NEW MICROCHEMISTRY OF PLANT COMPONENTS—HISTOCHEMISTRY AND ENFLEURAGE CHROMATOGRAPHY[1]

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ABSTRACT.—In histochemical chromatography, plant cell components such as crystals, oil droplets, and concentrated substances of reaction products are removed from tissue sections by the full automatic micromanipulator system and analyzed by gc, hplc, and ms. In addition to chromatography of components from plant cells, uric acid was identified from kidney. Enfleurage (pomade method) is a classical procedure in the preparation of perfumes, in which volatile components are absorbed into natural fresh fat, extracted with EtOH, and concentrated under reduced pressure. In enfleurage chromatography, volatile components are absorbed into the synthetic pure glycerides, extracted with MeCN, and analyzed by gc, hplc, and ms.

The author was engaged in the microanalysis of medicinal and poisonous plants as an apothecary officer in the Japanese Navy from 1943 to 1946, having constructed a small laboratory in a tunnel at Rabaul on New Britain Island (1). From 1953 to the present, he has continued his studies on the microanalysis of plant constituents, eventually making expeditions to the interior of the Amazon. In addition, he was in charge of orientation of chromatographic studies as a UNESCO expert on four occasions—1953, 1963, 1966, and 1969. These contracts were undertaken at Brazilian and Colombian universities or National Research Institutes and represent a total of over 5 years of study of microanalysis in South America. In 1971, he established in Japan the Symposium for Crude Drug Analysis.

Thus he has continually contributed to the analysis of crude drugs, which is important in quality control of drug manufacture. A monograph entitled "Crude Drug Analysis" was published by Hirokawa Publishing Company, Japan, in 1977 and was well received in China after it had been translated into Chinese in 1980. Increased versatility in the use of micro techniques can be expected from the methods of histochemical and enfleurage chromatographies applied to the analysis of living cellular tissue in situ.

HISTOCHEMICAL CHROMATOGRAPHY.—H. Molisch (2) in Austria in 1914 and O. Tunmann and L. Rosenthaler (3) in Switzerland in 1931 studied plant histochemistry by observation of tissue sections treated with suitable reagents to produce colors or characteristic crystalline precipitates under a microscope. However, by those methods it was difficult to identify individual cell constituents. A new technique having application to classical histochemistry is referred to as histochemical chromatography (4). Cell components such as crystals, oil droplets, and concentrated substances of reaction products are removed from tissue sections by means of a computerized micromanipulator. The micromanipulator was designed for the injection of compounds into cells, but it can be used for the removal of cell contents to put into the trap of a microtest tube by aspiration through a micropump. Cell contents then can be analyzed by an appropriate technique.

The apparatus for sampling of cell contents from tissue slices is shown in Figure 1, and a magnification of the suction part of the micromanipulator in Figure 2. Sliced tissue of appropriate thickness prepared by an automatic microslicer was put on the stage.
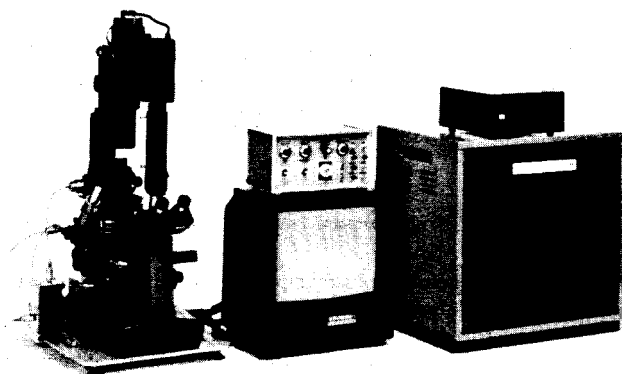
FIGURE 1.   Histochemical apparatus. From left to right: microscope with micromanipulator, controller
            on Braun Tube, computer set.

The capillary tube (0.01–0.04 mm i.d.), which was prepared like a microelectrode, was connected to a microtrap through a microsuction pump. The suctioned substances in the capillary tube and in the connected teflon tube were washed through with appropriate solvents to flow into the microtrap.

Automatic sampling of the contents in the tissue under the microscope is performed with the controller (Figure 3). One joy-stick circuit, table JS-1, moves the stage in eight horizontal directions with variable speed controlled by VR 1. The other joy-stick circuit, cursor JS-2, moves the cursor on the Braun tube in eight horizontal directions with variable speed controlled by VR 2. The capillary tube is moved vertically by a toggle switch, with speed controlled by VR 3. First, the tip of the microcapillary tube is aligned with the cursor on the Braun tube by operating JS-2. The "home set" position, where the tip of the needle is slightly above the slice, is set by the "home set" button. The "lower set" position is used when the needle is ready to aspirate. The target in a cell that is desired for analysis is positioned to the cursor by operating JS-1. When the target is aligned with the cursor, the aspiration of the target at that position is completed by pushing the "aspiration" button. The aspiration speed in seconds can be set by the timer, which is turned on by the lower toggle switch. After aspiration, the position of the capillary tube returns automatically to the home set position.
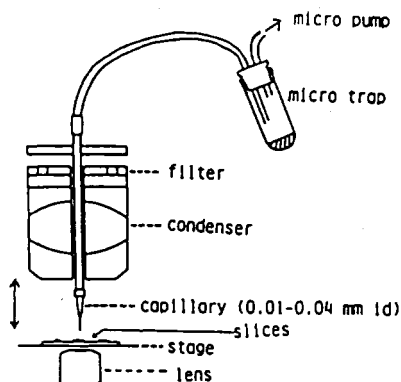

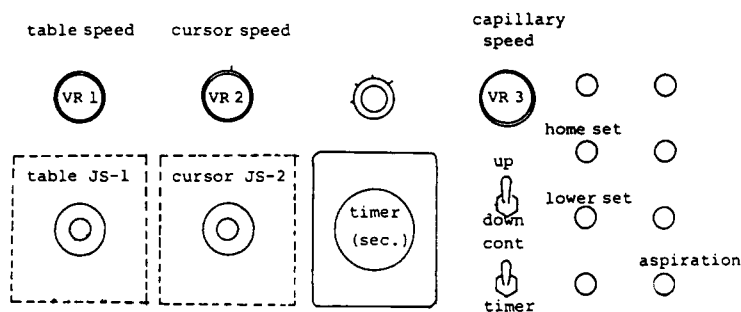FIGURE 2.   Suction part of micromanipulator.

FIGURE 3.   Controller for micromanipulator.

The first example is an analysis of clove, a widely used spice. The essential oil derived from clove buds is also employed as a toothache remedy. The clove flower buds and fruits were collected in the National Medicinal Plant Station, Izu, Japan, and classified according to their stage of development: stage I, the beginning of bud; stage II, bud; stage III, nearly ripe fruits with petals and filaments beginning to fall off; and stage IV, fully ripe fruit (4). From each bud or fruit, two transverse sections were prepared: the upper part, cut below the corolla, and the lower part, cut through the base. The clove oil was aspirated from oil sacs in the tissue at each stage using the micromanipulator. Figure 4 shows the hpl chromatogram for reference eugenol and acetyleugenol, which were known to be present in clove. The correlation between the ratio of acetyleugenol to eugenol content in the oil sacs and the weight of the raw bud or fruit is shown in Figure 5. The content of acetyleugenol decreases in order from stage I through stage IV. It is noteworthy that in stage IV, when the fruit is fully ripe, only eugenol was detected. Further, there was no difference in the data between the upper
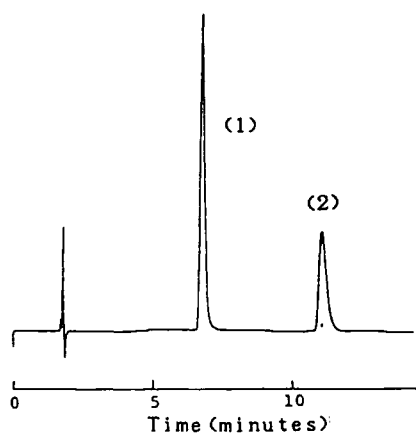

FIGURE 4.   Hplc separation of reference eugenol (1) and acetyleugenol (2) [column Zorbax Sil (4.6 mm × 150 mm); solvent system n-hexane–$CHCl_3$ (5:1); flow rate 1.3 ml/min; detection 254 nm].
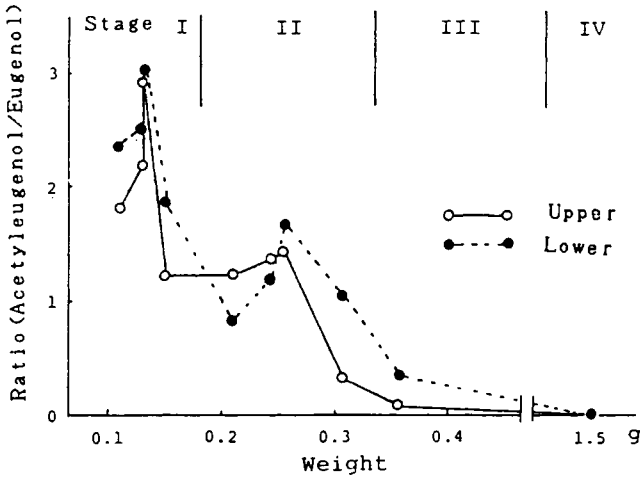
FIGURE 5. Decrease of acetyleugenol in oil sac of fresh clove from stage I to IV.

and lower parts of the fruit. In the oil from dried buds, the acetyleugenol content decreased as a function of maturation, although the oil at each stage consistently contained more eugenol than acetyleugenol (Figure 6).

Phellodendron bark is a Japanese medicinal plant used for stomach and intestinal diseases. The yellowish bark contained two major alkaloids, berberine and palmatine,
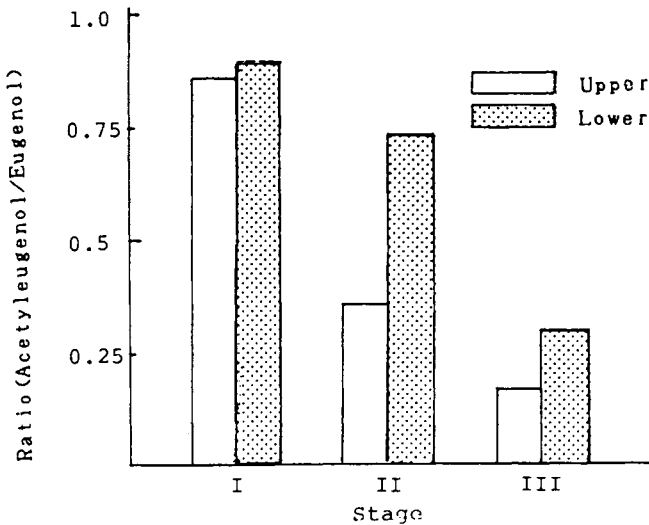
FIGURE 6. Decrease of acetyleugenol in oil sac of dried clove from stage I to III.

FIGURE 7.   Alkaloids crystallized in cells by soaking *Phellodendron* bark in 5% HNO$_3$.

yellow alkaloids in the tissue cannot be distinguished when viewed microscopically. However, when the bark was soaked in 5% HNO$_3$ for 3 days, the alkaloids were crystallized as nitrate salts in the phloem and medullary rays (Figure 7) (4). Crystals were readily removed by means of the micromanipulator and analyzed by hplc. The crystals were composed of about 95% berberine and 3% palmatine in both fresh and dried bark samples (Figures 8,9). Traces of an unknown compound were also found in both samples analyzed.

The papaveraceous plant *Macleaya cordata* is ubiquitous in Japan in summer, producing an orange liquid when cut. Orange-colored cells scattered in the tissue of the root are shown on the right side of Figure 10 and their fluorescence under uv on the left side of Figure 10. Colors in the cells in *M. cordata* depend on the proportion of reddish and yellowish benzo[c]phenanthridine-type alkaloids, sanguinarine and chelerythrine, respectively. The colorless alkaloids in this plant are known to be protopine and the protopine-type allocryptopine. The hplc separation of a reference mixture of the four alkaloids in this plant is shown in Figure 11. The contents of 200 colored cells were removed uniformly by aspiration under the microscope to be quantitatively analyzed by hplc. From the calibration curve, 1–6 ng of benzo[c]phenanthridine-type alkaloids (as chloride) and 10–40 ng of protopine-type alkaloids were found to be present per single

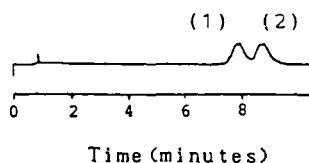
Time (minutes)

FIGURE 8.   Hplc separation of reference palmatine (1) and berberine (2) [column Lichrosorb RP8 (4.6 mm × 150 mm), solvent system 0.1 N tartaric acid-MeCN-MeOH-SDS (50:40:2:0.5), flow rate 2 ml/min, detection 345 nm].
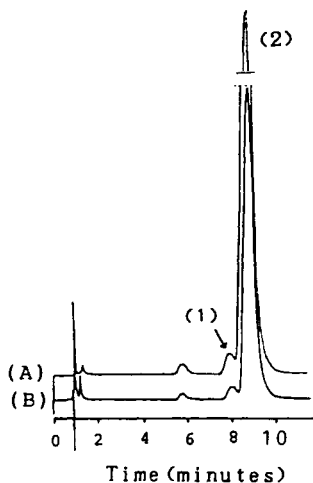
FIGURE 9.   Hplc separation of palmatine (1) and berberine (2) from crystals obtained from fresh (A) and
             dried (B) *phellodendron* cortex in situ; hplc conditions see Figure 8.

colored cell (Table 1) (5). The biosynthetic pathway for the alkaloids of *M. cordata*, which was elucidated by using callus culture (6), could also be proved by histochemical chromatography (7).

   Sanguinaria canadensis, which is called blood root because of the reddish color of the rhizome, was examined by histochemical chromatography. The right and left sides of Figure 12 show a transverse section of the rhizome before and after the suction of the cell
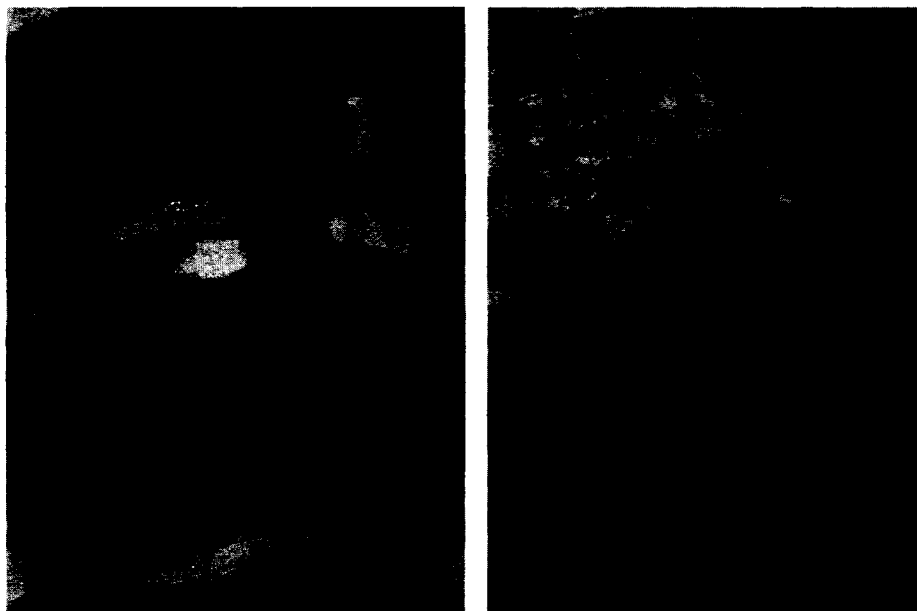
FIGURE 10.   The orange liquids in cells of *Macleaya cordata* (right) and their fluorescence under uv light
              (left). Right: darker cells are orange colored. Left: lighter cells are fluorescent.

cis-N-methyl   allocryptopine   chelerythrine
tetrahydro-
berberine salt
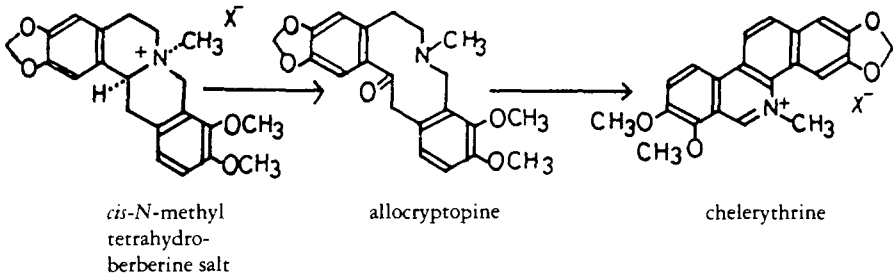
SCHEME 1.   Biosynthetic sequence for alkaloids of *Macleaya cordata*.

Time (minutes)
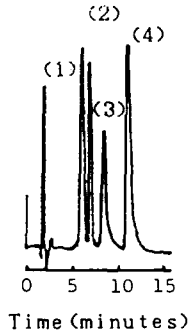
FIGURE 11.   Hplc separation of reference protopine (1), allocryptopine (2), sanguinarine (3), and
chelerythrine (4) [column NV-pack C-18 (5 μm) (8 mm × 100 mm), solvent system 0.1 N
tartaric acid containing SDS 0.125%-MeCN (45:55), flow rate 2.0 ml/min, detection 285
nm].

FIGURE 12.   The reddish liquid in a cell of *Sanguinaria canadensis* before aspiration (right) and a vacant
cell after aspiration (left). The + is the cursor.

TABLE 1.   Alkaloid Content and Proportion per Single Cell in the root of *Macleaya cordata* (1–2 years old).

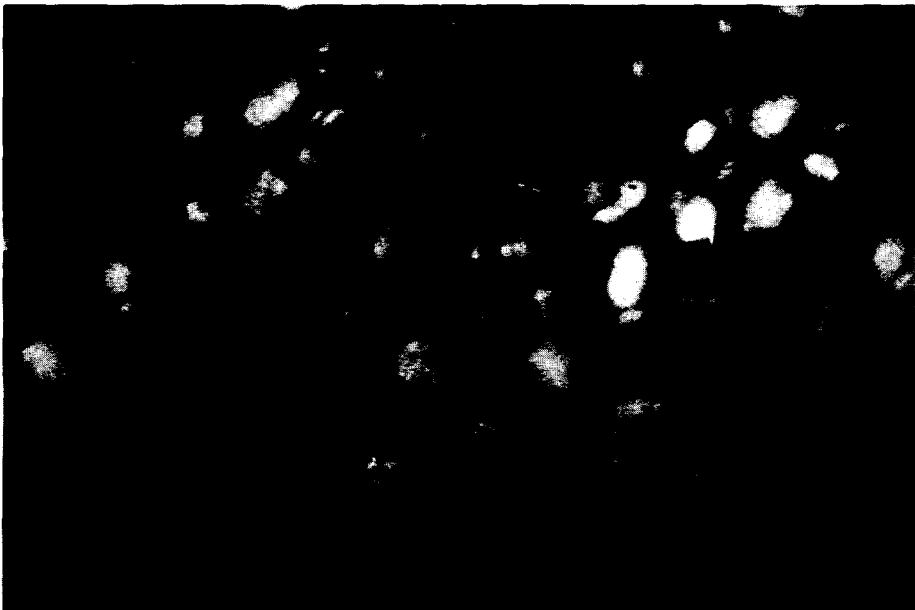| Alkaloid	Diameter of Root					
	ca. 3 mm[a]		5–7 mm[b]		ca. 14 mm[c]	
	ng	%	ng	%	ng	%
Protopine	9.7	43.9	16.0	33.1	25.9	33.7
Allocryptopine . .	11.4	51.6	26.3	54.3	40.6	52.8
Sanguinarine[d] . . .	0.6	2.7	3.2	6.6	4.9	6.4
Chelerythrine[d] . .	0.4	1.8	2.9	6.0	5.5	7.1

[a]Average of 1000 cells ($n = 5$).
[b]Average of 2800 cells ($n = 14$).
[c]Average of 2000 cells ($n = 10$).
[d]As chloride.

contents, respectively (magnification 400 ×) (8). The four alkaloids described in *M. cordata* and two unknown compounds were found in the rhizome of *S. canadensis* (Figure 13). The content of known alkaloids per single colored cell was analyzed and their ratios were calculated (Table 2). The reddish alkaloid sanguinarine is present in higher proportion in the reddish *S. canadensis* rhizome than in the orange-colored *M. cordata* root.
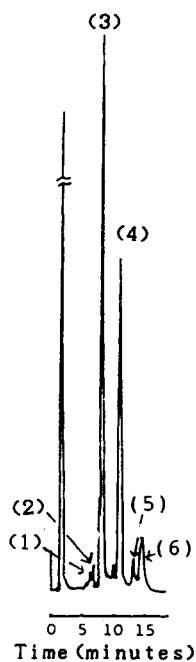

FIGURE 13.   Hplc separation of orange liquid from cells in *Sanguinaria canadensis* rhizome: protopine (1), allocryptopine (2), sanguinarine (3), chelerythrine (4), (5) and (6) unknown; hplc conditions see Figure 11.

TABLE 2.   Alkaloid Content per Single
Colored Cell and Ratio of Four Alkaloids.

Alkaloid	ng[a]	%
Protopine	9.3	4.1
Allocryptopine . .	12.7	5.6
Sanguinarine[b] . . .	136.9	60.2
Chelerythrine[b] . .	68.3	30.1

[a]Average of 2800 cells.
[b]As chloride, $n =$ 14.

The application of histochemical chromatography to animal sections involves the analysis of renal deposits found in hyperuricemic rats freely fed on a laboratory ration containing 3% uric acid and 2% potassium oxonate (9). After 3 weeks of this diet, the rats were killed with ether, and their kidneys were sliced for microscopic examination. The renal deposits were removed from the slices by means of the micromanipulator. The hplc separation of a reference mixture of purine derivatives and oxonate is shown in Figure 14. The major peak of the renal deposits was identified to be uric acid by hplc (Figure 15) and sims (10).

Thus, the technique of histochemical chromatography appears to have practical applications not only in histochemistry and pharmacognosy but also in the areas of biosynthesis, physiology, pathology, pharmacology, and biochemistry.

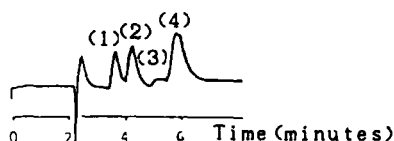FIGURE 14.   Hplc separation of reference xanthine (1), hypoxanthine (2), oxonate (3), and uric acid (4) [column Nucleosil NH_2 (4.6 mm $\times$ 150 mm), solvent system 1/15 M phosphate buffer pH 7.5, flow rate 1.5 m/min, detection 254 nm].
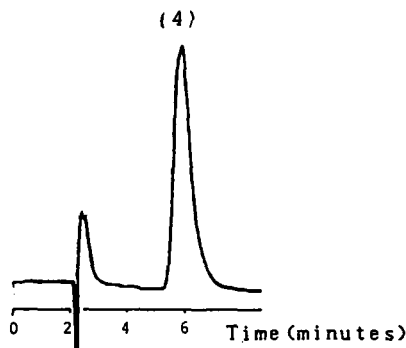

FIGURE 15.   Hplc separation of a renal deposit from a hyperuricemic rat (4 = uric acid); hplc conditions see Figure 14.

ENFLEURAGE CHROMATOGRAPHY.—Enfleurage is a method used in the commercial preparation of perfumes. Volatile compounds are absorbed into fresh fat such as a mixture of lard and tallow spread on glass plates in a closed box. The fat is then extracted with EtOH, and the solvent is removed under reduced pressure to obtain an "absolute" (11). The author has used this method for the analysis of the volatile components of materials and has named the technique "enfleurage chromatography" (12). The synthetic triglycerides such as triolein and tripalmitin were used for absorbents of the volatile compounds instead of natural fats; MeCN was used for their extraction. The components in the extract were obtained in sufficient concentration for analysis by hplc and gc without evaporation of the solvent. A minimum of 100 g was required to obtain volatile components by conventional extraction. On the other hand, the method newly developed by the author can be performed with only a few grams of sample (12).
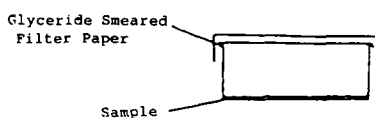
FIGURE 16.   Container for enfleurage method.

For enfleurage chromatography of Paeoniae Radix and Moutan Cortex, 1 g of pulverized sample was placed in a small Petri dish (4.5 cm i.d., 1.5 cm height) (Figure 16) (13). 4-Methoxyacetophenone dissolved in MeOH as an internal standard was added to the sample and stirred well. About 0.2 g of fat [triolein-tripalmitin (10:1)] was spread over filter paper (5.5 cm diameter), which was placed on the top of the Petri dish. The Petri dish was closed and placed in an oven at 100° for 2 h. The filter paper was extracted with 5 ml of 50% aqueous MeCN in a glass tube for 2 min with ultrasonification. The extract was filtered through a small filter paper and the filtrate was used for hplc analy-
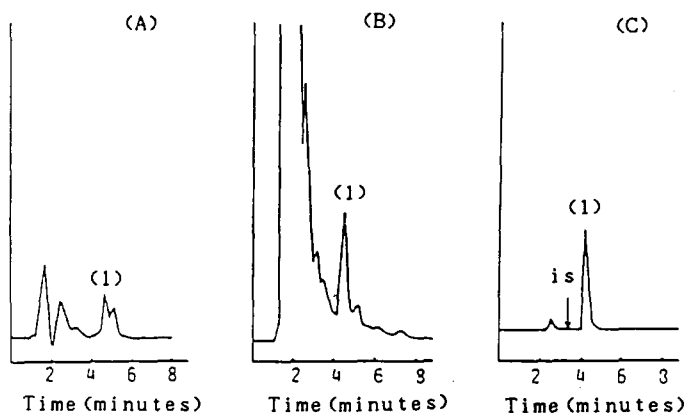
FIGURE 17.   Comparison of enfleurage and extraction method for Paeoniae Radix by hplc separation. A, Extracted with Et₂O. B, Extracted with MeOH. C, Enfleurage method. 1. Paeonol. Internal standard (IS), 4-methoxyacetophenone. Column, Spheriosorb ODS 5 μm (4.6 mm × 150 mm); solvent system, MeCN-H₂O-HOAc (100:10:0.2); flow rate, 1.2 ml/min; detection, 280 nm.

sis. The enfleurage extraction technique was compared with solvent extraction method. Paeoniae Radix was extracted with several solvents, such as Et_2O, MeOH, and EtOH, and each extract was subjected to hplc (13). Paeonol in solvent extracts was not clearly detected by hplc, which was seriously affected by coextracted substances (Figure 17). In contrast, the enfleurage method proved to be much superior and demonstrated the presence of paeonol in Paeoniae Radix without being affected by coexisting substances (Figure 17). The results suggested that volatile components and presumably sublimating components are both specifically absorbed into fat.

To determine the optimal temperature for the enfleurage method, samples were subjected to the enfleurage process in an oven kept at various temperatures for 2 h, and the content of paeonol in each extract was measured by hplc. The peak height of paeonol increased gradually as the enfleurage temperature increased and reached a maximum at 100°. Further increase in temperature brought about slight decrease in the peak height, and the samples colored a little brown. Therefore, the enfleurage was carried out at 100° (Figure 18). To determine the optimal time for the enfleurage process, enfleurage was conducted at 100° for various periods of time (0.5–3.0 h). A period of 2 h was found to be suitable (Figure 19). The content of paeonol in various Paeoniae Radix samples is
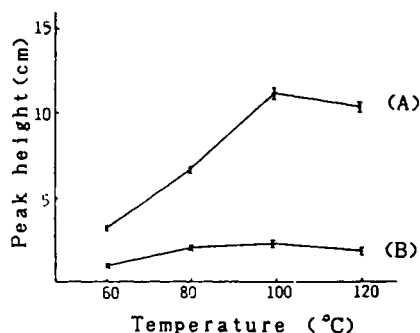

FIGURE 18.   Dependence of peak height of paeonol on temperature in enfleurage process. A, Mountan cortex (China), 2.5 µl. B, Paeoniae Radix (Japan No. 1 in Table 3), 20 µl. Mean of 5 samples.
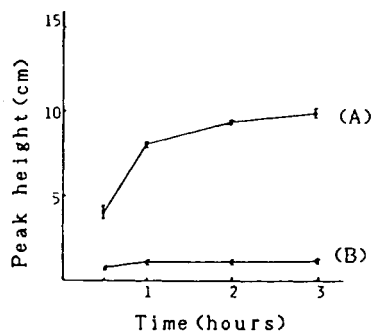

FIGURE 19.   Dependence of peak height of paeonol on heating time in enfleurage process. A, Mountan cortex (China), 2.5 µl. B, Paeoniae Radix (Japan No. 1 in Table 3), 20 µl. Mean of 5 samples.

given in Table 3. Paeoniae Radix (No. 6 in Table 3) was divided into three parts as shown in Figure 20, and the content of paeonol in each part was determined. Table 4 shows that a larger portion of paeonol exists near the cortex in Paeoniae Radix. The content of paeonol in Mountan Cortex of Chinese origin was found to be 16 mg/g.

TABLE 3.   Content of Paeonol in Various Paeoniae Radix.

No.	Crude drug samples	Paeonol content[b] (mg/g)
1	Japan (Nagano)	0.230 ± 0.009
2[a]	Japan (Nara)	0.006 ± 0.001
3	Korea	0.140 ± 0.002
4[a]	China (Bai-Shao-Yao)	trace
5	China (Chi-Shao-Yao)	0.240 ± 0.004
6	China (Chi-Shao-Yao)	0.234 ± 0.002
7	Japan (Hokkaido)	0.044 ± 0.005
8	North Korea	trace

[a]Scalded.
[b]Values are mean ± SE, $n = 5$.
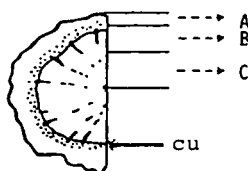
FIGURE 20.   Transverse section of Paeoniae Radix. cu: cumbium A, B & C: see text.

TABLE 4.   Distribution of Paeonol in Tissues of Paeoniae Radix.

Section[a]	Paeonol content[b] (mg/g)
A	0.062
B	0.016
C	0.031

[a]The location of sections A, B, and C is shown in Figure 20.
[b]Mean, $n = 5$.

Many crude drugs used for Chinese traditional medicine have characteristic odors, and odors are often closely connected with drug actions as in the case of aromatic stomachics. The analysis of volatile components in three crude drugs, the seeds of *Amomum xanthioides, Amomum tsao-ko,* and *Alpinia katsumadai,* were carried out by enfleurage chromatography (14). These crude drugs have a characteristic odor and have been used for various Chinese drugs and condiments. According to Yoshikawa *et al.* (15), the seeds of *Am. tsao-ko* have a foul smelling odor, which may be attributed to 4-indanecarbaldehyde. In enfleurage investigation, the presence of 2-dodecen-1-al, 2-decen-1-al, and 2-octen-1-al, which are the principal compounds of the stink odor of a stinkbug, was confirmed (Table 5) (14,16). Thus, it is probable that this odor may be partially attributable to these unsaturated aldehydes along with 4-indanecarbaldehyde. The essential oil content from the seeds of *Am. xanthioides* was found to be 0.75 ml/30 g when determined with the apparatus for essential oil determination by the *Japanese Pharmacopoeia XI* (JP XI) (17). Results of quantitative analysis by gc using 4-methoxy-

TABLE 5.   Comparison of Volatile Components Identified from the Seeds of *Amomum xanthioides*,
Alpinia katsumadai, and *Amomum tsao-ko*.[a]

Peak No.	Compounds	Am. xanthioides	Al. katsumadai	Am. tsao-ko	Evidence	
					gc (authentic compounds)	gc-ms
1	α-pinene	+	+	+	o	o
2	camphor	+ +	+	−	o	o
3	β-pinene	−	+	+	o	o
4	β-myrcene	+ +	−	−	o	o
5	α-phellandrene	+	+	+	o	o
6	p-cymene	+	+	+	o	o
7	1,8-cineol	−	+ + +	+ + +	o	o
8	limonene	+ +	+	+	o	o
9	2-octen-1-al	−	−	+	o	o
10	Δ³-carene	−	+	+		o
11	1,4-cineol	+	−	+		o
12	nonan-2-one	−	−	+	o	o
13	fenchone	−	−	+	o	o
14	linalool	+ + +	+	+	o	o
15	camphor	+ + +	+	−	o	o
16	iso-borneol	+	−	−	o	o
17	borneol	+ + +	+	−	o	o
18	citronellal	−	−	+	o	o
19	myrcenol	−	−	+		o
20	terpinolene	+	−	−	o	o
21	terpinen-4-ol	+	+ +	+ +	o	o
22	pulegone	+	−	−	o	o
23	α-terpineol	+	+ +	+ +	o	o
24	sabinol	−	+ +	+		o
25	d-carvone	+	−	−	o	o
26	citral	−	−	+	o	o
27	benzyl acetone	+	+ +	−	o	o
28	sabinyl acetate	−	+	−		o
29	2-decen-1-al	−	−	+ +	o	o
30	geraniol	+	−	+ +	o	o
31	geranial	−	−	+ +	o	o
32	thymol	−	+	+	o	o
33	bornyl acetate	+ + +	−	−	o	o
34	terpinyl acetate	+	−	−	o	o
35	α-methyl cinnamaldehyde	−	−	+ +	o	o
36	4-indanecarbaldehyde	−	−	+		o
37	geranyl acetate	+	−	+	o	o
38	iso-bornyl acetate	+	−	−	o	o
39	methyl cinnamate	−	+	−	o	o
40	α-copaene	+	+	−		o
41	β-elemene	+	−	−		o
42	caryophyllene	+ +	+ +	−	o	o
43	trans-farnescene	+	−	−		o
44	2-dodecen-1-al	−	−	+	o	o
45	humulene	+	+ + +	−	o	o
46	aromadendrene	+	+	−		o
47	β-chamigrene	+	−	−		o
48	γ-cadinene	+	+	+		o
49	γ-elemene	+	−	−		o
50	γ-patchoulene	−	+	−		o
51	α-muurolene	−	+	−		o
52	calamenene	−	+	−		o
53	δ-cadinene	−	+	−		o
54	α-himacharene	+	−	−		o
55	β-bisabolene	+	−	−		o
56	elemol	−	−	+		o
57	nerolidol	+ + +	+	+	o	o

[a]Volatile contents are classified into four degrees (−, +, + + and + + +) according to peak height appearing on gas chromatograms by enfleurage method at 100° for 1 h.

acetophenone as an internal standard showed that the contents of major components (linalool, camphor, borneol, bornyl acetate, and nerolidol) in enfleurage extract for the seeds of this plant were almost the same as the contents of seeds in essential oil by the JP XI determination (Table 6). The authors also carried out the analysis of the volatile

components in seeds of *Amomum cardamomum* and *Elettaria cardamomum* used as aromatic stomachics and condiments. Sixteen constituents, including α-pinene, 1,8-cineol, linalool, and nerolidol, were found to be common to these two seeds (Table 7) (18). However, the presence of terpinyl acetate, which is one of the main constituents in seeds of *E. cardamomum*, was not detected in seeds of *A. cardamomum*. The presence of many sesquiterpenoids, including caryophyllene, β- and α-cardinenes, and β-bisabolene, was confirmed in seeds of *A. cardamomum* by this method.

Enfleurage of *Forsythia suspensa* was conducted at 60° for 2 h (19). The analysis of essential oils in seeds was possible even when the sample amount was as small as 10 mg. The content of β-pinene analyzed by enfleurage gc in four *Forsythia* seed samples was

TABLE 6. Contents of Linalool, Camphor, Borneol, Bornyl Acetate, and Nerolidol in *Amomum xanthoides* Seeds.[a]

Compounds	Enfleurage-gc (%)	Japanese Pharmacopoeia XI-gc (%)
Linalool	0.09	0.09
Camphor	0.88	0.90
Borneol	0.15	0.14
Bornyl acetate	0.76	0.73
Nerolidol	0.37	0.37

[a]Values are percent dry wt; mean of 5 samples.

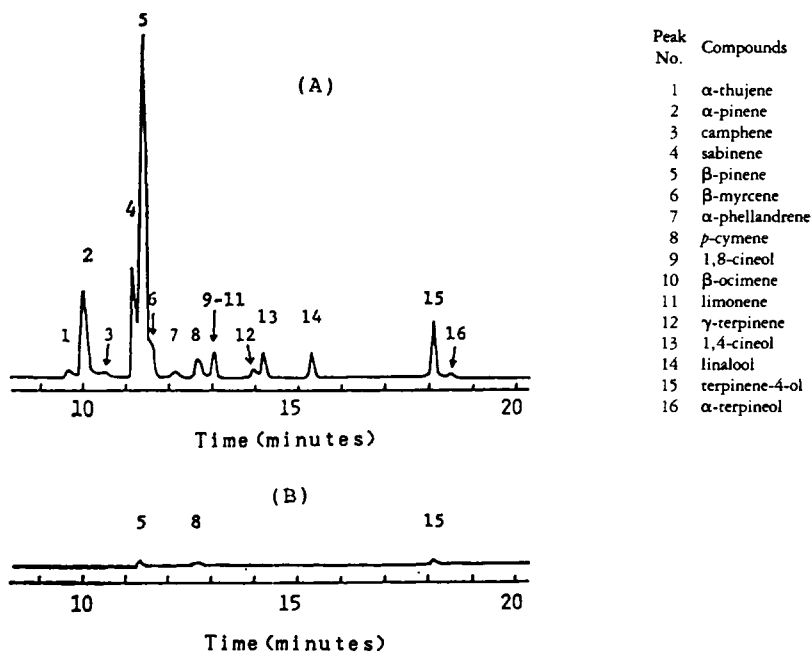| Peak No.	Compounds
1	α-thujene
2	α-pinene
3	camphene
4	sabinene
5	β-pinene
6	β-myrcene
7	α-phellandrene
8	p-cymene
9	1,8-cineol
10	β-ocimene
11	limonene
12	γ-terpinene
13	1,4-cineol
14	linalool
15	terpinene-4-ol
16	α-terpineol

FIGURE 21. Gas chromatograms of enfleurage extracts of *Forsythia suspensa* seed (A) and pericarp (B).

TABLE 7.   Comparison of Flavor Components Identified in the Seeds of *Amomum cardamomum* and *Elettaria cardamomum*.[a]

Peak No.	Compounds	Am. cardamomum	E. cardamomum	Evidence	
				gc	gc-ms
				(authentic compounds)	
1	α-pinene	+	+	o	o
2	camphene	−	+	o	o
3	sabinene	+ +	+ +		o
4	myrcene	+ +	+ +	o	o
5	1,8-cineol	+ + +	+ + +	o	o
6	limonene	+	+	o	o
7	Δ³-carene	+	−	o	o
8	1,4-cineol	+ +	+ +		o
9	linalool	+	+ + +	· o	o
10	β-terpineol	+	+ +		o
11	camphor	+	−	o	o
12	borneol	+	+	o	o
13	myrcenol	+ +	+		o
14	terpinen-4-ol	+	+ +	o	o
15	α-terpineol	+ + +	+ + +	o	o
16	geraniol	−	+ +	o	o
17	geranial	−	+	o	o
18	linalyl acetate	−	+ + +	o	o
19	bornyl acetate	−	+	o	o
20	neryl acetate	+	+	o	o
21	terpinyl acetate	−	+ + +	o	o
22	geranyl acetate	+	+	o	o
23	β-elemene	+	−		o
24	caryophyllene	+	−	o	o
25	γ-cadinene	+	−		o
26	γ-patchoulene	+	+		o
27	β-patchoulene	+	−		o
28	α-himacharene	+	−		o
29	β-cadinene	+	−		o
30	β-bisabolene	+	−		o
31	nerolidol	+	+ +	o	o

[a]Volatile contents are classified into four degrees (−, +, + + and + + +) according to their peak heights appearing on the gas chromatograms by the enfleurage method at 100° for 1 h.

found to be about the same (3.4, 3.3, 3.4, and 3.5%; 3.4% on average by wt). The essential oil content was determined to be 1.8 ml/30 g when assayed by the JP XI essential oil determination (17). The essential oil consisted of 3.3% β-pinene analyzed by gc (19). The fruits were divided into seeds and pericarps, and each portion was powdered in a porcelain mortar. Because the flavor of pericarps was faint, a fairly large amount of the sample (5.0 g) was necessary. Silicone-treated filter papers gave better results than untreated ones. The presence of seven compounds (α-thujene, sabinene, α-phellandrenene, 1,8-cineol, limonene, 1,4-cineol, and α-terpineol) was newly determined in the seeds by the enfleurage method (Figure 21A). Three compounds (β-pinene, *p*-cymene and terpinene-4-ol) were also found in pericarps (Figure 21B).

Enfleurage chromatography is also applicable for a versatile biogenetic study of the volatile components of plants such as *Crocus*. Safranal was biosynthesized from protocrocin through picrocrocin and hydroxy-β-cyclocitral (Scheme 2). Eight different oxidation derivatives were newly found from the enfleurage of *Crocus* along with safranal and
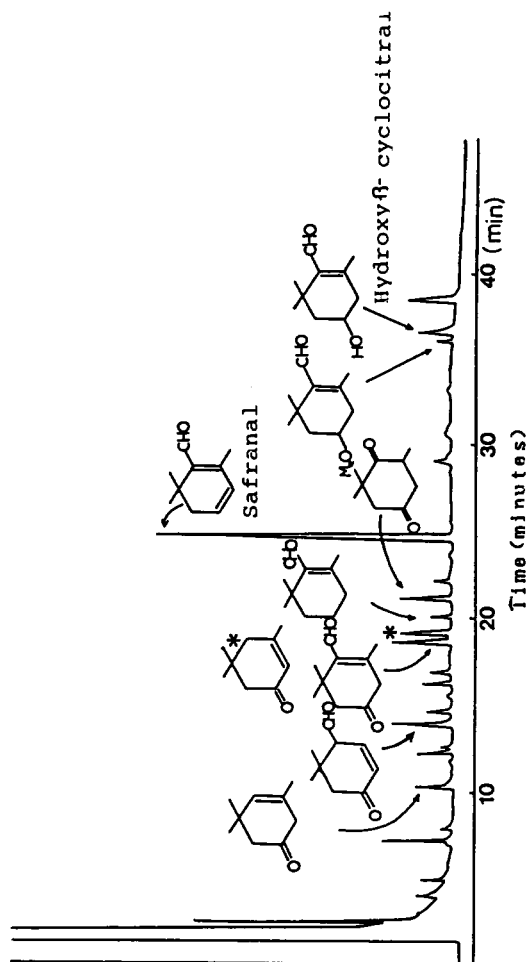
FIGURE 22.   Gc identification of safranal and related compounds from enfleurage extract of *Crocus*. Column, 0.24 mm i.d. × 25 m fused silica capillary column coated with OV101; oven temperature 60–200°/min; carrier gas N$_2$ (0.5 ml/min).

SCHEME 2.   Biosynthetic sequence of safranal in *Crocus*.

hydroxy-β-cyclocitral by gc-ms (Figure 22) (20). Crocin was known to be present in *Gardenia* fruits. Figure 23 showed the detection of safranal in *Gardenia* fruits (20). Only 1 mg and 5 mg for *Crocus* and *Gardenia* fruits, respectively, were necessary to detect safranal on hplc by this method. Contents of safranal in both materials were analyzed (Table 8) (20).

Fresh roots of shimotsuke (*Filipendula multijiga*) and onishimotsuke (*Filipendula kamtschatica*) have been studied by enfleurage chromatography to obtain the gc chromatograms illustrated in Figure 24 (12). Both samples contained methylsalicylate.

FIGURE 23.   Hpl chromatogram of enfleurage extract of *Gardenia* fruits. Column Nucleosil ODS, 5 μm (4.6 mm × 150 mm); solvent system MeCN-NH$_2$ (1:1); flow rate 1.0 ml/min, detection 243 nm; internal standard (is), eugenol.
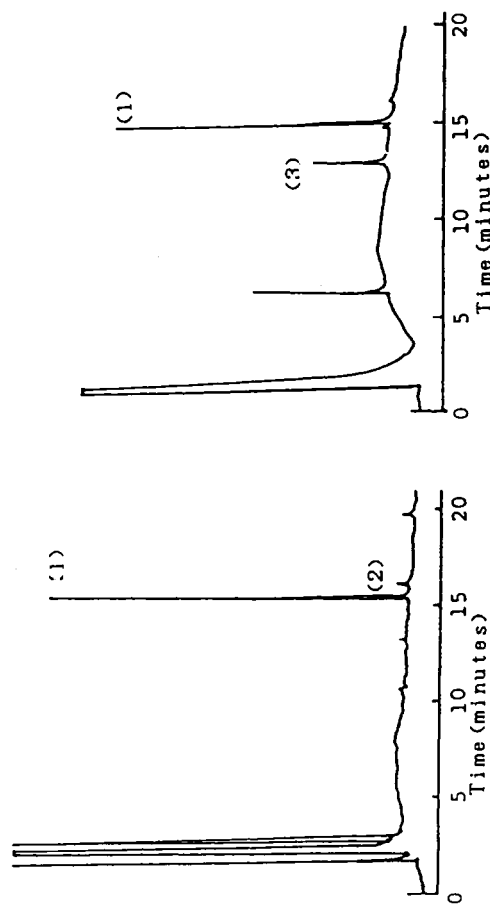
FIGURE 24. Gas chromatograms of enfleurage extract of *Filipendula multijuga* root (A) and *Filipendula kamtschatica* root and rhizome (B). Column 0.20 mm × 15 m fused silica capillary column coated with Carbowax 20M; oven temperature 80° (2 min), 200° at 5°/min; carrier gas He (0.6 ml/min; detection FID); methylsalicylate (1), ethylsalicylate (2), salicylaldehyde (3).

TABLE 8.   Contents of Safranal in *Gardenia*
fruits and *Crocus*.

Sample	mg/g[a]
Gardenia fruits	
A (China)	0.012
B (China)	0.021
C (Formosa)	0.033
D (China)	0.008
Crocus	
Japan	1.5

[a]Mean, $n = 5$.

The former contained a very small amount of ethylsalicylate as compared to methylsalicylate and the latter contained a fairly large amount of salicylaldehyde. Methylsalicylate was also found in dried senega root (*Polygala senega*) by enfleurage chromatography (Figure 25) (12,21). Dried bark of seironnikkei (*Cinnamomum zeylanicum*) and the fresh bark of nikkei (*Cinnamomum sieboldii*) were examined by enfleurage chromatography (12). The volatile compounds from the former consisted mainly of cinnamaldehyde, but volatile compounds from the latter consisted of cinnamyl alcohol and cinnamic acid as well as cinnamaldehyde (Figure 26). Two Chinese drugs containing *Cinnamomum cassia*, keishito and hachimijiogan, were analyzed. Cinnmamic acid, cinnamyl alcohol, and cinnamaldehyde were detected in both drugs; these compounds might be from *C. cassia* (Figure 27) (12).

Shimizu and Yoshihara (22) identified the components in the fruit of Japanese quince (*Cydonia oblonga*) with a strong and characteristic aroma, of which 20-kg samples were used. By enfleurage gc-ms, a quantity less than one fruit was used to detect the components, which were almost the same as the ones reported by Shimizu (Table 9) (23).

The volatile and sublimating components can be selectively assayed by enfleurage chromatography with high sensitivity. This technique accordingly provides very effective microdetermination for volatile components in materials, even when they are of very low content or coexisting with substances encountered in the usual extraction procedures. Thus, the enfleurage (pomade) method makes it possible to minimize the sample size and use only 1–0.1% as much sample as is needed for conventional methods.
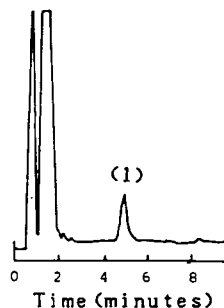
FIGURE 25.   Hpl chromatogram of enfleurage extract of *Polygala senega*; hplc conditions see Figure 24.
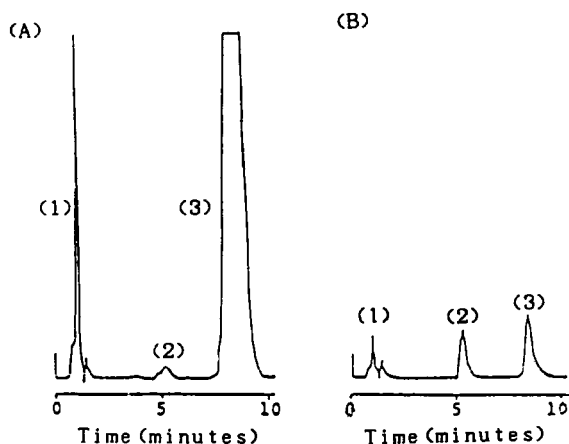
FIGURE 26. Hpl chromatograms of enfleurage extract of *Cinnamomum zeylanicum* (A) and *Cinnamomum sieboldii* (B). Column Chemcosorb ODS L 7 μm (4.6 mm × 150 mm); solvent system H_2O-MeCN (5:2); flow rate 1.5 ml/min; detection 265 nm; methylsalicylate (1), ethylsalicylate (2), salicylaldehyde (3).

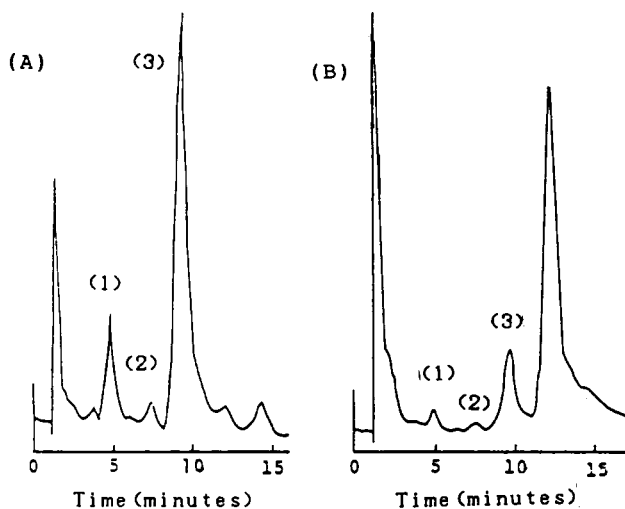FIGURE 27. Hpl chromatograms of enfleurage of Keishito (A) and Hachimijiogan (B). Column Chemcosorb ODS L, 7 μm (4.6 mm × 150 mm); solvent system H_2O-MeCN-HOAc (100:35:2); flow rate 1.2 ml/min; detection 280 nm; cinnamic acid (1), cinnamyl alcohol (2), cinnamaldehyde (3).

TABLE 9.   Gc-ms Identification of Components
of Enfleurage Extract of *Cydonia oblonga*.

Peak no.	Component	Mol wt
1	ethyl hexanoate	144
2	ethyl octanoate	172
3	ethyl octenoate	170
4	benzaldehyde	106
5	ethyl nonanoate	186
6	unknown	
7	ethyl decanoate	200
8	ethyl decenoate	198
9	ethyl decenoate	198
10	ethyl dodecanoate	228
11	ethyl dodecenoate	226
12	ethyl dodecenoate	226
13	ethyl dodecadienoate	224
14	unknown	
15	unknown	256
16	unknown	254
17	unknown	252
18 } 21	unknown	

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