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Lipophilic Constituents from Aerial and Root Parts of *Mercurialis perennis* L.

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ABSTRACT:

Introduction – Dog's mercury (*Mercurialis perennis* L.) is a perennial herb used in remedies for medicinal purposes. The plant is supposed to contain potentially active substances but its constituents have only been rarely studied.

Objective – Detailed studies on the phytochemical composition are of great interest to broaden the knowledge on the chemotaxonomy and pharmacognosy of *M. perennis*.

Methodology – Chloroform and hexane extracts from roots and aerial parts were investigated using GC/MS and LC/MS.

Results – The whole plant exhibited a broad spectrum of structurally diverse constituents, mainly alkaloids, terpenes, sterols and simple aromatic compounds. Closer inspection of the piperidine alkaloid hermidin revealed its inherent instability towards air oxygen. To obtain quantitative data on these alkaloids the synthesis of the more stable reference compound 4-methoxy-1-methylpyridine-2,6(1H,3H)-dione (MMPD) was required. In this study, MMPD was detected for the first time as a genuine compound in *Mercurialis*. Hermidine quinone and hermidin dimers originating from hermidin via a free anionic radical reaction were also confirmed by GC/MS. Moreover, volatile compounds such as benzylalcohol, 2-phenylethanol, 4-methoxy- and 3,4-dimethoxyphenol, (–)-*cis*- and (+)-*trans*-myrtanol, (–)-*cis*-myrtanal as well as squalene were predominantly present in *Mercurialis* roots. In contrast, aerial parts mainly contained phytol derivatives, sterols and tocopherols. By changing solvent polarity, lipid and wax-containing fractions were obtained. LC/MS-studies on hexane extracts showed the presence of several mixed triglycerides constituted by linolenic, linoleic, oleic, stearic and palmitic acids, as well as lutein, carotenes and pheophytins.

Conclusions – The phytochemical data presented complement our knowledge on the rarely studied plant *M. perennis* and may broaden its use in future phytotherapy. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: *Mercurialis*; alkaloids; carotenoids; hermidin; hermidin quinone; phenolics; terpenes; triacylglycerols; tocopherols; sterols

Introduction

Dog's mercury (*Mercurialis perennis* L.) is a perennial herbaceous plant belonging to the Euphorbiaceae family. Notably, as a member of the subfamily Acalypheae, it does not produce white sap, an otherwise common taxonomic feature of this plant family.

M. perennis may typically be encountered in shady woodlands throughout northern Europe, Russian Asia and some parts of North America (Jefferson, 2008). The fresh parts of the plant are malodorous and supposed to be poisonous to livestock (Bismarck and Floehr, 1974; Watson, 1998). Poisoning in humans caused by mistaken consumption of the herb has also been described in the literature (Rugman and Meecham, 1983) causing nausea, vomiting, haemorrhagic inflammation of the gastrointestinal tract and of the kidneys as typical symptoms of an intoxication.

On the other hand, positive effects can be found in the literature. For example, in the Middle Ages a tea made from aerial parts of the plant was recommended for dropsy, constipation, bronchial catarrh, loss of appetite, rheumatism and gout (Madaus, 1979). In phytotherapy dog's mercury was used for a long time as a purgative (laxative) and to treat women diseases such as menstrual molimina (Berger, 1954; Madaus, 1979). Nowadays, *M. perennis* is increasingly applied in homeopathy and anthroposophic remedies for treatment of inflammation, poorly healing wounds and sore, dry or inflamed eyes.

Leaves and roots are the source of an unstable blue dye formed upon air oxidation when plants are dried or freshly cut. Yet only a few constituents have been identified from *Mercurialis* sp.: piperidine alkaloids (Swan, 1984, 1985; Matsui *et al.*, 1986), flavonoid glycosides (Dumkow, 1969; Aquino *et al.*, 1987), maltose in the roots (Jeremias and Kull, 1966) and methylamine (Cromwell, 1949). However, the latter was most probably isolated as an artefact from the piperidine alkaloid hermidin under alkaline conditions (Swan, 1985). Moreover, dog's mercury is reported to

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contain saponins, cyanogenic glycosides and an essential oil (Hahn and Hahn, 2001). However, to the best of our knowledge their specific structures have not been identified. In the course of a phytochemical screening a comprehensive investigation of the chemical composition of *M. perennis* was performed. With the exception of *Acalypha indica* (Hungeling *et al.*, 2009) the phytochemical composition of members of Acalypheae has not been recently studied. Therefore chloroform and hexane extracts of the aerial and root parts from *M. perennis* were investigated using GC/MS and LC/MS techniques to assess the whole spectrum of lipid constituents. Special attention was devoted to the quantification of the piperidine-2,6-dione alkaloids in the plant since these neutral alkaloids may play a key role in biological and pharmacological functions.

Experimental

Chemicals and reagents

Reference standards of campesterol, glyceryl trilinolenate, linoleic acid, (–)-*cis*-myrtanol, (+)-*trans*-myrtanol, *β*–sitosterol, stigmasterol, squalene and (+)-*γ*–tocopherol were obtained from Sigma-Aldrich (Steinheim, Germany). *β*–Carotene, eicosane, linolenic acid, nonacosane, oleic acid, palmitic acid, phytol (*cis/trans*-mixture) and the silylating mixture Fluka I according to Sweeley were purchased from Fluka (Buchs, Switzerland). 2-Phenylethanol and DL-*α*–tocopherol were obtained from Carl Roth GmbH (Karlsruhe, Germany). 3,4-Dimethoxyphenol and benzylalcohol were obtained from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany) and Riedel-de-Haën (Seelze, Germany), respectively. (–)-*cis*-myrtanal was obtained by synthesis from (–)-*cis*-myrtanol via pyridinium chlorochromate oxidation (Corey and Suggs, 1975) in dichloromethane (GC-purity of the crude material: 70%, data not shown). All other chemicals of analytical or synthetic grade were purchased from VWR (Darmstadt, Germany), e.g. 4-methoxyphenol, 3-oxoglutaric acid, the aqueous solution of methylamine (40% w/v), trimethylformate, potassium peroxodisulfate and sodium dithionite (sodium hydrosulfite). A marigold extract (*Tagetes erecta* L.) used to prove identity of lutein via LC/MS was received from IMCD Deutschland GmbH (Cologne, Germany).

Plant material

Aerial and root parts from *M. perennis* were collected during the growing period between May and October 2008 in the mountain forest above Bad Boll/Eckwälden (Baden-Wuerttemberg, Germany). The fresh plant material was cleaned by rinsing with water, dried with tissue paper and kept at –80°C until analysis. Another portion of the plant material (herbal parts) was also air-dried in a shady place. *M. perennis* was identified by Professor O. Spring (Department of Botany, Hohenheim University, Stuttgart, Germany). Specimens of the plant were deposited at the herbarium of Hohenheim University (voucher numbers HOH-006229 to HOH-006232).

Extraction of the plant material

Method A. For analytical GC/MS profiling of the volatile constituents the deep frozen (–80°C) roots or aerial parts from *M. perennis* (20 g) were immersed in chloroform (200 mL). Subsequently, the plant material was minced for 1 min by an ultrathurrax (21,000 rpm; IKA-Werke GmbH & Co. KG, Staufen, Germany) and the slurry allowed to stand for 24 h. The sediment was recovered by vacuum filtration over Celite and the filter cake re-extracted in the same manner again and finally washed with chloroform (50 mL). Remaining water was removed from the combined filtrates and the chloroform fraction evaporated to dryness under vacuum rotovaporation. For quantitative GC/MS analyses, the residue was dissolved in chloroform (20 mL) containing the internal reference compound eicosane (*n*-C20). Three separate extractions were analysed by GC/MS each measured in triplicate ($n = 3$).

Method B. In a modified procedure a stream of nitrogen was bubbled through the extraction mixture for 10 min before and then after ultrathurrax treatment, to exclude atmospheric oxygen during the 24 h extraction process. The slurry was worked up in the same manner as described in method A.

Method C. The plant material was extracted according to method B but in the presence of sodium dithionite (1.20 g) dissolved in water (12 mL), for reduction of Herm-Q.

Method D. A total lipid fraction was derived from the air-dried, powdered (<2 mm) aerial parts (150.5 g) by extraction with hexane (2 L) for 24 h. After vacuum filtration over Celite the extract was concentrated by vacuum rotovaporation to yield an orange-brown tarry residue (1.4 g; 0.93% of the plant material).

Synthesis of the reference compound hermidin

Hermidin was synthesised from 4-methoxy-1-methylpyridine-2,6(1H,3H)-dione (MMPD) according to a modified procedure described by Swan (1985).

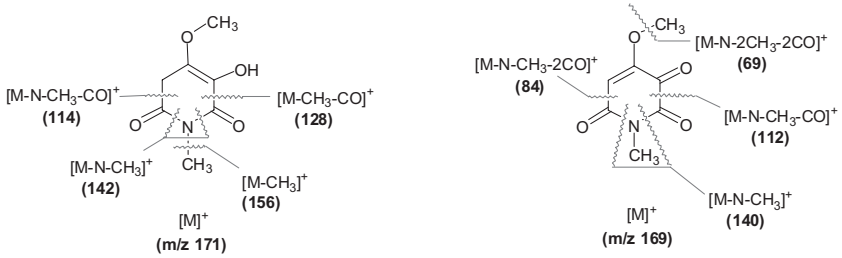
4-Methoxy-1-methylpyridine-2,6(1H,3H)-dione. Dimethyl-2-oxopropane-1,3-dicarboxylate (24.6 g; 0.131 mol), obtained as a *cis/trans* mixture according to Swan's (1985) procedure starting from 3-oxoglutaric acid and trimethylformate, was cooled to –80°C and treated under nitrogen atmosphere with an aqueous solution of methylamine (40% w/v, 39.5 mL). The mixture was stirred for 2 h, while the temperature was allowed to reach room temperature. The red-brownish liquid thus obtained was kept overnight in the refrigerator. After removing the solvent together with unreacted methylamine by vacuum rotovaporation, toluene (2 × 100 mL) was added and removed again under reduced pressure to azeotrope the water. The resulting product was refluxed for about 90 min with a sodium methoxylate solution, freshly prepared from metallic sodium (3.2 g; 0.139 mol) and methanol (150 mL). Subsequently, the solvent was distilled off and the residual solid dissolved in water (300 mL). Unreacted starting material was removed by extraction with diethyl ether (4 × 100 mL). Acetic acid (22 mL) was added to the aqueous solution, and the latter extracted with chloroform (3 × 100 mL). The strawberry-red chloroform extract was dried over sodium sulfate, filtered and the solvent evaporated under vacuum to yield crude MMPD (GC purity 80%). Repeated recrystallisation from a mixture of chloroform–ether yielded MMPD (11.26 g; 55.5% of the theoretical value) as faintly pink crystals, m.p. 111–112°C (m.p.$_{Lit}$ = 114–115°C; Swan, 1985); UV–vis 254 nm ($\log\varepsilon = 3.89$); GC purity 96%; GC/MS (t_R 17.2 min) *m/z* (%BPI): 155 (M^+, 100), 127 (M – CO^+, 11), 126 (M – NCH_3^+, 9), 112 (M – CO – CH_3^+, 15), 98 (M – NCH_3 – CO^+, 8), 69 (26), 68 (39). IR (KBr, cm^{-1}): 521 (w), 614 (w), 662 (w), 823 (m, C=C), 963 (w), 994 (w), 1102 (w), 1165 (w),1192 (w), 1237 (s), 1293 (s), 1390 (s), 1436 (s), 1636 (s), 1673 (s, C=O), 1722 (s), 2907 (w, C=CH), 3446 (br).

5-Hydroxy-4-methoxy-1-methylpyridine-2,6(1H,3H)-dione (hermidin). 4-Methoxy-1-methylpyridine-2,6(1H,3H)-dione (MMPD; 2.3 g; 14.82 mmol) was dissolved under nitrogen atmosphere in an ice-cold solution of 2.95 g (73.75 mmol) sodium hydroxide in water (53 mL). While stirring, potassium peroxodisulfate (potassium persulfate 4.9 g, 18.12 mmol) was added rapidly and stirring continued for 15 min during which the solution turned blue-green. To the solution kept for 2 days in the refrigerator (4°C), sulfuric acid (96% w/w, 4.4 mL) was added dropwise with cooling (ice water bath) under a nitrogen atmosphere. The

Table 1. Gas chromatography–mass spectrometric (GC/MS) data of compounds, identified in the chloroform extract from the roots and aerial parts of *M. perennis* L.

Compound	t_R (min)	Characteristic mass fragments, *m/z* (% BPI)
Benzylalcohol	7.0	108 (M^+, 100), 107 (64), 91 (14)[a], 79 (69), 77 (42)
2-Phenylethanol	8.6	122 (M^+, 33), 103 (5), 92 (51), 91 (100)[a], 77 (5), 65 (12)
(–)-*cis*-Myrtanal	10.6	152 (M^+, 1), 137 (43)[b], 123 (77)[c], 109 (51)[d], 82 (100), 67 (78)
4-Methoxyphenol	10.8	124 (M^+, 100), 109 (97)[b], 81 (40), 65 (4), 53 (15)
(–)-*trans*-Myrtanol	12.1	154 (M^+, 0.1), 136 (9)[e], 123 (100), 107 (12), 93 (54), 81 (52)
(+)-*cis*-Myrtanol	12.4	154 (M^+, 0.1), 136 (14)[e], 123 (97), 107 (24), 93 (100), 81 (66)
Hermidin[j]	16.5	171 (M^+, 99), 156 (36)[b], 142 (100), 128 (11), 114 (26), 69 (24)
3,4-Dimethoxyphenol	16.6	154 (100), 139 (80)[b], 111 (47), 93 (16), 81 (6), 69 (9)
Hermidin-quinone[k]	20.3	169 (M^+, 38), 140 (0.3), 112 (36), 84 (18), 69 (100)
Phytadiene isomers[j]	28.0–29.1	278 (3), 137 (11), 123 (53), 109 (27), 95 (74), 82 (64), 68 (100)
trans-Phytol	34.7	278 (2)[e,f], 123 (37), 111 (15), 95 (29), 81 (31), 71 (100)
Squalene	48.8	410 (M^+, 1), 341 (9)[g], 149 (18), 136 (29), 121 (23), 81 (54), 69 (100)
δ-Tocopherol	51.1	403 (M + 1, 68), 402 (M^+, 100), 281 (5), 177 (43), 137 (67)
γ-Tocopherol	52.8	417 (M + 1, 89), 416 (M^+, 100), 368 (9), 191(24), 151 (96)
α-Tocopherol	54.1	430 (M^+, 100), 205 (9), 165 (56), 136 (3), 121 (3)
Campesterol	56.0	400 (M^+, 100), 382 (75)[e], 367 (37)[h], 315 (77)[i], 289 (41), 255 (50), 213 (55)
Stigmasterol	56.4	412 (M^+, 89), 395 (11)[e], 379 (21)[h], 351 (29), 327 (7)[i], 300 (45), 271 (40), 255 (100), 213 (54)
β-Sitosterol	57.5	414 (M^+, 100), 396 (29)[e], 381 (20)[h], 329 (43)[i], 303 (24), 273 (26), 255 (28), 213 (32)

[a] $[C_6H_5CH_2]^+$; [b] $[M - CH_3]^+$; [c] $[M - C_2H_5]^+$; [d] $[M - C_3H_7]^+$; [e] $[M - H_2O]^+$; [f] molecular ion not detectable; [g] $[M - C_5H_9]^+$; [h] $[M - H_2O - CH_3]^+$; [i] $[M - C_6H_{13}]^+$; [j] tentatively assigned; MS data of one representative isomer are shown. [k] Proposed fragmentation for hermidin (left) and hermidin quinone (right) in the MS:



reaction mixture was heated under reflux for 1 h. Subsequently, solutions of anhydrous sodium sulfite (10.00 g; 79.34 mmol) in water (25 mL) and sodium dithionite (5.00 g; 28.72 mmol) in water (25 mL) were added at +4°C and the mixture stirred for 10 min. The resulting solution was treated with 50 mL water and extracted with oxygen free chloroform (3 × 100 mL). The combined chloroform extracts were dried (sodium sulfate) and the solvent removed by rotovaporation to yield 1.84 g crude material. By use of vacuum liquid chromatography (VLC) on a silica column (78 g TLC grade Merck silica 60, preconditioned with chloroform), hermidin was purified by elution with chloroform. The fractions containing the pure compound (checked by GC/MS) were combined, the solvent distilled off and the product recrystallised from a chloroform–ether mixture. Total yield: 0.255 g (9.2% of the theoretical value); pale yellow crystals, m.p. 124–126°C; UV–vis 292 nm ($\log\varepsilon = 3.795$); GC purity 98%; GC/MS (t_R 16.4 min) *m/z* (%BPI): see Table 1. IR (KBr, cm^{-1}): 610 (m), 760 (m), 1083 (m), 1132 (s), 1234 (s), 1292 (s), 1356 (m), 1456 (s), 1621 (s, C=O), 1692 (s, C=O), 1721 (s), 3387 (s, br, OH).

Analytical and spectral analyses

IR-analyses. The IR spectra were recorded on a Bruker Tensor 27-FT-IR spectrometer at the SGS Institute Fresenius (Taunusstein, Germany).

GC/MS-analyses. GC/MS was performed with a PerkinElmer Clarus 500 gas chromatograph with split injection (split ratio 30 : 1, injection volume 1.0 µL) coupled to a mass detector. The column used was a Zebron ZB-5ms capillary column (60 m × 0.25 mm inner diameter × 0.25 µm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; Phenomenex, Torrance, CA, USA). Helium was the carrier gas at a flow rate of 1 mL/min. The injector used was a PSS (programmed-temperature split/splitless injector, temperature 250°C). The temperature program for the column oven was 100–320°C at 4°C/min with a final hold time of 30 min. The mass spectrometer was run in the electron ionisation mode (70 eV). Alkaloids, aromatic alcohols, phenolics, terpenes, tocopherols and sterols were identified via reference standards or by the help of the NIST spectral database. The most abundant components were quantified by GC/MS in the TIC mode (*m/z* 50–610) via external calibration using eicosan (*n*-C20) as internal standard. Calibration curves were established for each single compound in the range of 0.001–0.2 mg/mL ($r^2 = 0.999$).

HPLC-DAD-MS/MS analyses. Chromatographic analyses were carried out with an Agilent 1200 HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA), equipped with a binary pump, a micro vacuum degasser, an autosampler, a thermostatic column compartment and a UV–vis diode array detector. A YMC™ Carotenoid reversed-phase column (5 µm, 250 × 3.0 mm i.d., Waters Corporation, Milford, MA USA) was used for

chromatographic separation at 25°C. The UV-detection of carotenoids and chlorophyll derivatives was performed at 419 nm.

The mobile phase consisted of methanol–*tert*-butyl methyl ether–water (81 : 15 : 4; v/v/v; mobile phase A) and methanol–*tert*-butyl methyl ether–water (6 : 90 : 4; v/v/v; mobile phase B) with a flow rate of 0.60 mL/min. Starting with 0% B at 0 min, a linear gradient was followed to 55% B at 50 min, keeping 55% B until 54 min, then increasing to 100% B at 55 min, continuing for 10 min, before re-equilibration to starting conditions.

The lipid fraction of *M. perennis* (2.7 mg) obtained via extraction method D was dissolved in 2-propanol (10 mL). The resulting clear solution was filtered through a 0.45 µm GHP acrodisc membrane (PALL Life Sciences, Dreieich Germany) before use. The injection volume of each sample was 20 µL.

The LC system was coupled to an HCT ultra ion trap (Bruker Daltonic GmbH, Germany) with an APCI source operating in the positive mode with the following parameters: HV voltage –4000 V; dry gas N_2 4 L/min with a dry temperature set at 300°C; nebuliser 35 psi, vaporiser temperature 350°C. Full-scan mass spectra of the HPLC eluates were recorded during the chromatographic separation yielding $[M + H]^+$ and $[M + H_2O]^+$ ions. In the case of triacylglycerols $[M + K+Na]^+$ were also observed. To obtain further structural information, these ions were trapped and fragmented to yield the precursor product patterns of the analytes. The mass range was recorded from *m/z* 50 to 1200 with a compound stability and trap drive level of 100%. MS^n data were acquired in the auto MS/MS mode. The instruments were controlled by an Agilent Chemstation and EsquireControl Software.

Determination of the dry mass amount

Fresh plant material (10 g) was dried at 50°C for 2 days. Afterwards the dry mass was determined [dry mass amount (%) = dry weight × 100/fresh weight]. A mean value of four measurements for each sample was ascertained ($n = 4$).

Determination of the fatty acid and sterol composition

For the qualitative analysis of the fatty acids and sterol composition the lipid fraction (obtained via extraction method D) was saponified and derivatised as follows: the lipid fraction (0.60 g) was treated with potassium hydroxide solution (50 mL, 10% in methanol, w/v). After sonication (1.5 min) the suspension was kept at room temperature for 24 h. Afterwards the mixture was acidified by addition of 1 M hydrochloric acid (85 mL) and then extracted with hexane (3 × 50 mL). The combined hexane extracts were dried over sodium sulfate. After filtration, the solvent was removed by rotovaporation to yield 0.51 g of an orange residue (stored at –20°C until analysis). For GC/MS analyses the saponified material (7.3 mg) was dissolved in chloroform (0.5 mL) and silylated at 105°C for 1 h, corresponding to a previously reported procedure (Lorenz *et al.*, 2008) using 0.2 mL silylating mixture Fluka I by Sweeley.

Results and Discussion

Profiling of lipophilic constituents by GC/MS

Roots and aerial parts from *M. perennis* were extracted by use of chloroform to yield a broad spectrum of lipophilic constituents. Typical total ion chromatograms (TIC) of aerial part and root extracts are shown in Fig. 1A and B, respectively.

The main constituents were identified by comparison of the mass spectra with the NIST spectral database, as well as via reference compounds (Table 1). It is obvious that the aerial part extracts (Fig. 1A) contain a larger variety of compounds than the roots (Fig. 1B). However monoterpenes, simple phenolics and squalene were present in much higher amounts in the root parts (for quantitative data see below).

At short retention times of the TIC (t_R 6–18 min) simple aromatic constituents like benzylalcohol (Benzyl), 2-phenylethanol (2-Phen), 4-methoxyphenol (4-MP) and 3,4-dimethoxyphenol (3.4-DMP) were identified (Fig. 2). Moreover, bicyclic monoterpenes of the pinane type such as (–)-*cis*-myrtanal (*cis*-MyrAl), (–)-*cis*- and (+)-*trans*-myrtanol (*cis*/*trans*-MyrOH) were detected and another monoterpene 6,6-dimethyl-bicyclo[3,1,1]heptan-2-one (DBH) tentatively assigned by its specific mass data. All of the compounds determined are typical essential oil constituents detected in *M. perennis* for the first time. Only 2-phenylethanol, myrtanol and myrtanal have been reported earlier as constituents in the essential oil of Yamaai (*Mercurialis leiocarpa*) (Kameoka *et al.*, 1988). In the intermediate and long retention time range of the TIC (t_R 23–63 min; Fig. 1A), various polyisoprene compounds were detected in the aerial parts of *M. perennis*. Between 27 and 30 min, three compounds with similar mass spectra were observed (*m/z* 278, 137, 123; Table 1). Because of MS similarities to *trans*-phytol (*trans*-Phyt; t_R 34.7 min), these compounds were tentatively assigned as phytadiene isomers (Phyt-D). Such phytadiene isomers (2,6,10,14-tetramethyl hexadienes) have been earlier reported as pyrolysis artefacts from pheophytin a (Hites, 1974). Furthermore, α-, γ- and δ-tocopherols (α-, γ-, δ-Toco), phytosterols like campesterol (Camp), stigmasterol (Stig) and β-sitosterol (β-Sito), as well as linolenic acid ethylester (Linolen-E), squalene (Squa) and nonacosane (n-C29) were assigned (Fig. 1). The hermidin quinone (Herm-Q) and hermidin dimers (Herm-D; co-elution in one peak at t_R 43.0 min) were formed from hermidin (Herm; see below).

Quantification of terpenoid, aromatic and phytosterol constituents

Quantitative data on the main constituents of aerial and root parts were obtained by GC/MS via external calibration with reference compounds. As mentioned before, some of the compounds with short retention times were more frequently found in the roots than in aerial parts. In roots collected in October, 4-methoxyphenol, (–)-*cis*-/(+)-*trans*-myrtanol and 3,4-dimethoxyphenol concentrations of 179.5, 35.8 and 99.6 mg/kg were determined, while in aerial parts 9.5, 13.2 and 7.4 mg/kg were found (see Fig. 3). Only 2-phenylethanol was present in slightly higher amounts in aerial (32.8 mg/kg in May and 43.2 mg/kg in October) than in root parts (17.4 mg/kg in October). The tentative phytadiene isomers (Phyt-D) and *trans*-phytol (*trans*-Phyt) were exclusively present in aerial parts (1185.2 and 1184.0 mg/kg Phyt-D and 77.4 and 108.0 mg/kg *trans*-Phyt in May and October, respectively Fig. 3), corroborating the hypothesis that these constituents are derived from chlorophylls (Hites, 1974) not present in the roots.

Also, α-Toco was 8–18-fold higher in the aerial (369.8 and 814.7 mg/kg collected in May and October, respectively) compared with root parts (44.2 mg/kg). Other tocopherol isomers (δ- and γ-Toco) were detected only in aerial parts (not quantified).

Considering the total phytosterol concentration (sum of campesterol, stigmasterol and β-sitosterol), approximately double was found in aerial parts (1363.7 mg/kg in May and 1101.2 mg/kg and October) compared with root (580.6 mg/kg in October). In summary, distinct qualitative and quantitative differences in the composition of root and aerial parts of *M. perennis* exist.

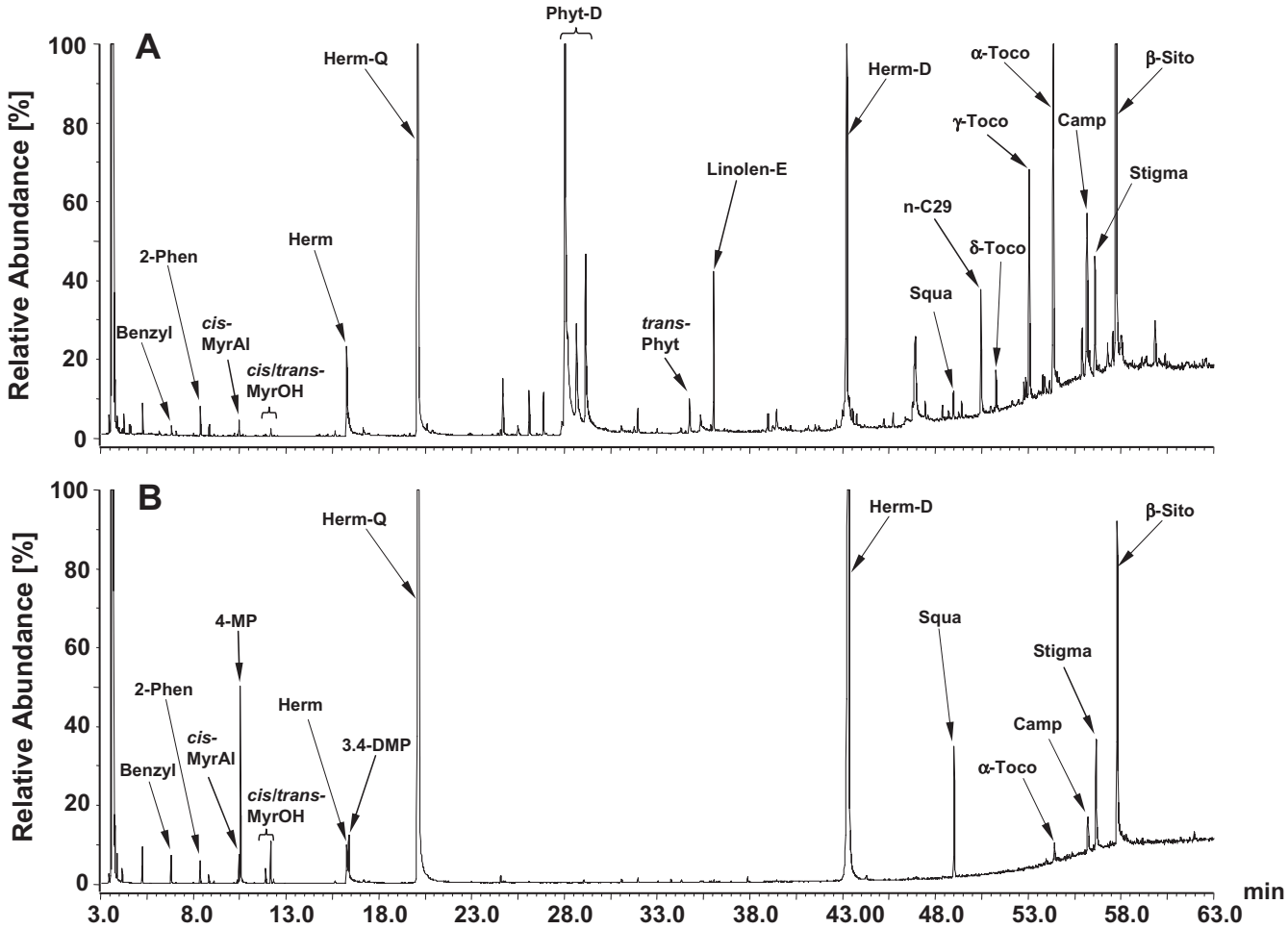


Figure 1. GC fingerprint (TIC) of chloroform extracts obtained from aerial parts (A) and roots (B) of *M. perennis*. Both extracts were obtained via extraction method A (see Experimental) in the presence of oxygen. Abbreviations: Benzyl, benzylalcohol; 2-Phen, 2-phenylethanol; *cis*-MyrAl, (–)-*cis*-myrtanal; 4-MP, 4-methoxyphenol; *cis*/*trans*-MyrOH, (–)-*cis*-/(–)-*trans*-myrtanol; 3.4-DMP, 3,4-dimethoxyphenol; Phyt-D, phytadiene isomers (tentatively identified); *trans*-Phyt, *trans*-phytol; Linolen-E, linolenic acid ethylester; Squa, squalene; *n*-C29, nonacosane, *δ*-Toco, *δ*-tocopherol; *γ*-Toco, *γ*-tocopherol; *α*-Toco, *α*-tocopherol; Camp, campesterol; Stigma, stigmasterol; *β*-Sito, *β*-sitosterol.

Qualitative characterisation of the alkaloids (hermidin derivatives)

By GC/MS investigation two nitrogen-containing compounds with odd numbered molecular ion peaks (M^+, $m/z = 171$ and 169) were detected in the chloroform extracts. The mass spectra of these molecules exhibited loss of methyl (CH_3^+) and *N*-methyl (*N*-CH_3^+)-fragments. Furthermore an *α*-cleavage of the bonds in the neighbourhood of the ionised nitrogen atom resulted in the split of carbon monoxide (CO^+) from the mother ions. The mass spectra have not been reported in the literature. However, the fragmentation patterns (see Table 1, footnote) allowed their identification as the piperidine-2,6-dione alkaloids hermidin (Herm) and Herm-Q. Both alkaloid structures have earlier been described from *M. perennis* by Swan (1984, 1985). In addition, the hermidin structure was verified with a synthetic reference compound we obtained via Elbs peroxodisulfate oxidation (Behrman, 2006) of MMPD. Both the synthetic and the natural hermidin were identical regarding their chromatographic and mass spectroscopic features. Notably, MMPD, the putative biosynthetic precursor of hermidin, was detected for the first time as a genuine metabolite in *Mercurialis* (see Fig. 2, at $t_R \approx 17.2$ min). The finding of MMPD complements recent studies related to the biosynthesis of hermidin alkaloids explored by Ostrozhenkova *et al.* (2007) as MMPD appears to be a biosynthetic intermediate on the way to hermidin starting from nicotinic acid.

It is known from the literature (Swan 1984, 1985) that hermidin easily oxidises in the presence of oxygen, yielding the yellow coloured 4-methoxy-1-methylpyridin-2,3,6-(1H)-trione, the so-called hermidin quinone. In fact, Herm-Q could be found as a main component when chloroform extraction was performed under aerobic conditions (extraction method A, see Table 2). A semiquinone-like radical anion, which has been characterised by ESR-spectroscopic studies (Forrester, 1984) as well as by measurement of the electrode potential (Cannan, 1926), is formed via single-electron transfer from Herm onto Herm-Q (see Fig. 4). The radical anion, also known as cyanohermidin, is responsible for the transient deep blue colour of aqueous hermidin solutions and is also apparent when roots or young shoots of *M. perennis* are cut or bruised. However, the blue radical anion is unstable

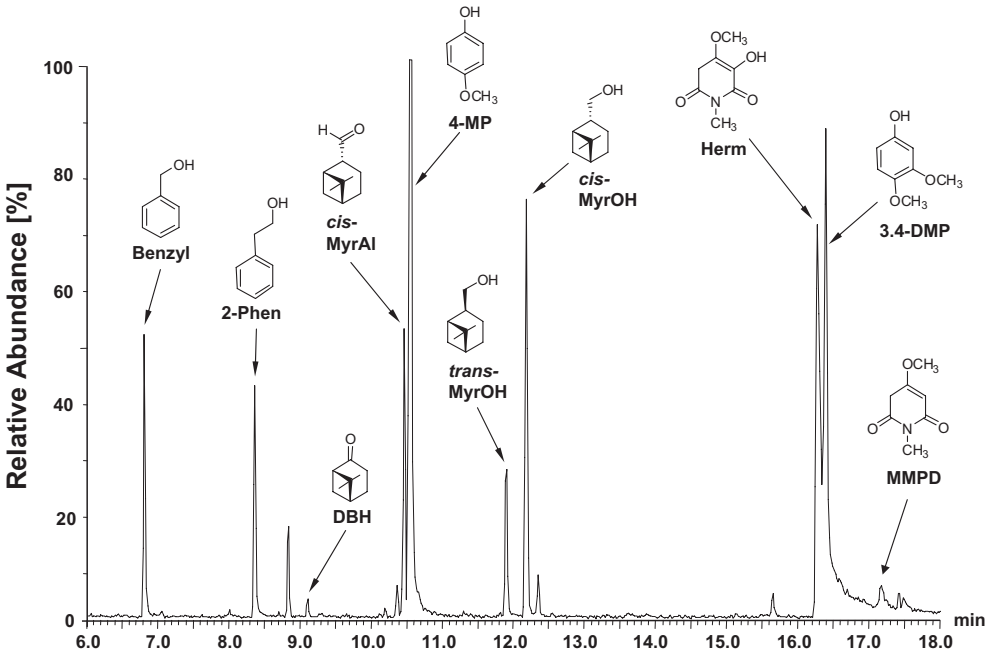


Figure 2. Section of the total ion chromatogram (TIC of the root chloroform extract, shown in Fig. 1B) showing aromatic alcohols, phenolics, terpenes and alkaloids. Abbreviations: analogous to Fig. 1; DBH, 6,6-dimethyl-bicyclo[3,1,1]heptan-2-one; MMPD, 4-methoxy-1-methylpyridine-2,6(1H,3H)-dione.

Table 2. Qualitative data of hermidin and hermidin derivatives in chloroform extracts of *M. perennis* dependent on the respective extraction procedure used (estimated via the relative abundance in the TIC)

		Component[a]		
Extraction method	Conditions	Herm	Herm-Q	Herm-dimers
A	Presence of O_2[b]	+	+++	++
B	Absence of O_2 (inert gas: N_2)[c]	+	++	+++
C	Absence of O_2 (inert gas: N_2) + sodium dithionite[d]	+++	(+)	(+)

[a] Concentrations expressed as: (+)/+ = very low/low, ++ = middle, +++ = high concentration; [b] oxidative; [c] inert; [d] reducing conditions.

and transforms into the hermidin dimer chrysohermidin by dimerisation (see Fig. 4). On the other hand, another hermidin dimer (hermidin dimer A) may be formed by radical addition of cyanohermidin with Herm-Q. Finally, the hermidin dimer A can be oxidised into chrysohermidin (see Fig. 4). Both dimers may appear simultaneously in a mixture (Swan, 1984, 1985; Boger and Baldino, 1993) as a quinhydrone-like compound (charge-transfer complex) as proposed by Swan (1985). In the present investigation the dimer mixture (Herm-D; see Fig. 4) could be detected in the TIC (GC/MS) as a main product when air oxygen was excluded during the extraction process (Table 2, nitrogen atmosphere, extraction method B). The MS of the dimers (Herm-D) exhibited weak molecular ion signals at *m/z* 338 and *m/z* 336 at t_R 43.0 min, indicating co-elution of both compounds (Fig. 5). Furthermore the characteristic fragmentation pattern corroborates the co-existence of both dimers (Fig. 5). A straightforward mechanism for the preferred formation of Herm-D under inert conditions (nitrogen atmosphere) is not easily comprehensible. It is speculated that the formed anionic radical (cyanohermidin) normally stabilised in aqueous solution by a water hydrate shell becomes unstable when chloroform is added. As a result intermolecular reactions are triggered, e.g. dimerisation of the anionic radical or a radical addition to Herm-Q (Fig. 4).

As is known from the literature, quinones and quinone-type structures are easily reduced by sodium dithionite (March, 1992). For that reason a reducing environment should convert Herm-Q into Herm and consequently suppress anionic radical and dimer formation. Haas and Hill (1925) demonstrated the reduction of Herm-Q into Herm by use of sodium dithionite. Thus, when the chloroform extraction of *M. perennis* was performed in the

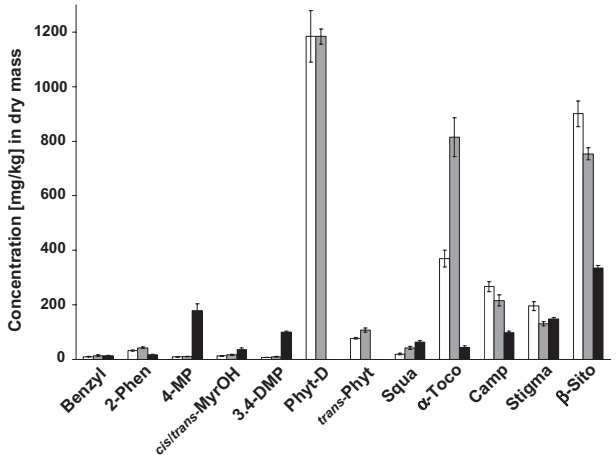


Figure 3. Quantitative data for aromatic alcohols, phenolics and terpenoids in aerial and root parts from *M. perennis*, based on chromatograms exemplarily shown in Fig. 1 (for peak assignment see Table 1). Compound quantification was performed via external calibration ($n = 3$, measured in triplicate; ± standard deviation; for measurement of *trans*-phytol [*trans*-Phyt) and the phytadiene isomers (Phyt-D) $n = 2$, measured in triplicate]. White bars, aerial parts (May); grey bars, aerial parts (October); black bars, root parts (October).

presence of dithionite (Table 2, extraction method C), mostly Herm was detected. As a consequence Herm-Q and Herm-D were observed only in low amounts, corroborating the oxidation pathway depicted in Fig. 4.

In summary, by applying three different extraction methods (oxidising, reducing and inert conditions), the sensitivity of the Herm–Herm-Q–Herm-D redox system on oxidising or reducing conditions in solution could be ascertained (Table 2). The findings presented allow the proposition that Herm, Herm-Q and Herm-D function as a redox system *in vivo* because of easy electron transfer over several oxidation stages—similar to ubiquinones which act as electron transporters in the respiratory chain of higher plants (Rich and Moore, 1976).

Quantitative analysis of hermidin derivatives

Since quantitative data on hermidin (Herm) in *M. perennis* were not available from the literature, an analytical approach was established. Because Herm is highly unstable in solutions, MMPD was chosen as a stable reference compound for external calibration. Roots and aerial parts were extracted with chloroform in the presence of oxygen (method A) to convert the bulk of Herm into the more stable compounds Herm-Q and Herm-D.

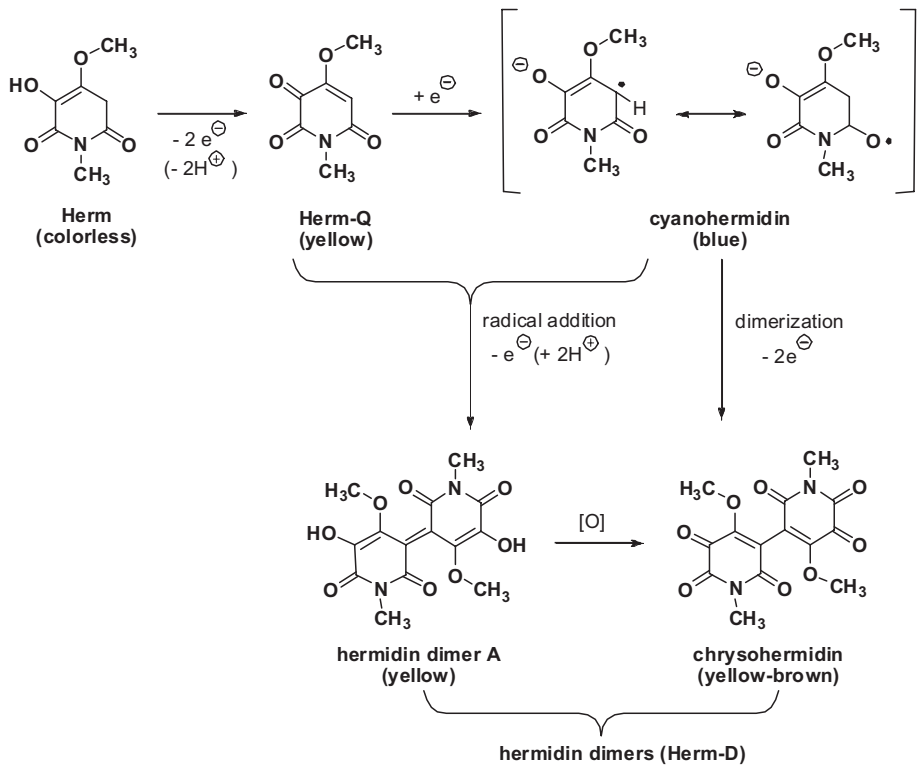


Figure 4. Oxidation pathway of hermidin. Colorless hermidin (Herm) oxidises to yield the yellow-coloured hermidin quinone (Herm-Q). Herm-Q can be converted by single electron transfer into the blue coloured radical anion (cyanohermidin), a reactive intermediate which is stabilised via mesomeric effects. Either by dimerisation of the radical anion or by radical addition, e.g. to Herm-Q, quinhydrone-like hermidin dimers are formed. Chrysohermidin could also arise by oxidation of the hermidin dimer A with oxygen.

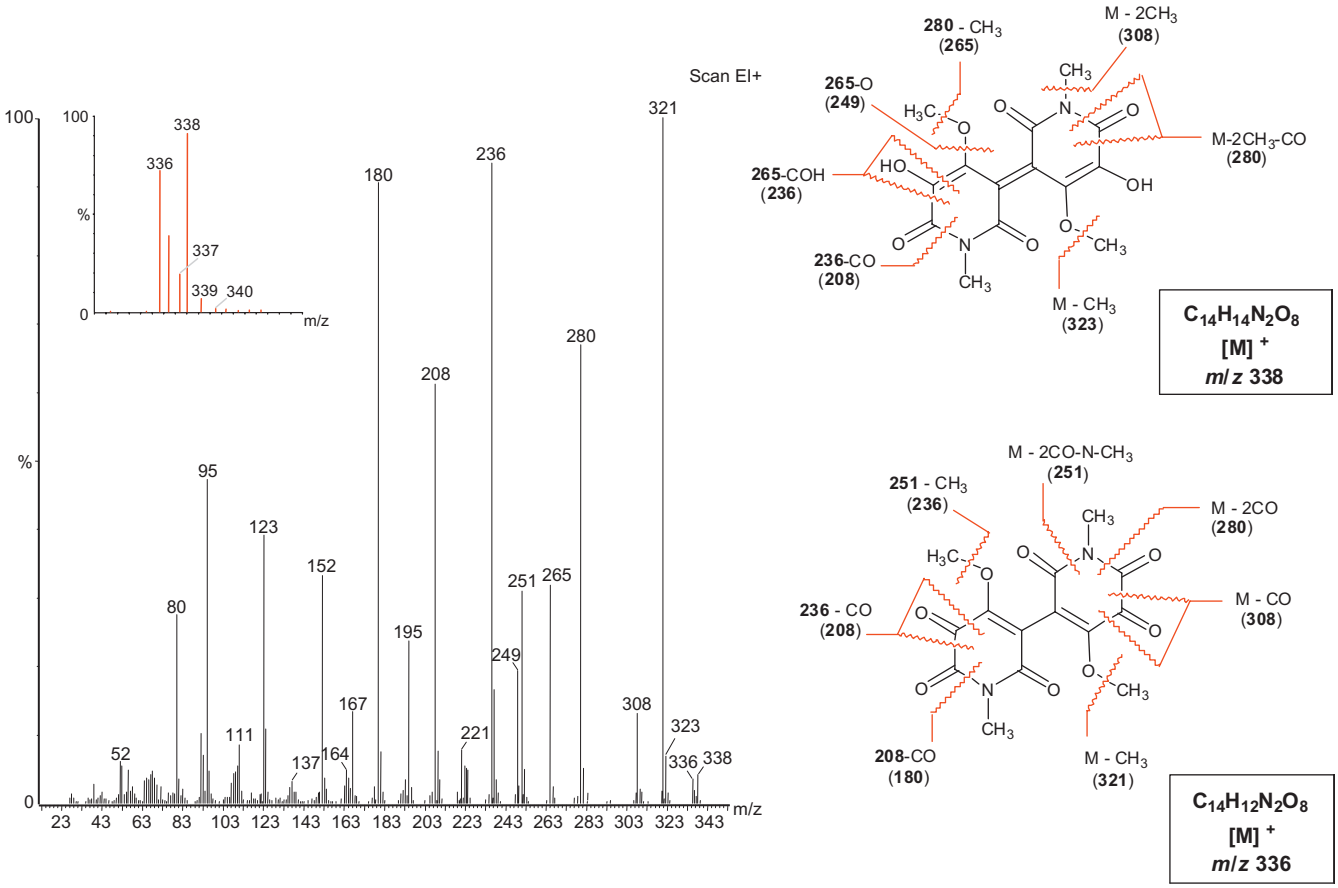


Figure 5. GC/MS spectrum of the peak at t_R 43.0 min (see Fig. 1) showing a mixture of two hermidine dimers. The chemical formulas of the two dimers depict a feasible decay into daughter ions. The insert exhibits the molecular ions $[M]^+$ at *m/z* 336 and 338 of the two dimers.

Thereafter the area percentages of all hermidin derivatives (Herm + Herm-Q + Herm-D) were determined and calculated as MMPD. In the roots of *M. perennis* collected in October the highest alkaloid content was found (2618.5±136.2 mg/kg). In aerial parts, lower total alkaloid amounts were measured, while there was a big difference between the plant material collected in May (1972.9±100.0 mg/kg) and October (404.2 ±33.1 mg/kg). Although these data were obtained from random samples, it is suspected that the highest alkaloid concentration will be found in the aerial parts in early spring. During the period between May and October, the alkaloid concentration dropped to a fifth or even lower concentrations compared with the original value. It may thus be suspected that a high alkaloid concentration will deter herbivores, e.g. insects, during the growing period in spring. However, chemo-ecological investigations are warranted to corroborate this suspicion.

Determination of fatty acids and sterols

Saturated and unsaturated fatty acids are widespread constituents in the plant kingdom, where they are found mainly in esterified form. Phytosterols are also common lipid components. To determine the fatty acid and sterol composition of *M. perennis*, a hexane-soluble fraction obtained from dried aerial parts was saponified, acidified and the organic material extracted. Afterwards the resulting mixture of free fatty acids and sterols was derivatised by silylation followed by GC/MS analyses.

A total ion chromatogram (TIC) of such a silylated mixture is shown in Fig. 6A. In the retention range of 32–38 min, a variety of different fatty acid trimethylsilyl esters were detected while between 55 and 60 min different sterol trimethylsilyl ethers were found (see insert, Fig. 6A). The distribution of all silylated constituents were determined by measurement of the peak areas (see TIC fingerprint, Fig. 6B). α-Linolenic and linoleic acids were assessed as main constituents (47.1 and 12.5%). Also *cis*-oleic, palmitic and stearic acids (7.5, 7.1 and 3.7%, respectively) were detected, accompanied by low amounts of *trans*-oleic acid and *trans*-phytol (0.9 and 0.8%; see Fig. 6B).

Campesterol, stigmasterol, β-sitosterol and cycloartenol amounted to 2.1, 1.5, 14.2 and 2.4% of the total chromatogram peak area respectively. Whereas campesterol, stigmasterol and β-sitosterol were detected in the unsaponified matter (see TIC, Fig. 1), the cycloartenol was only observed after saponification (see insert Fig. 6A). This implies that cycloartenol mainly occurs in esterified form in the *M. perennis* lipids.

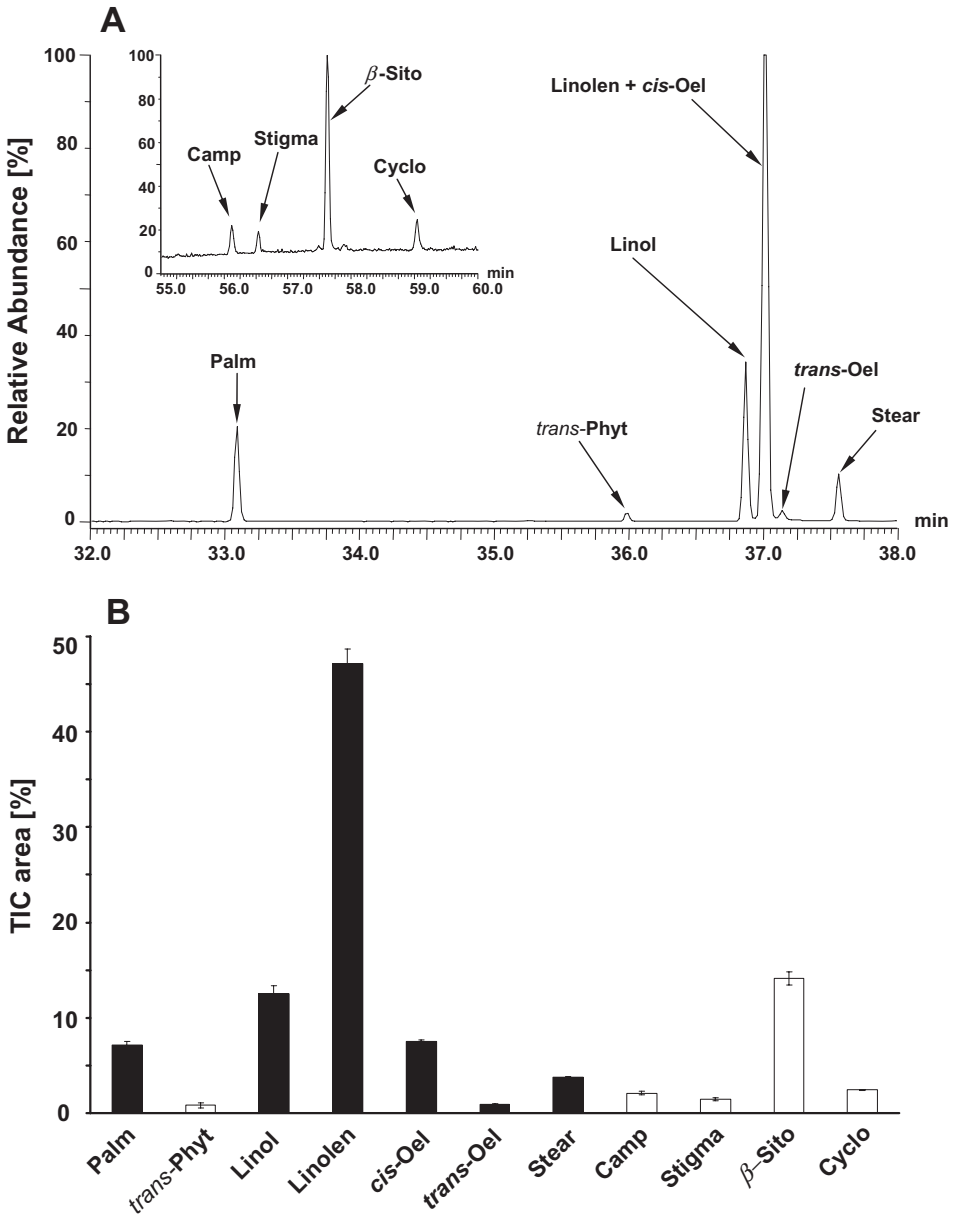


Figure 6. (A) Fatty acid and sterol distribution (TIC fingerprint) determined by GC/MS, after saponification and silylation of a hexane-extract obtained from dried aerial parts of *M. perennis*. In this TIC α-linolenic- and *cis*-oleic acid trimethylsilyl derivatives exhibit co-elution. The insert depict the sterol range section. (B) Distribution of chromatographic area percentages of the TIC (black bars = fatty acids; white bars = *trans*-phytol and sterols). Abbreviations: Camp, campesterol; Cyclo, cycloartenol; *cis*-Oel, *cis*-oleic acid (*cis*-C18:1); *trans*-Oel, *trans*-oleic acid (*trans*-C18:1); Palm, palmitic acid (*n*-C16); Linolen, α-linolenic acid (=all-*cis*-C18:3); Linol, linoleic acid (=all-*cis*-C18:2); *trans*-Phyt, *trans*-phytol; *β-Sito*, β-sitosterol; Stear, stearic acid (C18); Stigma, stigmasterol.

Characterisation of triacylglycerols, carotenoids and pheophytins by LC/MS

To characterise the non-volatile lipid constituents which were not detected by GC/MS techniques an unsaponified hexane extract from the aerial parts was analysed by LC/DAD, equipped with an APCI-source and a MS/MS-detector system. In the base peak chromatogram (BPC, Fig. 7A), a large number of triacylglycerols could be identified via their molecular ions and mass spectral patterns. A typical mass spectrum of a triacylglycerol (glycerol–linoleate–di-linolenate) recorded in the positive ion mode is shown in Fig. 7B. The protonated molecular ion $[M + H]^+$ and a water adduct signal $[M + H_2O]^+$ are the most prominent ions in the mass spectrum. Under the ionisation conditions adducts of several sodium and potassium ions with respective molecular ions such as $[M + K + Na]^+$ and $[M + H + 2K + Na]^+$ could also be observed and confirmed the identity of the detected triacylglycerols. By considering the fatty acids present (see above), some of the triacylglycerol structures were identified (Fig. 7A). The structure of glycerol–tri-linolenate was additionally

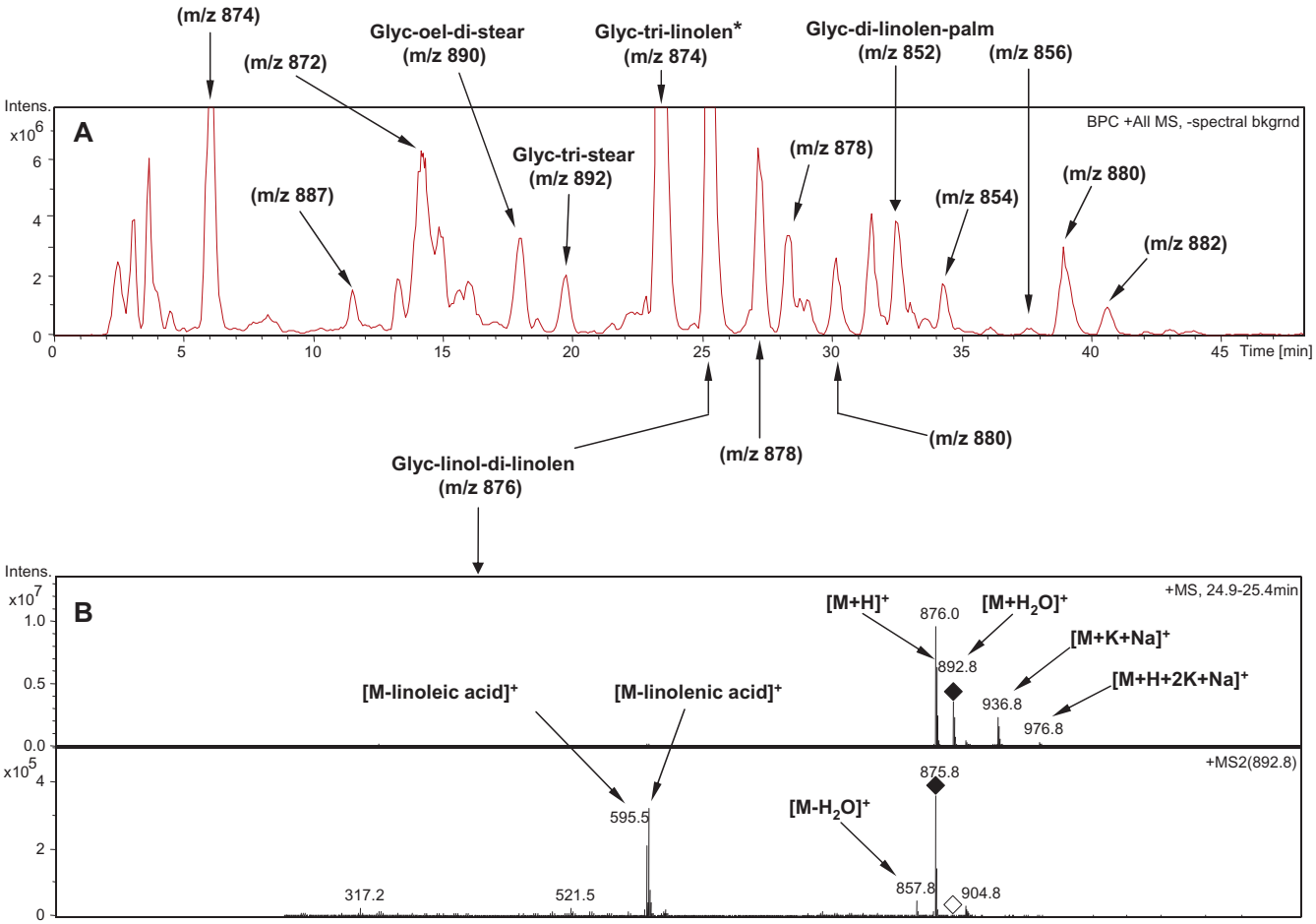


Figure 7. (A) Base peak chromatogram (LC/MS; *m/z* 50–1200) of a hexane extract obtained from aerial parts from *M. perennis* exhibit uniform and mixed triacylglycerols with linolenic (*linolen*), linoleic (*linol*), oleic (*oel*), stearic (*stear*) and palmitic acids (*palm*). Identified compounds are arranged with increasing retention time: Glyc-olea-di-stear = glyceryl monooleat distearate (M + H = 890), Glyc-tri-stear = glyceryl tristearate (M + H = 892), Glyc-tri-linolen = glyceryl trilinolenate (M + H = 874,* ascertained with a reference compound), Glyc-linol-di-linolen = glyceryl linoleate dilinolenate (M + H = 876), Glyc-di-linolen-palm = glyceryl dilinolenate palmitate (M + H = 852); the molecular ions of the peaks (M + H) 854, 856, 872, 874, 878, 880, 882 and 887 allow two or more possible triacylglycerol structures. (B) MS and MS^2 spectrum of an exemplary triacylglycerol: Glyc-linol-di-linolen = glyceryl linoleate dilinolenate ($C_{57}H_{94}O_6$, MW = 875.35).

proven via a commercially available reference compound. However, some of the mass spectra allow more than one possible triacylglycerol structure because of variable position of the fatty acids on the glycerol backbone, as shown by identical *m/z*-values (Fig. 7A).

Finally, carotenes and chlorophyll derivatives were identified via their characteristic UV absorption spectra (Fig. 8). At t_R 11.4 min a polar carotene with characteristic UV absorption at 270, 444 and 472 nm was detected (see insert Fig. 8, **Lut**). The UV absorption (Bauerfeind, 1981) together with the observed signal at 551.5 [M – H_2O+ H]$^+$ allowed identification of this compound as lutein. The structure was additionally proven by parallel measurement of a commercially available extract from marigold (*Tagetes erecta* L.) containing 10% lutein (data not shown). Furthermore, two β-carotenes ([M + H]$^+$ = 537.4) were detected at t_R 33.6 and 35.8 min. Their maxima at 450, 476 and 446, 472 nm allowed their identification as *trans*-β-carotene and 9-*cis*-β-carotene, respectively (Rodriguez-Amaya and Kimura, 2004). Moreover, at t_R 31.4 min pheophytin *a* was tentatively assigned based on the [M + H]$^+$ signal at *m/z* 871.7. Further support for the structure was obtained by comparison of its UV-spectrum (maxima at 410 and 666 nm, see insert Fig. 8, **Pheo a**) with literature data (Nakamura and Watanabe, 2001). In the retention time range between 22 and 31 min (Fig. 8) further carotenoids and another chlorophyll derivative were detected. However the low concentration of the analytes did not allow their closer characterisation.

In summary, the present study provides a comprehensive evaluation of the different spectrum of lipophilic constituents in aerial and root parts of *M. perennis*. In this course, several compounds not earlier found in this species were described for the first time. Investigations on polar compounds to complete the phytochemical spectrum of *M. perennis* are currently underway.

Acknowledgements

The authors wish to acknowledge Professor Otto Spring (Department of Botany, Hohenheim University, Stuttgart, Germany) for identification of the plant specimens.

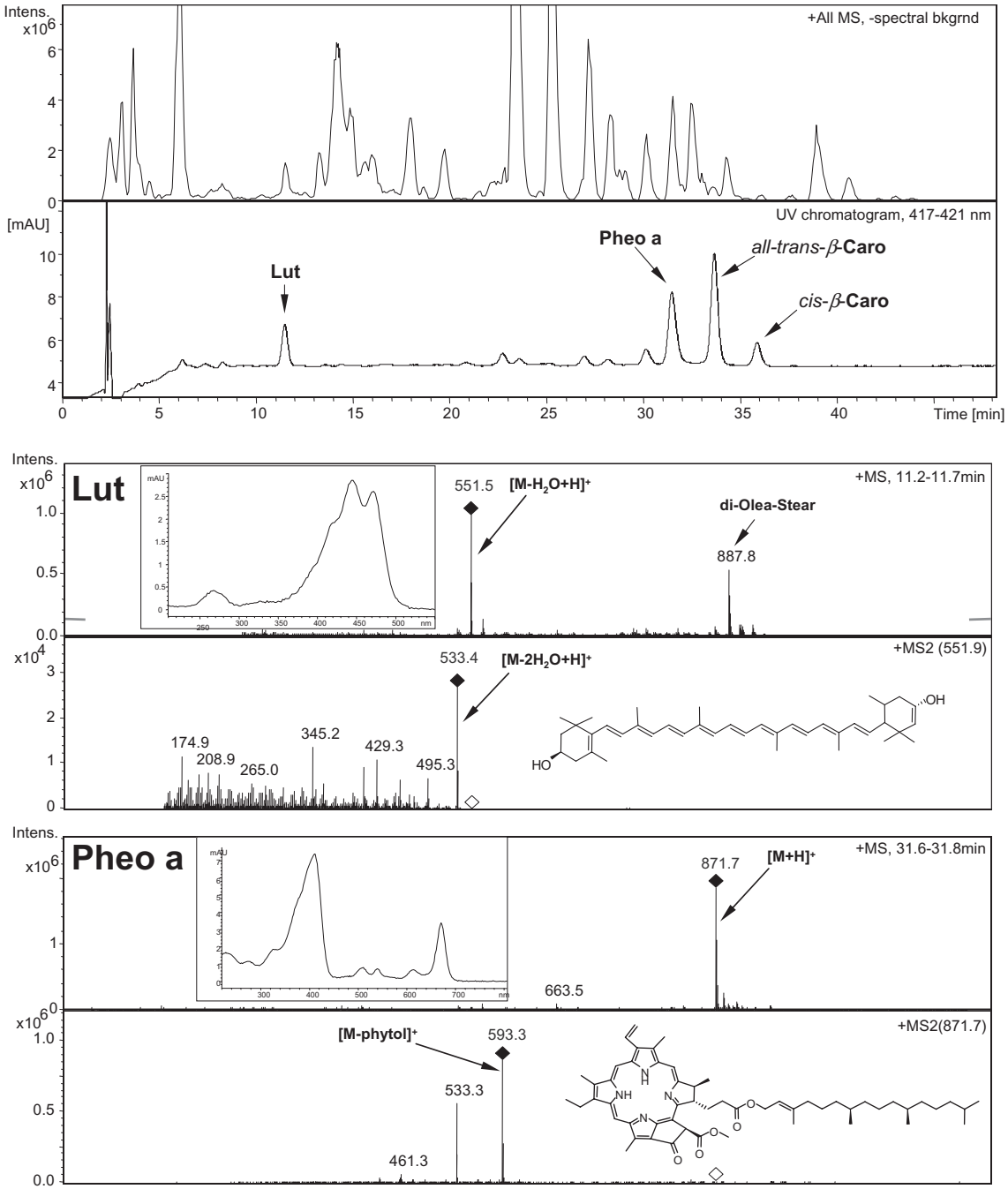


Figure 8. Base peak traces and UV traces (recorded at 419 nm) of a hexane extract obtained from dried aerial parts of *M. perennis*; MS^+ and MS^2 spectra of lutein (Lut, $C_{40}H_{56}O_2$, MW = 568.87) and pheophytin a (Pheo a, $C_{55}H_{74}N_4O_5$, MW = 871.20). The inserts show UV spectra (with UV maxima) of both compounds at the specified retention times in the LC.

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