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Isolation and characterization of methoxylated flavones in the flowers of *Primula veris* by liquid chromatography and mass spectrometry

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Abstract

Characterization of six flavones, which were named substances G1, G2, G3, G4, G5 and G6 according to their R_F values in normal-phase thin-layer chromatography, is reported. The pure flavones were purified after maceration with methanol by normal-phase solid-phase extraction, normal-phase medium-pressure liquid chromatography, normal-phase preparative thin-layer chromatography and preparative reversed-phase high-performance liquid chromatography (RP-HPLC). The collected fractions of several isolation steps were analyzed by normal-phase (NP) and RP-HPLC. Detection and identification of the substances G was accomplished by UV detection at 213–216 nm, diode array UV detection, or fluorescence detection (λ_{ex}=330 nm; λ_{em}=440 nm). The molecular mass, the elementary composition, and the structure of the six components was determined by electron-impact high-resolution mass spectrometry (EI-HRMS). Substance G4 was identified as 3′,4′,5′-trimethoxyflavone. The substances G1–G6 were shown to be mono-, di- tri- and pentamethoxyflavones. HPLC–electrospray ionization tandem mass spectrometry (ESI-MS–MS) of the flavones was carried out employing a 150×2 mm I.D. column packed with a 3 μm/100 Å octadecylsilica stationary phase and a mobile phase comprising 1.0% acetic acid in water–acetonitrile (50:50). Comparative RP-HPLC–ESI-MS of the raw methanol extract and the isolated substances G1–G6 proved that the isolated compounds were pure and were not artifacts. Finally, RP-HPLC–ESI-MS–MS was used to identify substances G1–G6 in phytopharmaceutical drugs.

Keywords: *Primulae veris*; Plant materials; Flavones

1. Introduction

Over 4000 chemically unique flavonoids have been identified in plant sources. These low-molecular-mass substances, found in all vascular plants, are phenylbenzopyrones (phenylchromones) with an assortment of basic structures [1]. On average, the daily western diet contains approximately 1 g of mixed flavonoids in fruits, vegetables, nuts, seeds, stems, flowers, as well as tea and wine [2]. This quantity can provide pharmacologically significant concentrations in body fluids and tissues. Flavonoids may have existed in nature for over one billion years [3]. Methoxyflavones have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, precursors of toxic substances and have long been recognized to possess antiallergic, anti-inflammatory, antiviral, antiproliferative and anticarcinogenic activities as well as to affect some aspects of mammalian metabolism [4–11].

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For the separation and quantification of flavones in plant materials gas chromatography [12–15], normal-phase thin-layer chromatography (NP-TLC) [16–19], reversed-phase high-performance liquid chromatography (RP-HPLC), and micellar electrokinetic capillary chromatography (MECC) [20] have been applied. UV-absorbance and fluorescence detection (FLD) are the most commonly used detection modes for liquid phase separations, FLD offering higher sensitivity and selectivity than UV-absorbance detection [21].

Nuclear magnetic resonance spectroscopy (NMR) [22] and mass spectrometry (MS) are among the most powerful techniques for the elucidation of flavonoid structures. Electron impact mass spectrometry (EI-MS) allows one to verify the structure of flavones and to identify certain structure elements [23,24]. During the past decade, atmospheric pressure chemical ionization (APCI) [25,26] and electrospray ionization (ESI) [27–30] MS have emerged as highly useful mass spectrometric methods that allow the direct conjugation with liquid phase separation techniques such as chromatography and electrophoresis [31]. However, these soft ionization techniques favorably produce protonated or deprotonated molecule ions with little fragmentation that can be used for structural analysis. Therefore, collisionally induced dissociation (CID) is used to obtain fragment ions of structural relevance [32]. Moreover, fragment-ion spectra can be used as ''fingerprints'' for identifying target compounds in highly complex mixtures. In this paper we report on a scheme for the isolation and identification of the six methoxylated flavones named substances G1, G2, G3, G4, G5 and G6 in the flowers of *Primula veris*. The utility of HPLC separation was greatly enhanced by mass spectrometric detection, which allowed to confidently identify the flavones in plant material and drugs made thereof.

2. Experimental

2.1. Materials and reagents

Acetic acid (analytical-reagent grade), acetonitrile (gradient grade), methanol (analytical-reagent grade), *n*-hexane (analytical-reagent grade), ethyl acetate (analytical-reagent grade) were purchased from Merck (Darmstadt, Germany). Water purified by a NanoPure-unit (Barnstead, Boston, MA, USA) was used. Aluminium oxide (100–125 mesh; for chromatography) was from Fluka (Buchs, Switzerland). Preparative TLC plates were from Merck (PSC-Plates Si 60 F254, 20×20 cm, layer thickness 1 mm).

2.2. High-performance liquid chromatography

The HPLC system consisted of a low-pressure gradient pump (Model 480 G, Gynkotek, Germering, Germany), a column heater (W.O. Electronics, Vienna, Austria), a vacuum degasser (Knauer, Berlin, Germany), an injection valve (Model 9125, Rheodyne, Cotati, CA, USA) with a 20-μl loop for analytical and a 400-μl loop for preparative separations and a UV-diode array detector (Model UVD 320, Gynkotek) with a 13-μl flow cell which was connected to a fluorescence detector (Model 474, Waters, Milford, MA, USA) with a 16-μl flow cell. Data was recorded on a personal computer-based data system (GynkoSoft, version 5.32, Gynktotek). For preparative RP-HPLC, a LiChrospher 100 RP-18 column (5 μm, 100 Å, 250×8 mm I.D., Knauer), for analytical RP-HPLC, an Inosil 100 RP-18 column (5 μm, 100 Å, 250×4 mm I.D., Innovex, Vienna, Austria) or LiChrospher 100 RP-18 column (5 μm, 100 Å, 250×4 mm I.D., Merck) were used. For analytical NP-HPLC, a Nucleosil 50-5 (5 μm, 50 Å, 250×4 mm I.D., Macherey–Nagel, Düren, Germany) was used.

2.3. Electron impact high-resolution mass spectrometry and electrospray mass spectrometry coupled to liquid chromatography

EI-HRMS was carried out with a Model MAT 95 double focusing mass spectrometer (Finnigan MAT, Bremen, Germany). For HPLC–ESI-MS–MS experiments a low-pressure gradient micropump (Model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (Knauer), a microinjector (Model CC00030, Valco, Houston, TX, USA) with a 5-μl internal loop, a variable-wavelength detector (Model Linear UV–Vis 200, Linear Instruments, Fremont, CA, USA) with a

1.2-μl detector cell connected to a quadrupole ion trap mass spectrometer (Model LCQ, Finnigan, San Jose, CA, USA) were used. Separations were carried out with a 150×2 mm I.D. column packed with Nucleosil 100 RP_{18} (3 μm, 100 Å, Macherey–Nagel). For ESI-MS the following parameters were used in all experiments: temperature of the heated capillary, 200°C; electrospray voltage, 4.0 kV. Nitrogen served as sheath gas (50 units).

2.4. Plant material and isolation of substances G1, G2, G3, G4, G5 and G6 [33]

2.4.1. Maceration

The flowers of *Primula veris* were identified and received from Plantamed Bionorica (Neumarkt/Oberpfalz, Germany). The plant material was pulverized in a vibration ball mill (Retsch, Hoon, Germany). An 11-kg amount of *Primula veris* flowers was macerated with 100 l of methanol on a waterbath (40°C for 36 h). After filtration of the united methanol extracts and evaporation of the solvent, 2 kg of spissum extract was obtained.

2.4.2. Normal-phase solid-phase extraction

Portions (200 g) of spissum extract were mixed with 400 g of aluminium oxide in a mortar and the mixture was filled into a glass column (1000×35 mm I.D.), which was connected to a second glass column filled with aluminium oxide (600×35 mm I.D.). A step-gradient from 100% *n*-hexane to 100% ethyl acetate (flow-rate 10 ml/min) in 10% steps was run within 10 days. Substances G1, G2, G3, G4, G5 and G6 were located as blue fluorescing zones by a UV lamp (detection wavelength λ=350 nm). The fluorescing fraction containing substances G1–G6 was collected.

2.4.3. Normal-phase medium-pressure chromatography

After evaporation of the solvent the residue was redissolved in 15 ml *n*-hexane–ethyl acetate (70:30, v/v). Aliquots of 5 ml were loaded onto a medium-pressure column (500×25 mm I.D.) packed with LiChroprep Si 60 (25–40 μm, Merck) and eluted with *n*-hexane–ethyl acetate (70:30, v/v) at a flow-rate of 8 ml/min and a pressure of 15 bar using a pneumatic HPLC pump (Knauer, Berlin, Germany). Five different blue fluorescing zones corresponding to substances G1, G2/G3, G4, G5 and G6 were detected by a UV lamp. Substances G2 and G3 coeluted. The five fluorescing zones were collected.

2.4.4. Preparative normal-phase thin-layer chromatography

The volume of the fractions containing substances G1–G6 was reduced to 4 ml. Each fraction was loaded separately onto a preparative TLC plate by a TLC applicator (Model Linomat III, Camag, Muttenz, Switzerland), and the chromatogram was developed with *n*-hexane–ethyl acetate (70:30, v/v) in a chromatographic chamber. After drying at room temperature the blue fluorescing zones [R_F(G1)=0.35; R_F(G2)=0.29; R_F(G3)=0.24; R_F(G4)=0.18; R_F(G5)=0.14; R_F(G6)=0.09] were scratched from the plates and extracted with 30 ml methanol. After evaporation of the solvent the residue was redissolved in 4.0 ml *n*-hexane–ethyl acetate (70:30, v/v). For the isolation of substances G1, G4, G5 and G6 a second and for substances G2 and G3 a third preparative TLC separation were performed as described above in order to increase the purity of the substances G.

2.4.5. Preparative reversed-phase HPLC

The extracts containing substances G1–G6 were filtered through a 0.2-μm membrane filter (Millipore, Bedford, MA, USA) and the volume was reduced to 10 ml. Portions (400 μl) of substances G1–G6 were injected onto a preparative RP-HPLC column (LiChrospher 100 RP-18, 5 μm, 100 Å, 250×8 mm I.D., Knauer) and eluted with a gradient of 0–38% acetonitrile in 10 min, followed by an isocratic hold at 38% acetonitrile for 45 min at a flow-rate of 2 ml/min. The purity and homogeneity of the target products were confirmed by peak purity analysis using a diode array detector. Finally, the column was washed with 100% acetonitrile for 1 min to elute strongly adsorbed compounds. The peaks containing the pure compounds were collected and the solvent was evaporated in vacuo. The total yields were as follows: substance G1: 8 mg; substance G2: 9 mg; substance G3: 8 mg; substance G4: 12 mg; substance G5: 10 mg; substance G6: 9 mg [34–36].

3. Results and discussion

Before their isolation and structural analysis, substances G1–G6 were characterized and identified on the basis of their retention behavior in NP- and RP-HPLC. Fig. 1 shows the isocratic NP-HPLC separation of substances G1–G6 in the raw methanol extract of *Flos Primulae veris* with *n*-hexane–isopropanol (92:8) as mobile phase at ambient temperature. Fluorescence detection (Fig. 1b) allowed more selective and sensitive detection than UV-absorbance detection (Fig. 1a) especially at the beginning of the chromatogram. The elution order of the substances G in NP-HPLC is G1<G2<G3<G4<G5<G6 and is determined by the increasing number of methoxy groups of the analytes. As expected, the elution order of substances G1–G6 is reversed in RP-HPLC applying an eluent comprising water–acetonitrile (60:40) (Fig. 2). UV spectra were recorded and allowed the correlation of the peaks in both chromatographic modes as well as the identification of the individual peaks after several steps of purification. A comparison of NP- and RP-HPLC reveals that higher resolution between substances G1 and G2 and substances G5 and G6 was feasible in the NP mode. Nevertheless, reproducibility of retention was worse in NP-HPLC because highly polar matrix

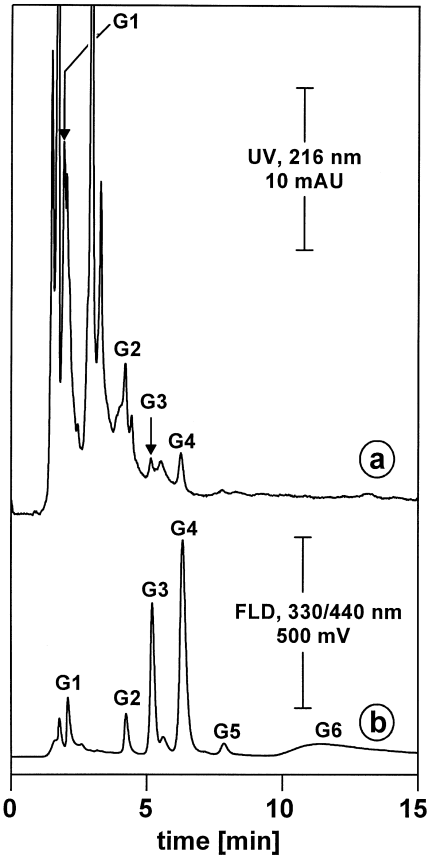

Fig. 1. Normal-phase HPLC of the *Flos Primulae veris* methanol extract with (a) UV-absorbance and (b) fluorescence detection (FLD). Column, Nucleosil 50-5 (5 μm, 50 Å, 250×4.0 mm); mobile phase, *n*-hexane–isopropanol (92:8); flow-rate, 1.4 ml/min; temperature, 21°C; detection, (a) UV, 216 nm, (b) FLD, λ_{ex}=330 nm, λ_{em}=440 nm; sample size, 5.0 μl.

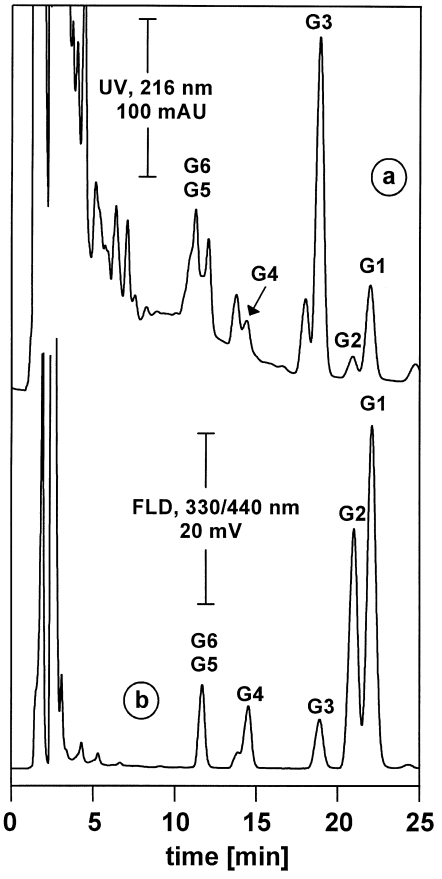

Fig. 2. Reversed-phase HPLC of the *Flos Primulae veris* methanol extract using (a) UV-absorbance and (b) fluorescence detection (FLD). Column, Inosil 100 RP-18 (5 μm, 100 Å, 250×4 mm); mobile phase, water–acetonitrile (60:40); flow-rate, 1.0 ml/min; temperature, 21°C; detection, (a) UV, 216 nm, (b) FLD, λ_{ex}=330 nm, λ_{em}=440 nm; sample size, 10 μl.

compounds strongly adsorbed to the silica stationary phase and could not be eluted by the mobile phase. Therefore, the NP column had to be washed with isopropanol for 20 min after every second run, but still, reproducibility of retention times was rather poor. In the RP mode, a 5-min wash with 100% acetonitrile after the chromatographic separation was used to elute strongly adsorbed compounds, which allowed reproducible chromatography of substances G1–G6 in the raw methanol extract for several months on a single column.

Because of the better reproducibility the RP-HPLC system was chosen to control the isolation procedure of substances G1–G6. The chromatograms of the reversed-phase chromatographic analysis of substance G4 after various steps of purification are depicted in Fig. 3. The chromatogram in Fig. 3a shows that substance G4 is a minor compound in the raw extract of *Flos Primulae veris* (approx. 0.4% of the total peak area). After NP-SPE on aluminium oxide the majority of matrix components as well as most of the other substances G have been removed. Substance G4 elutes at 39 min and the major impurity is substance G3, eluting at 42 min. The amount of highly polar matrix compounds was significantly reduced after NP-MPLC (Fig. 3c) and NP-TLC (Fig. 3d). Moreover, substance G3, which was still present after NP-MPLC, was not present after preparative NP-TLC. However, a number of small peaks eluting before substance G4 indicated, that these polar impurities cannot be removed by NP chromatography. Therefore, preparative RP-HPLC was applied to remove them and the success of this final purification step can be deduced from the chromatogram in Fig. 3e. The purity and homogeneity of the substance G4 peak was assessed using the peak purity analysis facilities of the diode array detection system.

The other substances G1, G2, G3, G5 and G6 were purified in the same manner as described above. Their purity was controlled after each purification step and confirmed by peak purity analysis using diode array detection. All isolated substances G1–G6 showed a homogenous overlay of UV spectra. The purity of the final products was additionally confirmed by RP-HPLC with UV-absorbance and FLD (Fig. 4). Only very small peaks representing less than 3% of the total peak area were detected in

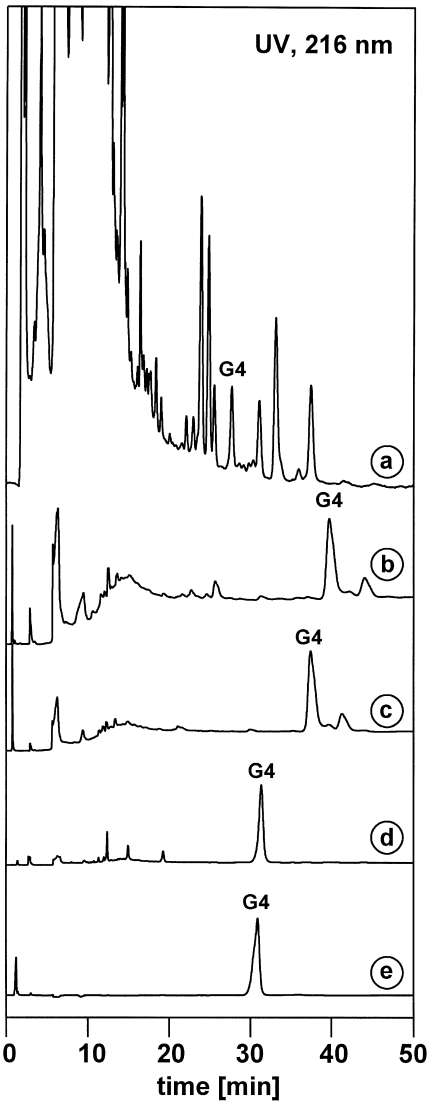

Fig. 3. Reversed-phase HPLC analysis of substance G4 from *Flos Primulae veris* after various steps of purification: (a) raw methanol extract; (b) substance G4 fraction after NP-SPE; (c) after preparative NP-MPLC; (d) after preparative NP-TLC; (e) after preparative RP-HPLC. Column, LiChrospher 100 RP18 (5 μm, 100 Å, 250×4 mm I.D.); gradient, 0–38% acetonitrile in water in 10 min, 38% acetonitrile for 45 min, 38–100% acetonitrile in 5.0 min; flow-rate, 1.0 ml/min; temperature, 21°C; detection, UV, 216 nm, height normalized to G4; sample size, 20 μl; in (b) and (c) a LiChrospher 100 RP18 pre-column (5 μm, 100 Å, 5×4 mm I.D.) was used.

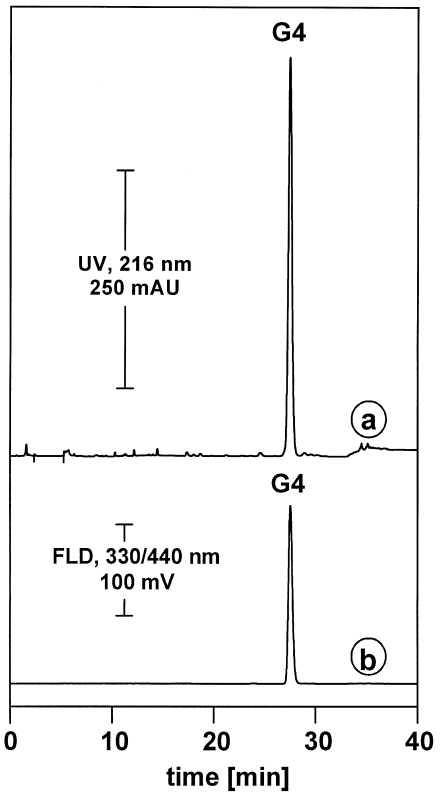

Fig. 4. Confirmation of the purity of isolated substance G by RP-HPLC. Column, Inosil 100 RP-18 (5 μm, 100 Å, 250×4 mm); gradient, 0–38% acetonitrile in water in 10 min, 38% acetonitrile for 45 min, 38–100% acetonitrile in 5.0 min; flow-rate, 1.0 ml/min; temperature, 21°C; detection, (a) UV, 216 nm, (b) FLD, λ_{ex}=330 nm, λ_{em}=440 nm; sample size, 230 μg in 5.0 μl.

the UV chromatogram of purified substance G4 (Fig. 4a) whereas FLD showed no other fluorescing compounds (Fig. 4b). Table 1 shows the mass, elementary composition, and suggested structures of substances G1–G6 as determined by EI-HRMS. Substance G1 and G2 were identified as monomethoxyflavones. Substance G3 was instable and converted into substance G6. Structural elucidation showed substance G4 to be 3′,4′,5′-trimethoxyflavone with identical UV and ^{13}C-NMR spectral data [36] as reported in the literature [37]. Substance G5 is a dimethoxyflavone, substance G6 a pentamethoxyflavone. Unfortunately, the low isolated amounts of substances G1, G2, G5 and G6 did not allow NMR measurements to obtain their substitution pattern in the course of this study.

Finally, the utility of RP-HPLC separation for the identification of substances G1–G6 was greatly enhanced by mass spectrometric detection. The RP-HPLC system was coupled to a quadrupole ion trap mass spectrometer by using an ESI interface, which allowed one to confidently identify the flavones in plant material and drugs made thereof. HPLC–ESI-MS analysis of the six methoxylated flavones was carried out employing a 150×2 mm I.D. column packed with a 3 μm/100 Å octadecylsilica stationary phase and a mobile phase comprising 1.0% acetic acid in water–acetonitrile (50:50). Under the soft electrospray ionization conditions all substances G1–G6 could efficiently be transformed into protonated molecules $[M+H]^+$. A comparison of the HPLC–ESI-MS spectra of the raw *Flos Primulae veris* methanol extract with that of the isolated and purified substances G1–G6 confirmed that the isolated compounds were pure and no artifacts. Moreover, the molecular masses of the Substances G in the raw methanol extract determined by RP-HPLC–ESI-MS (Fig. 5a) were identical to those of the isolated and purified substances G determined by EI-HRMS (Table 1). Substances G1–G6 were tracked in the *Flos Primulae veris* extract (Fig. 5a) from selected ion traces at *m/z* 253.2 (G1 and G2), *m/z* 313.3 (G4), 283.2 (G5) and *m/z* 374.2 (G6). Substances G2 and G1, which both have a molecular

Table 1
Measured and calculated exact masses, elementary compositions and structures of substances G1, G2, G3, G4, G5, G6

Substance	Exact mass	Calculated mass	Elementary composition	Structure
G1	252.07804	252.07864	$C_{16}H_{12}O_3$	Monomethoxyflavone
G2	252.07804	252.07864	$C_{16}H_{12}O_3$	Monomethoxyflavone
G3	–	–	–	Instable, converted into G6
G4	312.06810	312.09977	$C_{18}H_{16}O_5$	3′,4′,5′-Trimethoxyflavone
G5	282.08890	282.08921	$C_{17}H_{14}O_4$	Dimethoxyflavone
G6	372.12000	372.12090	$C_{20}H_{20}O_7$	Pentamethoxyflavone

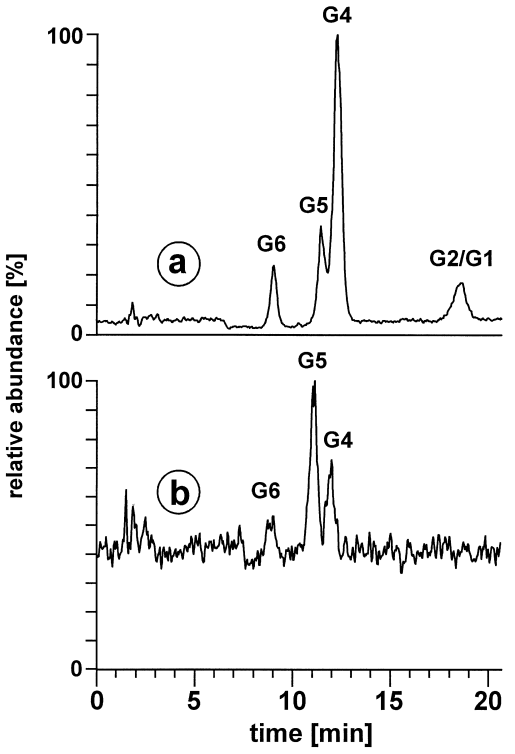

Fig. 5. RP-HPLC–ESI-MS of substances G1–G6 in (a) the *Flos Primulae veris* methanol extract and (b) a phytopharmaceutical drug containing *Flos Primulae veris*. Column, Nucleosil 100 RP_{18} (3 μm, 100 Å, 150×2 mm I.D.); mobile phase, water–acetonitrile (50:50) containing 1.0% acetic acid; flow-rate, 200 μl/min; temperature, 21°C; scan, selected ion monitoring at *m/z* 253.2, 283.2, 313.1, 373.1; sample volume, 5.0 μl.

mass of 252.1 and differ only in the position of the methoxy group were not separated under the chosen RP-HPLC conditions. The reconstructed ion chromatogram in Fig. 5a revealed that the most abundant flavone in the *Flos Primulae veris* methanol extract is substance G4 and that the instable substance G3 could not be detected in this particular extract.

The established HPLC–ESI-MS method also proved to be highly suitable for the identification of substances G1–G6 in complex samples used in the phytopharmaceutical industry. A liquid phytopharmaceutical drug containing *Flos Primulae veris* and four other plants, which is widely used to prevent Sinusitis as a consequence of a cold due to its secretolytic, expectorant, antiviral and antiinflammatory activities, was analyzed by RP-HPLC–ESI-MS (Fig. 5b). The reconstructed ion chromatogram showed that substances G2/G1 and G3 were not detectable in the drug (Fig. 5a). Substances G6, G5 and G4 could be identified by selected ion monitoring at the masses corresponding to the protonated molecules of the substances G.

Collisionally induced dissociation (CID) was used to obtain fragment ions of structural relevance to improve the selectivity of ESI-MS detection. Fragment ion spectra were used as ''fingerprints'' for fast identification of target compounds, which is demonstrated for substance G4 in Fig. 6. For this purpose, 5.0 μl of the raw methanol extract were injected onto the column and eluted isocratically with an eluent comprising 50% acetonitrile and 1.0% acetic acid in water. For ESI-MS detection the protonated molecule of substance G4 at *m/z* 313.3 was fragmented and daughter ion spectra were recorded at 25% collision energy (Fig. 6a). The daughter ion spectrum of substance G4 is illustrated in Fig. 6b. The fragmentation pathway of substance G4 was characterized by loss of a methyl group giving the product ion at *m/z* 298.1 (−15 u; $-CH_3$), further loss of H_2O (*m/z* 280.1; −18 u) and further loss of a carbonyl group (*m/z* 252.1; −28 u).

Fig. 7a depicts the tandem mass spectrum of substance G4 obtained by direct infusion of the isolated and purified substance G4, whereas Fig. 7b shows the mass spectrum extracted from the substance G4 peak in the reconstructed ion chromatogram of a phytopharmaceutical drug analyzed by RP-HPLC with ESI-MS–MS detection. Both spectra were compared to the tandem mass spectrum of a chemically sythesized substance G4 sample (Fig. 7c). The high similarity of all three mass spectra as well as the identical fragmentation patterns clearly corroborate the identity of the compounds found in extracts of *Flos primulae veris* and a phytopharmaceutical drug with the chemically synthesized substance G4. Tandem mass spectra of the other isolated substances G and the substances G1, G2, G5 and G6 in the raw methanol extract and in the phytopharmaceutical were recorded in the same way for identifying the compounds and in order to ensure that they were not artifacts. Substances G5 and G6 gave almost identical fragmentation patterns (Table 2) as substance G4, which indicates that the fragmentation of flavones with higher and lower degree

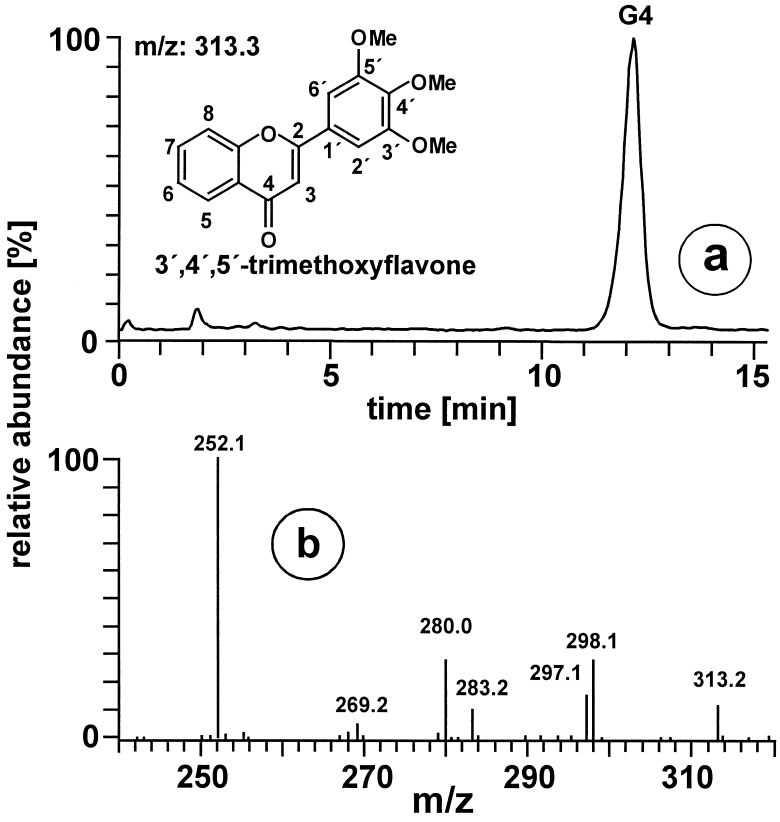

Fig. 6. RP-HPLC–ESI-MS–MS of substances G1–G6 in a phytopharmaceutical drug containing *Flos Primulae veris*. (a) Reconstructed ion chromatogram, and (b) tandem mass spectrum of substance G4. Column, Nucleosil 100 RP_{18} (3 μm, 100 Å, 150×2 mm I.D.); mobile phase, water–acetonitrile (50:50) containing 1.0% acetic acid; flow-rate, 200 μl/min; temperature, 21°C; scan, daughter ions of *m/z* 313.2, 85–400 u at 20% collision energy; sample volume, 5.0 μl.

of methoxylation also starts with the elimination of a methoxy group, followed by the elimination of water and elimination of a carbonyl group. Tandem mass spectra could not be recorded for substances G1 and G2 because of the relatively low abundances of these two flavones.

4. Conclusions

Extraction and several preparative chromatographic purification steps of 11 kg plant material yielded 12 mg of pure substance G4 and smaller amounts of substances G1, G2, G3, G5, G6, which were all identified as methoxyflavones by EI-HRMS. Comparison of NP- and RP-HPLC separations of substances G1–G6 in different sample matrices revealed that higher resolution between substances G1 and G2 and between substances G5 and G6 was feasible in the normal-phase mode. Nevertheless, RP-HPLC showed better reproducibility. For the recording of the chromatograms of the methoxyflavones fluorescence detection showed to be more selective and sensitive than UV-absorbance detection. It has been shown that reversed-phase column liquid chromatography combined with ESI-MS is a valuable tool for identification of methoxyflavones in different sample matrices. The high selectivity of ESI-MS allowed the confident identification of the flavones in crude

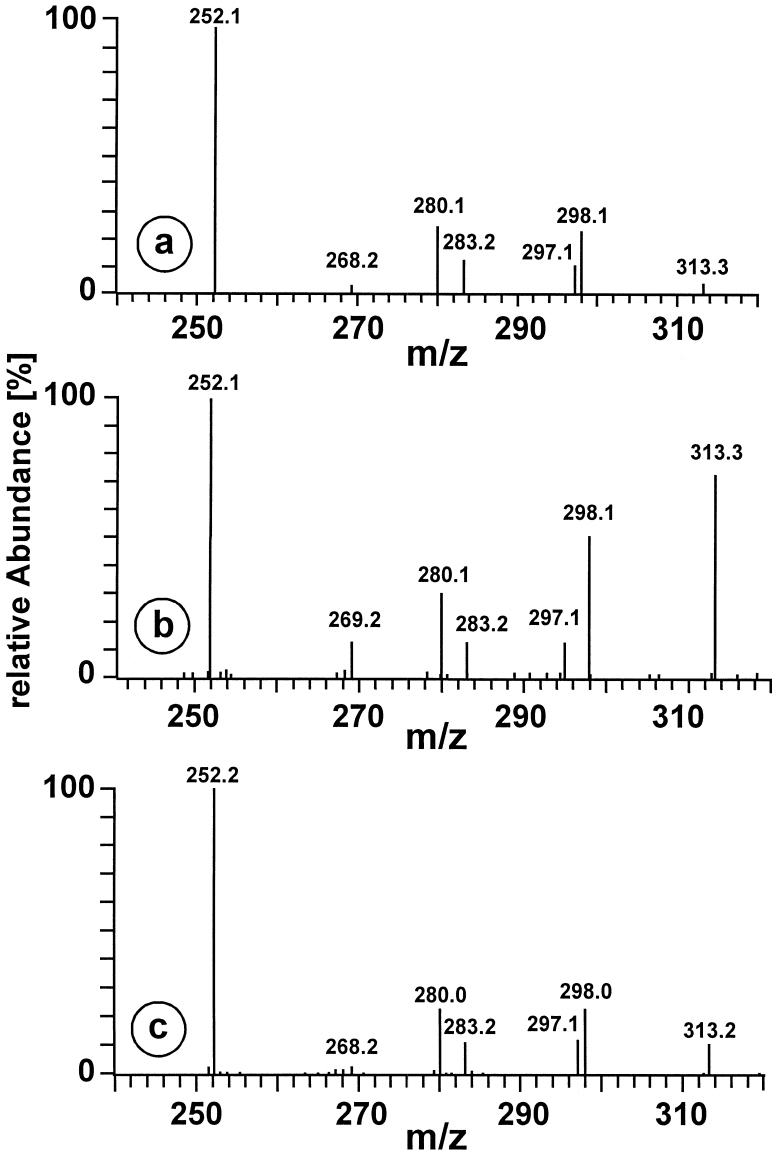

Fig. 7. Tandem mass spectra of (a) substance G4 isolated and purified from *Flos Primulae veris*, (b) substance G4 in a phytopharmaceutical drug containing *Flos Primulae veris*, and (c) chemically synthesized substance G4. (a) and (c) direct infusion at 50 μl/min of 0.20 mg/ml substance G4 in water–acetonitrile (50:50) containing 1.0% acetonitrile. (b) RP-HPLC–ESI-MS–MS; column, Nucleosil 100 RP_{18} (3 μm, 100 Å, 150×2 mm I.D.); mobile phase, water–acetonitrile (50:50) containing 1.0% acetic acid; flow-rate, 200 μl/min; temperature, 21°C; scan, daughter ions of *m*/*z* 313.2, 85–400 u at 20% collision energy; sample volume, 5.0 μl.

extracts of complex composition. Furthermore, HPLC–ESI-MS–MS was used to identify substances G1–G6 in phytopharmaceutical drugs and to ensure that the isolated flavones were not artifacts.

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Table 2
Product ions of substances G4, G5 and G6 observed in RP-HPLC–ESI-MS–MS

Substance	$-CH_3$ m/z	$-H_2O$ m/z	$-CO$ m/z
G4	298.1	280.1	252.1
G5	268.2	250.2	222.2
G6	358.1	340.0	312.1

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References

[1] J.B. Harborne, The Flavonoids – Advances in Research Since 1986, Chapman and Hall, London, 1993.
[2] J. Kuhnau, World Rev. Nutr. Diet 24 (1976) 117.
[3] T. Swain, The Flavonoids, Chapman and Hall, London, 1975.
[4] M. Gabor, The Pharmacology of Benzopyrone Derivatives and Related Compounds, Akademiai Kiado, Budapest, 1986.
[5] L. Farkas, M. Gabor, F. Kallay, Flavonoids and Bioflavonoids, Akademiai Kiado, Budapest, 1986.
[6] V. Cody, E. Middleton, J.B. Harborne, A. Beretz, Plant Flavonoids in Biology and Medicine – Biochemical, Pharmacological and Structure–Activity Relationships, Alan R. Liss, New York, 1988.
[7] M. Das, Flavonoids in Biology and Medicine III, National University of Singapore, 1989.
[8] B. Havsteen, Biochem. Pharmacol. 32 (1984) 1141.
[9] A.F. Welton, J. Hurley, P. Will, Plant Flavonoids in Biology and Medicine, Biochemical, Cellular and Medicinal Properties, Vol. II, Alan R. Liss, New York, 1988.
[10] J.W.T. Selway, Plant Flavonoids in Biology and Medicine – Biochemical, Pharmacological and Structure–Activity Relationships, Alan R. Liss, New York, 1986.
[11] S.M. Kupchan, J.R. Knox, M.S. Udayamurthy, J. Pharm. Sci. 54 (1965) 929.
[12] T. Katagi, A. Horii, Y. Omura, H. Miyakawa, T. Kyu, Y. Ikeda, K. Isoi, M. Makita, J. Chromatogr. 79 (1973) 45.
[13] N. Narasimhachari, E. Rudloff, Can. J. Chem. 40 (1962) 1123.
[14] M. Munekazu, S. Matsuura, K. Kurogochi, T. Tanaka, Chem. Pharm. Bull. 28 (1980) 717.
[15] E.M. Gaydou, T. Berahia, J.-C. Wallet, J.-P. Bianchini, J. Chromatogr. 549 (1991) 440.
[16] J.B. Harborne, Phytochemistry 7 (1968) 1215.
[17] J.B. Harborne, Phytochemical Methods, Chapman and Hall, London, 1997.
[18] J.P. Bianchini, E.M. Gaydou, J. Chromatogr. 190 (1980) 233.
[19] J.P. Bianchini, E.M. Gaydou, J. Chromatogr. 211 (1981) 61.
[20] P.G. Pietta, P.L. Mauri, A. Rava, G. Sabbatini, J. Chromatogr. 549 (1991) 367.
[21] B. Heimhuber, R. Galensa, K. Herrmann, J. Chromatogr. 439 (1988) 311.
[22] J. Wallet, E.M. Gaydou, Magn. Reson. Chem. 31 (1993) 518.
[23] D.C. Neckers, F.L. Wagenaar, J. Org. Chem. 46 (1981) 3939.
[24] O.N. Portner, Mass Spectrometry of Heterocyclic Compounds, 2nd ed., Wiley, Chichester, 1985, p. 244.
[25] A.P. Bruins, Trends Anal. Chem. 13 (1994) 37.
[26] A.P. Bruins, Trends Anal. Chem. 13 (1994) 81.
[27] X. He, L. Lin, L. Lian, J. Chromatogr. A 755 (1996) 127.
[28] C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Anal. Chem. 57 (1985) 675.
[29] A.P. Bruins, T.R. Covey, J.D. Henion, Anal. Chem. 59 (1987) 2642.
[30] D.J. Carroll, I. Dzidic, R.N. Stillwell, K.D. Haegele, E.C. Hornig, Anal. Chem. 47 (1975) 2369.
[31] R.B. Cole, Electrospray Ionization Mass Spectrometry, Wiley, New York, 1997.
[32] Y.Y. Lin, K.J. Ng, S. Yang, J. Chromatogr. 629 (1993) 389.
[33] K. Hostettmann, M. Hostettmann, A. Marston, Preparative Chromatography Techniques – Applications in Natural Product Isolation, Springer, Berlin, 1997.
[34] M. Mösmang, Ph.D. Thesis, University of Innsbruck, 1989.
[35] N. Hinterdorfer, Ph.D. Thesis, University of Innsbruck, 1995.
[36] C.W. Huck, Ph.D. Thesis, University of Innsbruck, 1998.
[37] M. Gaydou, Bull. Soc. Chim. France II (1978) 43.