PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **10**, 69–75, (1999)

Electrospray Liquid Chromatography–Mass Spectrometry of the Leaf Extract of *Rhamnus prinoides*

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Three naphthalenic derivatives, four flavonoids and two of the four anthraquinones previously isolated from the leaves of *Rhamnus prinoides* were successfully ionised under electrospray ionisation conditions. These compounds were subsequently detected in the leaf extract of the plant using electrospray liquid chromatography–mass spectrometry (LC–ESI–MS). The potential of LC–ESI–MS for screening a leaf extract, with the focus on the suitability of the method for assaying the bitter principle, geshoidin, of this commercially important plant, is presented.

Keywords: *Rhamnus prinoides*; anthraquinones; flavonoids; naphthalenic derivatives; geshoidin; bitter principles; liquid chromatography; mass spectrometry; electrospray ionisation.

INTRODUCTION

Rhamnus prinoides L'Herit (Rhamanceae) is a shrub or tree up to 6 m tall. It is widespread throughout Africa and is one of two taxa representing the genus in the continent. It has been used as a bittering agent in the traditional brewing process for hundreds of years in East Africa. The plant has recently received further attention as reports have become available dealing with such aspects as the role of the plant in the regulation of microbial flora (Sahle and Abegaz, 1991), and the secondary metabolites in the leaves (Abegaz and Kebede, 1995), and in the fruits (Abegaz and Dagne, 1988; Abegaz and Peter, 1995). It has also received positive assessment as a commercial hopping agent for beer (Tessema, 1994). Recently a naphthalenic glucoside, named geshoidin **(3)**, has been identified as the substance responsible for the bitter attributes of the plant (Abegaz and Kebede, 1995). It is also claimed that this substance is innocuous even to brine shrimps at high concentrations (up to 1 mg/mL) and also showed no toxicity to several cancer cell lines (R. Becker, personal communication). In view of the potential importance of this plant, we have investigated the electrospray ionisation of 11 secondary metabolites, including **3**, isolated from the leaves (Fig. 1). We have also conducted high performance liquid chromatographic (HPLC) analyses of each of these compounds, their total mixture and also the crude leaf extract.

Presently there are several 'soft' ionisation methods that are useful for the mass spectrometric (MS) study of highly polar and high molecular mass compounds such as glycosides, carbohydrates, etc. These include fast atom bombardment (FAB), continuous flow-FAB (Cf-FAB), matrix-assisted laser desorption ionisation (MALDI), thermospray ionisation (TSP) and atmospheric pressure ionisation (API), electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Some workers have demonstrated the application of, for example, Cf-FAB and TSP–MS in screening medicinal plants (Wolfender and Hostettmann, 1995). However, ESI–MS, is a gentle technique that has been shown to produce intact ions from many biological and chemical compounds with low or high molecular masses. Its most useful applications are in the study of supramolecular chemistry (Leize *et al.*, 1996), in the analysis of peptides (Yates *et al.*, 1995), oligonucleotides (Barry *et al.*, 1995) and organic salts (Aubagnac *et al.*, 1995), and in drug metabolism studies (Jackson *et al.*, 1995). In our laboratory we have studied a number of glycosides using ESI–MS and have found this method to be effective and versatile. The present study represents one example of the application of ESI–MS as a further screening method for natural products. Although nine of the eleven compounds present in the leaf extract of *R. prinoides* gave good MS under electron impact (EI) or chemical ionisation (CI) conditions, the glycosides, including the bitter substance geshoidin, are not amenable to analysis employing these common ionisation conditions.

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Contract/grant sponsor: Department of Chemistry, University of Botswana.
Contract/grant sponsor: International Programs in Chemical Sciences (IPICS) under the auspices of the Network of Analytical and Bioarray services in Africa (NABSA).

EXPERIMENTAL

Plant material

The plant material used in this study was purchased from a "Gesho tera" market in Addis Ababa, Ethiopia. The reference compounds used in this study (Fig. 1), namely

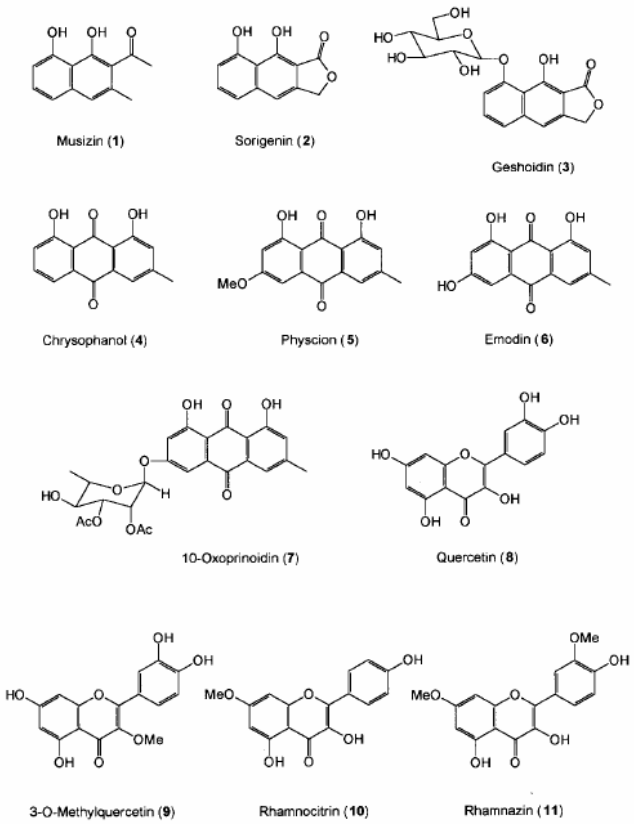

Figure 1. Structures of the compounds previously isolated from the leaves of *Rhamnus prinoides*.

musizin (**1**), sorigenin (**2**), geshoidin (**3**), chrysophanol (**4**), physcion (**5**), emodin (**6**), 10-oxoprinoidin (**7**), quercetin (**8**), 3-O-methylquercetin (**9**), rhamnocitrin (**10**), and rhamnazin (**11**), were previously isolated from the leaves of *R. prinoides* (Abegaz and Kebede, 1995).

Chemicals

HPLC grade solvents were used for all HPLC analyses (Romil, Cambridge, UK). Ultra pure water was produced using UIgaStat UHQPS (High Wycombe, UK) and Millipore Alpha Q (Bedford, MA, USA) equipment. All organic solvents were filtered through 0.45 μm filters (Millipore).

Sample preparation

The residue (20 mg) of the methanol extract of the leaves of *R. prinoides* was redissolved in 1 mL of HPLC grade methanol, and 10 μL of this solution was injected directly onto the narrow bore column (see below). Authentic natural products (1 mg of each) were dissolved in 1 mL of methanol, and 5 μL of each solution was injected and separated using the same column and mobile phases as employed for the leaf extract. The retention times and spectra of these substances were recorded. Approximately 1 mg of each of the 10 authentic samples (**1–3, 5–11**) was added to a small vial and the solid mixture dissolved in *ca.* 1 mL of methanol, and 10 μL of this solution was injected for analysis.

LC–ESI–MS Analyses

A Waters (Marlborough, MA, USA) model 600-MS multi-solvent pump with a model 490-MS programmable wavelength detector was interfaced to a Finnigan (San Jose, CA, USA) SSQ-7000 single quadrupole mass spectrometer coupled to a Digital Personal DEC 5000/25 workstation. The LC was carried out on a narrow bore column (250 × 2.0 mm i.d.) packed with YMC J'sphere ODS-M80 (S-4 μm; 80Å; Wilmington, NC, USA). The mobile phase consisted of two components, namely solvent **A** (acetonitrile with 6.25 mM trifluoroacetic acid (TFA)), and solvent **B** (water with 6.25 mM TFA). Samples were separated using a slow gradient from 20 to 80% **A** in 60 min at a flow rate of 0.25 mL/min. For LC–ESI–MS, the sheath gas of UHP nitrogen (Afrox, Germiston, South Africa) at 413.72 kPa and the auxiliary gas of nitrogen at 103.43 kPa were used to assist with nebulization. A potential of 4.5 kV was applied to the ESI needle: the metal capillary was maintained at 200°C in

Table 1. Molecular ions observed for authentic compounds 1–11 from *Rhamnus prinoides* analysed under electrospray ionisation

Compound[a]	Molecular weight	Observed ions $M^+ + 1$	$M^+ + Na$	$M^+ + K$
Musizin **(1)**	216	217	—	—
Sorigenin **(2)**	216	217	—	—
Geshoidin **(3)**	378	217[b]	401	417
Chrysophanol **(4)**	254	—	—	—
Physcion **(5)**	284	—	—	—
Emodin **(6)**	270	271	—	—
10-Oxoprinoidin **(7)**	500	—	523	—
Quercetin **(8)**	302	303	—	—
3-Methylquercetin **(9)**	316	317	—	—
Rhamnocitrin **(10)**	300	301	—	—
Rhamnazin **(11)**	330	331	—	—

[a] For structures see Fig. 1.

[b] This is a fragment ion.

order to provide optimum dissolution of the ions generated.

Flow injection analysis (FIA)–ESI–MS

The standards were introduced into the electrospray source by flow injection analysis (FIA) using a micro syringe pump (Harvard Apparatus, South Natick, MA, USA) at a flow rate of 3–20 μL/min. About 1 mg of standard was dissolved in 1 mL of methanol, and 50 μL of this solution was mixed with 200 μL of buffer. The resulting solution was infused as specified above. For FIA, a sheath gas of UHP nitrogen of 413.72 kPa and no auxiliary gas was used. A potential of 4.5 kV was applied to the ESI needle and the metal capillary was maintained at 200°C. Four different buffers (1–4) were prepared using acetonitrile:water (1:1) and the following: buffer 1, 6.25 mM TFA; buffer 2, 25 mM formic acid; buffer 3, 25 mM ammonium acetate; and buffer 4, 25 mM acetic acid. Averaged spectra were recorded over a period of 60 s: the scan range was 50–750 amu at a scan rate of 0.50 s. Musizin **(1)**, sorigenin **(2)** and geshoidin **(3)** were subjected to further fragmentation through collision induced dissociation (CID) by varying the offset voltages from +10 to +40 V. In a single quadrupole system, such as the one used here, fragmentation is induced by CID by manipulating octapole voltage offset, whereas in a triple quadrupole system (MS/MS technique) CID is induced by introducing an inert gas into the collision cell.

RESULTS AND DISCUSSION

The initial studies were aimed at determining the conditions under which it would be possible to ionise the 11 authentic natural products (three naphthalenic compounds **1, 2** and **3**, four anthracene derivatives **4–7**, and four flavonoids **8–11**) that had been previously isolated and characterised in the leaves of *Rhamnus prinoides* (Abegaz and Kebede, 1995). Two of the compounds studied, the bitter principle geshoidin **(3)** and 10-oxoprinoidin **(7)**, are glycosidic compounds. We have investigated the MS ionisation conditions and fragmentation patterns of the novel compound **3**, and the isomeric aglycones sorigenin **(2)** and musizin **(1)** which co-occur in the leaves.

ESI–MS investigations of authentic compounds

The ease of ionisation of the 11 authentic compounds was investigated using four different buffers containing TFA, formic acid, ammonium acetate or acetic acid (see Experimental section). A solution of each of the compounds was introduced into the electrospray capillary using an FIA method. All, except the anthraquinones chrysophanol **(4)** and physcion **(5)**, gave high intensity ions corresponding to either $[M+H]^+$, $[M+Na]^+$ or $[M+K]^+$ with little or no fragmentation (see Table 1). Quantitative comparison of the spectra obtained for each of the compounds using the various buffers revealed that high intensity ions were observed using all buffers, with the best results being obtained when TFA was used (buffer 1). Although the glucoside geshoidin **(3)** failed to yield a molecular ion under EI as well as CI ionisation conditions, the ESI–MS spectrum (Fig. 2a) gave major ions at *m/z* 401 ($[M+Na]^+$; 100%) and 217 (see below; 72%), and a less intense ion at *m/z* 417 corresponding to $[M+K]^+$ (10%). MS data for **3** is presented here for the first time. The ion at *m/z* 217 was initially assumed to be derived from the aglycone sorigenin **(2)**, presumably arising by hydrolysis, but it is probably a fragment derived from the molecular ion (as will be shown later). Additional spectra were obtained by applying a series of voltage offsets (of 10, 15, 25, 30, 35 and 40V) to investigate the CID of the major ions. This resulted in the formation of two principal ions at *m/z* 239 and 199. The ion at *m/z* 239 is assumed to be a fragment arising from $[M+Na\text{-glucose}]^+$, while the ion at *m/z* 199 results probably from the loss of water from the *m/z* 217 ion. These ions appeared at about 25V reaching an optimum at 30V and declining thereafter. The spectrum obtained at 30V is shown in Fig. 2b.

The MS of the isomeric naphthalenic compounds sorigenin **(2**; MW 216) and musizin **(1**; MW 216) are shown in Fig. 2c and d, respectively. Both compounds gave protonated molecular ions as the most intense ions; this is in contrast to **3** which shows the $[M+Na]^+$ ion.

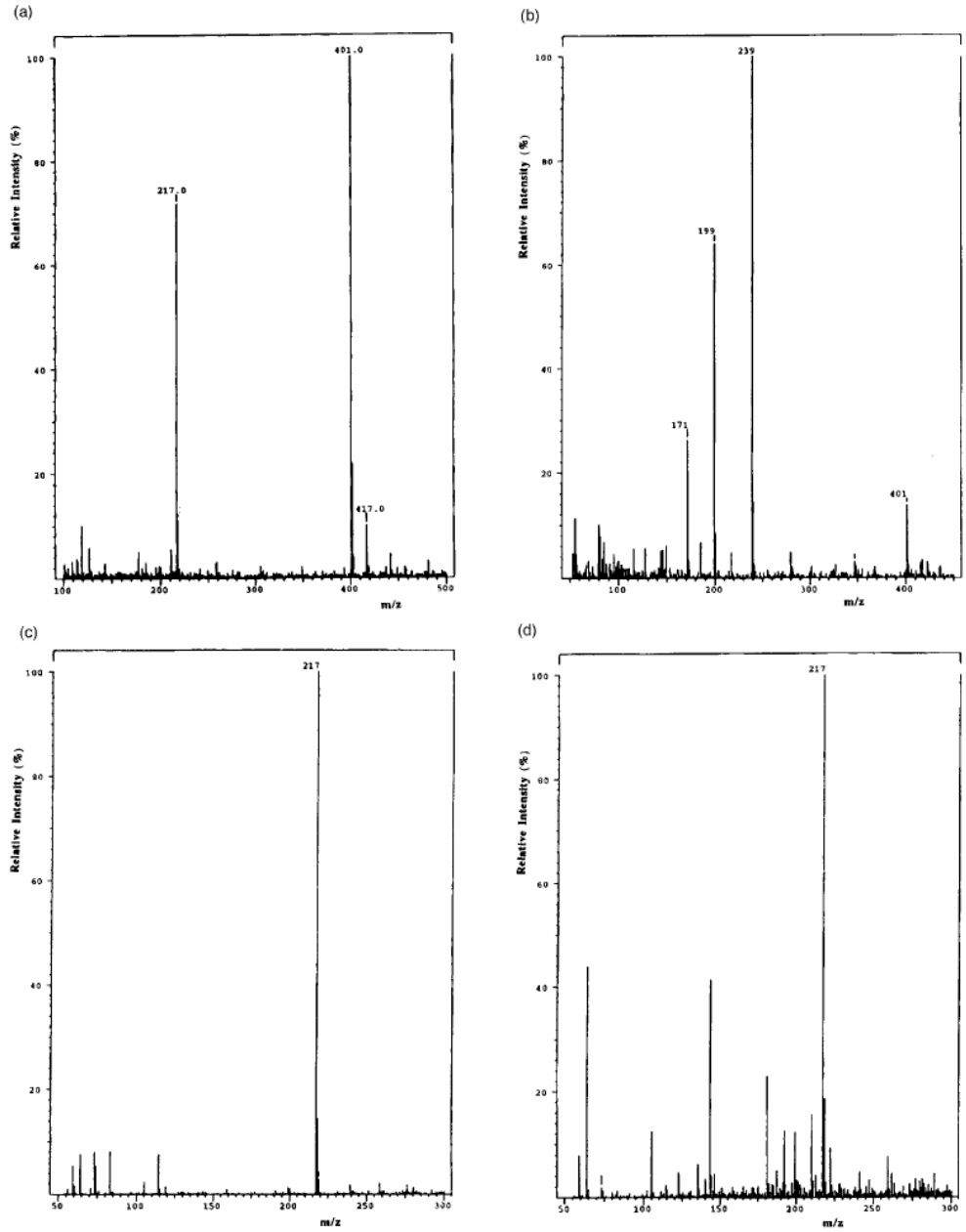

Figure 2. (a) FIA–ESI mass spectrum of geshoidin (**3**). (b) CID FIA–ESI mass spectrum of geshoidin (**3**) at 30 V. (c) FIA–ESI mass spectrum of sorigenin (**2**). (d) FIA–ESI mass spectrum of musizin (**1**).

The CID spectra of both **2** and **1** were measured under various voltage offset values. With increasing voltages, three ions (at *m/z* values of 199, 177 and 80) became prominent in the MS of both compounds. The intensities of these ions were, however, different: for **1** the ion at *m/z* 199 became the base peak within the voltage offset range 15–25V, while the fragment at m/z 80 was the dominant ion for **2**. This is in stark contrast to the characteristically

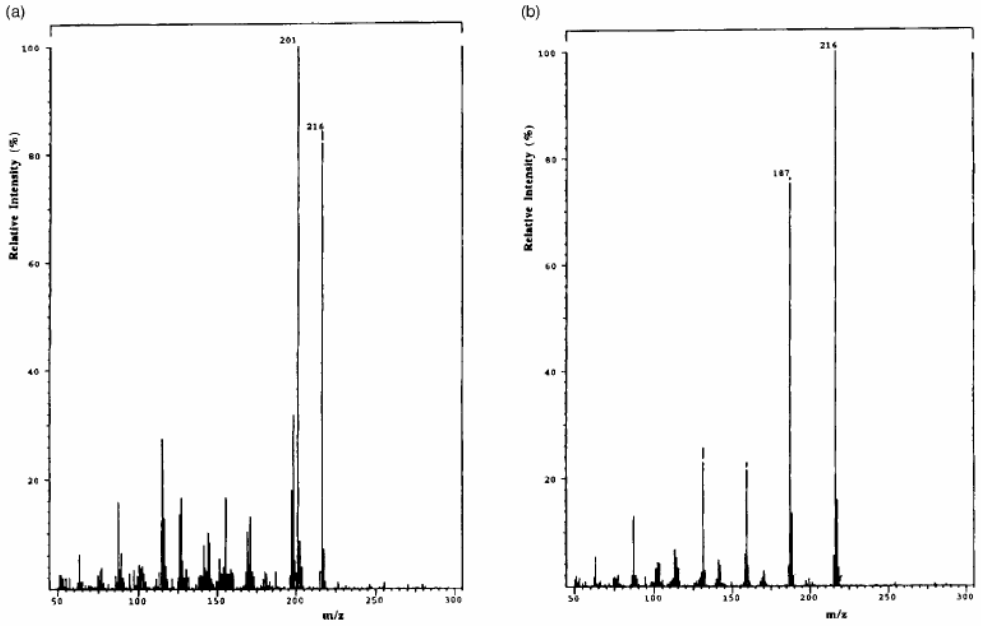

Figure 3. (a) EI mass spectrum of musizin **(1)**. (b) EI mass spectrum of sorigenin **(2)**.

different fragmentation patterns observed under EI conditions (see Fig. 3a and b): under EI, **1** loses a methyl group leading to a prominent $[M\text{-}CH_3]^+$ ion, while **2**, not having a methyl substituent, loses 29 amu giving rise to a $[M\text{-}CHO]^+$ ion. Under CID conditions, both compounds appear to each lose a molecule of water and thus the fragment ions formed cannot be used to characterise these isomeric compounds. Both of these compounds also gave $[M\text{-}H]^-$ ions at *m/z* 215 when run under negative ionisation conditions in contrast to geshoidin which failed to give a reasonable MS.

Attempts to induce electrospray ionisation of the anthraquinones were not successful for chrysophanol **(4)** and physcion **(5)**. These quinones completely failed to ionise in all four of the buffer systems. Negative ion ESI–MS also failed to show any ions attributable to these quinones. These substances furnish MS readily under EI as well as CI ionisation conditions. Emodin **(6)** was more amenable for investigation under positive and negative ESI–MS and in all four buffer systems. Positive ESI–MS furnished a $[M+H]^+$ ion only; no ions attributable to $[M+Na]^+$ or $[M+K]^+$ were observed. Negative ESI–MS consistently furnished a $[M\text{-}H]^-$ ion and a *m/z* 113 ion as the only two ions in the MS; the latter is due to a trifluoroacetate ion coming from the buffer. 10-Oxoprinoidin **(7)** showed an intense ion at *m/z* 523 corresponding to $[M+Na]^+$. No ions attributable to $[M+H]^+$ or $[M+K]^+$ were observed in any of the four buffers. Negative ESI–MS did not lead to any significant ion at $[M\text{-}H]^-$ mu.

The four flavonoids **(8–11)** were studied in all four buffers systems and yielded in every case intense positive ions at $[M+H]^+$. Apart from rhamnazin **(11)**, the remaining flavonoids also gave intense $[M\text{-}H]^-$ ions when run under negative ion ionising conditions.

HPLC–ESI–MS analysis of the leaf extract of *R. prinoides*

In order to demonstrate the potential of LC–ESI–MS, a methanolic solution of the leaf extract was separated by a slow gradient on a C_{18} column (see Experimental section). The eluent was sequentially monitored by UV at 254 nm and ESI–MS. Two chromatograms were generated, one trace arising from the reconstructed total ion chromatogram from the analyser and the second showing a trace of the active components detected by the UV detector (Fig. 4). A full scan of the chromatogram was obtained and the data were manipulated by selected ion monitoring (SIM) using the characteristic ions (see Table 1) observed for each of the authentic compounds. Fig. 4 shows the result of the search for the naphthalenic compounds **1** (*m/z* 217), **2** (*m/z* 217) and **3** (*m/z* 401, 217, 417). Trace **e** (Fig. 4) represents components in the mixture that give rise to ions with *m/z* of 217. SIM (trace **d**) of ion *m/z* 401 (which had been established from the earlier FIA studies to be that of the $[geshoidin+Na]^+$ adduct) coincides with the second of the three peaks in trace **e**. Further confirmation is given by the SIM search (trace **c**) for the $[geshoidin+K]^+$ ion (*m/z* = 417), which again coincides with the second peak in trace **e**. This observation led to the conclusion that the ion with *m/z* of 217 observed in the MS of **3** arises by fragmentation of the molecular ion, although the possibility of hydrolysis caused by the acid in the buffer used cannot be completely ruled out. The remaining two peaks in trace **e** (Fig. 4) are concluded to be those of compounds **1** and **2**. As mentioned earlier in the FIA study, it is difficult to distinguish between these two isomeric compounds on the basis of ESI–MS alone. As they show similar fragment ions under CID conditions, the only way to

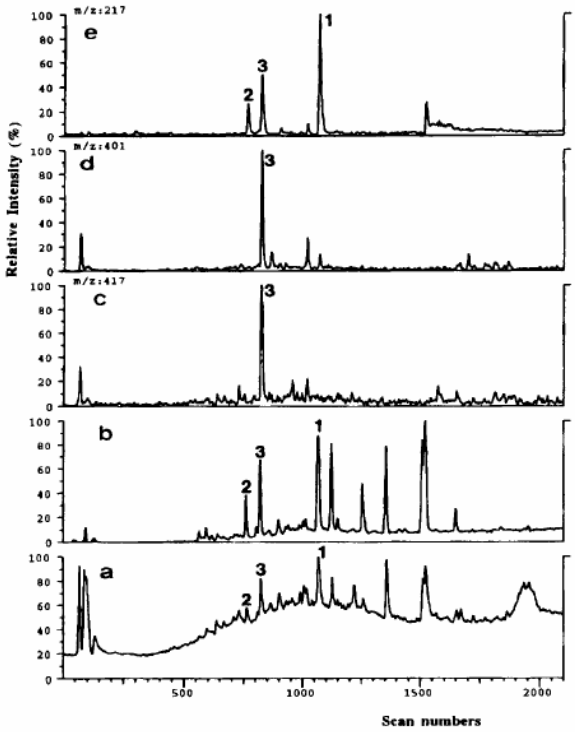

Figure 4. ESI–LC–MS analysis of the methanol extract of the leaf of *R. prinoides* showing (**a**) the reconstructed ion current chromatogram, and (**b**) the chromatogram obtained by UV detection at 254 nm. Traces **c**, **d** and **e** show SIM searches of the chromatogram for ions of *m/z* 417, 401 and 217, respectively. Key to peak identities: **1**, musizin; **2**, sorigenin; **3**, geshoidin.

establish the identity of the peaks in the chromatograms was by comparison of retention times with authentic samples.

Among the quinones, only emodin (**6**) and 10-oxoprinoidin (**7**) were detected in the ion chromatogram of the leaf extract. Chrysophanol (**4**) and physcion (**5**) were found to ionise poorly even under FIA conditions accounting for the failure to detect them in the ion chromatogram of the extract; all the quinones could clearly be observed by UV detection, however.

The flavonoids **8–11** were easily detected in the leaf extract (Fig. 5). Rhamnocitrin (**10**) and rhamnazin (**11**) gave rise to overlapping peaks but SIM established **10** as the early eluting peak (Fig. 5, trace **f**) followed immediately by **11** (trace **c**).

CONCLUSIONS

The ionisation and mass spectral behaviour of three naphthalenic derivatives, four anthraquinones and four flavonoids, previously isolated from *R. prinoides* leaves, have been studied under electrospray ionisation conditions. The naphthalenic derivatives, the flavonoids and the anthraquinone glycosides were detected easily, but two of the three common anthraquinone aglycones failed to ionise under ESI. LC–ESI–MS has been found to be quite suitable for the qualitative analysis of the bitter principle (geshoidin) and other secondary metabolites of the leaf extract of *R. prinoides*. A small aliquot (10 μL) of the methanol extract (20 mg dissolved in 1 mL) of the leaf material was required for direct injection into the HPLC column with the effluent being led into the electrospray ionising capillary. The entire analysis could be carried out in a relatively short time. Pilot studies by a commercial brewer have indicated that the extract of *R. prinoides* can be used as a hopping agent and thus the development of the method described in this report may provide a direct assay method for the level of the bitter substance in beer.

Acknowledgements

We are grateful to Mr Merhatibeb Mezabih for providing us with solutions and mixtures of the authentic compounds used in this study. We also acknowledge financial support given by the Department of

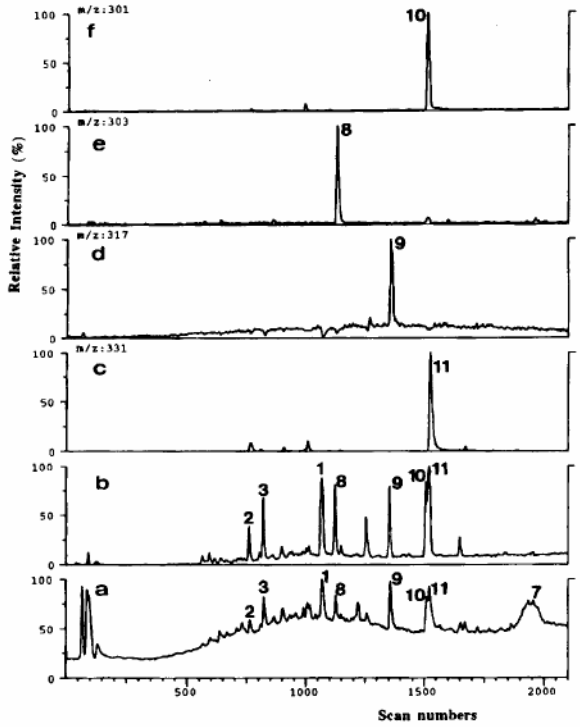

Figure 5. ESI–LC–MS analysis of the methanol extract of the leaf of *R. prinoides* showing (**a**) the reconstructed ion current chromatogram, and (**b**) the chromatogram obtained by UV detection at 254 nm. Traces **c, d, e** and **f** show SIM searches of the chromatogram for ions of *m/z* 331, 317, 303 and 301, respectively. Key to peak identities: **1**, musizin; **2**, sorigenin; **3**, geshoidin; **8**, quercetin; **9**, 3-O-methylquercetin; **10**, rhamnocitrin; **11**, rhamnazin.

Chemistry of the University of Botswana. Financial support by International Programs in Chemical Sciences (IPICS) under the auspices of the Network of Analytical and Bioassay Services in Africa (NABSA) is also acknowledged. Dr Jean-Luc Wolfender is grateful to Professor Hostettmann for a one-week travel grant to Botswana during the course of this study.

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