

# Simultaneous Quantitative Determination of Eight Triterpenoid Monoesters from Flowers of 10 Varieties of *Calendula officinalis* L. and Characterisation of a New Triterpenoid Monoester

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Dichloromethane extracts of dried flowers of *Calendula officinalis* contain eight known bioactive triterpenoid monoesters, namely, faradiol-3-*O*-palmitate, faradiol-3-*O*-myristate, faradiol-3-*O*-laurate, arnidiol-3-*O*-palmitate, arnidiol-3-*O*-myristate, arnidiol-3-*O*-laurate, calenduladiol-3-*O*-palmitate and calenduladiol-3-*O*-myristate. These pentacyclic terpenoids have been quantified simultaneously using reversed-phase HPLC with isocratic elution and internal standardisation. Of the 10 varieties of *C. officinalis* investigated, Calypso Orange Florensis produced the highest amounts of the bioactive monoesters, followed by Fiesta Gitana Gelb and May Orange Florensis. The lipophilic extract from the flowers of Calypso Orange Florensis variety also contained low levels of the newly characterised calenduladiol-3-*O*-laurate. Copyright © 2004 John Wiley & Sons, Ltd.

**Keywords:** Reversed-phase HPLC; triterpenoid ester; faradiol ester; calenduladiol ester; arnidiol ester; *Calendula officinalis*.

## INTRODUCTION

The flowers of marigold (*Calendula officinalis* L.; Asteraceae) are rich in pentacyclic mono-, di- and trihydroxy triterpenoids, which have been isolated in both free and ester form (Wilkomirski and Kasprzyk, 1979; Ukiya *et al.*, 2001). These compounds show various pharmacological activities, i.e. anti-inflammatory (Della Loggia *et al.*, 1994; Akihisa *et al.*, 1996; Yasukawa *et al.*, 1996a; Ukiya *et al.*, 2001), anti-oedematous (Zitterl-Eglseer *et al.*, 1997) and anti-tumour (Yasukawa *et al.*, 1996b). Moreover, extracts of flowers of *C. officinalis* possess anti-HIV properties of therapeutic interest (Kalvatchev *et al.*, 1997). Additionally, whole flowers have been used as food additives (as flavouring for soup and as yellow colouring for cheese), as well as in cosmetic and dermatological preparations (Isaac, 1994).

Currently, only the abundant faradiol-3-*O*-palmitate (1), faradiol-3-*O*-myristate (2) and faradiol-3-*O*-laurate (3) (Fig. 1) have been determined in their unmodified form (Zitterl-Eglseer *et al.*, 2001); quantitative estimation of the minor esters has been performed only through determination of the alcohol moieties following chemical hydrolysis of the esters. The scientific and economic interest in these terpenoids, and the lack of an up-to-date procedure to determine their relative and total amount in cultivated *C. officinalis*, has encouraged us to develop a rapid method for the quantitative evaluation of the

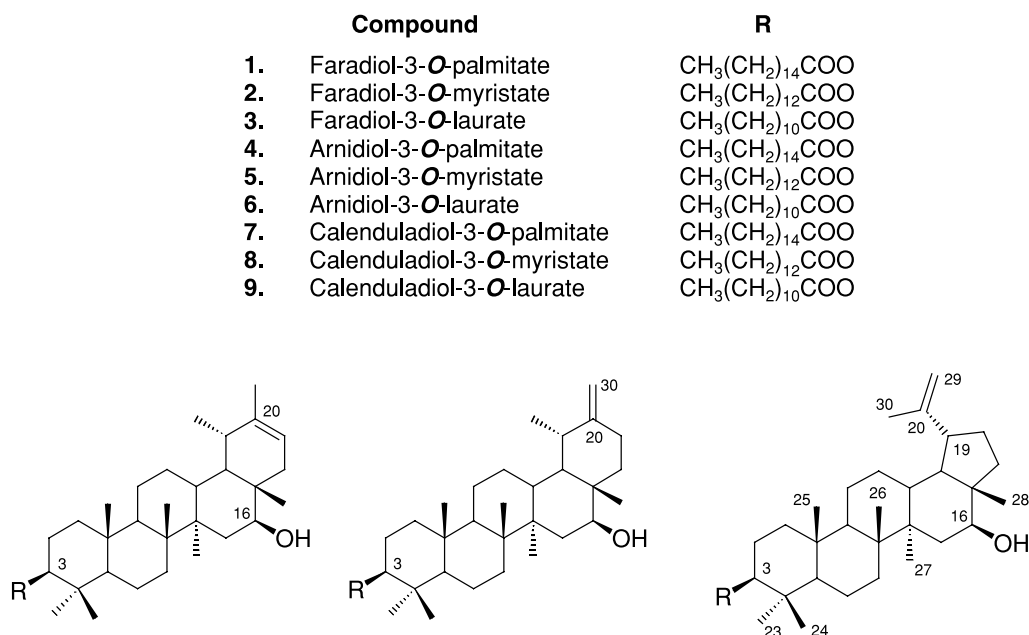
monoesters 1–8 (Fig. 1) without prior chemical modification. In this paper we report the appropriate conditions for the separation and simultaneous quantitative determination of 1–8 in lipophilic extracts from marigold flowers. In order to test the procedure, the variability of these esters has been studied in the flowers of ten varieties of locally cultivated *C. officinalis* plants. Further, we have isolated and structurally characterised a new analogue 9 which was detected in very small amounts in the extracts of the variety Calypso Orange Florensis.

## EXPERIMENTAL

**Spectroscopy.** <sup>1</sup>H-NMR (299.94 MHz) and <sup>13</sup>C-NMR (75.43 MHz) spectra were acquired in deuteriochloroform solution at room temperature using a Varian (Palo Alto, CA, USA) XL-300 spectrometer and chemical shifts were referenced to the residual solvent signals (CDCl<sub>3</sub>; δ<sub>H</sub> = 7.25 and δ<sub>C</sub> = 77.0 ppm); <sup>1</sup>H and <sup>13</sup>C-NMR signal assignments were obtained by DEPT and homonuclear <sup>1</sup>H–<sup>1</sup>H COSY experiments. Low- (EI) and high-resolution (HR) MS were recorded at 70 eV using a Kratos (Manchester, UK) MS80 instrument with a home-built acquisition system.

**Plant material.** Ten varieties of *Calendula officinalis* L. (Table 1) were cultivated in an experimental field (Gachhof; 600 m above sea level) of the Research Centre for Agriculture and Forestry at Laimburg (Alto Adige-Südtirol, Italy). The flowers were harvested during July and August 2001 and carefully dried at room

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**Figure 1.** Structures of the triterpenoid monoesters (**1–8**) from the 10 *Calendula officinalis* varieties studied (see Table 1) and of analogue **9** from the variety Calypso Orange Florensis.

**Table 1.** The 10 varieties of *Calendula officinalis* studied

Code <sup>a</sup>	Variety
A	Orange Waltz
B	Orange Florensis
C	Maya Orange Waltz
D	Gitana Orange Florensis
E	Fiesta Gitana Gelb
F	Calypso Orange Florensis
G	Parade Florensis
H	Kablauna Orange Gelb
I	May Orange Florensis
J	Erfurter Orange

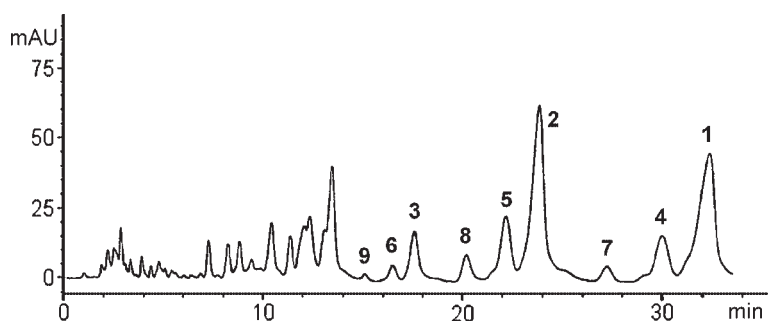
<sup>a</sup> Code letters correspond to those shown in Fig. 3.

temperature (25–30°C): the collected flowers were a representative sample of the whole harvest.

**Isolation and purification of the terpenoid 3-*O*-monoesters.** Dried flowers (5.0 g) of *C. officinalis* (variety Calypso Orange Florensis) were extracted with dichloromethane (100 mL) at room temperature for 30 min with sonication, the solvent evaporated under a stream of nitrogen and the resulting residue (330 mg) subjected to TLC on silica gel 60 F<sub>254</sub> layers (20 × 10 cm plates; 2 mm layer thickness; Merck, Darmstadt, Germany) using *n*-hexane:ethyl acetate (8:2) as mobile phase. Fractions A and B were recovered from two bands (*R<sub>f</sub>* ranges 0.48–0.55 and 0.55–0.70, respectively) which had been removed from the layers. Re-chromatography of fraction A by semi-preparative reversed-phase HPLC (detection at 210 nm) using a Machery-Nagel (Düren, Germany) Nucleosil® 100-5-C<sub>18</sub> column (250 × 10 mm i.d.) eluted with methanol (at a flow rate of 6.0 mL/min) gave a small amount of **9** (*R<sub>t</sub>* 15.03 min; 0.2 mg). Under similar analytical conditions, re-chromatography of fraction B by RP-HPLC gave compounds **1** (*R<sub>t</sub>* 32.16 min; 17.6 mg), **2** (*R<sub>t</sub>* 23.89 min; 22.5 mg), **3** (*R<sub>t</sub>* 17.71 min; 3.1 mg), **4**

(*R<sub>t</sub>* 29.72 min; 5.3 mg), **5** (*R<sub>t</sub>* 22.15 min; 4.2 mg), **6** (*R<sub>t</sub>* 16.43 min; 3.5 mg), **7** (*R<sub>t</sub>* 27.06 min; 4.0 mg), and **8** (*R<sub>t</sub>* 20.24 min; 4.1 mg). A chromatogram of the semi-preparative HPLC separation of a representative dichloromethane extract of flowers of *C. officinalis* (solvent evaporated under a stream of nitrogen and the residue dissolved in methanol) is shown in Fig. 2. Isolated compounds **1–8** were characterised by <sup>1</sup>H- and <sup>13</sup>C-NMR and by EI-MS spectra, and used as references for the construction of calibration curves. The spectroscopic analyses of these compounds indicated a purity grade greater than 97% for each.

**Extraction and HPLC analysis.** For each variety of *C. officinalis* studied (Table 1), the dried flowers were crushed to a fine powder using a grinding mill, and a sample (100.0 mg) was extracted with dichloromethane (10.0 mL) containing the internal standard (IS) lupeol acetate (1.00 mg; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature with sonication. The mixture was filtered and the residue extracted with pure dichloromethane (10.0 mL). The combined extracts were concentrated to ca. 200 µL and partially purified by semi-preparative TLC on silica gel 60 F<sub>254</sub> layers (20 × 10 cm plates; 0.25 mm layer thickness; Merck) using *n*-hexane:ethyl acetate (8:2) as mobile phase. The band of silica gel in the *R<sub>f</sub>* value range 0.35–0.90, between two yellow strips, was removed and extracted five times with dichloromethane. After evaporation of the combined eluates, the residues were dissolved in dichloromethane (400 µL) and subjected to quantitative analysis by RP-HPLC (five replicates for each sample). Analyses were performed using a Merck-Hitachi model L-7100 pump, L-7400 UV detector, D-7500 integrator and Rheodyne manual injector equipped with a 200 µL loop. The Nucleosil 100–5 C<sub>18</sub> reversed-phase column (250 × 4 mm i.d.) was eluted with methanol:water (97:3) at a flow rate of 1.5 mL/min with UV detection at 210 nm. The relative retention times (*RR<sub>t</sub>*) with respect to the IS of compounds **1–9** are reported in Table 2.



**Figure 2.** Semi-preparative RP-HPLC chromatogram of the dichloromethane extract of flowers of *Calendula officinalis* variety Calypso Orange Florensis. Key to peak identity: **1**, faradiol-3-*O*-palmitate; **2**, faradiol-3-*O*-myristate; **3**, faradiol-3-*O*-laurate; **4**, arnidiol-3-*O*-palmitate; **5**, arnidiol-3-*O*-myristate; **6**, arnidiol-3-*O*-laurate; **7**, calenduladiol-3-*O*-palmitate; **8**, calenduladiol-3-*O*-myristate; and **9**, calenduladiol-3-*O*-laurate. (The solvent was removed from the initial dichloromethane extract and the residue taken up in methanol prior to analysis: for chromatographic protocols see Experimental section).

**Table 2.** Nomenclature and relative retention times of compounds 1–9, and concentrations used for calibration

Compound <sup>a</sup>	Chemical Abstracts nomenclature (trivial names)	$RR_t^b$	Concentrations used for calibration (mg/mL)
1	(3 $\beta$ ,16 $\beta$ ,18 $\alpha$ ,19 $\alpha$ )-Urs-20-ene-3,16-diol 3-hexadecanoate (faradiol 3-monopalmitate; faradiol-3- <i>O</i> -palmitate)	2.75	1.2500; 1.8750; 2.5000; 5.0000
2	(3 $\beta$ ,16 $\beta$ ,18 $\alpha$ ,19 $\alpha$ )-Urs-20-ene-3,16-diol 3-tetradecanoate (faradiol 3-monomyristate; faradiol-3- <i>O</i> -myristate)	1.86	0.5000; 1.2500; 2.5000; 5.0000
3	(3 $\alpha$ ,16 $\beta$ ,18 $\alpha$ ,19 $\alpha$ )-Urs-20-ene-3,16-diol 3-dodecanoate (faradiol 3-monolaurate; faradiol-3- <i>O</i> -laurate)	1.28	0.3125; 0.6250; 0.9375; 1.2500
4	(3 $\beta$ ,16 $\beta$ ,18 $\alpha$ ,19 $\alpha$ )-Urs-20(30)-ene-3,16-diol 3-hexadecanoate (arnidiol 3-monopalmitate; arnidiol-3- <i>O</i> -palmitate)	2.56	0.1562; 0.3125; 0.6250; 1.2500
5	(3 $\beta$ ,16 $\beta$ ,18 $\alpha$ ,19 $\alpha$ )-Urs-20(30)-ene-3,16-diol 3-tetradecanoate (arnidiol 3-monomyristate; arnidiol-3- <i>O</i> -myristate)	1.73	0.3125; 0.6250; 1.2500; 1.8750
6	(3 $\beta$ ,16 $\beta$ ,18 $\alpha$ ,19 $\alpha$ )-Urs-20(30)-ene-3,16-diol 3-dodecanoate (arnidiol 3-monolaurate; arnidiol-3- <i>O</i> -laurate)	1.17	0.0625; 0.1250; 0.2500; 0.5000
7	(3 $\beta$ ,16 $\beta$ )-Lup-20(29)-ene-3,16-diol 3-hexadecanoate (calenduladiol 3-monopalmitate; calenduladiol-3- <i>O</i> -palmitate)	2.30	0.0625; 0.1250; 0.2500; 0.5000
8	(3 $\beta$ ,16 $\beta$ )-Lup-20(29)-ene-3,16-diol 3-tetradecanoate (calenduladiol 3-monomyristate; calenduladiol-3- <i>O</i> -myristate)	1.56	0.1250; 0.1562; 0.3125; 0.6250
9	(3 $\beta$ ,16 $\beta$ )-Lup-20(29)-ene-3,16-diol 3-dodecanoate (calenduladiol 3-monolaurate; calenduladiol-3- <i>O</i> -laurate)	1.01 <sup>c</sup>	(not quantified)

<sup>a</sup> Compound numbers correspond with structures shown in Fig. 1.

<sup>b</sup> Relative retention times with respect to lupeol acetate used as reference ( $RR_t = 1.00$ ).

<sup>c</sup> Compound **9** co-eluted with the internal standard hence its  $RR_t$  was estimated with respect to compounds **1–8**.

#### Recoveries, calibration curves and statistical evaluation.

The losses of the triterpenoid 3-*O*-monoesters during extraction, purification by TLC and RP-HPLC analysis were evaluated in comparison with lupeol acetate as IS. The recovery of each ester following five extractions of the silica gel band with dichloromethane was estimated to be 72% with respect to the IS. Calibration curves for esters **1–8** were obtained by linear fitting of the RP-HPLC responses for four different concentrations for each compound co-injected with 2.5 mg/mL of IS (see Table 2). Data analyses were performed by referring the measured 3-*O*-monoester:IS ratios to the calibration curves (five replicates). For statistical purposes, the mean percentage content of each component in each variety was compared with the mean value of the most abundant variety using the Mann–Whitney *U*-test ( $p < 0.05$ ) after Kruskal–Wallis analysis of variance ( $p < 0.01$ ). Analyses

were performed using an Abacus Concepts (Berkeley, CA, USA) StatView software package.

## RESULTS AND DISCUSSION

Until now, the analysis of triterpenoid esters from the flowers of *C. officinalis* has been described only for the abundant components **1–3** using an HPLC method with gradient elution (Zitterl-Eglseer *et al.*, 2001). These analyses required 70 min of elution plus equilibration time for each HPLC run. The modified RP-HPLC procedure reported in this paper allows the simultaneous quantitative determination of all of the major 3-*O*-ester components (**1–8**) in the flower extracts using isocratic elution with methanol:water (97:3) as the mobile phase.

**Table 3.** Amount of compounds 1–8 present in each of the ten varieties of *Calendula officinalis* studied

Variety <sup>a</sup>	Mean content (%) $\pm$ standard deviation <sup>b</sup>							
	1 <sup>c</sup>	2 <sup>c</sup>	3 <sup>c</sup>	4 <sup>c</sup>	5 <sup>c</sup>	6 <sup>c</sup>	7 <sup>c</sup>	8 <sup>c</sup>
A	0.675 $\pm$ 0.025	1.130 $\pm$ 0.040	0.337 $\pm$ 0.021	0.180 $\pm$ 0.012	0.466 $\pm$ 0.010	0.052 $\pm$ 0.004	0.034 $\pm$ 0.002	0.064 $\pm$ 0.004
B	0.621 $\pm$ 0.048	0.794 $\pm$ 0.049	0.245 $\pm$ 0.016	0.152 $\pm$ 0.008	0.320 $\pm$ 0.040	0.034 $\pm$ 0.004	0.030 $\pm$ 0.003	0.054 $\pm$ 0.005
C	0.916 $\pm$ 0.020	1.060 $\pm$ 0.010	0.245 $\pm$ 0.017	0.217 $\pm$ 0.009	0.443 $\pm$ 0.002	0.031 $\pm$ 0.002	0.038 $\pm$ 0.004	0.064 $\pm$ 0.002
D	0.820 $\pm$ 0.034	1.050 $\pm$ 0.010	0.338 $\pm$ 0.009	0.170 $\pm$ 0.010	0.351 $\pm$ 0.004	0.049 $\pm$ 0.006	0.049 $\pm$ 0.009	0.064 $\pm$ 0.005
E	1.220 $\pm$ 0.050	1.150 $\pm$ 0.020	0.411 $\pm$ 0.024	0.360 $\pm$ 0.048	0.416 $\pm$ 0.021	0.070 $\pm$ 0.009	0.058 $\pm$ 0.009	0.071 $\pm$ 0.005
F	1.340 $\pm$ 0.050	1.250 $\pm$ 0.020	0.390 $\pm$ 0.024	0.381 $\pm$ 0.030	0.477 $\pm$ 0.024	0.069 $\pm$ 0.007	0.060 $\pm$ 0.006	0.115 $\pm$ 0.010
G	0.810 $\pm$ 0.026	0.794 $\pm$ 0.087	0.235 $\pm$ 0.018	0.184 $\pm$ 0.018	0.320 $\pm$ 0.023	0.028 $\pm$ 0.002	0.043 $\pm$ 0.011	0.062 $\pm$ 0.014
H	0.660 $\pm$ 0.033	0.966 $\pm$ 0.022	0.291 $\pm$ 0.032	0.145 $\pm$ 0.013	0.380 $\pm$ 0.043	0.041 $\pm$ 0.005	0.031 $\pm$ 0.003	0.084 $\pm$ 0.007
I	1.010 $\pm$ 0.030	1.170 $\pm$ 0.030	0.271 $\pm$ 0.010	0.355 $\pm$ 0.026	0.549 $\pm$ 0.044	0.044 $\pm$ 0.003	0.045 $\pm$ 0.004	0.085 $\pm$ 0.013
J	0.930 $\pm$ 0.033	1.040 $\pm$ 0.020	0.279 $\pm$ 0.027	0.244 $\pm$ 0.027	0.435 $\pm$ 0.015	0.047 $\pm$ 0.005	0.039 $\pm$ 0.002	0.073 $\pm$ 0.005

<sup>a</sup> Variety code as defined in Table 2.<sup>b</sup> Values based on five replicates starting from 100 mg of dried flowers.<sup>c</sup> Compound numbers correspond with structures shown in Fig. 1.

A single HPLC run could be completed within 38 min and no equilibration of the column was required. The significantly shorter analysis time and contemporary quantitative evaluation of the eight triterpenoid esters are valuable improvements on the previously published protocol.

The modified method has been used to study the variability of esters 1–8 in the flowers of 10 varieties of locally cultivated *C. officinalis* plants (see Table 1). For calibration purposes, the triterpenoid esters were isolated (purity > 97%) from flowers of the variety Calypso Orange Florensis, and their structures confirmed by comparison of <sup>1</sup>H-, <sup>13</sup>C-NMR and EI-MS spectra with those previously reported (Oksuz and Topcu, 1987; Diaz *et al.*, 1992; Zitterl-Eglseer *et al.*, 1997; Ukiya *et al.*, 2001). Quantitative data could then be obtained by comparing experimental determinations with the calibration curves (linear regression coefficients > 0.99) for 1–8. In order to perform HPLC analyses on dichloromethane extracts of the flowers, it was found to be necessary to carry out a pre-purification step involving semi-preparative TLC on silica gel. During this process, strong interactions between silica gel and triterpene monoesters were demonstrated by the low recovery results (30%) obtained following one extraction with dichloromethane. Lupeol acetate could be totally recovered from silica gel by two extractions with the solvent and thus, for compounds 1–8, five extractions of the stationary phase were carried out giving a 72% recovery.

The results shown in Table 3 and Fig. 3 indicate variable levels of 1–8 in the 10 varieties of *C. officinalis* studied. It appears that the faradiol esters 1–3 may be much more abundant than previously reported (Zitterl-Eglseer *et al.*, 2001). It is worthy of note that, in the whole flowers of Calypso Orange Florensis, the content of 3 (or 1 and 2) is double (or similar) to that previously evaluated in the separated ray florets which were described as the richest source of esters compared with the other organs (disc florets and involucre bracts) of the flowers. Thus, either the ten varieties of *C. officinalis* studied in the present work are high-producing plants or, alternatively, previous workers have underestimated the difficulty of recovery of these compounds.

From the results obtained in this study it may be concluded that: (a) the faradiol-3-*O*-esters (1–3) are ca. four- and 20 times, respectively, more abundant than the arnidiol- (4–6) and calenduladiol- (7 and 8) 3-*O*-esters

(Fig. 3); (b) compounds 4–6 are present in significantly larger amounts than suggested previously (Della Loggia *et al.*, 1994); (c) the myristate esters are slightly more plentiful than the palmitate esters; (d) the palmitate and myristate esters of faradiol are far more abundant than the laurate ester; (e) arnidiol myristate (5) occurs at levels which are ca. twice those of arnidiol palmitate (4), whilst the content of calenduladiol myristate (8) and calenduladiol palmitate (7) are comparable. Figure 3 also shows that the total triterpenol esters are most abundant in varieties May Orange Florensis, Fiesta Gitana Gelb and Calypso Orange Florensis, with the latter containing the highest amounts. This would suggest that the flowers of Calypso Orange Florensis, followed by Fiesta Gitana Gelb, may be of most value in preparing extracts for topical use or, in general, for economic exploitation.

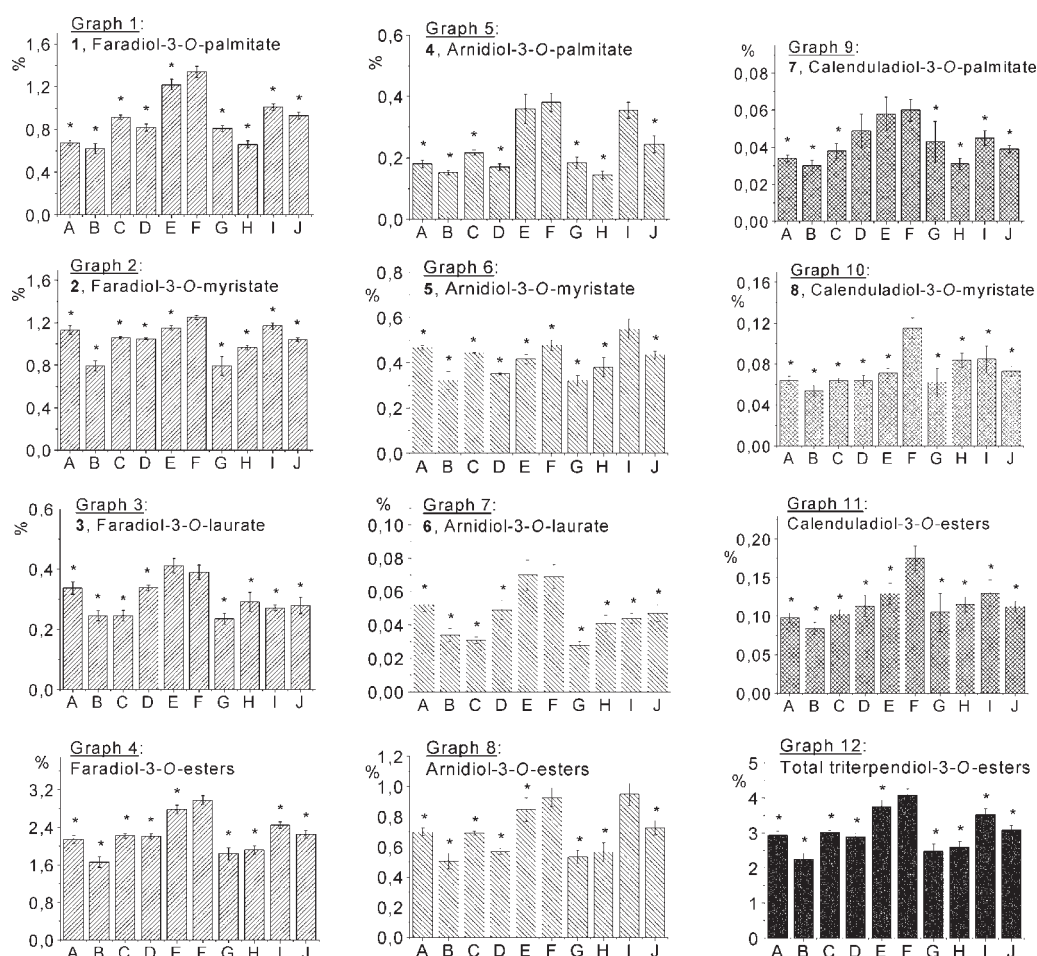
During the chromatographic analyses of the extract from Calypso Orange Florensis, a compound more polar than compounds 1–8 and at a shorter *R*<sub>f</sub> (15.03 min; Fig. 2) was detected. Semi-preparative RP-HPLC provided a small amount (0.2 mg) of this pure compound (9) which was subjected to <sup>1</sup>H-NMR and MS analysis.

#### Calenduladiol-3-*O*-laurate (9)

<sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta$  0.78 (s, 3H, H-28), 0.82 (s, 6H, H-23, H-24, or H-25), 0.84 (s, 3H, H-25, or H-23, or H-24), 0.87 (t, *J* = 7.0 Hz, 3H, Me-CH<sub>2</sub>), 0.97 (s, 3H, H-27), 1.02 (s, 3H, H-26), 1.25 [m, (CH<sub>2</sub>)<sub>n</sub>], 1.67 (bs, 3H, H-30), 1.85–2.05 (m, 1H), 2.27 (t, *J* = 7.5 Hz, 2H, CH<sub>2</sub>-COO), 2.49 (td, *J* = 10.5, 6.0 Hz, 1H, H-19), 3.60 (dd, *J* = 11.0, 5.0 Hz, 1H, H-16), 4.46 (dd, *J* = 11.0, 5.0 Hz, H-3), 4.59 (m, 1H, H-29), 4.69 (m, 1H, H-29). EI-MS: *m/z* (ion, relative intensity) 624 (M<sup>+</sup>, 0.2), 606 ([M-H<sub>2</sub>O]<sup>+</sup>, 0.2), 424 ([M-RCOOH]<sup>+</sup>, 0.9), 28 (100); HR-MS: *m/z* 624.54834  $\pm$  0.005 (C<sub>42</sub>H<sub>72</sub>O<sub>3</sub><sup>+</sup>, calculated 624.54816); 424.36922  $\pm$  0.005 (C<sub>30</sub>H<sub>48</sub>O<sup>+</sup> as [M-C<sub>12</sub>H<sub>24</sub>O<sub>2</sub>]<sup>+</sup>, calculated 424.37052).

The <sup>1</sup>H-NMR spectrum was similar to those reported for 7 and 8 (Oksuz and Topcu, 1987; Diaz *et al.*, 1992). The presence of a fatty acid ester portion was suggested by the triplet at  $\delta$  0.87 for the terminal methyl, the strong signal near 1.25 ppm for the (CH<sub>2</sub>)<sub>n</sub>, and the triplet at 2.27 ppm for the protons of the methylene in  $\alpha$  position to the carbonyl function. The alcohol moiety showed sharp singlet signals at  $\delta$  0.78 (3H), 0.82 (6H), 0.84 (3H),





**Figure 3.** Mean percentage content (with standard deviation) of the eight triterpenoid monoesters 1–8 (graphs 1–3, 5–7, 9 and 10), of faradiol- (graph 4), arnidiol- (graph 8), and calenduladiol- (graph 11) 3-*O*-esters, and of the total 3-*O*-esters (graph 12) in the 10 *Calendula officinalis* varieties A–J (detailed in Table 1). In each graph, a bar labelled with an asterisk indicates a mean content which is significantly different ( $p < 0.05$ ) from the mean value of the most abundant variety as determined by the Mann-Whitney  $U$ -test after Kruskal-Wallis analysis of variance ( $p < 0.01$ ).

0.97 (3H), and 1.02 (3H) for six  $\text{CH}_3$  at quaternary carbons, and a broad singlet resonance at 1.67 (3H) for the methyl protons coupled to vinylic protons at 4.59 and 4.69 ppm. Furthermore, the triplet of doublets at  $\delta$  2.49, assigned to H-19 $\beta$ , and the absorptions at 3.60 (H-16 $\alpha$ ) and 4.46 ppm (H-3 $\alpha$ ) supported the calenduladiol substructure. Finally, the structure of calenduladiol laurate (**9**) was confirmed by MS which showed two key-peaks at  $m/z$  624 and 424 due at the molecular ion  $[\text{C}_{42}\text{H}_{72}\text{O}_3]^+$  and the fragment  $[\text{M}-\text{C}_{12}\text{H}_{24}\text{O}_2]^+$ , respectively. The structure of **9** has been recently reported by Michelazzi (2001). The very small amount of **9** present in *C. officinalis* suggests that it is not of significant interest and the quantitative determination of this compound was not performed.

In summary, a fast analytic procedure for the simultaneous quantification of eight triterpenoid esters of *C.*

*officinalis* flowers has been developed. The RP-HPLC analyses of ten locally cultured varieties indicated that the variety Calypso Orange Florensis produces the largest amounts of the bioactive terpenoids, in particular of faradiol laurate which is present in the whole flowers of this variety at levels which are two-fold higher those previously determined in the specialised ray florets.

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