Plant Drug Analysis by Planar Chromatography

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Abstract

Optimal performance laminar chromatography and automated multiple development chromatography are relatively recent techniques of planar chromatography that can be applied with success in plant material analysis. Therefore, these methods are used to study plant extracts and constituents belonging to different chemical classes of secondary metabolism: heterocyclic oxygen compounds (coumarins, flavonoids, and anthocyanins), alkaloids and quaternary ammonium salts, cannabinoids, essential oils, ginsenosides, and cardiac heterosides. Generally, the results obtained with these methods are good, and in most cases they compare with those of thin-layer chromatography.

Introduction

Besides classical thin-layer chromatography (TLC), two other techniques of planar chromatography appeared in the 1980s: overpressured layer chromatography, or optimal performance laminar chromatography (OPLC) (1–4), and automated multiple development (AMD) (5–8). Few studies concerning OPLC and AMD had been reported in the literature at the beginning of this study.

These techniques were applied to the analysis and characterization of numerous natural compounds belonging to various chemical classes found in plant material, and the results were compared with those of TLC. All of the analyses were performed on crude plant extracts versus reference substances.

Numerous compounds found in plants have pharmacological and, for some of them, therapeutic activity. The principal classes of compounds include nitrogen-containing heterocycles such as alkaloids and derivatives; heterosides and aglycones such as those of cardiotonic steroids; and phenolic compounds such as coumarins, flavonoids, and anthocyanins, which embrace a wide range of plant substances and possess in common an aromatic ring bearing one or more hydroxyl groups or phenolic ether substituents (9).

All of the results with heterocyclic oxygen compounds, alkaloids and ammonium quaternary salts, cannabinoids, essential oils, ginsenosides, and cardiac heterosides are reported in this work.

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Experimental

A Linomat IV (Camag, Muttenz, Switzerland) was used for sample applications. A TLC–MAT (automated development in TLC) Desaga (Bionisis, Le Plessis-Robinson, France) was also used. A Chrompres 25 (OPLC-NIT Engineering Company, Budapest, Hungary) OPLC (Bionisis) was used. For AMD, a Densitometer Camag Model 76510 TLC–high-performance (HP) TLC scanner was used.

All solvents and reagents were analytical grade and obtained from Merck (Darmstadt, Germany). Before use, solvents were filtered through a 0.45-µm Millipore membrane after sonication.

All standards were commercially pure products.

Heterocyclic oxygen compounds aglycones (10)

Plant material samples

Powdered plant material (5 g) was mixed with 50 mL of methanol, and then the dry sample residue was hydrolyzed with 2M hydrochloric acid for 1 h at 100°C. Once cool, the solution was extracted three times with 50 mL of ethyl acetate, dried on anhydrous sodium sulfate, and the solvent removed under reduced pressure whereupon the residue was taken up with 1 mL of methanol.

Apparatus

The apparatus used was a Chrompres 25.

Plates

Silica gel F_{254} 20- ¥ 20-cm glass TLC plates (Merck, Art. 5715) were used. For OPLC, the chromatographic plates required a special preparation mode: three edges were obliquely scratched off and impregnated with a suitable polymer suspension Impress II (OPLC-NIT Engineering Company).

Chromatographic conditions

Three mobile phases were employed: ethyl acetate–chloroform (60:40, v/v) for coumarins and flavonoids, ethyl acetate–chloroform (90:10, v/v) for furanochromones, and ethyl acetate–methyl ethyl ketone–formic acid–2M hydrochloric acid (65:10:6:9, v/v) in

the case of anthocyanins.

During chromatography, the external pressure of the water cushion was 16 bars. The starting mobile phase pressure was 20 bars, and plates were developed on a distance of 17 cm with a mobile phase flow rate of 0.40 mL/min.

The development time was between 12 and 25 min depending upon the eluent.

The separations were checked first by visual observation under UV illumination (l = 365 nm) for coumarins and furanochromones, then by spraying with 1% methanolic diphenylboric acid b-ethylamino ester, followed by 5% ethanolic polyethyleneglycol 400 [NP/PEG reagent (11,12)]. Then, observation occurred at l = 365 nm for flavonoids and by direct visual observation without reagent treatment for anthocyanins.

Alkaloids and quaternary ammonium salts

Plant material samples

Powdered plant material (1 g) was mixed thoroughly with 60% ethanol (10 mL) by shaking for 4 h, followed by filtration through a No. 2 glass frit and adjustment of the volume to 10 mL. The quantity of the sample that was applied to the layer was calculated from the average content of the drug in the plant material extracted.

Plates

The plates were prepared with the special mode for OPLC (see Phenolic compounds section).

For alkaloids, aluminum oxide 60 F_{254} type E 20-¥20-cm glass plates (Merck, Art. 5713) were used. For quaternary ammonium salts, silica gel 60 F_{254} 20-¥20-cm glass plates (Merck, Art. 5715) were used. For semipreparative, aluminum oxide 60 F_{254} 20-¥20-cm glass plates (1.5-mm thickness for preparative layer chromatography) (Merck, Art. 5788) were used.

Chromatographic conditions

Analytical OPLC of alkaloids. Two mobile phases were employed: ethyl acetate (eluent A) for almost all of the alkaloids and methylene chloride–ethyl acetate (80:20, v/v) (eluent B) for *Rauwolfia* alkaloids.

During chromatography, the external pressure of the water cushion was 15 bars, the starting mobile phase pressure was 7 bars, and plates were developed on a distance of 16 cm at a mobile phase flow rate of 0.40 mL/min. The development time was 10 min.

The separations were checked by visual observation under UV illumination (l = 365 nm) and after being sprayed with either Dragendorff's or iodoplatinate reagent (11).

Densitograms were recorded at 540 nm after visualization with Dragendorff's reagent.

Semipreparative OPLC of alkaloids. Sample applications were performed by successive deposits in the line (8 times). For chromatography, the water cushion pressure was 12 bars and the flow rate 0.6 mL/min.

For each plant extract, the alkaloids are listed in the following elution order. For *Strychnos nux vomica* [ethyl acetate–isopropanol (4:1, v/v)], it was 1-brucine then 2-strychnine. In the case of opium and *Datura stramonium*, a gradient of eluents was used. For opium [hexane–ethyl acetate (1:1, v/v); then ethyl acetate; and finally ethyl acetate–isopropanol (4:1, v/v)], it was 1-noscapine, 2-papaverine, 3-codeine, 4-thebaine, and then 5-morphine. For *D. stramonium* [ethyl acetate, then ethyl acetate–28% ammonia (100:1, v/v)], it was 1-scopolamine and then 2-atropine. Finally, for *Lupinus mutabilis* (ethyl acetate), it was lupanine.

The eluted fractions were controlled by TLC on silica gel 60 F_{254} plates (Merck, Art. 5715), and separations were checked after being sprayed with Dragendorff's or iodoplatinate reagent (11). The hRf for *S. nux vomica* [chloroform–methanol–28% ammonia (95:5:1, v/v)] was 63 for brucine and 75 for strychnine. The hRf for opium [toluene–acetone–ethanol–28% ammonia (45:45:7:3, v/v)] was 4 for morphine, 51 for codeine, 64 for thebaine, 88 for papaverine, and 96 for noscapine. The hRf for *D. stramonium* [acetone–water–28% ammonia (90:7:3, v/v)] was 25 for atropine and 78 for scopolamine. The hRf for *L. mutabilis* [chloroform–acetone–28% ammonia (25:24:1, v/v)] was 62 for lupanine.

Quaternary ammonium salts

Ethyl acetate–tetrahydrofuran–acetic acid (60:20:20, v/v) was used as an eluent. During chromatography the external pressure of the water cushion was 18 bars, the starting mobile phase pressure was 7 bars, and plates were developed upon a distance of 14 cm at a mobile phase flow rate of 0.40 mL/min. The development time was 10 min. The separations were checked by visual observation under UV illumination (l = 365 nm) and after dipping in Dragendorff's reagent (11). Quantitation was accomplished by spectrodensitometry.

Opiates and derivatives

Preparation of reference and extract solution

The opium extract was obtained from 2 g of opium powder by treatment with 0.1N hydrochloric acid and extraction by methylene chloride. The acidic solution was alkalized by 28% ammonia until pH 10 and extracted with methylene chloride. The alkaloidic extract was dried on anhydrous sodium sulfate and then evaporated. The residue was dissolved in 5 mL of methanol.

Each standard solution was prepared by dissolving 10 mg of reference substance (morphine, codeine, thebaine, papaverine, noscapine, ethylmorphine, pholcodine, heroin, buprenorphine, dextromethorphan, or methadone) in 1 mL of methanol.

Plates

For TLC and AMD, silica gel 60 F_{254} 10-¥20-cm plates (Merck, Art. 5729) were used. For OPLC, aluminum oxide 60 F_{254} 20-¥20-cm plates (Merck, Art. 5713) impregnated on three sides by Impress polymer suspension No. 2 (OPLC-NIT Engineering Company) were used.

Application

For TLC and AMD, a 5-µL opium extract (1-µL morphine, codeine, thebaine, noscapine, and papaverine); 1-µL heroin; 2-µL buprenorphine; 2-µL methadone; 2-?L ethylmorphine; 1-µL dextromethorphan; and 1-µL pholcodine were used. For OPLC, a 5-µL opium extract (2-µL morphine, codeine, thebaine, noscapine, and papaverine) was used.

Eluents

For TLC, toluene–acetone–ethyl alcohol (96º)–28% ammonia (45:45:7:1, v/v) was used. For OPLC, ethyl acetate 100 was used.

For AMD, elution gradient 1a was methanol 100, acetone 100, ethyl acetate 100, ethyl acetate–methylene chloride (50:50), methylene chloride 100, and methylene chloride 100, and elution gradient 1b was methanol saturated with ammoniac 100, acetone 100, acetone 100, ethyl acetate 100, ethyl acetate–methylene chloride (50:50), and methylene chloride 100.

Reagent

The reagents used were Dragendorff's and iodoplatinate reagents (11).

Cannabinoids

Plant material and reference samples preparation

Cannabis resin (0.1g) was extracted by shaking at room temperature for 20 min with 10 mL of hexane. The filtrate was evaporated to dryness and the residue dissolved in 1 mL of toluene.

Hemp samples (0.5 g) were extracted for 10 min with 20 mL of hexane. After filtration the extracts were evaporated under vacuum and the residue dissolved in toluene.

Standard solutions of D^8-tetrahydrocannabinol (THC) and D^9-THC were prepared in $1/10^e$ methanol.

Chromatographic conditions

TLC and AMD were performed on silica gel HP-TLC F_{254} 10-¥ 20-cm plates (Merck), and standards and samples were applied with a Linomat IV for a volume of 5 ?L.

TLC analysis on silica gel was conducted with methanol–chloroform (9:1, v/v) (13).

HP-TLC development was performed at a distance of 60 mm with the eluent diethyl ether–hexane (20:80, v/v).

OPLC separation was realized with hexane–ethyl acetate (70:30, v/v).

For AMD, two gradients based on the "universal gradient" were tested with the following composition. For elution gradient 1a, it was methylene chloride 100, methylene chloride–hexane (50:50), hexane 100, hexane 100, and hexane 100 migration for 25 steps. For elution gradient 1b, it was diethyl ether 100, ether–hexane (50:50), hexane 100, hexane 100, and hexane 100 migration for 20 steps.

The postchromatographic derivation by chemical visualization according to Wagner (11) was accomplished by spraying fast blue salt B reagent (0.5 p. 100 in methanol).

The different cannabinoids were identified by their hRf and the color of spots: purple for D^8-THC and D^9-THC, orange red for cannabidiol, and violet for cannabinol (CBN).

Essential oils

Plant material samples and standard preparations

Thymes and wild thyme essential oils were obtained from Myrtéa (St. Genès Champanelle, France). The samples studied were seven chemotypes of *Thymus vulgaris*: *T. vulgaris* b.s. (thymol), *T. vulgaris* b.s. (carvacrol), *T. vulgaris* b.s. (linalool), *T. vulgaris* b.s. (geraniol), *T. vulgaris* b.s. (thuyanol), *T. vulgaris* b.s. (limonene), *T. vulgaris* b.s. (borneol), and *T. serpyllum*.

Mint samples studied were *Mentha piperita, M. citrata*, and *M. pulegium*, and they were obtained from Myrtéa.

Eucalyptus essential oils studied were *Eucalyptus phellandra, E. radiata, E. maidenii, E. smithii, E. camaldulensis*, and *E. divers* from Myrtéa.

All of the essential oil samples were prepared by the dilution of 0.1 mL of essential oil/1 mL in pure toluene.

For thymes, thymol and carvacrol were prepared through a dissolution of 10 mg/10 mL in pure ethanol (96°). Thymol and carvacrol mixtures were prepared from the dissolution of 10 mg thymol–10 mg carvacrol in 10 mL pure ethanol (96°).

For mints, 1,8-cineole, menthone, carvone, and pulegone were prepared at 1% in methanol, and a mixture of reference samples according to European Pharmacopoeia was 50 mg menthol, 20 ?L 1,8-cineole, 10 mg thymol, and 10 ?L menthyl acetate in 10 mL toluene.

For eucalyptus, 1,8-cineole was at 1% in methanol and citronellal at 0.2% in methanol.

Chromatographic conditions

Thymes. For TLC, the plates were silica gel 60 F_{254} 20-¥ 20-cm on glass (Merck, Art. 5715). The eluent used was toluene–ethyl acetate (95:5, v/v).

For OPLC, the plates were silica gel 60 F_{254} 20-¥ 20-cm on an aluminum sheet (Merck, Art. 5554) sealed by OPLC-NIT Engineering Company. The external pressure was 50 bars, the start flash volume was 300 ?L, the flow rate 300 ?L, the elution volume 600 ?L, and the total elution time was 1210 s. The eluent used was hexane–ethyl acetate (95:5, v/v).

For AMD, the plates were silica gel 60 F_{254} 10-¥ 20-cm on glass (Merck, Art. 5729). The elution gradient 1 was chloroform 100, chloroform–cyclohexane (50:50), cyclohexane 100, cyclohexane 100, and hexane 100, and the number of steps was 20.

Mints. For TLC, the plates were silica gel 60 F_{254} 20-¥ 20-cm on glass (Merck, Art. 5715). The eluent was toluene–ethyl acetate (95:5, v/v).

For AMD, the plates were silica gel 60 F_{254} 10-¥ 20-cm on glass (Merck, Art. 5729). The elution gradient 7 was methylene chloride 100, methylene chloride–cyclohexane (50:50), cyclohexane 100, cyclohexane 100, hexane 100, and hexane 100, and the number of steps was 15.

Eucalyptus. For TLC, the plates were silica gel 60 F_{254} 20-¥ 20-cm on glass (Merck, Art. 5715). The eluent was toluene–ethyl acetate (90:10, v/v).

For AMD, the plates were silica gel 60 F_{254} 10-¥ 20-cm on glass (Merck, Art. 5729). The eluent gradient 1 was methylene chloride 100, chlorobenzene 100, benzene 100, cyclohexane 100, and hexane 100, and the number of steps was 20.

Reagents

Anisaldehyde-sulfuric acid and vanillin-sulfuric acid were prepared according to Wagner (11).

Heterosides

Ginsenosides

Plant material samples. Seven commercial samples from various provenances were studied: "red" ginseng (9) and *Panax ginseng* radix.

Preparation of reference samples and extract solutions. Powdered ginseng (1 g) was extracted with 75 mL of a mixture of methanol–water (80:20, v/v), shaken for 30 min, and then filtered. This extraction was repeated four times. The extracts were com-

Table I. Studied Compounds and Plants

Compounds	hRf	Fluorescence (365 nm)	Plants
Coumarins			
Simple coumarins			
Coumarin	86	—	Dipteryx odorata, Melilotus officinalis
Aesculetin	40	pale yellow	Aesculus hippocastanum
Scopoletin	51	pale blue	Aesculus hippocastanum, Anthemis nobilis, Ruta graveolens
Umbelliferone	68	pale blue	Angelica archangelica, Hieracium pilosella, Matricaria chamomilla, Ruta graveolens
6,7-Furanocoumarins			
Bergapten	85	yellow	Ruta graveolens
Methoxsalen (Xanthotoxin)	87	yellow	Ruta graveolens
Psoralen	82	blue-violet	Ruta graveolens
7,8-Furanocoumarins			
Angelicin	95	white	Angelica archangelica
Flavonoids			
Chalcone	98	brown-red	
Flavone	87	red	
Flavanone	85	blue	
Flavones			
Apigenin	56	blue	Crataegus monogyna
Diosmetin	52	orange	Barosma betulina
Luteolin	15	brown-orange	Citrus aurantium
Myricetin	7	yellow-brown	Tilia cordata
Flavonols			
Kaempferol	56	lemon yellow	Ginkgo biloba, Tilia cordata
Quercetin	48	yellow-brown	Crataegus monogyna, Ginkgo biloba, Sophora japonica, Tilia cordata
Rhamnetin	9	yellow	
Flavanones			
Eriodictyol	44	yellow	Citrus aurantium
Hesperetin	69	white	Citrus aurantium
Naringenin	74	pale yellow	Citrus aurantium
Isoflavones			
Genistein	69	blue	
Furanochromones			
Khellin	50	brown-orange	Ammi visnaga
Visnagin	95	blue-violet	Ammi visnaga
Anthocyanins			
Cyanidin	39	purple*	Centaurea cyanus, Papaver rhoeas, Ribes nigrum, Vaccinium myrtillus, Vitis vinifera
Delphinidin	28	blue*	Hibiscus sabdariffa, Ribes nigrum, Vaccinium myrtillus
Malvidin	34	mauve*	Malva sylvestris, Vaccinium myrtillus

* Visible color.

bined and the solvent evaporated under reduced pressure. The dry residue was taken up in 20 mL water.

One milliliter of this solution was extracted 6 times with 10 mL of butanol saturated with water. The butanolic solution was dried with anhydrous sodium sulfate and then evaporated under reduced pressure and taken up with methanol.

Each of the standard ginsenosides (Rb_1, Rb_2, Rc, Rd, and Rg_1) were diluted in 10 mL of methanol.

Chromatographic conditions. For TLC, silica gel 60 F_{254} 20-¥ 20-cm glass plates (Merck, Art. 5715) were used. The eluent was chloroform–methanol–water (65:35:4, v/v).

For AMD, HP-TLC kieselgel 60 10-¥ 20-cm plates (Merck, Art. 5641) were used. The elution gradient 1 was methanol 100, methanol–acetonitrile (50:50), acetonitrile 100, acetonitrile 100, and dichloromethane 100, and the number of steps was 20.

Reagent. Vanilline–phosphoric acid (11) was used as the reagent, and the heating was at 100–110°C.

Cardiac heterosides

Plant material samples and standards. *Digitalis purpurea* and *D. lanata* extracts were prepared (14) from 1 g of powdered dried leaves with a mixture of 20 mL ethanol (50%) and 10 mL lead acetate. Heating and boiling occurred for 2 min. After cooling and centrifugation, the solution was extracted twice with 15 mL of methylene chloride. The organic phases were collected and dried with anhydrous sodium sulfate. Then, 10 mL was evaporated to dryness and the residue taken up in 1 mL of methanol–chloroform (v/v).

The standards digitoxin; digoxin; digoxigenin; gitoxigenin; and lanatosides A, B, and C were prepared with 5 mg for 2 mL methy-

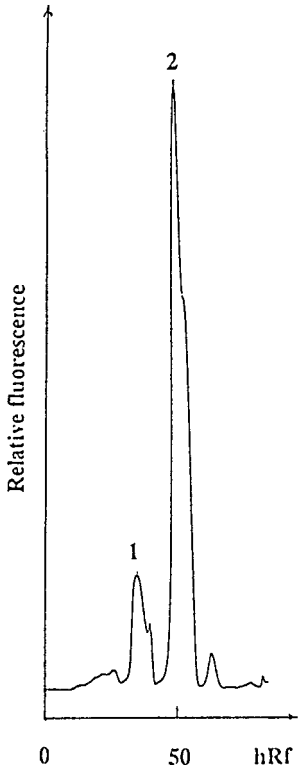

Figure 1. Densitogram of Aesculus hippocastanum extract: (1) aesculetin and (2) scopoletin.

lene chloride–ethanol (v/v).

Chromatographic conditions. For TLC, silica gel 60 F_{254} 20- ¥ 20-cm plates on glass (Merck, Art. 5715) were used. The eluent was ethyl acetate–methanol–water (81:11:8, v/v).

For AMD, silica gel 60 F_{254} 10- ¥ 20-cm plates on glass (Merck, Art. 5729) were used. The elution gradient 1 was ethanol 100, ethanol–methylene chloride (50:50), methylene chloride 100, methylene chloride 100, and hexane 100, and the number of steps was 20.

Reagent. Kedde's reagent (11) was used.

Results and Discussion

Phenolic compounds aglycones (15)

In the literature, there were few OPLC publications concerning coumarins and flavonoids and the eluent compositions were complex (16–18); therefore, it was interesting to improve them.

Coumarins

The most widespread plant, coumarin, is the parent compound coumarin itself. It is common in many grasses and folder crops. The active constituents of coumarin drugs are benzo a-pyrones. They can be classified in simple coumarins, furanocoumarins, and pyranocoumarins. Their compounds are very easily detected because they give characteristic fluorescent colors in UV light.

The results concerning coumarinic aglycones present in various medicinal plants (coumarin, umbelliferone, aesculetin, scopoletin, psoralen, bergapten, methoxsalen, and angelicin) are reported Table I. For the simple coumarins and furocoumarins, ethyl acetate as the mobile phase (eluent for alkaloids, as will be discussed) is too polar; therefore, it was necessary to modify the eluent polarity by adding chloroform in different proportions. An ethyl acetate–chloroform (60:40, v/v) mixture gave the best results. For some of these compounds (e.g., bergapten, methoxsalen, and psoralen), the hRf differences were weak and are explained by structure analogies. The same observation has been made in TLC analysis (19,20). However, the fluorescence at 365 nm exhibited a difference in the coloration for psoralen in comparison to bergapten and methoxsalen. For these last two compounds, the similarity in fluorescence arises because of structural isomerism.

Figure 1 reports the densitogram of *Aesculus hippocastanum* as an example.

Concerning relationships between hRf and structure, the presence of a furanic cycle at the 6,7 or 7,8 position did not influence the molecule polarity; this can be seen by comparing the hRf of coumarin, psoralen, and angelicin. As in the flavonoid series, the presence of a phenolic or methoxy group in the homocycle increased weakly the polarity if only one group was present on the 6,7-furocoumarin compounds and increased more strongly in simple coumarins.

Flavonoids

A large number of different flavonoids are known to occur in nature, and these yellow pigments are distributed widely throughout the higher plants. Apigenin, genistein, hesperetin,

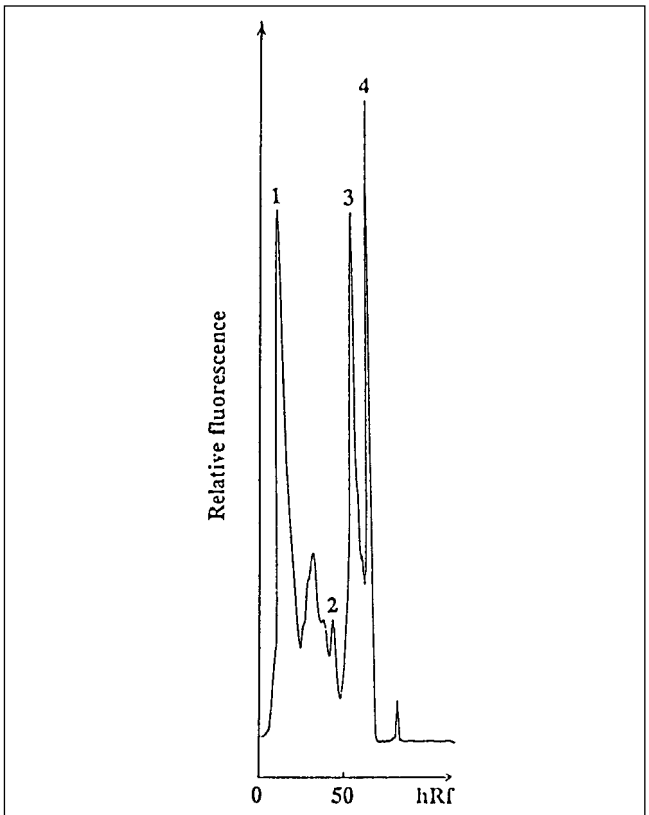

Figure 2. Densitogram of Citrus aurantium extract: (1) luteolin, (2) eridictyol, (3) hesperetin, and (4) narigenin.

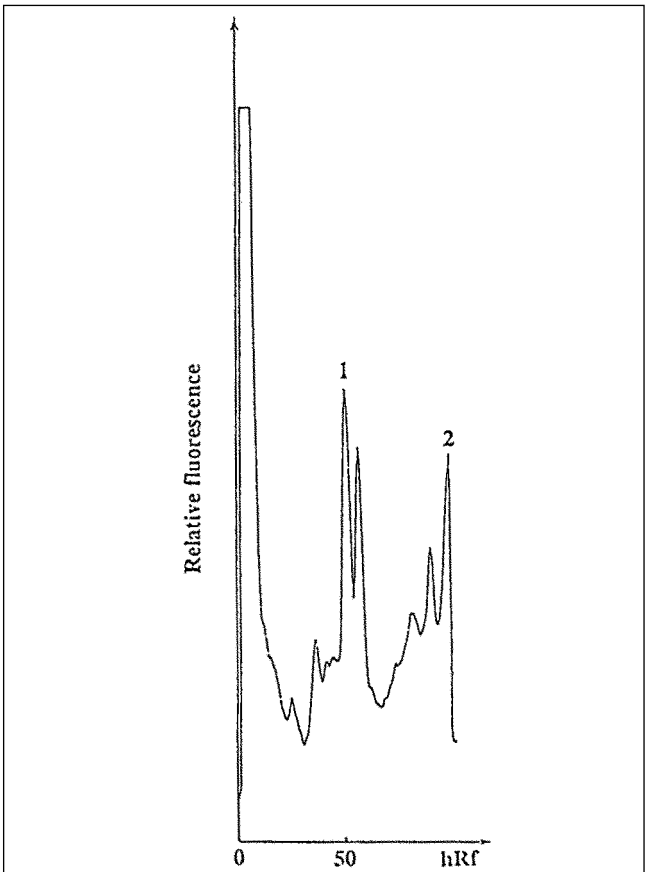

Figure 3. Densitogram of Ammi visnaga extract: (1) khellin and (2) visnagin.

kaempferol, luteolin, myricetin, naringenin, quercetin, and rhamnetin are among the best known and most frequently found flavonoid glycosides.

Only the chromatographic results concerning flavonoid aglycones that are characteristic and more easily determined than those of the corresponding glycosides are reported.

The various flavonoid aglycones investigated with their hRf and the plants that contain these products are reported in Table I.

For flavonoids, ethyl acetate gave a satisfactory separation, although it was too polar for an efficient separation. Thus, its polarity was modified by adding chloroform in different proportions. Two eluents proved interesting: ethyl acetate–chloroform (60:40, v/v) and ethyl acetate–chloroform (40:60, v/v).

The best separation was obtained with the first eluent mixture. However, for the unsubstituted compounds, flavone and flavanone do not frequently occur in nature. Thus, it was interesting to compare their chromatographic comportment in the same eluent with substituted analog derivatives in order to estimate the substituents' influence upon the polarity. The values of hRf are reported in Table I.

This method was then applied to different medicinal plant extracts (*Barosma betulina*, *Citrus aurantium* var. *amara*, *Crataegus monogyna*, *Ginkgo biloba*, *Sophora japonica*, and *Tilia cordata*). Before chromatographic analysis, the extracts were hydrolyzed by 2M hydrochloric acid, then the corresponding aglycones were extracted and characterization of these aglycones was effected by comparison with the hRf of authentic samples.

The densitogram obtained from an extract of orange flower (*C. aurantium* var. *amara*) (Figure 2) showed an efficient separation, which enabled the identification of the major flavonoid compounds luteolin (hRf = 15), eriodictyol (hRf = 44), hesperetin (hRf = 69), and naringenin (hRf = 74). A full list of the plant extracts studied and the characterized major constituents are given in Table I.

For furanochromones, better results were obtained with the eluent ethyl acetate–chloroform (90:10, v/v). Figure 3 reports the densitogram of the chromatography from *Ammi visnaga*, which is an interesting drug containing particularly khellin and visnagin identified by comparison with authentic samples.

Concerning the relationships between the structure and hRf of flavonoid derivatives, it should be noted that the presence of the C=C (flavone) or C=C–OH (flavonol) arrangement increased the polarity of the molecule. For example, apigenin and kaempferol had an hRf lower than that of naringenin. The same conclusion was valid when a phenol or methoxy group was present as a substituent; for example, the hRf of quercetin was lower than that of kaempferol. This was also the case with luteolin or diosmetin as compared with apigenin.

Concerning the flavone–isoflavone skeleton, the last type was a little less polar than the former. This was in relation to a lower conjugation within the molecule.

The same remarks formulated for flavonoid derivatives about the increase of polarity with the supplementary phenolic or methoxy group are valid in the furanochromone series (khellin and visnagin).

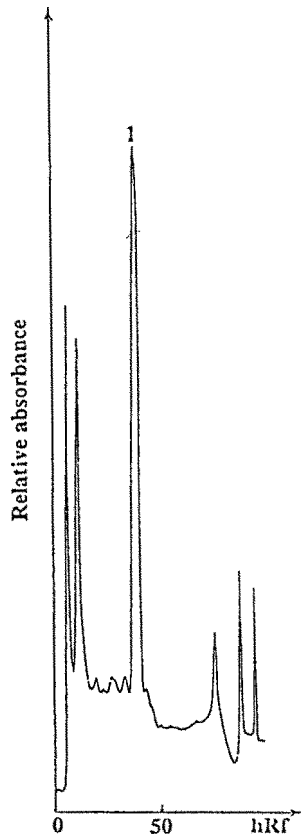

Figure 4. Densitogram of Malva sylvestris extract: (1) malvidol.

Table II. Alkaloids Classes and Plant Extracts Studied

Alkaloid class	Plant extracts
Pyridine–Piperidine	Areca catechu (seed), Conium maculatum (leaf, fruit), Lobelia inflata (flower head), Nicotiana tabacum (leaf)
b-Phenylethylamine	Ephedra sp.
Quinoline	Cinchona sp. (bark)
Isoquinoline	Hydrastis canadensis (root), Ipecacuanha sp. (root), Papaver somniferum (latex from capsule), Peumus boldus (leaf)
Quinolizidine	Lupinus sp. (seed), Cytisus scoparius (flower head)
Pyrrolidine	Physostigma venenosum (seed)
Imidazole	Pilocarpus microphyllus (leaf)
Indole	Claviceps purpurea (sclerotium), Rauwolfia sp. (root), Strychnos nux vomica (seed)
Tropane	Atropa belladonna (leaf), Datura stramonium (leaf), Erythroxylum coca (leaf), Hyoscyamus niger (leaf)
Purine	Coffea sp. (seed), Cola nitida (seed), Ilex paraguariensis (leaf), Thea sinensis (leaf), Theobroma cacao (seed)
Diterpene	Aconitum napellus (root)
Tropolone	Colchicum autumnale (seed)

Anthocyanins

Anthocyanins are responsible for the red, violet, or blue color of flowers and other plant parts. They are present in plants as glycosides of hydroxylated 2-phenylbenzopyrilium salts. Cleavage by acid hydrolysis gives the corresponding free flavylium salt, the most common being cyanidin, delphinidin, and malvidin (21).

Anthocyanins present a therapeutic interest as angiovascular protectors and are used in natural foodstuff as coloring agents (9). These compounds have been studied by TLC and characterized (22). Therefore, we have achieved the chromatographic study by OPLC of the different plants described previously containing anthocyanins. The separation was difficult because of it being necessary to modify eluents used for coumarins and flavonoids by adding methyl ethyl ketone to ethyl acetate for an increase in the polarity and formic acid and 2M hydrochloric acid to stabilize the color of these compounds. Based on this, a mixture of ethyl acetate–methyl ethyl ketone–formic acid–2M hydrochloric acid (65:10:6:9, v/v) was chosen as the best combination.

Many different anthocyanins can be recognized by their dis-

Table III. hRf Values of the Different Alkaloids and the Colors Obtained After Spraying with Iodoplatinate Reagent

Alkaloid	hRf	Source	Color
Aconitine	86	Aconitum napellus	pink brown
Anabasine	52	Nicotiana tabacum	blue
Ajmaline	7	Rauwolfia sp.	beige pink
Arecoline	79	Areca catechu	violet brown
Atropine	19	Mydriatic Solanaceae	violet blue
Boldine	72	Peumus bolbus	pink violet
Brucine	28	Strychnos nux vomica	violet brown
Caffeine	43	Coffea sp.	pink
Cephaeline	7	Ipecacuanha sp.	light yellow
Coniine	3	Conium maculatum	violet
Cinchonidine	50	Cinchona sp.	deep blue
Cinchonine	32	Cinchona sp.	gray beige
Cocaine	89	Erythroxylum coca	violet
Codeine	47	Papaver somniferum	pink violet
Colchicine	20	Colchicum autumnale	beige pink
Ellipticine	92	Ochrosia sp.	pink brown
9-Methoxyellipticine	88	Ochrosia sp.	pink brown
Emetine	33	Ipecacuanha sp.	violet brown
Ephedrine	3	Ephedra sp.	light brown
Ergotamine	60	Claviceps purpurea	pink
Eserine	80	Physostigma venenosum	violet pink
Harmaline	61	Peganum harmala	violet
Harman	76	Peganum harmala	violet
Harmine	65	Peganum harmala	violet
Harmol	83	Peganum harmala	pink violet
Hordenine	38	Hordenum vulgare	gray blue
Hydrastine	97	Hydrastis canadensis	brown violet
Lobeline	78	Lobelia inflata	brown
Lupanine	22	Lupinus sp.	pink violet
13-Hydroxylupanine	7	Lupinus sp.	pink violet
Lupinine	10	Lupinus sp.	pink violet
Morphine	15	Papaver somniferum	deep blue
Nicotine	72	Nicotiana tabacum	black blue
Nornicotine	10	Nicotiana tabacum	beige pink
Noscapine	95	Papaver somniferum	pink brown
Papaverine	82	Papaver somniferum	light pink
Pilocarpine	50	Pilocarpus microphyllus	violet brown
Quinidine	40	Cinchona sp.	beige pink
Quinine	32	Cinchona sp.	beige pink
Raubasine	79	Rauwolfia sp.	beige pink
Reserpine	40	Rauwolfia sp.	light beige
Scopolamine	36	Mydriatic Solanaceae	deep violet
Sparteine	3	Cytisus scoparius	violet blue
Strychnine	48	Strychnos nux vomica	violet brown
Thebaine	70	Papaver somniferum	brown violet
Theobromine	24	Theobroma cacao	light pink
Theophylline	30	Thea sinensis	light pink
Vincamine	80	Vinca minor	deep violet
Vincristine	15	Catharanthus roseus	deep violet
Yohimbine	13	Corynanthe yohimbe	light yellow

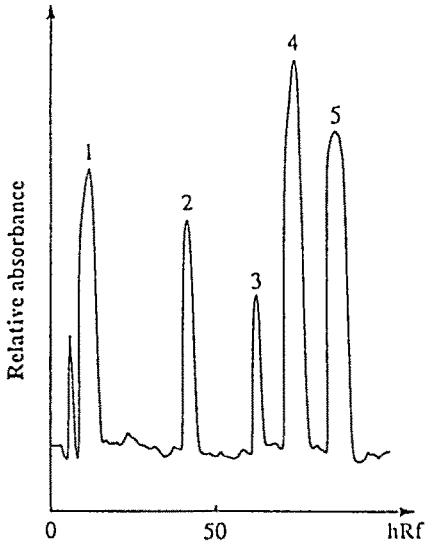

Figure 5. Densitogram of opium alkaloids by OPLC: (1) morphine, (2) codeine, (3) thebaine, (4) papaverine, and (5) noscapine.

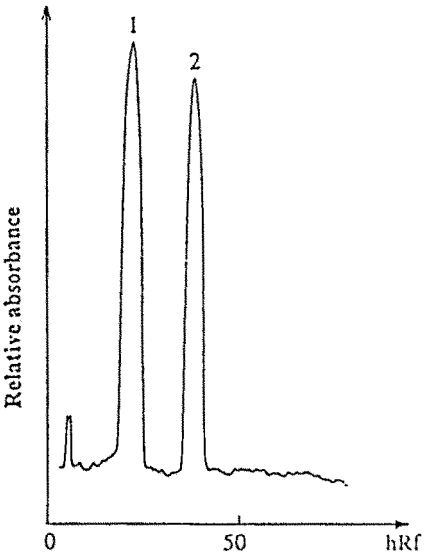

Figure 6. Densitogram of Datura stramonium extract: (1) hyoscyamine (atropine) and (2) scopolamine.

tinctive visual colors on chromatograms: cyanidin is purple, malvidin is mauve, and delphinidin is blue. However, it is important to visualize the chromatogram immediately because the colors of the spots fade fairly rapidly after separation (Table I).

The densitogram of *Malva sylvestris* (Figure 4) is shown as an example.

Concerning the structure and hRf relationships, it has been noted as in the coumarin and flavonoid series that a supplementary OH or OCH_3 on the phenyl group present in the 2 position of the benzopyrylium skeleton increases the polarity (i.e., compare delphinidin and malvidin with cyanidin).

Alkaloids (23–25)

The most interesting results with OPLC was the discovery that complete separations with OPLC of crude alkaloidal extracts of the most important classes of alkaloids could be obtained using only ethyl acetate as the mobile phase. This is a significant improvement over those separations by both TLC and OPLC, which require the use of three or more components in the mobile phase (26–30). Such mixtures are not easy to employ in preparative chromatography and may also disturb visualization by spray reagents.

In total, 80 natural and synthetic alkaloids were studied. Table II shows some representative reference compounds with their hRf and their natural source, and Table III reports the alkaloid classes and plant extracts tested.

The densitograms were recorded following the chromatography of plant extracts selected on the basis of their importance because of: (*a*) the frequency of their utilization either therapeutically or as a drug (e.g., opium, datura, or tobacco) (Figures 5–7); (*b*) the extent to which they were representative of a chemical class of alkaloid from isoquinoline, tropane, or pyridine, for example; and (*c*) the frequency of the occurrence of a given alkaloid in several plants (e.g., caffeine in the purine alkaloid plants such as *Camellia sinensis*) (Figure 8).

The densitogram of opium alkaloids (Figure 5) shows an efficient separation that enables the identification of all the major

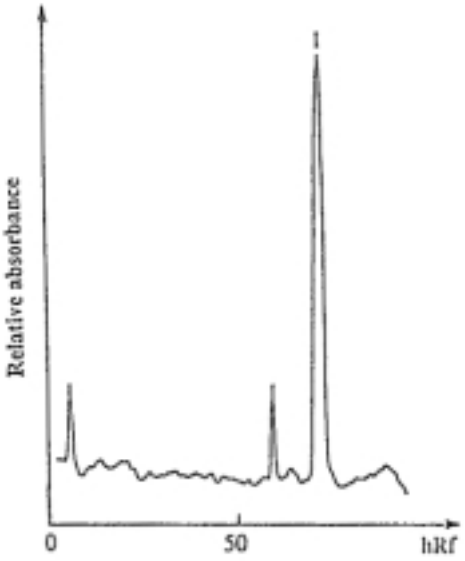

Figure 7. Densitogram of Nicotiana tabacum extract: (1) nicotine.

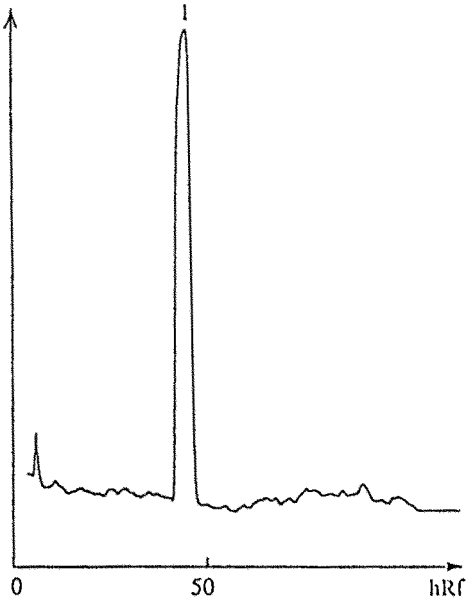

Figure 8. Densitogram of Thea sinensis extract: (1) caffeine.

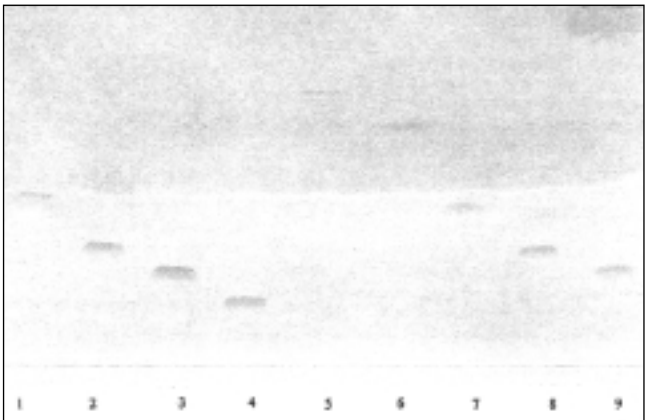

Figure 9. Chromatogram of opium alkaloids and derivatives by AMD: (1) heroin, (2) ethylmorphine, (3) dextromethorphan, (4) pholcodine, (5) noscapine, (6) papaverine, (7) thebaine, (8) codeine, and (9) morphine.

Table IV. hRf and Colors with the Iodoplatinate Reagent of Opium Alkaloids and Derivatives

Compounds	hRf TLC	hRf OPLC	hRf AMD	Colors (Iodoplatinate)
Opium alkaloids				
Codeine	41	47	19	pink violet
Morphine	21	15	15	deep blue
Noscapine	84	95	87	pink brown
Papaverine	68	82	73	light pink
Thebaine	59	70	26	brown violet
Analogs				
Buprenorphine	74	86	90	pink violet
Heroin	58	53	31	deep blue
Methadone	87	96	37	pink violet

alkaloids, which is not always the case in TLC separation of the alkaloids (26–31) because these alkaloids belong to two different groups: morphinan (morphine, codeine, and thebaine) and benzylisoquinoline (noscapine and papaverine). Their chromatographic behavior is thus very different, which generally led authors to use silica gel with complex eluents.

The separation of *Strychnos nux vomica* alkaloids on aluminum oxide or silica gel (26) requires the use of complex eluents containing three or more components, whereas by OPLC with the ethyl acetate as eluent, brucine, and strychnine they are clearly separated (hRf = 28 and 48, respectively).

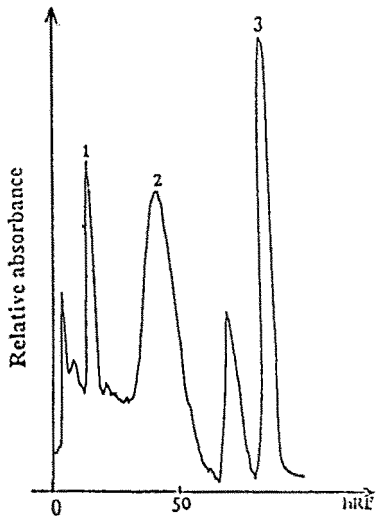

Figure 10. Densitogram of Hydrastis Canadensis extract: (1) hydrastinine, (2) hydrastine, and (3) berberine.

Table V. Plant Extracts Studied and Compounds Obtained

Plants	Compounds
Berberis vulgaris (bark)	berberine
Chelidonium majus (flowering plant)	chelidonine, sanguinarine
Jatrorrhiza palmate (root)	palmatine
Hydrastis canadensis (root)	berberine, hydrastine, hydrastinine
Rauwolfia serpentina (root)	ajmaline, serpentine

Table VI. hRf Values of the Compounds Studied

Compounds	hRf
Choline (chloride)	45
Betaine	20
Carnitine (hydrochloride)	43
Trigonelline (hydrochloride)	48
Hydrastinine	17
Hydrastine (hydrochloride)	41
Berberine (chloride)	86
Palmatine (chloride)	80
Chelidonine	94
Sanguinarine (chloride)	51
Serpentine	95
Ajmaline	90
d-Tubocurarine	28

When the hRf of some alkaloids in the same plant extracts were close (e.g., *Cinchona*), the identification was made possible by the observation of fluorescence under UV illumination and also by derivation with iodoplatinate reagent, which is better than Dragendorff's reagent because of its better color differentiation.

In AMD (25,33), opium alkaloids are studied as well as some derivatives and analogs such as heroin, methadone, and buprenorphine. A clean separation was obtained after 25 development steps with a gradient 1 consisting of methanol 100, acetone 100, ethyl acetate 100, ethyl acetate–methylene chloride (50:50), methylene chloride 100, and methylene chloride 100 (Figure 9).

The hRf and colors of opium alkaloids and analogs with iodoplatinate reagent are reported in Table IV.

Quaternary ammonium salts and alkaloids (34)

In contrast with the ternary compounds, there are few publications about quaternary alkaloids salts. Aluminum oxide and cellulose are the stationary phases most frequently employed (35–38). Silica gel is also used, but in these instances it is necessary to employ water, acidic solutions, or other polar solvents as mobile phases (39–41). The slow advance of such eluents leads to spot diffusion and stretching, with the result that sample hRf values cannot be determined clearly and precisely. OPLC could be the solution to these problems as reported by several authors (17,42).

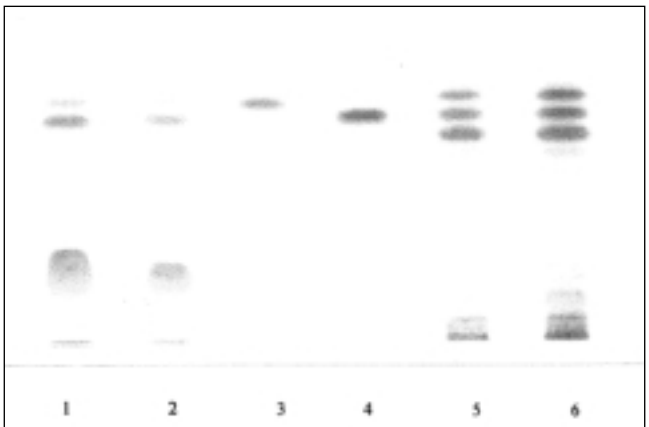

Figure 11. HP-TLC of cannabinoids: (1) 10 ?L cannabis extract, (2) 5 ?L cannabis extract, (3) 5 ?L D8-THC, (4) 10 ?L D9-THC, (5) 2 ?L cannabis resin, and (6) 4 ?L cannabis resin.

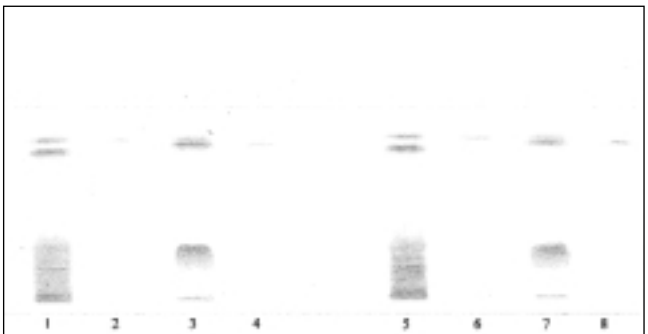

Figure 12. AMD of cannabinoids: (1) 10 ?L cannabis resin, (2) 4 ?L D9-THC, (3) 10 ?L cannabis extract, (4) 4 ?L ?8-THC, and (5) 5 ?L cannabis resin, and (6) 2 ?L D9-THC, (7) 2 ?L cannabis extract, and (8) 2 ?L D8-THC.

It was not possible in the chromatographic analytical conditions employed for alkaloids to separate quaternary ammonium salts because they are too polar. The use of silica gel was more suitable than aluminum oxide in that the diffusion of spots was reduced, thus promoting a clean separation. Similarly, the optimum mobile phase was selected by the addition of formic acid and tetrahydrofuran to ethyl acetate. With these analytical conditions a good separation of the quaternary alkaloids was obtained. All of the compounds investigated in the plant extracts could be separated by OPLC and identified by comparison with authentic samples.

The most interesting result was the clean separation of quaternary ammonium salts from crude vegetal extracts. The spots obtained were regular in form without diffusion or stretching. The densitogram of *Hydrastis canadensis* compounds (Figure 10) shows an efficient separation, which enables the identification of all the major alkaloids (berberine, hydrastine, and hydrastinine). The alkaloids from *Chelidonium majus*, sanguinarine, and chelidonine were cleanly separated, and the hRf values were 51 and 94, respectively.

The analysis of *Rauwolfias* is not easy because they contain alkaloids with a large diversity of polarity. In this study only two very polar compounds were selected: serpentine and ajmaline.

A full list of the plant extracts studied and major quaternary ammonium salts and alkaloids characterized are given in Tables V and VI.

Isolation of alkaloids by OPLC semipreparative mode (43)

Few works have been reported for the OPLC semipreparative mode (44,45). The main aim of this study was to obtain pure compounds from crude plant extract by coupling the chromatograph to a collector.

First, a plant extract containing few alkaloids was selected and very clearly separated by analytical OPLC, such as the *Strychnos nux vomica* extract. Then, an opium extract that had a more complex alkaloidal composition and finally *Lupinus* and *Datura stramonium* extracts were selected.

A *S. nux vomica* extract was deposited inline and eluted with ethyl acetate. The migration of the eluent was performed during the time required to obtain a start of the elution process. Upon observing that migration was linear with time, it was possible to determinate the time when brucine and strychnine will be eluted.

With this method it is possible to obtain pure strychnine. In order to obtain pure brucine, 20 mL of isopropanol was added to ethyl acetate. Methanol was not used because its use damages the impregnated polymer.

By this technique it was also possible to collect fractions of alkaloidal constituents of opium (papaverine, noscapine, thebaine, and codeine) and to elute morphine [ethyl acetate–isopropanol–28% ammonia (80:20:1, v/v)]. Alkaloids from *D. stramonium* and lupanine from *Lupinus* were separated.

The advantages of semipreparative OPLC are quickness, reproducibility, and small consumption of developing solvent. The disadvantage of scratching the plates off, as encountered in classical TLC, was also avoided by the direct collection of eluting compounds.

Cannabinoids (46,47)

In OPLC with ethyl acetate as the mobile phase, D^9-THC (a psychotropic phenolic compound) and other cannabinoids from *Cannabis sativa* var. *indica* migrate up to the solvent front, but it is possible to separate them by reducing the mobile phase polarity by adding hexane [hexane–ethyl acetate (70:30, v/v)]. The characterization of these compounds was effected with Fast Blue B salt reagent using a common procedure (11). In this instance a good separation of cannabinoids was obtained, particularly for CBN (which is found in all of the cannabis species) and D^9-THC (which is the major active euphoric substance). The hRf values were 79 for CBN and 84 for D^9-THC.

It is possible to separate the investigated compounds (D^8-THC and D^9-THC) from the resin of cannabis and from Indian hemp with TLC (48), but with traditional TLC we obtained only three or four spots for cannabis extract and eight spots for the resin (Figure 11).

With AMD the number of spots was more important in the case of resin than in cannabis extracts. Thus, this new technique can give more information about the composition of various samples of Indian hemp, and a continuation of this work can open possibilities to determine the geographic origin and operation mode of the preparation of different samples by the examination of different proportions of cannabinoids (Figure 12).

Essential oils (49)

Essential oils analysis is generally realized by gas chromatography; however, the reference method for Pharmacopoeia (and especially the European one) is always TLC because it is rapid, easy to use, and inexpensive. However, this technique is limited because the components of essential oils are volatile and thus a diffusion can be watched as an edge effect at the top of the plate.

Table VII. hRf Values of the Principal Constituents Present in Essential Oil of the Thyme Chemotypes Studied

	hRf (100 Rf)			
Compounds	Classical TLC	TLC-MAT*	OPLC	AMD
Borneol	22	20	30	21
Carvacrol	45	44	55	36
Citronellal	64	66	85	51
Citronellol	19	17	23	14
Geraniol	16	16	19	16
Linalool	29	27	44	27
Thymol	48	48	60	38

* TLC–MAT, TLC automated tank.

Table VIII. hRf Values of the Ginsenosides Studied

Ginsenosides	hRf
Rb1	16
Rb2	23
Rc	30
Rd	47
Rg	67

Therefore, OPLC and AMD can be applied because they do not lead to much diffusion and evaporation.

For thymes, with OPLC good separation of the different chemotypes constituents was obtained, particularly for the two phenol isomers thymol and carvacrol (49) An analysis of the chromatograms shows that the quickness of the migration limits the diffusion effect, thereby making the evaporation cleaner than in classical TLC.

This technique provides good information by highlighting the position of the different compounds included in the essential oils of the seven chemotypes of thyme studied. With the eluent hexane–ethyl acetate (95:5, v/v) the results obtained were better than in classical TLC and the two phenol isomers were cleanly separated [carvacrol (hRf = 55) and thymol (hRf = 60)]. The mixture of thymol and carvacrol and other constituents of thyme and wild thyme extracts were clearly separated without diffusion; therefore, OPLC was an interesting method of analysis for these compounds (Table VII).

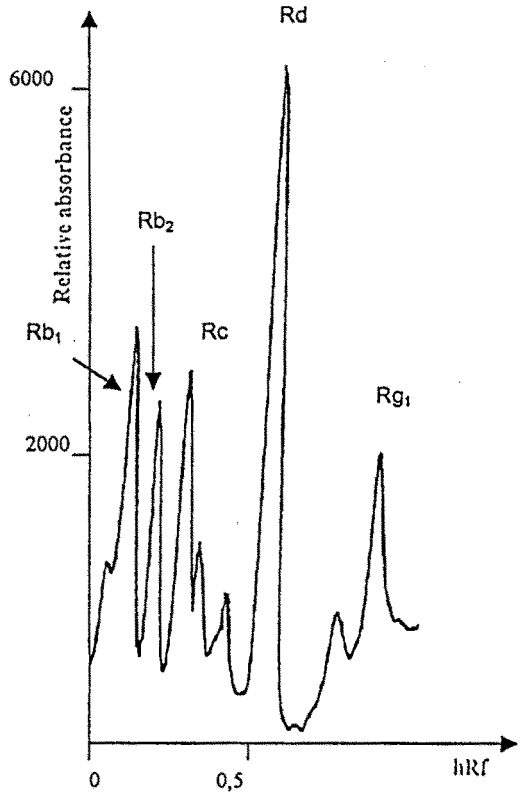

Figure 13. Densitogram from a sample of Panax ginseng.

Table IX. hRf Values of the Cardiac Heterosides Studied

Cardiac heterosides and aglycones	hRf
Digoxine	55
Digoxigenine	70
Digitalin	58
Digitoxigenine	82
Lanatoside A	35
Lanatoside B	32
Lanatoside C	30
Gitoxigenine	70

With the AMD technique, the diffusion on the plate was reduced and the evaporation was also very limited. Besides, the successive migrations took place under a nitrogen atmosphere, thus preventing oxidation. Therefore, it was possible to study essential oils that become degraded by heat and easily oxidizable. With this technique the separation of thymol and carvacrol is clearly defined. This is the reason why AMD could be useful for the chromatography of essential oils, because with very small quantities of sample the resolution is satisfactory and thus it is possible to envisage a quantitative analysis by scanner densitometry.

With thymes, the results obtained with OPLC were better than those currently reported by Pharmacopoeia. With this method it was possible to separate cleanly the two phenol isomers thymol and carvacrol with a classical binary eluent [hexane–ethyl acetate (95:5, v/v)] (Table VII). A single chromatographic plate permitted the analysis of approximately ten samples within 15 min as well as the easy identification of the different chemotypes of thymes.

With AMD, it was possible to satisfactorily separate thymol and carvacrol. This technique has the advantage of working with small quantities of samples. This method could be developed with a view to permit the identification of the typical compound of the chemotype and the quantitation of the different constituents of the essential oils studied.

For seven chemotypes of thymes, results were obtained by classical TLC and automated tank Desaga. The results obtained are listed in Table VII.

An observation of the two TLC chromatograms after derivation shows numerous compounds with very different shades of colors; therefore, it is easy to determine unambiguously which chemotype each belongs, particularly when the reference compound is available (except for thuyanol because the standard is not marketed). In this case, we have only the chromatographic profile of essential oil.

With AMD, the essential oils of several mints and eucalyptus were also studied. The first results show satisfactory separations with the different species studied, but a relative diffusion of compounds having an hRf of greater than or equal to 50 was observed, such as 1,8-cineole (eucalyptol) and citronellal in the case of eucalyptus oils. The analysis of the mints showed a better separation of the different constituents. The employment of gradients having 20 or 25 steps brought about a more important diffusion than 15 steps, perhaps because these monoterpenes constituents were volatile and as the experimental time increased so did the effect of the diffusion.

Heterosides

Ginsenosides

Classical TLC gives good separation for the different ginsenosides (51). The eluent described in the literature (52) has been lightly modified by decreasing the polarity using chloroform–methanol–water (65:35:4, v/v) (Table VIII).

In AMD, good results were obtained with all of the reference samples, but with the ginseng extracts studied the separation was less clean. Consequently, the quantitative evaluations by scanner densitometry were realized from TLC with different extracts (Figure 13).

Cardiac glycosides

TLC gives clean separation for *Digitalis purpurea* and *D. lanata* glycosides, according to Wagner and Bladt (53).

AMD gives a clean separation with all glycosides except lanatosides B and C, which have a similar hRf value because their chemical structures are nearly identical differing only by the position of the free hydroxyl group: 16b for lanatoside B and 12b for lanatoside C. An optimization of the elution gradient should be obtained for a better separation.

The hRf in TLC and AMD are reported in Table IX.

Conclusion

Planar chromatography is always the choice method for the analysis of plant material (natural products, medicinal plants, and hemisynthetic or synthetic analogs).

Planar chromatography has become a modern technique with the commercialization of a large choice of adsorbents and the arrival of automated apparatuses for the application of samples, an automated development chamber, OPLC, and AMD.

Classical TLC is also a choice method for the analysis of plant material, particularly to separate constituents in crude extracts. As such, in a control method such as screening for a preliminary study of new plant extracts, it is an effective and inexpensive technique.

OPLC opens up the quick analysis for an important number of samples. Also, it permits semipreparative separations allowing pure product obtention by direct elution, because in OPLC the migration is linear in time. The hRf values are reproducible and each compound is eluted at a defined time.

AMD presents the best resolution, no spots diffusion, elution without oxidation in a microchamber saturated with methanol under a nitrogen atmosphere, various possibility of elution gradients, and fully automated development of the plates. This makes it a very interesting method for densitometry.

OPLC and AMD are often more appropriate in many instances because their techniques give better reproducible hRf values in well-defined experimental conditions. AMD permits analysis on very small quantities and obtains sharper separations because of the absence of diffusion in the adsorbent.

The advantages of OPLC are efficiency, reproducibility, small consumption of developing eluent, and short analysis time. Consequently, the obtained spots are more regular in form, which decreases diffusion or stretching effects. This is not the case for TLC and particularly for quaternary ammonium salts. Other advantages compared to TLC are the possibility to extend this method to semipreparative chromatography; no scraping and eluting of bands are necessary because the components may be drained from the plate and obtained pure by coupling to a collector. Because the migration is linear with time, it is possible to determine the beginning of the elution of the studied compound. Alkaloids were separated from different plant extracts by this method.

This method can be certainly extended to numerous plant extracts. Therefore, by this technique it is also possible to collect fractions of the alkaloid constituent of opium (papaverine, noscapine, thebaine, and codeine). However, when the hRf value is nearly 20 (morphine), it is necessary to increase the polarity of the mobile phase by the addition of isopropanol to ethyl acetate. Methanol cannot be employed because it can damage the impregnated polymer.

It is not so easy to separate phenolic compounds in comparison to alkaloids, and for these products it is necessary to employ binary or more complex eluents, whereas with alkaloids a good separation can be obtained with a single solvent. However, OPLC is an interesting method to separate compounds from plant extracts such as the polyphenols, and an extension of this method to the semipreparative scale can be envisaged.

Therefore, semipreparative OPLC is a technique more efficient than preparative TLC, which will gain in importance with the arrival of the second generation of OPLC chromatographs.

This work could be made complete by the chromatographic study of phenolic compounds glycosides and perhaps other essential oils and saponins in order to cover the main vegetable origin compounds that possess a pharmacological activity.

Acknowledgments

The results on ginsenosides were obtained by Mathilde Glorieux and those on opiate alkaloids, in part, by Géraldine Salou during the preparation of their theses in our laboratory. We are very grateful to these two pharmacist students for these results. Our acknowledgments go also to Dr. Julien Pothier for the rereading of our English writing.

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