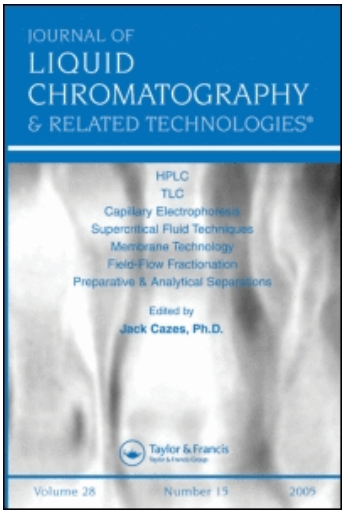

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DETERMINATION OF NAPHTHOQUINONES IN *DROSERAE HERBA* BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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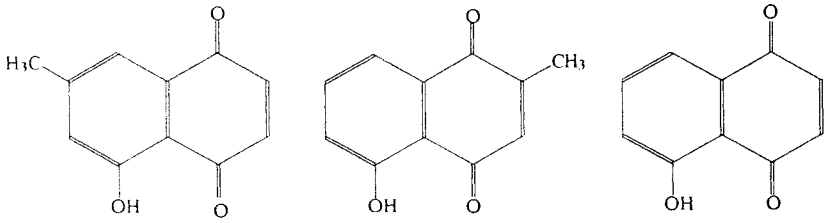
ABSTRACT

The naphthoquinones plumbagin and 7-methyljuglone are considered to be responsible for the spasmolytic and antimicrobial properties of sundew herb (*Hb. Droserae*), used mainly in pediatrics against whooping-cough. For the quality control of the drug a high performance liquid chromatographic method was established, which permits the simultaneous assay of the two substances within 16 minutes. The samples are processed with a base deactivated C_{18} reverse phase column by isocratic elution with 0.2M acetic acid (pH adjusted to 3) : acetonitrile : tetrahydrofuran (62:36,1:1,9 v/v) as mobile phase. The quantification is performed by internal standardization with juglone. Regression equations show linear relationships between the peak area ratios of each naphthoquinone to juglone and concentration. The relative standard deviation for plumbagin was ±1.3 % and for 7-methyljuglone ±4.0 %. The method was applied successfully in the analysis of commercial samples of Hb. Doserae.



7-methyljuglone plumbagin juglone

Figure 1. Chemical structures of the analytes.

INTRODUCTION

The dried aerial parts of different sundew species are mainly used in pediatrics in the therapy of infections of the respiratory tract like convulsive cough or whooping-cough.[1] Traditionally *Drosera rotundifolia* was the source for the drug Hb. Droserae. Due to their imminent extinction *Drosera rotundifolia* and the other European species *Drosera intermedia* and *Drosera anglica*, used as substitutes, are protected by law. Presently, the commercial source for the drug are species from Africa or Asia, e.g. *Drosera madagascariensis* or *Drosera peltata.*[2] 1,4-Naphthoquinones are considered to be responsible for the spasmolytic and antimicrobial effects of Drosera extracts.[3,4] The major ones are plumbagin and 7-methyljuglone (=ramentaceone)[5,6] (for structures see Fig. 1). Hb. Droserae and its extracts or preparations usually are standardized on the total content of 1,4-naphthoquinones by spectrophotometric assay.[7,8,9,10,11] *Drosera madagascariensis* and *Drosera rotundifolia* contain 7-methyljuglone as the major naphthoquinone; in *Drosera peltata* only plumbagin is detectable. Therefore, for the quality control of the drug or its preparations, the separate determination of the compounds is recommended, as this allows the detection of certain adulterations (see below). For the separation and quantification of the single substances only few methods have been published.[12,13,14,15] GLC[12] and HPLC methods[13,14] were used for the analysis of *Drosera* species from in vitro cultivation. GLC[12] seems to allow a sufficient determination of the naphthoquinones, however with LC, using a 254 nm detector, many interferences were found[13].

This paper presents a fast RP-HPLC method for the simultaneous and accurate determination of plumbagin and 7-methyljuglone from sundew by internal standardization with juglone. This new method was applied in the quality control of different commercial samples of Hb. Droserae.

EXPERIMENTAL

Materials

The commercial samples of Hb. Droserae containing *Drosera madagascariensis* were purchased from E. Ritzberger Kräuter-GH (Linz, Austria), A. Galke GmbH (Gittelde, Germany), Kräuter- und Drogenhaus Kottas-Heldenberg & Sohn (Vienna, Austria) and H. Klenk GmbH & Co. KG (Schwebheim, Germany). One sample of Hb. Droserae peltatae was obtained from P. Müggenburg GmbH & Co (Alveslohe, Germany) and one of Hb. Droserae rotundifoliae from A. Galke GmbH (Gittelde, Germany). The latter was identified botanically to be a mixture, *Drosera intermedia* being its main component.

Chemicals and Reagents

Juglone was obtained from Sigma Chemical Co. (St. Louis, USA) and Plumbagin from Carl Roth KG (Karlsruhe, Germany). Acetonitrile and tetrahydrofuran were HPLC grade, acetic acid and triethylamine analytical reagent grade.

Instruments

Instrumentation consisted of two Perkin-Elmer Series 10 Liquid Chromatographs, a Rheodyne 7125 sample injector, an Aston LC Controller and a Perkin-Elmer LC Auto Scan Diode Array Detector. The monitoring of the chromatographic parameters and the processing of the data was performed by Omega Software.

The determination of the total naphthoquinone content according to DAB 7 - DDR was performed on a Hitachi 200 UV/VIS spectrophotometer.

Chromatographic Conditions

The following analytical reversed phase columns, purchased from Shandon (Runcorn, Great Britain) were evaluated for separation: Hyperbond C18 (300 x 4.0 mm, 10 µm particle size), Hypersil ODS (125 x 4.0 mm, 3 µm particle size), Hypersil BDS (250 x 4.6 mm, 5 µm particle size). The columns were operated at ambient temperature (21 ± 1°C).

Mobile phase: acetonitrile with 5 % (v/v) tetrahydrofuran (A) and 0.2 M acetic acid (pH adjusted to 3.0 with triethylamine) (B); isocratic elution with 38% A and 62 % B at a flow rate of 1.0 mL/min with detection at 425 nm.

Standard Solutions

For the calibration of the system individual stock solutions of 7-methyljuglone (dissolved in tetrahydrofuran-toluene 10+3) and of plumbagin (dissolved in acetonitrile) were prepared. Accurately measured aliquots of the stock solutions were mixed with internal standard solution (juglone dissolved in acetonitrile) to give 10 different working solutions with 0,6mg/mL juglone, 0.08 - 0.8 mg/mL 7-methyljuglone and 0.1 - 1.0 mg/mL plumbagin.

Sample Preparation

Two methods were compared for sample preparation to obtain the maximum yield of naphthoquinones in minimum time:

A. To 1.0 g of Hb. Droserae madagascariensis and 0.1 g of Hb. Droserae peltatae resp., 5 g tartaric acid and 15 mL water were added and by the introduction of steam at a temperature of 125-135°C distillation was performed. 250 mL of distillate were collected, mixed with 5 g tartaric acid and partitioned with 30 mL chloroform. The aqueous layer was extracted a second time with 10 mL chloroform. The combined organic layers were filtered and the filter washed with chloroform up to a total volume of 50.00 mL. After evaporation at 240 mbar and 30°C the residue was dissolved in 1.00 mL of the solution of internal standard (6.00 mg juglone in 10.00 mL acetonitrile).

B. 1.0 g of Hb. Droserae madagascariensis and 0.1 g of Hb. Droserae peltatae resp., was moistened with 5 mL water. After addition of 50 ml petroleum ether (b.p. 40 - 60°C) the mixture was sonicated for 15 minutes. The extract was filtered over anhydrous sodium sulfate, which was washed with 25 mL petroleum ether. After evaporation at 240 mbar and 30°C the residue was dissolved in 2 mL of the solution of internal standard (6.00 mg juglone in 10.00 mL acetonitrile).

RESULTS AND DISCUSSION

The goals for development of a new LC assay for naphthoquinones in *Drosera* species were the simultaneous quantification of plumbagin and 7-methyljuglone by internal standardization, short analysis time and further minimization of HPLC time by isocratic elution.

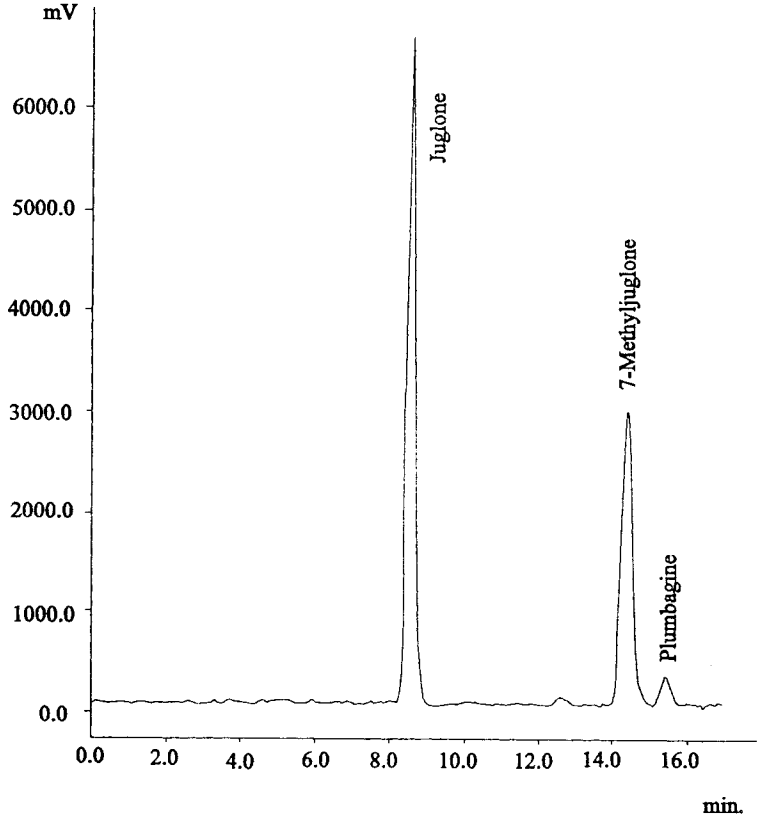


Figure 2. HPLC of an extract of *Drosera madagascariensis*. Column: Hypersil BDS (250 x 4.6 mm, 5 µm particle size).

Table 1

HPLC Data for Juglone, Plumbagin, and 7-Methyljuglone

Substance	Retention Time (min) ±S.D. (n=10)	R.S.D. in %	k'Value	Selectivity Factor α
Juglone	8.54 ± 0.06	0.65	2.42	---
Plumbagin	14.30 ± 0.12	0.86	4.72	1.95
7-Methyljuglone	15.30 ± 0.20	1.29	5.12	1.08

In HPLC analysis of naphthoquinones in Hb. Droserae RP-18 stationary phases have been applied by different working groups.[13,14] The detection usually was performed at 254 nm.[13,14] In our preliminary tests the system of Crouch et al.[13] proved to be insufficient: due to the detection at 254 nm massive interferences of the analytes with accompanying substances were observed. By changing the wavelength of detection to the second λ_{max} of the naphthoquinones at 425 nm the exclusive determination of these compounds was possible. Furthermore, in the analysis of the standard mixture no separation of plumbagin and 7-methyljuglone could be achieved in the described system.[13] Therefore, the identification of only plumbagin in *Drosera capensis* is false,[13] this species is known to contain 7-methyljuglone as the major naphthoquinone.[5,6]

3µm Hypersil ODS (125 x 4.0 mm) and 10µm Hyperbond C-18 (300 x 4.0 mm) stationary phases with large amounts of organic modifier in the mobile phase did not provide a good resolution of plumbagin and 7-methyljuglone. Less organic solvent in the mobile phase led to elongated retention times and a broad peak shape of the analytes, which did not allow a precise quantification.

As base deactivated stationary phases are known to suppress tailing effects of alkaline substances and also phenolics, a 5 µm Hypersil BDS (250 x 4.6 mm) column was applied to the system.

Mobile phases consisting of acetic acid and acetonitrile or methanol were used for separation of naphthoquinones from plant or microbial sources.[13,16,17] Although methanol due to its lower toxicity seems easier to handle, a better peak shape was reached by the use of acetonitrile. The performance could be optimized by addition of 5% (v/v) tetrahydrofuran to the organic modifier. The separation of the analytes was improved additionally by reduction of the pH of the acetic acid from 3.5[13] to 3.0.

Fig. 2 shows a typical separation of an extract from *Drosera madagascariensis* obtained under the optimized chromatographic conditions. For the HPLC data of juglone, plumbagin and 7-methyljuglone see Table 1.

Different extraction procedures were experimented with: Steam distillation according to ref. [10] was compared to exhaustive extraction by sonication with petroleum ether similar to ref.[18] The determination of both extracts, from *Drosera peltata* and from *Drosera madagascariensis,* resp., showed good correlation (see Table 2). Although the amount of accompanying substances in extracts produced by steam distillation is very small, we preferred extraction by sonication due to the simple handling and lower time consumption.

The quantitative extraction of the naphthoquinones was proved by recovery tests for each substance. Three different amounts of plumbagin and 7-methyljuglone resp., were added to a sample of *Drosera peltata* and *Drosera*

Table 2

Comparison of the Naphthoquinone Content in *Drosera Madagascariensis and Drosera peltata* Extracted by Steam Distillation or Sonication*

Drug	% Naphthoquinones ±S.D. Steam Distillation	% Naphthoquinones ±S.D.: Sonication
Drosera peltata	0.589 ± 0.013% plumbagin	0.614± 0.023% plumbagin
Drosera madagascariensis	0.015 ± 0.0003% 7-methyl-juglone	0.014 ±0.001% 7-methyl-juglone

* Mean values (n=2).

Table 3

Recoveries of Added Plumbagin and 7-Methyljuglone*

Drug (Amount Processed)	Substance Added	Amount Added (mg)	Recovery (%)
Drosera peltata (0.1 g)	plumbagin	0.04	100.8
	plumbagin	0.06	102.3
	plumbagin	0.12	102.2
Drosera madagascariensis (1.0 g)	7-methyljuglone	0.025	98.7
	7-methyljuglone	0.049	97.4
	7-methyljuglone	0.074	1002

* Mean values (n=2).

madagascariensis resp., of known content and the sample preparation and HPLC processed as described. For each concentration the determination was repeated twice. The results (see Table 3) showed a satisfying recovery. In repeated HPLC analysis of the petroleum ether extracts no problems occurred due to accompanying substances.

Juglone was chosen as internal standard. Due to the structural similarities to the analytes it shows similar chromatographic behavior and a similar λ_{max}. The substance is easily available, it has not yet been detected in *Drosera* species, and its short retention time in the proposed system differs sufficiently from those of the naturally occurring naphthoquinones.

Table 4

Data for Calibration Graphs

Substance	Concentration Range (mg/mL)	Correlation Coefficient	Intercept	Slope
Plumbagin	0.1 - 1.0	0.9997	0.015	1.02
7-methyljuglone	0.08 - 0.82	0.9998	-0.002	0.77

x = mg naphthoquinone

y = mg internal standard x area naphthoquinone/area internal standard

Table 5

Reproducibility for Plumbagin and 7-Methyljuglone

Drosera Species	Substance	% Naphthoquinones ± S.D.	R.S.D. %
Drosera peltata	plumbagin	0.614 ± 0.008	1.30
Drosera madagascariensis	7-methyljuglone	0.045 ± 0.0018	4.00
	plumbagin	0.004 ± 0.0002	5.00

* Mean values of five extractions ± standard deviation.

The system was calibrated for plumbagin and 7-methyljuglone. From stock standard solutions ten solutions were prepared and each was injected duplicately. Linearity between peak area and concentrations over the selected concentration range was observed for both compounds with correlation coefficients > 0.999 (see Table 4).

The calibration ranges adequately cover the variations in the naphthoquinone amounts of the samples. The detection limit for both substances was 0.02 μg/μL.

The reproducibility of the method was determined by repeated assay (n=5) of one commercial sample of *Drosera peltata* and *Drosera madagascariensis.* The standard deviations (see Table 5) proved the sufficient accuracy and reproducibility of the method even for samples with low naphthoquinone content. Additionally, standard deviations for the retention times were investigated (see Table 1).

Table 6

Naphthoquinone Content and Composition of Different Commercial Samples of Hb. Droserae*

Drug	% Plumbagin	% 7-Methyljuglone	% Total Content
Hb. Droserae (Ritzberger)	---	0.014	0.014
Hb. Droserae "longifoliae" (Galke)	0.009	0.054	0.063
Hb. Droserae (Kottas)	---	0.007	0.007
Hb. Droserae (Klenk)	0.008	0.022	0.030
Hb. Droserae "ramentaceae" (Kottas)	0.004	0.045	0.049
Hb. Droserae peltate (Müggenburg)	0.614	---	0.614
Hb. Droserae "rotundifoliae" (Galke)	0.620	---	0.620

* Mean values (n=2).

GLC analysis recently had proved that commercial samples of Hb. Droserae from the German and Austrian drug market show inferior quality in respect to their naphthoquinone content.[2] Therefore, another seven drug samples were obtained from different suppliers and their naphthoquinone concentrations investigated. Due to morphological and anatomical characteristics[19,20] five drugs were identified as *Drosera madagascariensis* and one as *Drosera peltata.* One sample, marked *Drosera rotundifolia*, contained mainly *Drosera intermedia* with small amounts of *Drosera rotundifolia.*

The determination of these samples showed, that the quality of Hb. Droserae madagascariensis, sold usually as Hb. Droserae longifoliae or Hb. Droserae ramentaceae or without any specification of the species at all, is still insufficient. The concentrations assayed ranged between 0.007 and 0.063 % (see Table 6). This fulfils only 5 - 45 % of the lower range of the naphthoquinone content (0.14 - 0.22 %) demanded in DAB 7-DDR for Hb. Droserae.

Table 7

Comparison of the Naphthoquinone Content Assayed by Spectrophotometry and HPLC*

Drug	Total Naphtoquinone Content-HPLC Analysis	Total Naphtoquinone Content According to DAB 7 - DDR
Drosera peltata	0.614 %	0.600 %
Drosera madagascariensis	0.041 %	0.042 %

* Mean values (n=2).

The sample of Hb. Droserae peltatae, containing 0,614 % plumbagin, met the demands of minimum 0.6 % plumbagin of the Pharm. Belg. VI for Hb. Droserae. The declaration of Hb. Droserae "rotundifoliae" was not only disproved by its botanical characteristics,[19,20] but also by its chemical properties: it contained only plumbagin, the main naphthoquinone of *Drosera rotundifolia,* however, is 7-methyljuglone.[12] Nevertheless, due to its content of 0,62 % plumbagin this drug would fulfil the demands of the Pharm. Belg. VI, which accepts *Drosera rotundifolia* and *Drosera intermedia* as well as *Drosera peltata* and *Drosera anglica* as a source for Hb. Droserae.

The total naphthoquinone content of one sample of *Drosera peltata* and of *Drosera madagascariensis* was determined additionally by the spectrophotometric method of DAB 7 - DDR and showed a good correlation with the HPLC assay (see Table 7).

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