BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 42 2333—2336 (1969)

An Automatic Chromatographic Method for the Separation of Flavonoid Compounds

Masatake HORI

Research and Development Division, Takeda Chemical Ind., Ltd., Juso, Higashiyodogawa-ku, Osaka

(Received Junuary 6, 1969)

A new chromatographic method for the separation of flavonoids was developed using Amberlite XAD-2 adsorption resin as the stationary phase and a gradient system of ethanol-water as the eluent. The advantage of the method lies in the fact that a number of flavonoids can be separated by a single operation with good reproducibility. The accuracy of this method determined with pure flavonoids was within ±2%. In addition, the method is useful for preparative purposes, because the solvents used are readily removable by evaporation. The elution time of 56 flavonoids was determined by this method; moreover, the method was applied to the determination of flavonoids in crude methanol extracts from plants (*Scutellaria baicalenisis, Citrus aurantium* and *Crataegus cuneata*).

The chemistry of flavonoids has been a subject of interest for many organic chemists, and hence a number of the compounds have been recorded, which differ considerably in structure. In general, flavonoids can be separated by the chromatography on Magnesol, silica gel, cellulose powder, or alumina, *etc.*[1] These adsorbents, however, have to be selected for each experiment, because the polarity of flavonoids differs considerably among these compounds. Moreover, the adsorbents mentioned above have to be refilled in each experiment.

In this paper the author wishes to report a new method for the separation of flavones, flavonols, flavanones, and flavanonols, *etc.* by the use of an Amberlite XAD-2 column an dethanol-water as an eluant, with a gradient increase of the ethanol concentration from 20% to 90%.

Experimental

Instruments. All the experiments in this work were carried out using Beckman Model-130 Spectrochrom Analyzer.

Procedure. A glass column (9×1600 mm, with jacket) was packed with Amberlite XAD-2 adsorption resin (200—400 mesh) up to 1500 mm in height, and was kept at 45°C by circulating water in the jacket, except when investigation of the effect of column temperatures was made. Then the column was allowed to equilibrate with 20% ethanol at a flow rate of 60 ml/hr.

A solution of two to four flavonoids, each less than 500 μg in 1 m*l* of 20% ethanol solution, was placed on top of the column. If the sample was sparingly soluble in 20% ethanol, a suspension of the sample in the same solvent was applied. In the case of morin, the UV-absorbancy of which is very low, 2 mg of the sample was applied; by the same reason myricetin was applied as much as 20 mg. As for the solvent, 100 m*l* of 20% ethanol was run first; a successive linear gradient elution then followed with a total volume of 1 *l* raising the ethanol concentration from 20 to 90%. The eluate was determined automatically at three wavelengths, 270, 300 and 330 mμ, and collected by a fraction collector every ten minutes.

Materials. The flavonoids used in this study, except morin (E. Merck), myricetin (Fluka A. G.), naringenin (Tokyo Kasei), were all supplied by courtesy of Drs. S. Imai and N. Morita.*1

Methanol extracts of *Scutellaria baicalensis, Citrus aurantium* and *Crataegus cuneata* were prepared by repeated extraction of the dried plants with methanol at 70°C, followed by evaporation of the solvent. The residue was then dissolved in 20% ethanol and subjected to chromatography. The yields of extracts are shown in Table 1.

Results and Discussion

As is clear from theoretical plate concept in chromatography that the use of smaller granules as a stationary phase results in more efficient separation of the peaks, the resin used in this investigation was granulated and sieved in water and 200—400 mesh granules were collected. It is also known that the temperature of a column affects the separation and the elution time of the peaks. By a preliminary investigation, it was found that the peaks are tailing at low temperatures such as 25°C and the elution time becomes shortened with the rise in temperature (Fig. 1). However, the temperature of the column was maintained at 45°C to prevent bubble formation in the column at higher temperatures.

The chromatograms obtained with 56 authentic

1) M. K. Seikel, "The Chemistry of Flavonoid Compounds," ed. by T. A. Geissman, Pergamon Press, New York (1962), p. 34.

*1 Faculty of Pharmacology, Toyama University.

Table 2. Flavonoids used in this investigation

Sample	Name	Type of compound*	$\frac{E_{270}\,m\mu}{E_{300}\,m\mu}$	$\frac{E_{330}\,m\mu}{E_{300}\,m\mu}$	Sample	Name	Type of compound*	$\frac{E_{270}\,m\mu}{E_{300}\,m\mu}$	$\frac{E_{330}\,m\mu}{E_{300}\,m\mu}$
1	Apigenine-7-glucuronide	E	1.55	2.16	29	Phellamurin	G	0.79	0.34
2	Scutellarin	E	1.38	2.10	30	Morin	A	3.13	0.88
3	Nelumboside	E	1.48	1.43	31	Aromadendrin	A	0.56	0.38
4	Baicalin	E	1.77	1.10	32	Myricetin	A	1.79	1.41
5	Homoorientin	G	1.80	1.82	33	Linarin	G	1.07	1.61
6	Saponarin	G	1.66	1.86	34	Pectolinarin	G	1.44	2.04
7	Robinin	G	1.79	1.79	35	Cirsimarin	G	2.28	2.81
8	Rutin	G	1.13	1.43	36	Pedalitin	A	1.32	1.98
9	Scoparin	G	1.44	1.72	37	Chrysosplenin	G	1.72	1.82
10	Lonicerin	G	1.75	1.85	38	Fisetin	A	0.89	1.42
11	Rhoifolin	G	1.69	2.00	39	Fukugetin	G	1.12	0.85
12	Hyperin	G	1.49	1.49	40	Chrysosplenetin	A	1.32	1.90
13	Liquiritin	G	1.79	0.57	41	Quercetin	A	1.67	1.33
14	Tagetin	G	2.32	2.57	42	Amurensin	G	2.28	1.50
15	Pedaliin	G	1.68	2.07	43	Luteolin	A	1.43	1.71
16	Plantaginin	G	1.31	2.10	44	Neolinarin	G	1.38	2.10
17	Quercitrin	G	0.60	0.53	45	Genistein	A	2.11	0.36
18	Luteolin-7-glucoside	G	1.73	1.87	46	Naringenin	A	0.67	0.34
19	Trifolin	G	1.49	1.31	47	Kaempherol	A	1.91	1.67
20	Narcissin	G	1.57	1.52	48	Apigenin	A	1.20	1.54
21	Avicularin	G	1.53	1.53	49	Hesperetin	A	0.65	0.31
22	Kaempheritrin	G	1.45	1.36	50	Baicalein	A	1.96	1.30
23	Bakkoside	G	1.56	1.79	51	Cirsimaritin	A	1.41	2.19
24	Reynoutrin	G	1.39	1.33	52	2-Methylthio-chromone	A	2.04	5.11
25	Hesperidin	G	1.84	0.53	53	Wogonin	A	1.62	0.64
26	Apiin	G	1.37	1.79	54	Swertianol	A	1.79	1.59
27	Cosmosiin	G	1.27	1.68	55	Flavone	A	0.49	0.18
28	Juglanin	G	1.33	1.34	56	Euparin	A	2.92	0.37

* A: Aglycone, G: Glycoside, E: Sugar acid ester.

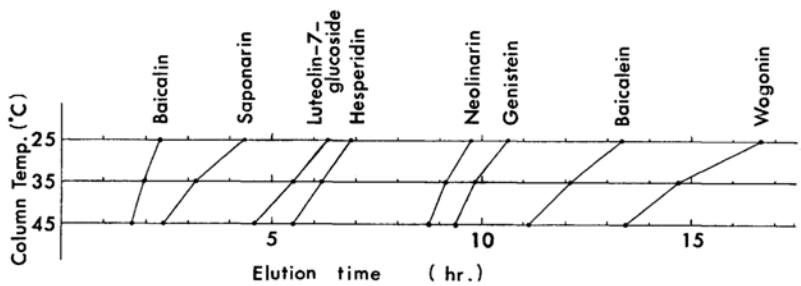

Fig. 1. Effect of the column temperature on the elution time of flavonoids.

flavonoids are summarized in Fig. 2. The number given to each peak corresponds to the sample number listed in Table 2.

In the actual chromatography each peak was drawn at three wavelengths. However, the peaks in Fig. 2 were drawn schematically to avoid complication of the chromatogram.

The elution curve of apiin (No. 26) consisted of two peaks, indicating that both peaks were due to an impurity. The broad peak of morin (No. 30) was far from being a Gaussian distribution. The fact suggests that the shape of the elution curve might be affected by an impurity.

Correlation of the elution time of these substances with their structure led to the conclusion that in this chromatography the polar flavonoids elute with shorter elution time. Thus, the following generalizations are possible.

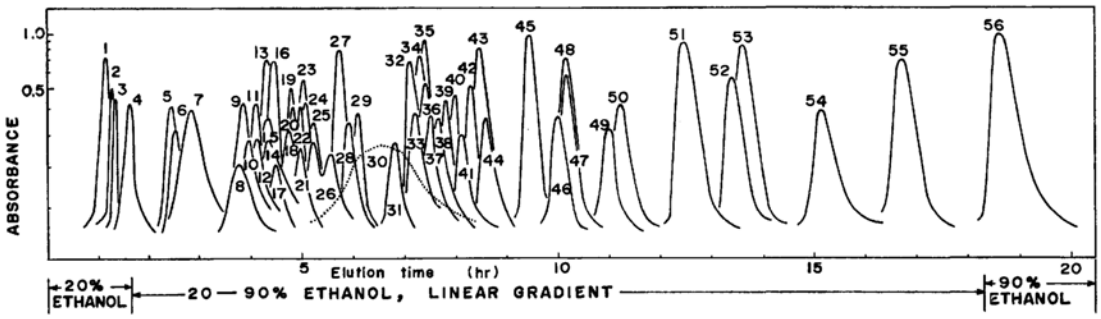

Fig. 2. The elution time of flavonoids.
Column: Amberlite XAD-2, 200—400 mesh, 9×1500 mm, 45°C
Solvent: Aqueous ethanol
Flow rate: 60 ml/hr

TABLE 1. YIELDS OF METHANOL EXTRACTS FROM PLANT SOURCES

Samples	Dry plant (g)	Methanol extract (g)	Amount applied to the column (mg)	Flavonoids determined	%/extract
Scutellaria baicarensis	3.0	0.8648	9.88	Baicalin	7.37
				Baicalein	3.38
				Wogonin	1.39
Citrus aurantium	1.2	0.1653	13.08	Hesperidin	39.6
Crataegus cuneata	3.0	0.2018	67.09	—	—

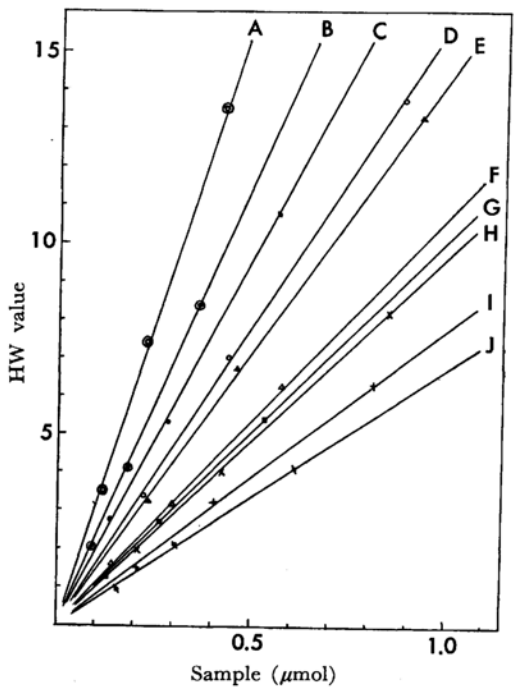

Fig. 3. Calibration curves determined by the HW method from the peak area.

A: Wogonin (53), B: Saponarin (6),
C: Flavone (55), D: Kaempherol (47),
E: Baicalein (50), F: Rutin (8),
G: Baicalin (4), H: Hesperidin (25),
I: Quercetin (41), J: Quercitrin (17).

i) The elution is in the order of sugar esters→glycosides→aglycones.

ii) Among the aglycones the compounds having more hydroxyl groups elute faster than the compounds having fewer hydroxyl groups. The stereochemical nature of the hydroxyl groups also affects the elution time of these aglycones.

iii) The elution time of glycosides varies considerably depending on the position where the sugar moiety is attached, even if the aglycone and the sugar are the same among the glycosides.

iv) The elution time for glycosides is greatly affected by the number of hydroxyl groups and sugars.

As is seen in Fig. 2, many of the glycosides and aglycones, which are eluted in the range of retention time, 4—8 hr, are difficult to be separated if they are present in one sample. In such a case, the measurement of the absorbances at three different wavelengths on the chromatogram becomes very significant, because the shape of the absorption spectra differs considerably from one flavonoid to another.[2] The ratios of the absorbance ($E_{270\,m\mu}/E_{300\,m\mu}$, $E_{330\,m\mu}/E_{300\,m\mu}$) calculated from chromatograms are listed in the right column of Table 2. The calibration curves of ten flavonoids calculated by the height-width

2) L. Jurd, "The Chemistry of Flavonoid Compounds," ed. by T. A. Geissman, Pergamon Press, New York (1962), p. 107.

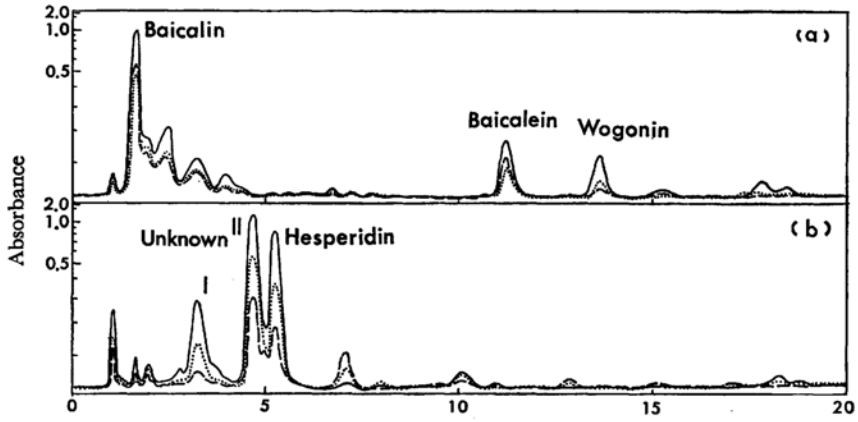

Fig. 4. Chromatograms of the methanol extracts of *Scutellaria baicarensis* (a) and *Citrus aurantium* (b).
——: 270 mμ ·····: 300 mμ — — —: 330 mμ

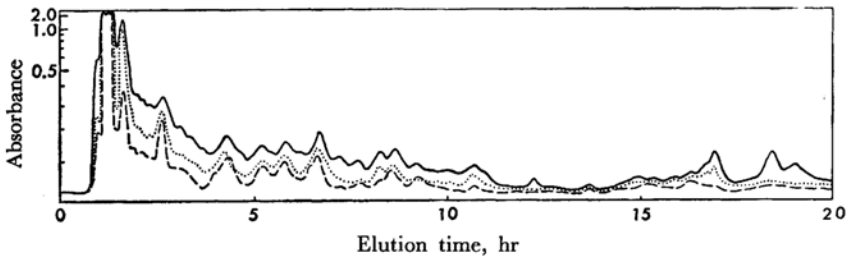

Fig. 5. Chromatogram of the methanol extract of *Crataegus cuneata*.

method*2 are shown in Fig. 3. The accuracy of these measurements lies within ±2%.

Application in the Chromatography of Plant Extracts. Methanol extracts of *Scutellaria baicalensis*, *Citrus aurantium* and *Crataegus cuenata* were prepared by the method as described above. The chromatogram of the extract from *Scutellaria baicalensis*, Fig. 4(a), indicates the presence of baicalin, baicalein and wogonin, all of which have been isolated from these plants. Similarly, the chromatogram of *Citurs aurantium* indicates the presence of hesperidin. Other peaks, which are seen in Fig. 4(b) and designated as peaks I and II, have not yet been characterized. These compounds, however, seem to be akin to hesperidin in their structures, because the absorption spectra resemble that of hesperidine. The chromatogram of the extract from *Crataegus cuneata* was complex as shown in Fig. 5, and the peaks of quercetin and vitexin, which have been isolated from this plant, were indistinguishable. The complexity is due to a large quantity of impurities extracted by methanol, and some pretreatment would be needed.

The author is grateful to Professor Hiroyuki Hatano (Kyoto University) and Dr. Katsura Morita (these laboratories) for valuable discussions. The author is also grateful to Drs. Shunji Imai and Naokata Morita for providing the samples. Thanks are due to Miss Michiyo Miyazaki for her assistance in the experimental work.

*2 The height-width method has been used in the amino acid analysis.