3μm [70].

Degradation of the column occurs under conditions such as major changes in solvent polarity and pH which are required to remove interfering compounds that are not eluted under the analytical chromatographic conditions. Although reverse phase C_{18} columns are used in most studies, some researchers [9, 12, 13, 34] have used the radial compression separation system (RCSS) which is considered more durable. Others [29, 100] suggested the use of a polystyrene-divinylbenzene column which is stable over a pH range of 1 to 13. A majority of chromatographers use guard columns to extend the life of the analytical column, and an increasing number [e.g., 27, 47, 63, 64, 89] purify the plant extracts by solid phase extraction techniques prior to injection for HPLC analysis.

SOLVENT SYSTEMS

Methanol:water followed closely by acetonitrile:water continued to be the preferred solvent systems. The addition of acetic or formic acid improves the separation and prevents tailing, but systems without acid were successful in a number of studies [e.g. 33, 34, 50, 60, 65, 67].

Ion-pairing was helpful when a methanol:water system was used to separate the neutral glycosides from flavonol sulphates [96]. This technique also proved essential in the separation of flavonoids extracted from Scutellariae radix. In this comprehensive study, a water:acetonitrile system with a variety of tetraalkyl ammonium bromides was investigated [116]. Two geometrical designs for solvent optimization were demonstrated with the separation of flavonoid standards [110, 117] and applied to chemotaxonomic studies [108-9]. An unusual tert-butanol:water (14:86) solvent system was used to separate the flavonoid glycosides of Dryas octopetala [89].

DETECTION

Although the availability and high sensitivity of ultraviolet-visible (UV) detectors make them the choice of most researchers, there has been some experimentation with other types of detection. Isoflavones in silage were analyzed, qualitatively and quantitatively, by the use of UV and fluorescence detectors in sequence. Confirmation of the isoflavone identity was possible by comparison of the UV and fluorescence responses of standards with samples [50, 64, 67]. Kitada et al. showed that amperometric detection was more sensitive in isoflavone HPLC analyses than UV or fluoresence detection [68].

Technology innovation in variable wavelength detectors allowed a choice of wavelength for quantitation of phenolic acids and flavonoids [5, 10, 15-6, 63, 70, 74], and type of flavonoid to be analyzed [9]. Absorbance ratios obtained by two variable wavelength detectors in series served as additional evidence for positive identification of flavonoids and phenolic acids [62, 80]. Both the stop-flow scanning technique [76] and absorbance ratios have been used for checking peak purity as well as for peak identification [35]. However, Law and Das [35] advocated the use of several absorbance ratios rather than relying on a single ratio.

Several other groups [17, 21, 35, 41, 44, 49, 81, 103, 109] have taken advantage of the innovative technology of photodiode-array spectrophotometric detectors for rapid, on-line peak identification. Sophisticated HPLC systems now include computers which control portions of the chromatographic procedure and also the storage of sample spectra for later analysis by comparison with reference compounds. This latest technology was combined with chemical characterization by Hostettmann et al. [36, 43]. In preference to collecting selected eluent fractions for reaction with classical shift reagents and determination of modified spectra in a UV spectrophotometer, Hostettmann, et al. [41] used a post column derivatization system to react column eluents with shift reagents while continuously monitoring spectral changes. Another technological innovation has been the simultaneous display of a contour line

map and three dimensional UV absorbance profile [84]. This technique can be used to detect minor components, even if they are hidden behind larger peaks in the three-dimensional HPLC trace. Hunte's recent combination of a dual-electrode amperometric detector and a photodiode-array detector classified a complex mixture of flavonoids on the basis of their conjugation pattern and hydroxyl substitutions [7].

Derivatization by benzoylation and the use of a photodiode-array detector gave a better estimation of the number of sugar units bound to an aglycone. Also, it allowed the qualitative and quantitative determination of all the glycosides as a result of partial and complete hydrolysis [90].

Separation of Flavonoids and Related Compounds

The flavonoids and the simple acidic phenols extracted from plant tissue were separated effectively by reverse-phase HPLC. The type of flavonoids included: dihydroflavonols and proanthocyanidins [26], proanthocyanidins [30, 32], proanthocyanidins and flavonols [28], and isoflavones [69]. The coumarins and flavones of sagebrush were separated by either an acetic acid:water: acetonitrile or acetic acid:water:methanol system [102]. The main phenolic compounds of olives (oleuropein, verbascoside, rutin, luteolin-7-glucoside) were quantitated [107]. The isoflavonoid phytoalexins and isoflavones of Bengalgram [58] were qualitatively analyzed and the isoflavonoid phytoalexins of soybeans [55, 70] cowpeas [72], and french bean [53] were quantitatively analyzed. Seo and Morr separated phenolic acids from isoflavonoids with a mini column. Although this technique was time consuming, the phenolic compounds were recovered in greater amounts and the resolution of the individual components was improved during HPLC [63].

Other studies reported the retention times of a wide variety of flavonoids and related compound (standards). This information was used to study the polyphenols in commodities such as soybeans [29, 80] and eggplant [75].

Effect of Structure on Retention

Castelle et al. [73] used reverse phase chromatography to make structure/retention time evaluations of 141 flavonoid standards in a single system. They subsequently extended this study to include anthocyanidins, anthocyanins and proanthocyanidins [4]. Synthetic isoflavones were used to determine the effect of the 2- and 6-methyl group, the 6- and 7-methoxyl, and the 7-hydroxyl group on elution time [61]. In a specialized study of quercetin, kaempferol, and myricetin glucosides, the effect of glycosylation and position of glycosyl groups was discussed [96]. More than thirty 5-hydroxyflavones, most of them bearing a tri- or tetrasubstituted A ring, were used to study how different positions of hydroxy or methoxy groups on the flavone nucleus affected HPLC behaviour [112]. Flavanones, flavones, C-glycosylflavones, and flavonols, a majority of them aglycones, were listed by structure and retention time [76].

QUANTITATIVE ANALYSIS

The availability of commercial standards and the use of semi-preparative columns as a means of isolating pure flavonoids from plant material [e.g. 15-6, 31, 69, 89] had a positive impact on quantitation of flavonoids. While peak heights or areas were used for percentages of total flavonoids [3, 38, 87], internal [56, 65] and/or external standards [21, 57, 94-5, 99, 104] more closely defined the concentration of each flavonoid in the various commodities. Most chromatographers, however, used calibration curves [27, 31, 42, 52, 106], some of which were established with an internal [33, 41, 76] or external [8, 10, 17, 22, 103] standard. In medicinal plants, the concentrations of O-glycoside flavonoids were similar when determined by HPLC or TLC. However, data from HPLC determinations of C-glycosides indicated higher concentrations of these compounds than TLC [39].

SUMMARY

The innovative technological developments in HPLC use in recent years has provided the researcher with extremely rapid procedures for the qualitative and

Flavonoid Analysis By HPLC

Flavonoid Type	Column	Mobile Phase	Commodity Studied	Reference
ANTHOCYANIDINS				
	µBondapak C_{18}	water:methanol:acetic acid (71:19:10)	Vaccinium myrtillus L.	2,3
	Lichrosorb RP-18	water:formic acid:methanol (GR[1])	Std.[2]	4
	µBondapak C_{18}	water:methanol:formic acid (73:17:10)	Crowberry (Empetrum nigrum coll.)	5
	Nucleosil-7 C_{18}	methanol:water:$HClO_4$ (50:49.9:0.1)	Rhododendron	6
	Altex C_{18}	0.05m$(NH_4)_3PO_4$ in water:acetonitrile (GR)	Grape Juice	7
ANTHOCYANINS 3-G[3];E[4]	Lichrosorb RP-18	methanol:water:formic acid (49:49:2)	Vitis vinifera	8
3-G;3,5 Di G[5]	Radial Pak C_{18}	1.5% H_3PO_4:20% acetic acid:acetonitrile (GR)	Roses	9
3-G	Aquapose RP-300	water:formic acid:methanol:acetonitrile (GR)	Vaccinium myrtillus L.	10
3-G;E	Lichrosorb 10, RP18	methanol:formic acid:water (50:10:40) (GR)	Petunia hybrida	11
3-G,3, 5 Di G	Lichrosorb RP-18	formic acid:water:methanol (GR)	Std. Geranium Florets Gerbera Flowers	4 12 13
3-G,E	µBondapak	acetic or formic acid:water:methanol (GR)	Wine	14
3-G,E	Spherisorb-Hexyl	0.6% $HClO_4$ in water:methanol (GR)	Wine (Grapes)	15,19,20
3-G	Dynamax C-18 Resolve C_{18}	0.1 M phosphate buffer:acetonitrile (GR)	Blackberry, Cranberry	16
3-G	Hypersil ODS	formic acid:water:methanol (GR)	Cowberry (Vaccinium vites-idaea L.)	17
G; Di G	µBondapak C_{18}	water:methanol:formic acid (73:17:10)	Crowberry (Empetrum nigrum coll.)	5,18
3-G	Lichrosorb RP-18	formic acid:water:methanol (GR)	Saskatoon Berry (Amelanchier Aknifolin Nutt.)	21
3-G	Supelcosil LC-18	methanol:formic acid:water (GR)	Bog Whortleberry (Vaccinium Uliginosum L.)	22
PROANTHOCYANIDINS				
	µBondapak C_{18}	methanol:water (7:3)	Douglas Fir Strawberry and Avocado Leaves	23 24
	Partisil 10 ODS3	acetic acid:water:methanol (GR)	Douglas Fir	25
	Lichrosorb RP-18	water:formic acid:methanol (GR)	Std.	4

	Brownlee RP-8 PXS-C_{18} Ultrasphere C_8	formic acid:water:methanol (GR)	Wine (Grapes)	26
	µBondapak C_{18}	water:methanol:acetic acid (87:8:5)	Cocoa Beans	27
	MCH 10 C_{18}	water:methanol:$HCLO_4$ (GR)	Wine	28
	Hamilton PRP-1	formic acid:water:methanol (GR)	Soybean	29
	Altech C_{18}	water:methanol:acetic acid (GR)	Pears	30
	Altech C_{18}	water:acetic acid (GR)	Barley, Hops, Beer	31
	µBondapak C_{18}	water:methanol:acetic acid (60:40:1)	Saint-John's Wort (Hypericun perforatum L.)	32
	µBondapak C_{18}	water:methanol (2:8)	Cassia Bark	33
	Radial Pak C_{18}	water:acetonitrile (GR)	Grapes	34
	MCH-5	acetonitrile:water:acetic acid (GR)	Uncaria elliptica	35
	Altex C_{18}	0.05 M $(NH_4)_3$ PO_4 in water:acetonitrile (GR)	Grape Juice	7
DIHYDROFLAVANOLS				
	Partisil 10ODS3	acetic acid:water:methanol (GR)	Douglas Fir	25
	Brownlee RP-8 PXS-C_{18} Ultrasphere C_8	formic acid:water:methanol (GR)	Grapes	26
	M9 C_8	methanol:water:formic acid (35:65:0.2)	Grapes	36
G	Lichrosorb 10RP 18	methanol:acetic acid:water (12.5:5:82.5)	Petunia hybrida	11
BIFLAVONOIDS				
	Lichrosorb-Diol	hexane:chloroform:tetrahydrofuran (GR)	Ginkgo biloba	37
C-GLYCOSYLFLAVONES				
A[6], G	Partisil 100 DS	methanol:0.033M KH_2PO_4 in water (GR)	Lupinus sericeus	38
A, G	µBondapak C_{18}	acetic acid:water:acetonitrile (GR)	Crataegus and Passiflora	39
A, G	HC-ODS-Sil X	water:acetic acid:acetonitrile (GR)	Crataegus Oxyacantha L.	40
A, G	Lichrosorb RP-8 Hypersyl RP-8	methanol:water (pH=3) (GR)	Gentiana	41
A, G	Lichrosorb RP-18	water:acetic acid:methanol (50:3:47)	Crataegus	42
A, G	Lichrosorb RP-18	acetonitrite:water:acetic acid (18:82:1)	Cayaponia Tayuya	43

(continued)

Flavonoid Analysis by HPLC (continued)

G	Hypersil ODS	water:acetic acid:acetonitrile (GR)	Fig	44,45
A	μBondapak C_{18}	water:methanol:acetic acid (65:30:5)	Combretum micranthum G. Don	46
A, G	μBondapak C_{18}	isopropanol:tetrahydrofuran:water (5:15:85)	Passiflora incarnata L. Crataegus monogyna	47
A	Senshu Pak SS-1251N	acetic acid:water:acetonitrile (GR)	Swertia Herb	48
A, G	Hypersil RP8	methanol:water:phosphoric acid (GR)	Gentiana	49
ISOFLAVONES				
A, G	Ultrasphere C_{18}	methanol:water (GR)	Soybean	50
A	μBondapak C_{18}	acid:methanol:water (27:73)	Clover	51
A, G	Partisil-10ODS-2	methanol:water (2.1:1)	Clover	52
G	Partisil 10 ODS	methanol:0.033M KH_2PO_4 in water (GR)	Lupinus sericeus	38
P	Lichrosorb-SI 100	hexane:chloroform:methanol (GR)	French bean (Phaseolus vulgaris)	53
A	μBondapak C_{18}	water:phosphoric acid:acetonitrile (GR)	Ononis Spinosa L.	54
A, P[7]	Hitochigel 3053	acetonitrile:water (40:60)	Soybean	55
A, G	Zorbax ODS	methanol:water (GR)	Soybean	56
A, G, E, P	Lichrosorb RP8 (RP18)	water:acetic acid:acetonitrile (GR)	Cicer arietinum	57, 58
G	Aquasil 452N	chloroform:methanol:ethanol:water (62:16:16:6)	Pueraria lobata	59
A, G	Partisil ODS-3	methanol:water (GR)	Soybean	60,66
A	Lichrosorb RP-18	methanol:water (7:3)	Std.	61
A	Lichrosorb RP-18	acetonitrile:water (GR)	Clover	62
A, G	Ultrasphere ODS	water:methanol:acetic acid (GR)	Soybean	63
A	μBondapak C_{18}	58% methanol in 10 mM phosphate buffer (pH=6.5)	Animal Feed	64
A	Hamilton PRP-1	formic acid:water:methanol (GR)	Soybean	29
A, G	Zorbax ODS	methanol:water (8:17)	Pueraria Root	65
A, G, E	Partisil ODS-3	methanol:water (GR)	Soybean	67
A, G	Irica ODS	acetonitrile:KH_2PO_4 buffer	Puerariae radix	68

A	Ultrasphere ODS	methanol:water (GR)	Soybean (root and leaves)	69,71
P	Ultrasphere ODS	methanol:water (GR)	Soybean	70
P	Si 100 Polyol RP 18	methanol:water:acetic acid (GR)	Cowpea	72
FLAVANONES				
A, G	Lichrosorb RP-18	water:formic acid:methanol (GR)	Std.	73
A, G	C_{18}	acetic acid:methanol:n-butanol (GR)	Prunus ovium L. P. cerasus L.	74
A	μBondapak C_{18}	methanol:acetic acid:water (GR)	Std.	75
A, G	Ultrasphere C_8	methanol:acetonitrile:water:acetic acid (GR)	Citrus, Std.	76
G	Lichrosorb 10 RP-18	methanol:acetic acid:water (30:5:65)	Petunia hybrida	11
A	Zorbax ODS	water:acetonitrile (GR)	Citrus natsudaidai	77
A	Hibar RP-18	acetonitrile:water (GR)	Citrus Juice	78
A, C8/	Cosmosil 5 C_{18}	acetonitrile:water (9:1)	Std.	79
A, G	Ultrasphere ODS	water:acetate buffer:methanol:n-butanol (GR)	Soybean, Std.	80
G	Spherisorb ODS II	tetrahydrofuran:water:methanol (GR)	Salix purpurea and daphnoides	81
A	Develosil ODS-5	tetrahydrofuran:dioxane:methanol:acetic acid:phosphoric acid 5%:water (145:125:50:20:2:658)	Scutellaria baicalensis Georgi	82
G	Hypersil ODS	variety of solvent systems	Orange, Grapefruit	83
	Senshu Pak 7C18H	chloroform:methanol:water:25% ammonia (200:30:1.L:0.3)	Licorice	84
A	MCH-5	acetonitrile:water:acetic acid (GR)	Uncaria elliptica	35
FLAVONOLS				
A	Altex C_{18}	methanol:water (45:55)	Eriastrum densifolium	85
A	ODS-HC-SIL-X-1	water:methanol:acetic acid (60:75:5)	Propolis	86
A,G	μBondapak C_{18}	water:acetic acid:tetrahydrofuran	Hops	87
G	MCH-10	methanol:citrate buffer (1:1)	Casimora edulis	88
G	μBondapak RP-18	water:tert-butanol (86:14)	Dryas octopetala	89
A, G, E	Lichrosorb Si 60	isooctane:diethyl ether:acetonitrile (150:90:30)	Std.	90

(continued)

Flavonoid Analysis by HPLC (continued)

G, E	Ultrasphere ODS Lichrosorb Si 60	acetic acid:water:acetonitrile isooctane:diethyl ether:acetonitrile	Red and Black Currants	91-2,98,105
G	μBondapak C_{18}	methanol:water:acetic acid (GR)	Leucaena leucocephala	93
A, G	Zorbax C_8	water:phosphoric acid:acetonitrile	Vicia faba (leaves)	94
A	μBondapak C_{18}	water:acetic acid:methanol (42:8:50)	Onion Lettuce, Kale, Chive, Garlic, Leek, Horseradish, Red Radish, Red Cabbage Blueberry, Cranberry, Blackberry	95 99 104
A, S[9], G	Spherisorb S5 ODS-2	methanol:water:acetic acid: .01N tetra-butylammonium phosphate (GR)	Oenanthe crocata Foeniculum valgare	96
A	ODS-HC-SIL-X-1	water:acetic acid:methanol (55:5:45)	Helianthemum squamatum	97
G	μBondapak C_{18}	methanol:water	Std.	100
A, G	μBondapak C_{18}	methanol:acetic acid:water (45:3:52)	Larch Bark	101
A	μBondapak C_{18}	acetic acid:water:acetonitrile (GR) acetic acid:water:methanol (GR)	Sagebrush (Artemisia)	102
G	Spherisorb C_8	methanol:acetic acid:water (GR)	Chondropetalum	103
A	Novapak C_{18}	water:methanol:acetic acid (65:30:5)	Wine	106
G	MCH-5	acetonitrile:water:orthophosphoric acid (GR)	Olive	107
G	Spherisorb ODS II	acetic acid:tetrahydrofuran:acetonitrile: water (GR)	Betulae folium	108
		see references:6-7, 9, 12, 13, 28, 29, 35, 38, 39, 42, 54, 74.		
FLAVONES				
A	Spherisorb ODS II	methanol:acetonitrile:tetrahydrofuran:water (2.5:2.03:11.56:83.91)	Stachys recta; Std.	109,110
A, G	Aquapore RP-3000	methanol:acetic acid:water (GR)	Sugar Cane	111
A	C_{18}	water:formic acid:acetonitrile (GR)	Sideritis, Std.	112, 113, 114
A	HC-ODS-SIL-X-1	methanol:water (GR)	Sideritis	115
A	TSK gel LS-410(ODS)	water:acetonitrile (68:32)-5mM tetra-n-amylammonium bromide; pH=4 phosphoric acid	Scutellariae radix	116
A	Lichrosorb RP-18	methanol:water:tetrahydrofuran:acetonitrile (solvent study) See references: 35, 38, 74, 82, 101, 107.	Std.	117

1/ GR=gradient 2/ Std.=Standards 3/ G=glycosides 4/ E=esters 5/Di G=diglycoside 6/ Aglycones 7/ Phytoalexins 8/ C =Chalcones 9/ Sulphates

quantitative analysis of flavonoids. The current sensitivity of this technique allows for the use of extremely small amounts of material-nanogram or picogram quantities can easily be used. The initial cost of HPLC instrumentation is still high, but small-scale, solid-phase extraction clean-up techniques coupled with inexpensive guard columns have extended the life of expensive analytical columns. As it becomes practical to resolve the more complex phenolic mixtures in plants by various combinations of these rapid, sensitive methods, HPLC may become the technique of choice for flavonoid analysis.

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