Journal of Chromatography A, 1225 (2012) 113–120

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development and application of high-performance liquid chromatography for the study of two new oxyprenylated anthraquinones produced by *Rhamnus* species

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ARTICLE INFO

Article history:
Received 4 October 2011
Received in revised form
19 December 2011
Accepted 19 December 2011
Available online 8 January 2012

Keywords:
Extraction procedure
HPLC–UV/vis
Method development
Oxyprenylated anthraquinones
Rhamnus spp.

ABSTRACT

Rhamnus spp. is known to contain biologically active anthraquinone secondary metabolites but the presence of oxyprenylated ones is not reported. To this aim, a new simple, and accurate analytical method was developed to reveal chemical fingerprint of these analytes in plant extracts. Plant samples were analysed after extraction with *n*-hexane (first step) and methanol (second step) using a C_{18} column with a mobile phase composed of 35% of water:65% of methanol (both with 1% formic acid, v/v) at 0.7 mL min^{-1} flow rate in gradient elution mode. For quantitative analyses, selective detection was performed at 435 nm. The limit of quantification (LOQ) was 0.5 μM, with the only exception of 3-geranyloxyemodin for which the LOQ value was 5.0 μM, and external matrix-matched standard curves showed linearity up to 125 μM. The within- and between-batch precision (RSD%) values ranged from 0.2% to 12.9% while within- and between-batch trueness (bias%) values ranged from 12.2% to 12.7%. The method was applied to evaluate for the first time the presence and the quantities of oxyprenylated anthraquinones in *Rhamnus* spp. barks as well as the anthraquinone profile of *Rhamnus pumila* Turra. The proposed method could be directly applied to the selective quantification of these analytes in natural sources.

1. Introduction

Anthraquinones are known to be present in many plant families such as Fabaceae (syn. Leguminosae), Liliaceae, Polygonaceae, Rubiaceae, and Rhamnaceae. Anthraquinone derivatives, including emodine, physcione, aloe-emodine, rhein, and chrysophanol, are nowadays well recognized as important biologically active compounds. Their presence is often used as criteria in the quality control of plants used for medicinal purposes.

Recently, it was reported that anthraquinones exert a wide range of biological activities including anti-fungal [1–3], antimicrobial [4–6], anti-cancer [7,8], anti-oxidant [9,10], and anti-viral ones [11], other than the well-known effects on the gastrointestinal apparatus [12]. Very recently, a survey on the analytical techniques used for the determination of "classic" anthraquinones from natural sources was also reported [13].

The main problem in the determination of these latter analytes is the similarity in their pK_a values that often leads to chromatographic separation difficulties (aloe-emodine pK_a = 8.49, rhein pK_a = 4.51 and 8.41, emodine pK_a = 5.70 and 7.94, chrysophanol pK_a = 8.51, physcione pK_a = 8.49) [14].

Due to this problem, several analytical assays were developed for the quantification of these compounds in different matrices, all based on HPLC–UV/vis [15,16], CZE [17,18] and with novel, sensitive, and selective instrumentation such as HPLC (or GC)–MS/MS [19,20]. Moreover, no information is available about the analytical and physicochemical properties of a naturally rare group of anthraquinones, namely oxyprenylated derivatives. So the research for an alternative and efficient analytical methodology for the qualitative and quantitative analysis of these secondary metabolites is of great and current interest. In particular the research field on these compounds has been recently implemented because their production and release has been recognized as key mechanism in plant physiology [21] and because these compounds represent a class of molecules of great interest in the natural product chemistry [22] especially concerning drug discovery and drug development process [23].

As a continuation of our studies on anthraquinones, with a particular reference on oxyprenylated derivatives, we wish to report herein a novel HPLC–UV/vis method for the

Abbreviations: CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantification; ODS, octadecylsilane; QC, quality control; TFA, trifluoroacetic acid; ULOQ, upper limit of quantification.

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doi:10.1016/j.chroma.2011.12.085

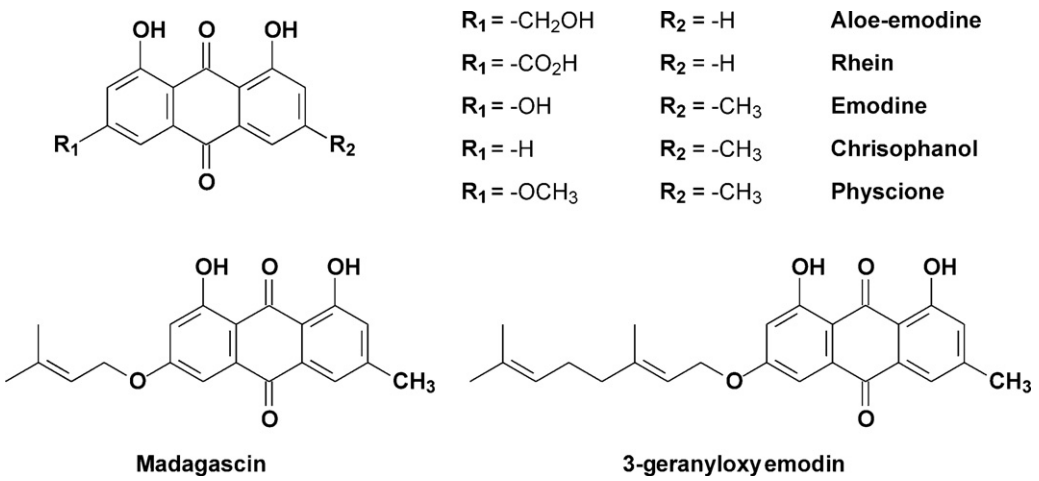

Fig. 1. Chemical structures.

quantification of two oxyprenylated compounds, madagascin (3-isopentenyloxyemodin) and 3-geranyloxyemodin (Fig. 1), in complex matrices derived from natural sources. It is noteworthy to point out that both madagascin and 3-geranyloxyemodin are described in our study for the first time as components of the anthraquinone tool of plants belonging to the genus *Rhamnus*. In fact previous studies reported the isolation of these two oxyprenylated secondary metabolites only from three genera (*Psorospermum*, *Vismia*, and *Cratoxylum*), all belonging to the *Clusiaceae* family [24]. Moreover, the anthraquinone profile of *Rhamnus pumila* Turra is also reported herein for the first time. In fact the only data from the literature concerning this plant only consist of TLC analysis of anthracene derivatives. HPLC methods for the direct quantification of these two oxyprenylated anthraquinones are not reported in the literature, although some authors described many assays for the extraction (such as ultrasonication [25,26] or fermentation broth [27]) and structure determination of several anthraquinone derivatives [28].

2. Materials and methods

2.1. Chemical and reagents

Emodine, rhein, chrysophanol, aloe-emodine, and physcione (all with >99% purity index) were purchased from Extrasynthese (Genay, France).

Oxyprenylated anthraquinones (madagascin and 3-geranyloxyemodin) were synthesized by means of an already reported procedure [29] and used as pure chemical standards (purity ≥ 98%) after NMR and MS/MS characterization.

Methanol (HPLC-grade) and formic acid (99%) were purchased from Carlo Erba Reagenti (Milan, Italy). Water is produced by Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).

2.2. Plant samples

Rhamnus saxatilis Jacq. and *R. alpinus* L. bark samples were collected in October 2008, in Campo Imperatore (Gran Sasso mountain, Abruzzo, Italy), and voucher specimens (GS-2008-1 and GS-2008-2) were deposited at the Herbarium of the Giardino dei Semplici of the University "G. d'Annunzio" of Chieti-Pescara. Samples of *R. pumila* Turra were collected in Mount Obruc (Croatia) in June 2009, and voucher specimens (No. 1361) were deposited at the Herbarium of the Department of Pharmaceutical Botany with "Fran Kušan" Pharmaceutical Botanical Garden, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia.

Bark was removed from the stem and dried for three weeks in a well-ventilated room, in one layer, protected from the direct solar light. Air-dried samples were placed in double paper bags, put in dark holder and stored in a dry place at room temperature (22° C) protected from light until the analysis.

2.3. Gradient chromatographic condition

HPLC analyses were performed on a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector, and the mobile phase was directly on-line degassed by using Degassex, mod. DG-4400 (Phenomenex, Torrance, CA, USA). Empower v.2 Software (Waters Spa, Milford, MA, USA) was used for data acquisition and elaboration.

An ODS reversed-phase packing column (GraceSmart RP18, 4.6 mm × 150 mm, 5 μm; Grace, Deerfield, IL, USA) was employed for the separation and the column was thermostated at 28 ± 1 °C using a Jetstream2 Plus column oven.

For quantitative analyses, selective detection was performed at 435 nm. Gradient elution mode was performed using a mobile phase containing a 35:65 water–methanol ratio both with 1% of formic acid (v/v) as starting conditions and gradient program as reported in Table 1.

All the sample solutions were previously centrifuged and 20 μL of the supernatant, after a filtration on Phenex-PTFE (4 mm, 0.45 μm) syringe filters (Phenomenex, Torrance, CA, USA), was injected into the HPLC–UV/vis system.

2.4. Calibration, linearity, and quality control samples

The seven chemical standards stock solutions were made at the concentration of 1 mM in a final volume of 10 mL of methanol.

Table 1
Chromatographic gradient program.

Time (min)	Flow (mL min^{-1})	% A[a]	% B[b]
0	0.7	35	65
15		35	65
20		10	90
45		10	90
47		35	65
60		35	65

[a] HPLC grade water + 1% formic acid (v/v).
[b] Methanol + 1% formic acid (v/v).

Combined working solutions of mixed standards at the concentrations of 0.5, 1, 5, 10, 25, 50, 75, 100, and 125 μM (corresponding to a linearity range from 0.127 to 50.78 μg mL^{-1}, depending on analytes) were obtained by dilution of a mixed stock solution at 1 mM in volumetric flasks containing the mobile phase. Finally the nine calibration standards were injected into the HPLC–UV/vis system. Calibration curves were calculated by analysing these nine non-zero concentration standards prepared in freshly spiked solution in triplicate.

All quantitative analyses were performed at 435 nm.

Calibration curves were plotted using weighted linear least-squares regression analysis according to the equation $y = a + bx$, where "y" is the analyte peak area, "x" represents the analyte concentration (μM) in the calibration samples, "a" is the intercept, and "b" is the slope of the regression line.

Concentrations of the QCs and unknown samples were calculated by interpolating their analyte peak area on the calibration curve.

2.5. Limit of detection (LOD) and limit of quantification (LOQ)

The LOQ of the method was defined according to International Guidelines [30–32] as the concentration of the lowest standard on the calibration curve for which (a) the analyte peak is identifiable and discrete, (b) the analyte response is at least ten times the response of the blank sample, and (c) the analyte response is reproducible with a precision less than 20% and trueness better of 80–120%.

The LOD was estimated at a signal-to-noise ratio of 3:1 by injecting a series of samples with known concentrations.

Precision and trueness studies were carried out at the LOQ and at three QC concentration levels by injecting six individual preparations of the analytes and calculating the RSD% and bias% of the back-calculated concentrations.

2.6. Recovery

The method efficiency was measured by the comparison of peak areas obtained from several samples pre-treatment extraction processes and different extraction solvent systems. Analysis of these results allowed to evaluate the better extraction procedure leading to the maximum recovery for the cited analytes, minimizing solvent and time consumptions.

3. Results and discussion

3.1. Optimisation of solvent extraction process

An important step in the determination of anthraquinones and especially of oxyprenylated anthraquinones in the bark of *Rhamnus* spp. and other plant products is the procedure employed to obtain herbal extracts. Set-up methodologies may enable exhaustive extraction of the secondary metabolites under investigation and avoid in the mean time their chemical modification or degradation.

In recent years, the development of environmental-friendly processes (ultrasound assisted extraction and supercritical CO_2-based methodologies) for the extraction of metabolites from raw plant materials has become a milestone in Phytochemistry and Pharmacognosy [33].

Two different extraction assays (maceration and ultrasonication) were examined for their extraction efficiency and/or selectivity.

Reported studies [34,35] suggested that the best extraction procedure for anthraquinones is the ultrasound assisted methods using methanol as the solvent. However, the evaluation of the presence of the oxyprenylated derivatives required a revision of the previously proposed extraction procedure, due to the enhancement in the lipophilicity of these secondary metabolites.

To this aim we first looked for the best solvent extraction system using maceration and ultrasonication. Several extraction procedures were tested, starting from already reported anthraquinone extraction methodologies [34,35]. These include different extraction solvent mixtures (ethanol, chloroform, *n*-hexane, and a mixture 1:1 chloroform–methanol) and procedures, in both extraction assays (maceration and ultrasonication). Upon the decrease of polarity, a reduction of extracted anthraquinones was observed, especially that of oxyprenylated ones, in terms of absolute chromatographic peak areas and overall recovery for the seven analytes. Using *n*-hexane only two classic anthraquinones were extracted (chrysophanol and physcione) with peak areas 80% lower than those recorded for the corresponding methanol extract. Similar data were obtained with chloroform as the extraction solvent. A better extraction process was obtained with ethanol due to an increase in polarity, although in this case only 50% of the five classic anthraquinones were recovered with respect to the corresponding methanol extract. In order to find the better conditions based on the solvents and processes examined, a 1:1 (v/v) mixture of chloroform–methanol was also investigated. In this case we observed an increase of the five classic anthraquinone peak areas (5% lower than the corresponding methanol extract). This last experimental procedure allowed us also to reveal the presence of the oxyprenylated anthraquinone peaks, although 60% lower than the corresponding methanol-based method.

Finally we disclosed that an exhaustive and reproducible extraction for all the seven analytes consisted in a multi-step process: an overnight maceration in *n*-hexane of barks, followed by separation and evaporation of the solution, filtration of the residual vegetable material and its overnight maceration using 100% methanol, followed by a second paper filtration of the latter.

The *n*-hexane extract was re-dissolved with methanol to avoid loss of the analytes.

The subsequent hydrolysis on the two combined extracts was carried out with different HCl concentrations ranging from 0.5 M to 6 M. A parallelism between the increase in the acid concentration and the analytes peak areas was recorded. To quantify the total anthraquinone contents (free + glycosilated), the two crude combined extracts were then treated with 6 M HCl for 1 h under reflux in order to hydrolyse glycosides. The resulting solutions were then submitted to liquid–liquid extraction (LLE) with ethyl acetate, the separated organic phases were dried under vacuum, and the obtained residues were stored at −20 °C until analysis as previously reported [34,35].

Extracts for HPLC–UV/vis analyses were exhaustively achieved on finely triturated bark (500 mesh) samples (1.0 g) at room temperature (25 ± 1 °C) with 10.0 mL of the extraction solvent. The maceration procedure was chosen because it allowed the same recovery although it required a longer extraction time (overnight) [35] but no chemical degradation of the oxyprenylated analytes was observed. It is noteworthy to underline that for these latter secondary metabolites extraction by ultrasonication led to an 80–90% decrease in peak areas.

Comparisons of the different extraction protocols are reported in Table 2.

3.2. HPLC separation

Several gradient mobile phases using different acids and buffers were examined in order to obtain the separation conditions of the seven anthraquinone standards.

Table 2
Comparison of the different extraction protocols.

Extraction solvent		Procedure	Time	Hydrolyse glycosides [a]	Recovery	Recovered analytes
One-step procedure						
Methanol		Maceration	Overnight	HCl 6M	Complete	Aloe-emodine, rhein, emodine, chrysophanol, physcione[c]
Ethanol		Maceration	Overnight	HCl 6M	50%	Aloe-emodine, rhein, emodine, chrysophanol, physcione
Chloroform		Maceration	Overnight	HCl 6M	20%	Chrysophanol and physcione
n-Hexane		Maceration	Overnight	HCl 6M	20%	Chrysophanol and physcione
Chloroform–methanol (1:1, v/v)		Maceration	Overnight	HCl 6M	40%	Aloe-emodine, rhein, emodine, chrysophanol, physcione, madagascin and 3-geranyloxyemodin
Methanol		Ultrasonication[b]	1 h[c]	HCl 6M	Complete	Aloe-emodine, rhein, emodine, chrysophanol, physcione[c]
Ethanol		Ultrasonication[b]	1 h[c]	HCl 6M	50%	Aloe-emodine, rhein, emodine, chrysophanol, physcione
Chloroform		Ultrasonication[b]	1 h[c]	HCl 6M	20%	Chrysophanol and physcione
n-Hexane		Ultrasonication[b]	1 h[c]	HCl 6M	20%	Chrysophanol and physcione
Chloroform–methanol (1:1, v/v)		Ultrasonication[b]	1 h[c]	HCl 6M	40%	Aloe-emodine, rhein, emodine, chrysophanol, physcione
Multi-step procedure						
1° step	*n*-Hexane	Maceration	Overnight			
2° step	Methanol	Maceration	Overnight			
3° step	Two crude methanol extracts were combined			HCl 6M	Complete	Aloe-emodine, rhein, emodine, chrysophanol, physcione, madagascin and 3-geranyloxyemodin

[a] Condition: 1 h under reflux (better condition found for hydrolysis).
[b] With ultrasonication procedure oxyprenylated compounds degradations were observed.
[c] According to previously reported procedure [35].

The chromatographic behaviour of the analytes was investigated employing several HPLC columns including Gemini C_{18} and Luna C_{18} (Phenomenex, Torrance, CA, USA); and finally a GraceSmart RP18 column (Grace, Deerfield, IL, USA) in mobile phases differing in organic modifier, buffer solution, and pH, i.e. (a) methanol–0.1% acetic acid (v/v), (b) methanol–water (both containing 1% TFA, v/v), (c) acetonitrile–30 mM phosphate buffer (pH = 3.0), and (d) methanol–water (both containing 1% formic acid, v/v), respectively [25].

The GraceSmart RP18 column was chosen to perform further experiments because it resulted in the better separation with respect to peak symmetry, resolution, and total analysis time using gradient elution with mobile phase system consisting of water (solvent A) and methanol (solvent B) both with 1% of formic acid (v/v). The choices of the column and the mobile phase were also addressed by the eventually future applications of this assay in HPLC-MS system, so that it would be possible to obtain lower LOD and LOQ values and a more complete structural information and identification for unmatched peaks in real extracted samples (i.e. peaks between 30 and 45 min that are probably 3-geranyloxyemodin degradation products). The baseline separation was achieved for nearly components, especially for emodine, chrysophanol, and physcione at 0.7 mL min^{-1} to avoid the peaks overlap and to avoid the co-elution of the aloe-emodin with other not-identified components in proximity of the void volume.

Fig. 2 shows the chromatographic separation of methanolic mixture containing seven anthraquinone standards at 435 nm (100 μM, 20 μL injected). A robust baseline analytes separation, column wash and re-equilibration, was achieved in 60 min. Under these conditions the analytes retention times were 8.5 (±0.6), 12.7 (±0.8), 22.7 (±0.7), 24.6 (±0.5), 27.7 (±0.2), 33.3 (±0.4), 44.8 (±1.0) min for aloe-emodine, rhein, emodine, chrysophanol, physcione, madagascin, and 3-geranyloxyemodin (n = 78, calibration and QC analyses), respectively.

3.3. HPLC method development

Calibration curves, obtained at 435 nm, were plotted using weighted ($1/x^2$ or $1/x$) linear least-squares regression analysis.

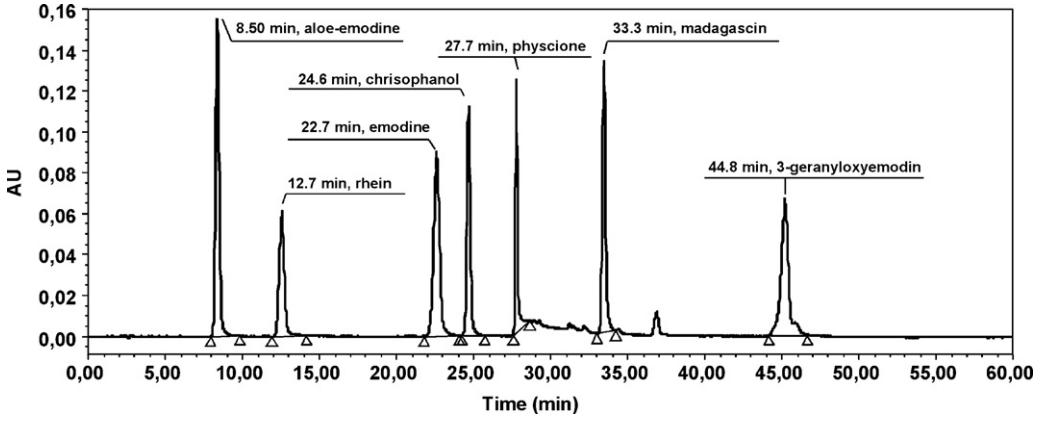

Fig. 2. Separation of standard solution mixtures containing seven anthraquinones at 435 nm (methanolic sample, 100 μM concentration level, 20 μL injected).

Table 3
Mean linear calibration curve parameters obtained by weighted linear least-squares regression analysis of three independent nine non-zero concentration point.

Analyte	Linearity range (μM)	Slope[a]	Intercept[a]	Determination coefficient (r^2)	Weighting factor
Aloe-emodine	0.5–125 (0.3 μM[b]) [0.135–33.78 (0.081 μg mL^{-1}[b])]	20,355–23,045	−1968–1487	0.9949	$1/x^2$
Rhein	0.5–125 (0.3 μM[b]) [0.142–35.53 (0.085 μg mL^{-1}[b])]	9869–10,878	5760–15,770	0.9856	$1/x$
Emodine	0.5–125 (0.3 μM[b]) [0.135–33.78 (0.081 μg mL^{-1}[b])]	19,286–20,588	420.8–2093	0.9983	$1/x^2$
Chrysophanol	0.5–125 (0.3 μM[b]) [0.127–31.78 (0.076 μg mL^{-1}[b])]	13,176–14,445	5.446–1634	0.9968	$1/x^2$
Physcione	0.5–100 (0.3 μM[b]) [0.142–35.53 (0.085 μg mL^{-1}[b])]	3673–4681	2335–3493	0.9929	$1/x^2$
Madagascin	0.5–100 (0.3 μM[b]) [0.169–33.82 (0.101 μg mL^{-1}[b])]	9619–12,209	−1551–1569	0.9850	$1/x^2$
3-Geranyloxyemodin	5–125 (1 μM[b]) [2.031–50.78 (0.406 μg mL^{-1}[b])]	14,574–16,937	−30,290–9320	0.9925	$1/x$

[a] Values at 95% confidence intervals on the mean of three independent calibration curves.
[b] In round bracket are reported LOD values obtained by signal-to-noise ratio = 3, while in square bracket are reported the limit values and ranges of linearity expressed in μg mL^{-1}; slope and intercept are reported for calibration in μM units.

The weighting factor was chosen to minimize deviation of back-calculated values from theoretical concentrations, especially for the lowest concentration levels, as permitted by the method validation guidelines, stating that "standard curve fitting is determined by applying the simplest model that adequately describes the concentration–response relationship using appropriate weighting ..." [30]. All calibration curves were linear over the concentration range tested with the determination coefficients $r^2 \geq 0.9850$ as reported in Table 3.

Table 4
Within-assay and between-assay precision (RSD%) and trueness (bias%) of the analytical method obtained from the analysis of QC samples.

	Aloe-emodine	Rhein	Emodine	Chrisophanol	Physcione	Madagascin	3-Geranyloxyemodin
Within assay							
Theoretical[b]				0.5[a]			5[a]
Mean back-calculated[b]	0.52	0.47	0.49	0.47	0.49	0.50	4.9
RSD%	8.4	8.3	8.1	8.6	12.4	11.5	7.9
Bias%	3.1	−5.5	−1.1	−5.5	−1.2	0.1	−1.3
Theoretical[b]				7.5			
Mean back-calculated[b]	7.51	6.65	7.53	6.96	7.40	7.21	6.66
RSD%	8.0	8.3	5.4	7.8	6.1	0.6	2.3
Bias%	0.2	−11.3	0.4	−7.2	−1.3	−3.8	−11.2
Theoretical[b]				60			
Mean back-calculated[b]	53.8	58.2	59.5	54.1	60.5	65.8	55.8
RSD%	4.8	12.9	4.3	4.4	11.4	5.5	9.0
Bias%	−10.4	−3.0	−0.9	−9.8	0.8	9.7	−6.9
Theoretical[b]				90			
Mean back-calculated[b]	79.2	81.0	81.2	83.3	100	98.0	88.8
RSD%	2.0	4.9	1.0	6.1	5.0	1.5	9.5
Bias%	−12.0	−10.0	−9.8	−7.5	11.5	8.9	−1.7
Between assay							
Theoretical[b]				0.5[a]			5[a]
Mean back-calculated[b]	0.54	0.47	0.49	0.47	0.49	0.50	4.7
RSD%	0.9	8.7	8.0	8.6	12.4	9.8	4.1
Bias%	8.2	−5.4	−1.1	−5.5	−1.1	0.1	−5.5
Theoretical[b]				7.5			
Mean back-calculated[b]	7.72	6.62	7.63	6.75	7.01	7.19	6.64
RSD%	6.8	12.4	0.3	3.6	1.6	0.2	1.6
Bias%	3.0	−11.7	1.7	−10.0	−6.6	−3.8	−11.5
Theoretical[b]				60			
Mean back-calculated[b]	56.6	52.7	55.6	55.6	60.3	67.6	61.2
RSD%	5.0	0.5	2.9	8.2	3.9	2.0	9.9
Bias%	−5.6	−12.2	−7.3	−7.3	0.5	12.7	2.0
Theoretical[b]				90			
Mean back-calculated[b]	83.6	83.0	86.8	83.1	98.0	93.6	88.3
RSD%	5.3	1.3	6.1	6.4	1.7	5.0	8.8
Bias%	−7.2	−7.7	−3.5	−7.7	8.9	4.0	−2.2
Over range sample diluted 1:20 (v/v) before analysis, within-assay							
Theoretical[b]				1500			
Mean back-calculated[b]	1473	1502	1483	1551	1437	1501	1459
RSD%	8.7	8.1	6.9	7.7	7.6	7.6	7.7
Bias%	−1.8	0.1	−1.1	3.4	−4.2	0.1	−2.7

Data are expressed as the mean values of six experiments.
[a] Concentration corresponding to the LOQ of the method.
[b] Concentration expressed as μM unit.

The within-assay precision (repeatability) of the method was determined by performing six consecutive assays in the same day on QC samples spiked at three different anthraquinone concentration levels, i.e. 7.5 (low level), 60 (medium level) and 90 (high level) μM, which are within the range of the calibration curve. The QC samples were also analysed in different days to assess the between-assay precision (intermediate precision) of the method.

The trueness of the method was evaluated at the same analyte concentration levels by comparing the measured anthraquinone concentrations of the QC samples with their nominal values. These data are summarized in Table 4.

As previously reported, the LOQ of the method was defined according to the Guidance for Industry on the validation of bio-analytical methods [30–32].

Following these criteria, the LOQ values for each analytes are 0.5 μM (except for 3-geranyloxyemodin for which it is 5 μM). On the basis of the signal-to-noise ratio of the chromatograms, the LOD of the method could be also set at 0.3 μM (1 μM for 3-geranyloxyemodin), as reported in Table 3.

Selectivity was first tested on standard anthraquinone solution mixtures and analysed by HPLC–UV/vis assays. Under these conditions the analytes retention times in the standard solution confirmed the retention times obtained by the real *Rhamnus* spp. bark samples analyses.

The method efficiency was measured by the comparison of the peak areas obtained from several sample pre-treatment processes and different extraction solvents.

The extraction and analysis protocol described in Section 3.1 and in Table 2 allowed to establish the better condition based on extraction efficiency/extraction time ratio and solvent consumption.

Due to the possibility of the presence of high extract concentrations (over the upper calibration concentration point), a parallelism check was performed by analysing a higher concentration samples diluted 20-fold (v/v) with the same solvent used for the preparation of standards and QC samples.

The obtained results (Table 4) indicate that the seven anthraquinone concentration levels above the top calibration standard and up to 1500 μM can be measured upon dilution of the sample with precision and trueness comparable to those achieved for concentrations within the calibration range.

During the analyses period and under the usual storage conditions no decreases in the measured classic and oxyprenylated anthraquinone concentrations or change of their chromatographic behaviour due to chemical degradation (e.g. oxidation and/or photo-oxidation) of the analytes were observed in the stock solutions and extracts samples.

3.4. Application to Rhamnus spp. sample

The content of the five "classic" anthraquinones and the two oxyprenylated ones obtained by the previously described extraction procedure are reported in Table 5.

Rhamnus spp. samples were prepared as reported above and at least three independent extractions and analyses were performed to obtain the final reported concentrations.

Fig. 3 reports the chromatograms obtained for a sample containing all the seven analytes (a, *R. saxatilis*), six analytes (b, *R. pumila*) or only the classic five anthraquinones (c, *R. alpinus*) at 435 nm.

t-Test with 95% confidence level ($\nu = 2$) showed that the quantitative results for the classical five anthraquinones in *R. saxatilis*, *R. alpinus*, and *R. pumila* obtained with the new HPLC–UV/vis assay are not significantly different from the previously reported ones [34,35] and in all cases experimental *t*-values are lower than tabulated *t*-value.

Table 5
Anthraquinone contents in *Rhamnus* spp. bark.

	Total content (μM)		
	R. saxatilis[a]	*R. alpinus*[a]	*R. pumila*[b]
Aloe-emodine	0.94 ± 0.28	35 ± 11	4.9 ± 1.5
Rhein	34 ± 10	91 ± 27	1.0 ± 0.3
Emodine	5.1 ± 1.6	231 ± 69	148 ± 44
Chrysophanol	18.6 ± 5.6	35 ± 11	46 ± 14
Physcione	95 ± 29	49 ± 15	50 ± 15
Madagascin	1.8 ± 0.5	ND	5.9 ± 1.8
3-Geranyloxyemodin	1.9 ± 0.6[c]	ND	ND
Total	158 ± 47	441 ± 32	255 ± 77
	Total content (mg g^{-1} of drug)		
	R. saxatilis[a]	*R. alpinus*[a]	*R. pumila*[b]
Aloe-emodine	0.003	0.097	0.013
Rhein	0.094	0.250	0.003
Emodine	0.013	0.603	0.387
Chrysophanol	0.046	0.087	0.112
Physcione	0.261	0.135	0.138
Madagascin	0.006	–	0.019
3-Geranyloxyemodin	0.007	–	–
Total	0.430	1.172	0.672

Data are reported as concentration (μM) ± SEM. ND: not detected.
[a] Raw sample material analysed after 3 years from collection.
[b] Raw sample material analysed after 1 year from collection.
[c] The value was obtained on concentrated sample (5-fold) after detection (>1 μM) of 3′-geranyloxy-emodine peak in the chromatogram obtained with the described method.

In comparison with the previously described methodologies [34–37], the one reported herein has undoubted advantages in terms of the overall analytical performance, mainly related to the relatively simple analytical pre-treatment (although longer due to the two overnight maceration, but more selective for the seven analytes), better LOQ (lower than 20-fold than 10 μM reported in [34–37]), and to shorten chromatographic separation run (complete gradient separation, wash and re-equilibration in 60 minutes instead of 80 minutes), thus allowing to identify and quantify the analytes with selectivity and sensitivity, especially concerning the two new oxyprenylated anthraquinones, madagascin and 3-geranyloxyemodin. To this aim it is noteworthy to underline another substantial feature of novelty of this paper, namely the discovery for the first time of the presence of isopentenyloxy and geranyloxyanthranoids in *Rhamnus* species. In our opinion, such a finding would greatly contribute to a re-examination of the whole anthraquinones profile of this pharmacologically important genus of plants as well as of other plants that are known to contain anthraquinones (e.g. *Aloe* L., *Cassia* L., and *Rheum* L. spp.). Moreover oxyprenylated anthraquinones were recently found to be efficient *in vitro* as cytotoxic and anti-protozoal agents [38] thus suggesting novel potential therapeutic uses of *Rhamnus* plants. This kind of consideration enforces the concept to provide a reliable and simple analytical methodology, like the one we described herein, for the detection of this kind of oxyprenylated secondary metabolites. Finally we depicted a preliminary anthraquinones profile of *R. pumila* Turra, a typical species of mountain regions of Europe (Iberian, Italian and Balkan Peninsula, France, central Europe) that was a virtually unknown plant up to now from a phytochemical point of view.

4. Conclusions

The new HPLC–UV/vis method for the determination of these seven anthraquinones fulfils the acceptance criteria generally established for bio-analytical assays when they are applied in pharmaceutical analysis. In the explored range the method is

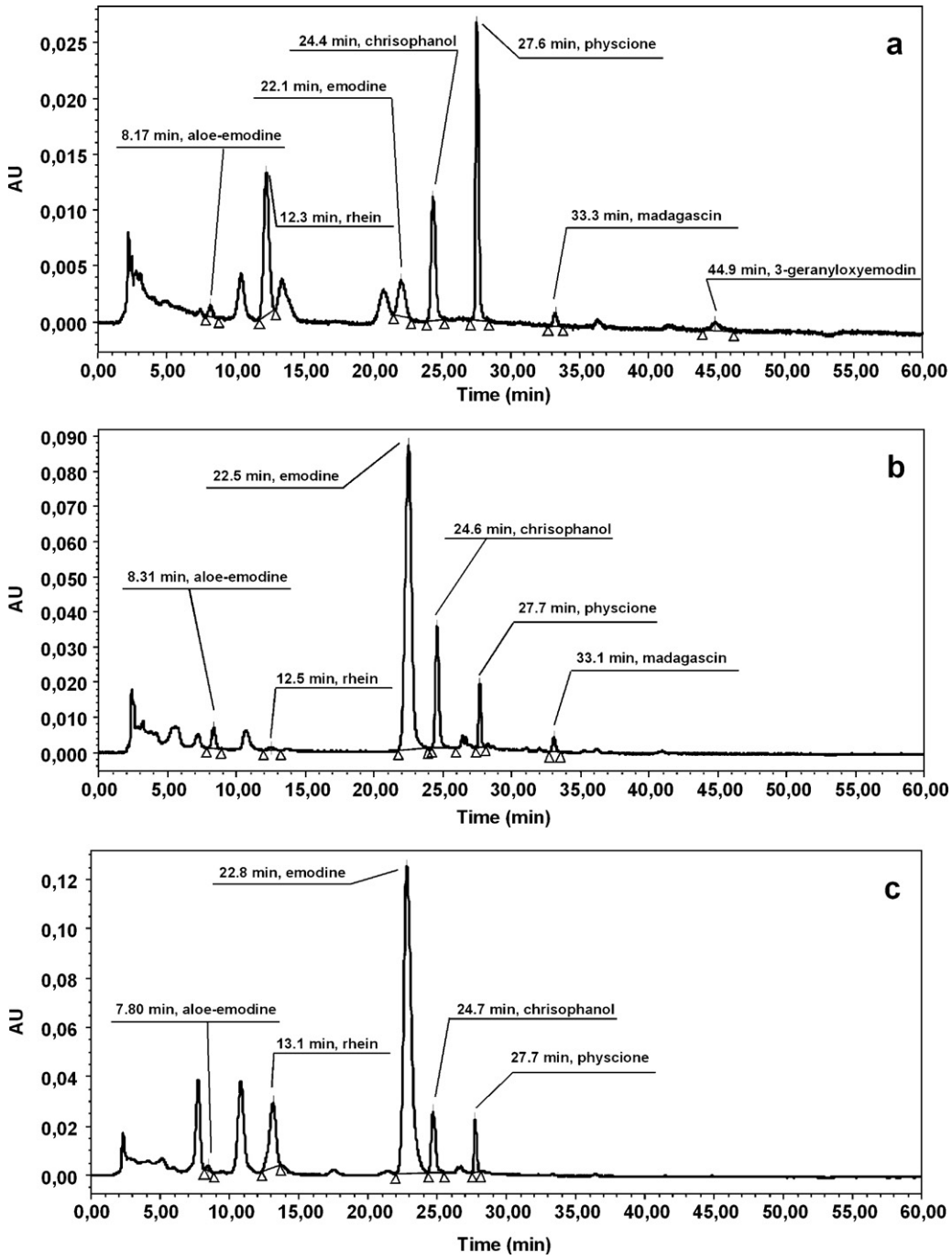

Fig. 3. Chromatogram of a sample containing all the seven analytes (a, *R. saxatilis*), six analytes (b, *R. pumila*) or only the classical five anthraquinones (c, *R. alpinus*) at 435 nm.

accurate (precision and trueness), selective, and sensitive enough to allow the analysis in methanol extract.

The analysis can be carried out by means of a relatively simple procedure, with a reduction of analytical variability and sample handling time.

Our results suggest that the reported analytical methodology is a suitable mean for the efficient detection, identification, and quantification of anthraquinones, e.g. in other *Rhamnus* spp. or in other anthranoids containing plants. These studies are now ongoing in our laboratories.

Acknowledgement

Authors wish to thank Anna Bruno for her friendly collaboration and for her active support to the experimental data collection.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.085.

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