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A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts

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Due to their substitution with an isoprenoid group, prenylated flavonoids have an increased affinity for biological membranes and target proteins, enhancing their potential bioactivity. Although many prenylated flavonoids have been described, there are no methods that specifically screen for their presence in complex mixtures, prior to purification. We describe a method based on ultra-high-performance liquid chromatography (UHPLC) with electrospray ionisation mass spectrometry (ESI-MS) that allows rapid screening for prenylated flavonoids in multi-component plant extracts. Identification of the prenylated flavonoids is based on screening for neutral losses of 42 u and 56 u in the positive-ion mode MS^2 and MS^3 spectra within the MS chromatograms. In addition, this method discriminates between a prenyl chain and a ring-closed prenyl (pyran ring), based on the ratio of the relative abundances of the ions that lose 42 u and 56 u (42:56). The application of this screening method on a 70% aq. ethanol, ethanol and ethyl acetate extract of the roots of *Glycyrrhiza glabra* indicated the presence of 70 mono- and di-prenylated flavonoids. In addition, of each prenylated flavonoid the type of prenylation, chain or pyran ring was determined. **Copyright © 2009 John Wiley & Sons, Ltd.**

Flavonoids are a class of naturally occurring compounds ubiquitous in plants, with a broad variety of bioactivities.[1] Flavonoids can be decorated with one or more C_5 isoprenoid substituents, and these are referred to as prenylated flavonoids.[2–4] Prenyl groups can be attached to a flavonoid in the form of a prenyl chain (3-methyl-2-butenyl or dimethylallyl) or in the ring-closed form of a pyran ring (2,2-dimethylpyran). In general, most flavonoids are *C*-prenylated, whereas *O*-prenylation is less common.[5] Prenylation increases the lipophilicity of flavonoids leading to an increased affinity to biological membranes and to an improved interaction with target proteins. This results in enhanced biological activities such as anti-microbial and estrogenic activities.[5,6] Examples of prenylated flavonoids with estrogenic activities are 8-prenylnaringenin and xanthohumol from hop (*Humulus lupulus*).[7,8] Prenylated compounds seem to offer interesting possibilities for developing new food supplements, e.g. for the alleviation of osteoporosis and menopausal complaints, which are both under hormonal control.

In the past two decades, many prenylated flavonoids have been reported, mainly in plants belonging to the *Leguminosae* and *Moraceae*.[2] Most of these flavonoids have been structurally elucidated by means of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) upon their isolation and purification. A screening method to directly detect prenylated flavonoids in multi-component samples is unavailable until now, but might offer opportunities in facilitating the discovery of potential bioactivities.

Flavonoids have been extensively investigated with electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), both in negative-ion (NI) and in positive-ion (PI) mode, each with their own advantages. In general, NI mode is considered more sensitive and selective for the analysis of flavonoids.[9] An essential part of the identification of flavonoids by MS is the fragmentation of the deprotonated molecules in MS^n, in which the flavonoid is degraded into fragment ions. So far, there are no clear indications for the degradation of the prenyl group of prenylated flavonoids in the MS^2 spectra in NI mode. This supports the hypothesis that due to the nucleophilic nature of the prenyl group degradation is highly unfavourable.[10]

Licorice refers to the roots from *Glycyrrhiza glabra* and have been traditionally used in herbal medicine for over 4000 years.[11,12] In the past decades, extensive chemical studies have resulted in the identification of many flavonoids in the roots of *Glycyrrhiza glabra*, including about 75 prenylated flavonoids.[13–22]

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The aim of the present study was to develop an ultra-high-performance liquid chromatography (UHPLC)/MS method that allows rapid screening of prenylated flavonoids in multi-component plant extracts. Licorice roots were used as a source because of the presence of many different prenylated and non-prenylated flavonoids.

EXPERIMENTAL

Materials

The roots of *Glycyrrhiza glabra*, collected in Afghanistan, were provided by Frutarom US (North Bergen, NJ, USA). Glabridin and glycyrrhizinic acid were purchased from Wako Chemicals GmbH (Neuss, Germany). UHPLC/MS grade acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were purchased from Merck (Darmstadt, Germany).

Sample preparation

The roots were milled with a model ZM 200 Retsch Ultra centrifugal mill (Haan, Germany) to yield a root powder with a particle size of 1 mm. The root powder (0.01 g powdered root/mLsolvent) was extracted with either 70% (v/v) aq. ethanol (EtOH), EtOH or ethyl acetate (EA). The compounds were extracted by a two-step sequential extraction with each solvent for 30 min in a sonication bath at 30°C. The extracts were centrifuged at 2500 *g* for 15 min. The dried extracts were obtained after evaporation (EtOH and EA) or after subsequent lyophilisation (70% (v/v) aq. EtOH). The extracts were resolubilised in methanol (MeOH) and stored at −20°C. All samples were thawed and centrifuged before analysis.

RP-UHPLC analysis

Samples were analysed on a Thermo Accela UHPLC system (San Jose, CA, USA) equiped with pump, autosampler and photo-diode array (PDA) detector. Samples (1 μL) were injected on a Waters Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm particle size) with a Waters Acquity UPLC BEH C18 Vanguard pre-column (2.1 × 5 mm, 1.7 μm particle size; Waters, Milford, MA, USA). Water acidified with 0.1% (v/v) acetic acid (eluent A) and acetonitrile (ACN) acidified with 0.1% (v/v) acetic acid (eluent B) were used as eluents. The flow rate was 300 μL/min, and the PDA detector was set to measure in the range of 205–400 nm. The following elution profile was used: 0–18 min, linear gradient from 10%–100% (v/v) B; 18–22 min, isocratic on 100% B; 22–23 min, linear gradient from 100%–10% B; 23–25 min, isocratic on 10% B.

Electrospray ionisation mass spectrometry (ESI-MS)

Mass spectrometric data were obtained by analysing samples on a Thermo Scientific LTQ-XL (San Jose, CA, USA) equipped with an ESI probe coupled to the reversed-phase (RP)-UHPLC system. Of the flow from the RP-UHPLC system, 150 μL/min was directed to the mass spectrometer. Helium was used as sheath gas and nitrogen as auxiliary gas. Data were collected over an *m/z*-range of 150–1500. Data-dependent MS^n analysis was performed with a normalised collision energy of 35%. The MS^3 fragmentation was always performed on the most intense product ion in the MS^2

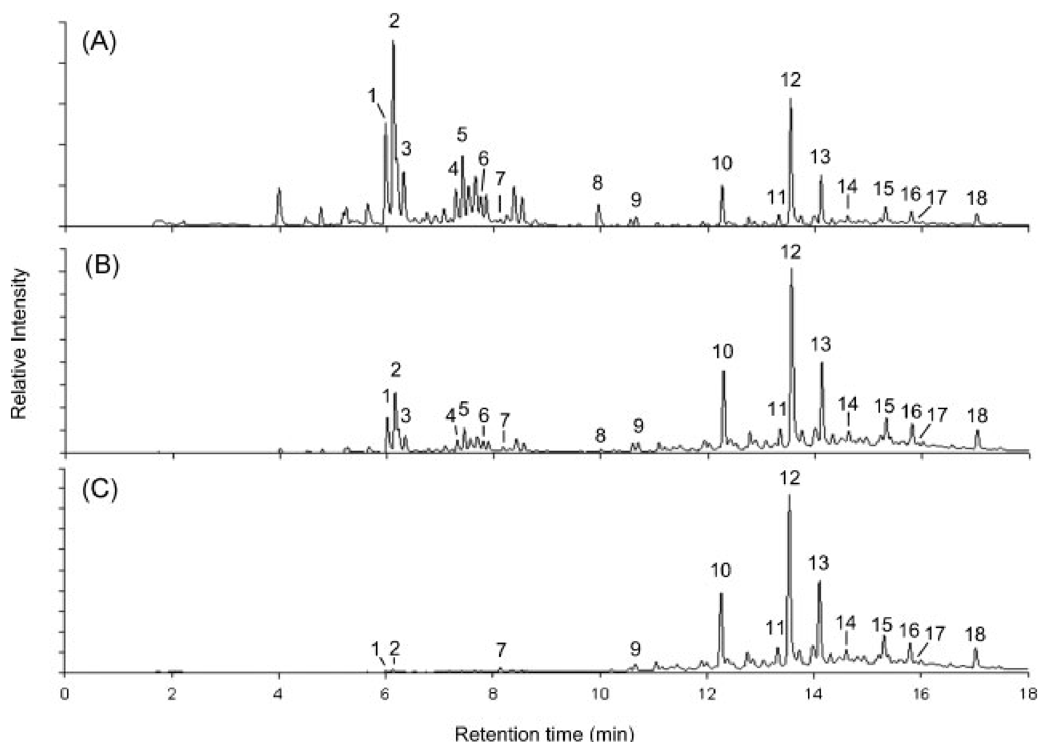

Figure 1. RP-UHPLC-UV profile of *Glycyrrhiza glabra* roots extracted with 70% aq. EtOH (A), EtOH (B) and EA (C). Peak numbers refer to compounds in Table 1.

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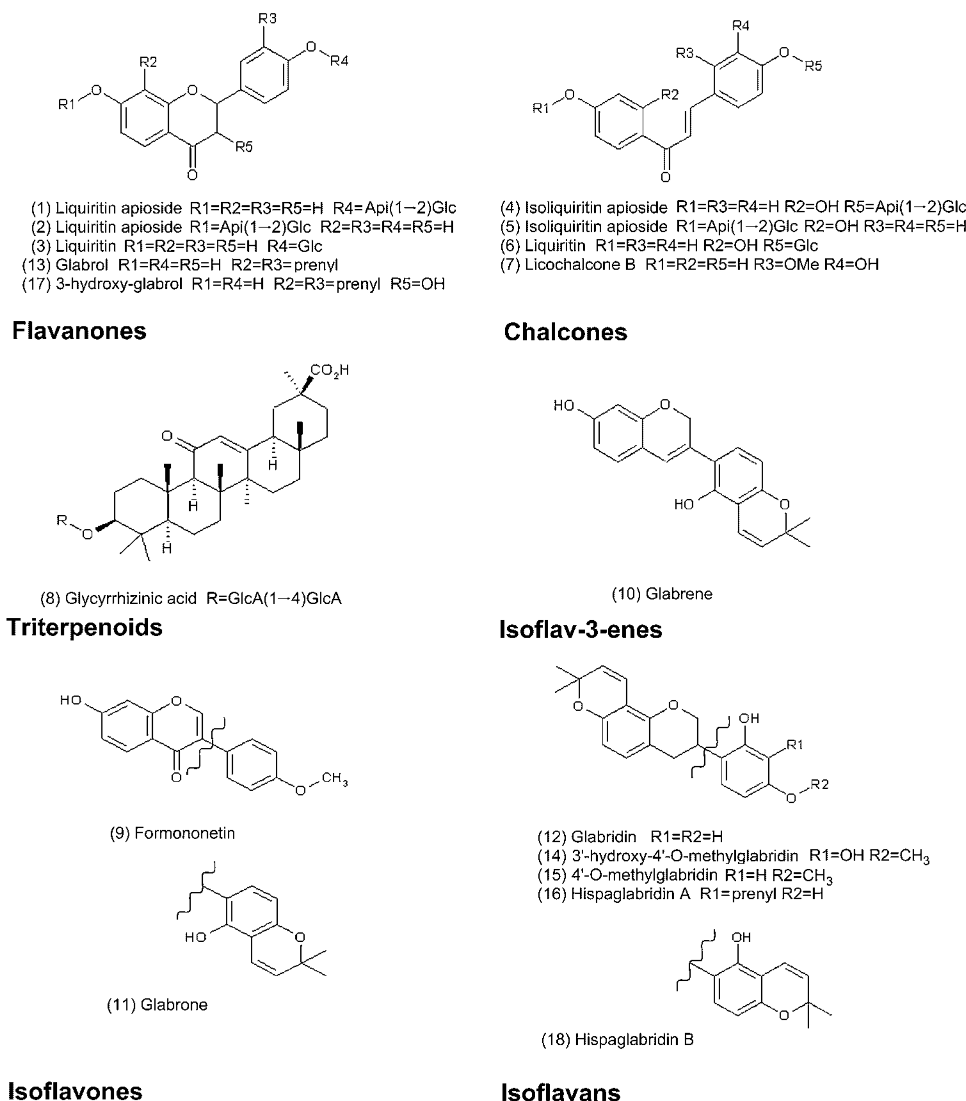

Figure 2. Structures of compounds assigned in a *Glycyrrhiza glabra* root extract.

spectrum. Most settings were optimised using 'tune plus' (Xcalibur 2.07, Thermo Scientific) via automatic tuning.

The system was tuned with glabridin in both PI and NI mode. In the NI mode, the ion transfer tube temperature was 350°C and the source voltage 4.5 kV. In the PI mode, the ion transfer tube temperature was 350°C and the source voltage 5.0 kV. Data acquisition and reprocessing were performed with Xcalibur 2.07 (Thermo Scientific).

Mass spectral data interpretation and peak determination were performed with Mass Frontier 4.0 (Highchem, Bratislava, Slovakia).

RESULTS AND DISCUSSION

Chromatographic analysis of root extracts from *Glycyrrhiza glabra*

The extraction of the licorice roots resulted in extraction yields of 23.7, 6.3 and 4.7 g/100 g for 70% aq. EtOH (v/v), EtOH and EA, respectively. Each of the UHPLC-UV profiles of the three licorice root extracts indicated a complex mixture of phenolic compounds (Fig. 1). Preliminary chemical analysis of the roots confirmed the presence of glabridin, which is a marker compound for *G. glabra* roots.[23] The 70% aq. EtOH (v/v) extract contained compounds eluting in the first 10 min of the chromatogram. Extracting with EtOH resulted in an extract with relatively less polar compounds and relative more apolar compounds, eluting in the second half of the chromatogram. With EA, mainly apolar compounds were extracted. A total of 18 peaks could be tentatively assigned in the UHPLC profiles by means of UV spectra, the *m/z* values of the precursor ion, and collision-induced dissociation (CID) fragmentation of these precursor ions in PI and NI mode (Fig. 2 and Table 1).

Peaks 1–9

Peaks **1–6** represent the flavonoid glycosides in the 70% (v/v) aq. EtOH and EtOH extracts, i.e. the three glycosidic forms of both liquiritigenin and isoliquiritigenin. The fragmentation of liquiritigenin, isoliquiritigenin and their corresponding glycosides have been described previously in PI and NI modes.[24,25] Our observations are in line with these results (Table 1).

The fragmentation patterns of both liquiritgenin (flavanone) and isoliquiritigenin (chalcone) in ESI were very

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Table 1. Compounds tentatively assigned in roots *Glycyrrhiza glabra* by UHPLC/ESI-MS

No	Rt. (min)	UV_{max} (nm)	Identification	Class	Molecular formula	$[M-H]^-$	MS^2 product ions (*relative intensity*)	$[M+H]^+$	MS^2 product ions (*relative intensity*)
1	6.01	216, 271, 316	Liquiritin apioside	Flavanone	$C_{26}H_{30}O_{13}$	**549**	429(84), 417(4), 297(13), 255(100)	**551**	419(35), 257(100)
2	6.15	214, 277, 312	Liquiritin apioside	Flavanone	$C_{26}H_{30}O_{13}$	**549**	429(8), 417(14), 297(13), 255(100)	**551**	419(100), 257(84)
3	6.34	215, 275, 310	Liquiritin	Flavanone	$C_{21}H_{22}O_9$	**417**	255(100)	**419**	257(100)
4	7.32	240, 361	Isoliquiritin apioside	Chalcone	$C_{26}H_{30}O_{13}$	**549**	429(10), 417(15), 297(15), 255(100)	**551**	419(88), 257(100)
5	7.45	240, 369	Isoliquiritin apioside	Chalcone	$C_{26}H_{30}O_{13}$	**549**	429(80), 417(4), 297(12), 255(100)	**551**	419(100), 257(40)
6	7.80	240, 370	Isoliquiritin	Chalcone	$C_{21}H_{22}O_9$	**417**	255(100)	**419**	257(100)
7	8.17	215, 360	Licochalcone B	Chalcone	$C_{16}H_{14}O_5$	**285**	270(100), 253(8), 225(1), 209(1), 191(7), 150(2)	**287**	245(100), 193(10), 167(7), 147(7), 121(6)
8	10.00	219	Glycyrrhizinic acid	Triterpenoid	$C_{42}H_{62}O_{16}$	**822**	803(15), 759(7), 645(10), 351(100)	**823**	647(30), 471(16), 453(100)
9	10.70	216, 248, 297	Formononetin	Isoflavone	$C_{16}H_{12}O_4$	**267**	252(100), 223(1), 208(1), 191(1)	**269**	254(100), 237(39), 213(33), 107(10)
10	12.26	217, 284, 324	Glabrene	Isoflav-3-ene	$C_{20}H_{18}O_4$	**321**	306(100), 303(27), 293(18), 277(27), 175(18), 145(14)	**322**	*Not determined*
11	13.50	222, 250, 302	Glabrone	Isoflavone	$C_{20}H_{16}O_5$	**335**	320(18), 307(10), 292(11), 291(100), 213(17)	**337**	295(41), 283(100), 239(17), 137(22)
12	13.53	230, 281	Glabridin	Isoflavan	$C_{20}H_{20}O_4$	**323**	213(37), 201(71), 147(28), 135(100), 121(35)	**325**	269(14), 215(8), 203(21), 189(100), 123(35)
13	14.09	220, 283	Glabrol	Flavanone	$C_{25}H_{28}O_4$	**391**	203(100), 187(24), 159(5)	**393**	337(100), 205(15), 203(10)
14	14.59	223, 271, 323	3′-Hydroxy-4′-*O*-methylglabridin	Isoflavan	$C_{21}H_{22}O_5$	**353**	338(31), 201(100), 175(28), 165(45)	**355**	215(4), 189(100), 153(73), 147(5)
15	15.35	208, 226, 281	4′-*O*-Methylglabridin	Isoflavan	$C_{21}H_{22}O_4$	**337**	322(47), 213(11), 201(100), 175(54), 149(10), 123(11)	**339**	215(4), 189(100), 147(3), 137(52)
16	15.83	206, 228, 280	Hispaglabridin A	Isoflavan	$C_{25}H_{28}O_4$	**391**	215(36), 203(100), 201(49), 189(40), 177(63)	**393**	337(100), 189(79), 191(79)
17	15.93	215,265, 280,383	3-hydroxy-glabrol	Flavanone	$C_{25}H_{28}O_5$	**407**	203(100), 221(3), 159(2)	**409**	353(100), 335(29), 229(6), 205(21), 177(6)
18	17.06	228, 280	Hispaglabridin B	Isoflavan	$C_{25}H_{26}O_4$	**389**	201(100), 187(5), 175(13)	**391**	189(100), 147(10)

similar, making it difficult to discriminate between chalcone and flavanone. This is caused by thermal isomerisation by which the C-ring of the flavanone is opened to form its corresponding chalcone.[26] Based on the UV spectra described by Fu *et al.*,[24] the peaks were assigned as either liquiritigenin glycoside or isoliquiritigenin glycoside.

Peaks **7**, **8** and **9** were assigned as licochalcone B, glycyrrhizinic acid and formononetin, respectively. Peaks **7** and **9** were confirmed by UV spectra and MS fragmentation patterns in NI mode, comparable to those found by Wang *et al.*[27] Peak **8** was assigned as the main triterpenoid glycoside in the roots of *Glycyrrhiza glabra* by means of the authentic standard.

Peaks 10–18

Peaks **10–18**, eluting in the second half of the chromatogram, were assigned as prenylated flavonoids. The predominant prenylated flavonoid in the chromatogram was glabridin (**12**). There were four other prenylated isoflavans assigned with the same skeleton as glabridin: 3′-hydroxy-4′-*O*-methylglabridin (**14**), 4′-*O*-methylglabridin (**15**), hispaglabridin A (**16**) and hispaglabridin B (**18**). The fragmentation patterns of these five isoflavans are shown in Fig. 3. The C-ring is cleaved in several places resulting in different retro-Diels Alder (RDA) fragments. The nomenclature for the RDA fragmentation was adapted from Ma *et al.*[28] and is shown in Fig. 4. The RDA fragments dominate the MS^2 spectra of the isoflavans (**12**, **14–16** and **18**). In addition, a number of small neutral losses were observed, such as 28 u (CO) and 44 u (CO_2). This indicated that the C-ring of the isoflavans studied is less stabile, and more prone to degradation, than that of, for example, glabrene (isoflav-3-ene) and glabrone (isoflavone) (see Supplementary Figs. S1 and S2, respectively, Supporting Information).

The fragmentation patterns of **12**, **14–16** and **18** were basically the same in NI mode. Fragments ${}^{1,3}B^-$ and ${}^{2,3}A^-$ were the most abundant ions in all spectra. The other RDA fragments were present in all MS^2 spectra of these isoflavans, with the exception of fragment ions ${}^{1,4}B^-$ and ${}^{2,3}B^-$. These ions were only formed in the MS^2 of **12** and **16**. This might be due to the conversion of the *para*-hydroxyl group of the B-ring into a carbonyl, which is only possible if the *para*-hydroxyl group is unsubstituted.

Both fragmentation patterns of glabrol (**13**) and 3-hydroxyglabrol (**17**) in NI mode were characterised by a predominant ${}^{1,3}A^-$ ion and a much smaller ${}^{1,3}B^-$ ion (as shown in Supplementary Fig. S3, see Supporting Information). Furthermore, the ${}^{1,3}A^-$ ion underwent a further loss of CO_2 yielding a $[{}^{1,3}A{-}CO_2]^-$ ion, a mechanism that has been described for flavonoids in NI mode.[9]

The fragmentation of glabrene (**10**) is characterised by the losses of a methyl radical $CH_3^{\cdot}$, CO and CO_2. Furthermore, the fragment ions $[B_{ring}{-}H]^-$ and $[M{-}H{-}B_{ring}]^-$ were observed, as well as four RDA fragments (Supplementary Fig. S1, see Supporting Information).

The main fragment ions in the MS^2 spectrum of the isoflavone glabrone (**11**) were due to small neutral losses such as a methyl radical ($CH_3^{\cdot}$) and CO_2. The most prominent RDA fragment observed was $[{}^{1,4}B{-}H_2O]^-$ (Supplementary Fig. S2, see Supporting Information).

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m/z 323 (**12**: R1=R2=H)
m/z 353 (**14**: R1=OH, R2=CH_3)
m/z 337 (**15**: R1=H, R2=CH_3)
m/z 391 (**16**: R1=prenyl, R2=H)

m/z 389 (**18**)

$^{1,3}A^-$
m/z 187 (**12,14,15,16,18**)

$^{1,3}B^-$
m/z 135 (**12**: R1=R2=H)
m/z 165 (**14**: R1=OH, R2=CH_3)
m/z 149 (**15**: R1=H, R2=CH_3)
m/z 203 (**16**: R1=prenyl, R2=H)

or

$^{1,3}B^-$
m/z 201 (**18**)

$^{1,4}A^-$
m/z 175 (**12,14,15,16,18**)

$^{1,4}B^-$
m/z 147 (**12**: R=H)
m/z 215 (**16**: R=prenyl)

$^{2,3}A^-$
m/z 201 (**12,14,15,16,18**)

$^{2,3}B^-$
m/z 121 (**12**: R=H)
m/z 189 (**16**: R=prenyl)

or

$^{2,3}B^-$
m/z 121 (**12**: R=H)
m/z 189 (**16**: R=prenyl)

$[M-H-B_{ring}]^-$
m/z 213 (**12,14,15,16,18**)

$[B_{ring}-H]^-$
m/z 109 (**12**: R1=R2=H)
m/z 139 (**14**: R1=OH, R2=CH_3)
m/z 123 (**15**: R1=H, R2=CH_3)
m/z 177 (**16**: R1=prenyl, R2=H)

or

$[B_{ring}-H]^-$
m/z 175 (**18**)

Figure 3. Proposed fragmentation pathway of glabridin (**12**), 3′-hydroxy-4′-*O*-methylglabridin (**14**), 4′-*O*-methylglabridin (**15**), hispaglabridin A (**16**) and B (**18**) in NI mode. The numbers inside the parentheses refer to the compounds in Table 1.

This is the first report on the fragmentation of prenylated flavonoids **10**–**18** by ESI-MS. The fragmentation patterns indicate that prenylated flavonoids follow the same degradation pattern, like the formation of RDA fragments and small neutral losses like CO and CO_2. Furthermore, it appeared that the prenyl groups are not directly involved in the MS^2 fragmentation in NI mode.

Neutral losses 42 u and 56 u in PI mode MS^n spectra of prenylated flavonoids

The MS^2 spectra of prenylated flavonoids **13**, **16** and **17** in PI mode were characterised by the dominant fragment ion $[M+H–56]^+$. Furthermore, the fragmentation of the isoflavans **12**, **14**, **15** and **18** all had similar degradation patterns in PI mode characterised by the presence of RDA fragments

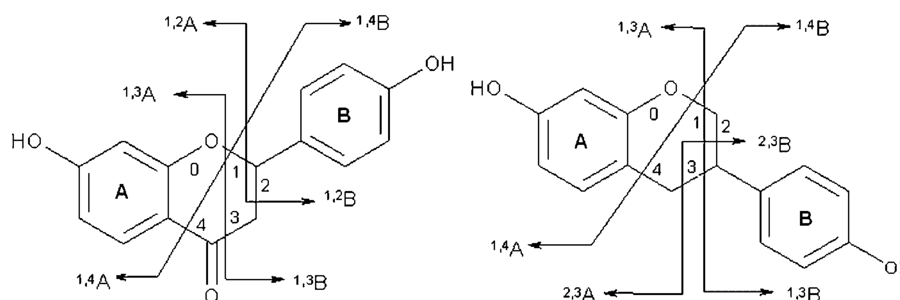

Figure 4. Nomenclature adopted for the different retrocyclisation cleavages on a flavanone and an isoflavan.

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Table 2. The presence of neutral losses 42 u [C_3H_6] and 56 u [C_4H_8] originating from the degradation of the prenyl group in PI mode

		MS^2		MS^3	
No[a]	Name	42 u	56 u	42 u	56 u
10	Glabrene[b]	*n.d.*	*n.d.*	*n.d.*	*n.d.*
11	Glabrone	++	+	*n.d.*	*n.d.*
12	Glabridin	−	±	++	±
13	Glabrol	−	++	+	+
14	3′-Hydroxy-4′-*O*-methylglabridin	−	−	++	±
15	4′-*O*-Methylglabridin	−	−	++	±
16	Hispaglabridin A	−	++	+	±
17	3-Hydroxyglabrol	−	++	−	+
18	Hispaglabridin B	−	−	++	±

[a] Number refers to the compounds in Table 1.
[b] Glabrene was tentatively assigned in NI mode. The $[M–H]^-$ precursor ion had an *m/z* value of 321. In PI mode, however, the $[M+H]^+$ of the peak at the same retention time had an *m/z* value of 322, while 323 was expected. Additional experiments with high-resolution MS showed that two compounds were co-eluting with an *m/z* value of 321 and 323. This resulted in an average *m/z* value of 322 in the UHPLC/MS chromatogram. It was not possible to determine the fragmentation of glabrene.
− Fragment ion not determined, less than 0.1%.
± The relative abundance of this fragment ion is less than 5%.
+ The relative abundance of this fragment ion is between 5 and 100%.
++ The relative abundance of this fragment ion is 100% (dominating fragment in the MS^n spectrum).
n.d. not determined.

(Supplementary Fig. S4, see Supporting Information). The dominant RDA fragment ion $^{1,3}A^+$ is substituted with a pyran ring. Upon fragmentation of $^{1,3}A^+$, a predominant loss of 42 u was observed in the MS^3 spectra.

The MS^2 spectrum of glabrone (**11**) in PI mode was characterised by neutral losses of 42 u, 54 u and 56 u, next to RDA fragment ions $^{1,3}A^+$ and $^{1,3}B^+$.

Table 2 summarises the prenylated flavonoids, assigned by us in the licorice root extract, for which neutral losses 42 u and 56 u were observed. The $[M+H–56]^+$ ion is the most abundant ion in the MS^2 spectra of compounds **13**, **16** and **17**, which have in common that they are substituted with at least one prenyl chain. Furthermore, in the MS^3 spectra of **12**, **14**–**16** and **18** a dominant neutral loss of 42 u was observed. These five prenylated flavonoids have in common that they are substituted with at least one pyran ring.

From the results shown in Table 2, it appeared that the degradation of a prenyl chain in PI mode in MS^2 preferably resulted in a neutral loss of 56 u, sometimes accompanied by a minor loss of 42 u. In contrast, the degradation of the pyran ring appeared to be characterised by a predominant neutral loss of 42 u in combination with a minor loss of 56 u. The loss of 56 u is suggested to be attributed to the loss of C_4H_8, whereas the loss of 42 u corresponds with the loss of C_3H_6.[15] This characteristic difference in fragmentation of both prenyl groups is supported by observations of Stevens *et al.*[10] and Da Costa *et al.*[29] in PI mode APCI. Based on our observations and the literature, a proposed fragmentation pathway for both the prenyl chain and the pyran ring is shown in Fig. 5.

The fragmentation of prenyl groups of prenylated polyphenolic compounds has been reviewed by Takayama *et al.*[26] This review focused on the fragmentation patterns in electron ionisation (EI), PI mode fast-atom bombardment (FAB)[9] and chemical ionisation (CI) and summarised the neutral losses encountered upon fragmentation of the prenyl group (Table 3). This overview shows that the neutral losses of 42 u and 56 u are commonly observed upon fragmentation of prenylated flavonoids. In a more recent study using ESI-MS,[4] a neutral loss of 56 u was also observed. These observations are consistent with our findings on the fragmentation of prenylated flavonoids obtained with ESI-MS.

Screening method for prenylated flavonoids based on neutral losses 42 u and 56 u

The screening method for prenylated flavonoids is based on the detection of neutral losses. In addition to the detection of prenyl substituents, it is possible to discriminate between the prenyl chain and the pyran ring, based on the relative abundances of the ions that lose 56 u and 42 u, respectively. From these relative abundances for the compounds shown in Table 2 it appears that if the ratio (42:56) is <1.0, the prenyl group is a prenyl chain. If the ratio is >1.0, the prenyl group is a pyran ring.

Neutral losses 42 u and 56 u have also been observed in the fragmentation of non-prenylated flavonoid aglycones. The fragmentation of licochalcone B (**7**) was characterised by a predominant neutral loss of 42 u, and in the MS^2 of formononetin (**9**) a neutral loss of 56 u was observed (Table 1). This indicated that the occurrence of these neutral losses in the MS^n spectra of flavonoids in PI mode does not exclusively point to the presence of prenyl groups. Losses of 42 u have been observed by Ma *et al.*[28] upon fragmentation of flavonoid aglycones in the PI mode, and were attributed to the loss of C_2H_2O, originating from the C-ring of the flavonoid aglycones. The neutral loss of 56 u has been observed by Kuhn *et al.* in the fragmentation patterns of different isoflavonoid aglycones.[30] In their study the authors concluded that a loss of two CO molecules accounted for the loss of 56 u.

To rule out the possibility that flavonoid aglycones were assigned as prenylated flavonoids, a criterion was set to selectively exclude flavonoid aglycones on the basis of their molecular weight. The prenylated flavonoids of the lowest molecular weight isolated from *Glycyrrhiza glabra* are glabrene and 2′,4′-dihydroxy-6″,6″-dimethylpyrano[2″,3″:7,8]isoflav-3-ene (both isoflav-3-enes) and have a molecular weight of 322 u ($C_{20}H_{18}O_4$).[12] According to an extensive review on prenylated flavonoids by Barron and Ibrahim, the smallest prenylated flavonoid isolated from a natural source is 7,8-(2,2-dimethylchromeno)flavone, with a molecular weight of 304 u ($C_{20}H_{16}O_3$).[5] The *m/z* of the precursor ion of this molecule in PI mode would be 305. To exclude non-prenylated flavonoid aglycones from the screening method, an *m/z* threshold of 305 was set.

In plants, flavonoids often appear as *O*-glycoside conjugates. The *O*-glycoside conjugates of non-prenylated flavonoid aglycones that lose 56 u upon fragmentation exceed the *m/z* threshold of 305, and they might be

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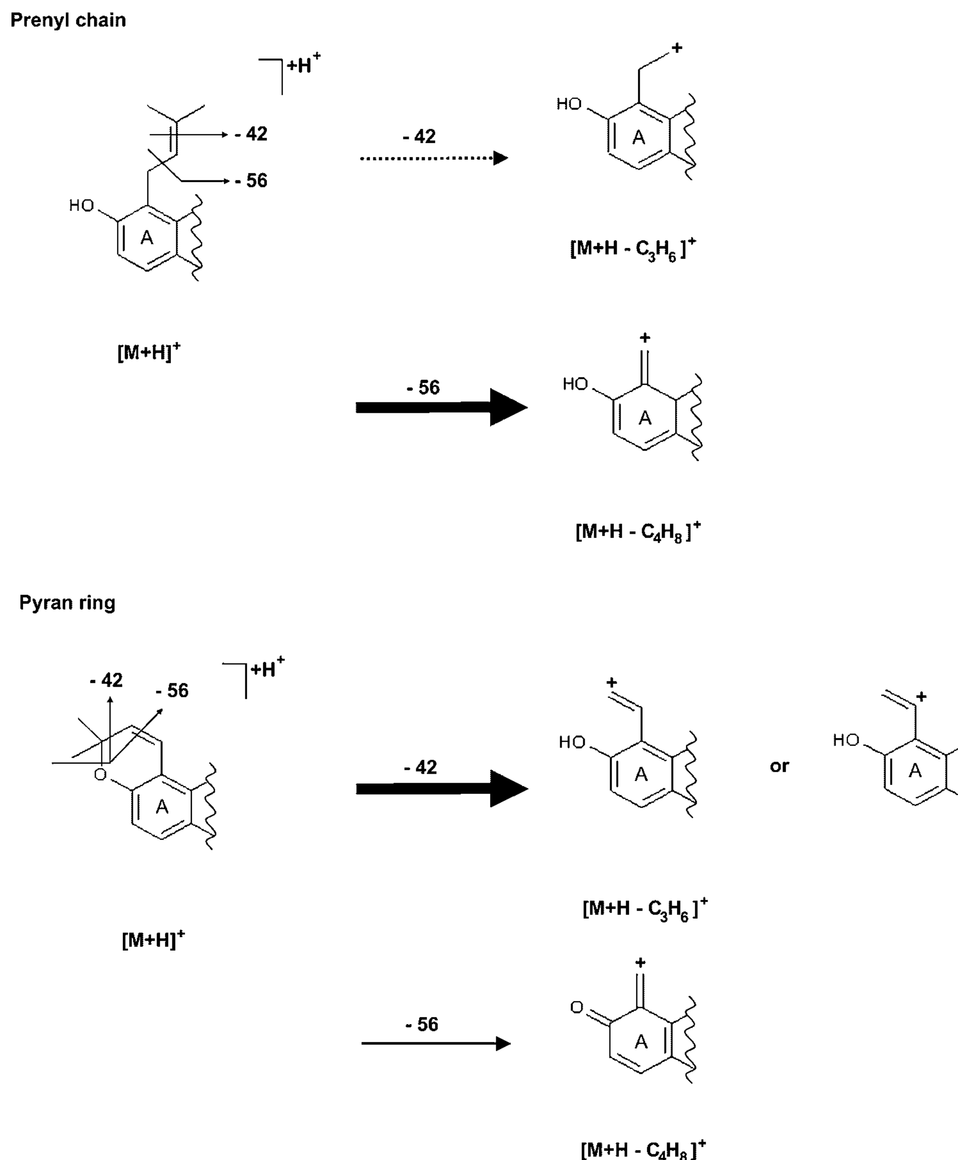

Figure 5. Proposed degradation pathways of a prenyl chain and a pyran ring attached to the A-ring of a flavonoid in PI mode. The same degradation pathways apply for a B-ring prenylation. Bold arrow indicates preferred fragmentation pathway, dotted arrow indicates less likely pathway. The loss of 56 u is always observed in both prenyl chain and pyran ring degradation. The loss of 42 u is always observed in the degradation of a pyran ring, but not necessarily in that of a prenyl chain.

inappropriately scored as prenylated flavonoids: in MS^2 the glycoside moiety is cleaved off, after which the aglycone loses 56 u in MS^3. In order to prevent that non-prenylated flavonoid *O*-glycosides are assigned as prenylated, the MS^2 spectra are checked for neutral losses corresponding with the loss of a sugar moiety. The predominant loss of 132 u (pentose), 146 u (deoxyhexose), 162 u (hexose) or 176 (uronic acid), or a loss of more than 264 u (2 pentoses), in the MS^2 spectrum is diagnostic for a flavonoid *O*-glycoside, and these compounds are, therefore, assigned as non-prenylated. The exclusion of flavonoid *O*-glycosides may consequently lead to the possibility that prenylated flavonoid *O*-glycosides are not detected. Five prenylated flavonoid *O*-glycosides have been described occurring within the plant families of the *Leguminosae*, *Rutaceae*, *Rhamnaceae* and *Pteridaceae*.[31] However, most prenylated flavonoid *O*-glycosides have been isolated from species within the genera *Epimedium* and *Vancouveria* belonging to the family of *Berberidaceae*.[5]

Our screening procedure, developed on the basis of fragmentation of the prenylated flavonoids from *Glycyrrhiza glabra* in the PI mode, is summarised in Fig. 6. The first selection criterion is the presence of a neutral loss of 56 u, alone or in combination with a neutral loss of 42 u, in the MS^2 spectrum. If a neutral loss is not present in the MS^2 spectrum, the MS^3 spectrum is analysed. The next step is to verify whether the *m/z* value of the precursor ion $[M+H]^+$ is above 305 to rule out non-prenylated flavonoid aglycones. In case neutral losses 56 u, or both 56 u and 42 u, are detected in MS^3, the MS^2 spectrum of this peak is analysed for neutral losses corresponding to the loss of sugar moieties. If the MS^2

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Table 3. Characteristic fragments for the fragmentation of a prenyl group reported for different ionisation techniques in PI mode

		EI[a]		(+)FAB[a]		(+)APCI[b,c]		(+)ESI[d]
Neutral Loss	Loss	Chain	Pyran	Chain	Pyran	Chain	Pyran	Chain
42 u	C_3H_6				✓		✓	
43 u	$C_3H_7^{\bullet}$	✓	✓					
54 u	C_4H_6						✓	
55 u	$C_4H_7^{\bullet}$	✓						
56 u	C_4H_8	✓		✓	✓	✓		✓
57 u	$C_4H_9^{\bullet}$	✓						
68 u	C_5H_8	✓		✓				
69 u	$C_5H_9^{\bullet}$	✓						
71 u	C_5H_{11}	✓						

EI – electron ionisation; APCI – atmospheric pressure chemical ionisation; ESI – electrospray ionisation
[a] Takayama *et al.*[26]
[b] Stevens *et al.*[10]
[c] Da Costa *et al.*[29]
[d] Zhang *et al.*[4]

spectrum indicates loss of glycosyl residues, the peak is considered non-prenylated. If these criteria are met, the compound is considered to be prenylated.

The second prenyl group of double prenylated flavonoids can be determined as well. The minimum molecular weight of a double prenylated flavonoid is 372 u, calculated by the addition of a prenyl chain (68 u) to the single prenylated flavone, 7,8-(2,2-dimethylchromeno)flavone (304 u), described earlier. Therefore, if the *m/z* value of the precursor ion $[M+H]^+$ is higher than 373, the MS^3 spectrum is checked. This can only be done in the case where MS^3 is performed on the $[M+H–42]^+$ or the $[M+H–56]^+$ ion.

Table 4. Number of individual peaks detected in 70% aq. EtOH, EtOH, and EA extracts and their classification based on the screening method. The 'Total' column summarises the total number of unique peaks in all three extracts

Group	70% EtOH	EtOH	EA	Total
Total number of peaks	66	82	60	118
Screening for neutral losses 56 u and 42 u	45	58	48	88
• *m/z < 305 (non-prenylated)*	*5*	*5*	*1*	*8*
• *Sugar loss detected in MS*^{2}	*6*	*6*	*3*	*10*
Total prenylated	*34*	*47*	*43*	*70*
Total non-prenylated	*32*	*35*	*17*	*48*
Mono-prenylated	*26*	*34*	*33*	*52*
• *Prenyl chain*	*20*	*26*	*26*	*41*
• *Pyran ring*	*6*	*8*	*7*	*11*
Di-prenylated	*9*	*14*	*10*	*18*
• *Prenyl chain + penyl chain*	*5*	*10*	*6*	*12*
• *Prenyl cain + pyran ring*	*4*	*4*	*4*	*6*

Verification of the screening method proposed

To verify the screening method proposed, the 70% aq. EtOH, EtOH and EA extracts of *Glycyrrhiza glabra* roots were screened for the presence of prenylated flavonoids in addition to the tentatively assigned compounds **10–18**. Based on the analysis of the MS chromatograms of the three different extracts by Mass Frontier software, 118 individual peaks were detected (see Table 4 and Supplementary Table

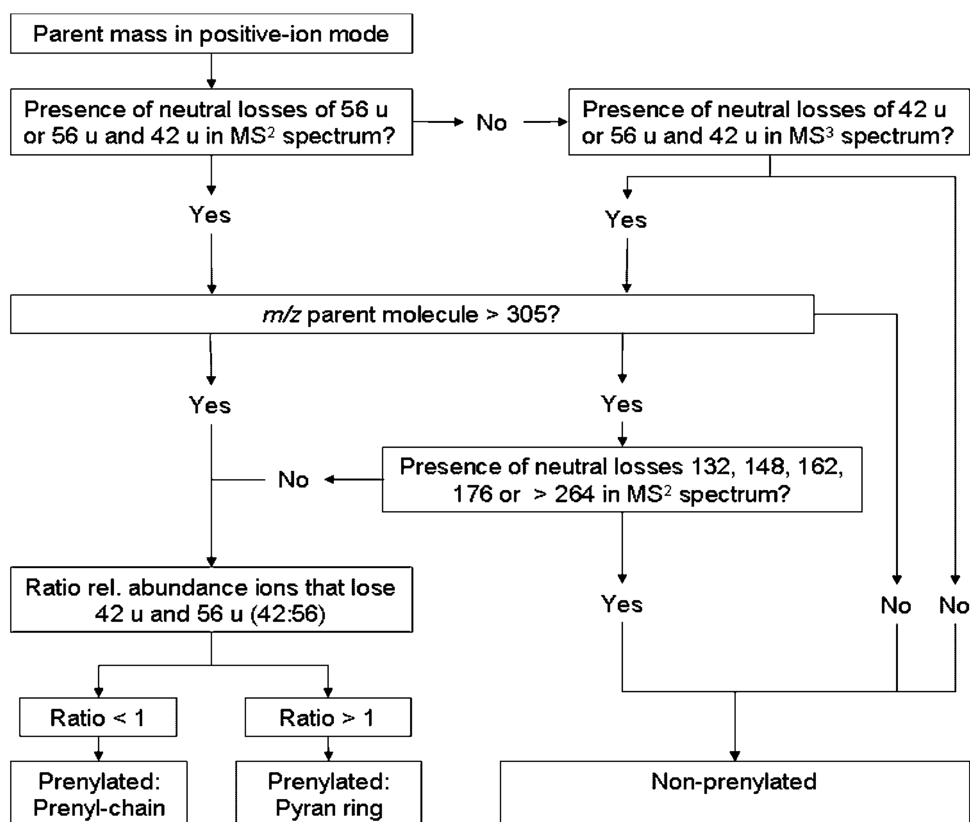

Figure 6. Interpretation guideline for identification of prenylated flavonoids.

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Table 5. Screening results for prenylated flavonoids in the MS chromatogram of the EA extract of *Glycyrrhiza glabra* in PI mode (**C** – prenyl chain; **P** – pyran ring; **np** – non-prenylated)

				MS^2				MS^3			
No*	Rt** (min) MS	Identification	$[M+H]^+$	R.A. neutral loss 42 u	R.A. neutral loss 56 u	Ratio (42:56)	Prenyl -type	R.A. neutral loss 42 u	R.A. neutral loss 56 u	Ratio (42:56)	Prenyl -type
S23	10.44		327	—	—	—	—	3	1	3.0	**P**
S26	10.79		321	24	25	1.0	C				
S27	10.89	Formononetin	269	—	33	0.0	**np**				
S29	11.27		341	<1	100	<0.1	**C**				
S31	11.38		339	<1	100	<0.1	**C**				
S32	11.51		323	2	100	<0.1	**C**				
S33	11.57		341	—	100	0.0	**C**				
S34	11.67		323	1	100	<0.1	**C**				
S35	11.79		355	41	100	0.4	**C**				
S36	11.90		337	1	7	0.1	**C**				
S37	12.10		355	<1	100	<0.1	**C**				
S38	12.20		325	3	100	<0.1	**C**				
S39	12.28		321	24	28	0.9	**C**				
S41	12.53		322	—	7	0.0	**C**				
S42	12.68		337	28	57	0.5	**C**				
S43	12.72		339	5	17	0.3	**C**				
S45	12.97		308	—	—	—	—	3	4	0.8	**C**
S47	13.25		355	—	100	0.0	**C**				
S48	13.31		341	—	100	0.0	**C**				
S49	13.52		409	—	21	0.0	**C**				
S50	13.64	Glabrone	337	100	6	16.7	**P**				
S51	13.77	Glabridin	325	—	<1	0.0	—	100	1	100	**P**
S53	13.91		321	18	64	0.3	**C**				
S54	14.08		391	—	100	0.0	**C**	1	100	<0.1	**C**
S55	14.13		645	1	100	<0.1	**C**	21	10	2.1	**P**
S57	14.29	Glabrol	393	—	100	0.0	**C**	21	22	1.0	**C**
S58	14.49		409	—	100	0.0	**C**	19	100	0.2	**C**
S59	14.66		395	—	82	0.0	**C**				
S60	14.72		647	—	60	0.0	**C**				
S62	14.79	3′-Hydroxy-4′-*O*-methylglabridin	355	—	—	—	—	100	1	100	**P**
S64	14.89		409	—	100	0.0	**C**	30	20	1.5	**P**
S66	15.04		647	—	14	0.0	**C**				
S67	15.08		661	—	100	0.0	**C**	1	35	<0.1	**C**
S69	15.27		391	—	18	0.0	**C**				
S72	15.42		645	<1	100	<0.1	**C**	35	18	1.9	**P**
S74	15.52	4′-*O*-Methylglabridin	339	—	—	—	—	100	1	100	**P**
S76	15.73		631	<1	100	<0.1	**C**	34	6	5.7	**P**
S77	15.88		391	—	100	<0.1	**C**	6	7	0.9	**C**
S78	15.97	Hispaglabridin A	393	—	100	<0.1	**C**	21	3	7.0	**P**
S80	16.08	3-Hydroxyglabrol	409	—	100	0.0	**C**	1	9	0.1	**C**
S81	16.18		391	1	39	<0.1	**C**				
S82	16.34		409	—	42	0.0	**C**				
S83	16.44		643	2	63	<0.1	**np**				
S84	16.53		393	—	100	0.0	**C**	10	8	1.3	**P**
S85	16.72		659	—	26	0.0	**np**				
S86	16.81		469	—	—	—	—	4	14	0.3	**C**
S87	16.89		388	1	2	0.5	**C**	11	6	1.8	**P**
S88	17.21	Hispaglabridin B	391	—	3	—	—	100	1	100	**P**

R.A. – Relative abundance of ion within MS^n spectrum
<1 – 0.5% to 1.0%
n.d. – not detected, <0.5%
* The 'S' refers to a peak found with the screening method in the EA extract.
** The retention time in MS has a delay of 0.20 ± 0.02 min compared to the UV retention time (Rt).

S1, see Supporting Information). Out of these 118 peaks, 88 showed a neutral loss of 56 u, alone or in combination with a neutral loss of 42 u, in either the MS^2 or MS^3 spectrum. Of these 88 peaks, 8 were assigned as non-prenylated aglycones, because their *m*/*z* value did not exceed 305. Another 10 peaks were assigned as flavonoid glycosides, as their MS^2 fragmentation indicated a loss of one or more sugar moieties. In total, 70 unique peaks were assigned as prenylated flavonoids present in the three different extracts of *Glycyrrhiza glabra*. Of these 70 peaks, 52 peaks were assigned as

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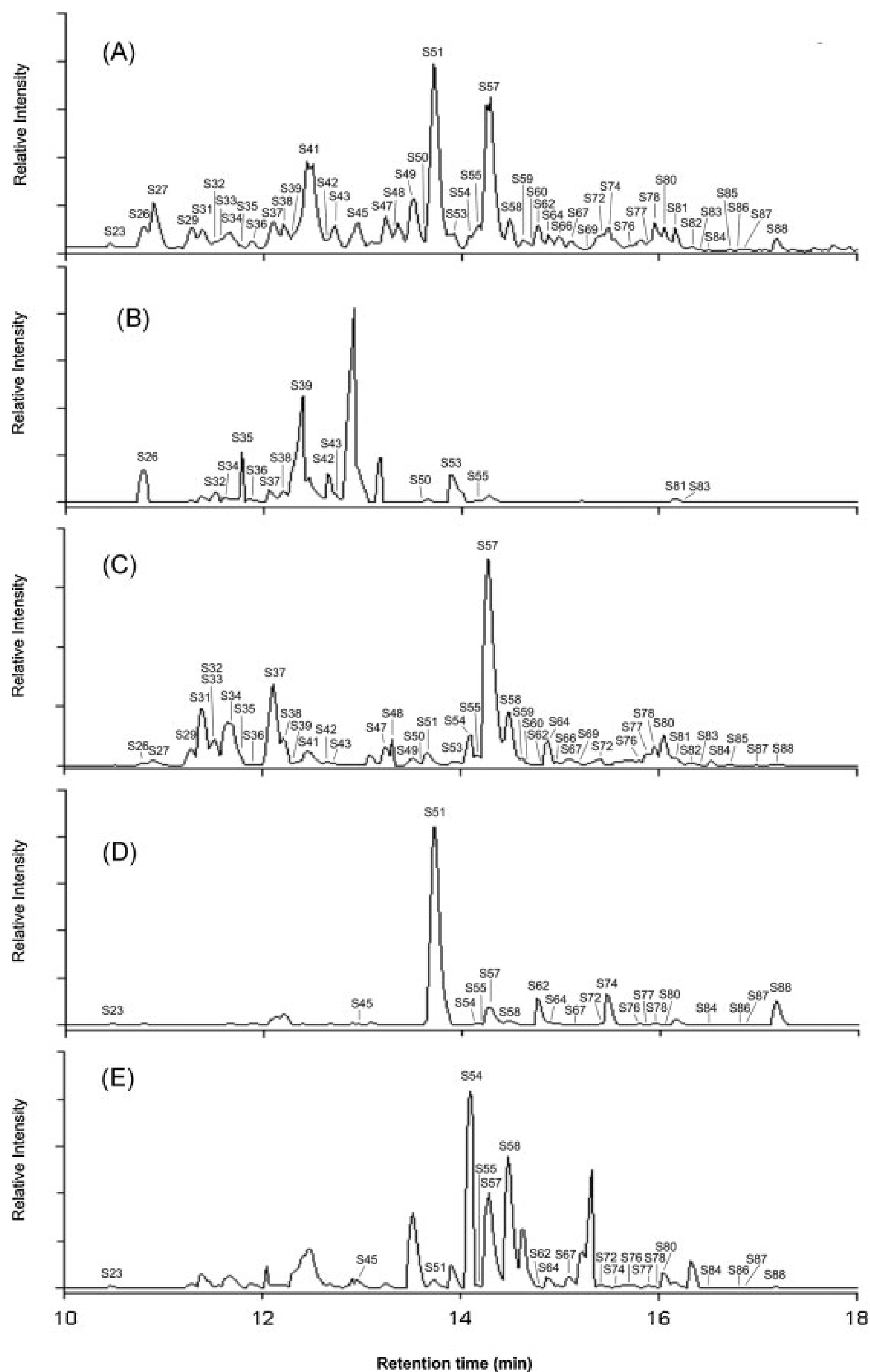

Figure 7. Screening results for neutral losses 42 u and 56 u in the PI mode MS^2 and MS^3 spectra. RP-UHPLC/MS basepeak chromatogram in PI mode (A) of the EA extract of *Glycyrrhiza glabra* roots. The MS chromatograms were screened for neutral losses in MS^2 of 42 u (B) and 56 u (C), and for neutral losses in MS^3 of 42 u (D) and 56 u (E). The numbers in the figure relate to the numbers given in Table 5.

mono-prenylated and 18 as di-prenylated. Based on the ratio (42:56), the majority of the 70 peaks have a prenyl chain (see Supplementary Table S1, Supporting Information).

The EA extract appeared to selectively extract prenylated flavonoids, which may be related to the non-polar properties of this solvent. For this reason, the EA extract was chosen as an illustrative example in Table 5 and Fig. 7. Table 5 shows an overview of the 48 peaks in the MS chromatogram of the EA extract that exhibit a loss of 56 u, alone or in combination with 42 u, in MS^n. The 43 peaks finally assigned as being prenylated are shown in the chromatograms in Fig. 7.

Roughly half of the peaks in Table 5 assigned as prenylated had the $[M+H–56]^+$ ion as their most abundant fragment in their MS^2 spectrum and, in addition, most of the peaks in Table 5 had a ratio (42:56) smaller than 1, indicating the presence of a prenyl chain. Peak **S50** was the only peak with a

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pyran ring determined by the MS^2 spectrum. The other peaks that were assigned as having a pyran ring were based on their ratio (42:56) in their MS^3 spectrum. This observation indicated that a pyran ring is more stable to MS degradation than a prenyl chain, as the fragmentation of the prenyl chain mostly occurred in MS^2, whereas the pyran ring was mostly fragmented in MS^3.

Around 75 prenylated flavonoids have been isolated from the roots of *Glycyrrhiza glabra*.[13–22] The application of the screening method described here indicated the presence of 70 unique prenylated flavonoids in three different extracts of the roots of *Glycyrrhiza glabra* without any purification or fractionation steps. This suggests that many of the prenylated flavonoids present can be detected in a crude extract by the application of the described screening method. A next step would be the application of this screening method for the detection of prenylated flavonoids in other plant extracts and, furthermore, to provide a greater number of definitive assignments of the prenylated flavonoids detected in complex extracts using the current screening method.

CONCLUSIONS

A rapid screening method is described to screen for prenylated flavonoids. The method is based on the fragmentation of prenyl substituents of flavonoids in PI mode MS^n, involving a neutral loss 56 u, alone or in combination with 42 u. In addition to the detection of prenyl substituents, it is possible to discriminate between a prenyl chain and a pyran ring, based on the relative abundances of the ions that lose 42 u and 56 u (42:56). A ratio (42:56) of <1.0 indicates the presence of a prenyl chain, a ratio >1.0 that of a pyran ring. The screening of three different extracts of roots from *Glycyrrhiza glabra* resulted in the detection of 70 peaks that are likely to be prenylated.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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