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Quantitative analysis of anti-inflammatory lignan derivatives in Ratanhiae radix and its tincture by HPLC–PDA and HPLC–MS

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ABSTRACT

Root preparations of *Krameria lappacea* (Dombey) Burdet et Simpson are traditionally used against oropharyngeal inflammation. Besides antimicrobial and astringent procyanidines, lignan derivatives, including ratanhiaphenol I, II, III and (+)-conocarpan, contribute to the activity of Ratanhiae radix, exerting a significant topical anti-inflammatory activity *in vivo*, and *in vitro* by inhibiting NF-κB and the formation of inflammatory prostaglandins and leukotrienes. Besides gravimetrical analysis of the ratanhiaphenols I, II and III, the content of these compounds in the herbal drug has never been determined. The developed HPLC method enables the quantification of twelve active lignan derivatives in the roots, and is also suitable for the determination of the constituents in Tinctura Ratanhiae. Separation was achieved on a phenyl-hexyl column material using a solvent gradient consisting of 0.02% aqueous TFA and a mixture of acetonitrile/methanol (75:25, v/v). Sensitivity, accuracy (recovery rates were between 95% and 105.6%), repeatability (RSD ≤ 4.6%), and precision (intra-day precision ≤ 4.8%; inter-day precision ≤ 3.4%) of the method were determined. HPLC–MS experiments in positive and negative electrospray ionization mode confirmed identity and peak purity of analytes. The analysis of several root and tincture samples revealed that (+)-conocarpan and ratanhiaphenol II dominated with contents of 0.49–0.71% and 0.51–0.53% in the roots and 0.66–0.68 mg/ml and 0.70–0.71 mg/ml in the commercial tinctures, respectively.

1. Introduction

Krameria lappacea (Dombey) Burdet et Simpson; (syn. *K. triandra* Ruiz et Pavon), Krameriaceae, is a hemiparasitic shrub, which is native to South America [1]. The plant is known as "raiz de dientes" because it has traditionally been used in South America as chewing sticks for cleaning and strengthening teeth. Other traditional indications are diarrhoea and mouth ulcer [1]. The drug was introduced into European medicine over 200 years ago as a remedy against stomach aches, diarrhoea, menstrual problems, nose bleeds and oropharyngeal inflammation [2,3]. Today the drug and its preparation, an ethanolic tincture, are listed in the European Pharmacopoeia, and the ESCOP Supplement 2009, respectively. The therapeutic indications comprise mild inflammations of mouth and throat such as stomatitis, gingivitis and pharyngitis [4].

The roots comprise 2 prominent compound classes: medium-to-high molecular weight oligomeric proanthocyanidins [5], with a content of approximately 10%, and a set of lignan derivatives [6–8]. Besides already known antimicrobial, antioxidant, and photoprotective properties [3,5,7,9] of root extracts, we could demonstrate in a previous study the pronounced anti-inflammatory activity of a dichloromethane extract as well as of 11 isolated lignan derivatives (compounds **1**–**6** and **8**–**12**). The topical anti-inflammatory properties of the secondary metabolites were comparable to the drug indomethacin (ID_{50} value 0.3 μmol/cm^2) *in vivo*, with ID_{50} values ranging from 0.3 μmol/cm^2 to 0.6 μmol/cm^2. The *in vitro* evaluation revealed NF-κB, cyclooxygenase-1/2, 5-lipoxygenase, and microsomal prostaglandin E synthase-1 as responsible targets for the activity against inflammation [10].

These pharmacological findings make a quality assessment of Ratanhiae radix and its preparations with an adequate analytical method with focus on the class of lignan derivatives, necessary. The content of these compounds in the drug, besides a gravimetrical analysis of the ratanhiaphenols I, II and III (content of 0.3%) [11] has never been determined to our knowledge. Two HPLC–MS or GC–MS studies published until now enabled only the identification of a limited number of lignan derivatives in different extracts or tinctures of *K. lappacea* [9,12]. Therefore we developed an efficient and according to the ICH guidelines validated HPLC method for the quantification of the active constituents in the roots as well as in the ethanolic tincture. One prominent constituent of the investigated extracts (3-formyl-2-(4-hydroxyphenyl)-5-(*E*)-propenylbenzofuran; compound 7), an additional lignan derivative, was isolated in course of the present study. HPLC–MS experiments

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were carried out in order to confirm purity and identity of the analytes.

2. Experimental

2.1. Materials

All solvents used for isolation were purchased from VWR International (Darmstadt, Germany). Acetonitrile, methanol, and trifluoroacetic acid (TFA) were of HPLC grade, and purchased from Merck (Darmstadt, Germany). Ultrapure water was produced by a Sartorius Arium® 611 UV water purification system (Göttingen, Germany). Polyamide 6 S was purchased from Riedel-de Haën (Seelze, Germany).

Quality of root samples of *K. lappacea* complied with the European Pharmacopoeia. RR-1 (batch number 212 965) and RR-2 (batch number A902194-002) were purchased from Mag. pharm. Kottas-Heldenberg and Sohn (Vienna, Austria). Sample RR-3 (batch number 913075) was obtained from Kwizda Kräutergroßhandel (Linz, Austria). Voucher specimens are deposited at the Institute of Pharmacy/Pharmacognosy, University of Innsbruck. Tincture 1 (T-1), a kind gift from the Institute of Pharmacy/Pharmaceutical Technology, University of Innsbruck, was prepared according to the Austrian Pharmacopoeia but with a reduced maceration time of 3 days. Tinctures 2–4 (commercial tinctures; T-2 to T-4; batch numbers 80516159, 80516419, and 1403/0409 1509) were purchased from different pharmacies in Austria. The quality of those complied with the Austrian Pharmacopoeia.

2.2. Standards

Compounds **1**–**6**, and **8**–**12** were isolated and their structures elucidated in a previous study [10]. Compound 7 was isolated as following: ground roots of *K. lappacea* (RR-1; 300 g) were exhaustively extracted with dichloromethane (DCM) in a Soxhlet apparatus (30 °C, 5 days). The extract was evaporated to dryness yielding 16.32 g. 15.50 g of the obtained extract were separated by flash silica gel 60 (40–63 μm, Merck, Darmstadt, Germany) column chromatography (270 g, 50 × 4 cm) using a gradient with petroleum ether (PE) and increasing the amount of a fixed mixture of DCM–ethyl acetate (EtOAc) (95:5, v/v), followed by increasing the EtOAc amount (for detailed gradient see supplementary Table S-1) yielding 29 fractions (A1-A29). The methanol insoluble part (110.71 mg) of fraction A18 (PE-(DCM–EtOAc) 40:60, v/v; 415.03 mg) was recrystallized in acetone yielding 14.91 mg of compound 7. Identity was confirmed by the analysis of spectroscopic and spectrometric data (1D- and 2D-NMR, MS) and comparison with published data [7], see supplementary Table S-2 for NMR data. Purity of all standards was ≥96% (determined by HPLC).

NMR: 1D- and 2D-experiments were measured on a Bruker DRX 300 (Bruker Biospin Rheinstetten, Germany) operating at 300.13 MHz (^{1}H) and 75.47 MHz (^{13}C) at 300 K; NMR solvent: acetone-d_6 with 0.03% TMS (Eurisotop Gif-Sur-Yvette, France), which was used as internal standard.

2.3. HPLC sample preparation

Extract preparation. Dry plant material was grounded and sieved (mesh size 750 μm). Extraction of the powdered roots (around 100 mg) was performed by using an Accelerated Solvent Extractor (ASE 100, DIONEX) with a mixture of DCM–acetone (95:5, v/v) as solvent. Following parameters were applied: temperature 100 °C, 130% flush volume, 5 cycles, static time 5 min, purge time 70 s. For an exhaustive extraction the procedure was carried out twice. The 2 extracts were combined and evaporated to dryness under reduced pressure. Subsequently the residue was redissolved in methanol and quantitatively transferred into a volumetric flask, adjusted to a final volume (5 ml) with methanol and filtered through a 0.45 μm nylon membrane filter (Phenomenex). Each sample was assayed in triplicate. The amount found in the samples was calculated as percent of the initial weight of the dried roots. In order to check if this extraction protocol is exhaustive, another 5 cycles (after the normal extraction protocol) were conducted and the obtained extract analyzed by HPLC. None of the compounds **1**–**12** could be quantified since their content was below the LOQ and even below the LOD, indicating an exhaustive extraction.

Preparation of tinctures. Tinctura Ratanhiae (0.50 ml) samples were directly applied on a small column filled with 300 mg polyamide and eluted with methanol (5 ml). The eluate was evaporated under reduced pressure, redissolved in methanol (1 ml) and filtered through a 0.45 μm nylon membrane filter (Phenomenex). In order to fit the calibration range the obtained samples were assayed directly for quantification of compounds **1**–**7**, **9**–**10**, **12**. For quantification of compound **8** and **11** the obtained solutions were further diluted with methanol 1:4 (v/v). Each sample was assayed in triplicate.

2.4. HPLC and HPLC–MS conditions

The quantitative analysis was performed on a Shimadzu UFLC XR (Kyoto, Japan) equipped with an auto sampler, PDA, an on-line degasser and column thermostat. As stationary phase a Phenomenex Luna Phenyl-Hexyl column (150 mm × 3 mm i.d., 3 μm particle size) was used. The composition of the mobile phase consisted of 0.02% (v/v) TFA in water (solvent A) and acetonitrile-methanol 75:25 (v/v; solvent B). Separation was achieved by a gradient analysis starting with 55A – 45B, increasing the amount of solvent B in 30 min to 75% and 30.1 min to 100% B, stop time 40 min. For equilibration a post time of 15 min was applied. Other parameters: flow rate 0.30 ml/min, injection volume 5 μl, detection wavelength 280 nm; column temperature 35 °C.

HPLC–MS experiments were performed on an Esquire 3000 ion-trap mass spectrometer (Bruker-Daltronics, Bremen, Germany) coupled to an Agilent HPLC system type HP 1100. MS parameters: ESI, alternating mode; spray voltage: 4.5 kV, 365 °C; dry gas: 9 l/min; nebulizer 40 psi; full scan mode: m/z 100–1500. Solvent A was changed from 0.02% aqueous TFA to water for analysis of extract samples, and to water with 0.9% formic acid and 0.1% acetic acid for analysis of tincture samples. All other HPLC parameters were maintained.

2.5. Calibration and validation

Individual stock solutions of standard compounds were prepared in methanol. According to solubility different starting concentrations were used: 2 mg/ml for compound **2**, 1 mg/ml for compounds **1**, **4**–**6**, **8**–**10**, and **12**, 0.5 mg/ml for compound **11**, and 0.3 mg/ml for compound 7. Five additional calibration levels were prepared by dilution with methanol, and each level was assayed in triplicate (see Table 1 for calibration data).

Limits of Detection (LOD) and Limits of Quantitation (LOQ) were calculated based on the calibration curve according to the ICH guidelines. Peak purity was assured by evaluating UV data with the "peak purity" option in the software and by HPLC–MS experiments. Accuracy was determined in sample RR-1 as well as in sample T-1 (T-2 in case of compound **4**) by spiking experiments at two different concentrations. Known amounts of the standard compounds were added either to the dry powdered root material, followed by extraction or were applied on the polyamide column together with the tincture.

Repeatability of the method was assured by relative standard deviations of multiple injections. Precision of the method was

Table 1
Calibration data of compounds **1**–**12**, including regression equation, correlation coefficient (R^2), linear range (μg/ml), limit of detection (LOD; (μg/ml), and limit of quantitation (LOQ; (μg/ml).

	Regression equat.	R^2	Linear range	LOD	LOQ
1	$y = 49358.75x + 96.39$	0.9998	1–500	0.46	1.40
2/3	$y = 9156.30x + 186.80$	0.9993	3–1650	1.65	4.99
4	$y = 21287.27x + 95.29$	0.9997	30–1000	12.47	37.78
5	$y = 42402.89x + 70.31$	0.9996	1.5–600	0.86	2.61
6	$y = 58933.29x + 224.43$	0.9991	0.7–400	0.65	1.97
7	$y = 33407.79x + 39.08$	0.9997	1–300	0.56	1.70
8	$y = 35470.63x + 167.03$	0.9982	1.5–480	0.75	2.27
9	$y = 71252.44x + 376.59$	0.9984	4–500	1.70	5.15
10	$y = 54630.94x + 142.76$	0.9995	3.5–500	1.45	4.38
11	$y = 84781.86x + 200.42$	0.9982	1–180	0.41	1.25
12	$y = 50084.95x + 187.11$	0.9994	1.5–600	0.74	2.23

determined by preparing and assaying 5 replicate samples of RR-1 and T-1 on the same day; the same procedure was repeated on two more days. By comparing variations within the same days intra-day precisions were determined, by observing differences within the 3 days inter-day precision was deduced.

3. Results and discussion

3.1. Isolation and identification of standards

Compounds **1**–**6** and **8**–**12** were obtained and identified in a previous study as:

5-(3-hydroxypropyl)-2-(2-methoxy-4-hydroxyphenyl)benzofuran (**1**), (−)-larreatricin (**2**), *meso*-3,3′-didemethoxynectandrin B (**3**), (2*S*,3*S*)-2,3-dihydro-3-hydroxymethyl-2-(4-hydroxyphenyl)-5-(*E*)-propenylbenzofuran (**4**), 2-(2-hydroxy-4-methoxyphenyl)-5-(3-hydroxypropyl)benzofuran (**5**), 2-(2,4-dihydroxyphenyl)-5-(*E*)-propenylbenzofuran (**6**), (+)-conocarpan (**8**), 2-(4-hydroxyphenyl)-5-(*E*)-propenylbenzofuran (**9**), rataniaphenol III (**10**), rataniaphenol II (**11**), and rataniaphenol I (**12**) (see Fig. 1 for structures) [10]. In the course of the present work compound 7, one additional prominent peak, was isolated out of the roots of *K. lappacea*. By mass spectrometry, 1- and 2D-NMR experiments and comparison with published data [7] this lignan derivative was identified as 3-formyl-2-(4-hydroxyphenyl)-5-(*E*)-propenylbenzofuran.

3.2. Method development

The major problem in the development of a HPLC method for the simultaneous detection of all active lignan derivatives was the structural similarity of the analytes. In order to accomplish a satisfactory separation, all parameters had to be carefully assessed. The initial screening experiments (stationary phases with RP-12, RP-18, ether-linked phenyl, phenyl-hexyl column material) clearly showed that the stationary phase had to be a phenyl-hexyl column, since this phase exclusively enabled the baseline separation of ratanhiaphenols II (**11**) and I (**12**), which represent the most prominent constituents in Ratanhiae radix. Moreover it was obvious that by using just methanol or acetonitrile alone no satisfactory separation was achieved. Several mixtures thereof (25:75, 50:50, 75:25; all v/v) were assayed; best resolution was achieved with acetonitrile-methanol 75:25 (v/v) at 35 °C. An acidic mobile phase was not mandatory, but improved peak shape of analytes and separation of unwanted constituents (tannins) in the tincture samples. Therefore 0.02% TFA was added to solvent A (water). The wavelength was set to 280 nm, since it enabled the sensitive detection of all analytes. With this method a baseline separation of compounds **1**–**12** (besides the stereoisomers **2** and **3**) was possible in less than 30 min (see Fig. 2). One problem emerged at the beginning of sample analysis when evaluating purity of all analytes by the use of the UV purity function of the used HPLC software: stereoisomers 2/3 showed in the root samples as well as in the tinctures a co-elution with small amounts of an additional compound. Attempts to improve the resolution with the aid of additives, such as tetrahydrofuran, 1-, and 2-propanol or tert-butylmethyl ether, or a variation of temperature showed no effect. Separation was possible by changing the stationary phase from the phenyl-hexyl material to a RP-12 column material, but as highlighted before this made a separation of compounds **11** and **12** impossible. Since these two constituents are very prominent compounds in the roots of *K. lappacea*, the impurity was accepted and the content of the 2 7,7′-epoxylignan derivatives was only estimated in the presented study.

For HPLC–MS experiments solvent A was changed from 0.02% aqueous TFA to water in case of root samples, and to 0.9% formic acid and 0.1% acetic acid for analysis of tinctures. All other parameters were maintained.

3.3. Method validation

Suitability of the developed method for the quantification of the major lignan derivatives in Ratanhiae radix and Tinctura Ratanhiae can be deduced from several analytical parameters. The detector signal was linear in the tested range with a correlation coefficient higher than 0.9982, calibration data for compounds **1**–**12** is shown in Table 1. Limits of detection (LOD) and limits of quantitation (LOQ), indicating the sensitivity of the method, ranged from 0.41–1.70 μg/ml to 1.25–5.15 μg/ml, respectively. Only for compound 4 LOD (12.47 μg/ml) and LOQ (37.78 μg/ml) were higher. MS and UV spectra showed that all analytes, besides **2**/**3**, were free of co-eluting compounds or impurities. Together with the fact that all compounds, except the stereoisomers **2** and **3**, were baseline separated, selectivity of the assay is indicated.

Accuracy was determined in recovery experiments where 2 different concentrations of standard compounds were added either to the plant material (RR-1) before extraction or applied on top of the polyamide column together with the tincture (normally T-1; T-2 in case of compound 4). All results were within the usually required recovery range of 100 ± 5%, as shown in supplementary Tables S-3 and S-4. Maximum deviations were reached for compound **1** (recovery at low spike in tincture 95%) and compound **6** (recovery rate at low spike in extract 105.2%), respectively. Relative standard deviations below 4.55% and very stable retention times during the whole study implicate the repeatability of the developed method. Precision of the whole assay was determined on three following days (see Table 2A and B). Maximum deviations within one day (intra-day precision) were 4.67% for compound **12** in the extract and 4.78% for compound 7 in the tincture. Compound 7 showed highest inter-day variance with maximum deviations of 2.98% in the extract and 3.38% in the tincture, respectively.

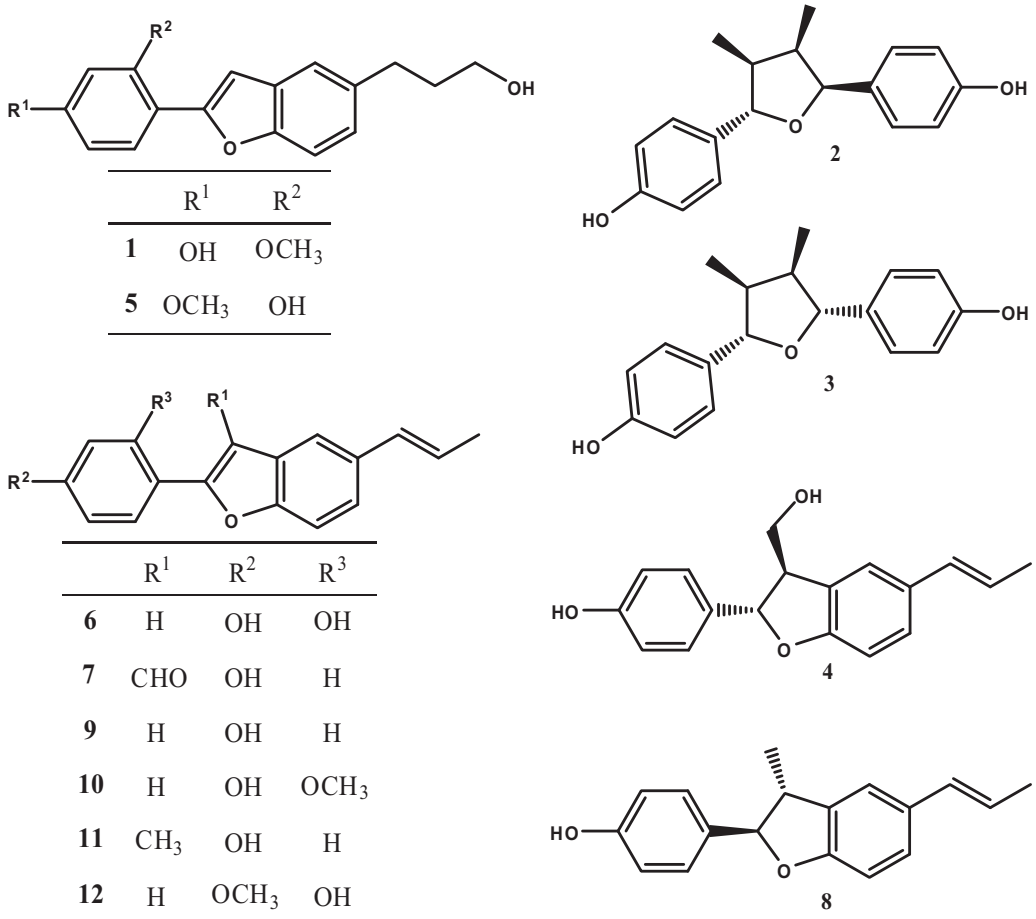

Fig. 1. Chemical structures of analyzed lignan derivatives.

3.4. Sample analysis

Preliminary tests were performed in order to determine optimum sample preparation conditions for the root as well as the tincture samples. In the case of root samples, different solvents (methanol, acetone, DCM and mixtures thereof) and extraction procedures (sonication, ASE extraction) were evaluated. ASE extraction (2 times 5 cycles for 5 min at 100 °C) with a mixture of DCM–acetone (95:5, v/v) was most efficient. Following these conditions the lignan derivatives were exhaustively extracted

Table 2
Intra- and inter-day precision of the developed HPLC-assay using sample RR-1 (A) and T-1 (B); results are based on peak area, relative standard deviation in parenthesis.

(A)	Intra-day ($n = 5$)			Inter-day ($n = 3$)
	Day 1	Day 2	Day 3	
1	2110.6 (2.9)	2169.4 (3.1)	2114.9 (3.5)	2131.6 (1.3)
4	<LOQ	<LOQ	<LOQ	<LOQ
5	1978.6 (2.5)	1974.2 (2.9)	1992.6 (3.3)	1981.8 (0.4)
6	3403.7 (4.4)	3224.4 (4.2)	3210.3 (3.3)	3279.5 (2.7)
7	1156.4 (3.2)	1115.5 (4.4)	1074.9 (3.5)	1115.6 (2.9)
8	5079.3 (2.4)	5036.5 (2.9)	4998.2 (2.2)	5038 (0.7)
9	1174.1 (3.8)	1149.7 (4.1)	1123.5 (4.1)	1149.1 (1.8)
10	2808.3 (3.5)	2780.6 (2.9)	2719.9 (3)	2769.6 (1.3)
11	9185 (2.5)	9152.1 (3.6)	9102.1 (3.1)	9146.4 (0.4)
12	2252.7 (4.1)	2252.5 (4.2)	2217.7 (4.7)	2241 (0.7)
(B)				
1	1556.1 (1.3)	1517.1 (1.5)	1510.7 (1.5)	1528 (1.3)
4	<LOQ	<LOQ	<LOQ	<LOQ
5	1617.9 (1)	1567.3 (1.5)	1587.5 (1.4)	1590.9 (1.3)
6	2711.8 (3.1)	2632.9 (1.4)	2646.5 (2.3)	2663.7 (1.3)
7	736.5 (1.3)	713 (4.8)	678.1 (3.2)	709.2 (3.4)
8	2937.7 (2.9)	2848.6 (2)	2863.2 (2.4)	2883.2 (1.4)
9	761.2 (1)	734.7 (4.5)	738.2 (1.7)	744.7 (1.6)
10	1115.7 (1.3)	1061.9 (0.9)	1079.7 (1.4)	1085.8 (2.1)
11	6263 (0.5)	6117.1 (0.7)	6121.6 (1.4)	6167.2 (1.1)
12	1855 (1.5)	1889.9 (1.8)	1938.8 (1.6)	1894.6 (1.8)

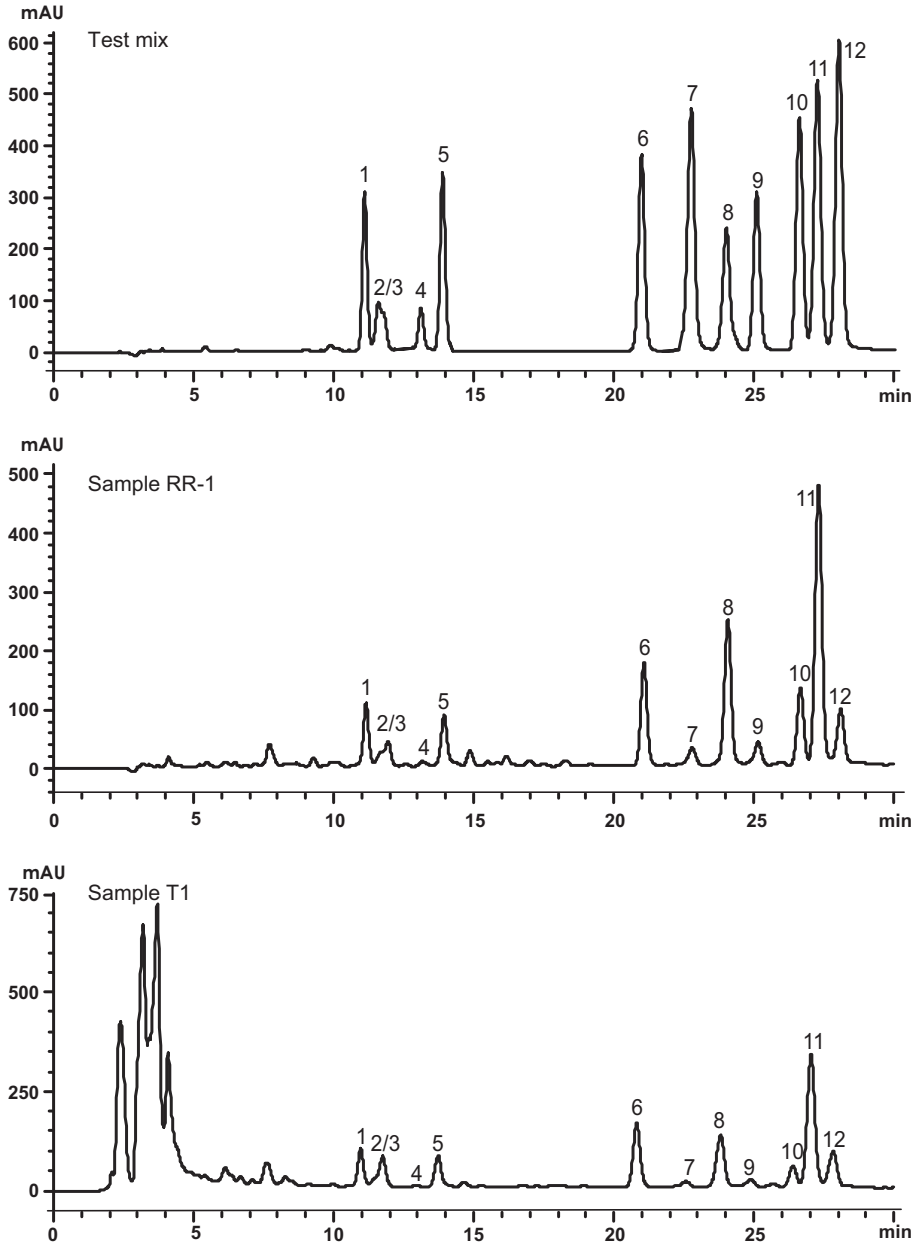

Fig. 2. Separation of a standard mixture of compounds **1**–**12**, and samples RR-1, and T-1 under optimized HPLC conditions (column Luna Phenyl-hexyl, 150 mm × 3 mm, 3 μm; mobile phase: 0.02% (v/v) TFA in water (A), acetonitrile:methanol 75:25 (v/v) (B); gradient: 55A-45B in 30 min to 25A-75B, in 0.1 min to 100B, stop time 40 min; flow rate: 0.3 ml/min; injection volume: 5 μl; temperature: 35 °C; detection: 280 nm).

but the yield of undesired tannins could be minimized. For the analysis of Tinctura Ratanhiae samples (containing ≥1% (m/m) tannins according to the Ph. Eur.) interfering tannins had to be eliminated. For this purpose 2 methods, liquid–liquid extraction (DCM–water) and separation over polyamide, were evaluated. Optimal results were obtained by applying the tincture samples directly on top of a polyamide column followed by elution with methanol. Chromatograms of the standard mix, a typical root and a tincture sample under optimized conditions are depicted in Fig. 2.

All compounds were well resolved and could be assigned by comparison of their retention times, UV-spectra of the respective standards, and by HPLC–MS experiments (ESI, alternating mode). In order to present the results in a clear form the extracted ion chromatograms (EIC) were selected for presentation (see Fig. 3). The MS signals of the 7,7′-epoxylignans **2**/**3** were assigned as $[M+H]^+$, and compound **9** as $[M]^-$. All other benzofuran derivatives were assignable as $[M-H]^-$.

Three root samples (RR-1 to RR-3) and four ethanolic tinctures (T-1 to T-4), 3 of them were purchased in pharmacies around Austria, were analyzed during this study, the obtained results are illustrated in Table 3. The major compounds in all samples were (+)-conocarpan (**8**) and ratanhiaphenol II (**11**) with contents of 0.49–0.71% and 0.51–0.53% in the roots as well as 0.19-0.68 mg/ml and 0.16-0.71 mg/ml in the tinctures, respectively. The content of the stereoisomer mixture **2**/**3**, ranging from 0.11–0.23% in the roots to 0.20–0.29 mg/ml in the commercial tinctures (T-2 to T-4), can only be estimated due to the observed co-elution. Interestingly, root sample RR-1 showed a higher content of all benzofurans, except for compounds **6**, and **11**. Highest deviations within the root samples were found for compounds **5** and **12** with contents ranging from 0.06–0.22% to 0.08–0.20%, respectively. Compound **4** was

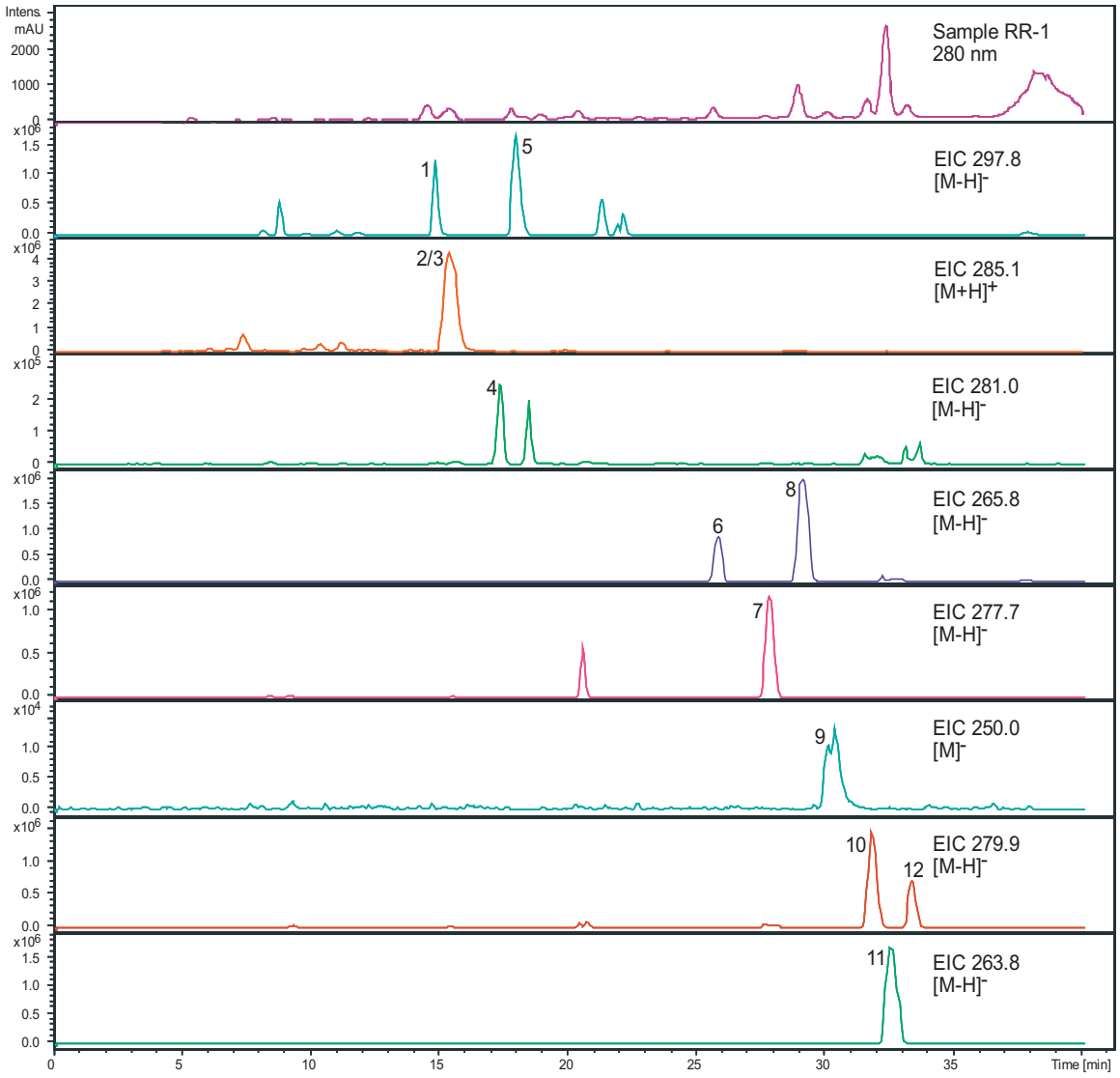

Fig. 3. HPLC–MS analysis of sample RR-1; HPLC-conditions according to Fig. 2 except solvent A (water); MS-conditions: ESI alternating mode, nebulizer 40 psi, spray voltage 4.5 kV, 365 °C, dry gas: 9 l/min; EIC: extracted ion chromatogram.

Table 3
Quantitative results for compounds **1** and **4–12** in Tinctura Ratanhiae samples T-1 to T-4 and Ratanhiae radix samples RR-1 to RR-3; relative standard deviations in parenthesis ($n = 3$).

	T-1 (mg/ml)	T-2 (mg/ml)	T-3 (mg/ml)	T-4 (mg/ml)	RR-1 mg/100 mg	RR-2 mg/100 mg	RR-3 mg/100 mg
1	0.07 (1)	0.21 (1.07)	0.21 (2.05)	0.19 (0.81)	0.20 (2.18)	0.15 (4.43)	0.15 (2.32)
4	<LOQ	0.08 (2.23)	0.08 (3.75)	0.07 (1.24)	<LOQ	<LOQ	<LOQ
5	0.09 (0.87)	0.10 (1.39)	0.09 (2.81)	0.07 (0.96)	0.22 (1.53)	0.07 (0.33)	0.06 (2.79)
6	0.10 (2.11)	0.34 (1.78)	0.34 (1.55)	0.31 (4.06)	0.26 (1.48)	0.29 (2.85)	0.29 (1.89)
7	0.05 (1.17)	0.15 (0.68)	0.15 (2.12)	0.10 (1.85)	0.17 (4.35)	0.10 (3.30)	0.11 (3.52)
8	0.19 (1.03)	0.66 (3)	0.66 (1.66)	0.68 (2.87)	0.71 (2.43)	0.51 (4.55)	0.49 (2.59)
9	0.01 (2.01)	0.06 (0.68)	0.06 (1.61)	0.07 (1.42)	0.05 (1.52)	0.03 (3.42)	0.03 (4.20)
10	0.04 (1.60)	0.32 (1.08)	0.33 (0.73)	0.35 (0.73)	0.22 (3.77)	0.27 (0.96)	0.29 (3.55)
11	0.16 (0.44)	0.71 (1.91)	0.70 (1.80)	0.71 (1.74)	0.53 (3.20)	0.52 (4.40)	0.51 (2.39)
12	0.08 (2.82)	0.12 (1.40)	0.12 (0.83)	0.11 (0.97)	0.20 (4.34)	0.08 (2.88)	0.09 (2.98)

below LOQ in all root samples and sample T-1, but could be quantified in the commercial tinctures (T-2 to T-4; 0.07–0.08 mg/ml). As expected T-1 contained 2-4 times lower amounts of the lignan derivatives. This issue could be explained by the shorter maceration time (3 days), since regular Tinctura Ratanhiae is prepared by a double maceration over a period of two weeks. The content of the lignan derivatives in the tincture samples 2–4 was very consistent with the highest variation for compound 7 (0.10–0.15 mg/ml).

4. Conclusions

In a previous study we could show that not only tannins with their astringent and antimicrobial properties, but also the neglected compound class of lignan derivatives with their pronounced anti-inflammatory activities play an important role in the use of the herbal drug against inflammatory disorders of mouth and throat. Thus, the HPLC-method, developed in the present study, is a sig-

nificant improvement and innovation, since it enables for the first time the quantitative analysis of lignan derivatives in Ratanhiae radix as well as in Tinctura Ratanhiae. This validated assay will be a useful tool for quality control of the plant material and its preparations, focusing on the compound class of lignan derivatives.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.06.016.

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