Journal of Chromatography, 213 (1981) 113–127
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,841

PLANT SCREENING BY CHEMICAL AND CHROMATOGRAPHIC PROCEDURES UNDER FIELD CONDITIONS*

G. B. MARINI-BETTÒLO*, M. NICOLETTI and M. PATAMIA
Centro Chimica dei Recettori e delle Molecole Biologicamente Attive, Via della Pineta Sacchetti, 644 Rome, and Cattedra di Chimica Generale ed Inorganica, Istituto dell'Orto Botanico, Università di Roma, Largo Cristina di Svezia, 24 Rome (Italy)

and

C. GALEFFI and I. MESSANA
Laboratorio Chimica del Farmaco, Istituto Superiore di Sanità, Rome (Italy)
(Received April 5th, 1981)

SUMMARY

The plant kingdom constitutes an invaluable source of new chemical products which may be important due to their biological properties and in particular because of their potential use in medicine. In many countries, mainly in the Tropics, systematic surveys of plants are currently in progress. It is therefore important to have a simple and universal method available for preliminary chemical screening of plants under field conditions.

The procedure suggested here is based on the identification in raw plant extracts of some chemical groups of substances (alkaloids, quinones, saponins, polyphenols, anthranoids) by precipitation and colour reactions. To obtain more accurate results, a second stage, based on thin-layer chromatographic separation of extracts of plant material, can be applied. Solvent systems, specific spray reagents and reference substances are proposed.

A number of groups of products, including flavonoids, coumarins, cardenolides and steroids can be rapidly identified in plants by this method.

INTRODUCTION

The growing interest in natural products from plants in relation to the search for new active principles (pharmacologically active substances, insecticides, etc.) and precursors in biosynthesis has promoted considerable work in the chemical screening of plants in the last decade.

A number of general tests have been proposed in the past to ascertain the presence of biologically active principles in medicinal plants. The pioneer work in this

* Based on WHO document DPM/80.5 by G. B. Marini-Bettòlo entitled "Preliminary Chemical Screening of Medicinal Plants in Field Conditions".

0021-9673/81/0000–0000/$02.50

field was carried out by Webb[1] in Australia and Wall *et al.*[2] in the U.S.A. A comprehensive report on this subject was prepared in 1966 by Farnsworth[3] with 854 references which cover, in a critical form, practically all previous literature. More recent reports on screening methods were published by Bandoni *et al.*[4] and Odebiyi and Sofowora[5].

These tests generally did not take into account rapid separation techniques, for example, thin-layer chromatography (TLC). If we compare critically the various systems so far adopted, we can make the following comments:

(a) only a few groups of products can be detected with sufficient reliability;

(b) not all of the reactions proposed, even those which can be considered as classical, are general for all members of a given group of products;

(c) since the last papers on the chemical screening of plants were published a great number of different procedures and reagents have been introduced. These facts indicate the necessity for a new unified or standardized method for screening.

In general, the older test methods do not take into consideration the presence of other substances in plants which may interfere and thus lead to false results. For example, the alkaloid test with Dragendorff's reagent may give a positive response in the presence of water-soluble proteins and α,β-unsaturated ketones. On the other hand, the Dragendorff reaction, characteristic of alkaloids, can be negative in the presence of less basic alkaloids (*e.g.*, amido derivatives). The same situation may occur with other active constituents; for example, the cyanidin test for flavonoids may be masked by other substances present in the plant extract.

The extraction systems used were generally poorly defined, water, dilute acetic acid and dilute ethanol being used in the tests referred to in the methods quoted above.

The possibility of an erroneous evaluation of the plant in preliminary tests must be borne in mind in order to prevent the discarding and rejection of a plant that could be important, or collecting for further study a material that does not contain any active principle.

These considerations indicate the necessity for a thorough, critical examination of the results of screening before rejecting a plant from further study, because this may lead to a loss to science for many years. However, the study of plants which later, on more exact examination, show the absence of active principles does not involve any loss of information, but only a waste of time and resources.

All this makes necessary a complete experimental revision of all the tests so far used which have proved to be insufficiently accurate and specific. Moreover, a modern approach to the screening of medicinal plants makes it necessary to establish rapid and simple procedures for use in the field.

These procedures, which are required mainly in tropical areas in order to be able to make rapid surveys of the potentiality of the local flora, have to take into account that even simple laboratory facilities, which may make the work easier, may not be available.

EXTRACTION OF NATURAL PRODUCTS FROM PLANT MATERIAL AND THEIR IDENTIFICATION BY PRECIPITATION AND COLOUR REACTIONS [BY TEST-TUBE METHOD]

We studied a rapid and simple system for the screening of plants based on a

limited number of reagents and feasible in field conditions. For this purpose a portable kit was constructed for submitting part of the plants to chemical tests.

We report here procedures proposed for the rapid screening under field conditions of some large chemical groups such as quinones, tannins, glycosides, alkaloids and flavonoids. The literature on this topic is extensive but it would be difficult to bring together the various methods that have been proposed. A number of plants, both known and unknown, were examined under different conditions in order to establish the present proposals for carrying out field screening, to ascertain the best method for rapid tests of the groups of substances considered important for their biological properties.

The first point to be considered was the limited possibilities of carrying out extractions under field conditions in extreme locations such as the rain forest or the bush. In such environments it may even be difficult to move, because the vegetation and numerous animals and insects make difficult even the simplest operations. However, the material, leaves, flowers, fruits, roots, barks and seeds collected from the plants must be identified. This implies a considerable amount of work in difficult conditions by one or more qualified collectors. A direct check on the plant under these conditions is often very difficult. In practice it is not possible, except in a few instances, to extract the active principles in a short time and at ambient temperature, *i.e.*, without using heat; thus the use of a heating device must be considered, but this makes operations in the forest even more complicated.

A portable case containing the essential tools is shown in Fig. 1. This kit is easily transportable and is adequate at accessible sites, *e.g.*, open areas*.

Nevertheless, the collection and preliminary examination of the plants should be made locally in a tent or a hut with a roof (to avoid rain), or in a car or jeep, in which a portable table with the kit is located. This permits the tests to be performed under better conditions than in the open field. However, under these conditions it is possible only to carry out rapid extractions with water, dilute acids and ethanol, but more facilities are needed to make a successful extraction of the plant material with solvents of increasing polarity, which is an effective and selective procedure.

The principal aim of the preliminary screening is to ascertain the presence of the active principles in plants before the collector leaves the area. This permits, if interesting positive results are obtained, more material to be gathered immediately for further study.

It is also suggested that the preliminary examination, the botanical classification of the plant and the preparation of specimens should be made at the same time. It is sometimes possible that the collector himself may be expert in plant systematics and be able to perform the chemical tests. Even then, tests based on direct extraction of plant material with very simple techniques can be performed.

---

* *Note added in proof:* The above methodology was tested in a scientific expedition of the University of Florence in the Sahara oasis in southern Tunisia and Algeria by Professor Vinceri, Istituto di Chimica Farmaceutica, Università, Firenze, Italy (April–May 1981). The performance of the method and the material (reagents and tools) proved to be quite adequate and sufficient for the rapid chemical screening of the plants in the field. Although the conditions were quite severe (50 days journey in the desert in a jeep for 7000 km, at temperatures from 5 to 47°C, and sand storms) the material and the kit were always efficient.

Fig. 1. Transportable kit for field chemical screening of plants.

Collection of plant material

The collection of plant material should include various parts of the plant: leaves, stem bark, roots, root bark; when possible, seeds, flowers and fruits should be collected for identification and classification of the plant. A voucher specimen must be kept for each plant, bearing the name of the collector, the number of the Institute Herbarium or the reference Herbarium, the site where the specimen was collected and other relevant data.

A rapid identification of the plant on the basis of chemotaxonomy can sometimes be an important indicator, as particular groups of substances (see Table I) have been demonstrated to be present in a limited number of plant families only. The importance of the season during collection should also be underlined owing to the turnover, during the vegetation period, of many secondary plant metabolites.

Extraction

A number of methods of extraction have been suggested and are very promising, but cannot be taken into account here because they require adequate training,

TABLE I

DISTRIBUTION OF CHEMICAL GROUPS IN PLANT FAMILIES

Compounds	*Plants*
Anthranoids	Bignoniaceae, Leguminosae, Liliaceae, Polygonaceae, Rhamnaceae, Rubiaceae, Verbenaceae
Alkaloids	Papaveraceae, Amaryllidaceae, Rubiaceae, Apocynaceae, Leguminosae, Liliaceae, Solanaceae, Rutaceae, Annonaceae, Menispermaceae, Ranunculaceae, Cactaceae, Compositae, Coprinaceae, Sapindaceae, Berberidaceae, Campanulaceae, Magnoliaceae, Lycopodiaceae, Labiateae, Gentianaceae, Loganiaceae
Cardenolides	Apocynaceae, Asclepiadaceae, Cactaceae, Celastraceae, Compositae, Cruciferae, Moraceae, Euphorbiaceae, Labiatae, Ranunculaceae, Rhamnaceae, Sterculiaceae, Tiliaceae
Flavonoids	Bryophytae, Chlorophytae, Cupressaceae, Cyatheaceae, Equisetaceae, Dicksoniaceae, Gleicheniaceae, Hymenophyllaceae, Lycopodiaceae, Pinaceae, Podocarpaceae, Polypodiaceae, Psilotaceae, Schizaeaceae, Selaginellaceae, Taxodiaceae, Araceae, Graminaceae, Zingiberaceae
Steroids	Ubiquitous
Saponins	Ubiquitous
Tannins	Ubiquitous

accuracy and certain equipment that is not in accordance with the simplicity required for our tests.

Identification

The groups of substances that can be tested under field conditions include alkaloids, anthranoids, cardiac glycosides, tannins, polyphenols and saponins[6]. The detection of flavonoids and steroids by test-tube methods was not considered possible for the reasons discussed below.

Alkaloids

Although rapid tests for alkaloids are considered to be amongst the easiest for the detection of active principles in plants, the literature shows that even the most accurate tests may be erroneous because of interferences. Moreover, many tests fail to recognize the presence of quaternary alkaloids, which are of great practical importance. The proposed test is based on an acid extraction of plant material, followed by the use of the Dragendorff's reagent. In order to establish the total alkaloid content semi-quantitatively a reference standard is provided, consisting of 0.05 % strychnine sulphate solution.

Polyphenols

Polyphenols can be detected by oxidation reaction with iron(III) chloride and potassium hexacyanoferrate(III). Although non-specific and not always certain, this reaction allows, in many instances, the detection of a number of substances belonging to different categories but having as a common feature the presence of one or more phenolic hydroxy groups, *e.g.*, phenols, phenolic acids, tannins, hydroxyflavonoids and hydroxystilbenes.

Tannins

Tannins, being polyphenols (belonging to two distinct chemical groups, gallotannins and catechutannins), are affected in the same way by reagents which cause oxidation of phenolic groups. A specific test consists of the use of gelatin precipitation. A 0.5% solution of tannic acid is used as a standard.

Flavonoids

The reaction for the detection of flavonoids can be based on the oxidation of the hydroxy groups with Fe^{3+} or on the reduction of the pyrone ring, according to Willstätter, to anthocyanidins which give blue to violet colours. The first reaction is not specific and the second is so delicate that a positive response can be obtained only in the presence of a pure substance or of a large amount of flavonoids in the extract. Unfortunately, the interfering substances present in a non-purified extract mask the colour and the reaction therefore cannot be used for the detection of flavonoids in crude extracts. Other reactions, such as that with antimony(III) chloride, also require pure substances and even anhydrous conditions.

Quinones

With the exception of hydroxyanthraquinones, it is not possible at present to recommend any rapid, easy test for the detection of quinones. Quinones are ubiquitous in plant cells, although in small amounts, and many plants contain as secondary metabolites not only quinones but also the corresponding hydroquinones (quinols) which, after cutting and exposure of the plant to air, may be partially transformed into the corresponding quinones (walnut, *Mansonia altissima*). On the other hand, when quinones are present in the plant they are found together with the corresponding quinols. The presence of quinones can be inferred indirectly from the colour of the plant extract in water or alcohol. Quinols may be detected through the common reactions used for polyphenols.

Anthranoids (anthracene derivatives)

These substances occur in plants at various oxidation levels and thus should be completely oxidized to anthraquinones before performing the test. A 0.05% solution of emodin (1,3,8-trihydroxy-6-methylanthraquinone) in toluene is proposed as a reference standard.

Steroids

Steroids are ubiquitous in all cell membranes and in the seeds of plants. The search for steroids has therefore to be limited to those parts of plants which contain the largest amounts. The detection of steroids without a preliminary separation (successive solvent extraction and chromatography) is not feasible under field conditions. Moreover, colour reactions reported for their identification (Liebermann or Salkowski reactions) are reliable only if a pure substance is used. The tests given here pertain to the detection of only particular groups of steroids, *viz.*, cardenolides and steroidal saponins.

Cardenolides (cardiac glycosides)

The detection of cardenolides in the aqueous extract of the plant is carried out with Kedde reagent (3,5-dinitrobenzoic acid). This reaction detects the α,β-unsaturated γ-lactone ring and is specific. A 0.025% solution of digitoxin is used as a reference standard.

Saponins

The data reported in the literature which are based on the formation of a foam by shaking an aqueous solution containing saponins are not reliable and are excessively subjective. The haemolysis determination is complicated and, moreover, the occurrence in plant extracts of various other substances may contribute to errors. A very simple method based on the tensioactivity of saponins on water is therefore proposed here. This test is based on the relationship between the height of a solution in a capillary and the surface tension.

SEPARATION AND IDENTIFICATION OF NATURAL PRODUCTS FROM PLANTS BY THIN-LAYER CHROMATOGRAPHY UNDER FIELD CONDITIONS

Simple chromatographic procedures have been studied for the screening of active principles in plants, including anthranoids, flavonoids, cardenolides, alkaloids, steroids, saponins, tannins and phenols. Although the procedures have been simplified for field use, they require more sophisticated tools than simple test-tubes, more accuracy and time from the operator and the presence of some facilities. The screening should be run on a table in a protected place, for example in a tent or in a room, and the investigator should have at his disposal an electric current.

The principal advantage of the procedure is the identification of substances in different fractions previously submitted to chromatographic separation. Thus colour reactions can be more specific and reliable. The use of general and specific reagents makes it possible to attain a good specificity of identification.

The simplest system is the use of TLC with pre-coated plates with a thin layer of silica gel, alumina or cellulose (without a fluorescent indicator) on aluminium foil. The plant sample is selectively extracted with a series of solvents of increasing polarity, which produces a first fractionation. For the collection of plant material, the same criteria above mentioned are pertinent. The plant is sun-dried and then ground or, if fresh (*e.g.*, leaves), cut into small pieces and subjected to extraction in a test-tube for 5 min in a water-bath at 50°C with the following solvents: light petroleum (b.p. 60–80°C); chloroform–acetic acid (99:1), giving solution A; methanol–chloroform–acetic acid (49.5:49.5:1), giving solution B; and methanol–water (1:1), giving solution C.

For alkaloids another procedure is also suggested, based on the extraction of the material with 1 % hydrochloric acid (R-5) under mild heating conditions followed by alkalinization with sodium carbonate (R-8) and extraction with chloroform. This procedure allows the separation of tertiary alkaloids (solution D), which are soluble in chloroform, from quaternary alkaloids, which are soluble in the aqueous phase (solution E), and their separate submission to TLC for identification. The five solutions (A, B, C, D and E), which contain different classes of substances (Table V), are submitted to chromatographic separation using the solvent systems reported in Table VI and detected with the reagents reported in Table VII.

EXPERIMENTAL

Test-tube method

Materials, reagents and solvents are reported in Tables II and III. When reagents or solvents are added to the tubes it is necessary to stir the solution with a glass rod.

TABLE II

MATERIALS FOR A FIELD LABORATORY (TEST-TUBE METHOD)

These materials can be fitted in a metal case (51.5 × 36 × 21.5 cm) and transported by hand (or on the shoulders) for use in the field (Fig. 1).

Burner (camping gas, or alcohol burner, or meta burner)
Tripod
Metal bowl
Mortar (for seeds)
Grinding device
Shears
Wood pincers
50 test-tubes (10 ml)
10 funnels (diameter 8 cm, 2 cm)
100 capillaries (15 cm long × 1 mm O.D.)
10 Pasteur pipettes, 5 and 10 ml; 5 each size)
4 beakers: 200, 100, 50, 25 ml; one each size
4 capsules: diameter 10, 8, 5, 3 cm; one each size
5 graduated test-tubes, 10 ml
20 glass stirrers
4 erlenmeyer flasks: 250, 120 ml; two each size
1 erlenmeyer flask, 50 ml
15 polythene bottles for reagents (50 ml)
5 polythene bottles for solvents (250 ml)
2 bottles for distilled water (1 l)
100 paper filters, diameter 6 cm
Cotton-wool
Glass-wool

Preliminary tests. Well ground material (2 g) is heated with S-1 (10 ml) for 30 min on a water-bath and the solution is filtered through cotton (WS). A coloured solution, generally yellow to red, indicates the presence of soluble substances containing chromophores (quinoids, flavonoids, anthranoids substances, etc.) with lyophilic groups (sugar moieties, acid and phenolic groups, etc.). On addition to the solution of R-1 (3 drops) the colour should become more intense.

Well ground material (2 g) is heated with S-2 (10 ml) for 10 min on a water-bath and the solution is filtered through cotton (AS). A coloured filtrate indicates the presence of chromophores which may be either lyophilic (see above) or lipophilic (such as carotenoids). The presence of the former compounds is confirmed by adding R-1 (4 drops) to the filtrate: the colour becomes deeper. Do not use the green parts of the plant in this test.

Test for alkaloids. Thoroughly ground plant material (2 g) is treated in a test-tube with R-5 (10 ml) for 30 min in a water-bath. The suspension is filtered through cotton into a test-tube and the filtrate is divided into two parts, A and B.

R-D (5 drops) is added to filtrate A. The formation of a precipitate indicates the presence of alkaloids.

Tertiary and quaternary alkaloids: R-D (3 drops) is added to the standard solution ST-1 (1 ml) to evaluate the above tests. The filtrate B is treated with R-8 until a drop of the solution turns the universal indicator paper (UIP) blue (pH 8–9), and is then mixed with S-3 (4 ml). After separation of the layers, the upper layer is re-

TABLE III

REAGENTS AND SOLVENTS FOR A FIELD LABORATORY (TEST-TUBE METHOD)

Abbreviation	*Specification*
R-1	KOH, 0.5 *N*
R-2	H_2O_2, 5%
R-3	Acetic acid
R-4	Toluene
R-5	HCl, 1%
R-6-D	Dragendorff A: add 10 ml of acetic acid to 0.85 g of bismuth subnitrate in 40 ml of distilled water
R-7-D	Dragendorff B: add 20 ml of distilled water to 8 g of potassium iodide
R-D	Mix 5 ml of R-6-D and 5 ml of R-7-D with 20 ml of acetic acid (R-3) and 100 ml of distilled water (S-1). Store in a dark glass bottle
R-8	Saturated sodium carbonate solution; 21.52 g of $Na_2CO_3 \cdot 10\ H_2O$ dissolved in 100 ml of water
R-9	3,5-Dinitrobenzoic acid (1 g in 50 ml of methanol)
R-10	KOH, 1 *N* in methanol*
R-K	Kedde reagent: mix 0.4 ml of R-9 and 0.6 ml of R-10 (to be prepared extemporarily)
R-11	NaCl, 2%
R-12	Gelatin, 1%. The solution is obtained by prolonged heating on a water-bath
R-13	$FeCl_3$, 1%
R-14	$K_3Fe(CN)_6$, 1%
S-1	H_2O (distilled or deionized)
S-2	C_2H_5OH
S-3	$CHCl_3$
S-4	CH_3OH
UIP	Universal indicator paper
Reference solutions:	
ST-1	Strychnine sulphate, 0.05% in water
ST-2	Emodin, 0.05% in toluene
ST-3	Digitoxin, 0.025% in methanol
ST-4	Tannic acid, 0.5% in water

* Use KOH pellets (average weight 100 mg each) to prepare the reagent extemporarily.

moved with a pipette and treated with R-3 until a yellow colour forms with UIP (pH 5). Then, R-D (5 drops) is added; a precipitate indicates the presence of quaternary alkaloids. The lower layer is then treated with R-5 (2 ml). On separation of the layers, the upper layer is removed with a pipette and treated with R-D (3 drops): a precipitate indicates the presence of tertiary alkaloids.

Test for anthranoids. Well ground plant material (0.2 g) is boiled for 2 min with R-1 (5 ml) and R-2 (0.5 ml). After cooling, the suspension is filtered through glass-wool. The filtrate is treated with R-3 (6 drops) (check with UIP if the colour is yellowish) and the resulting solution is mixed with R-4 (5 ml). The upper layer is separated with a pipette and transferred into a test-tube and R-1 (2ml) is added. If a red colour appears in the aqueous layer, anthranoids are present. R-1 (2 ml) is added to the standard solution ST-2 to compare the colour.

Test for polyphenols. Three drops of a mixture of R-13 (1 ml) and R-14 (1 ml) prepared immediately before the reaction are added to solution WS. The formation of a green-blue colour indicates the presence of polyphenols. The test should be repeated with solution (AS) (2 ml).

Test for tannins. R-11 (5 ml) is added to solution WS (5 ml). If precipitation occurs, the suspension is filtered through paper, then R-12 (5 ml) is added to the clear solution. A precipitate indicates the presence of tannins. As a reference, R-11 (2 ml) and R-12 (5 ml) are added to standard solution ST-4. The polyphenol test is also positive for tannins.

Test for cardenolides (cardiac glycosides). R-K (4 drops) is added to solution WS (2 ml): a blue-violet colour indicates the presence of cardenolides.

For further confirmation, solution WS (2 ml) is mixed with S-3 (2 ml) and S-2 (2 ml). The upper layer is removed with a pipette and the lower layer is treated with R-K (4 drops). A blue-violet colour indicates the presence of cardenolides.

As a reference, standard ST-3 solution (2 ml) was treated under the same conditions.

Test for saponins. The WS solution is transferred into a capillary (15 cm × 1 mm O.D.) up to a height of 10 mm. The capillary is kept vertical, then the liquid is allowed to flow out freely. The level of the liquid is compared with the level obtained with distilled water in an identical capillary at the same temperature. If the level of the test liquid is half or less than that of the distilled water, the presence of saponins may be inferred.

Concentration has little influence on the height of the liquid in the capillary and therefore no reference substance is used.

Thin-layer chromatography

Materials, solvents and reagents and the distribution of different substances in the various solutions are reported in Tables IV–VII.

Extraction

The material from the whole plant or from a part of it (about 2–3 g), finely ground, is heated in a 20-ml test-tube for 5 min in a water-bath at 50°C with light petroleum (b.p. 60–80°C) (S-6) (10 ml). The solvent is discarded and the extracted material (A) submitted to subsequent operations.

The material is treated with chloroform–acetic acid (S-3–S-5) (99:1) (10 ml) at 50°C on a water-bath. Lipophilic substances are extracted under these conditions. The extract is then filtered through filter-paper or cotton to give solution A for TLC and exhausted material (B) for further extraction.

The material B is extracted with methanol–chloroform–acetic acid (S-4–S-3–S-5) (49.5:49.5:1) (10 ml) for 5 min at 50°C on a water-bath, lipophilic and slightly hydrophilic substances being extracted. The extract is then filtered as indicated above to give solution B for TLC, and a residue (C).

The material C is extracted with methanol–water (S-4–S-1) (1:1) (10 ml) for 5 min on a water-bath at 50°C, hydrophilic substances being extracted. The extract is then filtered to give solution C for TLC (*cf.*, Table V).

Extraction of alkaloids

The plant or a part thereof (2 g) is finely ground and extracted as described below. It is advisable to treat the material previously with light petroleum as mentioned above. The material is extracted with 1 % hydrochloric acid (10 ml) at 40°C on a water-bath for at least 30 min. The solution is filtered through filter-paper or cotton.

TABLE IV

MATERIALS FOR TLC FIELD PROCEDURES

These materials can be fitted in a metal case (51.5 × 36 × 21.5 cm) and transported by hand (or on the shoulders) for use in the field.

No.	*Material*
3	TLC chambers for 20 × 20 cm plates
3	Cylinders, 5, 50, 100 ml
100	Capillaries (15 cm × 1 mm O.D.)
20	Pasteur pipettes
4	Sprayers
–	Cotton-wool or glass-wool
–	Filter-papers
5	Funnels (diameter 5 cm)
20	Test-tubes, 20 ml
–	Separating funnels, 25 and 250 ml
–	Silica gel TLC plates, 0.2 mm, 20 × 20 cm
–	Cellulose TLC plates, 0.2 mm, 20 × 20 cm
5	Beakers, 100 ml
1	Centrifuge tubes, 20 cm
2	Universal indicator paper, UIP
1	Stove (100–120°C)
1	Thermometer (110°C)
1	Balance (0.1 g sensitivity)
1	Grinder
1	Rack for test-tubes
1	Longwave UV lamp
2	Pipettes, 0.1–0.2 ml
Solvents:*	
S-5	Acetic acid
S-6	Light petroleum ether
SC-1	Acetone
SC-2	Acetic acid
SC-3	*n*-Butanol
SC-4	*tert.*-Butanol
SC-5	Cyclohexane
SC-6	Diethylamine
SC-7	Ethyl acetate
SC-8	Methyl ethyl ketone
SC-9	Pyridine
SC-10	Chloroform
SC-11	Toluene
SC-12	Methanol
SC-13	Formic acid

* S = Standard grade solvent; SC = chromatographic standard solvent.

The presence of alkaloids is ascertained by adding Dragendorff's reagent (1 drop) to the solution (1 drop) on a porcelain plate: a red colour indicates a positive reaction. If the previous assay is positive the solution is adjusted to pH 8–9 with 1 *M* sodium carbonate solution and extracted with 10 ml of chloroform. If an emulsion is formed a centrifuge should be used. The lower chloroform phase is separated and extracted

TABLE V

DISTRIBUTION OF THE VARIOUS GROUPS OF SUBSTANCES IN THE DIFFERENT FRACTIONS

These solutions are submitted to TLC separation, as in Table VI, and the substances are detected with the spray reagents listed in Table VII.

Solution	*Substances*
A	Anthraquinones, phenols
A	Flavonoids, steroids, coumarins
B	Flavonoid glucosides, cardenolides
B	Anthraquinone glucosides
B	Tannins, saponins
B	Coumarins
C	Flavonoid glucosides, anthraquinone glucosides, cardenolides, saponins
D	Tertiary alkaloids
E	Quaternary alkaloids

TABLE VI

SOLVENT SYSTEMS USED FOR TLC

The various groups are identified by spraying the plates with the reagents listed in Table VII.

Abbreviation	*Solvent system*	*Adsorbent*	*Solution tested*
E-1	Toluene–chloroform (9:11) SC-11 SC-10	Silica gel	A
E-2	Toluene–acetone–chloroform (40:25:35) SC-11 SC-12 SC-10	Silica gel	A
E-3	*n*-Butanol–acetic acid–water (4:1:5)–upper phase SC-3 SC-2	Silica gel Cellulose	B, C C, E
E-4	2-Butanone–toluene–methanol–acetic acid–water (80:10:5:2:6) SC-8 SC-11 SC-13 SC-2	Silica gel	B
E-5	Ethyl acetate–2-butanone–formic acid–water (50:30:10:10) SC-7 SC-8 SC-14	Silica gel	B
E-6	Chloroform–acetic acid–water (50:45:5) SC-10 SC-2	Silica gel	B
E-7	Ethyl acetate–pyridine–water (50:10:40) SC-7 SC-9	Cellulose	C, E
E-8	Cyclohexane–diethylamine (9:1) SC-5 SC-6	Silica gel	D
E-9	*tert.*-Butanol–chloroform–diethylamine (2:7:1) SC-4 SC-10 SC-6	Silica gel	D

with 1% hydrochloric acid (5 ml). The aqueous solution so obtained (upper layer, solution D) is submitted to TLC. The upper phase, neutralized with acetic acid, is solution E, to be submitted to TLC for identification (*cf.*, Table V).

Thin-layer chromatography

TLC is carried out with solutions A and B (0.1 ml) and C, D and E (0.05 ml)

TABLE VII

SPRAY REAGENTS

Abbreviation	*Reagent*	*Products*	*Reactions*
RC-1	Antimony(III) chloride	Flavonoids, steroids	Fluorescence, UV
RC-2	Diphenylboric acid β-aminoethyl ester	Flavonoids,	Fluorescence, UV
RC-3	Fast Blue B salt	Phenols, tannins, flavonoids	Various colours
RC-4	Potassium hexacyanoferrate(III)-iron(III) chloride	Phenols, tannins, flavonoids	Blue colour
RC-5	Acetic anhydride-sulphuric acid	Steroids, saponins	Dark spots or fluorescence
RC-6	Trifluoroacetic acid	Steroids, saponins	Dark spots
RC-7	Dragendorff's reagent	Alkaloids	Reddish brown colour
RC-8	3,5-Dinitrobenzoic acid	Cardenolides	Blue-violet colour
RC-9	Potassium hydroxide in methanol	Coumarins, anthranoids, phenols	Fluorescence
RC-10	Magnesium acetate	Anthraquinoids	Orange-violet colour

using different adsorbents and solvent systems according to Table VI. The plates, after development and drying, are sprayed with the reagents given in Table VII. The composition of each reagent and the procedure to be used are given below.

Flavonoids.

RC-1: antimony (III) chloride. A 10% solution of antimony (III) chloride in chloroform; flavonoids yield spots which fluoresce in longwave UV light; glucoside flavonoids yield yellow spots.

RC-2: diphenylboric acid β-aminoethyl ester. A 1% methanolic solution of the β-aminoethyl ester of diphenylboric acid. Test for flavonoids. The plate is sprayed with about 10 ml of reagent and the fluorescence spots observed in longwave UV light.

Phenols, tannins, flavonoids.

RC-3: Fast Blue B salt (diazonium reagent). Spray reagent I: freshly prepared 0.5% solution of Fast Blue B salt. Spray reagent II: 0.1 *N* sodium hydroxide solution. The plate is sprayed with I, then II. Various colours are formed.

RC-4: potassium hexacyanoferrate(III)–iron(III) chloride. Solution A: 1% potassium hexacyanoferrate(III) solution. Solution B: 1% iron(III) chloride solution. Equal amounts of A and B are mixed just before use. The colours are intensified by subsequent spraying with 2 *N* hydrochloric acid.

Steroids and saponins.

RC-5: acetic anhydride–sulphuric acid (Liebermann–Burchard reagent). A 5-ml volume of acetic anhydride is carefully mixed, with cooling, with 5 ml of sulphuric acid; this mixture is added cautiously to 50 ml of absolute ethanol with cooling. Prepare freshly before use. The plate is heated for 10 min at 100°C; it yields dark spots or fluorescing spots in longwave UV light.

RC-6: trifluoroacetic acid. A 1% solution of trifluoroacetic acid in chloroform. Dark spots are obtained after heating for 5 min at 120°C.

Test for alkaloids.

RC-7: Dragendorff's reagent (after development, the plates are heated at 110°C for 2 h). Solution A: 0.85 g of basic bismuth nitrate is dissolved in a mixture of 10 ml of acetic acid and 40 ml of water. Solution B: 8 g of potassium iodide are dissolved in 20 ml of water. Stock solution: a 1:1 mixture of solutions A and B is prepared. This may be kept several months in a refrigerator. A 1-ml volume of the stock solution is mixed with 2 ml of acetic acid and 10 ml of water before use. An orange-red colour is formed.

Cardenolides.

RC-8: 3,5-dinitrobenzoic acid (Kedde reagent). Spray reagent I: 2% methanolic solution of 3,5-dinitrobenzoic acid. Spray reagent II: 5.7 g of potassium hydroxide are dissolved in methanol and diluted to 100 ml with methanol. The plate is first lightly sprayed with I and then with excess of II. Blue-violet spots appear.

Coumarins and anthranoids.

RC-9: 5% methanol potassium hydroxide solution. After drying, the plate is inspected in daylight and longwave UV light.

Anthraquinones.

RC-10: spray reagent, 0.5% methanolic solution of magnesium acetate. The plate is heated for 5 min at 90°C; the spots are orange-violet.

REFERENCES

1 Y. Webb, *Aust. CSIRO Bull.*, (1949) 241.
2 M. E. Wall, C. S. Fenske, H. E. Kenney, J. J. Willaman, J. S. Correll, B. G. Schubert and H. S. Gentry, *J. Amer. Pharm. Ass. Sci. Ed.*, 46 (1957) 653.
3 N. R. Farnsworth, *J. Pharm. Sci.*, 55 (1966) 225.
4 A. L. Bandoni, M. E. Mendiondo, R. V. D. Rondina and J. Coussio, *Lloydia*, 35 (1972) 69.
5 D. D. Odebiyi and E. A. Sofowora, *Lloydia*, 41 (1978) 234.
6 G. B. Marini-Bettòlo, *Reazioni Organiche*, Sansoni Edizioni Scientifiche, Florence, 1964, pp. 557–611.