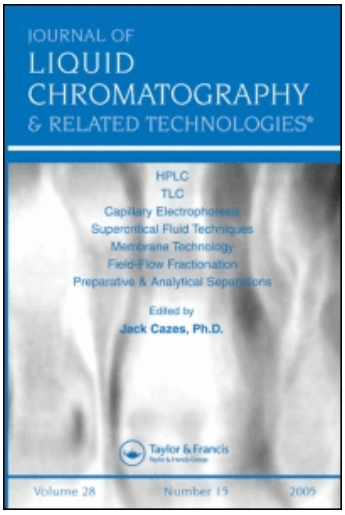

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Analysis of Flavonoids by HPLC

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ANALYSIS OF FLAVONOIDS BY HPLC

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INTRODUCTION

During the past ten years there has been a dramatic increase of interest in the field of flavonoid chemistry. This was mainly due to the application of HPLC to the study of flavonoids. These compounds, natural plant constituents with a structure based on the aromatic heterocycle, 3-phenylbenzopyrone, are ideally suited for analysis by HPLC. The basic complex structure can be varied by the number and position of hydroxyl substituents and other derivatives (sugars, methyl). These variations cause changes in the way the compounds react to solvents and columns commonly used in HPLC allowing separation and identification.

Since 1979 some pertinent and excellent reviews have been reported: Kingston [88], the application of HPLC to secondary metabolites; Adams and Nakanishi [89], selected examples of HPLC separation of natural products; Van Sumere et al [90], use of HPLC in the separation of plant phenolics; Roston and Kissinger [91], HPLC determination of phenolic acids of vegetable origin;

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Schwartz and von Elbe [92], HPLC of plant pigments; Rouseff and Ting [93], analysis of polymethoxyelated flavones in citrus; Hrazdina [94], analyses of anthocyanins in fruits and beverages; and K. and M. Hostettmann [95], the application of HPLC techniques to flavonoids analysis.

SILICA GEL COLUMNS

Various types and brands of columns have been used for flavonoid analysis using isocratic or gradient solvent systems. Early work was done on silica gel columns with and without derivatization prior to analysis. Although a major advantage of HPLC is the lack of derivatization prior to analysis, Hermann and co-workers cited several advantages to HPLC analysis of acetylated flavonoids. Among these were: durability of the column, definite identification and quantitation, and isocratic separation of the flavonoids. The isocratic run does not require solvent re-equilibration for each sample and this increases the rate of analysis.

Although silica gel columns have not been used often, they are well suited for the separation of non-polar or weakly polar flavonoid aglycones such as: polymethoxylated flavones [86,87], isoflavones [38,48,49,52], and biflavonoids [38]. The normal phase liquid chromatography on Lichrosorb Si 60 of the acetates of numerous flavonoids permitted the application of flavonoid analysis to celery [77-79], orange juice [81,82], tomatoes [79], plums [80], and cherries [83].

Reverse Phase Versus Normal Phase. By using the above discussed packing materials, (columns) the stationary phase is less polar than the mobile phase and the procedure is called reverse phase chromatography (RPLC). Thus highly polar solutes possess shorter retention times than less polar solutes. The retention time of the same polymethoxylated flavones shows this difference between RPLC [84,85] and normal phase HPLC (NPLC) [86]. However, with both types of columns, excellent separation was obtained in less than 30 minutes.

The use of RPLC [46] for the resolution of isoflavones was criticized [48] for the observed band spreading which would make the separation of multicomponent mixtures virtually impossible. A number of recent reports [53-56], however, have established a RPLC procedure for the separation and quantitation of the naturally occurring soybean isoflavone glycosides and aglycones.

REVERSE PHASE COLUMNS

In reverse phase columns, the stationary phases are prepared by bonding various organosilane molecules to the hydroxylic groups of a silica type surface. The most common of the organosilanes are octadecyltrichlorosilane, octyltrichlorosilane, and phenyltrichlorosilane. The simple procedures for preparing octadecylsilyl bonded stationary phases have been described [96,97] and may be employed, for example, for the preparation of preparative scale columns. Currently there are a number of commercially available columns possessing a high degree of reproducibility as reflected in the table.

Although the C_{18} or more specifically the μBondapak C_{18} column has been the dominant choice for RPLC of flavonoids, another type of column may be better dependent upon the class of flavonoid. In a comparison of Lichrosorb RP-18 and RP-8, Strack and Krause [41] obtained better resolution of glycosylflavone aglycones and glycosides on the RP-8 with a gradient methanol : acetic acid : water solvent system. Becker et al [39] in the only reported use of a Lichrosorb NH_2 column, achieved an excellent separation of isomeric O-glycosides of C-glycosylflavones. This type of column had been used for the separation of monosaccarides and for this reason chosen as the ideal phase for separation of C-glycosylflavones. There seemed to be no apparent differences in efficiency of separation of the flavonoids of Silybum marianum between the Lichrosorb RP-18 [33] and RP-8 [34,35] columns. However, two different solvent systems were used and an objective comparison cannot be made.

A μBondapak C_{18} with a acetonitrile : water solvent system failed to adequately separate methoxylated flavones but a Zorbax C_8 column with a tetrahydrofuran : water system did [84].

The difference in columns with the same generic name and a lack of consistent specifications make the choice of reverse phase columns difficult. The initial choice of a column for a particular reverse phase separation would involve matching the type of substance (class of flavonoid) to be separated to the column capable of providing good retentivity and selectivity characteristics toward the particular sample. Because few papers contain negative information on columns and this field is relatively new, the researchers should be aware of any available commercial literature [98].

Aromatic Acids and Flavonoid Separation. Extraction of phenolics from plant tissue usually means that both single ring phenolics and the flavonoids (C_6-C_3-C_6 carbon skeleton) are isolated in the same mixture. Two reports [72,74] advised carrying out prior separation of these groups for HPLC. Where a μBondapak alkylphenyl column was used, some of the aromatic acids and phenols interfered with separation of the flavonoids [72]. However, RPLC on a μBondapak C_{18} column proved to be effective in the separation of both the plant phenols and flavonols of tobacco [65]. A gradient elution system was found to be most effective and necessary for the separation of the aromatic acids, phenols, and flavonoids in the same mixtures [63]. There are also two other communications that have dealt with the separation of aromatic acids, phenols, and a few flavonoid compounds [66,67].

Solvent Systems. Methanol : water containing small amounts of acetic acid is one of the more commonly used solvent systems for RPLC of flavonoids. The addition of acetic or any acid improves the separation, but the amount of acid or pH of the eluting system is dependent upon the column's stability as a Chrompack Nucleosiel C_{18} column ruptured probably due to a low pH [31]. In lieu of acetic acid other compounds which have been used are: phosphoric acid

[34,35,50,64,71], perchloric acid [16,21,24], potassium dihydrogen phosphate [5,22,61,65], ammonium dihydrogen phosphate [36], and formic acid [4,7]. The absence of acid in the methanol : water system is rare [42] but such a solvent system appears to be very successful in the separation of isoflavones [51,53-56].

The acetonitrite : water system was successful in the quantitative determination of naringin and hesperidin in citrus juice [58-60,62] and in the separation of both isomeric C-glycosylflavones [39] and isomeric isoflavones [46]. The acetonitrite : acetic acid : water solvent system was most popular for the separation of flavonoids of flowers [44,70].

Other solvent systems use in RPLC are: tetrahydrofuran (THF) : acetonitrile : water [86]; THF : water [45]; acetone : acetic acid : water [8,9,11]; methanol : dimethylformamide : acetic acid : water [29]; and ethanol : acetic acid : water [72].

Effect of Structure on Retention. The effect of structure of flavonoids on their elution behaviour was first disclosed by Wulf and Nagel [57]. These data were confirmed and extended by Daigle and Conkerton [37]. In both cases a μBondapak C_{18} and a methanol: acetic acid : water solvent system was used. The influence of isomerization and the glycosylation pattern of some C-glycosylflavones on retention time was studied [40] and some of these findings were confirmed in a later report [43]. The chromatographic behavior of proanthocyanidins was investigated using several packing materials (columns) [21,24]. Lea concluded that RPLC offered the greatest potential for the separation of proanthocyanidins.

GENERAL

Detection. The high sensitivity of ultraviolet-visible detectors make analysis of sub-microgram samples possible. The wavelength must be compatible with the solvent system and, of course, suitable for the compound to be

Flavonoid Analysis By HPLC

Flavonoid Type	Column	Mobile Phase	Commodity Studied	Reference
ANTHOCYANIDINS	μBondapak C_{18}	methanol:acetic acid:water (20:5:75)	Std.[1]	1
	μBondapak C_{18}	methanol:acetic acid:water (19:10:71)	Muscadine grape skins (Vitis rotundifolia) Roselle (Hebiscus sabdariffa)	2
ANTHOCYANINS				
3-G[2] (AC)[3]	Pellidon	chloroform:methanol (87:13)	Grapes	3
3-G, E[4]	Lichrosorb ODS	10% formic acid in water:methanol (GR[5])	Grapes (Cabernet Sauvignon and Pinot noir)	4
3-G	Lichrosorb RP-18	0.1 M KH_2PO_4 in water:methanol (GR)	Wine	5
3-G; 3,5-Di G[6], E	μBondapak C_{18}	acetic acid:water (15:85) 0.1% H_3PO_4 in acetic acid:water (10:90) methanol:acetic acid:water (20:15:65)	Grapes (concord, Ives, DeChaunac)	6
3-G, E	Lichrosorb ODS	10% formic acid in water:methanol (GR)	Wine (Merlot and Cabernet Sauvignon grapes)	7
3-G	μBondapak C_{18}	acetone:acetic acid:water (7:10:83)	Poinsettia (Amy, V-10)	8
		" " " " (")	Poinsettia (28 cultivars)	9
3-G	μBondapak C_{18}	methanol:acetic acid:water (37:10:53)	Cranberry	10
3-G, 3,5-Di G	μBondapak C_{18}	acetone:acetic acid:water (7:10:83)	Rhododendron Simsii Planch (Azalea Indica L.), 7 cultivars	11
A	Lichrosorb RP-8	acetonitrile:acetic acid:H_3PO_4:water (GR)	Gladiolus	12
3-G; 3,5-Di G; E	Lichrosorb RP-18	acetonitrile:acetic acid:H_3PO_4:water (GR)	Std.	13
3-G, 3,5-Di G; E	μBondapak C_{18}	10% formic acid in water:methanol (GR)	Perilla	14
3-G; 3,5-Di G; E	Whatman ODS	methanol:formic acid 5 or 10%:water (GR)	Wine (Zinfandel and Cabernet Sauvignon)	15
3-G, Chalcones	Spherisorb Hexyl	0.6% $HClO_4$ in water:methanol (GR)	Std.	16
3-G, 3,5-Di G	Lichrosorb RP-18	acetonitrile:acetic acid:H_3PO_4:water	Gladiolus	17
Proanthacyanidins	VYDAC	hexane:methanol:chloroform:acetic acid (GR)	Beer	18,19
	SiC_{18} (RSL-Belgium)	acetic acid:water (GR)	Beer	20
	SAS Hypersheres	methanol:water:$HClO_4$ (20:80:0.1)	Cider	21
	Spherisorb S5-ODS	methanol:0.075 M KH_2PO_4:water (GR)	Beer	22
	Zorbax ODS	1% acetic acid in water:acetonitrile (GR)	Wine (Merlot and Cabernet Sauvignon)	23
	Spherisorb Hexyl	.1% $HClO_4$ in water:methanol (GR)	Cider	24
	SiC_{18} (RSL-Belgium)	acetic acid:water (GR)	Barley	25

	μBondapak C_{18}	methanol:water (GR)	Barley (Hordeum vulgare, 10 cultivars) Hops (Humulus lupulus, 8 cultivars)	26
	Lichrosorb RP-8 Lichrosorb Si 60	methanol:water (GR) tetrahydorfuran:methanol:acetic acid:hexane (1:3:0.4:4)	Beer Sorghum	27 28
theaflavins	μBondapak C_{18}	methanol:dimethylformanide:acetic acid:water (2:1:40:157)	Tea	29
	Partilsil 5 C_{22}	methanol:acetone:water (GR)	Tea	30
Dihydroflavonols	μBondapak C_{18}	methanol:0.1% acetic acid:water (GR)	Larix (7 species)	31
	μBondapak C_{18}	methanol:acetic acid:water (40:5:60)	Silybum marianium Gaertn.	32,33
	Lichrosorb RP-18	methanol:acetic acid:water (40:5:60)	Silybum marianum Gaertn.	33
	Lichrosorb RP-8	0.02 M H_3PO_4 in water:methanol (GR)	Silybum marianum	34,35
	Partisil-10 ODS	2% $NH_4H_2PO_4$ in water:methanol (1:1)	Virginia pine (Pinus virginiana Mill.)	36
	μBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
Biflavonoids	Pellosil-HC	isopropylether containing 8% methanol	Std.	38
C-Glycosylflavones				
G	Lichrosorb NH_2	acetonitrile:water (GR)	Std.	37
A,G	Zorbax ODS	methanol:0.1% acetic acid:water (GR)	Larix (seven species)	31
	Zorbax ODS	0.1M H_3PO_4 in water:ethanol (GR)	Std.	40
A,G	Lichrosorb RP-8	methanol:acetic acid:water (GR)	Duckweed (Spirodela), Buchwheat (Fagopyrum)	41
A,G	Lichrosorb RP-18			
A	Zorbax ODS	methanol:water (GR)	Passiflora incarnata	42
A,G	Lichrosorb RP-8	methanol:acetic acid:water (GR)	Oats (Avena sativa)	43
A	μBondapak C_{18}	acetonitrile:acetic acid:water (GR)	Passion Flower; Hawthorn	44
A,E	Copell ODS	tetrahydrofuran:water (4:53)	Zizyphus vulgaria var. spinosus	45
Isoflavones				
A	Merkosorb Si 60	hexane:tetrahydrofuran (2:1) or (9:1)	Std.	38
A	Partisil-10 ODS	acetonitrile:water (1:4)	Soybeans	46
GAc	μBondapak C_{18}	methanol:water (GR)	Soybeans	47
A	μPorasil	dichloromethane:ethanol:acetic acid (97:3:2)	Soybeans :hexanes (8:2)	48,49

(continued)

Flavonoid Analysis By HPLC

Flavonoid Type	Column	Mobile Phase	Commodity Studied	Reference
A	Lichrosorb RP-8	water (pH = 2.8, H_3PO_4):acetonitrile (GR)	Std.	50
G	μBondapak C_{18}	methanol:water (GR)	Soybeans	51
A	μPorasil	dichloromethane:ethanol:acetic acid (97:3:2):hexanes (8:2)	Garden peas (Pisum sativum)	52
A,G	Zorbax ODS	methanol:water (GR)	Soybeans	53,54,55
A	Sperisorb-5 ODS	methanol:water (GR)	Bengalgram	56
Flavanones				
A,G	μBondapak C_{18}	methanol:acetic acid:water (30:5:65)	Std.	57
G	μBondapak C_{18}	acetonitrile:water (20:80)	Grapefruit	58,59,60
A,G	Zorbax ODS	methanol : 0.1%:acetic acid:water (GR)	Larix (7 species)	31
A,G	μBondapak C_{18}	0.03M KH_2PO_4 in water:methanol (GR)	Orange	61
G	Lichrosorb RP-18	acetonitrile:water (20:80)	Orange, Grapefruit	62
A	μBondapak C_{18}	butanol:methanol:acetic acid:water (5:25:2:6)	Soybeans (Glycine max L., Forrest)	63
A,G	μBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
Flavonols				
A,G	μBondapak C_{18}	methanol:acetic acid:water (30:5:65)	Std.	57
G,E	Zorbax ODS	ethanol:0.1% H_3PO_4:water (GR)	Cedrus atlantica c.v. Glauca	64
A,G	μBondapak C_{18}	0.1N KH_2PO_4 in water:methanol (GR)	Tobacco (Nicotiana tabacum L.)	65
G	Zorbax ODS	methanol:0.1% acetic acid:water (GR)	Larix (7 species)	31
G	μBondapak C_{18}	2% acetic acid in water:tetrahydrofuran (GR)	Poinsettia (2 cultivars)	66
G	μBondapak C_{18}	methanol:water:acetic acid:tetrabutylammonium-phosphate (GR)	Std.	67
G	Zipax HCP	0.5M KH_2PO_4:ethanol:ethyl acetate (GR)	Geranium Thunbergii Sieb. et Zucc.	68
G	Zorbax ODS	methanol:water (GR)	Tetrapanax papyriferum	69
G	μBondapak C_{18}	2% acetic acid in water:acetonitrile (GR) 2% acetic acid in water:tetrahydrofuran (GR)	Poinsettia (38 cultivars)	70
G,E	Zorbax ODS	methanol:0.1% H_3PO_4:water (GR)	Larix (L. gmelinii)	71
A	μBondapak alkylphenyl	ethanol:acetic acid:water (47.5:5.0:47.5)	Std.	72

A	Partisil-10 ODS	methanol:NH_4 H_2PO_4:water (49:1:49)	Virginia pine (Pinus virginiana mill.)	36
A	μBondapak C_{18}	methanol:acetic acid:water (GR)	Opuntia (fulgida, spinosior, and acarthocarpa	73
A,G	Lichrosorb RP-8	water (pH = 2.8, H_3PO_4):acetonitrile (GR)	Std.	50
G	Zorbax ODS	1% acetic acid in water:acetonitrile (GR)	Wine (Merlot and Cabernet Sauvignon)	23
A	μBondapak C_{18}	acetonitrile:acetic acid:water (30:2:68)	Pine (Pinus elliotti)	74
A,G	μBondapak C_{18}	2.5% acetic acid in water:tetrahydrofuran (GR)	Hops (10 cultivars) and Barley (8 cultivars)	26
A,G,E	HC-ODS-Sil-X	2% acetic acid in water:acetonitrile (GR)	Matricaria chamomilla L.	75
A,G	μBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
Flavones				
A	μBondapak C_{18}	methanol:acetic acid:water (30:5:65)	Std.	57
A,G	Zorbax ODS	methanol:0.1% acetic acid:water (GR)	Larix (7 species)	31
A,G	μBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
G	μBondapak phenyl	methanol:acetic acid:water (GR)	Sugar cane	76
Flavonoid Acetates flavonols, flavonones, and their G	Lichrosorb Si 60	benzene:acetonitrile (85:22) benzene:acetone (90:15) benzene:ethanol (80:0.7)	Celery, Orange, and Tomatoe	77,78,79
flavonol G	Lichrosorb Si 60	benzene:acetonitile (80:20)	Plum	80
flavanone G	Lichrosorb Si 60	benzene:acetonitrile (85:20)	Orange	81,82
flavonols and G	Lichrosorb Si 60	isooctane:ethanol:acetonitrile (70:16:5.5) benzene:acetonitrile (80:20)	Cherries (Prunus avium L. and Prunus cerasus L., 7 cultivars)	83
Methoxylated Flavones				
A A	Micropak C_{18} Zorbax C_8	acetonitrile:water (40:60) tetrahydrofuran:water (25:75)	Orange (Valencia), Tangerine (Dancy)	84
A	Zorbax C_8	tetrahydrofuran:acetonitrile:water (22:6:72)	Orange	85
A	Lichrosorb Si 60	heptane:isopropanol (60:40)	Orange, Tangerine	86
A	Lichrosorb Si 60	heptane:ethanol (90:10) and (75:25) heptane:isopropanol (70:30) and (60:40)	Orange, Tangerine	87

1/ Std. = Standards 2/ G = glycosides 3/ Ac = acetates 4/ E = esters 5/ GR = gradient 6/ Di G = diglycosides 7/ Aglycones

detected at maximum sensitivity. For the flavonols, flavanones, flavones, and isoflavones and their respective glycosides, the range of wavelength between 254 nm and 280 nm was the most popular. A few researchers have also chosen the 340 nm to 360 nm range [8,26]. The anthocyanins and proanthycyanidins were detected either in the range between 520 nm and 546 nm or at 280 nm. The acetate derivatives of anthocyanidins were detected at 254 nm [3] and their chalcones at 340 nm [16]. The acetates of flavonols, and flavones and flavanones, however, were detected at 300 nm (77-83). Rouseff and Ting [10,93] employed a dual UV-fluorescence detector to determine the presence of interfering substances. The wavelength, 313 nm, was for all five polymethoxylated flavones since the impurity absorbed weakly at this wavelength. The C-glycosylflonones have been detected at a variety of wavelengths: 254 nm [39,40]; 270 [42]; 312 [41]; 330 [45]; 335 [22]; and 365 [43].

Qualitative Analysis of Flavonoids. In the HPLC analyses of complex mixtures researchers have used more than retention time data to identify, positively or negatively, the compound of interest. However, off line techniques are relatively time consuming. Recently on line techniques such as HPLC-UV/Vis and HPLC/MS spectroscopy have been found, in some cases, to be sufficient to obtain postive identification of compounds with an overall reduction in analyses time [50].

The identification of the chalcones of malvidin 3-glucoside and malvidin 3,5-diglucoside was carried out by using detectors monitoring simultaneously first at 280 nm and 525 nm, then 280 nm and 340 nm. As a result, these chalcones could be collected in sufficient amounts to measure their UV-visible absorption spectra directly and to follow their conversion to their corresponding flavylium cations [16].

SUMMARY

In the flavonoid field, HPLC has been used for the quantitative determination of plant constituents, purity verification of isolated compounds, and

chemotaxonomical comparisons. The practical applications in the beer, wine, and citrus industries also attest to its potential as an analytical tool.

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