

ARTICLE

HPLC analysis of carotenoids in four varieties of *Calendula officinalis* L. flowers

Adela Pinteaa*, Constantin Bele, Sanda Andrei, Carmen Socaciu

University of Agricultural Sciences and Veterinary Medicine, Cluj Napoca, Romania

ABSTRACT *Calendula officinalis* L. is a medicinal plant that accumulates large amounts of carotenoids in its inflorescences. The yellow-to-orange colour of inflorescences is mostly due to carotenoids and the shade is dependent on pigments content and profile. We investigated the carotenoid content and profile in four selected varieties of *Calendula*: Double Esterel Orange, Radio Extra Selected, Bonbon Abricot and Double Esterel Jaune. The total carotenoid content was evaluated spectrophotometrically and pigments were separated using chromatographic methods (CC, TLC, HPLC). An HPLC gradient system with a Nucleosil C₁₈ column and a Waters PDA detector was used for separation and identification of carotenoids. The carotenoid content was higher in orange varieties: 276 mg/100 g fresh flowers for Double Esterel Orange and 111 mg/100 g fresh flowers for Radio variety. All varieties contain the same pigments but there are significant differences for the ratio between individual pigments. Orange varieties contain higher amounts of hydrocarbons: 44.5% of total carotenoid in Double Esterel Orange; while yellow varieties contain mostly oxygenated derivatives: 97% of total carotenoids in Double Esterel Jaune. The main pigments identified were: flavoxanthin, lutein, rubixanthin, β -carotene, γ -carotene and lycopene. The cultivation of orange varieties is recommended especially when the pharmacological products for skin protection are envisaged.

Acta Biol Szeged 47(1-4):37-40 (2003)**KEY WORDS***Calendula officinalis* L.
carotenoids
HPLC
chromatography

Calendula officinalis L. is one of the most known and used medicinal plants, that is widely cultivated both for obtaining extracts used in phytotherapy and ornamental purposes. The chemical composition of the species and of its varieties is very complex. Among the biologically active compounds we can mention the isoprenoids and derivatives, the phenolic compounds, the polysaccharides, the nitrogen compounds, etc. The extracts obtained from *Calendula* possess a wide range of pharmacological effects: wound-healing, anti-inflammatory, antibacterial, immunostimulative, antitumoral, etc.. The shade depends on the carotenoids' composition; the yellow varieties are rich in xanthophylls (oxygenated derivatives), while orange varieties contain higher amounts of hydrocarbons (Toth and Szabolcs 1981; Milborrow et al. 1982; Petri and Lemberkovics 1994). Some works refer to qualitative aspects (separation and identification of carotenoids) and others to quantitative determination (total carotenoid content; Bomme et al. 1997; Piccaglia et al. 1997). Recently, the carotenoids composition of petals, pollens, leaves and stems of *Calendula* was investigated by HPLC (Bako et al. 2002). However, there are no systematic studies yet using a genetically controlled material regarding both aspects.

Carotenoids are known as biologically active compounds with multiple applications in therapy. Beside the provitamin A activity (of some pigments), it was proved that carotenoids have a favorable effect on the epitelisation process, influencing the cell cycle progression of the fibroblasts (Stivala et al. 1996). Carotenoids act as photoprotective agents (depending on the dose) and may reduce the risk of sunburns, photoallergy and even some types of skin cancer (Fuchs 1998; Lee et al. 2000). Lycopene is an active inhibitor of tumour cells proliferation (Levy et al. 1995), but oxygenated carotenoids can also have biological properties due to their antioxidant properties (Woodall et al. 1997; Smith 1998).

The aims of our study were to determine the carotenoid content in four selected varieties of *Calendula officinalis* L., the separation and identification of carotenoids and the determination of the carotenoids profile in these varieties by HPLC-PDA.

Materials and Methods

Biological material

The carotenoids analyses were made on four selected varieties, differentiated by colour, bought in specialized stores in France: "Esterel Double Orange" – with big, dark orange inflorescences, "Esterel Double Jaune" – with big, lemon yellow inflorescences, "Radio Extra Selected" - tall, with

Accepted September 25, 2003

*Corresponding author. E-mail: apinteaa@usamvcluj.ro

Table 1. The total carotenoid content in some *Calendula officinalis* L. varieties.

Variety	Colour	Carotenoid amount (mg/100g fresh flowers)
Bonbon Abricot	Yellow-orange	48.2
Double Esterel Jaune	Lemon yellow	97.0
Radio Extra Selected	Orange	111.8
Double Esterel Orange	Dark orange	276.0

orange inflorescences, “Bonbon Abricot” – small, with yellow-orange inflorescences. The plants were cultivated in experimental fields of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.

Total carotenoids were extracted from 10 g fresh inflorescences with a mixture of methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v) containing BHT as antioxidant and calcium carbonate. The total carotenoid content was estimated spectrophotometrically at 450 nm using a Perkin-Elmer Spectrophotometer.

The extract was dissolved in diethyl ether and saponified with 30% methanolic KOH at room temperature in dark. For the removal of soaps and alkalies, the solution was washed many times with a sodium chloride-saturated solution and distilled water. The organic layer containing carotenoids was dried over anhydrous sodium sulphate and evaporated to dryness. The samples were kept under nitrogen, at -20°C until further utilization and were filtered through 0.45 μm Whatman filters prior HPLC analysis (Britton et al. 1995).

A part of oleoresin dissolved in light petroleum was subjected to column chromatography on aluminium oxide grade III (100x10 mm). For removal of neutral lipids, the column was washed twice with light petroleum. Three fractions were collected separately 1) with petroleum ether, 2) with 50% diethylether in petroleum ether and 3) with 100% diethyl ether to 20% ethanol in diethyl ether. The fraction collected from alumina column was further subjected to TLC on silica thin layer plates using in parallel standards

of β -carotene, β -cryptoxanthin, and lutein. The bands separated on silica plates were scratched out and separated on magnesium oxide-kieselguhr plates, in order to obtain pure compounds.

HPLC-DAD of all samples was performed on a system with Waters 990 PDA detector, Kontron 322 pumps and controller, a Rheodyne 7152 injection valve with a 20 ml loop and a reversed phase C 18 column Nucleosil ODS (250 x 4,6 mm), 5 μm . The mobile phase consisted of mixtures of acetonitrile: water (9:1, v/v) with 0.25% triethylamine (A) and ethyl acetate with 0.25% triethylamine (B). The gradient started with 90% A at 0 min to 50% A at 10 min. The percentage of A decreased from 50% at 10 min to 10% A at 20 min. The flow rate was 1 ml/min and the chromatogram was monitored at 450 nm.

Identification of carotenoids was based on co-chromatography using standards, chemical tests on pure compounds and characteristics of UV-VIS spectra recorded by PDA detector and Perkin-Elmer spectrophotometer for pure compounds.

Results and Discussion

Quantitative determination of carotenoids

The results of quantitative determination of total carotenoid contents in the four investigated varieties are presented in Table 1.

The richest variety in carotenoids content from flowers was Double Esterel Orange, with a total content of 276 mg/

Table 2. Carotenoid composition in inflorescences of *Calendula officinalis* L. varieties.

Pigment	Nr. of Pigment on HPLC chromatogram	Double Esterel Orange %	Radio Extra Selected %	Bonbon Abricot %	Double Esterel Jaune %
Neoxanthin	1	0.92	1.71	2.84	1.74
Luteoxanthin + Auro	8	8.9	11.3	15.43	18.97
Antheraxanthin	9	2.09	4.31	4.56	6.83
Flavoxanthin	10	14.1	17.4	35.42	42.05
Mutatoxanthin	11	0.38	-	2.17	-
Lactucaxanthin	12	4.49	8.02	-	11.31
Lutein	3	9.18	11.38	8.27	12.29
Zeaxanthin	4	0.11	0.23	-	0.15
Rubixanthin	13,14	14.36	7.27	4.58	-
Lycopene	15	14.03	5	0.57	-
γ -carotene	16	12.15	6.15	5.11	-
α -carotene	17	0.98	1.15	1.89	0.2
β -carotene	7	16.68	17.51	10.31	2.37

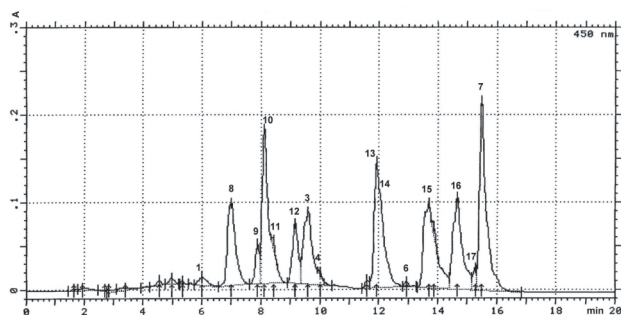


Figure 1. The HPLC chromatogram of total saponified extract from Double Esterel Orange variety.

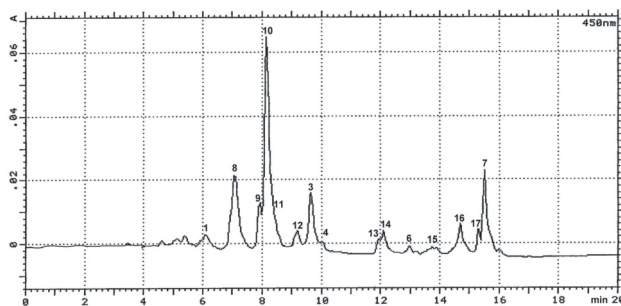


Figure 3. The HPLC chromatogram of total saponified extract of Bonbon Abricot variety.

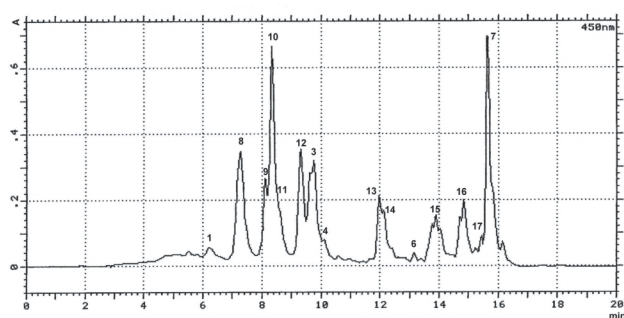


Figure 2. The HPLC chromatogram of total saponified extract of Radio Extra Selected variety.

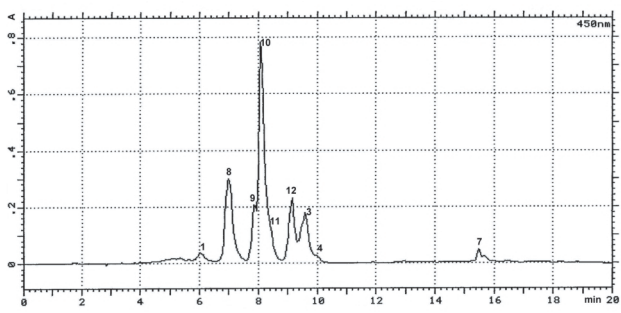


Figure 4. The HPLC chromatogram of total saponified extract of Double Esterel Jaune variety.

100 g fresh flowers. The carotenoid content in petals and tubular flowers of Radio Extra Selected was also determined. The highest amount of carotenoids was found in petals (103,8 mg/100 g), while tubular flowers contains only 8 mg/100 g fresh material. The values of quantitative determinations are very different from one variety to another. The amount of carotenoids increases with colour intensity, the dark orange variety being the richest one. The values we obtained are comparable to those indicated in literature (Goodwin 1980; Neamtu et al. 1981).

The separation and analysis of carotenoid pigments

The total saponified extracts obtained from the four varieties were separated using chromatographic methods (LC – Al_2O_3 , TLC - silica and magnesium oxide, HPLC-PDA) in order to identify the carotenoid pigments. The carotenoids separated and identified by HPLC and the percentages resulted from peak integration are presented in Table 2.

We observed that all the varieties contain the same pigments, but there are significant differences for the ratios between individual pigments.

The first observation is the existence of a direct relationship between the colour of the inflorescences and the

nature of carotenoids. The variety Double Esterel Orange (dark orange) is the richest in hydrocarbons and rubixanthin. We also observed a high content of lycopene (14%), and γ -carotene (12%). In Radio variety (orange), these two hydrocarbons have significant lower values: 5% for lycopene and 6% for γ -carotene. Bonbon Abricot variety contains important amounts of β -carotene, but very low amounts of γ -carotene and it is especially low in lycopene (0.57% from total carotenoids).

The lemon yellow variety, Double Esterel Jaune, does not contain lycopene and γ -carotene while α -carotene and β -carotene together represent 3% of total carotenoid contents.

The rubixanthin (3-hydroxy- β , ψ -carotene, a derivative of γ -carotene) content varies in the same direction as hydrocarbon contents. The percentages of γ -carotene and rubixanthin are directly proportional, but we cannot observe the same relationship between β -carotene and their monohydroxy derivative, β criptoxanthin. The intensity of the orange colour of *Calendula* is determined by the amount of lycopene, γ -carotene, β -carotene and rubixanthin; these pigments are responsible for the orange or even red colour of vegetal tissues.

We have found a wide range of oxygenated carotenoids in all varieties investigated. The most important ones, by

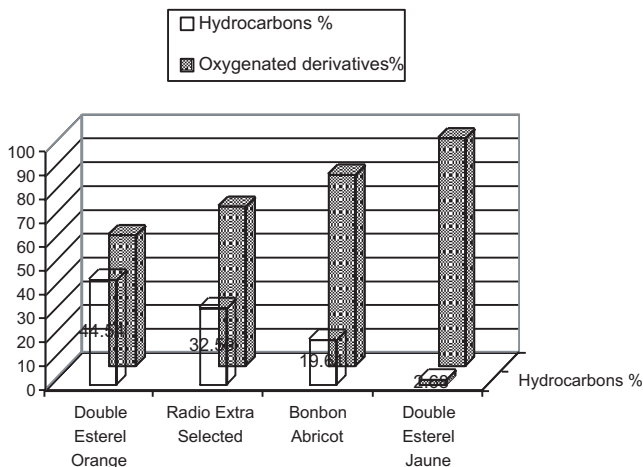


Figure 5. Hydrocarbons and oxygenated compounds content in different varieties of *Calendula*.

quantitative point of view, are the flavoxanthin and the lutein. Except for the rubixanthin, all varieties contain the same xanthophylls. The great majority of xanthophylls have a β - ϵ structure: flavoxanthin, lutein, luteoxanthin. Zeaxanthin (3,3'- dihydroxy- β , β - carotene) is present in small amount, as well as their epoxides: anteraxanthin, mutatoxanthin and auroxanthin.

The increasing percentage of the oxygenated compounds is accompanied by a decrease in hydrocarbons (Fig. 5). From a quantitative point of view, orange varieties are the richest in carotenoids and they contain both hydrocarbons and oxygenated derivatives.

Conclusion

All varieties of *Calendula* are rich in carotenoids. Double Esterel Orange and Radio varieties contain the most important amounts of pigments. The content and the distribution of carotenoids seem to be strongly influenced by the nature of biological material. The orange varieties contain important amounts of hydrocarbons, with provitamin A activity, while yellow varieties contain mainly oxygenated compounds. In the orange varieties, a preferential biosynthesis of hydrocarbons with ψ - ψ and β - ψ structure and of monoxanthophylls with β - ψ (or α - ψ) structure was noted.

The Double Esterel Orange variety has a special profile of carotenoids and contains a great amount of pigments. Their composition recommends this variety for using in pharmacological products designated to the skin protection.

Acknowledgments

This research was funded by grant 39/583 from the Romanian National Committee for Sciences Innovation and Technology. We want to thank to Dr. George Britton for his valuable advices.

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