Yasuhiko Hayashiba,[1] *B.S.; Takeaki Nagata,*[2] *M.D.;*
Ikuo Miyajima,[3] *B.S.; Kojiro Kimura,*[2] *M.D.; and*
Keiko Kudo,[2] *Ph.D.*

Identification of Plant Stains Using High Performance Liquid Chromatography

REFERENCE: Hayashiba, Y., Nagata, T., Miyajima, I., Kimura, K., and Kudo, K., **"Identification of Plant Stains Using High Performance Liquid Chromatography,"** *Journal of Forensic Sciences*, JFSCA, Vol. 34, No. 2, March 1989, pp. 328-335.

ABSTRACT: A study was designed to identify stains derived from plant pigments. Using high performance liquid chromatography (HPLC), 13 species of common weeds were examined, and 12 flavonoids were detected from as low as 100 to 200 μg of dried leaves and from their stains. Identification of plant species could be made, based on the various chromatographic patterns of the flavonoids with different retention indices, using rutin and rhamnetin as the reference standards. A brief practical case of application is described.

KEYWORDS: criminology, plant pigments, stain identification, chromatographic analysis, flavone, flavonoids, flavonol, high performance liquid chromatography, myricetin, quercetin, luteolin, apigenin, rutin, rhamnetin, kaempferol

Identification of stains on the surface of human bodies, clothes, or other materials has to be made in cases of criminal investigation. Despite a large volume of literature on bloodstains, little information is available with regard to plant components from the criminological aspect. In attempts to identify plant stains, our attention was directed to their pigments. Flavonoid was selected for analysis because of its chemical stability. Two other major plant pigments, chlorophyl and carotinoid, readily decompose.

Although paper chromatography, thin-layer chromatography [*1,2*], and high performance liquid chromatography (HPLC) [*3-7*] have been used to examine flavonoid, these studies do not relate to criminological cases. We, therefore, searched for trace amounts of flavonoids as indicators of plant stains.

Materials and Methods

Standard Flavonoids

Apigenin was a gift from the Department of Plant Chemistry, Faculty of Pharmaceutical Sciences, Kyushu University. Myricetin and kaempferol were purchased from Sigma, St.

Received for publication 22 April 1988; accepted for publication 25 May 1988.

[1]Guest researcher, Department of Forensic Medicine, Faculty of Medicine, Kyushu University, and forensic chemist, Forensic Science Laboratory, Fukuoka Prefectural Police Headquarters, Fukuoka, Japan.

[2]Professor, lecturer, and lecturer, respectively, Department of Forensic Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

[3]Department of Horticultural Science, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.

Louis; quercetin and rutin were from Nakarai Chemicals Ltd., Kyoto, Japan; luteolin was from K&K Labs, New York; and rhamnetin was from Extrasynthese (SSK), Genay, France. The chemical structures of the standard flavonoids are shown in Fig. 1.

Plant Samples for Testing

Thirteen weeds always present in the suburbs of Fukuoka, Japan, were collected in April 1987 and are listed in Table 1.

The leaves were dried at room temperature for two weeks, and then ground to a powder form. Samples of stain from the leaves were prepared by pressing the fresh leaves onto filter

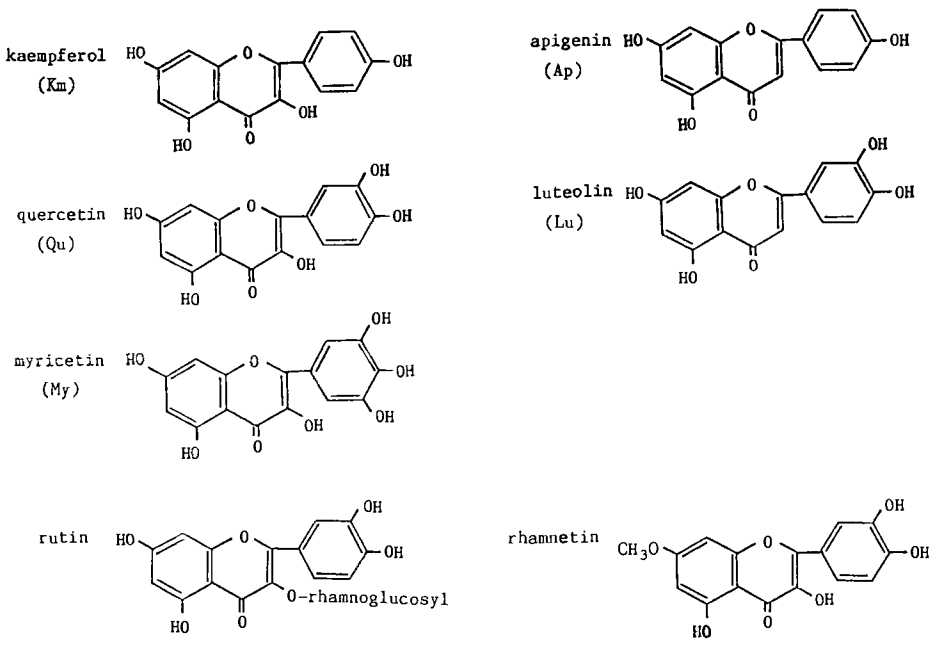


FIG. 1—*Chemical structures of standard compounds.*

TABLE 1—*Thirteen weeds collected for analysis.*

Nomenclature	Common Name	Family
Astragalus sinicus	Chinese milk vetch	Leguminosae
Medicago hispida	Bur-clover	Leguminosae
Trifolium repens	White clover	Leguminosae
Vicia hirsuta	Tyne grass	Leguminosae
Vicia sativa	Tare	Leguminosae
Vicia tetrasperma	Smooth tare	Leguminosae
Ranunculus cantoniensis	Japanese buttercup	Ranunculaceae
Ranunculus japonicus	Butter daisy	Ranunculaceae
Oxalis corniculata	Creeping Lady's sorrel	Oxalidaceae
Oxalis corymbosa	Dr. Martius' wood sorrel	Oxalidaceae
Veronica persica	Bird's-eye	Scrophulariaceae
Equisetum arvense	Horsetail	Equisetaceae
Capsella bursa-pastoris	Shepherd's purse	Cruciferae

paper. The samples of both leaf and stain were stored in a desiccator at room temperature until analysis. When small amounts of leaves were weighed, the samples were mixed with clean soil powder so that the weight could be measured fairly accurately. The soil was used after washing three times with a diluted hydrochloric acid solution and water, alternately.

Extraction Procedure

Extraction of flavonoid was carried out based on Harborne's method [*8*]: Each of the leaf samples (1.0 mg, corresponding to about 2 mm^2 of dry leaf) was mixed with 0.2 mL of 2*N* hydrochloric acid in a 10-mL test tube and the preparation heated at 100°C for 60 min for hydrolysis. The preparation was then cooled and extracted twice with 1 mL of ethyl acetate. The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 50 μL of ethanol, passed through a filter unit (Columnguard FH 4, Millipore Ltd., USA), and 2-μL aliquots of the solution were used for the HPLC. The flavonoids were selectively detected by an ultraviolet (UV) detector adjusted to the wavelength at 365 nm. The extraction process is summarized in Table 2. The stain (1 cm^2) was extracted in the same manner as for the leaf samples.

HPLC Conditions

The apparatus used was a Shimadzu LC-6A model high performance liquid chromatograph equipped with a Shimadzu SPD-6AV UV detector and an integrator, a Shimadzu chromatopac C-R3A. The column is a 15-cm by 6-mm inside diameter (id) stainless tube packed with Shim-pack CLC-ODS (octadecyl silica).

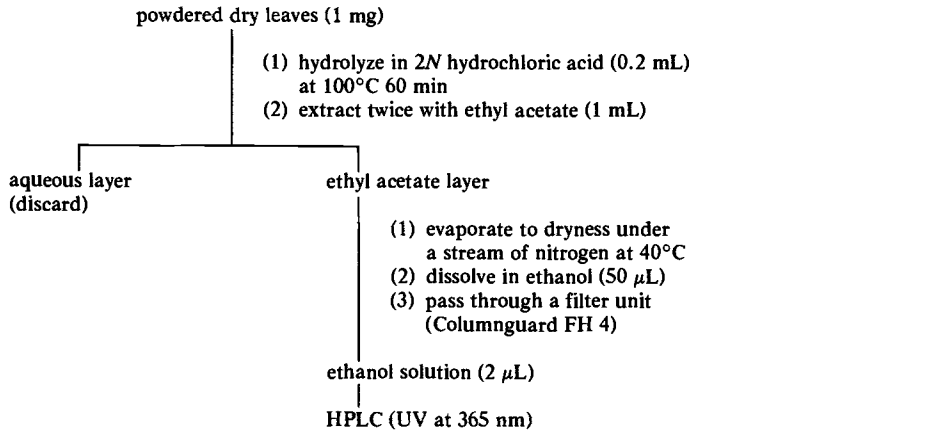
The eluent was water : acetic acid : methanol = 47 : 10 : 43 (v/v/v) and the flow rate was 1.0 mL/min. All chromatographic procedures were carried out at room temperature.

Results

Retention Indices (RIs) for Flavonoids

Peak identification was often different as a result of slight variations in the retention time (RT) when the eluting solvents were renewed for each experiment. The variation apparently

TABLE 2—*Procedure for analysis of flavonoids.*



increased as the RT became longer. To resolve this problem, retention index (RI) in gas chromatography [9] was introduced and was partially modified to identify the flavonoids. Two reference standards (RS) were selected out of the analog chemicals with flavonoids: rutin with the smallest RT and rhamnetin with the largest one to cover the main peaks appearing on the chromatogram. Flavonoid retention indices were obtained from the following formula, setting rutin at 0 and rhamnetin at 100.

$$RI(A) = [RI(Y)\text{-}RI(X)] \times \frac{\log t(A)/t(X)}{\log t(Y)/t(X)} + RI(X)$$

where

$t(A)$ = retention time of flavonoid A,
$t(X)$ = retention time of rutin,
$t(Y)$ = retention time of rhamnetin,
$RI(A)$ = retention index of flavonoid A,
$RI(X)$ = retention index of rutin = 0, and
$RI(Y)$ = retention index of rhamnetin = 100.

The value of RI(A) was set as a whole number between 0 and 100, counting fractions of 5 and over as a unit and disregarding the rest.

Five standard flavonoids were analyzed five times and the solvents were renewed for each experiment. The RTs and RIs of five standard flavonoids are shown in Fig. 2. Despite the varied RT, stable values of RI were obtained: 15 $\pm$ 1 for myricetin, 40 for quercetin, 48 for luteolin, 67 for kaempferol, and 71 for apigenin.

Qualitative Determination

The chromatograms of extracts from 3 weeds belonging to the same genus, *Vicia hirsuta, Vicia sativa,* and *Vicia tetrasperma* are shown in Figs. 3 through 5. The characteristic peaks of flavonoid aglycones appeared with the RT over 5 min. The distribution of flavonoids in 13 weeds is summarized in Table 3. Under the present chromatographic conditions, 12 separated peaks were observed between those of rutin and rhamnetin. RIs of 5 peaks were identical to those of myricetin, quercetin, luteolin, kaempferol, and apigenin. Seven other peaks with the RIs of 31, 34, 56, 73, 76, 79, and 89 remained unidentified, and were tentatively named F1 (flavonoid-1) to F7 in order of increasing RT. The flavonoids listed in Table 3 were chosen as indicators of the plant pigment.

Most of the leaf samples were found to contain two to eight recognizable flavonoids, while *Oxalis corniculata* and *Oxalis corymbosa* presented no peaks included in the indicators. *Vicia sativa* and *Ranunculus cantoniensis* shared four flavonoids: quercetin, luteolin, kaempferol, and F3. In nine other leaf samples, flavonoids were present in different combinations. The plants belonging to the same genus and with a similar form were differentiated, based on the peak pattern. For example, in this study, *Vicia sativa, Vicia hirsuta,* and *Vicia tetrasperma* in the same *Vicia* group were clearly distinguished, and *Ranunculus japonicus* could be differentiated from *Ranunculus cantoniensis.*

Change in the Flavonoids Depending on the Season

Seasonal change in flavonoids was examined using *Trifolium repens.* The samples were collected four times in March, June, August, and November. Three peaks of the flavonoids, F2, quercetin, and kaempferol, were found in all samples, with no variation in the peak number.

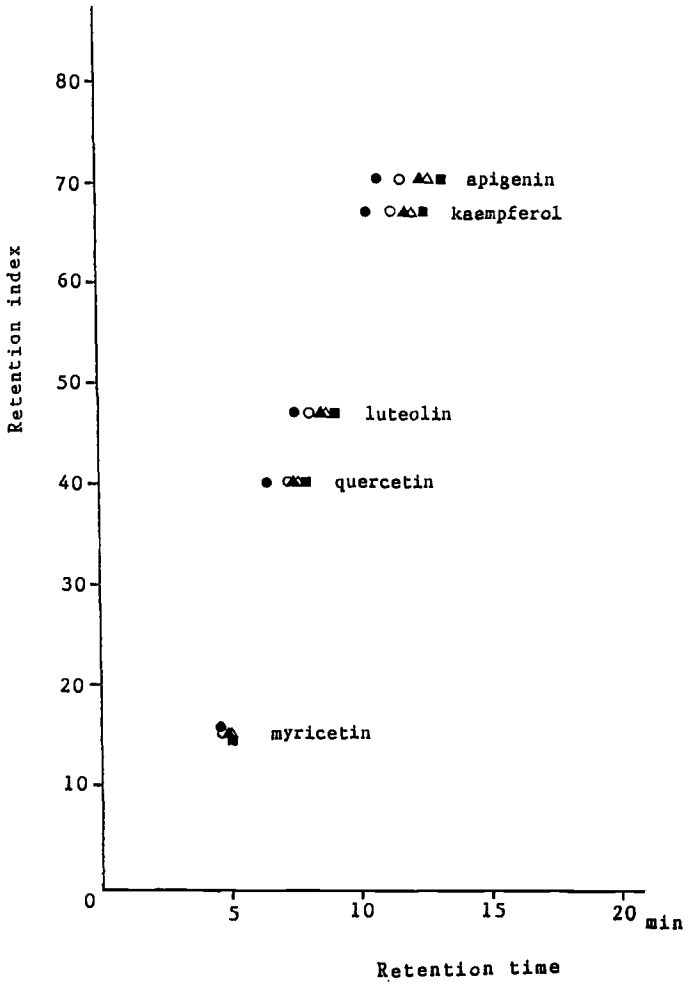


FIG. 2—*Retention indices of the standard compounds.* ●○▲△■*: series of experiment.*

The Lower Limit of Detection

The minimum level of sample amounts was examined to observe the same peak patterns as those when 1 mg of leaf sample was used. The least amount required for analysis was found to be 100 μg in *Medicago hispida* and *Vicia hirsuta* and 200 μg in the other eleven plant samples. When the amount was decreased to 50 μg, only the smaller peaks disappeared.

A Case of Application

A suspected bloodstain of brownish color on a shirt found at the scene of a crime was brought in for examination. When all blood tests proved to be negative, our presently described method was used to identify the stain. Chromatography presented the peaks of F2, quercetin, and kaempferol with the RIs of 34, 40, and 67, respectively. The stain was apparently derived from *Trifolium repens.*

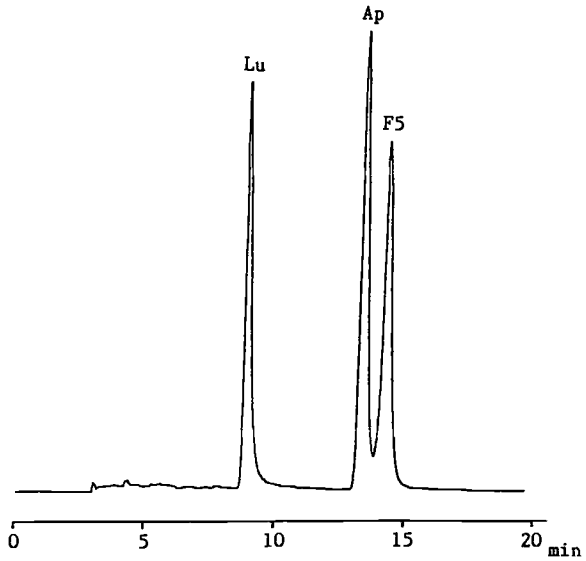


FIG. 3—*Chromatogram of the extract from* Vicia hirsuta.

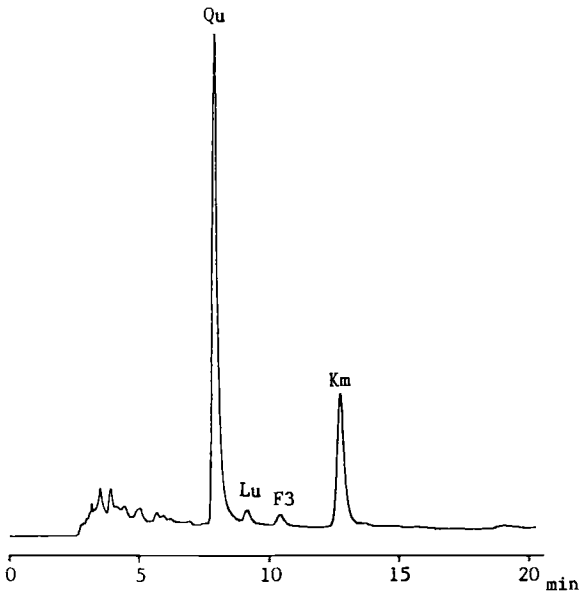


FIG. 4—*Chromatogram of the extract from* Vicia sativa.

Discussion

Flavonoids are substances of plant origin and are widely distributed in flowers, leaves, stems, and grains [*10*]. This compound seems favorable for use in identifying plants and is superior to unstable chlorophyl and carotinoid. The flavonoid is usually present in the form of glycoside [*11*] and can be analyzed by the gradient elution method [*12*]. The chromato-

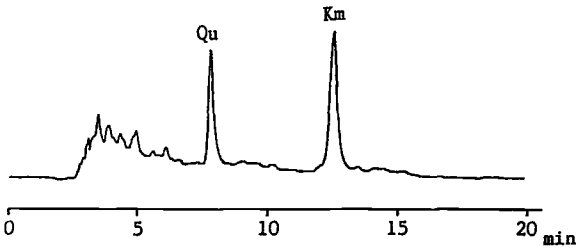


FIG. 5—*Chromatogram of the extract from* Vicia tetrasperma.

TABLE 3—*Distribution of flavonoids in weeds.*

Species of Weeds	Flavonoids[a]											
	My	F1	F2	Qu	Lu	F3	Km	Ap	F4	F5	F6	F7
Astragalus sinicus	−	−	−	+	−	−	+	−	+	−	−	+
Medicago hispida	−	−	−	+	+	−	−	+	−	−	−	−
Trifolium repens	−	−	+	+	−	−	+	−	−	−	−	−
Vicia hirsuta	−	−	−	−	+	−	−	+	−	+	−	−
Vicia sativa	−	−	−	+	+	+	+	−	−	−	−	−
Vicia tetrasperma	−	−	−	+	−	−	+	−	−	−	−	−
Ranunculus cantoniensis	−	−	−	+	+	+	+	−	−	−	−	−
Ranunculus japonicus	−	−	−	+	+	+	+	+	−	−	−	−
Oxalis corniculata	−	−	−	−	−	−	−	−	−	−	−	−
Oxalis corymbosa	−	−	−	−	−	−	−	−	−	−	−	−
Veronica persica	+	+	+	−	+	−	+	+	−	−	+	+
Equisetum arvense	−	−	+	+	−	+	+	−	−	−	−	−
Capsella bursa-pastoris	−	−	−	+	+	−	−	−	−	+	−	−
Retention index	15	31	34	40	48	56	67	71	73	76	79	89

[a]My: myricetin, Qu: quercetin, Lu: luteolin, Km: kaempferol, Ap: apigenin, and F1-7: unidentified flavonoids.

graphic patterns of plant pigments obtained by this technique, however, are generally complicated as a result of various combinations of sugar and aglycone. We, therefore, used a simpler method in which the sugar is eliminated from the glycoside by hydrolysis before extraction procedures so that only aglycones were analyzed and the time required was greatly diminished.

The findings of no seasonal change in composition and of the varied combinations ranging from two to eight flavonoids in each plant are advantageous for identifying plant stains independent of seasonal factors. The finding of the constant RI of each flavonoid using rutin and rhamnetin as the RS, independent of experimental conditions, is most useful for filing and comparing numerous data on plant species. The characteristic combinations of flavonoids in each plant suggest of a kind of "fingerprint" for each plant.

Our method makes feasible an analysis of as low as 100 to 200 μg of dry leaves and is adequate for practical examinations of plant stains.

Conclusion

Analyses of plant pigment were made feasible using HPLC. Twelve flavonoids were detected, including myricetin, quercetin, luteolin, kaempferol, and apigenin as characteristic indicators. Retention indices using rutin and rhamnetin as RS were introduced and proved

to be of practical value for identifying flavonoids. The detection of flavonoids was feasible from as low as 100 to 200 μg of dry leaves.

Acknowledgments

We thank Professor S. Uemoto of the Department of Agriculture, Kyushu University, and Dr. H. Iki of Fukuoka Prefectural Police Headquarters for advice, Dr. Y. Hieda of Fukuoka University School of Medicine for technical assistance, and M. Ohara for comments.

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Address requests for reprints or additional information to
Takeaki Nagata, M.D.
Department of Forensic Medicine
Kyushu University
3-1-1, Maidashi, Higashi-ku
Fukuoka, Japan, 812