Journal of Chromatography, 322 (1985) 236–239
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,521

Note

High-performance liquid chromatographic analysis of coumarins and flavonoids from section Tridentatae of *Artemisia*

RAMA V. TAMMA and GLENN C. MILLER*
Department of Biochemistry, University of Nevada, Reno, NV 89557 (U.S.A.)
and
RICHARD EVERETT
Intermountain Research Station, United States Forest Service, Reno, NV 89557 (U.S.A.)
(Received January 2nd, 1985)

Flavonoids and coumarinss are frequently used for chemotaxonomic identification of plant species, and have been described as the most favoured of all plant constituents as taxonomic markers[1]. The methods used to separate and detect these compounds for chemotaxonomic purposes have usually been by one or two dimensional thin layer or paper chromatography. Although simple and inexpensive, these systems lack the resolution, quantitation and sensitive detection capabilities of high-performance liquid chromatography (HPLC). HPLC of flavonoids has been described previously in several publications utilizing both reversed-phase[2–5], and silica columns[6]. Previous examples of use of HPLC for chemotaxonomic purposes for higher plants analyzed anthocyanins[7] and flavonols[8] in poinsettia cultivars.

Sagebrush (*Artemisia* section Tridentatae) (Compositae) is a dominant species over much of the western United States for which phenolics are frequently used for taxonomic identification[9–11]. Many of the coumarins and flavonoids of *Artemisia* have been identified by Brown *et al.*[12], Rodriguez *et al.*[13] and Shafizadeh and Melnikoff[14]. This paper describes an HPLC method for separation and quantitation of flavonoids and coumarins from *A. nova*, *A. tridentata* ssp. *tridentata* and *A. tridentata* ssp. *wyomingensis* and discusses the potential for utilizing HPLC for chemotaxonomy of sagebrush species and subspecies.

EXPERIMENTAL

Preparation of standards and samples

Standards of the flavonoids and coumarins were obtained from Roth (Atomergic Chemetals, Plainview, NY, U.S.A.). Standard solutions of each phenolic compound (1.0 mg/ml) were prepared in ethanol. The sagebrush samples were extracted by soaking 0.5 g of crushed, air dried leaves of each species/subspecies in 5 ml of ethanol–water (70:30, v/v) for 24 h. The liquid was decanted and the leaves were reextracted three additional times with 5 ml of ethanol–water (70:30, v/v). The total amount was filtered through No. 42 Whatman filter paper and the volume then adjusted to 20 ml. Aliquots (10 μl) were then injected onto the HPLC system after a second filtration through a 0.45-μm filter.

0021-9673/85/$03.30

Chromatographic system

Gradient elutions were performed with a Waters Assoc. (Milford, MA, U.S.A.) HPLC system composed of a Model 660 solvent programmer, in conjunction with two Model M6000A pumps and a U6K injector. The component elution was monitored with a Model 440 fixed-wavelength UV detector at 340 nm. Chromatograms were recorded on a Hewlett-Packard Model 3390A recorder/integrator. A μBondapack C_{18} (10-μm particles) 30 cm × 3.9 mm I.D. column (Waters Assoc.) was used at ambient temperatures.

Analytical method

Separation was accomplished by gradient elution: solvent A, acetic acid–water (1:99, v/v); solvent B, acetonitrile. The gradient profile was linear from 20 to 90% B in 20 min at a flow-rate of 1.5 ml/min and a column pressure of 1200 p.s.i. A second solvent system consisted of the above system with the substitution of methanol for acetonitrile.

RESULTS AND DISCUSSION

Esculin and methylesculin along with an unidentified compound are the major constituents of the species and subspecies of the section Tridentatae of *Artemisia* examined. Other coumarins, including esculetin, umbelliferone, isoscopoletin, scopoletin and scoparone along with the flavonoids quercetagetin, quercetin, axillarin, kaempferol, apigenin and penduletin are present in smaller amounts. In addition to the compounds reported in Table I it is evident from Fig. 1 that a number of other minor constituents have not yet been identified. These peaks can, however, still be

TABLE I

RETENTION TIMES OF COUMARINS AND FLAVONOIDS ON μBONDAPAK C_{18} USING TWO SOLVENT SYSTEMS

No.	*Compound*	*Chemical name*	*Retention time (min)*	
			Acetic acid–water (1:99); acetonitrile	*Acetic acid–water (1:99); methanol*
1	Esculin	6,7-Dihydroxycoumarin-6-glucoside	2.70	6.00
2	Methylesculin	6-β-D-Glucosyl-7-methoxycoumarin	3.02	6.26
3	Esculetin	6,7-Dihydroxycoumarin	3.98	8.12
4	Quercetagetin	3,3',4',5,6,7-Hexahydroxyflavone	4.57	11.34
5	Isoscopoletin	6-Hydroxy-7-methoxycoumarin	5.45	12.02
6	Quercertin	3,3',4',5,7-Pentahydroxyflavone	5.99	13.48
7	Umbelliferone	7-Hydroxycoumarin	6.32	10.34
8	Scopoletin	7-Hydroxy-6-methoxycoumarin	6.32	10.92
9	Scoparone	6,7-Dimethoxycoumarin	8.58	12.02
10	Axillarin	3,6-Dimethoxy-3',4',5,7-tetrahydroxyflavone	10.57	16.50
11	Kaempferol	3,4',5,7-Tetrahydroxyflavone	11.70	17.50
12	Apigenin	4',5,7-Trihydroxyflavone	11.48	17.88
13	Penduletin	3,6,7-Trimethoxy-4'-5-dihydroxyflavone	13.09	18.44

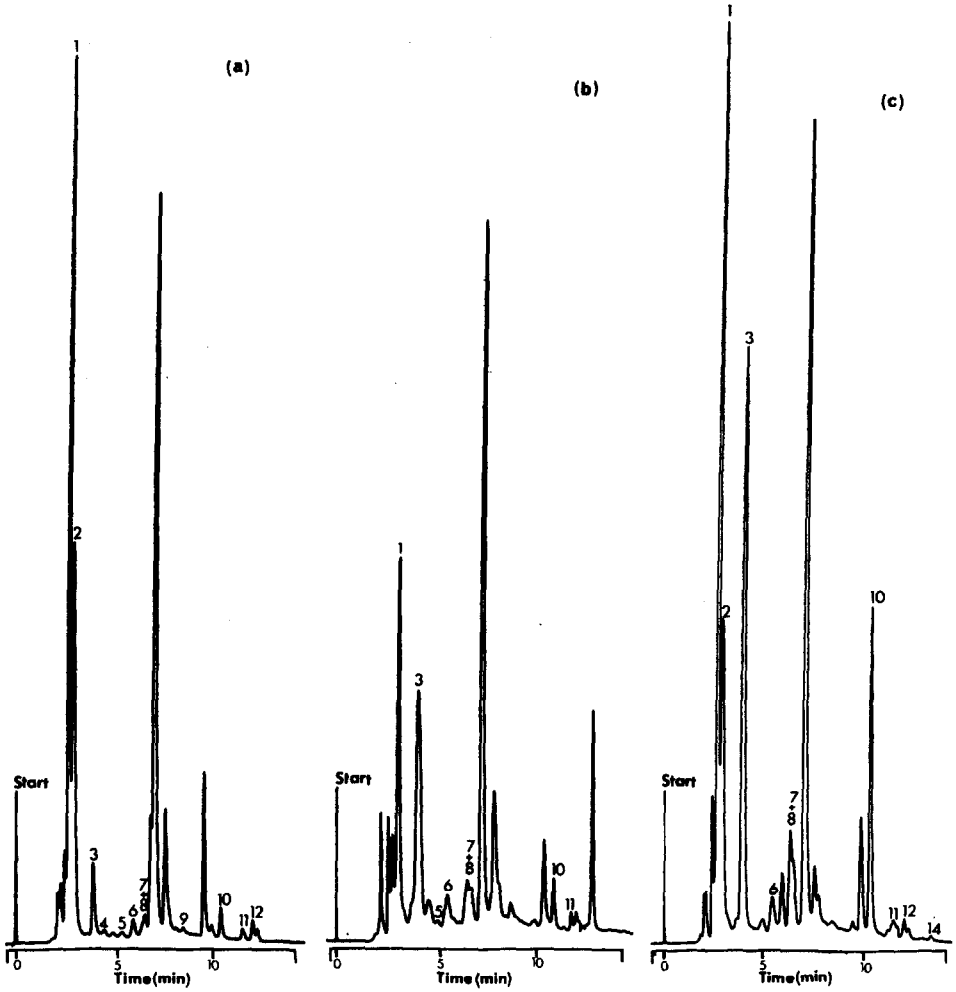

Fig. 1. Separation of coumarins and flavonoids from section Tridentatae of *Artemisia* on μBondapak C_{18} using an acetonitrile–water–acetic acid gradient. Peak identifications as in Table I. (a) *Artemisia nova*; (b) *A. tridentata* ssp *tridentata*; (c) *A. tridentata* ssp. *wyomingensis*.

used taxonomically, provided the chromatographic separation is reproducible and relative retention times can be established with known compounds.

Fig. 1 shows the chromatographic profile of the alcohol extractable content of the leaves of *A. nova, A. tridentata* ssp. *tridentata* and *A. tridentata* ssp. *wyomingensis* and a list of the compounds identified is given in Table I. The data refer to coumarins, coumarin glycosides, flavonoids and flavanoid glycosides which were detected in the leaf tissue by comparison of the retention times with standards in the two solvent systems. Significant differences in the chromatography were noted between the different species and subspecies of sagebrush tested, and provide a basis for their chemical identification.

The reversed-phase system gave reasonably well resolved and symmetrical

peaks, and separation of isomeric forms was achieved. The acetic acid was demonstrated to be necessary for protonation of the various phenols, and its elimination resulted in uninterpretable chromatograms. Careful equilibrium with the mobile phase was also essential for run to run reproducibility. Umbelliferone and scopoletin could not be separated by the acetonitrile solvent system but were resolved using methanol in the place of acetonitrile (Table I). The flavonoids and coumarins absorb 340 nm light, which conveniently excludes most other aromatic chromophores, and enables selective detection of the desired compounds.

The present work demonstrates that HPLC can be successfully used to separate and quantitate coumarins and flavonoids in extracts of sagebrush. Further work is ongoing to establish statistically the extent to which these compounds can be used as taxonomic markers using various pattern recognition programs. Preliminary data suggest that chromatographic differences characteristic of Fig. 1 are consistent in several samples (unpublished results). The high resolving capability and sensitivity of HPLC, in conjunction with the quantitative nature of the method offers substantial advantages over either thin-layer or paper chromatography for chemotaxonomic purposes. Not only the simple presence or absence of the compounds, but also relative concentrations of each constituent can be determined, allowing multiple differences between species and subspecies to be used for taxonomic identification.

REFERENCES

1 J. B. Harborne, in J. B. Harborne, T. J. Mabry and H. Mabry (Editors), *The Flavonoids*, Academic Press, New York, 1975, p. 1056.
2 L. W. Wulf and C. W. Nagel, *J. Chromatogr.*, 116 (1976) 271.
3 D. Strack and J. Krause, *J. Chromatogr.*, 156 (1978) 359.
4 K. Vande Casteele, H. Geiger and C. F. Van Sumere, *J. Chromatogr.*, 240 (1982) 81.
5 R. Galensa and K. Herrmann, *J. Chromatogr.*, 156 (1978) 358.
6 R. Galensa and K. Herrmann, *J. Chromatogr.*, 189 (1980) 217.
7 R. N. Stewart, S. Asen, D. R. Massie and K. H. Norris, *Biochem. Syst. Ecol.*, 7 (1979) 281.
8 R. N. Stewart, S. Asen, D. R. Massie and K. H. Norris, *Biochem. Syst. Ecol.*, 8 (1980) 119.
9 D. L. Hanks, E. D. McArthur, R. Stevens and A. P. Plummer, *Intermountain Forest and Range Experimental Station Research Paper INT-141*, (1973) 1.
10 H. R. Holbo and H. N. Mozingo, *Amer. J. Bot.*, 52 (1965) 970.
11 A. Young, *Wyo. Range Manage*, 198 (1965) 2.
12 D. Brown, R. O. Asplund and V. A. McMahon, *Phytochemistry*, 14 (1975) 1083.
13 E. Rodriguez, N. J. Carman, C. Vander Velde, J. H. McReynolds, T. J. Mabry, M. A. Irwin and T. A. Geissman, *Phytochemistry*, 11 (1972) 3509.
14 F. Shafizadeh, and A. B. Melnikoff, *Phytochemistry*, 9 (1970) 1311.