กรมวิทยาศาสตร์การแพทย์
DEPARTMENT OF MEDICAL SCIENCES

THAI HERBAL COMPENDIUM ON PHYSICO-CHEMICAL SPECIFICATIONS

VOLUME I

Department of Medical Sciences, Ministry of Public Health, Thailand

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VOLUME I

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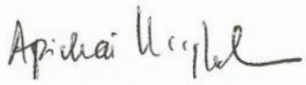
Preface

Nowadays, the trend on the consumption of herbal drugs and their preparations according to Thai traditional medicine wisdom in Thailand rapidly increases. Furthermore, Thai government policy continuously promotes the use of herbal medicines into Thai health system to reduce the cost of imported modern medicines. However, the quality of herbal medicines is a matter of concern for the Ministry of Public Health not only to protect the consumers from the substandard and adulterated herbal drugs and their preparations, but also to support governmental and private sectors to have a substantial herbal drugs standard. Realizing the herbal quality problem, the Department of Medical Sciences, therefore, has established the Thai Herbal Compendium on Physico-chemical Specifications Volume I to provide the physico-chemical specifications which are the important part of the standard and the requirement of herbal drugs and their preparations for quality assurance. The aims in establishing this first volume of the Thai Herbal Compendium on Physico-chemical Specifications are to compile physico-chemical specifications of potential herbal drugs and their preparations obtained from our research projects of the Medicinal Plant Research Institute, Department of Medical Sciences and some related appendices from Thai Herbal Pharmacopoeia, and also to provide it as one of the reference books on the quality assurance of herbal drugs. The herbal drugs and their preparations in the compendium may be adopted or adapted into Thai Herbal Pharmacopoeia. This publication contains 10 monographs of herbal drugs and their preparations, namely *Aegle marmelos* (L.) Corrêa, *Coriandrum sativum* L., *Eurycoma longifolia* Jack, *Gynostemma pentaphyllum* (Thunb.) Makino, *Gynostemma pentaphyllum* (Thunb.) Makino Dry Extract, *Hyptis suaveolens* (L.) Poit., *Hyptis suaveolens* (L.) Poit. Dry Extract, *Kaempferia parviflora* Wall. ex Baker Volatile Oil, *Morinda citrifolia* L., and *Portulaca grandiflora* Hook. This publication has been established by the cooperation between the Medicinal Plant Research Institute and the Bureau of Drug and Narcotic, Department of Medical Sciences. The contributors from the Medicinal Plant Research Institute who have provided their valuable work and expertise in making this task possible are gratefully acknowledged. Hopefully, the essential information obtained from this publication would not only immensely benefit private sectors, industries, and academics relevant in the research and development of herbal drug preparations, but also be importantly useful as the crucially basic tool in the quality assurance of herbal drugs for regulatory authorities to provide good herbal drug preparations in Thai markets for the good health of Thai people.



(Dr. Apichai Mongkol)

Director-General of the Department of Medical Sciences

Advisors

Director-General of the Department of Medical Sciences

Apichai **Mongkol**, M.D.

Assistant Director-General of the Department of Medical Sciences

Angkana **Herunsalee**, B.Sc. Hons (Chemistry), M.Sc. (Organic Chemistry), Dr.Agri.Sc. (Organic Chemistry)

Director of Medicinal Plant Research Institute

Nuchattra **Chansuvanich**, B.Sc. (Biology), M.S. (Env. Sci.)

Director of Bureau of Drug and Narcotic

Suratchanee **Savetsila**, B.Sc. in Pharm., M.Sc. in Pharm.

Editors

Medicinal Plant Research Institute

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Sakwichai **Ontong**, B.Sc. (Biology)

Sununta **Srisopon**, B. Pharm., M. Pharm. (Community Pharmacy), Ph.D.

Bureau of Drug and Narcotic

Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL.B., Barrister at Law

Kornvika **Charupant**, B.S. Pharm., M.Sc. in Pharm., Ph.D.

Sasiwimon **Patasema**, B. Pharm., M.Sc. in Pharm.

Santi **Nimnoi**, B.S. in Pharm.

Sasiphimol **Boontavee**, B. Pharm.

Contributors

Aegle marmelos (L.) Corrêa

Apirak **Sakpetch**, B.S. Pharm.

Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Sirikarn **Thana-ariyaroj**, B.S. (Chemistry)

Peradhama **Thiemthieprat**, B.S. (Chemistry)

Sakwichai **Ontong**, B.Sc. (Biology)

Nuchattra **Chansuvanich**, B.Sc. (Biology), M.S. (Env. Sci.)

Coriandrum sativum L.

Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Sekrachatakorn **Buabao**, B. Pharm.

Peradhama **Thiemthieprat**, B.S. (Chemistry)

Sorapetch **Marsud**, B.Sc., M.S.

Sakwichai **Ontong**, B.Sc. (Biology)

Nuchattra **Chansuvanich**, B.Sc. (Biology), M.S. (Env. Sci.)

Eurycoma longifolia Jack

Jiranuch **Mingmuang**, B. Pharm., M.Sc. in Pharm.

Somchit **Niumsakul**, B.Sc., M.Sc.

Sekrachatakorn **Buabao**, B. Pharm.

Sirikarn **Thana-ariyaroj**, B.S. (Chemistry)

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Prathom **Thongsrirak**, B.Sc. (Agriculture)

Sakwichai **Ontong**, B.Sc. (Biology)

Nuchattra **Chansuvanich**, B.Sc. (Biology), M.S. (Env. Sci.)

Gynostemma pentaphyllum (Thunb.) Makino

Warunee **Jirawattanapong**, B.S. Pharm., M.Sc. in Pharm., Ph.D.

Jaree **Bansiddhi**, B.Sc., M.Sc.

Yenchit **Techadamrongsin**, B.Sc., B.S. Phar.

Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Gynostemma pentaphyllum (Thunb.) Makino Dry Extract

Warunee **Jirawattanapong**, B.S. Pharm., M.Sc. in Pharm., Ph.D.

Thanawat **Thongchin**, B.S. (Science)

Nawarat **Chadchen**, B. Pharm.

Thidarat **Boonruad**, B.Sc., M.Sc. in Pharm.

Hyptis suaveolens (L.) Poit.

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Jaree **Bansiddhi**, B.Sc., M.Sc.

Thidarat **Boonruad**, B.Sc., M.Sc. in Pharm.

Yenchit **Techadamrongsin**, B.Sc., B.S. Phar.

Pranee **Chavalittumrong**, B.Sc. in Pharm., M.Sc. in Pharm.

Hyptis suaveolens (L.) Poit. Dry Extract

Apirak **Sakpetch**, B.S. Pharm.

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Atita **Pannanusorn**, B.Sc. in Pharm., M.Sc. in Pharm.

Jaree **Bansiddhi**, B.Sc., M.Sc.

Prathom **Thongsrirak**, B.Sc. (Agriculture)

Nongnuch **Maneechai**, B.Sc.

Kaempferia parviflora Wall. ex Baker Volatile Oil

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Narumole **Mongkholchaipak**, B. Pharm., M.Sc. (Biochem.)

Nuchattra **Chansuvanich**, B.Sc. (Biology), M.S. (Env. Sci.)

Yenchit **Techadamrongsin**, B.Sc., B.S. Phar.

Thidarat **Boonruad**, B.Sc., M.Sc. in Pharm.

Morinda citrifolia L.

Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Apirak **Sakpetch**, B.S. Pharm.

Sirikarn **Thana-ariyaroj**, B.S. (Chemistry)

Peradhama **Thiemthieprat**, B.S. (Chemistry)

Vilailuk **Chuennangchee**, B.Sc. (Chemistry)

Assama **Jiraputtarom**, B.Sc. (Veterinary Technology)

Sorapetch **Marsud**, B.Sc., M.S.

Sakwichai **Ontong**, B.Sc. (Biology)

Nuchattra **Chansuvanich**, B.Sc. (Biology), M.S. (Env. Sci.)

Portulaca grandiflora Hook.

Somchit **Niumsakul**, B.Sc., M.Sc.

Apirak **Sakpetch**, B.S. Pharm.

Jaree **Bansiddhi**, B.Sc., M.Sc.

Thidarat **Boonruad**, B.Sc., M.Sc. in Pharm.

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Pranee **Chavalittumrong**, B.Sc. in Pharm., M.Sc. in Pharm.

General Notices

General Notices

The information given in the general notices provides the basic guidelines for the interpretation and applications of the standards, tests and other specifications of the Thai Herbal Compendium on Physico-chemical Specifications.

Printing Types

In the text, words which refer to reagents, which conform to the requirements specified in the appendices of other parts of the book, and the systematic names of plants are usually printed in italics to distinguish them from the other words in that portion of the text.

Freshly and Recently Prepared

The direction that a preparation must be freshly prepared indicates that it must be made not more than 24 hours before it is issued for use. The direction that a preparation should be recently prepared indicates that deterioration is likely if the preparation is stored for longer than about 4 weeks at 15° to 25°.

Tests

When the solvent used for a solution is not named, the solvent is Purified Water.
In stating the appropriate quantities to be taken for quantitative tests, the use of the word "about" indicates a quantity within 10% of the specified weight or volume. However, the weight or volume taken is accurately determined, and the calculated result is based upon the exact amount taken. The same tolerance applies to specified dimensions.

Drying to Constant Weight

The specification "dried to constant weight" means that the drying shall be continued until two consecutive weighings do not differ by more than 2.5 mg per g of substance taken, the second weighing following an additional hour of drying at the prescribed conditions.

Ethanol

The term "ethanol" used without other indication means ethanol 95% v/v. Where other strengths are intended, the term "ethanol" is used followed by the statement of the strength.

Filtration

Where it is directed to "filter", without further qualification, the intent is that the liquid be filtered through suitable filter paper or equivalent device until the filtrate is clear.

Ignition to Constant Weight

The specification "ignite to constant weight" means that the ignition shall be continued until two consecutive weighings do not differ by more than 0.5 mg per g of substance taken, the second weighing following an additional 15-minute ignition period.

Percentage Expressions

Percentage concentrations are expressed as follows:

Percent weight in weight (w/w) expresses the number of g of a constituent in 100 g of solution or mixture.

Percent weight in volume (w/v) expresses the number of g of a constituent in 100 ml of solution, and is used regardless of whether water or another liquid is the solvent.

Percent volume in volume (v/v) expresses the number of ml of a constituent in 100 ml of solution.

The term "Percent" used without qualification means, for mixtures of solids and semisolids, percent weight in weight; for solutions or suspensions of solids in liquids, percent weight in volume; for solutions of liquids in liquids, percent volume in volume; and for solutions of gases in liquids, percent weight in volume. For example, a 1% solution is prepared by dissolving 1 g of a solid or semisolid, or 1 ml of a liquid, in sufficient solvent to make 100 ml of the solution.

Reagents

The proper conduct of the analytical procedures described in the Compendium and the reliability of the results depend, in part, upon the quality of the reagents used. The reagents, including the solutions required for the tests of the Compendium, are defined in the Appendices.

Solutions

Unless otherwise specified in the individual monograph, all solutions called for in tests are prepared with water.

An expression such as "(1 in 10)" means that 1 part by volume of a liquid is to be diluted with, or 1 part by weight of a solid is to be dissolved in, sufficient amount of the diluent or solvent to make the volume of the finished solution 10 parts by volume.

An expression such as "(20:5:2)" means that the respective numbers of parts, by volume, of the designated liquids are to be mixed, unless otherwise indicated.

Temperatures

Unless otherwise specified, all temperatures in this Compendium are expressed in Celsius degrees, and all measurements are made at 25°. Where "room temperature" is stated, a temperature from 20° to 30° is intended.

Water-Bath

Where the use of a water-bath is directed without qualification with respect to temperature, a bath of vigorously boiling water is intended.

Monographs

Aegle marmelos (L.) Corrêa

Thai Name มะตูม

Definition Dried mature fruits of *Aegle marmelos* (L.) Corrêa (Family Rutaceae). It contains not less than 0.2% w/w of imperatorin.

Synonyms *Belou marmelos* (L.) Lyons
Bilacus marmelos (L.) Kuntze
Crateva marmelos L.
Feronia pellucida Roth

Part Used Fruits

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 1 mg/ml of *imperatorin* in *methanol*

Sample solution: Reflux 1 g of the sample, in powder, with 20 ml of n-*hexane* for 20 min, filter, and concentrate to small volume (2 ml) by using a rotary evaporator.

Chromatographic system

Adsorbent: *Silica gel GF254*

Application volume: 5 µl of *Standard solution* and 20 µl of *Sample solution*

Developing solvent system: *Toluene* and *diethyl ether* (3:2)

Developing distance: 10 cm

Analysis

Samples: *Standard solution* and *Sample solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Sample*s as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Examine the plate under ultraviolet light (254 nm and 366 nm) (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in Hexane Extract of the Fruits of *Aegle marmelos* (L.) Corrêa

Spot	hR_f Value	Detection	
		UV 254	UV 366
1	7-8	quenching	yellow
2	14-18	–	violet
3	18-19	–	yellow
4	21-25	–	yellow
5	26-32	quenching	yellow
6	33-38	–	violet
7	38-43	quenching	–
8	43-48	quenching	violet
9	55-60	quenching	yellow
10*	63-70	quenching	yellow
11	72-74	–	violet
12	77-81	–	violet

*imperatorin

B. Colour reaction

• Reflux 500 mg of the sample, in powder, with 20 ml of *ethanol* for 10 min and filter (solution 1). Evaporate 2 ml of solution 1 to dryness. Dissolve the residue in 1 ml of *acetic anhydride* and then slowly add 1 ml of *sulfuric acid* to form two layers: a red-brown ring forms at the zone of contact and the upper layer is green.

• To 2 ml of solution 1, add a few drops of a 1% w/v solution of *iron(III) chloride* and shake well: a green colour is produced.

• To 2 ml of solution 1, add 2 or 3 pieces of *magnesium ribbon* and mix with a few drops of *hydrochloric acid*: a pink-orange colour develops.

• To 2 ml of solution 1, add a few drops of 0.02 M *potassium permanganate* and shake well: the colour of potassium permanganate solution is decolorized.

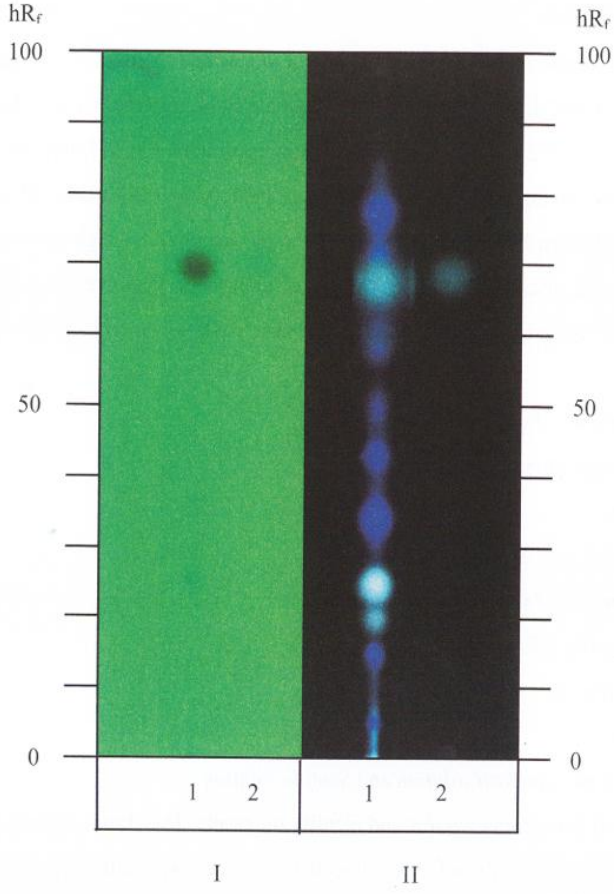


Fig. 1 Thin-layer Chromatogram of Hexane Extract of the Fruits of *Aegle marmelos* (L.) Corrêa

1 = sample solution

2 = standard solution

I = detection under UV light (254 nm)

II = detection under UV light (366 nm)

Content of imperatorin

Standard solution: Dissolve about 20 mg of *imperatorin*, accurately weighed, with sufficient *methanol* and dilute with *methanol* to obtain a stock solution having a known concentration of about 0.2 mg/ml. Dilute this solution quantitatively, and stepwise with *methanol* to obtain six solutions having known concentrations of 0.04, 0.08, 0.12, 0.16, 0.20 and 0.24 mg/ml.

Sample solution: Reflux about 1 g of the sample, accurately weighed, with 50 ml of n-*hexane* for 30 min, filter, and evaporate the filtrate to dryness. Dissolve the residue in sufficient *methanol*. Transfer quantitatively to a 25-ml volumetric flask, dilute with *methanol* to volume and mix.

Mobile phase: *Methanol* and *water* (60:40)

Chromatographic system

Mode: LC

Detector: UV 302 nm

Column: C18 (2.1×50 mm; 1.7 µm)

Flow rate: 0.5 ml/min

Injection size: 3 µl

Analysis

Samples: *Standard solution* and *Sample solution*

Record the chromatograms and identify the peaks. The chromatogram of the *Sample solution* exhibits the peak at the retention time of 1.2 min corresponding to the peak due to imperatorin (Fig. 2).

Acceptance criteria: Not less than 0.2% w/w of imperatorin.

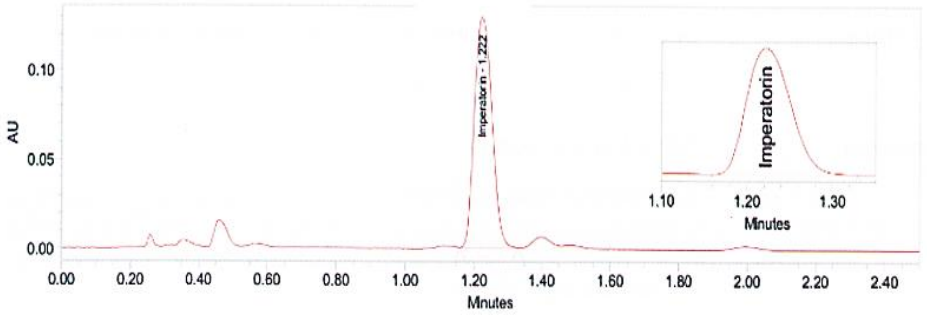


Fig. 2 LC chromatogram of *Aegle marmelos* (L.) Corrêa

Loss on drying Not more than 9.0% w/w after drying at 105° to constant weight (Appendix 3.5).

Acid insoluble ash Not more than 1.0% w/w (Appendix 4.3).

Total ash Not more than 3.0% w/w (Appendix 4.4).

Ethanol (50%)-soluble extractive Not less than 37.0% w/w (Appendix 4.5).

Ethanol-soluble extractive Not less than 14.0% w/w (Appendix 4.5).

Water-soluble extractive Not less than 45.0% w/w (Appendix 4.5).

Coriandrum sativum L.

Thai Name ผักชีลา

Definition Dried mature fruits of *Coriandrum sativum* L. (Family Apiaceae). It contains not less than 2.0% v/w of volatile oil.

Synonyms *Bifora loureiroi* Kostel.
Coriandropsis syriaca H.Wolff
Coriandrum globosum Salisb.
Coriandrum majus Gouan
Selinum coriandrum Krause

Part Used Fruits

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 5% v/v solution of *linalool* in *methanol*

Sample solution: Reflux 1 g of the sample, in powder, with 10 ml of *methanol* for 20 min, filter, and concentrate to small volume (2 ml) by using a rotary evaporator.

Chromatographic system

Adsorbent: *Silica gel G*

Application volume: 1 µl of *Standard solution* and 10 µl of *Sample solution*

Developing solvent system: *Toluene* and *ethyl acetate* (93:7)

Developing distance: 10 cm

Spray reagent: *Anisaldehyde TS*

Analysis

Samples: *Standard solution* and *Sample solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Samples* as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Spray the plate with *Spray reagent*, heat at 105-110° for 5 min, and immediately examine the plate under daylight (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in Methanolic Extract of the Fruits of *Coriandrum sativum* L.

Spot	hR_f Value	Detection
		Anisaldehyde TS
1	0-30	purple
2*	35-40	purple
3	65-70	violet
4	75-80	violet

*linalool

B. Colour reaction

Macerate 500 mg of the sample, in powder, with 5 ml of *ethanol* for 1 hr and filter. Evaporate the filtrate to dryness, dissolve the residue in 1 ml of *acetic anhydride* and then slowly add 1 ml of *sulfuric acid* to form two layers: a red-brown ring forms at the zone of contact and the upper layer is green.

Volatile oil Not less than 2.0% v/w (Appendix 4.2). Use 50 g, in *coarse powder*, freshly prepared and accurately weighed. Use 200 ml of *water* as the distillation liquid and a 500-ml round-bottomed flask. Distil at a rate of 2 to 3 ml/min for 4 hr. Use 2.0 ml of *xylene* in the graduated tube.

Water Not more than 8.0% v/w, using 20 g of the sample (Azeotropic Distillation Method, Appendix 3.3).

Acid-insoluble ash Not more than 1.0% w/w (Appendix 4.3).

Total ash Not more than 8.0% w/w (Appendix 4.4).

Ethanol-soluble extractive Not less than 17.0% w/w (Appendix 4.5).

Water-soluble extractive Not less than 18.0% w/w (Appendix 4.5).

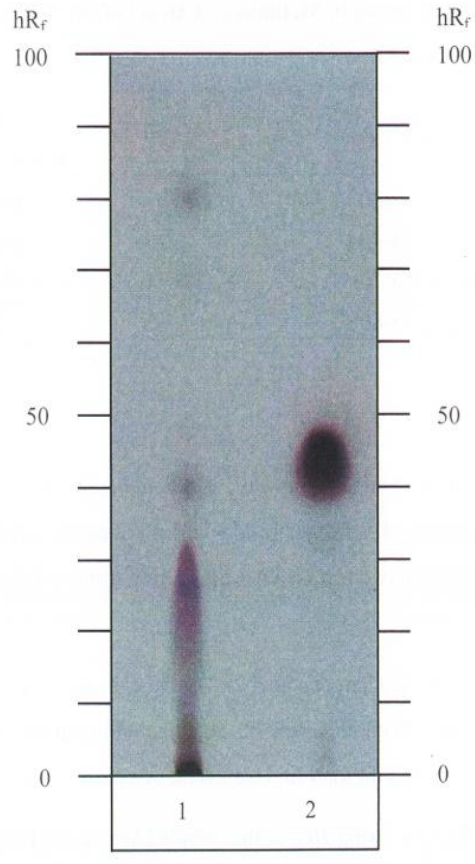


Fig. 1 Thin-layer Chromatogram of Methanolic Extract of the Fruits of *Coriandrum sativum* L., detected with *Anisaldehyde TS*

1 = sample solution

2 = standard solution

Eurycoma longifolia Jack

Thai Name ปลาไหลเผือก

Definition Dried roots of *Eurycoma longifolia* Jack (Family Simaroubaceae).

Synonyms *Eurycoma latifolia* Ridl.

Eurycoma merguensis Planch.

Part Used Roots

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 0.5 mg/ml of *eurycomanone* in *ethanol*

Sample solution: Reflux 1 g of the sample, in powder, with 20 ml of *ethanol* for 15 min and filter. Evaporate the filtrate to dryness under reduced pressure at 40° and add 2 ml of *ethanol* to dissolve the residue.

Chromatographic system

Adsorbent: *Silica gel GF254*

Application volume: each of 5 µl of *Standard Solution* and *Sample solution*

Developing solvent system: n-*Hexane*, *acetone*, and *ethyl acetate* (50:45:5)

Developing distance: 10 cm

Spray reagent: *Anisaldehyde TS*

Analysis

Samples: *Standard Solution* and *Sample Solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Samples* as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Examine the plate under ultraviolet light (254 nm and 366 nm). Subsequently, spray the plate with *Spray reagent*, then heat at 105° for 5 min and immediately examine the plate under daylight (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in Ethanolic Extract of the Roots of *Eurycoma longifolia* Jack

Spot	hR_f Value	Detection		
		UV 254	UV 366	*Anisaldehyde TS*
1	10-12	quenching	–	purple
2	20-24	quenching	–	green
3	36-38	quenching	–	brown
4	46-48	quenching	–	–
5	63-65	–	blue	–
6	75-77	quenching	blue	–
7	80-81	quenching	blue	–
8*	85-86	quenching	–	purple
9	87-88	quenching	blue	purple
10	91-93	quenching	yellow	purple
11	92-97	quenching	blue	purple
12	97-98	quenching	blue	purple

*eurycomanone

B. Colour reaction

• Reflux 1 g of the sample, in powder, with 10 ml of *ethanol* on a water-bath for 15 min and filter (solution 1). Evaporate 2 ml of solution 1 to dryness under reduced pressure. Dissolve the residue in 2 ml of *acetic anhydride*, slowly add 1 ml of *sulfuric acid*: a brownish red ring develops

• To 2 ml of solution 1, add 1 or 2 pieces of *magnesium ribbon* and a few drops of *hydrochloric acid,* warm in a water-bath: a pink-orange colour is produced.

• To 2 ml of solution 1, add a few drops of a 2% w/v solution of *3,5-dinitrobenzoic acid* in *methanol*: a brownish purple colour is produced.

Loss on drying Not more than 8.0% w/w after drying at 105° to constant weight (Appendix 3.5).

Foreign matter Not more than 2.0% w/w (Appendix 4.1).

Acid-insoluble ash Not more than 2.0% w/w (Appendix 4.3).

Total ash Not more than 4.0% w/w (Appendix 4.4).

Ethanol-soluble extractive Not less than 2.0% w/w (Appendix 4.5).

Water-soluble extractive Not less than 7.0% w/w (Appendix 4.5).

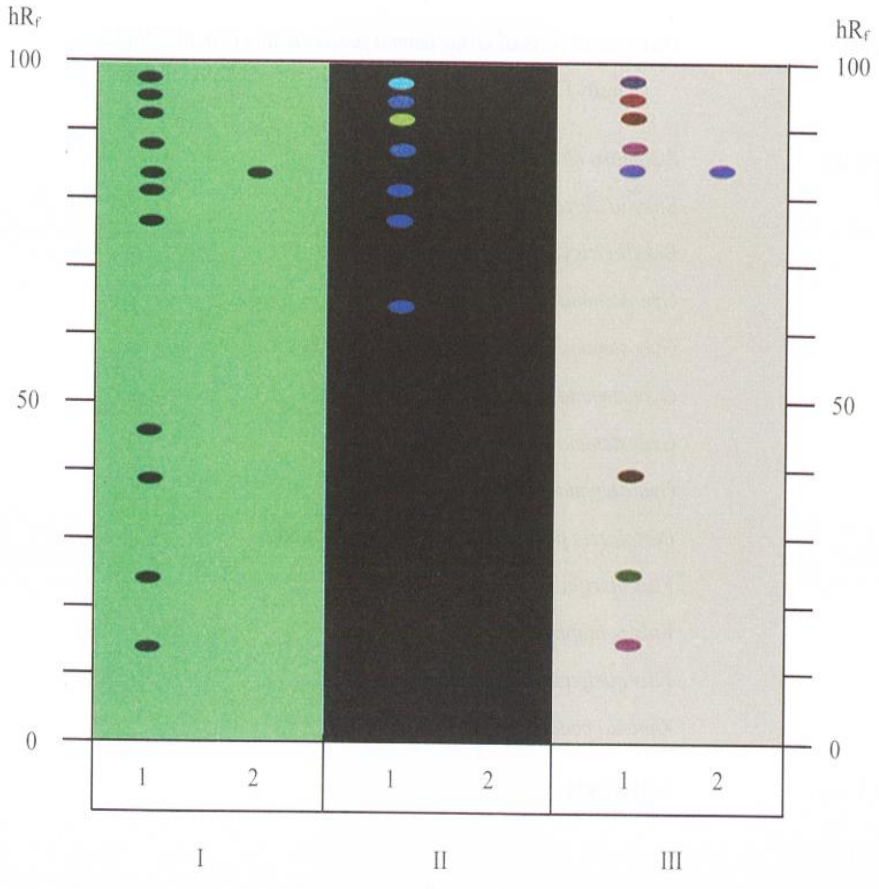


Fig. 1 Thin-layer Chromatogram of Ethanolic Extract of the Roots of *Eurycoma longifolia* Jack

1 = sample solution

2 = standard solution

I = detection under UV light (254 nm)

II = detection under UV light (366 nm)

III = detection with *anisaldehyde TS*

Gynostemma pentaphyllum (Thunb.) Makino

Thai Name ปัญจขันธ์

Definition Dried aerial parts of *Gynostemma pentaphyllum* (Thunb.) Makino (Family Cucurbitaceae).

Synonyms

Alsomitra cissoides M.Roem.

Enkylia digyna Griff.

Enkylia trigyna Griff.

Gynostemma blumei Hassk.

Gynostemma cissoides Benth. & Hook.f.

Gynostemma pedatum Blume

Gynostemma siamicum Craib

Gynostemma trigynum M.A.Lawson

Pestalozzia pedata (Blume) Zoll. & Moritzi

Vitis martini H. Lév. & Vaniot

Vitis pentaphylla Thunb.

Vitis quelpaertensis H. Lév.

Zanonia pedata (Blume) Miq.

Part Used Aerial parts

Identification

A. Thin-layer Chromatography Identification Test

Sample solution: Reflux 1 g of the sample, in powder, with 50 ml of *water* for 2 hr, filter, wash with hot *water*, then transfer to a 100-ml volumetric flask, and adjust to volume. Transfer 10 ml of the solution to a separatory funnel, add 15 ml of *water* and extract the mixture with three 10-ml portions of 1-*butanol*. Evaporate the butanol extract to dryness, then dissolve the residue in 1 ml of *methanol*.

Chromatographic system

Adsorbent: *Silica gel G*

Application volume: 10 µl of *Sample solution*

Developing solvent system: *Chloroform*, *methanol*, and *water* (65:35:10, lower phase)

Developing distance: 10 cm

Spray reagent: 20% v/v solution of *sulfuric acid* in *methanol*

Analysis

Sample: *Sample solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Sample* as spot to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Spray the plate with *Spray reagent*, heat at 105° for 5 min, and immediately examine the plate under daylight (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in the Extract of the Aerial Parts of *Gynostemma pentaphyllum* (Thunb.) Makino

Spot	hR_f Value	Detection
		20% v/v Solution of *Sulfuric Acid* in *Methanol*
1	11-12	violet
2	14-15	violet
3	17-18	deep violet
4	20-21	deep violet
5	25-26	violet
6	28-29	violet
7	31-32	violet
8	34-35	violet
9	39-41	deep violet
10	43-44	violet
11	47-48	violet
12	53-54	violet
13	58-59	violet
14	67-69	violet

B. Colour reaction

• To 500 mg of the sample, in powder, add 10 ml of *water*, heat on a water-bath for 15 min, and filter. Transfer the filtrate to a separatory funnel and extract with the equal volume of 1-*butanol*. Add 100 mg of *decolorizing charcoal* to the butanol layer, stir, and filter (solution 1). Evaporate 2 ml of solution to dryness, add dropwise a saturated solution of *antimony trichloride* in *chloroform*: a violet colour is produced.

• Evaporate 2 ml of solution 1 to dryness, add a few drops of *sulfuric acid*: a red colour is produced.

• To 500 mg of the sample, in powder, add 10 ml of *water*, heat on a water-bath for 15 min, and filter. Transfer 1 ml of the filtrate to a stoppered test-tube and shake for a while: the persisting foam for over 30 min is produced.

Content of total crude saponins

Analysis

Accurately weigh about 500 mg of the sample, in *No.180 powder*, place in a 250-ml round-bottomed flask, then reflux with 50 ml of *water* for 2 hr, and filter. Wash the marc with a proper volume of hot *water*. Combine the washing and the filtrate, transfer to a 100-ml volumetric flask and adjust to volume. Transfer 20 ml of this solution to a separatory funnel and extract with three 10-ml portions of 1-*butanol*. Combine the butanol extracts and wash with two 10-ml portions of *water*. After evaporating the extract to dryness, dry the residue to constant weight at 105°. Calculate the total crude saponins content on the water free basis.

Acceptance criteria: Not less than 8.0% w/w.

Content of total gypenosides

Analysis

Accurately weigh about 500 mg of the sample, in *No.180 powder*, place in a 250-ml round-bottomed flask, then reflux with 50 ml of *water* for 2 hr, and filter. Wash the marc with a proper volume of hot *water*. Combine the washing and the filtrate, transfer to 100-ml volumetric flask and adjust to volume. Transfer 20 ml of this solution to a separatory funnel and extract with three 10-ml portions of 1-*butanol*. Combine the butanol extracts and wash with two 10-ml portions of *water*. After evaporating the extract to dryness, dissolve the residue in *water* and make up to volume of 10.0 ml. Apply 5 ml of the resulting solution to Sep-Pak C-18, then wash with 10 ml of *water*, 5 ml of 50% v/v solution of *methanol*, 2 ml of a 60% v/v solution of *methanol,* and elute with 2 ml of *absolute methanol*, respectively. Evaporate the eluate to dryness and dry to constant weight at 105°. Calculate the total gypenosides content on the water free basis.

Acceptance criteria: Not less than 4.0% w/w.

Foaming index Not less than 242 (Appendix 3.1).

Water Not more than 8.0% v/w (Azeotropic Distillation Method, Appendix 3.3).

Acid-insoluble ash Not more than 2.0% w/w (Appendix 4.3).

Total ash Not more than 14.0% w/w (Appendix 4.4).

Ethanol-soluble extractive Not less than 9.0% w/w (Appendix 4.5).

Water-soluble extractive Not less than 21.0% w/w (Appendix 4.5).

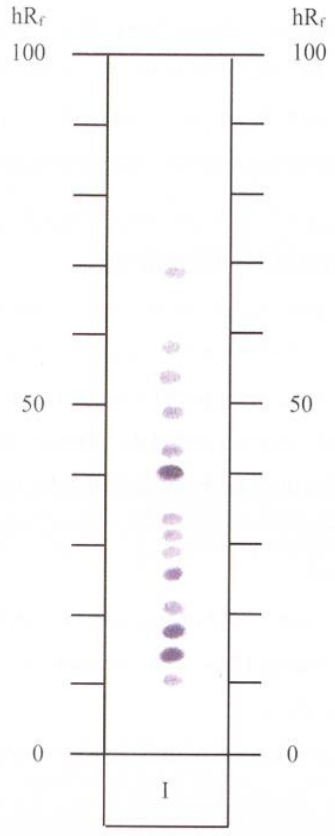


Fig. 1 Thin-layer Chromatogram of the Extract of the Aerial Parts of *Gynostemma pentaphyllum* (Thunb.) Makino

I = detection with 20% v/v solution of *sulfuric acid* in *methanol*

Gynostemma pentaphyllum (Thunb.) Makino Dry Extract

Thai Name สารสกัดปัญจขันธ์

Definition *Gynostemma pentaphyllum* (Thunb.) Makino Dry Extract is prepared from dried aerial parts of *Gynostemma pentaphyllum* (Thunb.) Makino by extraction with *water*, concentration under reduced pressure, and using a freeze-dried apparatus until dryness.

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 1 mg/ml of *Ginsenoside* Rb_1 in *methanol*.

Sample solution: Dissolve 500 mg of the extract, in powder, with 50 ml of *water*, then transfer to a 100-ml volumetric flask, and adjust to volume. Transfer 10 ml of the solution to a separatory funnel, add 15 ml of *water* and extract the mixture with three 15-ml portions of 1-*butanol*. Evaporate the butanol extract to dryness and dissolve the residue in 1 ml of *methanol*.

Chromatographic system

Adsorbent: *Silica gel G*

Application volume: each of 5 µl of *Standard solution* and *Sample solution*

Developing solvent system: *Chloroform*, *methanol*, and *water* (65:35:10, lower phase)

Developing distance: 10 cm

Spray reagent: 20% v/v solution of *sulfuric acid* in *methanol*

Analysis

Samples: *Standard solution* and *Sample solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Samples* as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Spray the plate with *Spray reagent*, heat at 105° for 5 min, and immediately examine the plate under daylight (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in the Extract of *Gynostemma pentaphyllum* (Thunb.) Makino Dry Extract

Spot	hR_f Value	Detection
		20% v/v Solution of *Sulfuric Acid* in *Methanol*
1	10-11	violet
2	13-14	violet
3	17-19	deep violet
4*	21-23	deep violet
5	23-24	magenta
6	26-28	magenta
7	29-31	violet
8	34-36	deep violet
9	36-37	violet
10	37-39	yellow
11	39-41	violet
12	42-46	deep violet
13	49-50	magenta
14	50-52	yellow
15	56-57	violet
16	58-59	violet
17	61-62	violet
18	64-65	violet
19	69-71	violet
20	77-78	violet
21	81-84	violet
22	91-92	violet
23	95-96	violet
24	98-99	green

*ginsenoside Rb_1

B. Colour reaction

• To 200 mg of the sample, in powder, add 10 ml of *water*, heat on a water-bath for 15 min, then transfer the filtrate to a separatory funnel, and extract with the equal volume of 1-*butanol*. If necessary, add 100 mg of *decolorizing charcoal* to the butanol layer, stir, and filter (solution 1). Evaporate 2 ml of solution 1 to dryness, add dropwise a saturated solution of *antimony trichloride* in *chloroform*: a violet colour is produced.

- Evaporate 2 ml of solution 1 to dryness, add a few drops of *sulfuric acid*: red colour is produced.

- To 200 mg of the sample, in powder, add 10 ml of *water* and heat on a water-bath for 15 min. Transfer 1 ml of the solution to a stoppered test-tube and shake for a while: the persisting foam for over 30 min is produced.

Content of total crude saponins

Analysis

Accurately weigh about 400 mg of the extract, in powder, dissolve in 50 ml of *water*, transfer to a 100-ml volumetric flask, and adjust to volume. Transfer 10 ml of this solution to a separatory funnel, add 10 ml of *water* and extract with three 15-ml portions of *1-butanol*. Combine the butanol extracts and wash with two 10-ml portions of *water*. After evaporating the extract to dryness, dry the residue to constant weight at 105°. Calculate the total crude saponins content on the water free basis.

Acceptance criteria: Not less than 22.0% w/w.

Loss on drying Not more than 9.0% w/w (Appendix 3.5).

Acid-insoluble ash Not more than 1.0% w/w (Appendix 4.3).

Total ash Not more than 18.0% w/w (Appendix 4.4).

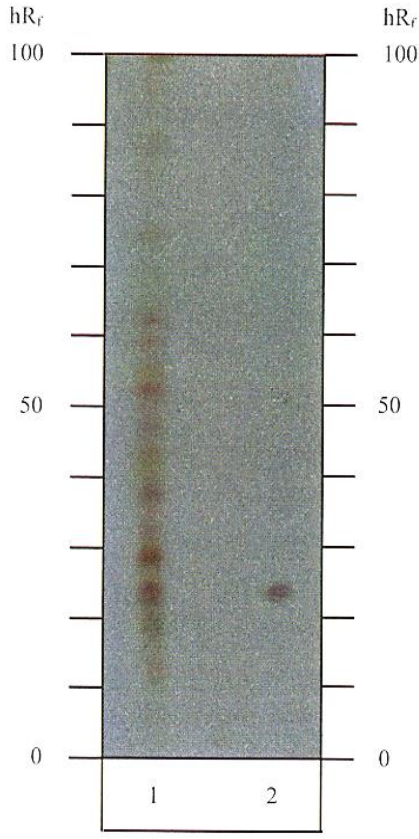


Fig. 1 Thin-layer Chromatogram of the Extract of *Gynostemma pentaphyllum* (Thunb.) Makino Dry Extract, detected with 20% v/v Solution of *Sulfuric Acid* in *Methanol*

1 = sample solution

2 = standard solution

Hyptis suaveolens (L.) Poit.

Thai Name แมงลักคา

Definition Dried aerial parts of *Hyptis suaveolens* (L.) Poit. (Family Lamiaceae-Labiatae)

Synonyms

Ballota suaveolens L.

Bystropogon graveolens Blume

Bystropogon suaveolens (L.) L'Hér.

Gnoteris cordata Raf.

Gnoteris villosa Raf.

Hyptis congesta Leonard

Hyptis ebracteata R.Br.

Hyptis graveolens Schrank

Hyptis plumieri Poit.

Marrubium indicum Blanco

Mesosphaerum suaveolens (L.) Kuntze

Schaueria graveolens (Blume) Hassk.

Part Used Aerial parts

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 2 mg/ml of *ursolic acid* in *methanol*

Sample solution: Reflux 1 g of the sample, in powder, in 20 ml of *methanol* for 30 min and immediately filter. Evaporate the filtrate to dryness under reduced pressure and add 10 ml of *methanol* to dissolve the residue.

Chromatographic system

Adsorbent: *Silica gel G*

Application volume: 2 µl of *Standard solution* and 5 µl of *Sample solution*

Developing solvent system: *Dichloromethane*, *ethyl acetate* and *water* (70:30:1)

Developing distance: 12 cm

Spray reagent: 20% v/v solution of *sulfuric acid* in *ethanol*

Analysis

Samples: *Standard solution* and *Sample solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Samples* as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Spray the plate with *Spray reagent*, heat at 105° for 5 min, and immediately examine the plate under daylight (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in Methanolic Extract of the Aerial Parts of *Hyptis suaveolens* (L.) Poit.

Spot	hR_f Value	Detection
		20% v/v Solution of *Sulfuric Acid* in *Ethanol*
1	3-4	violet
2	13-14	brown
3	26-27	violet
4	33-36	violet
5	38-40	brown
6	46-48	brown
7	49-50	brown
8	51-52	brown
9*	73-74	red
10	76-77	brown
11	88	violet
12	89-90	violet
13	97-98	green
14	99	violet

*ursolic acid

B. Colour Reaction

• Reflux 500 mg of the sample, in powder, with 10 ml of *methanol* on a water-bath for 15 min and filter. Add 300 mg of *decolorizing charcoal* in the filtrate and filter. Evaporate the filtrate to dryness under reduced pressure. Dissolve the residue in 1 ml of *acetic anhydride*, slowly add 1 ml of *sulfuric acid*: a pinkish red colour ring develops.

• Reflux 2 g of the sample, in powder, with 20 ml of *water* for 30 min, and filter. To 2 ml of the filtrate, add a few drops of a freshly prepared 1% w/v solution of *iron(III) chloride*: a greenish blue colour is produced.

Loss on drying Not more than 9.0% w/w after drying at 105° to constant weight (Appendix 3.5).

Foreign matter Not more than 2.0% w/w (Appendix 4.1).

Acid-insoluble ash Not more than 2.0% w/w (Appendix 4.3).

Total ash Not more than 10.0% w/w (Appendix 4.4).

Ethanol-soluble extractive Not less than 4.0% w/w (Appendix 4.5).

Water-soluble extractive Not less than 7.0% w/w (Appendix 4.5).

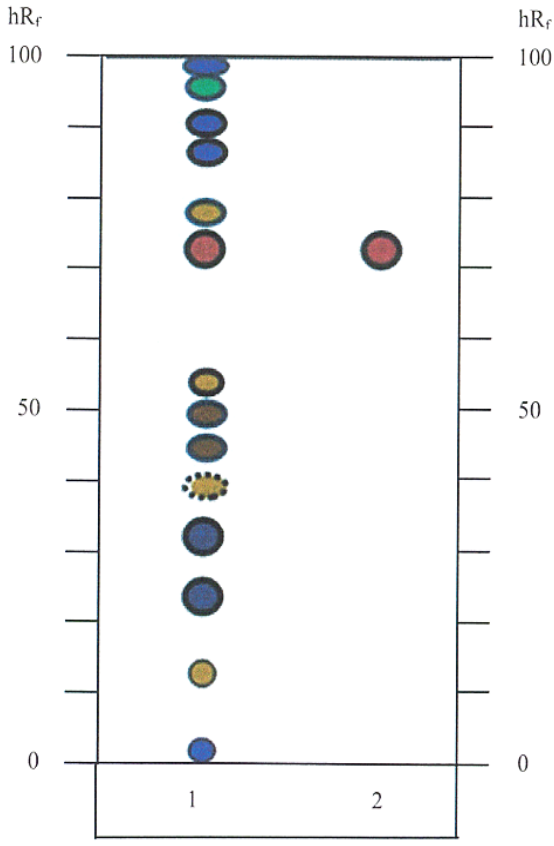


Fig. 1 Thin-layer Chromatogram of Methanolic Extract of the Aerial Parts of *Hyptis suaveolens* (L.) Poit., detected with 20% v/v Solution of *Sulfuric Acid* in *Ethanol*

1 = sample solution

2 = standard solution

◌ = spot developed in some samples

Hyptis suaveolens (L.) Poit. Dry Extract

Thai Name สารสกัดแมงลักคา

Definition *Hyptis suaveolens* (L.) Poit. Dry Extract is prepared from the dried aerial parts of *Hyptis suaveolens* (L.) Poit by extraction with *water*, concentration under reduced pressure, and using a freeze-dried or a spray-dried apparatus until dryness. The ratio of plant material to extract is between 6:1 and 11:1.

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 1 mg/ml of *caffeic acid* in *methanol*

Sample solution: Shake 100 mg of the sample, in powder, in 2 ml of a 50% v/v solution of *methanol* and filter.

Chromatographic system

Adsorbent: *Silica gel GF254* (HPTLC plates)

Application volume: 2 µl of *Standard solution* and 5 µl of *Sample solution*

Developing solvent system: *Dichloromethane*, *ethyl acetate*, and *formic acid* (24:6:1)

Developing distance: 6 cm

Spray reagent: 5% v/v solution of *sulfuric acid* in *ethanol*

Analysis

Samples: *Standard solution* and *Sample Solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Samples* as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Examine the plate under ultraviolet light (254 nm and 366 nm). Subsequently, spray the plate with *Spray reagent*, heat at 105° for 5 min, and immediately examine the plate under daylight and ultraviolet light at 366 nm (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in Methanolic Extract of *Hyptis suaveolens* (L.) Poit. Dry Extract

Spot	hR_f Value	Detection			
		UV 254	UV 366	5% v/v Solution of *Sulfuric Acid* in *Ethanol*	
				Daylight	UV 366
1	24-28	–	–	brown	red
2*	45-50	quenching	blue	violet	blue
3	64-68	quenching	blue	violet	blue

*caffeic acid

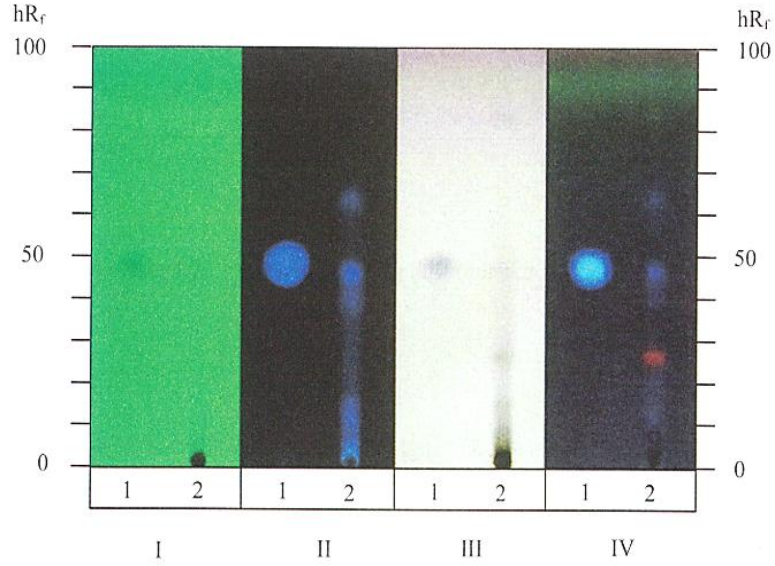


Fig. 1 Thin-layer Chromatogram of Methanolic Extract of *Hyptis suaveolens* (L.) Poit. Dry Extract

1 = standard solution

2 = sample solution

I = detection under UV light (254 nm)

II = detection under UV light (366 nm)

III = detection under daylight after spraying with 5% v/v solution of *sulfuric acid* in *ethanol*

IV = detection under UV 366 after spraying with 5% v/v solution of *sulfuric acid* in *ethanol*

B. High-pressure Liquid Chromatography Identification Test

Standard solution (A): 1 mg/ml of *rosmarinic acid* in *methanol*

Standard solution (B): 1 mg/ml of *caffeic acid* in *methanol*

Sample solution: Sonicate 500 mg of the sample, accurately weighed, in 20 ml of *water* for 30 min and filter. Adjust the filtrate to 25.0 ml with *water* and filter with a 0.45 µm nylon filter.

Mobile phase: 1% v/v solution of *glacial acetic acid*, *methanol*, and *water* (See Table 2)

Table 2

Time (min)	*Glacial acetic acid* (1% v/v)	*Methanol*	*Water*
0	80	20	0
10	70	30	0
15	50	40	10
35	50	30	20
40	80	20	0

Chromatographic system

Detector: UV 324 nm

Column: C18 (4.6×150 mm; 5 µm) with guard column (3.9 × 20 mm; 5 µm)

Flow rate: 1.0 ml/min

Injection size: 10 µl

Analysis

Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 2.3). Record the chromatograms and identify the peaks. The chromatogram of the *Sample solution* exhibits the peaks at the retention time of 13.8 and 28.0 min corresponding to the peaks due to caffeic acid and rosmarinic acid, respectively (Fig. 2).

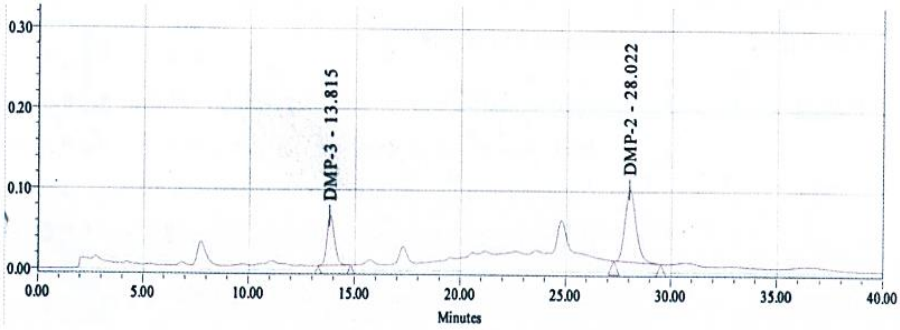


Fig. 2 HPLC chromatogram of *Hyptis suaveolens* (L.) Poit Dry Extract

(Note: DMP-2 = rosmarinic acid; DMP-3 = caffeic acid)

C. Colour Reaction

Sonicate 100 mg of the sample with 20 ml of *water* for 30 min and filter. To 2 ml of the filtrate, add a few drops of a freshly prepared a 1% w/v solution of *iron(III) chloride*: a greenish blue colour is produced.

Loss on drying Not more than 9.0% w/w after drying at 105° to constant weight, using 1.0 g of the sample (Appendix 3.5).

Ethanol-soluble extractive Not less than 14.0% w/w, using 1.0 g of the sample (Appendix 4.5).

Water-soluble extractive Not less than 85.0% w/w, using 1.0 g of the sample (Appendix 4.5).

Kaempferia parviflora Wall. ex Baker Volatile Oil

Thai Name น้ำมันระเหยง่ายกระชายดำ

Definition *Kaempferia parviflora* Wall. ex Baker Volatile Oil is prepared from the dried rhizomes of *Kaempferia parviflora* Wall. ex Baker (Family Zingiberaceae) by distillation with *water* in a clevenger apparatus. The ratio of plant material to extract is between 500:1 and 2,000:1.

Synonyms *Kaempferia rubromarginata* (S.Q.Tong) R.J.Searle

Stahlianthus rubromarginatus S.Q.Tong

Part Used Rhizomes

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 4 mg/ml of (+)-*borneol* in *ethyl acetate*

Sample solution: 5% v/v solution of the volatile oil in *ethanol*

Chromatographic system

Adsorbent: *Silica gel G*

Application volume: each of 1 µl of *Standard solution* and *Sample solution*

Developing solvent system: *Hexane*, *ethyl acetate*, and *glacial acetic acid* (90:10:1)

Developing distance: 15 cm

Spray reagent: *Vanillin-phosphoric acid TS*

Analysis

Samples: *Standard solution* and *Sample Solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Samples* as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Spray the plate with freshly prepared *Spray reagent*. Then heat at 120° for 10 min and immediately examine the plate under daylight and under ultraviolet light (366 nm) (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components of the Volatile Oil of the Rhizomes of *Kaempferia parviflora* Wall. ex Baker in *Ethanol*

Spot	hR_f Value	Detection	
		Vanillin-Phosphoric Acid TS and Daylight	*Vanillin-Phosphoric Acid TS* and UV 366
1	9-12	violet	–
2	21-23	–	blue
3	26-28	blue violet	orange
4*	31-34	brown violet	yellow
5	41-43	wine red	orange
6	69-72	violet	–
7	71-77	yellow	–
8	80-83	–	yellow
9	92-96	red violet	–

*borneol

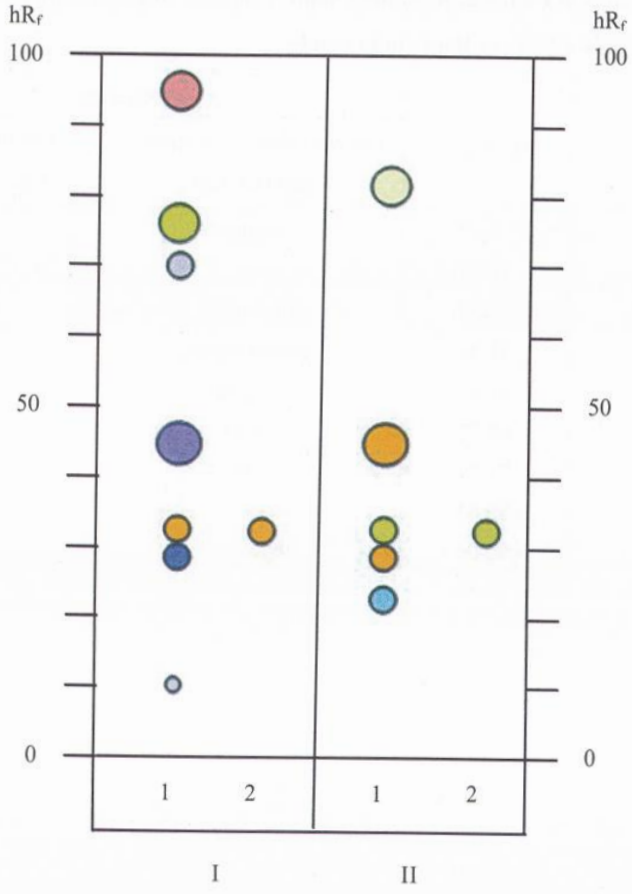


Fig. 1 Thin-layer Chromatogram of the Volatile Oil of the Rhizomes of *Kaempferia parviflora* Wall. ex Baker in *Ethanol*

1 = sample solution

2 = standard solution

I = detection under daylight after spraying with *vanillin-phosphoric acid TS*

II = detection under UV 366 after spraying with *vanillin-phosphoric acid TS*

B. Gas Chromatography Identification Test

Sample solution: 3.3% v/v solution of the volatile oil in *hexane*.

Chromatographic system

Detector: Mass Spectrometer

Column: DB-1 (30 m×0.25 mm×0.25 μm)

Oven temperature program: 50° for 3 min, 100° for 1 min (10.0° /min), 150° for 1 min (2.5° /min), 200° for 3 min (15.0°/min).

Injector temperature: 200°

Injection size: 0.1 μl

Split ratio: 100:1

Analysis

Carry out the test as described in the "Gas Chromatography" (Appendix 2.2). The chromatogram of the *Sample solution* exhibits the major peaks at the retention time of 9.51 and 11.08 min corresponding to the peaks due to linalool and borneol, respectively, using NIST library (Table 2); see also Fig. 2.

Table 2 Components of the Volatile Oil of the Rhizomes of *Kaempferia parviflora* Wall. ex Baker in *Hexane*

No.	Components	Retention Time (min)	Relative Area (%)
1	β-pinene	7.230	1.91
2	eucalyptol	8.143	4.92
3	linalool oxide	8.915	5.13
4	linalool oxide	9.225	5.03
5	linalool	9.513	24.81
6	camphor	10.327	3.95
7	borneol	11.084	32.60

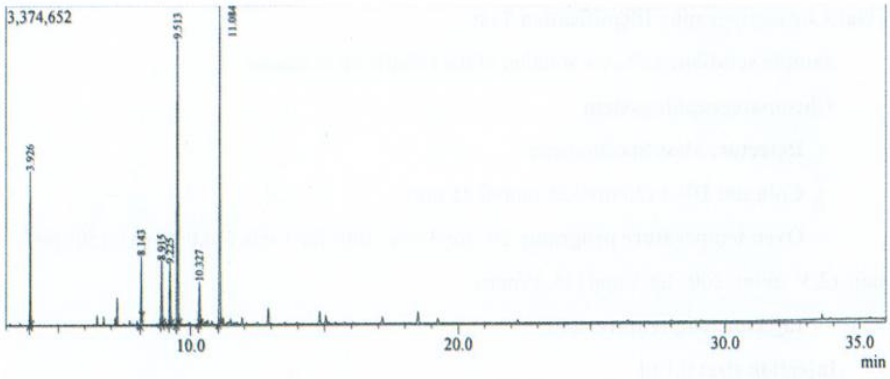


Fig. 2 GC-MS Chromatogram of the Volatile oil of the Rhizomes of *Kaempferia parviflora* Wall. ex Baker

C. Colour reaction

To one drop of the sample, add 0.5 ml of *ethanol* and mix well. Add a few drops of a freshly prepared *vanillin-sulfuric acid TS*: a purple colour is produced.

Solubility It is very soluble in *chloroform*, *ethanol*, *ethyl acetate*, and *hexane*. It is slightly soluble in *water*.

Refractive index 1.471 to 1.476, determined at 20° (Appendix 3.2).

Relative density 0.979 to 0.984, determined at 20° (Appendix 3.4).

Morinda citrifolia L.

Thai Name ยอ

Definition Dried unripe mature fruits of *Morinda citrifolia* L. (Family Rubiaceae). It contains not less than 40 mg% w/w of scopoletin.

Synonym *Morinda bracteata* Roxb.

Part used Fruits

Identification

A. Thin-layer Chromatography Identification Test

Standard solution: 0.5 mg/ml of *scopoletin* in *methanol*

Sample solution: Reflux 500 mg of the sample, in powder, with 20 ml of *methanol* for 20 min, filter, and concentrate to small volume (2 ml) by using a rotary evaporator.

Chromatographic system

Adsorbent: *Silica gel GF254*

Application volume: 2 µl of *Standard solution* and 10 µl of *Sample solution*

Developing solvent system: *Toluene* and *diethyl ether* (2:3)

Developing distance: 10 cm

Spray reagent: *Anisaldehyde TS*

Analysis

Samples: *Standard solution* and *Sample solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Samples* as spots to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Examine the plate under ultraviolet light (254 nm and 366 nm). Subsequently spray the plate with *Spray regent*, heat at 105-110° for 5 min, and immediately examine the plate under daylight and ultraviolet light at 366 nm (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components in Methanolic Extract of the Fruits of *Morinda citrifolia* L.

Spot	hR_f Value	Detection			
		UV 254	UV 366	*Anisaldehyde TS*	
				Daylight	UV 366
1*	28-34	blue	blue	–	blue
2	60-69	–	–	violet	yellow
3	70-80	–	–	red	red
4	83-87	–	–	red	red
5	92-95	–	–	grey	grey
6	97-100	–	–	red	red

*scopoletin

B. Colour reaction

• Macerate 500 mg of the sample, in powder, in 10 ml of *ethanol* for 1 hr and filter (solution 1). Observe the solution 1 under ultraviolet light (366 nm): a blue fluorescence appears.

• Evaporate 2 ml of solution 1 to dryness. Dissolve the residue in 1 ml of *acetic anhydride* and then slowly add 1 ml of *sulfuric acid* to form two layers: a red-brown ring forms at the zone of contact and the upper layer is green.

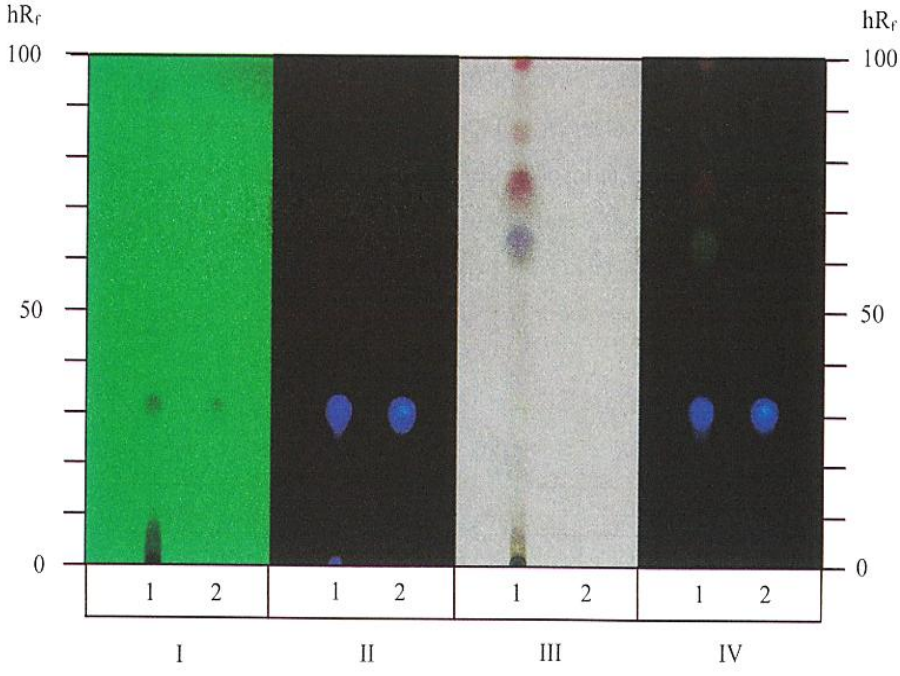


Fig. 1 Thin-layer Chromatogram of Methanolic Extract of the Fruits of *Morinda citrifolia* L.

1 = sample solution

2 = standard solution

I = detection under UV light (254 nm)

II = detection under UV light (366 nm)

III = detection under daylight after spraying with *anisaldehyde TS*

IV = detection under UV 366 after spraying with *anisaldehyde TS*

Content of scopoletin

Standard solution: Dissolve about 20 mg of *scopoletin*, accurately weighed, with sufficient *methanol* and dilute with *methanol* to obtain a stock solution having a known concentration of about 0.2 mg/ml. Dilute this solution quantitatively and stepwise with *methanol* to obtain six solutions having known concentrations of 0.008, 0.016, 0.024, 0.032, 0.040 and 0.048 mg/ml.

Sample solution: Reflux about 1 g of the sample, accurately weighed, with 30 ml of *methanol* for 30 min, filter, and evaporate the filtrate to reduced volume. Transfer quantitatively to a 25-ml volumetric flask, dilute with *methanol* to volume and mix.

Solution A: *Methanol* and *acetonitrile* (1:1)

Solution B: *water*

Mobile phase: See Table 2

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	30	70
1.50	35	65
1.70	30	70

Chromatographic system

Mode: LC

Detector: UV 345 nm

Column: C18 (2.1×100 mm; 1.7 µm)

Flow rate: 0.45 ml/min

Injection size: 3 µl

Analysis

Samples: *Standard solution* and *Sample solution*

Record the chromatograms and identify the peaks. The chromatogram of the *Sample solution* exhibits peak at the retention time of 1.09 min corresponding to the peak due to scopoletin (Fig. 2).

Acceptance criteria: Not less than 40 mg% w/w of scopoletin.

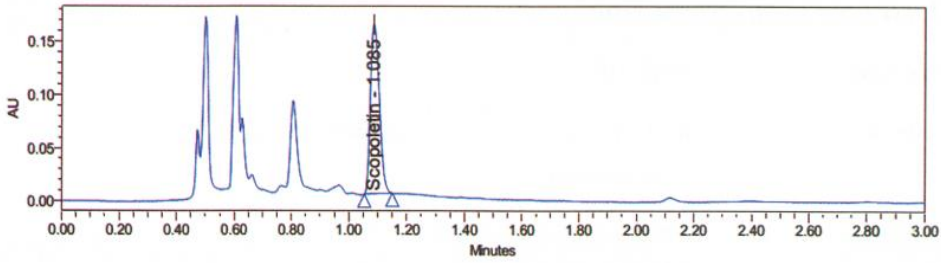


Fig. 2 LC chromatogram of *Morinda citrifolia* L.

Loss on drying Not more than 9.0 % w/w after drying at 105° to constant weight (Appendix 3.5).

Acid insoluble ash Not more than 1.0% w/w (Appendix 4.3).

Total ash Not more than 8.0% w/w (Appendix 4.4).

Ethanol (50%)-soluble extractive Not less than 28.0% w/w (Appendix 4.5).

Ethanol-soluble extractive Not less than 18.0% w/w (Appendix 4.5).

Water-soluble extractive Not less than 33.0% w/w (Appendix 4.5).

Portulaca grandiflora Hook.

Thai Name แพรเซี่ยงไฮ้

Definition Dried aerial parts of *Portulaca grandiflora* Hook. (Family Portulacaceae).

Synonyms *Portulaca hilaireana* G. Don

Portulaca immersostellulata Poelln.

Portulaca mendocinensis Gillies ex Hook.

Part Used Aerial parts

Identification

A. Thin-layer Chromatography Identification Test

Sample solution: Reflux 5 g of the sample, in powder, with 50 ml of *methanol* for 10 min and immediately filter. Evaporate the filtrate to dryness under reduced pressure at 45° and add 3 ml of *methanol* to dissolve the residue.

Chromatographic system

Adsorbent: *Silica gel GF254*

Application volume: 5 µl of *Sample solution*

Developing solvent system: *Hexane*, *acetone,* and *ethyl acetate* (50:45:5)

Developing distance: 10 cm

Spray reagent: *Anisaldehyde TS*

Analysis

Sample: *Sample Solution*

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1). Apply the *Sample* as spot to the plate and dry in air. Develop the chromatogram in a saturated chamber. Remove the plate from the chamber and dry in air. Examine the plate under ultraviolet light (254 nm and 366 nm). Subsequently, spray the plate with *Spray reagent*, then heat at 105° for 5 min, and immediately examine the plate under daylight (Table 1); see also Fig. 1.

Table 1 hR_f Values of Components of Methanolic Extract of the Aerial Parts of *Portulaca grandiflora* Hook.

Spot	hR_f Value	Detection		
		UV 254	UV 366	*Anisaldehyde TS*
1	3-5	–	blue	grey
2	11-12	–	blue	grey
3	20-21	quenching	–	purple
4	24-28	–	blue	grey
5	33-34	quenching	–	purple
6	38-42	–	blue	grey
7	44-48	–	blue	green
8	50-51	quenching	–	purple
9	52-53	–	blue	grey
10	61-62	–	blue	green
11	63-65	–	blue	violet
12	70-71	–	blue	violet
13	82-84	–	blue	violet
14	88-90	–	blue	violet
15	96-98	–	blue	violet

B. Colour reaction

• Reflux 500 mg of the sample, in powder, with 10 ml of *methanol* on water-bath for 5 min and filter. Evaporate the filtrate to dryness under reduced pressure. Dissolve the residue in 2 ml of *acetic anhydride* and slowly add 1 ml of *sulfuric acid*: a brownish red colour ring develops.

• Reflux 1 g of the sample, in powder, with 10 ml of *water* for 5 min and filter. Evaporate the filtrate to 1 ml under reduced pressure. Add 1 or 2 pieces of *magnesium ribbon* and a few drops of *hydrochloric acid,* warm in a water-bath: a brownish red-green colour is produced.

Loss on drying Not more than 9.0% w/w after drying at 105° to constant weight (Appendix 3.5).

Foreign matter Not more than 2.0% w/w (Appendix 4.1).

Acid-insoluble ash Not more than 1.0% w/w (Appendix 4.3).

Total ash Not more than 19.0% w/w (Appendix 4.4).

Ethanol-soluble extractive Not less than 7.0% w/w (Appendix 4.5).

Water-soluble extractive Not less than 20.0% w/w (Appendix 4.5).

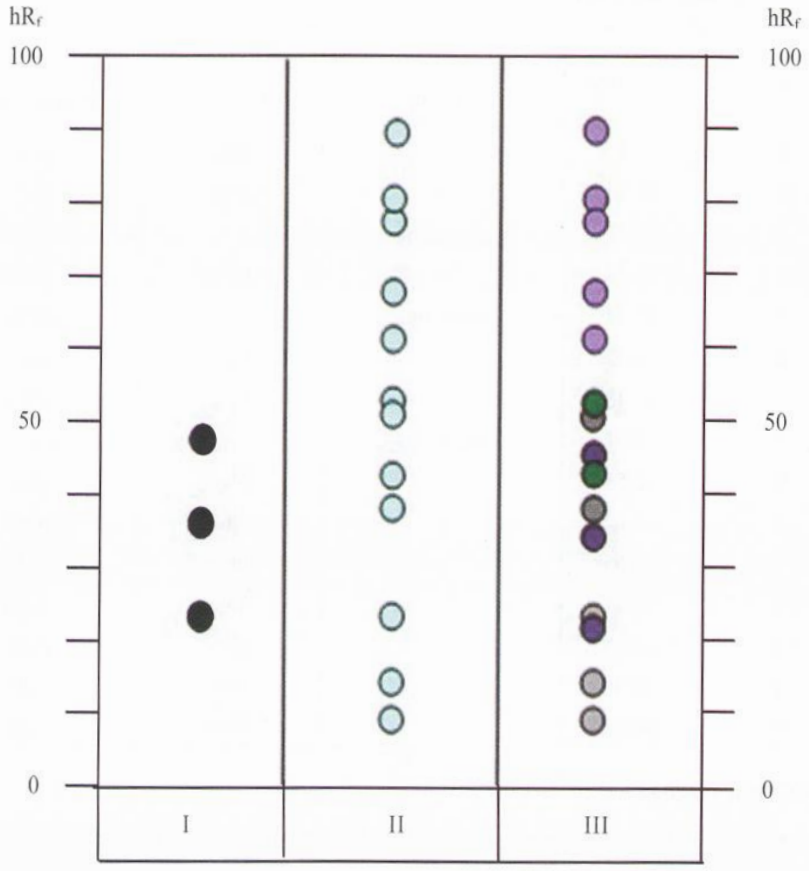


Fig. 1 Thin-layer Chromatogram of Methanolic Extract of the Aerial Parts of *Portulaca grandiflora* Hook.

I = detection under UV light (254 nm)

II = detection under UV light (366 nm)

III = detection with *anisaldehyde TS*

Appendices

Content of the Appendices

Appendix 1 General Information

The specifications given below are strictly for the use of the materials as reagents. The inclusion of a material in this Appendix does not imply that it is suitable for use in medicines. Exceptionally, a trademark or supplier may be indicated for certain reagents whose availability is limited. It is however acceptable to use reagents from another source provided that they comply with the standards of the Pharmacopoeia.

1.1 Reagents

The name of a substance or solution indicates a reagent included in the following list. The specifications given for reagents do not necessarily guarantee their quality for use in medicines. Some of the reagents included may be injurious to health. Important cautions have been stated for these reagents. They should be handled in accordance with good laboratory practice and any relevant regulations. Reagents in aqueous solution are prepared using water. Where the name of the solvent is not stated, an aqueous solution is intended. Unless otherwise specified, the reagents and reagent solutions are to be stored in well closed containers. The labelling should comply with the relevant national legislation.

Acetic Acid, Glacial $C_2H_4O_2$ = 60.05

Contains not less than 99.0% w/w of $C_2H_4O_2$

DESCRIPTION Clear, colourless liquid; odour, pungent. Crystallizes at about 10° and does not completely remelt until warmed to about 15°.

FREEZING TEMPERATURE Not lower than 14.8°.

WEIGHT PER MILLILITRE 1.048 to 1.051 g (Appendix 3.4).

IRON Not more than 1 ppm. To 9.50 ml add 0.1 ml of sodium carbonate TS and evaporate to dryness on a water-bath. Dissolve the residue by heating to boiling with a mixture of dilute hydrochloric acid and 10 ml of water, cool, and dilute to 50 ml with water. The solution complies with the "Limits Test for Iron".

CHLORIDE Not more than 67 ppm. A 5.0-ml sample shows no more chloride than that corresponds to 1.0 ml of 0.020 M hydrochloric acid.

SULFATE Not more than 0.023% w/w. A 4.0-ml sample shows no more sulfate than that corresponds to 1.0 ml of 0.010 M hydrochloric acid.

CERTAIN ALDEHYDIC SUBSTANCES To 10.0 g add 50 ml of water and 10.0 ml of a 1.25% w/v solution of sodium metabisulfite, allow to stand for 30 min, and titrate the excess of sodium metabisulfite with 1.05 M iodine VS. Repeat the operation without the glacial acetic acid; the difference between the titrations does not exceed 2.3 ml.

FORMIC ACID AND OXIDIZABLE IMPURITIES Mix 5.0 ml with 10.0 ml of water. To 5.0 ml of the solution add 6 ml of sulfuric acid and cool at 20°. Add 2.0 ml of 0.0167 M potassium dichromate and allow to stand for 1 min. Dilute with 25 ml of water, add 1 ml of potassium iodide TS, and titrate the liberated iodine with 0.10 M sodium thiosulfite, using starch TS as indicator. Not less than 1.0 ml of 1.0 M sodium thiosulfite is required.

ODOROUS IMPURITIES Dilute 1.5 ml to 5 ml with water and neutralize to litmus paper with 5 M sodium hydroxide: not more than a faint acetous odour is discernible.

NON-VOLATILE MATTER When volatized, leaves not more than 0.01% of residue.

ASSAY Weigh accurately about 2 g into a stoppered flask containing 50 ml of water and titrates with 1 M sodium hydroxide VS, using phenolphthalein TS as indicator. Each ml of 1 M sodium hydroxide VS is equivalent to 60.05 mg of $C_2H_4O_2$.

Acetic Anhydride $C_4H_6O_3 = 102.09$

Use analytical reagent grade of commerce containing not less than 97.0% w/v of $C_4H_6O_3$.

DESCRIPTION Colourless liquid.

Acetone $C_3H_6O = 58.08$

DESCRITION Clear, colourless, mobile, volatile liquid; odour, characteristic. Flammable.

SOLUBILITY Miscible with water, with chloroform, with ethanol, and with ether.

BOILING RANGE Not less than 95% distils between 55.5° and 57.0°.

WEIGHT PER MILLILITRE 0.790 to 0.792 g (Appendix 3.4).

ACIDITY Dilute 10.0 ml with 10.0 ml of carbon dioxide-free water. The solution requires for neutralization not more than 0.20 ml of 0.10 M sodium hydroxide, using phenolphthalein TS as indicator.

ALKALINITY Dilute 10 ml with 10 ml of carbon dioxide-free water. The solution is not alkaline to litmus paper.

WATER Shake 10.0 ml with 40.0 ml of carbon disulfide: a clear solution is produced.

OXIDIZABLE SUBSTANCES To 20.0 ml add 0.10 ml of 0.020 M potassium permanganate, and allow to stand for 15 min: the solution is not completely decolorized.

NON-VOLATILE MATTER When evaporated on a water-bath and dried at 105° to constant weight, leaves not more than 0.01% w/v of residue.

Anisaldehyde (4-Methoxybenzaldehyde) $C_8H_8O_2 = 136.15$

Use general reagent grade of commerce.

DESCRIPTION Colourless to pale yellow, oily liquid; odour, aromatic.

SOLUBILITY Slightly soluble in water; miscible with ethanol and with ether.

WEIGHT PER MILLILITRE About 1.125 g (Appendix 3.4).

Antimony trichloride $SbCl_3 = 22.811$

Contains not less than 97.0% of $SbCl_3$.

DESCRIPTION Colourless crystals, fuming in moist air.

SOLUBILITY Very soluble in absolute ethanol and in chloroform, forming solutions which are not more than slightly turbid.

ASSAY Dissolve about 500 mg, accurately weighed, in 30 ml of water containing 4 g of potassium sodium tartrate, add 2 g of sodium hydrogencarbonate and titrate with 0.05 M iodine VS, using starch TS as indicator near the end of the titration. Each ml of 0.05 M iodine VS is equivalent to 11.41 mg of $SbCl_3$.

(+)-Borneol $C_{10}H_{18}O = 154.25$

Use analytical reagent grade of commerce containing not less than 95% of $C_{10}H_{18}O$.

1-Butanol (*n*-Butyl Alcohol; *n*-Butanol) $C_4H_{10}O = 74.12$

Use analytical reagent grade of commerce.

DESCRIPTION Clear, colourless liquid.

SOLUBILITY Soluble in 11 parts of water, at 15.5°.

BOILING RANGE Not less than 95% distils between 116° and 119°.

WEIGHT PER MILLILITRE 0.807 to 0.810 g (Appendix 3.4).

Caffeic Acid $C_9H_8O_4 = 180.16$

Use analytical reagent grade of commerce containing not less than 95% of $C_9H_8O_4$.

Charcoal, Decolorizing

DESCRIPTION Black, light powder free from grittiness.

SOLUBILITY Practically insoluble in all usual solvents.

DECOLORIZING POWER Dissolve 100 mg of strychnine sulfate in 50 ml of water, add 1 g of the test substance, shake during 5 min, and pass through a dry filter, rejecting the first 10 ml of the filtrate. To a 10-ml portion of the subsequent filtrate add 1 drop of hydrochloric acid and 5 drops of mercury(II) iodide TS: no turbidity is produced.

ACID-SOLUBLE MATTER Not more than 3% w/w. To 1.0 g add 25 ml of dilute nitric acid and boil for 5 min. Filter whilst hot through a sintered-glass filter of porosity of 4 to 10 μm and wash with 10 ml of hot water. Evaporate the combined filtrate and washings to dryness on a water-bath, add to the residue 1 ml of hydrochloric acid, evaporate to dryness again and dry the residue to constant weight at 100° to 105°. The residue weighs not more than 30 mg.

Chloroform $CHCl_3 = 119.38$

Caution Care should be taken not to vaporize chloroform in the presence of a flame, because of the production of harmful gases.

Use analytical reagent grade of commerce containing 0.4 to 1.0% w/v of ethanol.

DESCRIPTION Colourless, volatile liquid; odour, characteristic.

SOLUBILITY Slightly soluble in water; miscible with absolute ethanol, with ether, with fixed and volatile oils, and with most organic solvents.

BOILING TEMPERATURE About 60°.

RELATIVE DENSITY 1.475 to 1.481 (Appendix 3.4).

Store protected from light.

Diethyl Ether, Anhydrous (Ether, Anhydrous; Ether, Absolute)

Ether, having a weight per milliliter of not more than 0.7079 g, complies with the following requirement.

ALCOHOL Transfer 100 ml to a separator and shake with five successive portions, 20 ml, 10 ml, 10 ml, 5 ml, and 5 ml, respectively, of water at about 25°. Shake each portion for 2 min and separate the aqueous layer carefully. Finally pour the combined aqueous extracts from one flask to another six times to assure minimum contamination with ether. Transfer 1 ml of the aqueous extract to a comparison tube and add 4 ml of water. For a standard take 5 ml of a solution of 0.2 ml of absolute ethanol in a litre of water. Add 10 ml of nitrochromic acid TS to each solution, mix, and allow to stands for 1 hr. At the end of this time the colour of the sample solution shows no more change from yellow to green or blue than is shown by the standard. The test and the standard must be kept at the same temperature.

WATER Not more than 0.041% w/v (Karl Fischer Method).

Dichloromethane (Methylene Chloride) $CH_2Cl_2 = 84.93$

DESCRIPTION Clear, colourless, mobile liquid.

SOLUBILITY Soluble in 50 parts of water; miscible with ethanol and with ether.

BOILING RANGE Not less than 95% distils between 39° and 41°.

WEIGHT PER MILLILITRE 1.323 to 1.325 g (Appendix 3.4).

NON-VOLATILE MATTER When evaporated on a water-bath and dried at 105° to constant weight, leaves not more than 0.05% w/v of residue.

3,5-Dinitrobenzoic Acid $C_7H_4N_2O_6 = 212.12$

DESCRIPTION Practically colourless crystals.

SOLUBILITY Slightly soluble in water; very soluble in ethanol.

MELTING POINT 205°.

Ethanol Use Ethanol (95%).

Ethanol (95%) (Ethyl Alcohol)

A mixture of ethanol and water. Contains not less than 92.3% w/w and not more than 93.8% w/w, corresponding to not less than 94.9% v/v and not more than 96.0% v/v, at 15.56°, of C_2H_6O.

DESCRIPTION Colourless, clear, mobile and volatile liquid; odour, characteristic and spirituous. Flammable, burning with a blue smokeless flame. Boils at about 78°.

SOLUBILITY Miscible with water, with chloroform and with ether.

IDENTIFICATION

A. Mix 5 drops in a small beaker with 1 ml of potassium permanganate TS and 5 drops of dilute sulfuric acid and cover the beaker immediately with a filter paper moistened with a solution recently prepared by dissolving 100 mg of sodium nitroferricyanide and 500 mg of piperazine hydrate in 5 ml of water: an intense blue colour is produced on the filter paper, the colour becoming paler after a few min.

B. To 5 ml of a 0.5% v/v solution, add 1 ml of 0.1 M sodium hydroxide; then slowly add 2 ml of iodine TS: the odour of iodoform develops and a yellow precipitate is produced.

ACIDITY OR ALKALINITY To 20 ml add 5 drops of phenolphthalein TS: the solution remains colourless and requires not more than 0.20 ml of 0.10 M sodium hydroxide to produce a pink colour.

CLARITY OF SOLUTION Dilute 5 ml to 100 ml with water in a glass cylinder: the solution remains clear when examined against a black background.

ALDEHYDES AND KETONES Heat 100 ml of hydroxylamine TS in a loosely stoppered flask on a water-bath for 30 min, cool, and, if necessary, add sufficient 0.050 M sodium hydroxide to restore the green colour. To 50 ml of this solution add 25 ml of the sample and heat on a water-bath for 10 min in a loosely stoppered flask. Cool, transfer to a Nessler cylinder, and titrate with 0.050 M sodium hydroxide until the colour matches that of the remainder of the hydroxylamine solution contained in a similar cylinder, both solutions being viewed down the axis of the cylinder. Not more than 0.90 ml or 0.050 M sodium hydroxide is required.

OXIDIZABLE SUBSTANCES To 20 ml add 1 ml of 0.002 M potassium permanganate. Allow the solution to stand at 20° for 10 min protected from light: the colour is not completely discharged.

NON-VOLATILE MATTER A 100-ml sample, when evaporated and dried at 100° to 105° to constant weight, leaves not more than 2.5 mg of residue.

SPECIFIC GRAVITY 0.805 to 0.821, at 25° (Appendix 3.4), using this result to ascertain the percentage of C_2H_6O contained in the liquid examined by reference to the Alcoholometric Table.

VOLATILE IMPURITIES Carry out the test as described in the "Gas Chromatography" (Appendix 2.2).

Reference solution (a) Dilute 100 ml of anhydrous methanol to 50.0 ml with the test substance. Dilute 5.0 ml of the solution to 50.0 ml with the test substance.

Reference solution (b) Dilute 50 ml of anhydrous methanol and 50 ml of acetaldehyde to 50.0 ml with the test substance. Dilute 100 ml of the solution to 10.0 ml with the test substance.

Reference solution (c) Dilute 150 ml of acetal to 50.0 ml with the test substance. Dilute 100 ml of the solution to 10.0 ml with the test substance.

Reference solution (d) Dilute 100 ml of benzene to 100.0 ml with the test substance. Dilute 100 ml of the solution to 50.0 ml with the test substance.

Test solution (a) The test substance.

Test solution (b) Add 150 ml of 4-methyl-2-pentanol to 500.0 ml of the test substance.

Chromatographic system A gas chromatograph equipped with (a) a glass (fused silica) column (30 m × 0.32 mm) packed with porous poly[(cyanopropyl)(phenyl)][dimethyl]-siloxane (1.8 mm), maintained as the following table, (b) a flame ionization detector maintained at 280°, and (c) helium as the carries gas.

	Time (min)	Temperature (°)
Column	0-12	40
	12-32	40→240
	32-42	240
Injection port		200

System suitability Chromatograph *Reference solution (b)* and record the peak response as directed for *Procedure*: the resolution between the first peak (acetaldehyde) and the second peak (methanol) is not less than 1.5.

Procedure Inject separately suitable volumes of each of *Reference solution (a), Reference solution (b), Reference solution (c), Reference solution (d), Test solution (a),* and *Test solution (b).*

Limits:

- methanol in the chromatogram obtained from test solution (a): not more than half the area of the corresponding peak in the chromatogram obtained from reference solution (a) (200 ppm v/v),
- cetaldehyde + acetal: maximum 10 ppm v/v, expressed as acetaldehyde.

Calculation Calculate the sum of the contents of acetaldehyde and acetal in parts per million (v/v) using the following expression:

$$\frac{10 \times A_E}{A_T - A_E} + \frac{30 \times C_E}{C_T - C_E}$$

where A_E = area of the acetaldehyde peak in the chromatogram obtained from test solution (a),

A_T = area of the acetaldehyde peak in the chromatogram obtained from reference solution (b),

C_E = area of the acetal peak in the chromatogram obtained from test solution (a), and

C_T = area of the acetal peak in the chromatogram obtained from reference solution (c).

- benzene: maximum 2 ppm v/v.

Calculate the content of benzene in parts per million (v/v) using the following expression:

$$\frac{2B_E}{B_T - B_E}$$

B_E = area of the benzene peak in the chromatogram obtained from the test solution (a), and

B_T = area of the benzene peak in the chromatogram obtained from reference solution (d).

If necessary, the identity of benzene can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).

- total of other impurities in the chromatogram obtained from test solution (b): not more than the area of the peak due to 4-methyl-2-pentanol in the chromatogram obtained from test solution (b) (300 ppm v/v),

- disregard limit: 0.03 times the area of the peak corresponding to 4-methyl-2-pentanol in the chromatogram obtained from test solution (b) (9 ppm v/v).

Ethyl Acetate $C_4H_8O_2 = 88.11$

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid; odour, fruity-like.

BOILING RANGE 76° to 78°.

WEIGHT PER MILLILITRE 0.901 to 0.904 g (Appendix 3.4).

Eurycomanone $C_{20}H_{24}O_9 = 408.40$

Use analytical reagent grade of commerce.

Formic Acid $CH_2O_2 = 46.03$

Use analytical reagent grade of commerce containing about 90% w/w of CH_2O_2 and about 23.6 M in strength.

DESCRIPTION Colourless, corrosive liquid; odour, pungent.

Ginsenoside Rb_1 $C_{54}H_{92}O_{23} = 1103.29$

Use analytical reagent grade of commerce.

Hexane $C_6H_{14} = 86.18$

The hexane fraction from petroleum.

DESCRIPTION Colourless, mobile, highly flammable liquid.

BOILING RANGE Not less than 95% distils between 67° and 70°.

WEIGHT PER MILLILITRE 0.670 to 0.677 g (Appendix 3.4).

NON-VOLATILE MATTER When evaporated on a water-bath and dried at 105° to constant weight, leaves not more than 0.01% w/v of residue.

***n*-Hexane** $C_6H_{14} = 86.18$

Use analytical reagent grade of commerce usually containing not less than 99% of the pure isomer, *n*-C_6H_{14}.

DESCRIPTION Colourless, flammable liquid.

BOILING RANGE Distils completely over a range of 1° between 67.5° and 69.5°.

REFRACTIVE INDEX 1.034 to 1.375 (Appendix 3.2).

WEIGHT PER MILLILITRE 0.658 to 0.659 g (Appendix 3.4).

Hydrochloric Acid $HCl = 36.46$

Use analytical reagent grade of commerce.

When no molarity is indicated, use analytical reagent grade of commerce with a relative density of about 1.18, containing not less than 35% w/w and not more than 38% w/w of HCl and about 11.5 M in strength.

DESCRIPTION Clear, colourless, fuming liquid; odour, pungent.

SOLUBILITY Miscible with water.

Solutions of molarity *x*M should be prepared by diluting 85x ml of hydrochloric acid to 1000 ml with water.

Store in a container of polyethylene or other non-reacting material at a temperature not exceeding 30°.

Imperatorin $C_{16}H_{14}O_4 = 270.28$

Use analytical reagent grade of commerce containing not less than 95% of $C_{16}H_{14}O_4$.

Iron(III) Chloride (Ferric Chloride) $FeCl_3 \cdot 6H_2O = 270.30$

Use analytical grade of commerce.

DESCRIPTION Yellowish orange or brownish, crystalline masses; deliquescent.

Store in well-closed containers.

Linalool $C_{10}H_{18}O = 154.25$

Use analytical reagent grade of commerce containing not less than 95% of $C_{10}H_{18}O$.

Magnesium Ribbon

Use a suitable grade.

Methanol (Methyl Alcohol) $CH_4O = 32.04$

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid.

SOLUBILITY Miscible with water, forming a clear colourless liquid.

BOILING RANGE 64° and 65°.

RELATIVE DENSITY 0.791 to 0.793° (Appendix 3.4).

Phosphoric Acid (Orthophosphoric Acid) $H_3PO_4 = 98.00$

Caution Avoid contact, as phosphoric acid rapidly destroys tissues.

Use analytical reagent grade of commerce containing not less than 84% w/w of H_3PO_4 and about 15.7 M in strength.

DESCRIPTION Corrosive, clear colourless, syrupy liquid.

SOLUBILITY Miscible with water and with ethanol.

WEIGHT PER MILLILITRE About 1.75 g (Appendix 3.4).

Potassium Permanganate $KMnO_4 = 158.03$

Use analytical reagent grade of commerce.

Rosmarinic Acid $C_{18}H_{16}O_8 = 360.31$

Use analytical reagent grade of commerce containing not less than 95% of $C_{18}H_{16}O_8$.

Scopoletin $C_{10}H_8O_4 = 192.17$

Use analytical reagent grade of commerce containing not less than 95% of $C_{10}H_8O_4$.

Sulfuric Acid $H_2SO_4 = 98.07$

When no molarity is indicated, use analytical reagent grade of commerce containing about 96% w/w of sulfuric acid and about 18 M in strength.

DESCRIPTION Colourless, oily, corrosive liquid.

WEIGHT PER MILLILITRE About 1.84 g (Appendix 3.4).

When solutions of molarity *x*M are required, they should be prepared by carefully adding 54*x* ml of sulfuric acid to an equal volume of water and diluting to 1000 ml with water. When "sulfuric acid" is followed by a percentage figure, an instruction to add, carefully, sulfuric acid to water to produce the specified percentage v/v (or, if required, w/w) proportion of sulfuric acid is implied.

Toluene (Methylbenzene) $C_7H_8 = 92.14$

Use analytical grade of commerce.

DESCRIPTION Clear, colourless liquid; odour, characteristic. Flammable.

SOLUBILITY Miscible with water and with ethanol.

BOILING TEMPERATURE About 110°.

WEIGHT PER MILLILITRE 0.865 to 0.870 g (Appendix 3.4).

Ursolic Acid $C_{30}H_{48}O_3 = 456.70$

Use analytical reagent grade of commerce containing not less than 95% of $C_{30}H_{48}O_3$.

Water $H_2O = 18.02$

Use Purified Water of the Official Pharmacopoeia.

Xylene $C_8H_{10} = 106.17$

DESCRIPTION Clear, colourless liquid, consisting mainly of *m*-xylene with smaller proportions of *o*- and *p*-xylenes. Flammable.

SOLUBILITY Insoluble in water; Miscible with absolute ethanol.

BOILING RANGE Not less than 90 per cent distils between 136° and 140°.

WEIGHT PER MILLILITRE 0.85 to 0.86 g (Appendix 3.4).

SULFUR COMPOUNDS Boil 10 ml with 1 ml of absolute ethanol and 3 ml of potassium plumbite TS for 15 min under a reflux condenser, and allow to stand for 5 min; the aqueous layer remains colourless.

REACTION WITH SULFURIC ACID Shake 5 ml with 5 ml of sulfuric acid; the xylene remains colourless and the acid may become yellow but not brown.

NON-VOLATILE MATTER When evaporated on a water-bath and dried at 105° to constant weight, leaves not more than 0.01 per cent w/v of residue.

1.2 Test Solutions

Anisaldehyde TS Mix, in order, 0.5 ml of anisaldehyde, 10 ml of glacial acetic acid, 85 ml of methanol, and 5 ml of sulfuric acid.

Iron(III) Chloride TS Dissolve 9 g of iron(III) chloride in water to make 100 ml.

Vanillin-Phosphoric acid TS Dissolve 1.0 g of vanillin in 25 ml of ethanol, and then add 25 ml of water and 35 ml of phosphoric acid, respectively, and mix well. Prepare freshly before use.

Vanillin-Sulfuric Acid TS Dissolve 1 g of vanillin in 100 ml of ethanol, add 2 ml of sulfuric acid dropwise and mix.

1.3 Materials for Chromatography

Gas Chromatography

SUPPORTS

Diatomaceous Support (Diatomaceous Earth; Siliceous Earth) White or almost white, fine granular powder made up of siliceous frustules of fossil diatoms or debris of fossil diatoms. It may be identified by microscopic examination with a magnification of × 500.

Diatomaceous Support, Acid-washed Diatomaceous support that has been purified by treatment with hydrochloric acid and washed with water to remove metallic impurities, and to reduce surface activity and peak-tailing.

Diatomaceous Support, Alkali-washed Diatomaceous support that has been treated with potassium hydroxide solution to reduce peak-tailing of basic compounds.

Diatomaceous Support, Silanized Diatomaceous earth for gas chromatography, silanized acid-washed diatomaceous support that has been silanized with dimethyldichloro-silane or other suitable silanizing agents.

STATIONARY PHASES

A wide range of chemical substances is used, including polyethylene glycols, high-molecular weight esters and amides, hydrocarbons, silicone gums and fluids (polysiloxanes often substituted by methyl, phenyl, nitrilo, vinyl, or fluoroalkyl groups, or mixtures of these), and micro-porous cross-linked polyaromatic beads. Care should be taken to select grades specifically intended for use in gas chromatography. In most cases reference is made to a particular commercial brand which has been found to be suitable for the determination in question, but such statements do not imply that a different but equivalent commercial brand may not be used.

INTERNAL STANDARDS

Reagents used as internal standards should not contain any impurity which would produce a peak likely to interfere in the determination described in the monograph.

Thin-layer Chromatography

The coating substances described below are used to prepare thin-layer chromatoplates in accordance with the procedure described in Appendix 2.1. Prepare suspensions of the coating substances as recommended by the manufacturer unless otherwise prescribed. Commercial pre-coated chromatoplates may be used for Pharmacopoeial tests provided they comply with the test for chromatographic separation described for the corresponding coating substance.

Silica Gel G A fine, white, homogeneous powder of an average particle size between 10 and 40 µm containing about 13% w/w of calcium sulfate hemihydrate and complying with the following requirements.

CONTENT OF CALCIUM SULFATE To about 250 mg, accurately weighed, add 3 ml of 2 M hydrochloric acid and 100 ml of water and shake vigorously for 30 min. Filter, wash the residue with water and carry out the "Complexometric Titration of Calcium" on the combined filtrate and washings. Each ml of 0.1 M disodium edetate VS is equivalent to 14.51 mg of $CaSO_4 \cdot 1/2H_2O$.

SEPARATING POWER Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1), using toluene as the mobile phase. Apply to the plate, 10 µl of a solution in

dichloromethane containing 0.1 mg per ml of each of indophenol blue, sudan red G and dimethyl yellow in toluene. Allow the mobile phase to ascend 10 cm. The chromatogram shows three clearly separated spots of the indophenol blue, sudan red G and dimethyl yellow in order of increasing R_f value.

ALKALINITY pH of a suspension prepared by shaking 1 g with 10 ml of carbon dioxide-free water for 5 min, about 7.

Silica Gel GF254 A fine, white, homogeneous powder of an average particle size between 10 and 40 μm containing about 13% w/w of calcium sulfate hemihydrate and about 1.5% w/w of a fluorescent indicator having a maximum intensity at 254 nm. It complies with the tests for Content of Calcium Sulfate, Alkalinity and Separating Power stated under Silica gel G and with the following test.

FLUORESCENCE Carry out the test as described in the "Thin-layer Chromatography" (Appendix 2.1), using a mixture of 90 volumes of 2-propanol and 10 volumes of anhydrous formic acid as the mobile phase. Apply separately to the plate, increasing quantities from 1 to 10 μl of a 1 mg per ml solution of benzoic acid in the same solvent mixture. Develop the plate and dry in a current of warm air. Examine the chromatogram under ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram at levels of 2 μg and greater.

1.4 Powder Fineness and Sieves

Powder

The degree of coarseness or fineness of a powder is differentiated and expressed by reference to the nominal mesh aperture size of the sieves used.

The following terms are used in the description of powders:

COARSE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 1.70 mm and not more than 40.0% through a sieve with a nominal mesh aperture of 355 μm.

MODERATELY COARSE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 710 μm and not more than 40.0% through a sieve with a nominal mesh aperture of 250 μm.

MODERATELY FINE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 355 μm and not more than 40.0% through a sieve with a nominal mesh aperture of 180 μm.

FINE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 180 μm.

VERY FINE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 125 μm.

When the fineness of a powder is described by means of a number, it is intended that all the particles of the powder shall pass through a sieve of which the nominal mesh aperture, in μm, is equal to that number.

When a batch of a vegetable drug is being ground and sifted, no portion of the drug shall be rejected, but it is permissible, except in the case of assays, to withhold the final tailings, if an approximately equal amount of tailings from a preceding batch of the same drug has been added before grinding.

When the use of sieves is inappropriate, the definition is expressed in terms of the particle size as determined by suitable microscopical examination.

Sieves

Wire mesh sieves used in sifting powdered drugs are identified by numbers indicating the nominal mesh aperture.

The sieves should be made of wires of uniform circular cross-section. The wires may be of stainless steel or of other suitable material except that plated wire is not permitted. Sieves should conform to the specifications which are concordant with the recommended International Standard ISO 3310-1:2000 (E), shown in the following table.

Calibration and recalibration of test sieves is in accordance with the most current edition of ISO 3310-1. Sieves should be carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212 to 850 μm, Standard Glass Spheres are available from the national or international organization, e.g. NIST[1]. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and a relative humidity between 20% and 70%.

CLEANING TEST SIEVES Ideally, test sieves should be cleaned using only an air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort. Washing sieves in hot water is not recommended since the sieves can distort and rupture

[1] US National Institute of Standards and Technology

during heating and cooling. If it is necessary to use water, it should be used at ambient temperature and the sieve dried by first using a volatile water-miscible solvent to remove the water and then a low-pressure air jet to remove the solvent. This procedure should be carried out in a fume hood or cabinet that conforms to local regulations.

Method for Determining Powder Fineness

Place the specified quantity of the test powder upon the appropriate sieve having a close-fitting receiving pan and cover. Shake the sieve in a rotary horizontal direction and vertically by tapping on a hard surface for not less than the specified time or until sifting is practically complete. Avoid prolonged shaking that would result in increasing the fineness of the powder during the testing. In the case of oily or other powders that tend to clog the openings, carefully brush the screen at intervals during the test. Breaking up lumps that form during the sifting. Weigh accurately the amount remaining on the sieve and in the receiving pan.

The fineness of a powdered drug or chemical may be determined also by screening through the sieves in mechanical sieve shaker, which reproduces the circular and tapping motion given to testing sieves in hand sifting but with a uniform mechanical action, following the directions provided by the manufacturer of the shaker.

Number of Sieve*	Nominal Mesh Aperture Size	Preferred Average Wire Diameter	Percentage Sieving Area	US Sieve No.**
µm	mm	mm		
4000	**4.00**	**1.40**		
3350	3.35			6
2800	**2.80**	**1.12**	**51**	7
2360	2.36	1.00	49	8
2000	**2.00**	**0.90**	**48**	**10**
1700	1.70	0.80	46	12
1400	**1.40**	**0.71**	**44**	**14**
1180	1.18	0.63	43	16
1000	**1.00**	**0.56**	**41**	**18**
µm	µm	µm		
850	850			20
710	**710**	**450**	**37**	**25**
600	600	400	36	30
500	**500**	**315**	**38**	**35**
425	425	280	36	40
355	**355**	**224**	**38**	**45**
300	300	200	36	50
250	**250**	**160**	**37**	**60**
212	212	140	36	70
180	**180**	**125**	**35**	**80**
150	150	100	36	100
125	**125**	**90**	**34**	**120**
106	106	71	36	140
90	**90**	**63**	**35**	**170**
75	75	50	36	200
63	**63**	**45**	**34**	**230**
53	53	36	35	270
45	**45**	**32**	**34**	**325**

*Entries in bold are ISO "principal sizes".

**The list of United States standard sieves is included for information purposes.

Appendix 2 Chromatography

2.1 Thin-layer Chromatography

Thin-layer chromatography (TLC) is used for the rapid separation of compounds by means of a uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate. The coated plate can be considered as an "open chromatographic column". Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or on combinations of these mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase).

The retardation factor (R_f) is defined as the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application. As R_f values may vary significantly with the experimental conditions, it is always necessary to prepare chromatograms of authentic specimens or reference substances; preferably in varied quantities, alongside the chromatogram of the sample. Positive identification may be effected by observation of two spots of identical R_f value and about equal magnitude. A visual comparison of the size of the spots may serve for semi-quantitative estimation. More accurate quantitative measurements can be made by densitometry, fluorescence, and fluorescence quenching, or careful removal of the spots from the plate, followed by elution with a suitable solvent and spectrophotometric measurement. For two-dimensional thin-layer chromatography, the chromatographed plate is turned at a right angle and again chromatographed, usually in another chamber equilibrated with a different solvent system.

APPARATUS

Plate The chromatography is carried out using the TLC plate (typically 20 cm × 20 cm) of which the stationary phase has an average particle size of 10 to 15 µm, and that of high performance thin-layer chromatography (HPTLC) plates (typically 10 cm × 10 cm) has an average particle size of 5 µm. Commercial plates with a pre-adsorbent zone can be used if they are specified in a monograph.

Spreader A spreader, which, when moved over the plate, will apply a uniform layer of adsorbant, 250 to 300 µm thick, over the entire surface of the plate. Other thicknesses might be desirable in some procedures, and an adjustable spreader would be particularly useful in such cases.

Preparation of the TLC plate Use flat glass plates of convenient size typically 20 cm × 20 cm. The adsorbent consists of finely divided solid material, normally 10 to 15 µm in diameter, suitable for chromatography. It can be applied directly to the plate or can be bonded to the plate by means of plaster of Paris (hydrated calcium sulfate) at a ratio of 5 to 15%, or with starch paste or other binders. The former will not yield as hard a surface as will the starch, but it is not

affected by strongly oxidizing spray reagents. The adsorbent may contain fluorescing material to aid in the visualization of spots that absorb ultraviolet light. Clean the plates scrupulously, as by immersion in chromic acid cleansing mixture, rinsing them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, and then dry. It is important that the plates be completely free from lint and dust when the adsorbent is applied.

Arrange the plate or plates on the aligning tray, place a 5-cm × 20-cm plate adjacent to the front edge of the first square plate and another 5-cm × 20-cm plate adjacent to the rear edge of the last square, and secure all of the plates so that they will not slip during the application of the adsorbant. Position the spreader on the end plate opposite to the raised end of the aligning tray. Mix 1 part of adsorbent with 2 parts of water (or in the ratio suggested by the supplier) by shaking vigorously for about 30 seconds in a glass-stoppered conical flask, and transfer the slurry to the spreader. Usually 30 g of adsorbent and 60 ml of water are sufficient for five 20-cm × 20-cm plates. Complete the application of adsorbents using plaster of Paris binder within 2 min of addition of the water, since thereafter the mixture begins to harden. Draw the spreader smoothly over the plates towards the raised end of the aligning tray, and remove the spreader when it is on the end plate next to the raised end of the aligning tray. (Wash away all traces of adsorbant from the spreader immediately after use). Allow the plates to remain undisturbed for 5 min, then transfer the square plates, layer side up, to the storage rack and dry at 105° for 30 min. Preferably place the rack at an angle in the drying oven to prevent the condensation of moisture on the back side of the plates in the rack. When the plates are dry, allow them to cool to room temperature, and inspect the uniformity of the distribution and the texture of the adsorbant layer; transmitted light will show uniformity of texture. Store the satisfactory plates over self-indicating silica gel in a suitable chamber.

Pre-treatment of the plate It may be necessary to wash the plates prior to separation. This can be done by migration of an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 120° for 20 min.

Developing chamber A developing chamber with a flat bottom or twin trough, of inert, transparent material, of a size suitable for the plates is used and provided with a tightly fitting lid. For horizontal development, the chamber is provided with a trough for the mobile phase and it additionally contains a device for directing the mobile phase to the stationary phase.

Micropipette, microsyringe, calibrated disposable capillary A micropipette, microsyringe, calibrated disposable capillary or other application devices suitable for the proper application of the solutions are used.

Template A template (generally made of plastic) is used to aid in placing the test spots at definite intervals, to mark distances as needed, and to aid in labelling the plate.

Detection/Visualization device An ultraviolet (UV) light source suitable for observations under short-(254 nm) and long-(366 nm) wavelength UV light and a variety of other spray reagents to make spots visible are used.

A device may be used to provide documentation of the visualized chromatogram, for example a photograph or a computer file.

PROCEDURE

Sample application Apply the prescribed volume of the solutions at a distance of at least 15 mm (5 mm on HPTLC plates) from the lower edge and from the sides of the plate and on a line parallel to the lower edge; allow an interval of at least 10 mm (5 mm on HPTLC plates) between the centres of circular spots and 5 mm (2 mm on HPTLC plates) between the edges of bands.

Apply the solutions in sufficiently small portions to obtain circular spots of 2 to 5 mm in diameter (1 to 2 mm on HPTLC plates) or bands of 10 to 20 mm (5 to 10 mm on HPTLC plates) by 1 to 2 mm (0.5 to 1 mm on HPTLC plates) and allow to dry. Avoid physical disturbance of the adsorbant during the spotting procedure (by the pipette or other applicator) or when handling the plates. The template will aid in determining the spot points and the specified distance through which the solvent front should pass.

Development Line the walls of the developing chamber with filter paper. Pour into the developing chamber a sufficient quantity of the mobile phase for the size of the chamber to give after impregnation of the filter paper a layer of appropriate depth related to the dimension of the plate to be used. For saturation of the developing chamber, replace the lid and allow to stand for 1 hr. Unless otherwise indicated in the monograph, the chromatographic separation is performed in a saturated chamber. Apply the prescribed volume of solutions as described above. When the solvent has evaporated from the applied solutions, place the plate in the developing chamber, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase. Close the developing chamber. Remove the plate when the mobile phase has moved over 15 cm, or over three-quarters of the length of the plate, above the initial spots or bands, unless otherwise indicated in the monograph. Dry the plate and visualize the chromatog-rams as prescribed.

Horizontal development can be used in place of vertical development, if specified in the monograph. For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

Detection/Visualization Observe the principal spot or band in the chromatogram first under short-wavelength ultraviolet light (254 nm) and then under long-wavelength ultraviolet light

(366 nm). Measure and record the distance of each spot or band from the point of origin, and indicate for each spot or band the wavelength under which it was observed. If further directed, spray the spots or bands with the reagent specified, observe, and compare the sample with the standard chromatogram.

2.2 Gas Chromatography

Gas chromatography is a method of chromatographic separation in which the mobile phase is a gas (the carrier gas) and the stationary phase is a solid or liquid coated on a suitable solid support contained in a column. On emerging from the column the carrier gas is passed through a suitable detector.

Apparatus

The apparatus consists of an injector, a chromatographic column contained in an oven, a detector and data collection devices. The carrier gas flows through the column at a controlled rate or pressure and then through the detector. The chromatography is carried out either at a constant temperature or according to a given temperature programme.

INJECTORS Sample injection devices range from simple syringes to fully programmable automatic injectors. Direct injections of solutions are the usual mode of injection, unless otherwise prescribed in the monograph. Injection may be carried out either directly at the head of the column using a syringe or an injection valve, or into a vaporization chamber which may be equipped with a stream splitter. The amount of sample that can be injected into a capillary column without overloading is small compared to the amount that can be injected into packed columns and may be less than the smallest amount that can be manipulated satisfactorily by syringe. Capillary columns, therefore, often are used with injectors able to split samples into two fractions, a small one that enters the column and a large one that goes to waste. Such injectors may be used in a splitless mode for analyses of trace or minor components.

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are desorbed into the carrier gas by rapid heating of the temperature programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample heating chamber. Solid or liquid samples in tightly closed containers are heated in the chamber for a fixed period of time allowing the volatile components in the sample to reach an equilibrium between the nongaseous phase and the gaseous or headspace phase.

After this equilibrium has been established, the injector automatically introduces a fixed amount of the headspace in the sample container into the gas chromatograph.

COLUMNS Capillary columns which are usually made of fused silica, are typically 0.2 to 0.53 mm in internal diameter and 5 to 60 m in length. The liquid or stationary phase, which is sometimes chemically bonded to the inner surface, is 0.1 to 1.0 μm thick, although nonpolar stationary phases may be up to 5 μm thick.

Packed columns, made of glass or metal, are 1 to 3 m in length with internal diameters of 2 to 4 mm. Those used for analysis typically are porous polymers or solid supports with liquid phase loadings of about 5% (w/w). High-capacity columns, with liquid phase loadings of about 20% (w/w), are used for large test specimens and for the determination of low molecular weight compounds such as water. The capacity required influences the choice of solid support.

Supports for analysis of polar compounds on low capacity, low-polarity liquid phase columns must be inert to avoid peak tailing. The reactivity of support materials can be reduced by silanizing prior to coating with liquid phase. Acid-washed, flux-calcined diatomaceous earth is often used for drug analysis. Support materials are available in various mesh sizes, with 80- to 100-mesh and 100- to 120-mesh being most commonly used with 2- to 4-mm columns.

Helium or nitrogen is usually employed as the carrier gas for packed columns, whereas commonly used carrier gases for capillary columns are nitrogen, helium and hydrogen. Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly proportional to column length, while resolution is proportional to the square root of the column length. For packed columns, the carrier gas flow rate is usually expressed in ml/min at atmospheric pressure and room temperature. It is measured at the detector outlet with a flowmeter while the column is at operating temperature. The linear flow rate through a packed column is inversely proportional to the square of the column diameter for a given flow volume. Flow rates of 60 ml/min in a 4-mm column and 15 ml/min in a 2-mm column give identical linear flow rates and thus similar retention times. Unless otherwise specified in the monograph, flow rates for packed columns are about 30 to 60 ml/min. For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. At high operating temperatures there is sufficient vapour pressure to result in a gradual loss of liquid phase, a process called bleeding.

DETECTORS Flame-ionization detectors are used for most pharmaceutical analyses, with lesser use made of thermal conductivity, electron-capture, nitrogenphosphorous (alkali flame-ionization), mass spectrometric, Fourier transform infrared spectrophotometric detectors, and others, depending on the purpose of the analysis. For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of compound present in the detector over a wide range of concentrations. Flame-ionization detectors have a wide linear range and are

sensitive to most organic compounds. Detector response depends on the structure and concentration of the compound and on the flow rates of the combustion, air, makeup, and carrier gases. Unless otherwise specified in individual monographs, flameionization detectors with either helium or nitrogen carrier gas are to be used for packed columns and helium or hydrogen is used for capillary columns.

DATA COLLECTION DEVICES Modern data stations receive the detector output, calculate peak areas and peak heights, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation. Data can also be collected for manual measurement on simple recorders or on integrators whose capabilities range from those providing a printout of peak areas and peak heights calculated and data stored for possible reprocessing.

The design of a particular chromatograph may require modification of the conditions detailed in the monograph. In such a case, the analyst should be satisfied that the modified conditions produce comparable results. If necessary, adjust the flow rate of the carrier gas to improve the quality of the chromatogram or to modify the retention times of the peaks of interest.

Performance

Criteria for assessing the suitability of the system are described in the "Chromatographic Separation Techniques" (Appendix 2.4). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given.

Procedure

Equilibrate the column, the injector and the detector at the temperatures and the gas flow rates specified in the monograph until a stable baseline is achieved. Prepare the test solution(s) and the standard solution(s) as prescribed in the monograph. The solutions must be free from solid particles. Using standard solution determine experimentally suitable instrument settings and volumes of the solutions to be injected to produce an adequate response.

In applications where an internal standard is used, an injection of sample solution containing only the substance being examined should be made to determine whether any peak is present that will interfere with that of the internal standard. If an interfering peak is present, a suitable correction should be made.

Inject the selected volumes of the solutions prescribed in the monograph and record the resulting chromatograms. Repeat the determinations to ensure a consistent response.

For qualitative analysis, the retention time for a peak in the chromatogram obtained for a test specimen is "the same as," or "corresponding to" that obtained for a standard preparation under the conditions specified in the individual monograph.

For quantitative analysis, determine the peak areas or, alternatively, when the symmetry factor is between 0.80 and 1.20, determine the peak heights corresponding to the components of interest. From the values obtained calculate the content of the component or components being determined.

Assays require quantitative comparison of one chromatogram with another, and lack of control of the specimen size injected is a major source of error. Addition of an internal standard to the test specimen minimizes this error. The ratio of peak response of the components of interest to the internal standard is compared from one chromatogram to another. Where the internal standard is chemically similar to the substance being examined, minor variations in column and detector parameters are controlled also. In some cases, the internal standard may be carried through the assay procedure prior to gas chromatography to control other quantitative aspects of the procedure.

Materials

Supports, stationary phases and internal standards for gas chromatography are stated in the "Materials for Chromatography" (Appendix 1.3).

Solvents and reagents used in the preparation of solutions for examination should be of a quality suitable for use in gas chromatography.

2.3 High-pressure Liquid Chromatography

High-pressure liquid chromatography (HPLC), sometimes called high-performance liquid chromatography, is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion-exchange processes, depending upon the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of organic compounds. Compounds to be analyzed are dissolved in a suitable solvent, and most separations take place at room temperature. Thus, most drugs, being nonvolatile or thermally unstable compounds, can be chromatographed without decomposition or the necessity of making volatile derivatives. Most pharmaceutical analyses are based on partition chromatography and are completed within 30 min.

As in gas chromatography, the elution time of a compound can be described by the capacity factor, k', which depends on the chemical nature of the analyte, the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an

important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

Apparatus

A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device such as a computer, integrator, or recorder. Short, small-bore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary phases. In addition to receiving and reporting detector output, computers are used to control chromatographic settings and operations, thus providing for long periods of unattended operation.

PUMPING SYSTEM HPLC pumping systems deliver metered amounts of mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Modern systems consist of one or more computer-controlled metering pumps that can be programmed to vary the ratio of mobile phase components, as is required for gradient chromatography, or to mix isocratic mobile phases (i.e., mobile phases having a fixed ratio of solvents). However, the proportion of ingredients in premixed isocratic mobile phases can be more accurately controlled than in those delivered by most pumping systems. Operating pressures up to 34,474 kPa (about 5000 psi) or higher, with delivery rates up to about 10 ml per min are typical. Pumps used for quantitative analysis should be constructed of materials inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended periods of time.

INJECTORS After dissolution in mobile phase or other suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by a syringe or loop injectors, or automatically by autosamplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a loop from which it is loaded into the chromatograph. Some autosamplers can be programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables.

A syringe can be used for manual injection of sample through a septum when column head pressures are less than 6897 kPa (about 1000 psi). At higher pressures an injection valve is essential. Some valve systems incorporate a calibrated loop that is filled with the test solution for transfer to the column in the mobile phase. In other systems, the test solution is transferred to a cavity by a syringe and then switched into the mobile phase.

COLUMNS For most pharmaceutical analyses, separation is achieved by partition of compounds in the test solution between the mobile phase and stationary phase. Systems consisting of polar

stationary phases and nonpolar mobile phases are described as normal phase, while the opposite arrangement, polar mobile phases and nonpolar stationary phases, is called reverse-phase chromatography. Partition chromatography is almost always used for hydrocarbon-soluble compounds of molecular weight less than 1000. The affinity of a compound for the stationary phase, and thus its retention time on the column, is controlled by making the mobile phase more or less polar. Mobile phase polarity can be varied by the addition of a second, and sometimes a third or even a fourth, component.

Stationary phases for modern, reverse-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3 to 10 μm in diameter, but sizes may range up to 50 μm or more for preparative columns. Small particles thinly coated with the organic phase provide for low mass transfer resistance and, hence, rapid transfer of compounds between the stationary and mobile phases. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups. Liquid, nonbound stationary phases must be largely immiscible in the mobile phase. Even so, it is usually necessary to presaturate the mobile phase with stationary phase to prevent stripping of the stationary phase from the column. Polymeric stationary phases coated on the support are more durable.

Columns used for analytical separations usually have internal diameters of 2 to 5 mm; larger diameter columns are used for preparative chromatography. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential stationary phase degradation or mobile phase volatility. Unless otherwise specified in the individual monograph, columns are used at ambient temperature.

Ion-exchange chromatography is used to separate water-soluble, ionizable compounds of molecular weight less than 1500. The stationary phases are usually synthetic organic resins; cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines, while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers affect the equilibrium, and these variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column. Smaller molecules enter the pores and are

increasingly retained as molecular size decreases. These columns are typically used to measure aggregation and degradation of large molecules.

DETECTORS Many compendial HPLC methods require the use of spectrophotometric detectors. Such a detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes.

Fixed, variable-, and multi-wavelength detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp, variable-wavelength detectors contain a continuous source such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. The wavelength accuracy of a variable-wavelength detector equipped with a monochromator should be checked by the procedure recommended by its manufacturer, if the observed wavelengths differ by more than 3 nm from the correct values, recalibration of the instrument is indicated. Modern variable-wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Diode array detectors usually have lower-signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate and temperature so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done prior to chromatographic separation or, alternatively, the reagent can be introduced into mobile phase just prior to its entering the detector.

Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. A pulseless pump must be used, and care must be taken to ensure that the pH, ionic strength, and temperature of the mobile phase remain constant. Working electrodes are prone to contamination by reaction products with consequent variable responses.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols. New detectors continue to be developed in attempts to overcome the deficiencies of those being used.

DATA COLLECTION DEVICES Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation. Data also may be collected on simple recorders for manual measurement or on standalone integrators, which range in complexity from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible subsequent reprocessing.

Performance

Criteria for assessing the suitability of the system are described in the "Chromatographic Separation Techniques" (Appendix 2.4). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given.

Procedure

The mobile phase composition significantly influences chromatographic performance and the resolution of compounds in the mixture being chromatographed. For accurate quantitative work, high-purity reagents and "HPLC grade" organic solvents must be used.

Water of suitable quality should have low conductivity and low UV absorption, appropriate to the intended use.

Reagents used with special types of detectors (e.g., electrochemical, mass spectrometer) may require the establishment of additional tolerances for potential interfering species. Composition has a much greater effect than temperature on the capacity factor, k'.

In partition chromatography, the partition coefficient, and hence the separation, can be changed by addition of another component to the mobile phase. In ion-exchange chromatography, pH and ionic strength, as well as changes in the composition of the mobile phase, affect capacity factors. The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. It is sometimes used to chromatograph complex mixtures

of components differing greatly in their capacity factors. Detectors that are sensitive to change in solvent composition, such as the differential refractometer, are more difficult to use with the gradient elution technique.

The detector must have a broad linear dynamic range, and compounds to be measured must be resolved from any interfering substances. The linear dynamic range of a compound is the range over which the detector signal response is directly proportional to the amount of the compound. For maximum flexibility in quantitative work, this range should be about three orders of magnitude. HPLC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or an internal standardization procedure.

Reliable quantitative results are obtained by external calibration if automatic injectors or autosamplers are used. This method involves direct comparison of the peak responses obtained by separately chromatographing the test and reference standard solutions. If syringe injection, which is irreproducible at the high pressures involved, must be used, better quantitative results are obtained by the internal calibration procedure where a known amount of a noninterfering compound, the internal standard, is added to the test and reference standard solutions, and the ratios of peak responses of drug and internal standard are compared.

2.4 Chromatographic Separation Techniques

Interpretation of Chromatograms

Figure 1 represents a typical chromatographic separation of two substances, 1 and 2, where t_1 and t_2 are the respective retention times. h, $h/2$, and $W_{h/2}$ are the height, the half-height, and the width at half-height, respectively, for peak 1. W_1 and W_2 are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.

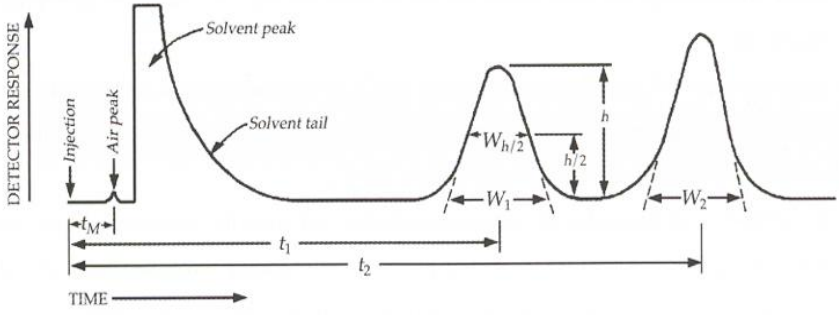


Fig. 1 Chromatographic Separation of Two Substances

Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next.

Because in most procedures there is no need to identify an unretained peak, comparisons are normally made in terms of relative retention times, R_r:

$$R_r = \frac{t_2}{t_1}$$

where t_2, and t_1 are retention times, measured from the point of injection, of the test and reference substances, respectively, determined under identical experimental conditions on the same column.

Other procedures may identify the peak position using the relative retention, r:

$$r = \frac{t_2 - t_M}{t_1 - t_M}$$

where t_M is the retention time of a non-retained marker, which needs to be defined in the procedure.

The number of theoretical plates, N, is a measure of column efficiency. For Gaussian peaks, it is calculated by the equation:

$$N = 16(\frac{t}{W})^2$$

where t is the retention time of the substance and W is the width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. The value of N depends on the substance being chromatographed as well as the operating conditions such as mobile phase or carrier gas flow rates and temperature, the quality of the packing, the uniformity of the packing within the column and, for capillary columns, the thickness of the stationary phase film, and the internal diameter and length of the column.

The separation of two components in a mixture, the resolution, R, is determined by the equation:

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$$

in which t_2 and t_1 are the retention times of the two components, and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Where electronic integrators are used, it may be convenient to determine the resolution, R, by the equation:

$$R = \frac{2(t_2 - t_1)}{1.70(W_{1,h/2} + W_{2,h/2})}$$

and to determine the number of theoretical plates, N, by the equation:

$$N = 5.54(\frac{t}{W_{h/2}})^2$$

where $W_{h/2}$ is the peak width at half-height, obtained directly by electronic integrators. However, in the event of dispute, only equations based on peak width at baseline are to be used.

Peak areas and peak heights are usually proportional to the quantity of compound eluting. These are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. For manual measurements, the chart should be run faster than usual, or a comparator should be used to measure the width at half-height and the width at the base of the peak, to minimize error in these measurements. For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak tailing and fronting and the measurement of peaks on solvent tails are to be avoided.

Chromatographic purity tests for drug raw materials are sometimes based on the determination of peaks due to impurities, expressed as a percentage of the area due to the drug peak. It is preferable, however, to compare impurity peaks to the chromatogram of a standard at a similar concentration. The standard may be the drug itself at a level corresponding to, for example, 0.5% impurity, or in the case of toxic or signal impurities, a standard of the impurity itself.

System Suitability

System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the detection sensitivity, resolution, and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

The detection sensitivity is a measure used to ensure the suitability of a given chromatographic procedure for the complete detection of the impurities in the Chromatographic purity or Related compounds tests by injecting a volume of a quantitation limit solution equal to that of the Test solution. Unless otherwise specified in the individual monograph, the quantitation limit solution may be prepared by dissolving the Drug Reference Substance in the same solvent as that used for the Test solution at a 0.05% concentration level relative to the amount of drug substance in the Test solution for drug substances, and a 0.1% level relative to the amount of drug substance in the Test solution for drug products. The signal-to-noise ratio for the drug substance peak obtained with the quantitation limit solution should be not less than 10.

The resolution, *R*, (**Note** All terms and symbols are defined in the Glossary of Symbols) is a function of column efficiency, *N*, and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation, *RSD*, if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

The symmetry factor (or tailing factor), *T*, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced (Fig. 2). In some cases, values less than unity may be observed. As peak asymmetry increases, integration and hence precision, becomes less reliable.

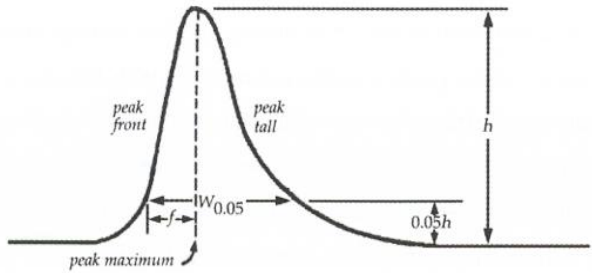


Fig. 2 Asymmetrical Chromatographic Peak

These tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions. Adjustments of operating conditions to meet system suitability requirements may be necessary.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

Relative retention times may be provided in monographs for informational purposes only, to aid in peak identification. There are no acceptance criteria applied to relative retention times.

To ascertain the effectiveness of the final operating system, it should be subjected to suitability testing. Replicate injections of the standard preparation required to demonstrate adequate system precision may be made before the injection of samples or may be interspersed among sample injections. System suitability must be demonstrated throughout the run by injection of an appropriate control preparation at appropriate intervals. The control preparation can be a standard preparation or a solution containing a known amount of analyte and any additional materials useful in the control of the analytical system, such as excipients or impurities. Whenever there is a significant change in equipment or in a critical reagent, suitability testing should be performed before the injection of samples. No sample analysis is acceptable unless the requirement of system suitability have been met. Sample analyses obtained while the system fails system suitability requirements are unacceptable.

Adjustment of Chromatographic Conditions

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are listed below for information. The chromatographic conditions described have been validated during the elaboration of the monograph. The system suitability tests are included to ensure the separation required for satisfactory performance of the test or assay. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reverse-phase liquid chromatographic methods, in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g., octadecylsilyl silica gel) which exhibits the desired chromatographic behaviour.

For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

Multiple adjustments which may have a cumulative effect in the performance of the system are to be avoided.

Thin-layer Chromatography and Paper Chromatography

COMPOSITION OF THE MOBILE PHASE; pH OF THE AQUEOUS COMPONENT OF THE MOBILE PHASE; CONCENTRATION OF SALTS The adjustments can be made as described under High-pressure Liquid Chromatography.

APPLICATION VOLUME The application volume can be adjusted to 10 to 20% of the prescribed volume if using fine particle size plates (2 to 10 μm).

MIGRATION DISTANCE The migration distance of the solvent front is to be not less than 50 mm or 30 mm on high-performance plates.

High-pressure Liquid Chromatography

COMPOSITION OF THE MOBILE PHASE The following adjustment limits apply to minor components of the mobile phase (specified at 50% or less). The amount(s) of these component(s) can be adjusted by ±30% relative. However, the change in any component cannot exceed ±10% absolute (i.e., in relation to the total mobile phase), nor can the final concentration of any component be reduced to zero. Adjustment can be made to one minor component in a ternary mixture. Examples of adjustments are given below.

Specified ratio of 50:50 Thirty% of 50 is 15% absolute, but this exceeds the maximum permitted change of ±10% absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60 to 60:40.

Specified ratio of 2:98 Thirty% of 2 is 0.6% absolute. Therefore, the maximum allowed adjustment is within the range of 1.4:98.6 to 2.6:97.4.

Specified ratio of 60:35:5 For the second component, 30% of 35 is 10.5% absolute, which exceeds the maximum permitted change of ±10% absolute in any component. Therefore, the second component may be adjusted only within the range of 25% to 45% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100%. Therefore, mixture ranges of 50:45:5 to 70:25:5 or 58.5: 35:6.5 to 61.5:35:3.5 would meet the requirement.

pH OF THE AQUEOUS COMPONENT OF THE MOBILE PHASE The pH of the aqueous buffer used in the preparation of the mobile phase can be adjusted to within ±0.2 units of the value or range specified, or ±1.0 units when neutral substances are to be examined.

CONCENTRATION OF SALTS The concentration of the salts used in the preparation of the aqueous buffer used in the mobile phase can be adjusted to within ±10%, provided the permitted pH variation is met.

DETECTOR WAVELENGTH No adjustment permitted.

STATIONARY PHASE
- column length: ±70%,
- column internal diameter: ±25%,
- particle size: maximal reduction of 50%, no increase permitted.

FLOW RATE The flow rate can be adjusted by as much as ±50%.

COLUMN TEMPERATURE The column temperature can be adjusted by as much as ±10°. Column thermostating is recommended to improve control and reproducibility of retention time.

INJECTION VOLUME The injection volume can be reduced as far as is consistent with accepted precision and detection limits.

Gas Chromatography

STATIONARY PHASE column length: ±70%, column internal diameter: ±50%, particle size: maximal reduction of 50%, no increase permitted, film thickness: ±50% to 100%.

FLOW RATE The flow rate can be adjusted by as much as ±50%.

OVEN TEMPERATURE The oven temperature can be adjusted by as much as ±10%.

OVEN TEMPERATURE PROGRAM Adjustment of temperatures is permitted as stated above. For the times specified for the temperature to be maintained or for the temperature to be changed from one value to another, an adjustment of up to ±20% is permitted.

INJECTION VOLUME The injection volume can be reduced as far as is consistent with accepted precision and detection limits.

Glossary of Symbols

To promote uniformity of interpretation, the following symbols and definitions are employed where applicable in presenting formulas in the individual monographs. Where a different symbol or definition is used in an individual monograph, the monograph text takes precedence. (**Note** Where the terms W and t both appear in the same equation, they must be expressed in the same units.)

f distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

k' capacity factor,

$$k' = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}}$$

$$k' = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}} = \frac{t}{t_M} - 1$$

N number of theoretical plates in a chromatographic column,

$$N = 16\left(\frac{t}{W}\right)^2 \text{ or } N = 5.54\left(\frac{t}{W_{h/2}}\right)^2$$

r relative retention,

$$r = \frac{t_2 - t_M}{t_1 - t_M}$$

R resolution between two chromatographic peaks,

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2} \text{ or } R = \frac{2(t_2 - t_1)}{1.70(W_{1,h/2} + W_{2,h/2})}$$

R_r relative retention time,

$$R_r = \frac{t_2}{t_1}$$

RSD (%) relative standard deviation in percentage,

$$RSD(\%) = \frac{100}{\bar{X}} \left[\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{n-1} \right]^{1/2}$$

where X_i is an individual measurement in a set of n measurements and $\bar{X}$ is the arithmetic mean of the set.

T symmetry factor (or tailing factor),

$$T = \frac{W_{0.05}}{2f}$$

t retention time measured from time of injection to time of elution of peak maximum.

t_M retention time of non-retained component, air with thermal conductivity detection.

V retention volume,

$$V = \text{flow rate} \times t$$

V_o retention volume of non-retained component (void volume of the column)

W width of peak measured by extrapolating the relatively straight sides to the baseline.

$W_{h/2}$ width of peak at half height.

$W_{0.05}$ width of peak at 5% height.

Appendix 3 Physical Tests

3.1 Determination of Foaming Index

Many herbal materials contain saponins that can cause a persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of herbal materials and their extracts is measured in terms of a foaming index.

Sample preparation Transfer about 1g of the sample, in coarse powder, accurately weighed, into a 500-ml conical flask containing 100 ml of boiling water. Maintain at moderate boiling for 30 min. Cool, filter and dilute the filtrate with water to 100.0 ml.

Procedure Transfer Sample preparation into 10 stoppered test-tubes (16 mm × 16 cm) in a series of successive portions of 1, 2, 3, up to 10 ml and dilute each tube, if necessary, with water to 10 ml. Stopper and shake in a lengthwise motion for 15 seconds, 2 frequencies per second. Allow to stand for 15 min and measure the height of the foam.

If the height of the foam in every tube is less than 1 cm, the foaming index is less than 100.

If in any tube a height of foam of 1 cm is measured, the dilution in this tube (a) is the index sought. If this tube is the first or second tube in a series, it is necessary to have an intermediate dilution prepared in a similar manner to obtain a more precise result.

If the height of the foam is more than 1 cm in every tube, the foaming index is over 1000. In this case the determination needs to be made on a new series of dilutions of the Sample preparation in order to obtain a result.

Calculation

$$\text{Foaming index} = 1000/a$$

where a is the volume in ml of the Sample preparation used for preparing the dilution in the tube where foaming is observed.

3.2 Determination of Refractive Index

The refractive index (n) of a substance is the ratio of the velocity of light in a vaccum to its velocity in the substance. It varies with the wavelength of the light (λ) used in its measurement and with the temperature (t). It is therefore necessary to specify these conditions (n_λ^t). In practice it is usually convenient to measure the refraction with respect to air and the substance, rather than with respect to a vacuum and the substance, since, for Pharmacopoeial purposes, this has no significant influence on the observed values.

The refractive index may also be defined as the ratio of the sine of the angle of incidence to the sine of the angle of refraction.

The measurement of the refractive index is employed for Pharmacopoeial purposes mainly to establish the identity of liquid substances. It may also be used to test the purity of such substances.

Refractive indices are usually stated in terms of the wavelength of the sodium D line (589.3 nm) and at a temperature of 25° (n_D^{25}) unless otherwise specified in the individual monograph.

The accuracy of the measurement should be related to the requirements of the monograph. For Pharmacopoeial purposes it is usually adequate to express the refractive index to three decimal places.

Apparatus

Commercial refractometers are normally constructed for use with white light but are calibrated to give the refractive index in terms of the sodium D wavelength (589.3 nm).

The optical parts of the apparatus should be kept brilliantly clean. The working surfaces of prisms should be free from scratches.

Subject to the directions given above, the manufacturer's instructions relating to a suitable light source should be followed.

The instrument should be calibrated against a standard provided by the manufacturer; the temperature control of the liquid being examined and the cleanliness of the prism should be checked frequently by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25°.

3.3 Determination of Water

Azeotropic Distillation Method

Apparatus The apparatus (see figure) consists of a glass flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and a reflux condenser (C). The receiving tube (E) is graduated in 0.1-ml subdivisions so that the error of reading is not greater than 0.05 ml. The source of heat is preferably an electric heater with rheostat control or an oil-bath. The upper portion of the flask and the connection tube may be insulated with asbestos.

Method Clean the receiving tube and the condenser of the apparatus by a suitable method, thoroughly rinse with water, and dry.

Introduce 200 ml of toluene and about 2 ml of water into the dry flask. Distil for about 2 hr, allow to cool to room temperature and read the water volume to an accuracy of 0.05 ml. Place in the flask a quantity of the substance, weighed to the nearest centigram, expected to give about 2 to 3 ml of water. If the substance is of a pasty character, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 min. When the toluene begins to boil,

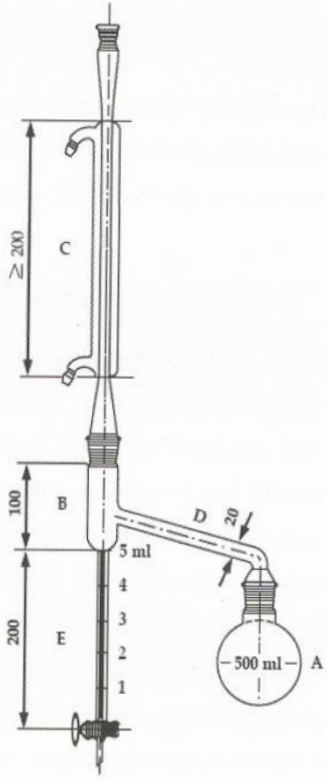
distil at the rate of 2 drops per second until most of the water has distilled over, and then increase the rate of distillation to about 4 drops per second. When the water has all distilled over, rinse the inside of the condenser tube with toluene. Continue the distillation for 5 min, remove the heat, allow the receiving tube to cool to room temperature, and dislodge any droplets of water which adhere to the walls of the receiving tube. When the water and toluene have completely separated, read the volume of water and calculate the percentage present in the substance using the expression:

$$\frac{100(n'-n)}{p}$$

where p = the weight in g of the substance to be examined,

n = the volume in ml of water obtained in the first distillation, and

n' = the total volume in ml of water obtained in the two distillations.



Apparatus for Determination of Water by the Azeotropic Distillation Method

Dimensions in mm

3.4 Determination of Weight per Millilitre, Specific Gravity and Relative Density

Weight per Millilitre

The weight per millilitre of a liquid is the weight in g of 1 ml of a liquid when weighed in air at 20°, unless otherwise specified in the monograph.

The weight per millilitre is determined by dividing the weight in air, expressed in g, of the quantity of liquid that fills a pycnometer at the specified temperature by the capacity, expressed in ml, of the pycnometer at the same temperature. The capacity of the pycnometer is ascertained from the weight in air, expressed in g, of the quantity of water required to fill the pycnometer at that temperature. The weight of a litre of water at specified temperatures when weighed against brass weights in air of density 0.0012 g per ml is given in the following table. Ordinary deviations in the density of air from the above value, here taken as the mean, do not affect the result of a determination in the significant figures prescribed for Pharmacopoeial substances.

Temperature (°)	Weight of a Litre of Water (g)
20	997.18
25	996.02
30	994.62

Specific Gravity

Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids, and, unless otherwise stated, is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of water at the same temperature. Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of liquid in air at the specified temperature to that of an equal volume of *water* at the same temperature. When the substance is a solid at 25°, determine the specific gravity of the melted material at the temperature directed in the individual monograph, and refer to *water* at 25°.

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic metre or grams per cubic centimetre ($1\ kg.m^{-3} = 10^{-3}\ g.cm^{-3}$).

Unless otherwise directed in the individual monograph, use Method I.

Method I Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recently boiled *water* contained in it at 25°. Adjust the temperature of the liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess of the liquid, and weigh. When the monograph

specifies a temperature different from 25°, filled pycnometers must be brought to the temperature of the balance before they are weighed. Subtract the tare weight from the filled weight.

The specific gravity of the liquid is the quotient obtained by dividing the weight of the liquid contained in the pycnometer by the weight of water contained in it, both determined at 25° unless otherwise directed in the individual monograph.

Method II The procedure includes the use of the Oscillating transducer density meter. The apparatus consists of the following:

— a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;

— a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;

— a means of measuring the oscillation period *(T),* which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants *A* and *B* described below; and

— a means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested.

The oscillation period is a function of the spring constant *(c)* and the mass of the system:

$$T^2 = \left(\frac{M}{c} + \frac{\rho \times V}{c} \right) \times 4\pi^2,$$

where ρ is the density of the liquid to be tested, M is the mass of the tube, and V is the volume of the filled tube.

Introduction of two constants, $A = c/(4\pi^2 \times V)$ and $B = M/V$, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the expression:

$$\rho_L/\rho_W,$$

where ρ_L and ρ_W are the densities of the liquid and water, respectively, both determined at 25°, unless otherwise directed in the individual monograph.

CALIBRATION The constants *A* and *B* are determined by operating the instrument with the U-tube filled with two different samples of known density (e.g., degassed water and air). Perform the control measurements daily, using degassed water: the results displayed for the control measurement using degassed water do not deviate from the reference value (ρ_{25} = 0.997043 g.cm^{-3}) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator frequency. Density meters are able to achieve measurements with an error on the

order of 1×10^{-3} g.cm^{-3} to 1×10^{-5} g.cm^{-3} and a repeatability of 1×10^{-4} g.cm^{-3} to 1×10^{-6} g.cm^{-3}. For example, an instrument specified to $\pm1\times10^{-4}$ g.cm^{-3} must display 0.9970±0.0001 g.cm^{-3} in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

PROCEDURE Using the manufacturer's instructions, perform the measurements using the same procedure as for Calibration. If necessary, equilibrate the liquid to be examined at 25° before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

— temperature uniformity throughout the tube,

— nonlinearity over a range of density,

— parasitic resonant effects, and

— viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence.

Relative Density

The relative density $d_{t_2}^{t_1}$ of a substance is the ratio of the mass of a certain volume of a substance at temperature t_1 to the mass of an equal volume of water at temperature t_2. Unless otherwise indicated, the relative density d_{20}^{20} is used. Relative density is also commonly expressed as d_4^{20}.

Density ρ_{20}, defined as the mass of a unit volume of the substance at 20°, may also be used, expressed in kilograms per cubic metre or grams per cubic centimetre (1 kg.m^{-3} = 10^{-3} g.cm^{-3}). These quantities are related by the following equations where density is expressed in grams per cubic centimetre:

$$\rho_{20} = 0.998203 \times d_{20}^{20} \qquad \text{or} \qquad d_{20}^{20} = 1.00180 \times \rho_{20}$$

$$\rho_{20} = 0.999972 \times d_4^{20} \qquad \text{or} \qquad d_4^{20} = 1.00003 \times \rho_{20}$$

$$d_4^{20} = 0.998230 \times d_{20}^{20}$$

Relative density or density is measured with the precision to the number of decimals prescribed in the monograph using a density bottle (solids or liquids), a hydrostatic balance (solids), a hydrometer (liquids) or a digital density meter with an oscillating transducer (liquids and gases). When the determination is made by weighing, the buoyancy of air is disregarded, which may introduce an error of 1 unit in the third decimal place. When using a density meter, the buoyancy of air has no influence.

Proceed as directed under "Specific Gravity, Method II."

3.5 Loss on Drying

The procedure set forth in this section determines the amount of volatile matter of any kind that is driven off under the conditions specified. For substances appearing to contain water as the only volatile constituent, the procedure given in "Determination of Water" (Appendix 3.3), is appropriate, and is specified in the individual monograph.

Unless otherwise directed in the monograph, conduct the determination on 1 to 2 g of the substance (2 to 5 g in case of crude drugs), previously mixed and accurately weighed. If the test substance is in the form of large crystals, reduce the particle size to about 2 mm by quickly crushing. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 min under the same conditions to be employed in the determination. Put the test substance in the bottle, replace the cover, and accurately weigh the bottle and the contents. By gentle, sidewise shaking distribute the test substance as evenly as practicable to a depth of about 5 mm generally, and not over 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber. Dry the test substance at the temperature and for the time specified in the monograph. The temperature of heating is within the range of ±2° of the stated figure in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of Loss on drying, maintain the bottle with its contents for 1 to 2 hr at a temperature 5° to 10° below the melting temperature, then dry at the specified temperature.

Where the sample under test is Capsules, use a portion of the mixed contents of not less than 4 capsules.

Where the sample under test is Tablets, use powder from not less than 4 tablets ground to a fine powder.

Where the individual monograph directs that loss on drying be determined by thermogravimetric analysis, a sensitive electrobalance is to be used.

Where drying in vacuum over a desiccant is directed in the individual monograph, a vacuum desiccator or a vacuum drying pistol, or other suitable vacuum drying apparatus, is to be used.

Where drying in a desiccator is specified, exercise particular care to ensure that the desiccant is kept fully effective by frequent replacement.

Where drying in a capillary-stoppered bottle in vacuum is directed in the individual monograph, use a bottle or tube fitted with a stopper having a 225±25 µm diameter capillary, and maintain the heating chamber at a pressure not exceeding 0.7 kPa (about 5 Torr). At the end of the heating period,

admit dry air to the heating chamber, remove the bottle, and with the capillary stopper still in place allow it to cool in a desiccator before weighing.

Appendix 4 Crude Drugs

4.1 Foreign matter

Vegetable drugs should be free from moulds, insects and other animal contamination. Foreign matter is material consisting of any or all of the following:

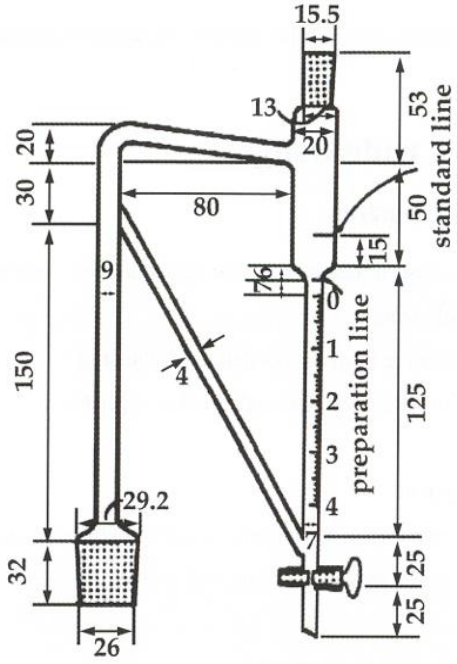
1. *Foreign organs*: matter coming from the source plant but not defined as the drug.
2. *Foreign elements*: matter not coming from the source plant and of either vegetable or mineral origin.

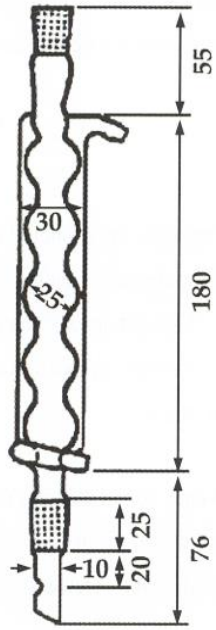
Method

Weigh 100 to 500 g of the substance being examined or the quantity specified in the monograph and spread it in a thin layer. Separate the foreign matter by hand as completely as possible, weigh it and calculate the percentage present.

4.2 Determination of Volatile Oil

Carry out the determination according to the nature of the drug to be examined. Place the prescribed quantity of the drug in the round-bottomed flask of suitable capacity as specified in the monograph and add the prescribed volume of distillation liquid. Set up the apparatus (Fig. 1), inserting a reflux condenser (Fig. 2). Fill the graduated tube with water to the standard line and add the prescribed volume of xylene. Heat the liquid in the flask in an oil-bath between 130° and 150° to boiling and adjust the distillation rate as prescribed in the monograph. Unless otherwise specified, continue boiling for 5 hr at the same rate. Allow it to stand for some time, open the stopper of the apparatus and draw off the water slowly until the level of the xylene and volatile oil mixture corresponds to the preparation line, and allow it to stand for more than 1 hr at cool temperature. Then lower the level of the xylene and volatile oil mixture to the zero line, and read the volume in millilitre of the mixture of xylene and volatile oil at cool temperature. Subtract the volume in millilitre of xylene from the volume of the mixture of xylene and volatile oil and calculate the percentage of volatile oil content in the sample.





Dimensions in mm

Fig. 1 **Fig. 2**

4.3 Acid-insoluble Ash

Use Method I unless otherwise indicated in the monograph.

Method I

Boil the total ash for 5 min with 25 ml of dilute hydrochloric acid, collect the insoluble matter on an ashless filter paper, wash with hot water until the filtrate is neutral, and ignite at about 500°. Calculate the percentage of acid-insoluble ash with reference to the air-dried substance.

Method II

Place the total ash or the sulfated ash, as directed in the monograph, in a crucible, add 15 ml of water and 10 ml of hydrochloric acid, cover with a watch glass, and boil for 10 min; allow to cool. Collect the insoluble matter on an ashless filter paper, wash with hot water until the filtrate is neutral, ignite to dull redness (550° to 700°), cool in a desiccators and weigh. Reheat until the difference between two successive weighings is not more than 1 mg. Calculate the percentage of acid-insoluble ash with reference to the air-dried substance.

4.4 Total Ash

Use Method I unless otherwise directed in the compendium monograph.

Method I

For vegetable drugs Incinerate 2 to 3 g of the ground drug in a tared platinum or silica dish at a temperature not exceeding 450° until free from carbon, cool and weigh. If a carbon-free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ashless filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness and ignite at a temperature not exceeding 450°. Calculate the percentage of total ash with reference to the air-dried drug.

For other substances Carry out the above method using 1 g, unless otherwise stated. Calculate the percentage of total ash.

Method II

Heat a silica or platinum crucible to red heat for 30 min, allow to cool in a desiccators and weigh. Unless otherwise specified in the monograph, evenly distribute 1 g of the substance being examined in the crucible, dry at 100° to 105° for 1 hr and ignite to constant weight in a muffle furnace at 600°±25°. Allow the crucible to cool in a desiccators after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition a carbon-free ash cannot be obtained, take up with hot water, filter through an ashless filter paper and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant weight.

4.5 Extractives

Ethanol-soluble Extractive

Use Method I unless otherwise indicated in the monograph.

Method I Macerate 5 g of the air-dried drug, coarsely powdered and accurately weighed, with 100.0 ml of ethanol of the specified strength in a closed flask for 24 hr, shaking frequently during the first 6 hr and then allowing to stand for 18 hr. Filter rapidly, taking precautions against loss of ethanol, evaporate 20.0 ml of the filtrate to dryness in a tared, flat-bottomed, shallow dish and dry at 105° to constant weight. Calculate the percentage of ethanol-soluble extractive with reference to the air-dried drug.

Method II Transfer about 4 g of the air-dried drug, coarsely powdered and accurately weighed, to a glass-stoppered conical flask. Add 100 ml of ethanol of the specified strength, and weigh the flask. Shake and allow to stand for 1 hr. Attach a reflux condenser to the flask. Boil

gently for 1 hr, cool, and weigh. Readjust to the original weight with ethanol. Shake, and filter rapidly through a dry filter. Transfer 25.0 ml of the filtrate to a tared flat-bottomed dish, and evaporate on a water-bath to dryness. Dry at 105° for 6 hr, cool in a desiccator for 30 min, and weigh without delay. Calculate the percentage of ethanol-soluble extractive with reference to the air-dried drug.

Water-soluble Extractive

Use Method I unless otherwise indicated in the monograph.

Method I Proceed as directed in Method I under Ethanol-soluble Extractive but using chloroform water in place of ethanol.

Method II Proceed as directed in Method II under Ethanol-soluble Extractive but using chloroform water in place of ethanol.

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Department of Medical Sciences, Ministry of Public Health, Thailand
88/7 Tiwanon Rd. Amphoe Muang, Nonthaburi 11000
Tel. 0 2951 0000
www.dmsc.moph.go.th