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Non-volatile floral oils of *Diascia* spp. (Scrophulariaceae)

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Abstract

The floral oils of *Diascia purpurea*, *Diascia vigilis*, *Diascia cordata*, *Diascia megathura*, *Diascia integerrima* and *Diascia barberae* (Scrophulariaceae) were selectively collected from trichome elaiophores. The derivatized floral oils were analyzed by gas chromatography–mass spectrometry (GC–MS), whilst the underivatized samples were analysed by electrospray ionization mass spectrometry (ESI-MS) and Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS). The most common constituents of the floral oils investigated are partially acetylated acylglycerols of (3*R*)-acetoxy fatty acids (C_{14}, C_{16}, and C_{18}), as was proven with non-racemic synthetic reference samples. The importance of these oils for *Rediviva* bees is discussed in a co-evolutionary context.

Keywords: *Diascia*; Scrophulariaceae; Floral oils; Fatty acid profiling; Gas chromatography–mass spectrometry (GC–MS); Electrospray Fourier-transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS); Oxidized fatty acids-asymmetric derivatization

1. Introduction

Flowering plants have evolved a wide range of floral rewards to achieve pollination by insects. Nectar and/or pollen, containing carbohydrates, amino acids and proteins have long been recognized as the most common recompense for pollinating animals. Various specialized pollinators are, however, attracted to other substances, including feeding tissue, resins and gums, or floral scent compounds (Simpson and Neff, 1983). Non-volatile (fatty) oils are less well known floral rewards, despite of being significant for many plants and their pollinating bees, especially in the neotropics (Vogel, 1969; Simpson and Neff, 1981, 1983). Oil-collecting bees have special adaptations to gather the lipids, which are offered in so called elaiophores, e.g., in tube-like petal appendages. The bees use the lipid secretions (mostly instead of nectar) along with pollen as provision for their larvae and likely also for water-resistant cell lining of the soil nests (Simpson and Neff, 1981; Buchmann, 1987).

The main literature reports are focused on the floral morphology, ecology, taxonomy and evolution of several oil-secreting flowers (Vogel, 1974, 1986, 1990; Simpson et al., 1977, 1979; Seigler et al., 1978; Buchmann, 1987; Cane et al., 1983; Vinson et al., 1997; Seipold et al., 2004). The morphology and anatomy of elaiophores in the families Malpighiaceae, Krameriaceae, Scrophulariaceae, Iridaceae and Orchidaceae were initially described by Vogel (1974). He reported the diacylglycerol of acetic acid and β-acetoxy palmitic acid as a major component of *Calceolaria pavonii* (Scrophulariaceae) trichome exudates.

In most oil-secreting flowers studied, free β-acetoxy fatty acids, free fatty acids and unique acylglycerols containing

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β-acetoxy fatty acids were found as main lipid components (Vogel, 1969, 1974, 1986, 1990; Simpson et al., 1977, 1979; Seigler et al., 1978; Cane et al., 1983; Simpson and Neff, 1983; Buchmann, 1987; Vinson et al., 1997). The structure of a novel diacylglycerol of *Ornithophora radicans* was elucidated from spectroscopic data (Reis et al., 2000, 2003). Our group (Seipold et al., 2004) was first to report partially acetylated 3,7-dihydroxy fatty acids as a new type of constituent in *Malpighia coccigera* (Malpighiaceae) oil; and to discuss a possible relationship between floral oil and plant epicuticular wax biosynthesis. Recently, Reis et al. (2007) proved *R* configuration for both positions 3 and 7.

The genus *Diascia* comprises about 70 species, 20 of which are found in the eastern parts of Southern Africa (Fig. 1). Most of the *Diascia* spp., perennial or annual, are characterized by twin floral sacs (Steiner and Whitehead, 1988). The trichome elaiophores are located within the tips of paired spurs. The females of *Rediviva* bees have specially adapted elongated forelegs that allow only them to exploit the floral oils from the spurs of *Diascia* flowers. The bees transfer oil to their hind legs and then carry it to the nest, where it is used as larval food and presumably also for nest cell lining and construction (Steiner and Whitehead, 1990, 1991). The lipid-based association of *Diascia* and *Rediviva* has both historical and evolutionary implications beyond pollination ecology. Vogel (1974) assumed that *Rediviva* bees and *Diascia* hosts might have a co-evolutionary association. The morphological data strongly indicated that the *Rediviva* leg length is correlated to the length of the *Diascia* spurs (Steiner and Whitehead, 1990, 1991).

In the present study, we have extensively investigated the chemical composition of the floral oils of six *Diascia* spp. (Scrophulariaceae) by different mass spectrometric methods (GC/EI-MS, ESI-MS and ESI-FTICR-MS). The identification of acylglycerols of *Diascia* spp. oil is described in detail including the stereochemistry (see Fig. 2). The application of high resolution FTICR-MS of underivatized *Diascia* oils is presented. The co-evolutionary relation between *Diascia* flowers and *Rediviva* oil-collecting bees is discussed.

2. Results and discussion

2.1. FAME profiling of the Diascia species

Diascia oils from trichome elaiophores are yellowish by nature. Table 1 shows the fatty acid methyl ester (FAME) profiles of the six *Diascia* spp., and as an example, Fig. 3 illustrates the total ion chromatogram (TIC) of the FAME profiling of *Diascia vigilis*. Unbranched fatty acids and (3*R*)-hydroxy fatty acids with even-numbered chain length ranging from C_{14} to C_{18} represent the main compounds of the lipids. In all cases, there were no remainders of acylglycerols due to the complete transesterification reaction. The main compound of all derivatized *Diascia* oil samples was (3*R*)-hydroxypalmitic acid in a range of 55–75% (Table 1). Non-oxidized fatty acids could not be detected in *Diascia barberae* oil. EI mass spectra of trimethylsilyl (TMSi)-derivatives of 3-hydroxy fatty acid methyl esters exhibit a predominant peak at *m*/*z* 175 attributed to a cleavage at the C_3/C_4-linkage as well as a strong $[M-CH_3]^+$ ion (Mayberry, 1980; Mielniczuk et al., 1993). Experiments to determine the absolute configuration of the target compounds proved that the hydroxyl at C-3 shows (*R*)-configuration. Results are based on GC-comparison between the (2*S*)-phenylpropionyl derivative of a chiral standard and the samples (Hammarström, 1975; Weil et al., 2002).

2.2. EI-MS analysis of the acylglycerols of Diascia oils

A GC/EI-MS study of TMSi-derivatives of *Diascia* oils yielded diacylglycerols (DAGs) and monoacylglycerols (MAGs) along with small amounts of triacylglycerols (TAGs) as main constituents. According to the results obtained from the TMSi-derivatives, the detected acylglycerols of *Diascia* spp. contain one or two acetyl groups and an (3*R*)-acetoxy fatty acid attached to the glycerol backbone. Furthermore, the ESI-FTICR-MS profiling analysis of underivatized *Diascia* oils confirmed the (3*R*)-acetoxy fatty acids as long-chain moiety of the acylglycerols (see below). The acetylation of the 3-hydroxy acids may be related to the export of the floral oils out of the

Table 1
FAME profiling of TMSi-derivatives of *Diascia* spp.[a]

Number	Compound	t_R (min)	Relative composition (%)						t_R[b] (min)
			D. purpurea	*D. vigilis*	*D. cordata*	*D. megathura*	*D. integerrima*	*D. barberae*[b]	
1	Myristic acid (C14:0)	13.24	1.3	1.1	0.3	1.7	0.9	–	–
2	(3*R*)-Hydroxymyristic acid (C14:0)	14.12	0.2	6.6	24.5	3.7	9.2	3.1	13.63
3	Palmitic acid (C16:0)	15.69	6.7	5.0	2.0	11.3	8.4	–	–
4	(3*R*)-Hydroxypalmitic acid (C16:0)	16.71	56.5	72.1	65.0	55.8	61.1	75.6	16.07
5	Stearic acid (C18:0)	18.48	5.3	3.4	1.4	7.8	6.4	–	–
6	(3*R*)-Hydroxystearic acid (C18:0)	19.54	30.1	11.9	6.8	19.7	14	18.0	18.77

[a] GC/EI-MS analysis obtained from TMSi-derivatives of *Diascia* spp.
[b] *D. barberae* floral oil was measured on an Agilent 6890 series gas chromatograph equipped with a Micromass, GCT mass-sensitive reflectron time-of-flight (TOF) detector (DB-5 MS, 30 m × 0.25 mm i.d., 0.25 μm film thickness). Non-oxidized fatty acids were not detected.

cells. It has been reported that a hydroxy group in fatty acids reduces the lipid transporter affinity compared to unfunctionalized fatty acids (Zachowski et al., 1998). Therefore, the acetylation could be crucial for an improved transport (Seipold et al., 2004).

Fig. 4 shows the total ion chromatogram of the TMSi-derivatives of *D. vigilis*. The mass spectral data of the identified compounds are presented in Table 2. The two main components of *D. vigilis* were 2-[(3*R*)-acetoxypalmitoyl]glycerol (**14**) and 2-[(3*R*)-acetoxypalmitoyl]-1-acetylglycerol (**16**, Table 3).

Our results prove that in case of 3-acetoxy fatty acids the 1-monoacyl and 2-monoacyl glycerols (MAGs) can be distinguished by the EI-MS data of their bis-TMSi-derivatives. Fig. 5 shows a comparison of the EI mass spectra of the 1-monoacyl and 2-monoacyl isomer of (3*R*)-acetoxypalmitoylglycerol. The molecular weight of MAGs of (3*R*)-acetoxy fatty acids in *Diascia* spp. can be determined by the appearance of significant ions of type **a**, $[M-Me-HOAc]^+$ (Seipold, 2004). In case of the MAGs, the fragment $[M-CH_3]^+$, formed by loss of a methyl radical from the trimethylsilyl group, represents the peak of highest mass (Curstedt, 1974; Wood, 1980). Scheme 1 shows the characteristic fragmentation of bis-TMSi-derivatives of 2-[(3*R*)-acetoxypalmitoyl]glycerol (**14**) and 1-[(3*R*)-acetoxypalmitoyl]glycerol (**15**). An important key fragment of the 2-MAG isomer is the ion of type **e** at *m*/*z* 218 (compounds **7**, **14** and **20**), while the ion of the type (**b**−HOAc) is a significant ion for 1-MAGs (compounds **8**, **15** and **21**). As suggested by Johnson and Holman (1966) the 2-MAGs display a significant signal at *m*/*z* 218 which is formed by loss of the 3-acetoxy fatty acid from $[M]^+$. Rearrangement of a TMSi group from the acylglycerol backbone to the carboxy group of the fatty acids leads to a **c**-type ion at *m*/*z* 311 (after loss of a HOAc unit). The ion of type (**d**-HOAc) corresponds to the acylium ion after loss of the 3-acetoxy group. The fragment ion at *m*/*z* 203 (**e**−Me) is produced by both 1- and 2-monoacyl isomers. The most significant evidence of the 1-MAGs of (3*R*)-acetoxy fatty acid is the formation of an ion at *m*/*z* 369 (**b**−HOAc) as

Table 2
GC/EI-MS data of the identified compounds from *Diascia* spp.[a] (as TMSi-derivatives)

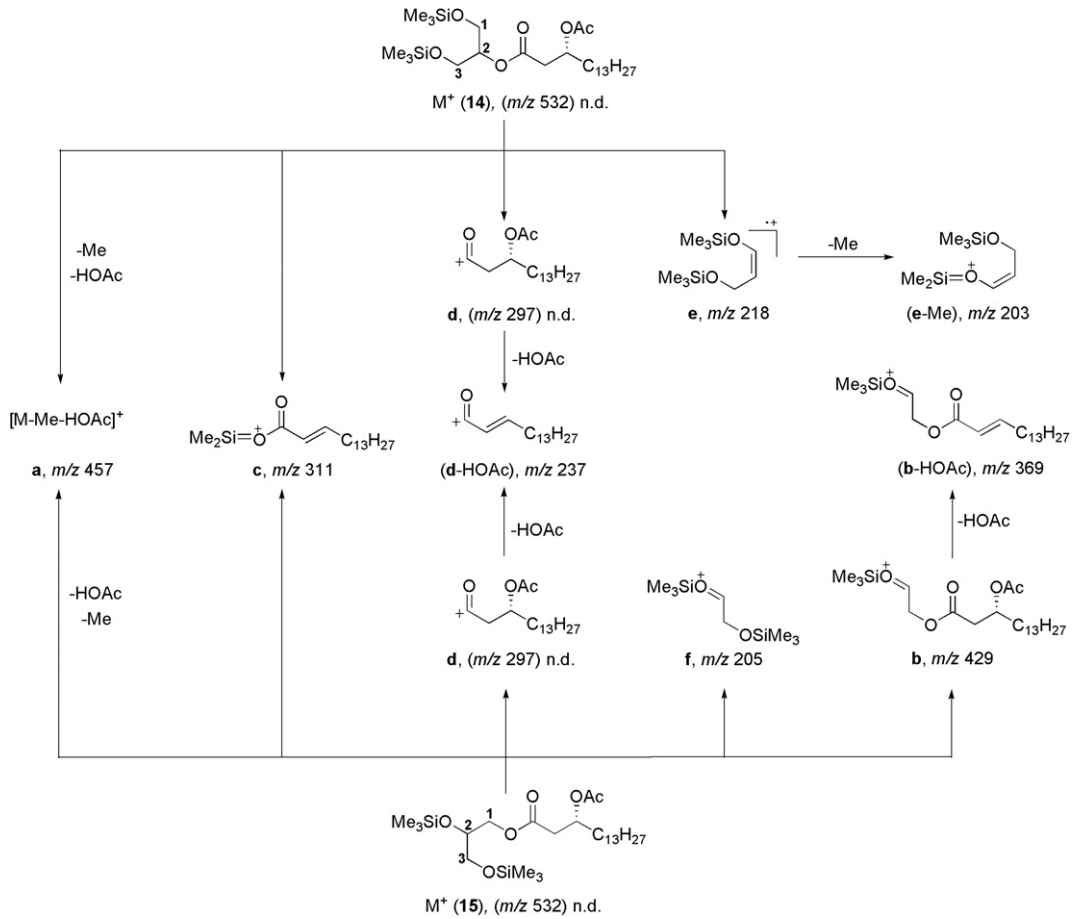
Number	t_R (min)	Compound	Characteristic fragmentation ions (*m*/*z* (relative intensity, %))
7	22.96	2-[(3*R*)-Acetoxymyristoyl]glycerol	504 (M^+, −), 429 (**a**, 15), 373 (9), 283 (**c**, 35), 209 (**d**-HOAc, 60), 218 (**e**, 85), 203 (**e**-Me, 39), 147 (66), 129 (100), 73 (55)
8	23.41	1-[(3*R*)-Acetoxymyristoyl]glycerol	504 (M^+, −), 429 (**a**, 10), 401 (**b**, 2), 341 (**b**-HOAc, 88), 283 (**c**, 5), 209 (**d**-HOAc, 58), 205 (**f**, 15), 203 (**e**-Me, 22), 147 (56), 129 (28), 73 (100)
9	23.93	2-[(3*R*)-Acetoxymyristoyl]-1-acetylglycerol	474 (M^+, −), 399 (**a**, 12), 283 (**c**, 48), 209 (**d**-HOAc, 95), 189 (**g**, 50), 188 (**e**, 23), 146 (10), 145 (**k**, 72), 129 (79), 117 (96), 73 (75), 43 (100)
10	24.22	1-[(3*R*)-Acetoxymyristoyl]-3-acetylglycerol	474 (M^+, −), 399 (**a**, 14), 341 (**b**-HOAc, 27), 283 (**c**, 5), 209 (**d**-HOAc, 44), 189 (**g**, 30), 188 (e_1, 3), 175 (**h**, 100), 146 (e_1-CH_2CO, 12), 117 (33), 43 (82)
11	24.64	2-[(3*R*)-Acetoxymyristoyl]-1,3-diacetylglycerol	444 (M^+, −), 324 (2), 269 (**d**, 5), 209 (**d**-HOAc, 48), 159 (88), 117 (11), 43 (100)
12	24.79	Not identified [isomer of **16**: an (acetoxypalmitoyl)-acetylglycerol?]	502 (M^+, −), 427 (**a**, 6), 311 (**c**, 56), 237 (**d**-HOAc, 72), 189 (**g**, 7), 188 (**e**, 31), 146 (12), 145 (**k**, 100), 129 (55), 117 (72), 73 (77), 43 (68)
13	25.18	Not identified [isomer of **17**: an (acetoxypalmitoyl)-acetylglycerol?]	502 (M^+, −), 427 (**a**, 5), 369 (**b**-HOAc, 10), 311 (**c**, 2), 237 (**d**-HOAc, 24), 189 (**g**, 2), 188 (e_1, 2), 175 (**h**, 100), 146 (e_1-CH_2CO, 12), 117 (20)
14	25.67	2-[(3*R*)-Acetoxypalmitoyl]glycerol	532 (M^+, −), 457 (**a**, 5), 401 (4), 311 (**c**, 15), 237 (**d**-HOAc, 36), 218 (**e**, 60), 203 (**e**-Me, 24), 147 (55), 129 (100), 73 (84)
15	26.14	1-[(3*R*)-Acetoxypalmitoyl]glycerol	532 (M^+, −),457 (**a**, 6), 429 (**b**, 2), 369 (**b**-HOAc, 92), 311 (**c**, 4), 237 (**d**-HOAc, 48), 205 (**f**, 17), 203 (**e**-Me, 24), 147 (60), 129 (32), 73 (100)
16	26.66	2-[(3*R*)-Acetoxypalmitoyl]-1-acetylglycerol	502 (M^+, −), 427 (**a**, 10), 311 (**c**, 43), 237 (**d**-HOAc, 83), 189 (**g**, 66), 188 (**e**, 26), 146 (9), 145 (**k**, 74), 129 (96), 117 (80), 73 (77), 43 (100)
17	26.96	1-[(3*R*)-Acetoxypalmitoyl]-3-acetylglycerol	502 (M^+, −), 427 (**a**, 12), 369 (**b**-HOAc, 22), 311 (**c**, 5), 237 (**d**-HOAc, 33), 189 (**g**, 40), 188 (e_1, 2), 175 (**h**, 100), 146 (e_1-CH_2CO, 11), 117 (44), 43 (91)
18	27.39	2-[(3*R*)-Acetoxypalmitoyl]-1,3-diacetylglycerol	472 (M^+, −), 352 (1), 297 (**d**, 4), 237 (**d**-HOAc, 37), 159 (96), 117 (10), 43 (100)
19	27.92	Not identified [isomer of **23**: an (acetoxystearoyl)-acetylglycerol?]	530 (M^+, −), 455 (**a**, 5), 397 (**b**-HOAc, 9), 339 (**c**, 1), 265 (**d**-HOAc, 13), 189 (**g**, 2), 188 (e_1, 1), 175 (**h**, 100), 146 (e_1-CH_2CO, 10), 117 (14)
20	28.28	2-[(3*R*)-Acetoxystearoyl]glycerol	560 (M^+, −), 485 (**a**, 4), 429 (3), 339 (**c**, 13), 265 (**d**-HOAc, 28), 218 (**e**, 60), 203 (**e**-Me, 23), 147 (54), 129 (100), 73 (85)
21	28.73	1-[(3*R*)-Acetoxystearoyl]glycerol	560 (M^+, −), 485 (**a**, 5), 457 (**b**, 1), 397 (**b**-HOAc, 60), 339 (**c**, 5), 265 (**d**-HOAc, 40), 205 (**f**, 15), 203 (**e**-Me, 19), 147 (48), 129 (40), 73 (100)
22	29.27	2-[(3*R*)-Acetoxystearoyl]-1-acetylglycerol	530 (M^+, −), 455 (**a**, 8), 339 (**c**, 34), 265 (**d**-HOAc, 60), 189 (**g**, 67), 188 (**e**, 25), 146 (9), 145 (**h**, 70), 129 (98), 117 (88), 73 (95), 43 (100)
23	29.56	1-[(3*R*)-Acetoxystearoyl]-3-acetylglycerol	530 (M^+, −), 455 (**a**, **7**), 397 (**b**-HOAc, 17), 339 (**c**, 2), 265 (**d**-HOAc, 21), 189 (**g**, 28), 188 (e_1, 2), 175 (**h**, 100), 146 (e_1-CH_2CO, 12), 117 (44), 43 (89)
24	30.00	2-[(3*R*)-Acetoxystearoyl]-1,3-diacetylglycerol	500 (M^+, −), 380 (−), 325 (**d**, 4), 265 (**d**-HOAc, 27), 159 (88), 117 (20), 43 (100)

[a] The retention time and relative abundance data were obtained from the mass spectrum of TMSi-derivatives of *D. vigilis*.

Table 3
Relative composition of the acylglycerols in *Diascia* spp. (obtained from the GC/EI-MS data of their TMSi-derivatives)

Number	t_R (min)	Relative composition (%)						t_R (min)[a]
		D. purpurea	*D. vigilis*	*D. cordata*	*D. megathura*	*D. integerrima*	*D. barberae*[a]	
7	22.96	1.2	1.3	1.7	–	1.7	–	–
8	23.41	0.3	0.2	9.5	0.8	–	–	–
9	23.93	0.3	5.8	1.2	1.6	3.1	1.4	23.00
10	24.22	0.1	0.2	–	4.7	–	–	–
11	24.64	0.9	0.6	–	0.2	6.6	0.9	23.70
12[b]	24.79	17.4	2.5	–	–	–	–	–
13[b]	25.18	6.5	1.1	39.8	–	–	–	–
14	25.67	16.2	35.6	8.7	13.6	40.2	8.0	24.70
15	26.14	9.2	3.6	4.2	12.5	5.6	0.5	25.15
16	26.66	17.6	35.7	25.8	24.9	17.9	80.6	25.72
17	26.96	6.8	2.0	1.5	19.6	1.7	0.3	25.98
18	27.39	3.2	0.1	–	5.5	9.2	1.4	26.40
19[b]	27.92	2.0	0.3	–	–	–	–	–
20	28.28	8.2	4.8	0.3	2.1	8.9	0.6	27.27
21	28.73	5.8	1.2	9.1	4.0	2.8	–	–
22	29.27	2.5	4.4	–	5.0	2.4	6.3	28.23
23	29.56	1.8	0.4	–	5.0	0.1	–	–
24	30.00	–	0.1	–	0.6	0.4	0.1	28.97

[a] *D. barberae* floral oil was measured on an Agilent 6890 series gas chromatograph equipped with a Micromass, GCT mass-sensitive reflectron time-of-flight (TOF) detector (DB-5, 30 m × 0.25 mm, i.d., 0.25 μm film thickness).
[b] Unidentified.



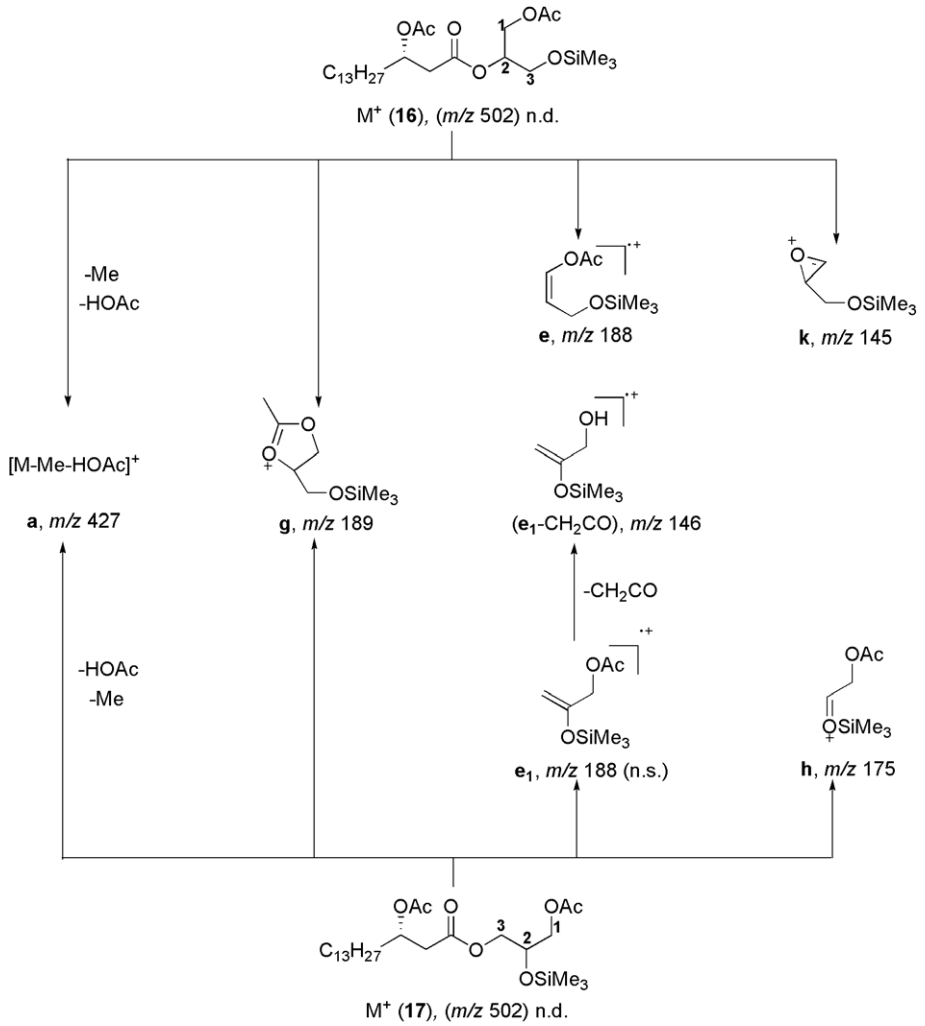
Scheme 1. Mass spectral fragmentation of TMSi-derivatives of the acylglycerols 2-[(3*R*)-acetoxypalmitoyl]glycerol (**14**) and 1-[(3*R*)-acetoxypalmitoyl]glycerol (**15**) (n.d. = not detected).

an unique peak, including the **f**-type ion, *m*/*z* 205. In most cases of 1-MAGs, the fragment of type **b** ($[M-103]^+$) corresponding to the loss of $CH_2OSi(CH_3)_3$ forms the base peak (Fig. 5) (Johnson and Holman, 1966; Curstedt, 1974; Myher et al., 1974; Wood, 1980).

Fig. 6 demonstrates the comparison of the EI mass spectra of TMSi-derivatives of the 1,2- and 1,3-diacylglycerols (DAGs) of (3*R*)-acetoxypalmitic acid and an acetic acid moiety, also displaying significant differences. Generally, the mass spectral behaviour of DAGs containing (3*R*)-acetoxy fatty acids and an acetyl moiety is similar to that of the MAGs. A signal at *m*/*z* 189 (**g**) in both the TMSi-derivatives of 1,2- and 1,3-DAGs can be explained as a cyclic structure (Curstedt, 1974) as shown in Scheme 2. The TMSi-derivatives of 1,2-diacylglycerols (compounds **9**, **16** and **22**) show an analogous signal at *m*/*z* 188 (type **e**) with moderate intensity (Table 2). On the other hand, $\mathbf{e}_1$ (*m*/*z* 188), in 1,3-DAGs is of low abundance. This ion can be a first hint indicating that the (3*R*)-acetoxy fatty acid is attached to the secondary hydroxy group of the glycerol backbone. A further key ion confirming the 1,2-DAG structure is **k** at *m*/*z* 145 (Curstedt, 1974) which is not observed in the TMSi-derivatives of 1,3-diacylglycerols (compounds **10**, **17** and **23**). It should be pointed out that the signals at *m*/*z* 175 (type **h**) and *m*/*z* 146 ($\mathbf{e}_1$-CH_2CO) are only present in the mass spectra of TMSi-derivatives of 1,3-diacylglycerols (Scheme 2 and Table 2; Seipold, 2004).

The TAGs of *D. vigilis* flowers (compounds **11**, **18** and **24**) consist of one (3*R*)-acetoxy fatty acid (C_{14}, C_{16}, or C_{18}) at C-2 and two acetyl moieties at C-1 and C-3 of the glycerol backbone. They were detected in amounts lower than 1% (Table 2). Some of these compounds were also reported from *Oncidium* oil (Reis et al., 2000). EI mass spectra of 1-acyl-2,3-diacetyl-glycerols were also previously described (Reis et al., 2003).

The unidentified compounds **12**, **13**, and **19** appear to be isomers of 2-[(3*R*)-acetoxypalmitoyl]-1-acetylglycerol (**16**), 3-[(3*R*)-acetoxypalmitoyl]-1-acetylglycerol (**17**) and 3-[(3*R*)-acetoxystearoyl]-1-acetylglycerol (**23**), respectively (Table 2), but no unequivocal assignment is possible due to the limitations of the MS-techniques with the amounts available.



Scheme 2. Mass spectral fragmentation of TMSi-derivatives of the acylglycerols 2-[(3*R*)-acetoxypalmitoyl]-1-acetylglycerol (**16**) and 1-[(3*R*)-acetoxypalmitoyl]-3-acetylglycerol (**17**) (n.s. = not significant; n.d. = not detected).

Table 4
Positive-ion ESI-FTICR mass spectral data of the floral oils of the *Diascia* spp.

Number	Elemental composition	*m/z* ($[M+Na]^+$)[a]	Error[a] (ppm)	M_r	Relative abundance (%)				
					D. purpurea	*D. vigilis*	*D. cordata*	*D. megathura*	*D. integerrima*
7, **8**	$C_{19}H_{36}O_6\ Na^+$	383.29800	+0.2	360	1.3	0.3	2.5	1.8	2.9
9, **10**	$C_{21}H_{38}O_7\ Na^+$	425.25130	+0.8	402	4.8	10.5	38.3	38.9	45.9
11	$C_{23}H_{40}O_8\ Na^+$	467.26179	+0.8	444	7.0	4.0	22.7	25.4	22.1
12, **13**, **16**, **17**	$C_{23}H_{42}O_7\ Na^+$	453.28256	+0.8	430	100	100	100	100	100
14, **15**	$C_{21}H_{40}O_6\ Na^+$	411.27216	+1.1	388	28.2	8.4	16.0	12.0	16.1
18	$C_{25}H_{44}O_8\ Na^+$	495.29381	+0.7	472	38.9	3.5	8.6	10.1	6.8
19, **22**, **23**	$C_{25}H_{46}O_7\ Na^+$	481.31404	+1.4	458	62.7	28.0	15.6	16.5	13.5
20, **21**	$C_{23}H_{44}O_6\ Na^+$	439.30342	+1.0	416	17.9	2.6	2.2	2.2	2.2
24	$C_{27}H_{48}O_8\ Na^+$	523.32383	−0.6	500	13.8	–	–	1.4	–

[a] The exemplary *m/z* ($[M+Na]^+$) and error (ppm) data were obtained from *D. integerrima* (exception: compound 24 from *D. purpurea*).

An isomerization via an acyl-migration might occur during the storage or during the analytical procedure (Lyubachevskaya and Boyle-Roden, 2000; Seipold, 2004; Christie, 2006). Therefore, compounds **12**, **13**, and **19** also may be artefacts.

Table 3 shows the relative composition of acylglycerols of the *Diascia* flower oils investigated. In most of the cases, the six *Diascia* spp. exhibited a similar pattern with respect to their MAG, DAG and TAG distribution. Exceptionally, in *Diascia cordata* no TAG could be detected.

By TMSi-derivatization of the floral oils of *Diascia* spp., fatty acids could not be detected. DAGs were the most abundant class (60–80%) along with MAGs (20–30%) and TAGs (<15%). MAGs and DAGs as well as a small amount of TAGs were also described as the main oil components in *Byrsonima crassifolia* (Malpighiaceae) elaiophores (Vinson et al., 1997). The dominance of MAGs and DAGs could be related to the insects' digestive system. It could be shown that both MAGs and DAGs are better digestible than TAGs (Vinson et al., 1997).

2.3. Analysis of acylglycerols of underivatized Diascia oils

The underivatized floral oils of the *Diascia* spp. were investigated by ESI-FTICR-MS to obtain high resolution mass data of the lipid compounds allowing a rapid profiling of the various oils. All measurements were performed in the positive-ion mode. In these cases the electrospray mass spectra of the investigated oils display sodium adducts ($[M+Na]^+$) of the corresponding compounds (Table 4). The positive ESI-FTICR mass spectrum of *Diascia integerrima* displays a DAG signal (base peak) comprising compounds **12**, **13**, **16** and **17** (*m/z* 453.28256) (Fig. 7). It should be noted that the ESI-FTICR-MS of *D. cordata* indicates the presence of TAGs with (3*R*)-acetoxy fatty acids with chain lengths of C_{14} and C_{16}, whereas the EI-MS data yield no evidence of such TAGs. However, all the significant acylglycerol components could be detected with both methods.

The underivatized *D. barberae* oil was investigated by positive ESI-MS experiments using ammonium acetate (Fig. 8). In that case, four types of adduct ions were detected, namely $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$. The ion type $[M+NH_4]^+$ represented the predominant adduct. The DAG of (3*R*)-acetoxypalmitic acid represents the compound with the highest abundance as shown by ions at *m/z* 448 $[M+NH_4]^+$ and *m/z* 453 $[M+Na]^+$. The results indicated the DAGs and MAGs as the predominant constituents along with a small amount of TAGs and were in good agreement with EI-MS experiments.

In conclusion, positive-ion ESI-FTICR mass spectra of the non-derivatized floral oils of *Diascia* spp. exposed essential results with respect to the acylglycerol profile. The determination of the elemental composition can yield a faster and simple look at the lipid pattern of the oil-secreting *Diascia* flowers. With respect to the relative composition of the floral oils the most prominent components are shown both in the FTICR-MS or ESI-MS and the GC–MS data (Tables 3 and 4).

3. Conclusions

Floral oils represent the vital floral reward of *Diascia* species. The variation in foreleg lengths of *Rediviva* could be explained as an evolutionary response to *Diascia* floral spur lengths (Steiner and Whitehead, 1990, 1991). The floral oils play an important role in larval provision and have also been suggested to be used in nest construction (Cane et al., 1983; Buchmann, 1987). Some hydroxylated fatty acids are reported to possess antibiotic properties (Valcavi et al., 1989; Weil et al., 2002). The prevalence of such chemical species in the *Diascia* flower oils instead of simple fatty acid oils – may be necessary to keep the larval food free of microbial decomposition, or for the nest cell lining. The additional acetylation may either be required by the plant for excretion (Seipold et al., 2004) or to reduce the oils water content, thereby avoiding, e.g., fungal decomposition. Also, there is no detailed report on the chemistry of *Rediviva* bee nest cell lining. Therefore, some further investigations have to be carried out to verify the chemical nature of the association between *Diascia* flower oil and *Rediviva* bee cell lining. Future questions concern the

natural variation of flower oil compositions within a species or with flower age, the absolute configuration of glycerols with a chiral center at the *sn*-2 position, and if stereochemistry has any relevance in the biological context.

4. Experimental

4.1. Oil-secreting flowers

Different specimens of *Diascia purpurea*, *D. vigilis*, *D. cordata*, *Diascia megathura*, *D. integerrima* (Scrophulariacece) were collected in the Drakensberg area of Southern Africa. *D. barberae* was cultivated in a green house of the Botanical Garden Munich (Germany). All samples were collected during the blooming stage between January and February 2006. Samples of each species were collected at single locations from 1 to 4 individuals with 2–10 flowers each, except for *D. purpurea*, of which two collections (S.D., Bayreuth: SA3, SA9) were made. In order to gather enough material for this first survey, all samples of a species were united, thereby averaging the composition.

4.2. Gathering of floral oils

Most *Diascia* species have twin spurs containing oil-secreting glands located within their tip. Fig. 1 shows *D. megathura* (Scrophulariaceae) as an example. Fresh flowers were cut and floral oils were collected from the trichome elaiophores in the field. Tiny pieces of filter papers were carefully manipulated to adsorb the oil from the inside of the twin spurs. The amounts of the lipids per flower varied from 1 to 5 μl. All samples were dissolved into 450 μl of *t*-butylmethylether (MTBE)–MeOH (2:1, v/v) and stored under N_2 at −18 °C (Seipold, 2004; Seipold et al., 2004; Neff and Simpson, 2005).

4.3. Derivatization of floral oils

Fatty acid methyl ester (FAME) profiling was carried out according to the previously described procedure by using the transesterification followed by trimethylsilylation (Seipold et al., 2004). Both the crude *Diascia* oils and the methanol transesterified oils were then converted to the trimethylsilyl (TMSi)-derivatives by using 2,2,2-trifluoro-*N*-methyl-*N*-(trimethylsilyl) acetamide (MSTFA) as reagent (2 h at room temperature, see Morrison and Smith, 1964; Seipold, 2004; Seipold et al., 2004). Diluted derivatized samples were analyzed by GC–MS as described below.

4.4. Synthesis of (3*R*)-hydroxypalmitic acid methyl ester (Fig. 2)

In a 50 ml two neck-flask, equipped with a drop funnel with pressure balance and reflux cooler, 1.44 g calcium chloride (144.13 g/mol, 10 mmol) were added to 1.38 g Meldrum's acid (0.983 g/ml, 79.10 g/mol, 17.4 mmol) in 12 ml CH_2Cl_2 and 1.4 ml pyridine at 0 °C. 2.9 ml or 2.64 g myristic acid chloride (246.82 g/mol, 0.91 g/ml, 10.7 mmol) were added dropwise. The solution was stirred for 1 h at 0 °C and additional 4 h at room temperature. The

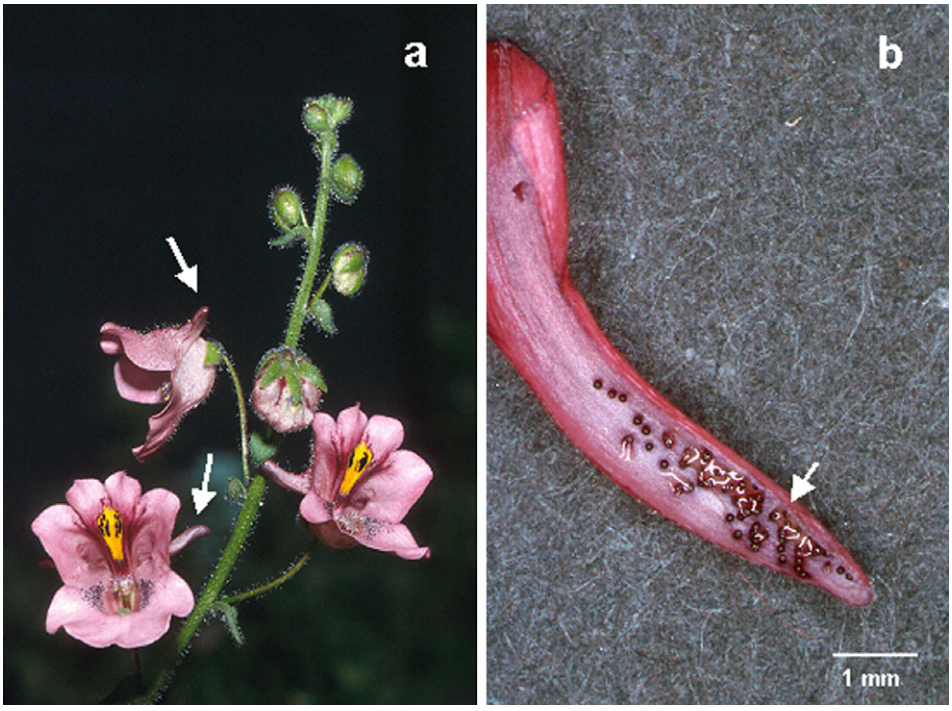


Fig. 1. *Diascia megathura* (Scrophulariaceae): (a) inflorescence showing spurs and (b) spur longitudinally split, showing the elaiophores with free oil. Arrows show the spurs (Photos: G. Gerlach).

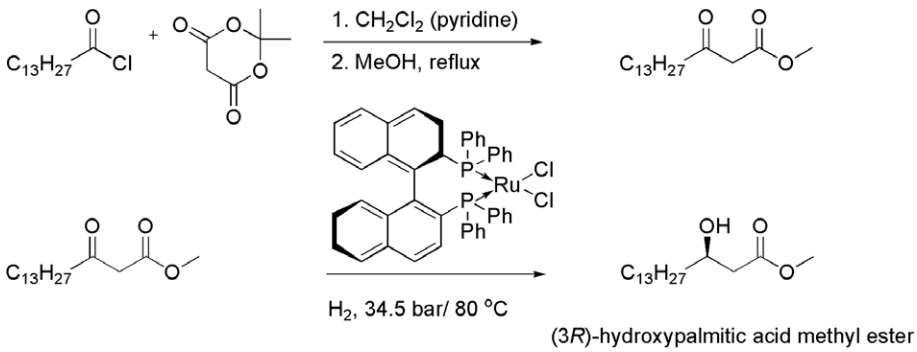


Fig. 2. Synthetic route to (3*R*)-hydroxypalmitic acid methyl ester.

orange solution was washed twice with 2 N HCl and then with 5% aq. $NaHCO_3$ solution. The upper layer was separated and the solvent evaporated under vacuum. The orange residue was dissolved in 25 ml MeOH and refluxed for 3 h. The crude 3-ketopalmitic acid methyl ester was purified by chromatography on silica gel 60 (particle size 0.040–0.063 mm (230–400 mesh), Merck, Darmstadt, Germany) (hexane–EtOAc (6:1, v/v)) to give an overall yield of 61% (1.61 g) (Valcavi et al., 1989; Oikawa et al., 1978; Seipold, 2004). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0. 88 (3H, *t*, $J = 6.8$ Hz), 1.23–1.30 (22H, *m*), 1.61 (2H, *m*), 2.53 (2H, *t*, $J = 7.31$ Hz), 3.45 (2H, *s*); 3.74 (3H, *s*) (Seipold, 2004).

Five hundred milligrams of 3-ketopalmitic acid methyl ester (284.44 g/mol, 1.76 mmol) in 15 ml of $MeOH/CH_2Cl_2$ (96:4 v/v) was degassed by triple freezing and thawing under vacuum. Under argon, 10 mg of dichloro[(*R*)(+)2,2′bis(diphenylphosphino)1,1′binaphthyl]-ruthenium(II) ($[RuCl_2(C_{44}H_{32}P_2)]_x$, 794.67 g/mol, 0.013 mmol, Strem Chemicals, USA) was added. The reduction was carried out in a pressure reactor under a hydrogen atmosphere of 34.5 bar (500 psi) for 24 h at 80 °C (Heiser et al., 1991). The solvent was evaporated under vacuum. The residue was dissolved in a mixture of benzene–EtOAc (4:1, v/v) and filtered through 2 g silica gel to eliminate the catalyst (Heiser et al., 1991). (3*R*)-Hydroxypalmitic acid methyl ester was obtained in a purity >99% (GC). The optical rotation was −14.2° at 24 °C (λ 589 nm) (literature −14.3°, Tulloch and Spencer, 1964). The (2*S*)-phenylpropionate derivative of (3*R*)-hydroxypalmitic acid methyl ester revealed in 93% ee (GC).

(3*R*)-Hydroxypalmitic acid methyl ester was obtained in 97% yield (m.p. 82 °C, literature: 83–85 °C, Valcavi et al., 1989). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, *t*, $J = 6.9$ Hz), 1.23–1.30 (22H, *m*), 1.44 (2H, *m*), 2.41 (1H, *dd*, $J_1 = 16.5$ Hz, $J_2 = 8.8$ Hz), 2.52 (1H, *dd*, $J_1 = 16.5$ Hz, $J_2 = 3.3$ Hz), 3.71 (3H, *s*), 4.0 (1H, *m*) (Seipold, 2004). The purity (%) and ee (%) measurements were obtained with a GC 8000 series (Fisons Instruments) gas chromatograph with MS-detector (DB-5 MS, 20 m × 0.18 mm, i.d., 0.18 μm film thickness) (J&W Scientific, Folsom, CA, USA). The column temperature was programmed for 1 min at 60 °C, increased 15 °C/min from 60 to 200 °C, and 5 °C/min to 300 °C and completed at 300 °C for 20 min. The mass spectrometer was operated with an electron impact (EI) 70 eV, ion source temperature 180 °C and mass range 40–800 a.m.u.

4.5. Synthesis of (2*S*)-phenylpropionyl chloride

Ninety milligrams (+)-(2*S*)-phenylpropionic acid (164.08 g/mol, 0.55 mmol) and 120 μl thionyl chloride were mixed at 0 °C and kept at 70 °C for 30 min. Dried benzene was added, and the mixture was evaporated to dryness. This step was repeated to remove traces of thionyl chloride. The residue was dissolved in 1 ml dry benzene and kept at 4 °C in a sealed flask (Hammarström, 1975; Seipold, 2004).

4.6. Determination of the absolute configuration

The procedures were carried out using GC–MS techniques, due to the limited amount of floral oil samples. A

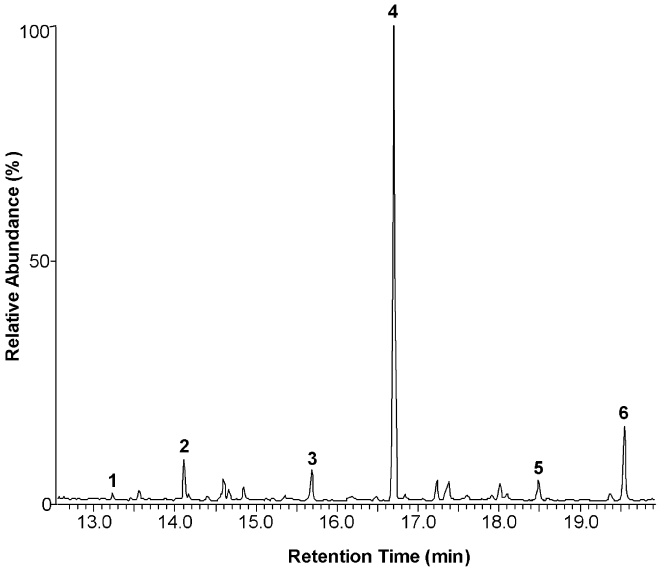


Fig. 3. Partial total ion chromatogram (TIC) of the FAME profiling of *D. vigilis* oil. (For the identified compounds of interest see Table 1; the non-labeled peaks correspond to fatty acid TMSi-derivatives, saturated hydrocarbons, artefacts, and not identified compounds: t_R 13.56 (min) = trace of non-silylated hydroxy fatty acid methyl ester (C_{14}), 14.60 = dibutyl phthalate, 14.66 = not identified, 17.23 = octadecanol, 17.37 = not identified, 18.01 = saturated hydrocarbon.)

comparison of retention times between the (2*S*)-phenylpropionyl derivatives of *Diascia* oils and of (3*R*)-hydroxypalmitic acid methyl ester as chiral standard were performed (Hammarström, 1975; Wollenweber et al., 1985; Gradowska and Larsson, 1994). Racemic mixtures were obtained directly from the *Diascia* oils through oxidation with potassiumdichromate (K_2CrO_4) and backward reduction with sodiumborohydride ($NaBH_4$) (Fabritius et al., 1996). (*2S*)-Phenylpropionyl derivatives of samples gave a complete separation of the *R*- and *S*-derivatives ($\alpha = 1.01$) under the GC–MS conditions described below (Hammarström, 1975; Seipold, 2004).

4.7. GC/EI-MS analysis of natural samples

The GC–MS investigations were performed with two GC–MS systems. Five floral oils (*D. purpurea*, *D. vigilis*, *D. cordata*, *D. megathura* and *D. integerrima*) were

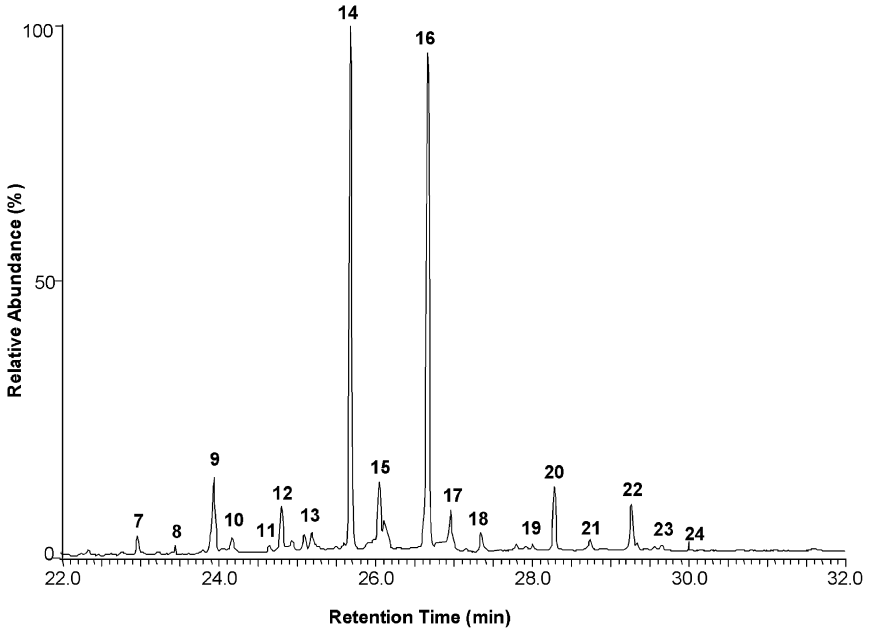


Fig. 4. Total ion chromatogram (TIC) of TMSi-derivatives of *D. vigilis* oil. (For the corresponding identified compounds see Table 2.)

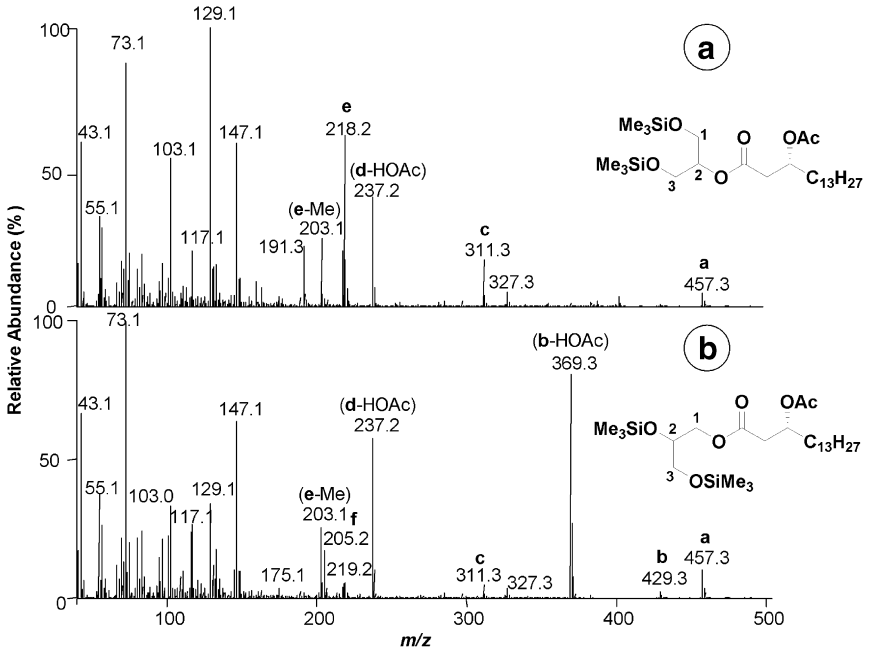


Fig. 5. Mass spectra of TMSi-derivatives of monoacylglycerols (MAGs): (a) 2-[(3*R*)-acetoxypalmitoyl]glycerol (**14**); and (b) 1-[(3*R*)-acetoxypalmitoyl]glycerol (**15**). The significant fragment ions are described in Scheme 1.

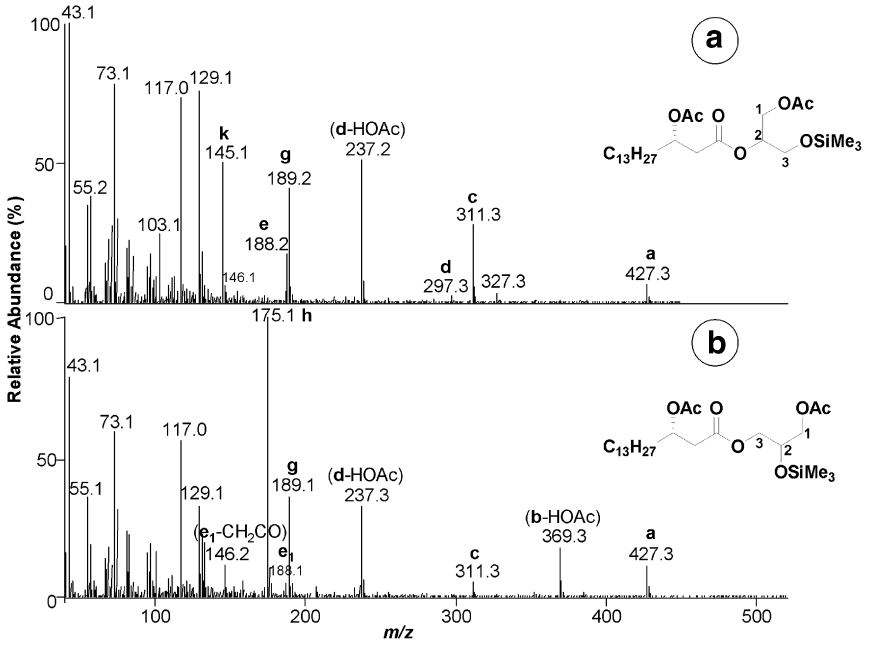


Fig. 6. Mass spectra of TMSi-derivatives of diacylglycerols (DAGs): (a) 2-[(3*R*)-acetoxypalmitoyl]-1-acetylglycerol (**16**); and (b) 1-[(3*R*)-acetoxypalmitoyl]-3-acetylglycerol (**17**). The significant fragment ions are described in Schemes 1 and 2.

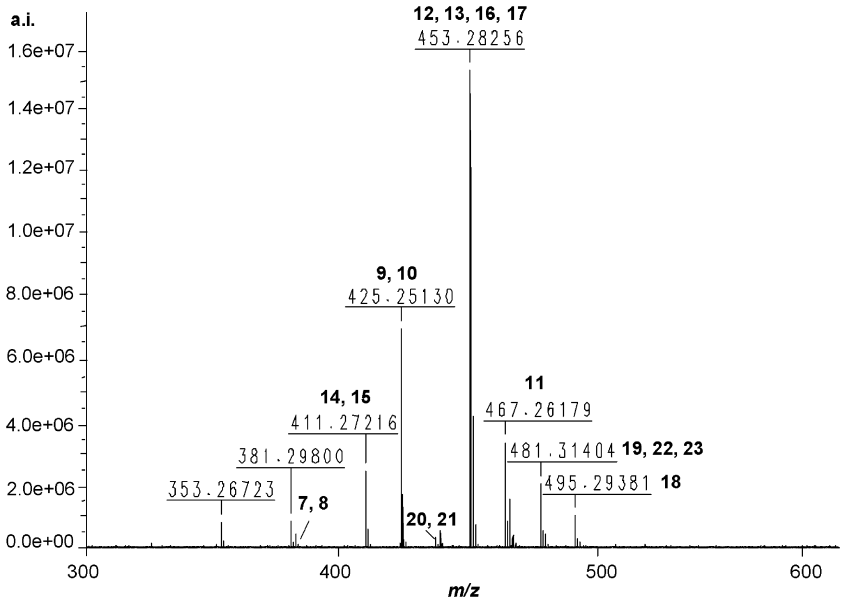


Fig. 7. Positive-ion ESI-FTICR mass spectrum of the acylglycerol profile of *D. integerrima*. Peak heights are scaled relative to the highest magnitude peak. (For the corresponding identified compounds see Table 4.)

separated using a Finnigan Voyager GC/MS system mounted with capillary column (DB-5 MS, 30 m × 0.25 mm i.d., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). The following temperature program was used: from 60 to 200 °C with a rate of 10 °C/min, followed by 5 °C/min to 300 °C and a hold at 300 °C for additional 20 min. Helium was used as the GC carrier gas at a constant flow of 1 ml/min. The mass spectrometer was operated with an electron impact (EI) ion source at 70 eV electron energy, ion source temperature 200 °C and a mass range of 40–800 a.m.u.

The investigation of the floral oil of *D. barberae* was carried out on an Agilent 6890 series (Wilmington, USA) gas chromatograph equipped with a Micromass (Manchester, UK) GCT mass sensitive reflectron time-of-flight (TOF) detector (DB-5 MS, 30 m × 0.25 mm, i.d., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA) (see above for the conditions).

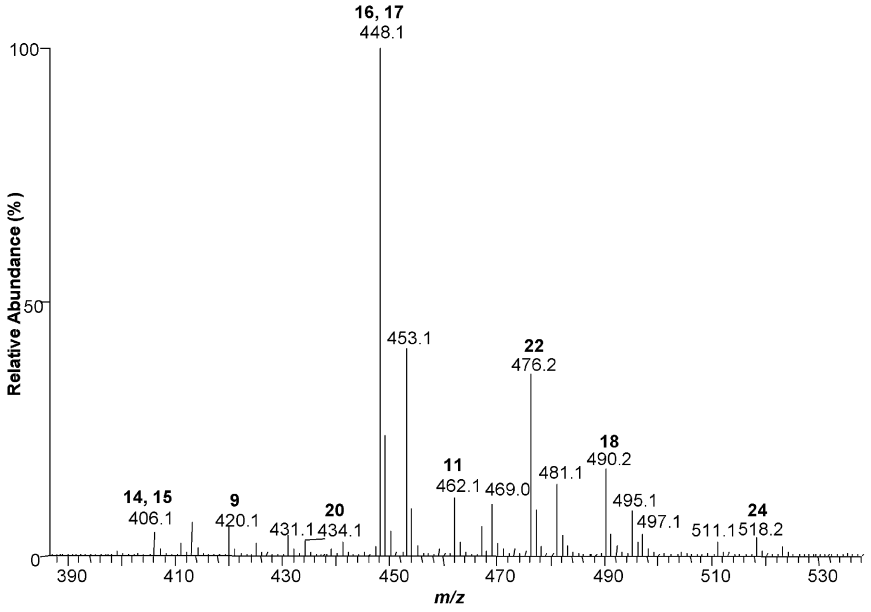


Fig. 8. Positive-ion ESI mass spectrum of the acylglycerol profile of underivatized *D. barberae* oil. (For the corresponding identified compounds see Tables 2 and 3.)

4.8. ESI-FTICR-MS analysis

The positive-ion high resolution ESI mass spectra of oils of all species except *D. barbarae* were obtained from a Bruker Apex III Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics, Billerica, USA) equipped with an Infinity™ cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an APOLLO electrospray ion source (Agilent, off axis spray, voltages: endplate, −3.700 V; capillary, −4.200 V; capillary exit, 100 V; skimmer 1, 15.0 V; skimmer 2, 10.0 V). N_2 was used as drying gas at 150 °C. 0.1 mg of *Diascia* oil in MeOH was introduced continuously via a syringe pump with a flow rate of 120 µl h^{-1}. All data were acquired with 512k data points and zero filled to 2048k by averaging 32 scans.

4.9. ESI-MS analysis

Positive-ion ESI mass spectra of *D. barberae* were obtained by using a Finnigan TSQ 7000 instrument. Fifty microlitres of *D. barberae* oil was dried in a constant stream of N_2. The oil residue was dissolved in 1 ml of 5 mM ammonium acetate in $CHCl_3$–MeOH (70:30, v/v). The sample was injected using a syringe pump at a constant flow 5 µl/min. The electrospray voltage was 4.5 kV, the capillary temperature 200 °C. N_2 was used as a sheath gas (Seipold, 2004; Seipold et al., 2004).

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