HPLC ANALYSIS OF CAROTENOIDS FROM *INULA HELENIUM* L. FLOWERS AND LEAVES

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Abstract

Inula helenium L. (elecampane), *Asteraceae*, is a perrenial herb mostly known for the high content of volatile oil rich in sesquiterpene lactones. The aim of our study was to determine the profile and the carotenoids composition of the aerial part (inflorescences and leaves). The separation and quantification of carotenoids was performed by high-performance liquid chromatography (HPLC), on a reversed-phase C18 column and photodiode array detector.

The carotenoid content of *Inula helenium* L. was determined in leaves, inflorescences, tubular flowers and ligulate flowers extracts, after solvent extraction and saponification. The carotenoid content varies from 4.87 mg % in leaves to 47.7 mg % in ligulate flowers. In ligulate flowers the major compound was lutein-5,6-epoxide, representing 73.8% of the total carotenoids. Tubular flowers contain higher amounts of antheraxanthin (36.9%) and lutein (28.4%), while in leaves β-carotene and lutein together represent more than 72% of the total carotenoids. All the aerial parts of the plant contain low amounts of neoxanthin and violaxanthin.

Rezumat

Inula helenium L. (iarba-mare), *Asteraceae*, este o plantă perenă cunoscută îndeosebi pentru conţinutul ridicat în ulei volatil bogat în lactone sesquiterpenice. Studiul de faţă a avut ca obiectiv determinarea profilului şi conţinutului în carotenoide a părţilor aeriene ale plantei (frunze şi inflorescenţe), în scopul valorificării terapeutice. Separarea şi cuantificarea carotenoidelor s-a efectuat prin cromatografie de lichide de înaltă performanţă (HPLC), pe o coloană cu fază inversă C18 şi detector cu şir de fotodiode.

Conţinutul în carotenoide al speciei *Inula helenium* a fost determinat în extracte de frunze, inflorescenţe, flori tubuloase şi flori ligulate după extracţie cu solvenţi şi saponificare. Concentraţia de carotenoide variază între 4,87 mg/100g în frunze şi 47,7 mg/100 g în flori ligulate. În florile ligulate compusul major este lutein-5,6-epoxidul, care reprezintă 73,8% din totalul de pigmenţi carotenoidici. Florile tubuloase conţin cantităţi mai ridicate de anteraxantină (36,9%) şi luteină (28,4%), în timp ce în frunze, β-carotina şi luteina împreună reprezintă peste 72% din totalul de carotenoide. În toate probele analizate au mai fost prezenţi, în cantităţi mai reduse, neoxantina şi violaxantina.

Keywords: *Inula helenium* L., carotenoids, HPLC-PDA

Introduction

Inula helenium L. (elecampane), *Asteraceae*, is a widely distributed perennial herb in Europe [2]. Its roots are known to contain up to 3% volatile oil rich in sesquiterpene lactones particularly eudesmanolide type such as alantolactone and isoalantolactone [14,17]. In our knowledge, there is only one reference regarding the carotenoids in *Inula*, which reports the presence of helenien (lutein dipalmitate) in the inflorescences [1]. The aerial part of *Inula helenium* L. is less studied, therefore in this study we aimed to determine the carotenoid content in flowers and leaves, by high-performance liquid chromatography.

Carotenoids are liphophilic compounds which belong to the class of isoprenoids. More than 600 naturally occuring carotenoids are known and they are derived from the common C40 isoprenoid skeleton. Carotenoids are produced by photosynthetic organisms, and in plants they are synthesized in both chloroplasts and chromoplasts, imparting color to photosynthetic tissues as well as fruits, storage organs and flowers [5,18]. Together with flavones and anthocyanins, carotenoids are responsible for petal colors. In petals, carotenoids are responsible for the yellow to orange color. The color and the shade is strongly influenced by the chemical nature of carotenoid pigments.

Carotenoids play a very important role in human health and nutrition. Some carotenoids have pro-vitamin A activity (β-carotene, β-cryptoxanthin) and the dietary intake of carotenoids has been inversely associated with the risk of different types of cancer and cardiovascular diseases. Carotenoids are also known as efficient lipophilic antioxidants acting as singlet oxygen quenchers and as scavengers of reactive oxygen intermediates. Lutein and zeaxanthin are present in the human retina where they act as filter pigments, thus preventing light-associated damage, such as the development of age-related macular degeneration and cataract [12, 15].

Materials and Methods

Plant material: the plant material consisting of leaves from the medial part of the stem and inflorescences were harvested at the beginning of August 2009 from the Collection of Medicinal Plants of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. Inflorescences were separated into ligulate flowers and tubular flowers. Samples were stored at -18ºC.

Extraction

The plant material was ground in an Ultraturax homogenizer and extracted with a mixture of ethyl acetate:methanol:petroleum ether (1:1:1/v/v) [3]. Extraction was carried out under continuous agitation, in diminished light, in the presence of buthylated hydroxytoluene as antioxidant and of sodium bicarbonate, added for the prevention of epoxidic rearrangements. The extraction was repeated until the material became colorless. The combined extracts were filtered and washed in a separation funnel with 5% NaCl solution. The upper phase, consisting in ethyl acetate and petroleum ether was then evaporated to dryness in a rotary evaporator (Heidolph), at 35ºC. The residue was dissolved in diethyl-ether for saponification and spectrophotometric quantification of carotenoids.

Saponification

Saponification is necessary to release carotenoids from their ester forms and to remove the saponifiable lipids. The diethyl-ether carotenoid extract was treated with an equal volume of 30% w/v KOH in methanol. Saponification was carried out in the dark and under permanent stirring, for 8 hours. The extract was washed with 5% w/v NaCl solution until the pH of the aqueous phase was neutral. The diethyl-ether fraction containing the carotenoids was separated, evaporated to dryness and kept at – 20 ºC until further chromatographic analysis.

Spectrophotometric determination of total carotenoids

The concentration of total carotenoids was calculated using the following relation: X= (A x Y x 1000)/ (2500 x 100), were: A= absorbance at λ max = 450 nm, $A^{1\%}_{1cm}$ = 2500, specific absorbance coefficient of colored carotenoids), X = weight of carotenoids in the sample (mg), Y = volume of the sample, mL [4].

HPLC analysis

The separation was performed on a HPLC system Shimadzu LC20 AT, a Hibar 250 – 4 Lichosorb RP18 (25 cm x 4,6 mm; 5 µm) column and a SPD – M20A Photodiode Array Detector.

The mobile phase consisted of solvent A: acetonitrile – water (9:1; v/v) + 0.5 % EPA (ethyl-isopropyl-amine) and solvent B: ethyl-acetate + 0.5% EPA.

The gradient started with 15 % B and increased to 60 % B at 25 min, decreased at 15 % at minute 27 and continued isocratically up to 32 minutes. The flow rate was 1 mL/min and the chromatogram was monitored at 450 nm.

Identification of carotenoids was made by comparing the retention times of sample compounds with retention times of available standard

compounds and according to UV-VIS absorption spectra recorded with PDA detector for not available carotenoids (lutein-5,6-epoxide and antheraxanthin). Carotenoid standards (β-carotene, β-cryptoxanthin and lutein) were kindly provided by Dr. George Britton. The purity of these standards was estimated by HPLC and was: 95% - β-carotene, 97.7% - β-cryptoxanthin and 98.5% - lutein. Neoxanthin and violaxanthin were purchased from LGC Standards, UK.

The quantitative analysis was performed using calibration curves. The calibration curves for lutein and β-carotene were obtained in the concentration range of 0-200 μg/mL, by plotting the peak area recorded on PDA *versus* the concentration. The linear regression factor of the calibration curves was higher than 0.98. All chemicals were of analytical grade or HPLC grade and provided by Merck KGaA, Darmstadt, Germany.

Results and Discussion

Quantitative analysis

The total carotenoids content of the plant samples were determined by UV-VIS spectrophotometry, in the saponified extract [3]. Our results showed that the carotenoids content is highly variable in aerial parts of the plant. The highest carotenoids content was found in ligulate flowers 47.7mg/100g wet plant material. The carotenoids concentrations in the wet plant material are presented in Table I.

Table I
Total carotenoid content in *Inula helenium*

No.	Sample	Total carotenoid content (mg/100 g wet plant material)
1	Ligulate flowers	47.7
2	Tubular flowers	9.9
3	Leaves	4.87
4	Inflorescences	11.78

Quantitative analysis performed by the same method revealed concentrations of 31 mg/100 g and 110 mg/100 g wet plant material in the yellow inflorescences of *Solidago canadensis* L. (*Asteraceae*), and respectively *Chelidonium majus* L. (*Papaveraceae*) [8].

Qualitative analysis

HPLC-PDA analysis allows the separation of seven main carotenoids in the aerial part of *Inula helenium*. Their retention time and spectral characteristics are presented in Table II. Absorption maxima of

identified carotenoids were in good agreement with values reported in literature [4, 5]. The profile of carotenoids was different in the analyzed samples.

Table II

VIS spectroscopic data of carotenoids identified in *Inula helenium*

No.	Identified compounds	Retention time (min)	Observed absorption maxima (HPLC)	Reported absorption maxima*	Ratio III/II [4, 5] **
1	**Neoxanthin**	7.29	416, 438, 467	415, 439, 467	80 %
2	**Violaxanthin**	7.67	418, 441, 472	419, 440, 470	95 %
3	**Lutein-5,6-epoxide**	9.08	418, 440, 471	419, 440, 470	85 %
4	**Antheraxantin**	10.15	422, 445, 472	422, 447, 475	55 %
5	**Lutein**	10.97	422, 446, 475	422, 445, 474	60 %
6	**β-cryptoxanthin**	16.64	428, 450, 474	428, 450, 478	27 %
7	**β-carotene**	19.465	426, 452, 480	425, 450, 478	25 %

*Reported in references [4, 5]

**Spectral fine structure of carotenoids is expressed as the ratio of peak heights III/II as percentage (% III/II).The peak height of the longest-wavelenght absorption band is designated as III and the peak height of the middle absorption band as II.

Ligulate flowers are characterized by a very high content of lutein-5,6-epoxide - 73.8%. Lutein, neoxanthin and violaxanthin were also found but in significantly lower amounts. Ligulate flowers do not contain β-carotene derivatives, β-carotene, β-cryptoxanthin or zeaxanthin (Table III). A similar profile was described for *Chelidonium majus* inflorescences, which were reported to contain (all-E)-lutein-5,6-epoxide at 69.7% of total carotenoids, but also 11% Z-isomers of lutein-5,6-epoxide [8]. A very detailed study showed that in petals of chrysanthemum (*Dendrathema grandiflorum* (Ramat.) Kitamura), lutein-5,6-epoxide occurs in the form of eight geometrical isomers, accounting for almost 50% of total carotenoids [10]. Using a C30 column, Melendez-Martinez *et al*. isolated six geometrical isomers of lutein epoxide from dandelion (*Taraxacum officinale*). Lutein-5,6-epoxide, also known as taraxanthin, can be present in the esterified form, with palmitic or stearic acid [13].

Tubular flowers contain higher amounts of antheraxanthin (36.9%) and lutein (28.4%), but also lutein-5,6-epoxide (17%). One can notice the presence of antheraxanthin, a β-carotene derivative (Table III). It is known that antheraxanthin is present in free and esterified forms in anthers and petals of flowers [4, 5, 18]. However, β-carotene was present only in trace amounts.

Typical green tissues carotenoids were identified in the extract of leaves: neoxanthin, violaxanthin, lutein, β-cryptoxanthin and β-carotene [4]. The major carotenoids in leaves were lutein and β-carotene, with 33.8% and respectively 38.7%. The analysis of whole inflorescences, including bracts, showed the presence of major epoxides: lutein-5,6-epoxides, antheraxanthin, neoxanthin and violaxanthin. Lutein, a dihidroxy carotenoid was also well represented in important amounts. Due to the bracts, in the extract of inflorescences β-carotene could also be quantified (Table III). It would be interesting to evaluate a crude, non-saponified extract, from *Inula helenium* flowers, in order to verify the presence of esterified forms of xanthophylls.

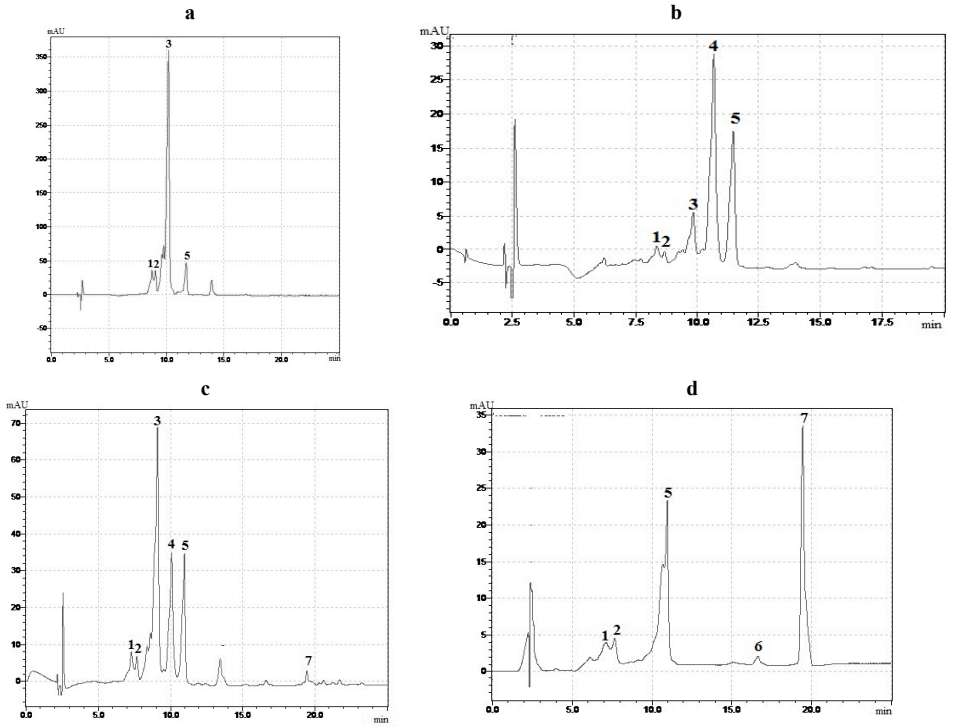

Figure 1

The HPLC chromatograms of the extracts of ligulate flowers (**a**), tubular flowers (**b**), inflorescences (**c**), leaves (**d**) of *Inula helenium* L. For peak numbers see Table III.

Table III

The concentration of carotenoids in samples (HPLC assay)

No.	Compound	Ligulate flowers (µg/g)	Tubular flowers (µg/g)	Inflorescences (µg/g)	Leaves (µg/g)
1	**Neoxanthin**	30.16	5.74	18.25	5.21
2	**Violaxanthin**	21.28	4.40	7.48	6.08
3	**Lutein-5,6-epoxide**	352.13	17.24	34.43	-
4	**Anteraxanthin**	-	36.56	20.29	-
5	**Lutein**	38.08	28.11	18.53	16.46
6	**β-criptoxanthin**	-	-	0.44	0.63
7	**β–carotene**	-	-	1.33	18.84
	Total	**441.62**	**92.34**	**100.75**	**47.22**

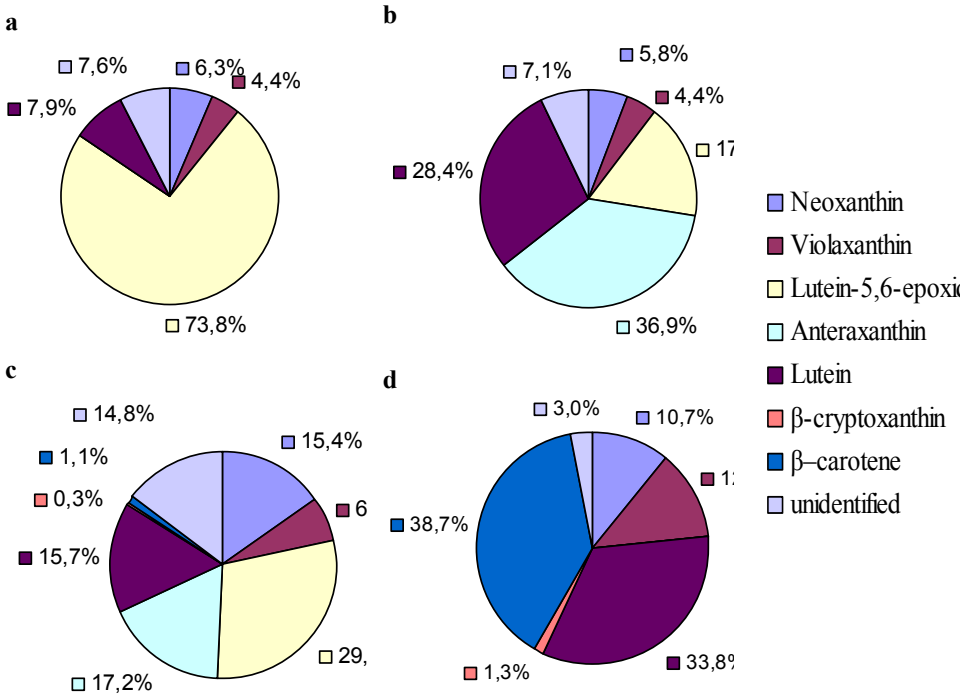

Figure 2

Carotenoid percentage in ligulate flowers (**a**), tubular flowers (**b**), inflorescences (**c**), leaves (**d**) of *Inula helenium* L.

The carotenoid profile in petals depends on the plant species. There are plants which preferentially accumulate α-carotene derivatives (lutein, lutein epoxide, etc.), like *Tagetes erecta*, as well as species producing β-carotene derivatives (zeaxanthin, antheraxanthin etc.) [5, 6]. Even in the

same plant species, there are important differences in carotenoid composition between different cultivars, as demonstrated for *Calendula officinalis* L. Yellow cultivars contain mainly flavoxanthin, while orange varieties contain a higher proportion of γ-carotene, lycopene and rubixanthin [11].

Conclusions

Inula helenium is an important source of carotenoids. Carotenoid content varies from 4.87 mg/100 g in leaves to 47.7 mg/100 g in ligulate flowers. The main components are lutein-5,6-epoxide in ligulate flowers (73.8%) and inflorescences (29.2%) and lutein in tubular flowers (28.4%) and leaves (33.4%). Considering ligulate flowers, we can assert that *Inula helenium* belongs to the class of plants specialized for the biosynthesis of α-carotene derivatives. Also, due to the high content of lutein-5,6-epoxide, *Inula helenium* petals can be used as a source of this reference compound.

Further investigations, using advanced spectroscopic methods, are needed in order to confirm the structure of carotenoid pigments in this plant.

References

1. Auster F., Schäfer J., Arzneipflanzen. *Inula helenium* L., VEB Thieme, 1955
2. Ball P.W., Tutin T.G., Flora Europaea, Vol.4. Cambridge: Cambridge University Press, 1976, 133–136
3. Breithaupt D.E., Schwack W., Determination of free and bound carotenoids in paprika (*Capsicum annuum* L.) by LC/MS, *Eur Food Res Technol,* 2000, 211, 52-55
4. Britton G., Liaaen-Jensen S., Pfander H., Carotenoids, Volume 1A: Isolation and Analysis, Birkhäuser Verlag, Basel, Boston, Berlin, 1995
5. Britton G., Liaaen-Jensen S., Pfander H., Carotenoids – Handbook, Birkhäuser Verlag, Basel, Boston, Berlin, 2004
6. Goodwin T.W (Ed.)., Plant Pigments, Academic Press, London, 1988
7. Hanganu D., Pintea A., Mărculescu A., Toma C., Mirel S., Chemical research of carotenoids from *Anthemis tinctoria* L. (Asteraceae), *Farmacia*, 2008, LVI(3), 88-95
8. Horvath G., Molnar P., Farkas A., Szabo L.G., Turcsi E., Deli J., Separation and identification of carotenoids in flowers of *Chelidonium majus* L. and inflorescences of *Solidago canadensis* L., *Chromatographia*, 2010, Suppl. Vol. 71, S103-S108
9. Ignasiak T., Lesins K., Carotenoids in petals of some perennial *Medicago* species, *Biochemical Systematics and Ecology*, 1973, 1(2), 97-100
10. Kishimoto S., Maoka T., Nakayama M., Ohmiya A., Carotenoid composition in petals of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura), *Phytochemistry*, 2004, 65(20), 2781-2787
11. Kishimoto S., Maoka T., Sumitomo K., Ohmiya A., Analysis of carotenoid composition in petals of calendula (*Calendula officinalis* L.), *Biosci. Biotechnol. Biochem*., 2005, 69(11), 2122-2128
12. Krinsky N.I., Johnson E. J., Carotenoid actions and their relation with health and diseases, *Molecular Aspects of Medicine*, 2005, 26, 459-516
13. Melendez-Martinez A.J., Britton G., Vicario I.M., HPLC analysis of geometrical isomers of lutein epoxide isolated from dandelion (*Taraxacum officinale* F. Weber ex Wiggers). *Phytochemistry*, 2006, 67(8), 771-777

14. Nan M., Veres K., Hohmann J., TLC-densitometric determination of sesquiterpene lactones from *Inula helenium* roots. *Acta Medica Marisiensis*, 2010, 56(3), 269-271
15. Stahl W., Sies H., Bioactivity and protective effects of natural carotenoids, *Biochim. Biophys. Acta*, 2005, 1740, 101-107
16. Tai C.Y., Chen B.H., Analysis and stability of carotenoids in the flowers of daylily (*Hemerocallis disticha*) as affected by various treatments. *J. Agric. Food. Chem*., 2000, 48(12), 5962-5968
17. Toiu A., Vlase L., Oniga I., Benedec D., Tămaş M., HPLC analysis of salicylic derivatives from natural products. *Farmacia*, 2011, 59(1), 106-112
18. Tămaş V., Neamţu G., Pigmenţi carotenoidici şi metaboliţi, Vol I, II, Editura Ceres, Bucureşti, 1986

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