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FREE AND ESTER-BOUND TRITERPENE ALCOHOLS AND STEROLS IN CELLULAR SUBFRACTIONS OF *CALENDULA OFFICINALIS* FLOWERS

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Abstract—In the chromoplast fraction and in the chromoplast-free fraction, obtained from *Calendula officinalis* ligulate flowers, the contents of individual free and ester-bound triterpene alcohols and sterols as well as the fatty acid components of the ester form were determined. It was shown that all sterols and triterpene monols in both forms occur in the two subfractions investigated, whereas all diols are localized only in the chromoplast fraction. The compositions of the fatty acids esterifying monols and sterols were similar to those esterifying diols in the chromoplasts. However, the fatty acids esterifying extra-chromoplast monols and sterols were different. This result indicates that triterpene monol esters are substrates for the biosynthesis of 3-monoesters of diols.

INTRODUCTION

In *Calendula officinalis* flowers the presence of pentacyclic triterpene alcohols has been reported. Five monols and five diols in both free form and ester-bound with higher fatty acids have been detected [1–3]. Adler and Kasprzyk [4] have shown that triterpene monols in both forms predominate in the chromoplast fraction, whereas in the microsome and cell wall fractions they occur at a lower concentration, and in the mitochondrial fraction they are present in a very small amount. Triterpene diols, which are 98% bound as 3-monoesters with higher fatty acids, are localized almost exclusively in the chromoplast fraction. In the above-mentioned study, monols and diols in both forms have been determined together. After having developed appropriate conditions of separation and quantitative determination of individual triterpene alcohols [5, 6], in the present study we determined them in free and esterified form in the chromoplast fraction and in the chromoplast-free fraction of *C. officinalis* flowers. Free and ester-derived sterols, as well as fatty acids esterifying sterols and triterpene alcohols, were also determined in these fractions.

RESULTS AND DISCUSSION

Observations under a light microscope showed that the chromoplast fraction obtained by the above-mentioned procedure exhibited high homogeneity, though a large proportion of the chromoplasts were broken, because, as shown by Adler *et al.* [4], chromoplasts of *C. officinalis* flowers belong to the class of globular chromoplasts and are characterized by a labile structure. On the other hand, the remaining chromoplast-free fraction was contaminated with the chromoplast fraction, as testified by its faintly orange colour. The amount of contaminants was assessed by the determination of carotenoids; 78% of carotenoids (expressed as β-carotene) occurred in the chromoplast fraction and 22% was in the chromoplast-free homogenate. The error resulting from the contamination of the homogenate with chromoplasts was taken into account in the calculation of the contents of the investigated compounds.

In both above-mentioned subfractions, as well as in complete non-fractionated flowers, the content of triterpene alcohols in free form and esterified with higher fatty acids was determined. In both subfractions the different groups of compounds were obtained in a mean yield of 70%, relative to their content in non-fractionated flowers.

Some discrepancies in the quantitative determinations of the investigated compounds between the non-fractionated and fractionated flowers were due to experimental error arising from the extraction and TLC. In GLC determinations errors were mainly caused by the impossibility of finding a suitable reaction time for monol oxidation with SeO_2, which would simultaneously be optimal for all the compounds tested.

It was found, as in earlier studies [7], that in flowers the sterol content was *ca* 0.06% of dry matter, the level of monols was *ca* ten times higher than that of sterols, and the amount of diols was three times higher than that of monols. In non-fractionated flowers, monol esters accounted for 11% of total monols and sterol esters and for 15% of total sterols.

Free and ester-bound monols and sterols occurred in both investigated subfractions, whereas diols were localized exclusively in chromoplasts (almost completely as 3-monoesters, and in trace amounts free diols).

Since a relatively small quantity of material was used only those sterols occurring in greatest amounts could be assayed. In non-fractionated flowers, determinations were made of four sterols: cholesterol, stigmasterol, sitosterol and campesterol. Results concerning free sterols and ester-bound sterols are given in Table 1.

Table 1. Contents of free and ester-bound sterols in *Calendula officinalis* total flowers and their chromoplast and extra-chromoplast fractions (μg/g of wet wt)

Compound	Non-fractionated flowers μg	%	Chromoplast fraction μg	%	Extra-chromoplast fraction μg	%
Free sterols						
Stigmasterol	30.2	49.3	11.4	63.7	15.8	70.9
Sitosterol	20.7	33.8	4.5	25.1	4.7	21.1
Campesterol	7.0	11.4	2.0	11.2	1.1	4.9
Cholesterol	1.0	1.6	trace	—	0.7	3.1
$\Delta^0 + \Delta^7$ sterols	2.4	3.9	—	—	—	—
Total	61.3	100.0	17.9	100.0	22.3	100.0
Ester-bound sterols						
Stigmasterol	2.8	22.6	1.35	27.0	1.6	31.4
Sitosterol	9.6	77.4	1.8	49.3	2.2	43.1
Campesterol	—	—	0.5	13.7	1.3	22.5
Total	12.4	100.0	3.65	100.0	5.1	100.0

Both forms of sterols, taken as a whole, exhibited a slight predominance in the extra-chromoplast localization. Stigmasterol predominated among the free sterols, and sitosterol among the ester-bound sterols. The remaining sterols occurred in smaller amounts.

C. officinalis flowers contain the following pentacyclic triterpene monols [1]: α-amyrin, β-amyrin, taraxasterol, ψ-taraxasterol and lupeol. All these monols were determined in non-fractionated flowers and in the subfractions obtained from them. Results concerning both forms of monols are recorded in Table 2.

Monols in both forms predominated in chromoplasts; this predominance was greater for free monols (*ca* three times) than for ester-bound monols (*ca* two times). The proportions between monols in both forms were similar.

Table 2. Contents of free and ester-bound monols in *Calendula officinalis* total flowers and their chromoplast and extra chromoplast fractions (μg/g wet wt)

Chromoplast	Non-fractionated flowers μg	%	Chromoplast fraction μg	%	Extra-chromoplast fraction μg	%
Free monols						
β-Amyrin	166.7	26.1	74.6	24.4	25.5	29.3
α-Amyrin	89.3	14.0	79.9	26.1	15.3	17.6
Taraxasterol	17.6	2.8	9.4	3.1	3.9	4.5
ψ-Taraxasterol	325.9	51.1	124.2	40.7	36.8	42.3
Lupeol	38.0	6.0	17.4	5.4	5.5	6.3
Total	638.5	100.0	305.5	100.0	87.0	100.0
Ester-bound monols						
β-Amyrin	19.5	42.2	10.3	25.5	5.0	26.2
α-Amyrin	15.6	19.4	9.5	23.6	4.4	23.0
Taraxasterol	5.1	6.3	2.2	5.5	1.1	5.8
ψ-Taraxasterol	35.1	43.5	16.0	39.7	6.7	35.1
Lupeol	5.3	6.6	2.3	5.7	1.9	9.9
Total	80.6	100.0	40.3	100.0	19.1	100.0

Table 3. Contents of diols from the 3-monoesters in *Calendula officinalis* total flowers and their chromoplast and extra-chromoplast fraction (μg/g wet wt)

Compound	Non-fractionated flowers μg	%	Chromoplast fraction μg	%	Extra-chromoplast fraction μg	%
Erythrodiol	9.3	0.4	trace	—	trace	—
Brein	98.2	4.9	84.0	5.5	1.6	7.1
Ursadiol	66.7	3.3	60.7	3.9	1.7	7.6
Calenduladiol	121.4	6.0	168.6	11.0	0.6	2.7
Faradiol	1725.3	85.4	1224.9	79.6	18.6	82.6
Total	2020.9	100.0	1538.2	100.0	22.5	100.0
Free diols						
Faradiol	15.0		8.8			
Other diols	trace		—			

The level of ψ-taraxasterol was greatest, contents of α-amyrin and β-amyrin were lower, whereas lupeol and taraxasterol occurred in the smallest amounts. The relatively high content of free and ester-bound β-amyrin suggested that in the flowers, this compound is metabolized by two pathways. On the one hand it is, as in the green parts of the plant, oxidized to oleanolic acid, while on the other it is esterified similarly to other triterpene monols present only in flowers and seeds. Cyclization of squalene to triterpene monols occurs in the microsomes; thus, the high content of the free and bound forms of these compounds in the chromoplasts testified to their transport to these organellae, without settling which one is the transported form.

Diols were localized exclusively in chromoplasts confirming the suggestion that they are synthesized in this compartment. The occurrence of 1.5% of extra-chromoplast diols is regarded as an error of the assay method.

Table 4. Contents of fatty acids from monol and sterol esters and 3-monoesters of diols in *Calendula officinalis* total flowers and their chromoplast and extra-chromoplast fractions (μg/g of wet wt)

Fatty acid	Non-fractionated flowers μg	%	Chromoplast fraction μg	%	Extra-chromoplast fraction μg	%
Fatty acids from monols and sterol esters						
Lauric	3.2	9.3	1.1	6.1	0.5	4.3
Myristic	10.7	31.1	7.5	41.9	3.0	25.6
Palmitic	15.6	45.3	7.5	41.9	3.2	27.3
Stearic	1.8	5.2	0.7	4.0	0.2	1.7
Oleic	2.6	7.6	1.1	6.1	3.4	29.0
Linolic	0.5	1.5	trace	—	0.7	6.0
Linolenic	trace	—	trace	—	0.7	6.0
Total	34.4	100.0	17.9	100.0	11.7	100.0
Fatty acids from 3-monoesters of diols						
Lauric	41.3	7.2	17.0	5.6	—	—
Myristic	277.3	48.3	163.0	55.3	—	—
Palmitic	193.8	34.0	109.3	35.8	9.3	—
Stearic	21.0	3.7	5.5	1.8	—	—
Oleic	37.2	6.5	10.6	3.5	—	—
Total	570.6	100.0	305.4	100.0	9.3	—

Results of diol determinations are presented in Table 3.

Faradiol was the most predominant diol. Calenduladiol, brein and ursadiol were present in much lower, comparable amounts, whereas erythrodiol being an intermediate in β-amyrin oxidation to oleanolic acid occurred only in a trace amount. The results of determinations of fatty acids esterfying monols and sterols jointly, as well as of fatty acids esterifying diols, are shown in Table 4.

The main fatty acids esterifying monols and sterols in ligulate flowers comprised palmitic, myristic and lauric acids. The same acids occurred in a similar proportion in the chromoplast fraction. The chromoplast-free homogenate contained substantial amounts of unsaturated fatty acids (oleic and linoleic acids). The great similarity in the quantitative composition between acids esterifying monols and sterols in chromoplasts, and those esterifying diol 3-monoesters in the same compartment may suggest that monol esters are the immediate precursors of diol 3-monoesters in these organellae.

EXPERIMENTAL

Preparation of cellular subfractions. Ligulate flowers of *C. officinalis* were ground in a Potter homogenizer with a 0.25% sucrose soln and filtered through 4 layers of cheesecloth; the filtrate was centrifuged for 10 min at 600 *g*. The supernatant was then centrifuged for 30 min at 20000 *g*. The pellet was suspended in 0.25% sucrose, applied onto a 1.6 M sucrose layer and centrifuged for 60 min at 105000 *g*. The pellet at the bottom of the tube, mainly containing mitochondria, was combined with the ppt. obtained after centrifugation at 600 *g* and with the 20000 *g* supernatant; this fraction was assumed to be the chromoplast-free homogenate. The orange layer localized at the 0.25 and 1.6 M sucrose interface was collected and considered to be the chromoplast fraction.

Determination of carotenoids. To 0.5 ml of the suspension of the subfractions, 5 ml 6% ethanolic KOH were added. Subsequently the sample was saponified for 10 min at 90°, diluted with H_2O and extracted with 2 ml (4 × 0.5 ml) of hexane. Absorption was measured at 450 nm [7].

Separation of various forms of triterpenoid alcohols. The lipid fraction isolated from both subfractions obtained was separated by TLC on Si gel in hexane–$CHCl_3$–MeOH (20:10:1) into five preliminarily purified fractions comprising, when listed in order of decreasing polarity, of free diols, free sterols, free monols, diol 3-monoesters as well as esters of monols and sterols jointly. Each fraction was rechromatographed using the above system. The band of free diols was acetylated before rechromatography. The band of sterols was acetylated and resolved on $AgNO_3$-impregnated plates into groups of compounds differing in the number or position of double bonds. Plates were developed in MeOH-free $CHCl_3$.

Gas chromatography. Triterpene alcohols were determined under previously reported conditions [5, 6]. Fatty acids were determined on a 15% EGSS-X column, and sterols on a 3% SE-30 column.

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