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QUANTITATIVE DETERMINATION OF BIOLOGICALLY ACTIVE CONSTITUENTS IN MEDICINAL PLANT CRUDE EXTRACTS BY THIN-LAYER CHROMATOGRAPHY–DENSITOMETRY

I. *AESCULUS HIPPOCASTANEUM* L., *ARCTOSTAPHYLLOS UVA-URSI* SPRENG, *FRAXINUS EXCELSIOR* L., *GENTIANA LUTEA* L., *GLYCYRRHIZA GLABRA* L., *HAMAMELIS VIRGINIANA* L., *HYPERICUM PERFORATUM* L., *OLEA EUROPEA* L., *SALIX ALBA* L. AND *SILYBUM MARIANUM* GAERTN

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SUMMARY

Quantitative determination by thin-layer chromatography–densitometry of the biologically active and/or characteristic constituents of spray-dried aqueous, hydroethanolic or ethanolic extracts of the following ten medicinal plants is reported: *Aesculus hippocastaneum* (aescin), *Arctostaphyllos uva-ursi* (arbutin), *Fraxinus excelsior* (fraxin), *Gentiana lutea* (gentiopicrin), *Glycyrrhiza glabra* (glycyrrhizic acid), *Hamamelis virginiana* (gallic acid, tannins), *Hypericum perforatum* (hypericin, pseudohypericin), *Olea europea* (oleuropein), *Salix alba* (salicin) and *Silybum marianum* (silybin). In all cases, variation coefficients of these rapid and reliable methods were lower than 3.5%.

INTRODUCTION

In recent years, the use of medicinal plants and crude extracts has widely progressed. It can be assumed that this trend will not continue unless standardization methods for these plant materials become available. Numerous methods of identification for plant extracts have been proposed[1–5]; on the other hand, the quantitative determination of their biologically active constituents has been less studied, especially with regard to drugs used in phytotherapy. The improvement of densitometric instruments and new developments of the thin-layer chromatography (TLC)–densitometric methods (*e.g.*, ref. 6) indicate that this analytical procedure could be convenient for the analysis of plant extracts.

Therefore, TLC–densitometry was proposed for the rapid quantitative determination of the biologically active and/or characteristic constituents of spray-dried

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aqueous, hydroethanolic or ethanolic extracts of *Aesculus hippocastaneum*, fruit (aescin), *Arctostaphyllos uva-ursi*, leaves (arbutin); *Fraxinus excelsior*, leaves (fraxin); *Gentiana lutea*, roots (gentiopicrin); *Glycyrrhiza glabra*, roots (glycyrrhizic acid); *Hamamelis virginiana*, leaves (gallic acid, tannins); *Hypericum perforatum*, flowering tops (hypericin, pseudohypericin); *Olea europea*, leaves (oleuropein); *Salix alba*, barks (Salicin); and *Silybum marianum*, seeds (silybin).

EXPERIMENTAL

Plant extracts

Spray-dried extracts Extrenorm were obtained from Expansion Aromatique Française (rue Ambroise Croizat 1, F-94800 Villejuif, France).

Standards

Aescin (a crystalline mixture of triterpenic saponins), arbutin and hypericin (1:1 mixture of hypericin and pseudohypericin) were obtained from Carl Roth (Schoemperlenstrasse 1–5, D-7500 Karlsruhe, F.R.G.), gallic acid, oleuropein and salicin from Sarsynthex (avenue Président J. F. Kennedy, BP 100, F-33701, Mérignac, France) and glycyrrhizic acid (ammonium salt) from Fluka (Buchs, Switzerland).

Silybin was kindly supplied by Dr. A. Bonati (Inverni de la Beffa), fraxin by Dr. J. Ripphan (Merck) and gentiopicrin by Dr. B. Meier (Pharmazeutisches Institüt of the ETH of Zürich.

In some cases, these standards were purified by semi-preparative high-performance liquid chromatography (HPLC).

Equipment

A Shimadzu high-speed TLC-scanner CS-920 was used with the following settings: beam size 0.4 × 0.4 mm; $X = 9$ mm; $Y = 9$ mm; linearizer on position 1 for absorption measurements and on position 0 for fluorometry, AZS off; wavelengths are given in the figure captions.

Semi-preparative HPLC was performed on Lichroprep RP-18 (particle size 5–20 μm, Merck) packed in a stainless-steel column (25 cm × 22 mm I.D.), connected to a Milton Roy pump (flow-rate 12 ml/min), a Valco valve, a Waters differential refractometer R-401 and a Shimadzu spectrophotometer UV-120-02 equipped with a Hellma flow-through cell.

Sample preparation

The solutions of the spray-dried extracts in water (5 ml) were diluted to 10 ml with methanol and filtered; solubilization was improved using an ultrasonic bath for 15 min. Water alkalized by two drops of 25% ammonia was used as solvent for Glycyrrhiza glabra, whereas pyridine was used for *Hypericum perforatum* extracts. The same solvents were used for the corresponding standards.

Thin-layer chromatography

TLC plates silicagel 60 or silicagel $60F_{254}$ (10 × 20 cm; normal and for HPTLC) and HPTLC precoated plates RP-8 (10 × 10 cm, Merck) were used for TLC (adsorbents specified in the figure captions) performed in unsaturated tanks (excepted for *Glycyrrhiza glabra*, saturated tank).

One μl of each solution (1 μl micropipettes, Drummond) was spotted 15 mm from the lower edge of the chromatoplate and developed with the solvent systems specified in the figure captions. The mobile phase was allowed to run a distance of 100 mm, except for *Hypericum perforatum* (40 mm). After development and solvent evaporation, spot areas were integrated by TLC–densitometry (UV or visible) or fluorometry.

Calculations

Each determination corresponded to the mean value calculated from the integration results of four chromatograms (one spot of the extract solution and three different standard concentrations; lower and upper limits given in Table I) repeated four times (10 × 20 cm plates) or twice (10 × 10 cm HPTLC RP-8 plates) on the plates.

RESULTS AND DISCUSSION

Quantitative determination of the biologically active constituents of the ten medicinal plants studied in this paper has not previously been achieved by TLC–densitometry, with the exception of *Glycyrrhiza glabra*[7]; in this case, the mobile phase composition was modified in order to improve the glycyrrhizic acid separation.

The determinations were performed on spray-dried aqueous, hydroethanolic or ethanolic extracts of these drugs which are, in our opinion, considered as the best actual forms for plants extracts (better stability of the constituents during industrial treatment and storage, lower microbiological contamination); they were related to the main biologically active constituent(s) selected according to current pharmacological knowledge. Gentiopicrin was considered as sufficiently characteristic of the roots of *Gentiana lutea;* other more important bitter constituents (amarogentin, amaroswerin, amaropanin) were not measurable by the proposed method without preliminary fractionation.

The measured constituents are chemically well defined. However, the tannins of *Hamamelis* leaves consist of a mixture of gallotannins[8], the chemical structures of which have not yet been completely elucidated. This means that the densitometric results obtained with this drug were arbitrarily expressed in comparison (UV absorption at 300 nm) with gallic acid, which (i) could be obtained by hydrolysis of the tannins mixture (ii) was simultaneously determined in order to control the possible hydrolysis of the tannins during the industrial treatment and the storage of the drug.

Moreover, experimental conditions used allowed the separation of "hypericin" into two constituents, hypericin and pseudohypericin[9]; this occurred with the standard as well as with the crude extract of *Hypericum perforatum.* Development was stopped at 40 mm in this case to avoid further diffusion of the two spots, which are readily measured because of the high specificity of the fluorescence emitted by hypericin and pseudohypericin under an excitation wavelength of 313 nm.

Under the selected experimental conditions, the constituents of aescin were intentionally not separated in order to allow comparison of the densitometric results with the commercially available standard.

The antihepatotoxic constituents of *Silybum marianum* consists mainly of a mixture of silybin, isosilybin, silydianin and silychristin (silymarin). Silybin has been

TABLE I

PREPARATION CONDITIONS OF THE EXTRACTS AND STANDARD SOLUTIONS: CORRELATION COEFFICIENTS OF THE CALIBRATION GRAPHS

Plant material	*Solvents for extracts and standards dissolution*	*Extracts concentration**	*Standards concentration lower and upper limits**	*r Value (correlation coefficient) of the calibration graphs*
Aesculus hippocastaneum	Water–methanol (1:1)	100 mg in 10 ml	7.5–30 mg aescin/10 ml	0.996
Arctostaphyllos uva-ursi	Water–methanol (1:1)	100 mg in 10 ml	8–40 mg arbutin/10 ml	0.994
Fraxinus excelsior	Water–methanol (1:1)	200 mg in 10 ml	0.4–1.2 mg fraxin/10 ml	0.994
Gentiana lutea	Water–methanol (1:1)	500 mg in 10 ml	7–25 mg gentiopicrin/10 ml	0.997
Glycyrrhiza glabra	Water alkalized by two drops of 25% ammonia	80 mg in 10 ml	12.5–25 mg glycyrrhizic acid (ammonium salt)/10 ml	0.993
Hamamelis virginiana	Water–methanol (1:1)	(A) Gallic acid assay 200 mg in 10 ml	4–16 mg gallic acid/10 ml	0.992
		(B) Tannin assay 40 mg in 10 ml	4–16 mg gallic acid/10 ml	0.992
Hypericum perforatum	Pyridine	800 mg in 10 ml	1.5–2.5 mg hypericin/10 ml	0.991
Olea europea	Water–methanol (1:1)	250 mg in 10 ml	25–50 mg oleuropein/10 ml	0.997
Salix alba	Water–methanol (1:1)	600 mg in 10 ml	20–70 mg salicin/10 ml	0.998
Silybum marianum	Methanol	70 mg in 10 ml	2–7.5 mg silybin/10 ml	0.997

* 1 μl of each solution (extracts or standards) was spotted.

shown to be more active than the other constituents[10], so it was selected for evaluation.

High resolution of the measured constituents from other compounds was obtained by selection of specific adsorbents, mobile phases and detection conditions (Table I, Figs. 1–10); R_F values are given in Table II. The use of HPTLC plates instead of usual plates (except for *Salix alba, Silybum marianum* and *Hypericum perforatum*) was not essential but increased the resolution and the reproducibility of the results. A better visualisation of the chromatograms was obtained using silicagel 60(F_{254}).

In contrast to the gas–liquid chromatography and HPLC methods, no derivatization or prepurification steps were required: the crude extracts were solubilized

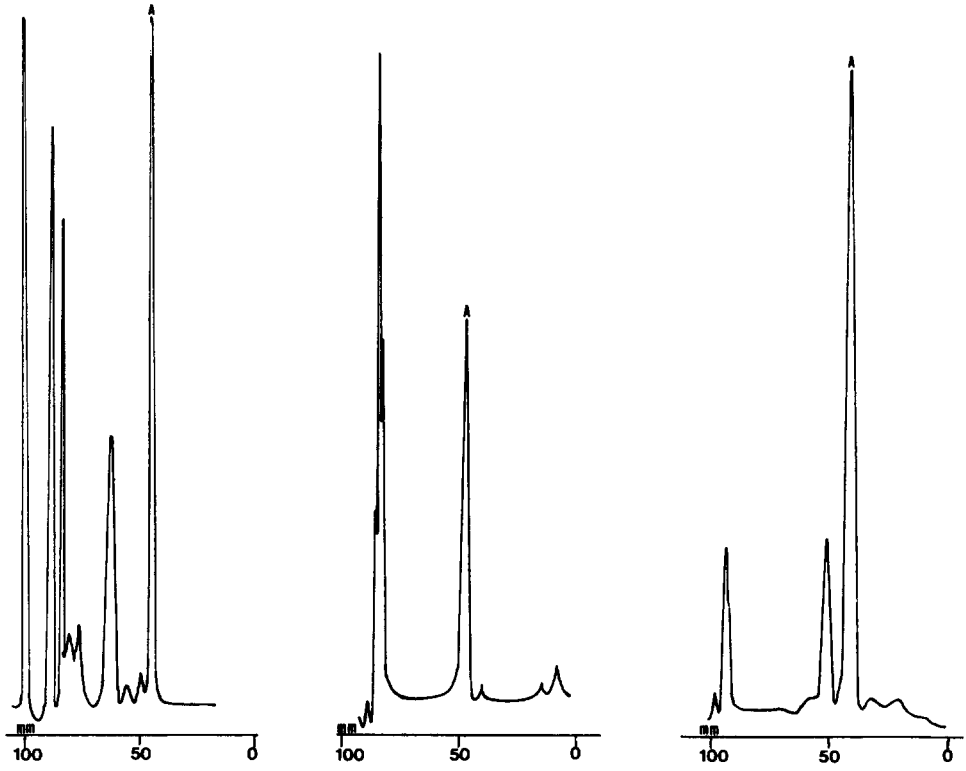

Fig. 1. Scanning profile of a spray-dried extract of *Aesculus hippocastaneum*. Adsorbent, silicagel 60(F_{254}); mobile phase, 1,2-dichloroethane–ethanol–methanol–water (50:20:20:6); detection, the chromatogram was sprayed with a mixture (1:1) of a 1% vanillin ethanolic solution and a 5% sulphuric acid ethanolic solution, heated at 120°C for 5 min and measured at 530 nm (absorption) after colour stabilization (10 min). Peak A = aescin.

Fig. 2. Scanning profile of a spray-dried extract of *Arctostaphyllos uva-ursi*. Adsorbent, silicagel 60(F_{254}); mobile phase, ethyl acetate–methanol–water (85:17;13); detection, 225 nm (absorption). Peak A = arbutin.

Fig. 3. Scanning profile of a spray-dried extract of *Fraxinus excelsior*. Adsorbent, silicagel 60(F_{254}); mobile phase, ethyl acetate–2-butanone–water–formic acid (5:3:2:1); detection, excitation wavelength 365 nm; filter 3 (fluorometry). Peak A = fraxin.

in an aqueous methanolic mixture, in water or in pyridine before chromatography (Table I).

The amounts of standards given in Table I allowed us to obtain a linear calibration graph with an r value (correlation coefficient) typically better than 0.990; these concentrations have been adjusted to take account of the amounts of extracts spotted onto the thin layers and the usual proportions of the measured constituents in the crude drug extracts. Other concentrations of pure standards could be used but the linearity of the response should be checked.

As far as possible (*Arctostaphyllos uva-ursi, Gentiana lutea, Glycyrrhiza glabra, Olea europea, Salix alba* and *Silybum marianum*), possible contaminants were excluded by densitometric absorption measurements at three different wavelengths in comparison with the standards.

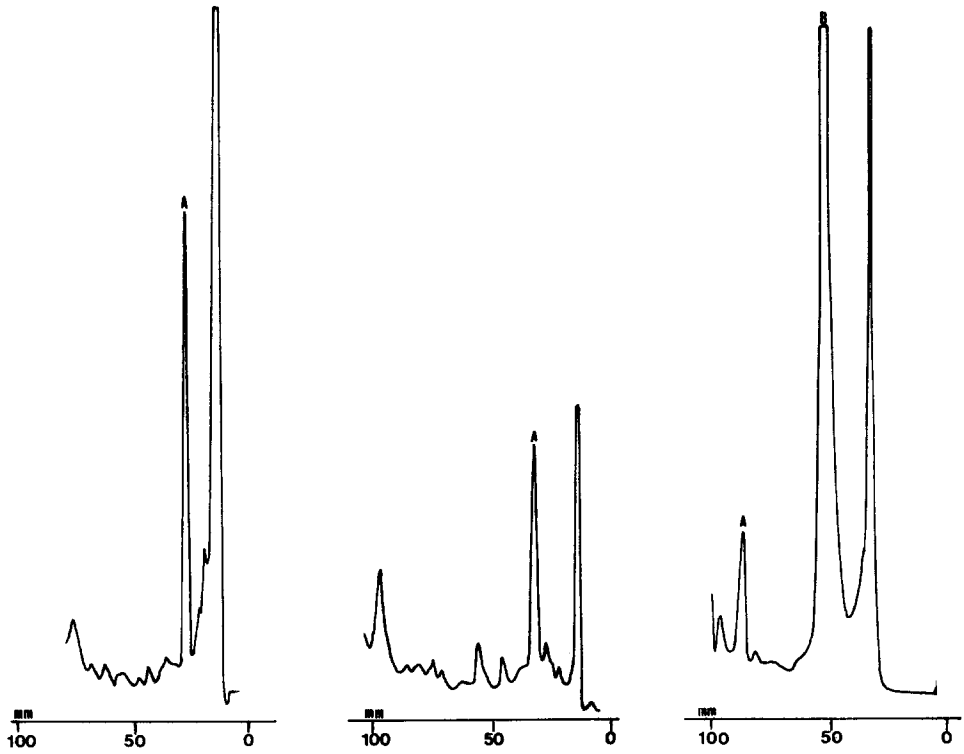

Fig. 4. Scanning profile of a spray-dried extract of *Gentiana lutea*. Adsorbent, silicagel 60(F_{254}); mobile phase, 1,2-dichloroethane–methanol–water (39:10:1); detection, 280 nm (absorption). Peak A = gentiopicrin.

Fig. 5. Scanning profile of a spray-dried extract of *Glycyrrhiza glabra*. Adsorbent, silicagel 60(F_{254}); mobile phase, dichloromethane–ethanol–methanol–25% ammonia (60:30:25:20), saturated tank; detection, 258 nm (absorption). Peak A = glycyrrhizic acid (ammonium salt).

Fig. 6. Scanning profile of a spray-dried extract of *Hamamelis virginiana*. Absorbent, silicagel 60(F_{254}); mobile phase, chloroform–ethanol–formic acid (5:4:1); detection, 300 nm (absorption). Peaks: A = gallic acid; B = tannins.

Solutions of crude extracts were diluted (1:1, v/v) with standard solutions (upper limit concentration, see Table I) in order to control the recovery of the measured constituents; in these experiments, the recovery was better than 95% (Table II).

The results of the quantitative analysis (repeated six times for each drug) and the corresponding variation coefficients (lower than 3.5%) are given in Table II. These results and the simple experimental conditions indicate that the proposed method is sufficiently precise and accurate to contribute to solving the problem of standardization of plant extracts. Moreover, the chromatograms obtained for the quantitative measurements were useful for the specific identification of the drug extracts and the detection of their eventual adulterations; qualitative analysis of the

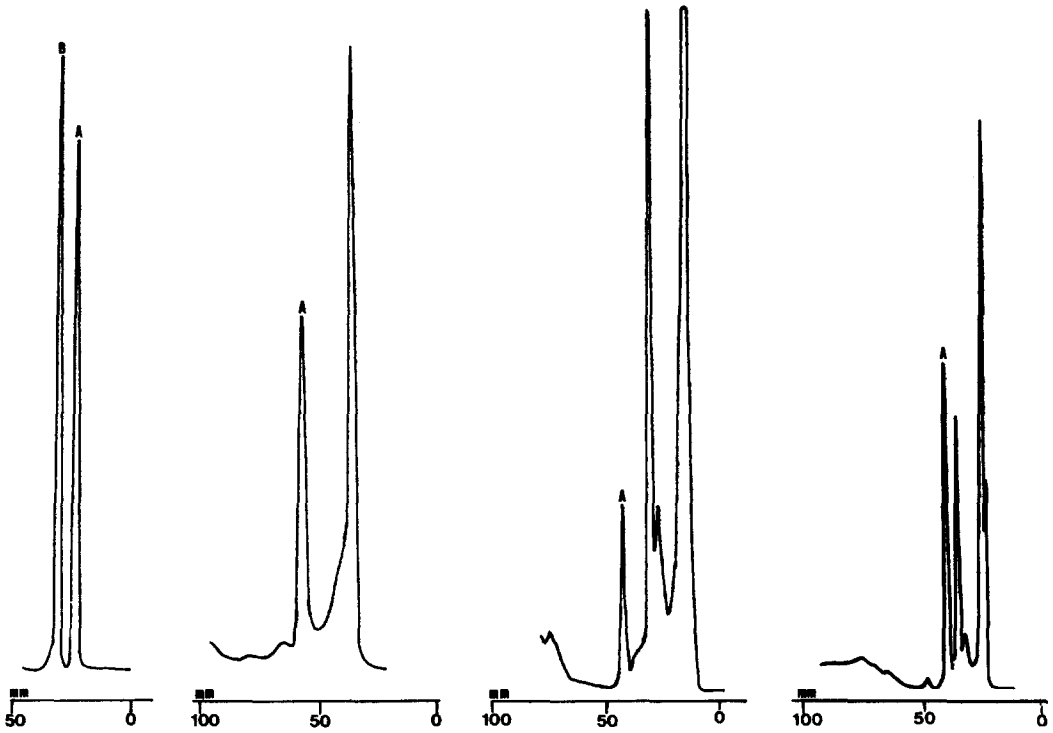

Fig. 7. Scanning profile of a spray-dried extract of *Hypericum perforatum*. Adsorbent, RP-8F_{254}; mobile phase, acetonitrile (development, 40 mm); detection, excitation wavelength, 313 nm; filter 4 (fluorometry). Peaks: A = hypericin; B = pseudohypericin.

Fig. 8. Scanning profile of a spray-dried extract of *Olea europea*. Adsorbent, silicagel 60(F_{254}); mobile phase, dichloromethane–methanol–water (85:15:1.5); detection, 245 nm (absorption). Peak A = oleuropein.

Fig. 9. Scanning profile of a spray-dried extract of *Salix alba*. Adsorbent, silicagel 60(F_{254}) HPTLC plate; mobile phase, dichloromethane–methanol–water (37:12:1); detection, 270 nm (absorption). Peak A = salicin.

Fig. 10. Scanning profile of a spray-dried extract of *Silybum marianum*. Adsorbent, silicagel 60(F_{254}) HPTLC plate; mobile phase, toluene–ethyl acetate–formic acid (40:10:5); detection, 295 nm (absorption). Peak A = silybin.

TABLE II

QUANTITATIVE RESULTS

Plant material	*Extracted yield (extract weight/dried plant material)*	*Quantitative determinations*				
		Constituent	R_F *values*	*Concentration in the extract (%)*	*Variation coefficient*(%)*	*Recovery (%)*
Aesculus hippocastaneum						
Ethanolic spray-dried extract	1/6	Aescin	0.47	18.83	3.2	99
Arctostaphyllos uva-ursi						
Aqueous spray-dried extract	1/3	Arbutin	0.50	18.17	3.3	97
Fraxinus excelsior						
Aqueous spray-dried extract	1/4	Fraxin	0.48	0.45	3.3	96
Gentiana lutea						
Aqueous spray-dried extract	1/4	Gentiopicrin	0.30	3.90	3.2	96
Glycyrrhiza glabra						
Aqueous spray-dried extract	1/5	Glycyrrhizic acid (ammonium salt)	0.18	24.5	3.3	98
Hamamelis virginiana						
Hydroethanolic spray-dried extract	1/6	Gallic acid	0.89	1.10	3.4	95
		Tannin mixture	0.35–0.68	15.94	3.4	–
Hypericum perforatum						
Hydro-ethanolic spray-dried extract	1/3	Hypericin	0.90	0.18	3.4	97
		Pseudohypericin	0.97			
Olea Europea						
Aqueous spray-dried extract	1/6	Oleuropein	0.65	16.86	3.0	99
Salix alba						
Aqueous spray-dried extract	1/10	Salicin	0.43	1.72	3.1	96
Silybum marianum						
Hydroethanolic spray-dried extract	1/6	Silybin	0.44	4.87	3.0	97

* Calculated from six determinations.

chromatograms could be completed after assay by using UV detection (254–360 nm) and/or by spraying reagents such as sulphuric vanillin solution (see *Aesculus hippocastaneum*, Fig. 1) for *Gentiana lutea* and *Olea europea*, 2,6-dichloroquinone-4-chlorimide for *Arctostaphyllos uva-ursi*, an ethanolic ferric chloride solution for *Hamamelis virginiana* and β-aminoethyldiphenylborinate (natural product reagent A) for *Fraxinus excelsior* and *Glycyrrhiza glabra*.

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