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Analysis of anthraquinones in *Rubia tinctorum* L. by liquid chromatography coupled with diode-array UV and mass spectrometric detection

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Abstract

A liquid chromatographic (LC) method for the separation of both anthraquinone glycosides and aglycones in extracts of *Rubia tinctorum* was improved. For on-line MS detection atmospheric pressure chemical ionisation as well as electrospray ionisation (ESI) were used. The glycosides were ionised in both positive and negative ionisation (NI) mode, the aglycones only in the NI mode. With ESI ammonia was added to the eluent post-column to deprotonate the compounds. The efficiency of mass detection of the hydroxyanthraquinone aglycones was found to depend on the pK_a value of the component. LC–diode-array detection and LC–MS provide useful complementary information for the identification of anthraquinones in plant extracts, which was proven with the identification of munjistin and pseudopurpurin.
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Keywords: *Rubia tinctorum*; Mass spectrometry; Plant materials; Anthraquinones; Alizarin; Glycosides; Aglycones

1. Introduction

Beside the main anthraquinone, alizarin, 36 other anthraquinones have been reported from *Rubia tinctorum* (Table 1) [1–4]. The anthraquinones found in *Rubia tinctorum* differ in the nature of their substituents and in their substitution pattern. Several liquid chromatography (LC) methods have been described for the characterisation of *Rubia tinctorum* extracts [5–10]. In all cases the anthraquinones were detected with ultraviolet–visible light (UV–Vis) spectroscopy.

LC–MS has become an important method for rapid identification of compounds in plant extracts. Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) have been used by different authors for the ionisation of phenols and their glycosides [11–21]. Literature is available on LC–MS of phenolic naphthoquinones and hydroxyfluorenones [14], xanthones [19,20], flavonoids [12,13,17,18], naphthalenes [18], gallotannins and ellagitannins [17], naphthodianthrones [12] and their glycosides [12,15,18–21]. In contrast to phenols like flavonoids, only few publications are available on the LC–MS of anthraquinones. Nindi et al. described a

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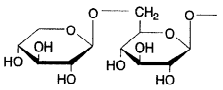
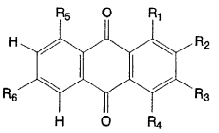
Table 1
Anthraquinone structures, the numbers in the table correspond with the numbers in the text and in the figures



primeverose



anthraquinone aglycone moiety

No:	Name	R_1	R_2	R_3	R_4	R_5	R_6	M_r
1	Lucidin primeveroside	OH	CH_2OH	Primeverose	H	H	H	564
2	Ruberythric acid	OH	Primeverose	H	H	H	H	534
3	Lucidin glucoside	OH	CH_2OH	Glucose	H	H	H	432
4	Alizarin glucoside	OH	Glucose	H	H	H	H	402
5	Pseudopurpurin	OH	COOH	OH	OH	H	H	300
6	Munjistin	OH	COOH	OH	H	H	H	284
7	Lucidin	OH	CH_2OH	OH	H	H	H	270
8	Alizarin	OH	OH	H	H	H	H	240
9	Xanthopurpurin	OH	H	OH	H	H	H	240
10	Purpurin	OH	H	OH	OH	H	H	256
11	Quinizarin	OH	H	H	OH	H	H	240
13	2-Hydroxymethylanthraquinone	H	CH_2OH	H	H	H	H	238
14	Anthraquinone	H	H	H	H	H	H	208
15	1,8-Dihydroxyanthraquinone	OH	H	H	H	OH	H	240
16	2,6-Dihydroxyanthraquinone	H	OH	H	H	H	OH	240

method for the analysis of a leaf extract of *Rhamnus prinoides* in which they applied positive ion (PI) or negative ion (NI) ESI-MS detection with addition of one of four different additives. Only two out of the four anthraquinones present in the extract were adequately ionised [18]. Mueller et al. applied NI-APCI-MS for detection of the anthraquinone aglycones emodin, chrysophanol and physcion in whole plant extracts, using gradient elution reversed-phase LC for separation of the compounds [16].

Due to the large number of anthraquinones that have been isolated from *Rubia tinctorum* [1–4], unambiguous identification solely by UV–Vis detection is not always possible. In this study an LC–MS method was developed for the separation and identification of anthraquinones in extracts of *R. tinctorum* roots.

2. Experimental

2.1. Chemicals

Acetonitrile (LC grade) and methanol (LC grade) were obtained from LAB-SCAN Analytical Sciences (Dublin, Ireland). Ultra pure water was obtained from a combined Seradest LFM 20 Serapur Pro 90 C apparatus (Seral, Ransbach, Germany). All LC solvents were degassed by vacuum filtration over a 0.45-μm membrane filter (Type RC, Schleicher & Schuell) prior to use. Ammonium formate, ammonium acetate, triethylamine, formic acid, and EDTA disodium salt dihydrate were obtained from Acros (Geel, Belgium), ammonia and acetic acid from Merck (Darmstadt, Germany).

2.2. Reference compounds

Alizarin (1,2-dihydroxyanthraquinone), purpurin (1,2,4-trihydroxyanthraquinone), quinizarin (1,4-dihydroxyanthraquinone), 2-hydroxymethylanthraquinone, 9,10-anthraquinone, 1,8-dihydroxyanthraquinone and 2,6-dihydroxyanthraquinone were purchased from Acros (Geel, Belgium). Lucidin primeveroside and ruberythric acid were not commercially available in pure form and were purified from ''crude ruberythric acid'' by droplet countercurrent chromatography (DCCC) [10]. Xanthopurpurin and lucidin were synthesised [10,22]. Corresponding structural formulas are depicted in Table 1.

2.3. Extraction and sample preparation

Dried and powdered 3-year-old root material of *Rubia tinctorum* (2.5 g) was refluxed with 100 ml water–ethanol (1:1, v/v). After 3 h the suspension was passed over a Büchner filter, and a sample of 500 μl of the filtrate was taken. The remainder of the filtrate was evaporated to dryness under reduced pressure. The resulting extract was suspended in 100 ml 2% (v/v) H_2SO_4 and refluxed for 48 h. At $t=0$, 0.5, 1, 2, 4, 12, 24 and 48 h a sample of 500 μl was taken. All samples (500 μl) were diluted with 2000 μl water–methanol (1:1, v/v). The diluted samples were filtered over a 0.45-μm membrane filter and analysed by LC.

2.4. LC conditions

LC separation was carried out at room temperature on an Alltima end-capped C_{18} column, 100 Å pore size, 5-μm particle size, 250 mm×4.6 mm I.D. column equipped with a pre-column. Prior to use, solvents were filtered over a 0.45-μm, diameter 50-mm membrane filter (Type RC, Schleicher & Schuell) and sonicated for 15 min in a Retsch Transsonic 570 (Emergo, Landsmeer, The Netherlands). Chromatography was carried out using two solvents: (A) ammonium formate–formic acid buffer (0.2 *M*, pH 3)+EDTA (30 mg/l) and (B) acetonitrile. A linear gradient programme was applied: 0–6 min 27% B; 6–20 min linear increase to 60% B; 20–23 min hold on 60% B; 23–25 min linear increase to 70% B; 25–35 min hold on 70% B, 35–40 min linear decrease to 27% B. The flow-rate during the experiment was 1.0 ml/min. Peaks were detected at 254 nm.

2.5. LC–diode-array detection (DAD) instrumentation

Analysis was performed with an LC system consisting of a 450G ternary low-pressure gradient LC pump and a 340S diode array detector. Injections were made by a Basic Marathon autosampler equipped with a 20-μl loop. The system was connected to a computer with CM PCS1 Chromeleon system control (Separations, H.I-Ambacht, The Netherlands).

2.6. LC–MS instrumentation

The LC–MS system consisted of TSP SpectraSystem including a SN4000 controller, an LC quaternary pump P4000, an autosampler AS3000, a UV2000 detector and a Finnigan LCQ ion trap mass spectrometer. The mass spectrometer was equipped with a Finnigan ESI or APCI interface. Data were processed by Finnigan Xcalibur software system (ThermoQuest, Breda, The Netherlands).

2.7. Continuous infusion

For tuning of the mass spectrometer, standard solutions of alizarin (1.3 m*M* alizarin in methanol) and ruberythric acid (1.3 m*M* ruberythric acid in methanol) were used. These solutions were separately introduced into the interface by continuous infusion using a syringe pump (Hamilton, NV, USA) at a flow of 3 μl/min. Averaged spectra were recorded over a period of 3 min: the scan range was m/z 50–2000 at a scan rate of 0.50 s. The syringe and outlet of the HPLC apparatus were coupled to the MS interface by a T-piece. During continuous infusion of alizarin or ruberythric acid with the syringe pump, also eluent was added with the HPLC pump, to mimic the circumstances during on-line LC–MS measurements. The eluent consisted of 73% solvent A and 27% solvent B, the composition for early eluting compounds of interest. Both PI and NI were used. When the ESI interface was used, various additives were introduced (by infusion from a second attached syringe at 3 μl/min) into the anthraquinone flow to promote the formation of ions. The additives, in order of increasing acidity, were: 6.25 m*M* triethylamine, 5% NH_3 in water, 25 m*M* ammonium acetate, acetic acid (2 m*M*), trifluoroacetic acid (0.5 m*M*).

2.8. HPLC–ESI-MS analysis

An Alltima end-capped C_{18} column (250 mm× 2.1 mm I.D., 100 Å pore size, 5 μm particle size) was used. The flow was 0.2 ml/min. The outlet of the LC column was connected by a T-piece with the syringe pump and the MS interface. The syringe pump added 5% NH_3 in water at a flow-rate of

3 μl/min.

The ESI parameters in the NI mode were as follows: spray voltage 4.5 kV (applied to the spray tip needle), sheath gas (N_2) 79 arbitrary units, auxiliary gas (N_2) 55 arbitrary units, heated capillary temperature 200 °C and capillary voltage −12.00 V.

2.9. HPLC–APCI-MS analyses

The conditions for the APCI interface were: vaporizer temperature 450 °C, sheath gas 80 arbitrary units, auxiliary gas 49 arbitrary units, discharge current 10 μA, heated capillary temperature 150 °C. The mass spectrometer was operated in the NI mode. Nitrogen was used as the nebulizing gas. The flow-rate was kept at 1.0 ml/min during LC analysis. No post-column addition was applied.

2.10. Multiple MS (MS^n) experiments

In MS^2 experiments helium was used as the collision gas. Only a single parent ion was kept in resonance (isolation width m/z 1–3), all other ions were ejected from the trap without mass analysis. The ion was then agitated and allowed to fragment by collision-induced dissociation (CID). The collision energy was adjusted experimentally to give >90% yield of fragmentation by varying the relative collision energy from 10 to 40%. During MS^n measurements ($n=3$) this procedure was repeated for one of the daughter ions.

3. Results and discussion

3.1. Improved RPLC separation of anthraquinones

In an earlier article [10] a simple water–acetonitrile gradient was used for the separation of anthraquinones in *Rubia tinctorum*. Using LC–MS in the current research two additional anthraquinones with a free carboxylic group were detected in *Rubia tinctorum*. If the pH of the eluent is not acidic, as is the case when pure water is used, these compounds will be ionised and elute near the dead time. For this reason instead of water a formate buffer of pH 3 was used in the eluent.

For the liquid chromatographic separations, described in this article, EDTA was added to the eluent. If not, the aglycones eluted as unidentifiable broad peaks. Alizarin forms complexes with certain metal ions. A calcium ion reacts with the 2-hydroxyl group while an aluminium ion forms a complex between the 1-hydroxyl and the carbonyl group [23,24]. Metal contamination can arise from the stainless steel parts and frits when the column is eluted or stored with pure acetonitrile or methanol [25–28]. Most likely some kind of complex formation between geminal silanol groups, metal ions and alizarin occurs during the analyses, which give rise to peak broadening.

3.2. Continuous infusion of pure standards

The initial continuous infusion studies were aimed at determining the conditions under which it is possible to ionise anthraquinone aglycones and anthraquinone glycosides present in madder roots. The two interfaces ESI and APCI were compared. Alizarin (an aglycone) and ruberythric acid (a glycoside) were used as test compounds (Table 1). Alizarin (1.3 m*M* alizarin in methanol) and ruberythric acid (1.3 m*M* ruberythric acid in methanol) were separately introduced into the ESI or the APCI interface. During introduction of the compound, also the eluent that was used for on-line LC–MS analysis was added with an LC pump.

With the ESI interface, continuous infusion of alizarin without further additives did not give any detectable ionisation in either the PI or the NI mode. To aid the formation of ions various additives (see Section 2) were used. The highest signal intensity for alizarin was obtained in NI-ESI with triethylamine solution as additive. A major drawback of this additive in MS applications is that it interferes with the ionisation of other samples and molecules over a long period of time. Therefore, ammonia instead of triethylamine was used. The MS and MS^2 results of alizarin are in Table 2. The mass spectrometer was tuned for maximum response with the peak at m/z 239.2.

Alizarin was also introduced into the APCI interface in the NI mode by continuous infusion, adding only the eluent that will be used for LC–MS measurements (Table 2). It turned out that APCI of the compounds under investigation did not require

Table 2
Results of continuous infusion experiments of alizarin and ruberythric acid

Ionisation method	m/z of major fragment	m/z obtained with MS^2	Additive
Compound alizarin			
ESI negative	239.3 $[M-H]^-$	211.2 $[M-H-C{=}O]^-$	$NH_4^+OH^-$, post-column
ESI positive	no ionisation		
APCI negative	239.3 $[M-H]^-$	211.2 $[M-H-C{=}O]^-$	NH_4^+ COO^-, in LC eluents
APCI positive	no ionisation		
	m/z of major ions		Additive
Compound ruberythric acid			
ESI negative	533.1 [M—H]$^-$, 1066.9 [2M—H]$^-$, 239.3 [M—primeverose-H]$^-$		$NH_4^+OH^-$, post-column
ESI positive	552.0 $[M+NH_4]^+$, 1085.9 $[2M+NH_4]^+$, 1619.6 $[3M+NH_4]^+$, 241.2 [M—primeverose+H]$^+$		NH_4^+ COO^-, in LC eluents
APCI negative	533.2 [M—H]$^-$, 239.4 [M—primeverose—H]$^-$		NH_4^+ COO^-, in LC eluents

eluent additives other than the buffer, an advantage over ESI. Another advantage of APCI is its compatibility with a higher eluent flow-rate. Alizarin could not be ionised with the APCI interface in PI mode with or without addition of various additives. Hence, alizarin is best analysed by APCI in NI mode.

The same experiments were carried out with a solution of the glycoside ruberythric acid (Table 2). In PI-ESI, the presence of ammonium ions in the LC eluent buffer provided the most intense signal. The main peak was the ammonium adduct ($[M+NH_4]^+$) at m/z 552.0. In NI-ESI, the best signal was obtained with ammonia as additive, with $[M-H]^-$ at m/z 533.1 as most abundant ion. Ionisation of ruberythric acid, in both the NI and PI ionisation modes, showed a pattern of M, 2M and 3M pseudomolecular ions (PI, $[M+NH_4]^+$; NI, $[M-H]^-$) at all concentrations [29]. This ionisation pattern is not seen for the aglycones. In this article on-line ESI-MS experiments were performed in the NI mode, given the observation that with ESI the aglycones can only be ionised in the NI mode.

For ionisation of ruberythric acid with NI-APCI the additive (ammonium formate) present in the LC eluent was sufficient. In contrast to ESI infusion experiments of ruberythric acid, with APCI not $[M-H]^-$ was the most abundant ion, but [M-primeverose-H]$^-$ at m/z 239.4. $[M-H]^-$ at m/z 533.2 was a minor ion (Table 2).

None of the MS or MS^n spectra applying either ESI or APCI with any additive in NI or PI mode showed an m/z signal corresponding to an ion of ruberythric acid minus xylose, the terminal sugar of the disaccharide primeverose. Thus these ionisation techniques are not suitable for determining the sugar sequence of ruberythric acid.

3.3. LC–MS analysis of a reference sample

For the development of an LC separation of the glycosides and aglycones in *Rubia tinctorum* extracts, a reference sample with the most reported anthraquinones, the glycosides lucidin primeveroside (8.8 μg/ml) and ruberythric acid (9.2 μg/ml) and the aglycones: lucidin (13.1 μg/ml), alizarin (25.4 μg/ml), purpurin (10.9 μg/ml) and quinizarin (12.1 μg/ml) was prepared. The standard mixture was analysed by LC–UV at 254 nm, LC–ESI-MS and LC–APCI-MS. For LC–NI-ESI ammonia was added post-column. Detection results of the different anthraquinones with APCI and ESI were similar. Not all the compounds in the standard mixture were detected equally well. The glycosides lucidin primeveroside (signal-to-noise, S/N of 21) and ruberythric acid (S/N 11) and the aglycone lucidin (S/N 28) could be easily detected whereas alizarin (S/N 5) and purpurin (S/N 5) were more difficult to observe and the peak for quinizarin could not be distinguished from the noise. Therefore the influence of the substitution pattern on the detectability was investigated in greater detail. Eight pure anthraquinones were separately injected and analysed. Lucidin, alizarin, xanthopurpurin, purpurin and 2,6-dihydroxyanthraquinone were easily detected. Quinizarin, 2-hydroxymethylanthraquinone, anthraquinone and

1,8-dihydroxyanthraquinone could not be detected at all. Summarising, anthraquinones with a hydroxy group γ to the carbonyl (position 2, 3, 6 and/or 7) could be detected. Anthraquinones with only one or more hydroxy groups β to the keto function of the molecule (positions 1, 4, 5 and/or 8) were not detected. These results agree with the results of Nindi et al., who reported that the anthraquinone emodin (1,6,8-trihydroxy-3-methylanthraquinone) was detected due to the hydroxy group at position 6 but not the anthraquinones chrysophanol (1,8-dihydroxy-3-methylanthraquinone) and physcion (1,8-dihydroxy-3-methyl-6-methoxyanthraquinone) [18]. A hydroxy group β to the carbonyl has a much higher pK_a value than a hydroxy group γ to the carbonyl group due to an internal hydrogen bond between the β-OH and the carbonyl group. In case of alizarin these pK_a values are 12 and 8.2 for the β and γ hydroxy group, respectively. In this research a 0.1 *M* acidic buffer solution (HPLC eluent) was mixed post-column with a 5% ammonia solution, which resulted in an overall pH of 9. All the pH values were measured before the addition of acetonitrile. Under these conditions a hydroxy group at the γ-position was deprotonated in the liquid phase, which was confirmed by the colour change of the solution (yellow→purple). The solution has insufficient basicity to abstract a proton from an anthraquinone with only hydroxy group(s) β to the keto group [14]. Also anthraquinones lacking any phenolic group like 2-hydroxymethylanthraquinone cannot be deprotonated because of the high pK_a value of the alcohol moiety. Thus the easy ionisation and mass spectral detection of lucidin is due to the γ hydroxy group and not the hydroxymethyl group.

3.4. LC–MS analysis of an ethanol–water extract of madder roots

An ethanol–water (1:1) extract of *Rubia tinctorum* was analysed with LC–ESI-MS and with LC–DAD. The UV 254 nm chromatogram and the mass spectra for individual chromatographic peaks are depicted in Fig. 1. Lucidin primeveroside and ruberythric acid are the major anthraquinone components in the extract. Lucidin primeveroside showed MS signals for $[M-H]^-$ at *m/z* 563.0, $[2M-H]^-$ at *m/z* 1126.9, $[3M-H]^-$ at *m/z* 1691.7, [M-primeverose]$^-$

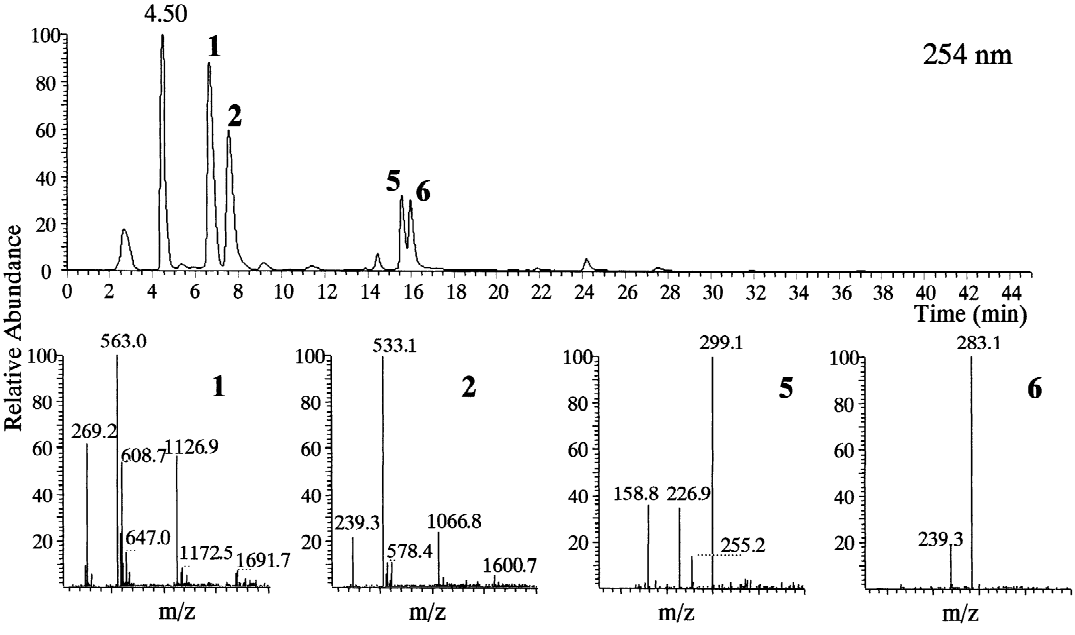


Fig. 1. UV (254 nm) trace of a crude extract of *Rubia tinctorum* roots and mass spectra (single MS) of the chromatographic peaks for individual anthraquinones lucidin primeveroside (**1**), ruberythric acid (**2**), pseudopurpurin (**5**), and munjistin (**6**). Mass spectra were obtained with NI-ESI with post-column addition of ammonia.

at m/z 269.2, and $[M+HCOO^-]^-$ at m/z 608.7. Ruberythric acid showed MS signals for $[M-H]^-$ at m/z 533.1, $[2M-H]^-$ at m/z 1066.8, $[3M-H]^-$ at m/z 1600.7, [M-primeverose]$^-$ at m/z 239.3, and $[M+HCOO^-]^-$ at m/z 578.4.

Two unknown anthraquinone peaks appeared at $t_R=15.6$ (peak **5**) and $t_R=16.0$ (peak **6**) min. Absorption maxima were found at 259 and 494 nm for peak **5** and at 248, 288 and 420 nm for peak **6**. In Fig. 1 the mass spectra of both peaks are depicted. The mass spectrum for chromatographic peak **5** showed a base peak at m/z 299.1 and for chromatographic peak **6** at m/z 283.1. Assuming that both anthraquinones ionise like the anthraquinones of the standard solution, these peaks would correspond with the $[M-H]^-$ ions. In that case the corresponding molecular masses of both peaks were, respectively, 300 and 284. MS^2 spectra showed a fragment at m/z 255.3 for the parent ion at m/z 299.1 and a fragment at m/z 239.3 for the parent ion at m/z 283.1. This agreed with decarboxylation of the parent ion (i.e. $[M-H-CO_2]^-$). The data obtained with MS, MS^2 and DAD were compared with data from literature [1,30]. From this comparison, the unknown peaks were tentatively identified as pseudopurpurin (2-carboxy-1,3,4-trihydroxyanthraquinone, 15.6 min) and munjistin (2-carboxy-1,3-dihydroxyanthraquinone, $M_r=284$, $t_R=16.0$ min) (Table 1). To confirm this assignment, the ethanol–water (1:1, v/v) extract was refluxed in sulphuric acid and the solution was analysed with LC–MS. If the peaks were really pseudopurpurin and munjistin these components should give purpurin and xanthopurpurin after decarboxylation with sulphuric acid. Indeed, after acidic hydrolysis the two peaks had disappeared and two new peaks had appeared in the chromatogram. The elution times and masses of these new peaks corresponded with those of commercially available purpurin ($M_r=256$) and synthesised xanthopurpurin ($M_r=240$).

The UV-254 nm chromatogram of the ethanol–water extract (Fig. 1) showed a large peak, not numbered, at $t_R=4.5$ min. This peak was separated by means of LC with an eluent with a higher water content, giving a number of minor peaks (not shown). From analysis of the DAD spectra, only one minor peak turned out to be an anthraquinone, which was however not further analysed.

During the first hour of refluxing of the extract with sulphuric acid two peaks appeared at $t_R=10.4$ min (peak **3**) and $t_R=13.1$ min (peak **4**). The chromatogram and corresponding MS and UV–Vis spectra are depicted in Fig. 2. The UV–Vis spectra of both unknown peaks were identical to the UV–Vis spectra of lucidin primeveroside (maxima at 247, 266 and 406 nm, match factor 999.25) and ruberythric acid (maxima at 231, 260 and 416 nm, match factor 999.15), respectively. MS^2 of the ions at m/z 431.0, 476.7 and 862.8 of the compound eluting at $t_R=10.4$ min (**3**) gave a fragment ion at m/z 269.1. MS^2 of the ion at m/z 401.0 of the compound eluting at $t_R=13.1$ min (**4**) gave a fragment ion at m/z 239.1. From these data it was concluded that the two peaks were the monoglucosides of lucidin and alizarin, respectively, lucidin glucoside ($t_R=10.4$ min) and alizarin glucoside ($t_R=13.1$ min). Lucidin glucoside gave the ions $[M-H]^-$ at m/z 431.0, $[2M-H]^-$ at m/z 862.8, [M-glucoside]$^-$ at m/z 269.1, $[M+HCOOH-H]^-$ at m/z 476.7 and [M-glucose-H]$^-$ at m/z 269.1. Alizarin glucoside gave the ions $[M-H]^-$ at m/z 401.1 and [M-glucose-H]$^-$ at m/z 239.1.

4. Conclusion

For anthraquinones on-line UV detection was found to be more sensitive than on-line mass detection. Thus, LC–UV remains the method of choice for quantitative analysis of anthraquinones.

For deprotonation of anthraquinones with only hydroxy group(s) β to the keto function, the pH of the solution has to be increased to above 10. Addition of concentrated ammonia post-column gave a pH 9. If a four times diluted buffer eluent was used the pH of the solution was 10.3 after addition of ammonia, which is still too low for deprotonation of β-hydroxyl groups. To preserve the separation of the different anthraquinones, it is not possible to change the pH of the eluent buffer to higher than 10. So the developed LC–MS method is not suitable for the identification of all the anthraquinones. Possibly identification of the anthraquinones can be achieved with stronger bases or by applying exotic ESI conditions to generate ions from these neutral anthraquinones. However the goal of this research was identification of anthraquinones in *Rubia tinctorum*

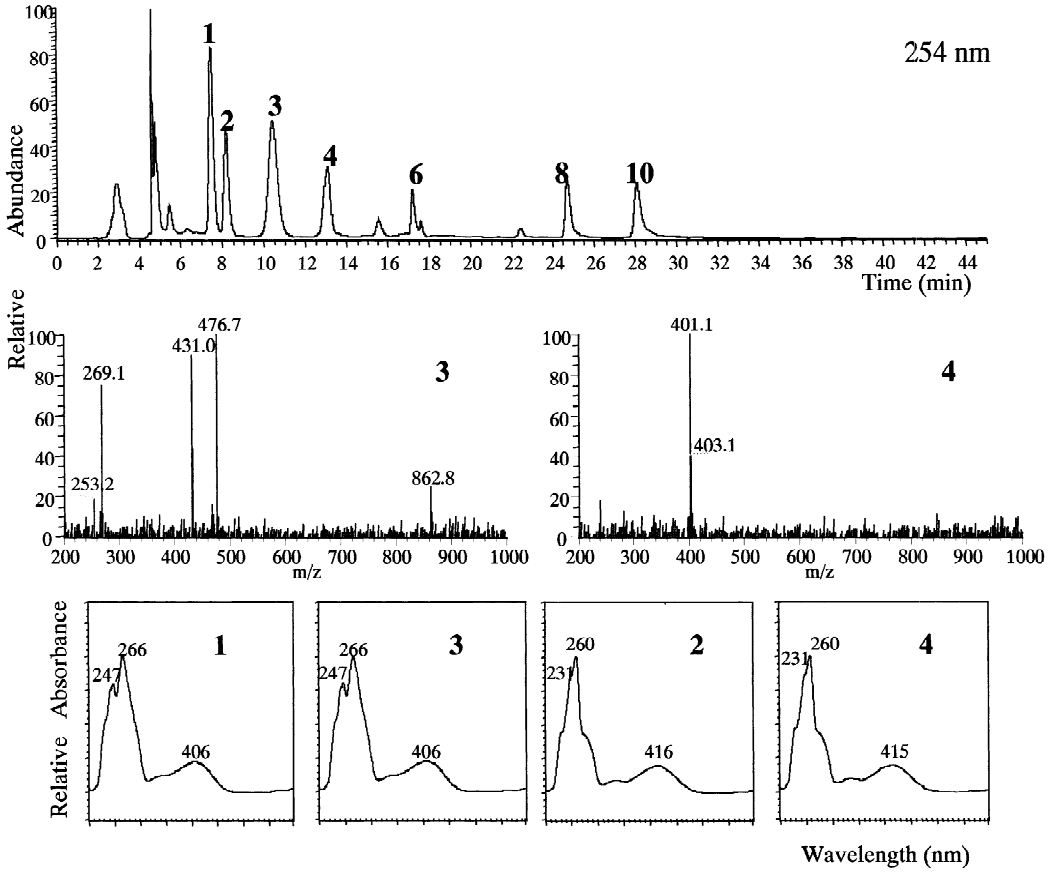


Fig. 2. UV (254 nm) profile of an acidic hydrolysate of an aqueous alcoholic extract of *Rubia tinctorum* roots (after 30 min). The mass spectra (single MS) obtained with NI-ESI with ammonia added post-column and the UV–Vis spectra of the peaks **3** and **4** are depicted, lucidin primeveroside (**1**), ruberythric acid (**2**), lucidin glucoside (**3**), alizarin glucoside (**4**), munjistin (**6**), alizarin (**8**), and purpurin (**10**).

and according to the literature most identified anthraquinones in this plant contain a hydroxy group at the 2 or 3 position γ to the keto function, which can be easily deprotonated by post-column addition of ammonia. But more important is the fact that mass spectrometry is not the only method used for detection. Anthraquinones that are missed with MS detection will still be recognised as a possible anthraquinone by Vis at 430 nm or DAD. In case of *Rubia tinctorum* all peaks detected by Vis at 430 nm also gave a signal with NI-ESI detection.

LC–DAD and LC–MS are helpful for the on-line identification of unknown anthraquinones in extracts. This is proven in this study with the identification of the anthraquinones: pseudopurpurin, munjistin, lucidin glucoside and alizarin glucoside. It can be concluded that the main anthraquinones in madder root are the glycosides lucidin primeveroside and ruberythric acid and the carboxylic anthraquinones pseudopurpurin and munjistin. Also a small amount of the aglycones alizarin and purpurin is present.

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