Approach for Analyse Stability of Lutein from *Tropaeolum majus*

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The main goal of the study was to reveal the carotenoid antioxidants by spectrophotometric methods in order to identify and determine nasturtium petals (Tropaeolum majus) lutein through extinction coefficients known or experimentally estimated from calibration curves (solubilised in diethyl ether). The selection of extraction solvents, doses, and lutein recovery confirm efficacy of diethyl ether (lutein extractability is 60%). To establish diethyl ether efficacy in lutein conditioning, the stability of diethyl ether extracts was tested. The study of preservation schedules for antioxidant lutein was highlighted by out parameters determining stability of lutein, i.e. a temperature of 0°C. The results revealed that spectral method combined with spectrophotometric analysis techniques can provide efficient, accurate information for routine monitoring of carotenoid compounds as quality indicators for identifying and quantification lutein.

Keywords: lutein, nasturtium, stability test, spectral and spectrophotometric analysis

Carotenoids are an organic pigment that are found in chloroplasts/chromoplasts of plants and photosynthetic organisms and is polyisoprenic compounds made up of isoprene units (eight units, forty carbon atoms) [1, 2]. Carotenoid pigments play a role in photosynthesis [3] and in protection of self-photo-destruction of chlorophyll molecules [4] and other active substances [5, 6]. This photo-protection process can be achieved by carotenoid compounds that are associated with chlorophylls [7; 8], since they can fix oxygen forming unstable oxygenated compounds [9], through oxydoreduction processes.

Carotenoids can also form intermediary metabolites that stimulate or inhibit plant development [10], which is important for their activity in the photo-tropes and photoaxes [11].

Lutein is one of most valuable antioxidants [12-16]: its antioxidant activity is superior to that of tocopherol [17], ascorbic acid, â–carotene, lycopene [18]. Lutein has same chromophore and same spectrum as its parent carotenoid, β-carotene [19]. Lutein differs of zeaxanthin only in location of one of terminal conjugated double bonds [20]; however, chromatographic separation of these compounds [21], while difficult, it protects oxidation photosynthesis apparatus [22, 23]. In organic solutions, lutein is a trap for singlet oxygen, an inhibitor of peroxyl radicals that result by lipid oxidation [24].

The study was focused on overview of specific metabolic profiles of nasturtium petals's lutein. Was found that chromatography gives qualitative and quantitative information related to individual molecules. Such complementary procedures have a good performance/price ratio, and may be used either in laboratories (food, phytopharmaceuticals' control) or in food research in order to evaluate their quality, authenticity, traceability in production and marketing chain. A spectrum scan has not been seized in this procedure and the structure of a mix of carotenoids can only be detected after HPLC separation.

The aim of the study was the obtaining of lutein in high yields purities, isolating xanthophylls of interfering materials such as waxes, oils, and fats.

Experimental part

Tissue extraction by nasturtium petals.

To prepare a homogeneous, representative sample for investigations and to facilitate extraction, nasturtium petals samples have been cut into small pieces or minced. Lutein distribution in nasturtium petals was quantified by extraction in different solvents. Lutein extraction was done through direct saponification with a solution of 30 % KOH in diethyl ether. Was used a PG Instruments UV–VIS spectrophotometer. For extraction, analytical purity solvents have been used. In the solvents ascorbic acid was added [4].

Extraction and saponification.

To get carotenoid extract (lutein), a quantity of 50.00 g of nasturtium petals sample was weighed and subjected it to solvent extraction, by adding 0.2 g of ascorbic acid to prevent carotenoid oxidation. The extractions were repeated on several times with fresh portions of solvents until material was exhausted [6]. Reunited extracts were concentrated at 35°C in a rotary evaporator (for the efficient and gentle removal of solvents from samples by evaporation). Latter extracts were subjected to saponification with 30 mL of solution of KOH 15 % in diethyl ether at room temperature in dark schedules for 16 h. Non-saponifiable fraction was then extracted with petroleum ether, then washed several times with a concentrated solution of NaCl and then with water until complete removal of soaps and alkali. An aliquot of 1 mL was drawn of reaction mixture every 1 h and the sample was analysed by HPLC to determine completion of saponification [7] which is indicated by complete disappearance of lutein ester peaks. After saponification reaction the mixture was

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cooled to about -50–60°C and neutralized with a 10–30 % aqueous acetic acid. Then 50–60 mL of distilled water were added to reaction mixture and temperature increased to about 60–70°C; the mixture was stirred continuously for a period of 15–30 min. Resultant mixture was then centrifuged for a period of 100–200 min in a tubular centrifuge, centrifugation continued for 2–3 cycles with continuous replenishment of distilled water until supernatant became clear. Precipitate was collected, washed with warm distilled water 2–3 times to eradicate impurities and dried under vacuum for 3 h to produce a fine crystalline powder.

Chemical tests

Iodine-catalysed cis-trans Isomerization: Few crystals of iodine were dissolved in petroleum ether and the spectrum of carotenoid recorded and a drop of iodine solution was added too. The spectrum was measured after 1–5 min. of exposure to light.

This reaction can be done directly in spectrophotometer cuvette. An λmax quantities of trans carotenoids will shift 3–5 nm to a lower wavelength whereas those of cis carotenoids will shift by same quantity to longer wavelengths. The 9-cis-β-Carotene does not change λmax [4]. Lutein extract was dissolved in diethyl ether and subjected to spectrophotometric investigations (445/ 474 nm). Dosing total lutein was done spectrophotometrically.

$$\text{Lutein } (\mu g/g) = A \cdot V \cdot 10^4 / A^{1\%}_{1cm} \cdot m \quad (1)$$

Calculation was done according with relation (1) where: A–absorbance; V–total extract volume (mL); $A^{1\%}_{1cm}$ –lutein absorption coefficient in solvents; m–sample mass (g).

Stabilising lutein diethyl ether extracts

Upon initiation the study selection of solvent for lutein extraction was made. The solvent quality for later conditioning was taken in account. The diethyl ether lutein extracts stability was made and latter ascorbic acid (pro-oxidant) to nasturtium petals sample was added [3].

HPLC analyses

Analytic procedures for carotenoids are chromatographic technique into open-column or HPLC methods. Chromatographic schedules for carotenoid determination were executed according to standard procedure with certain modifications: instrument HP1090.

HPLC procedure has been developed for separation/ quantification of lutein.

Experimental schedules selected for test are a binary gradient system based on acetonitrile-containing solvents; a non-endcapped C_{18}-column is used for chromatography of obtained extracts. Pigments are detected by monitoring absorbance at 445 nm, by on-line recorded absorption spectra with, flow-rate 3 mL/min. On-line coupling is suitable for differentiation/identification of carotenoids (similar carotenoids) [6].

Results and discussions

Tissue extraction

In *T. majus*, xanthophylls are mostly found in nasturtium petals. In order to break cell wall and obtain experimental compounds, acid hydrolysis procedure was applied (alkaline hydrolysis can cause irreversible degradation of lutein) [6].

Extraction of lutein carotenoid

Lutein extractability reaches acceptable quantities of a biotechnological viewpoint only with self–clavation of *acid mixture. To* prevent lutein degradation through saponification diethyl ether was used. The saponification of lutein esters to yield free lutein may yield lutein in any ratio from 1:1 to 1:2 molar ratios with the saponifying fatty acid. Were used also organic acids 2N to achieve acid hydrolysis [9]. By this procedure, were extracted small quantities (only 4.8 %–17.2 %) of lutein accumulated in nasturtium petals.

$$\textit{Extractability (\%)} = \textit{(Free lutein } \mu g \cdot mL^{-1} / \textit{Total lutein } \mu g \cdot mL^{-1}) \; 100 \quad (2)$$

To test the efficacy of procedures were used repeatedly notion of lutein extractability. The quantity of extractability was appraised according to the formula (2) where "free lutein" is quantity of nasturtium petals sample lutein in diethyl ether following 1 h of stirring, and "total lutein" is quantity of nasturtium petals lutein obtained by diethyl ether extraction under identical schedules of same sample (fig. 1, λ_{max} were obtained at 445/ 474). Carotenoids are polyene compounds, which makes existence of *cis*/*trans* (E/Z) isomerism possible [7].

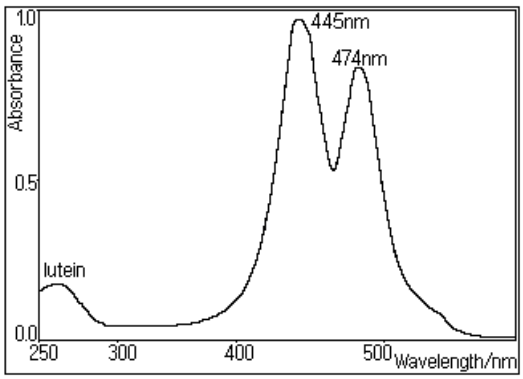

Fig. 1. Lutein spectrum, absorption spectrum of lutein solubilised in diethyl ether nasturtium petals sample

Lutein esters were quantified on basis of their respective molecular masses using lutein for calibration; total lutein was determined after saponification of aliquots of extracts.

The extraction of lutein and geometrical isomers is carried out in a homogenisation process with diethyl ether/ water (9:1, v/v) and leads to small extraction volumes without further quantity steps during sample preparation [12].

HPLC method of lutein carotenoid

Complete liberation of lutein esters to free or non–esterified forms is helpful to effectively isolate and concentrate lutein to a desired extent. To verify type and position of substituent's in xanthophylls, appropriate chemical reactions were made [23].

To verify geometric configuration iodine–catalyzed isomerization was completed. Their physical properties, especially wavelength shift in spectrum allow for their detection by HPLC with detector (fig. 2).

The dihydroxy carotenoid is reflected in its behavior on open–column and high-performance liquid chromatography methods (RF is around 0.21). Temperature regulation is recommended to maintain day-today reproducibility. Variations in column temperature result in substantial fluctuation of carotenoids' retention times. The presence nonallylic positions of these groups are shown by positive response to acetylation with acetic anhydride

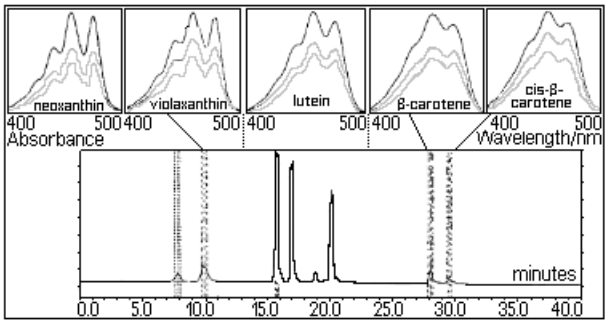

Fig. 2. HPLC chromatogram and photodiode array spectra of carotenoids, wavelength shift in spectrum allow for their detection by HPLC with detector

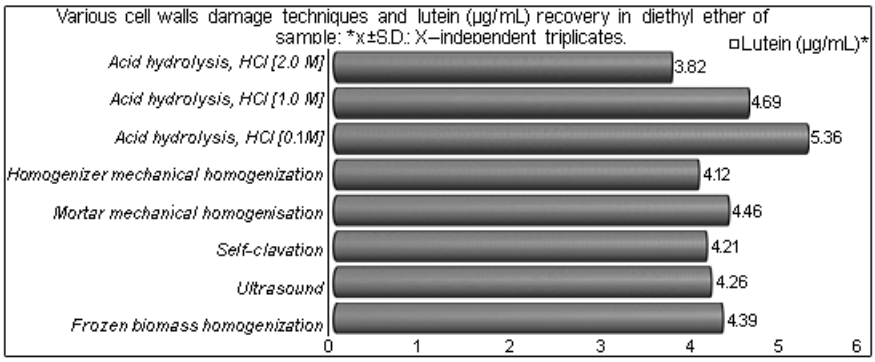

Fig. 3. Various cell walls damage techniques and lutein (µg/mL) recovery in diethyl ether of nasturtium petals, sample.

and negative response to methylation with acidified methanol, respectively. Partial acetylation will yield two acetylated products, corresponding to acetylation of only one of hydroxyl groups [5]. Complete acetylation yields one product with both hydroxyls acetylated. Most efficient procedure to break cell wall was found to be use of HCl (fig. 3.).

In diethyl ether, highest quantities of lutein quantity obtained were under acid hydrolysis of cell wall (5.36 µg/mL). Decline lutein recovery may be explained by profound damage of wall allowing lutein molecules to degrade by temperature impact (3.82 and 4.69 µg/mL in HCl 1N and 2N, respectively). Separation and quantification of lutein was done through spectrophotometric method which supposes extraction of liposoluble pigments, removal of chlorophylls and of hydroxy–carotenes, and then spectrophotometric determination of carotenes expressed as lutein (λ–445 nm) [24]. FDA Draft guideline recommends acceptable quantities of residual solvents in pharmaceuticals for safety of patients as well as use of less toxic solvents in manufacture of drug materials and dosage forms [2]. In spectrum, λ_{max} were obtained at 445/474 (422, 445 474) nm, that are characteristic absorption peaks of chemical structure of lutein. This dihydroxy carotenoid is reflected on its behaviour in HPLC (RF is ~0.21). Presence and non-allylic position of hydroxy groups are shown by its positive response to acetylation with acetic anhydride and negative response to methylation with acidified methanol, respectively.

Calibration curve

A standard lutein (purity > 98 %) was used for calibration curve. Calibration curve was recorded for quantity quantities of standard lutein in interval 1.0–8.0 µg/mL.

Then quantity of lutein was theoretically appraised, of initial absorbance of 1.443; of absorption coefficients A = 2480 or 2600 according to below equation (3) determined concentration of 6.8 µg/mL lutein. Calibration curve was done for recorded absorbance 445 nm, for which we know extinction coefficient A = 2480 or 2600. Lutein was detected on basis of absorbance/acceptance criteria, as described by Compendial Approvals for FCC, 2010 [3].

$$Lutein\ (\mu g/mL) = Absorbance \cdot 10.000/2540 \quad (3)$$

Calibration curve lutein for recorded absorbance 445 nm and absorption coefficients A=2480/2600. Establish calibration curves based on peak area for standard for lutein and use these curves to determine quantities. Appraised lutein quantity in diethyl ether, according to absorbance, using absorption coefficients, was 1.4 times higher than lutein quantity in diethyl ether appraised by calibration curve. Lutein quantity results are higher than those obtained under similar schedules, but appraised of calibration curve.

To solubilise lutein in diethyl ether, dissolved 10 mg of lutein in 0.5 mL of chloroform, and then matrix solution of lutein was set up by diethyl ether passage. Calibration curve was designed for quantities of lutein in diethyl ether within 0.5 to 4.0 µg/mL intervals (n=8), correlation coefficient being r^2 = 0.9896 for measurements made at 445 nm.

Selection conditions for stabilisation of diethyl ether extracts of lutein by nasturtium petals.

Factors that influenced stability of lutein are light and high temperature. Pure crystalline lutein degrades under influence of atmospheric oxygen and light. As for carotenoid extract in diethyl ether, optimal storing schedules were freezing ones. By adding ascorbic acid to lutein extract it lowered carotenoid oxidation process. Lutein quantity diminished with only 50% in 30 days. In stabilisation research, we used samples of 100 mL diethyl ether solution each with a quantity of 50 µg/mL of lutein that were stored in following schedules in different conditions. Quantitative test was repeated after one day, seven days, and 30 days of extraction. Room temperature oscillated between 18 and 22°C. Samples exposed to daylight were kept away of direct sunlight/artificial light. Ascorbic acid in quantity of 1.0–100 mg was added [4]. No matter schedules, lutein content during first storage days did not change at all practically, lutein quantity in samples was between 54.56 and 59.80 µg / mL.

After 30 days of storage, lutein quantity transformed. In samples stored at room temperature, lutein quantity decreased with 42 % in light schedules and with 32 % in darkness schedules. In ascorbic acid schedules, during 30 days, lutein content decreased with 29 % when it was kept in light and at room temperature. In diethyl ether solutions lutein with ascorbic acid was kept in dark schedules and thermal factor did not modified solution stability [24]. Both

samples of at lutein content remained at initial level for 0°C, +20°C.

Conclusions

The most efficient procedure of damaging cell walls was chemical one, with chlorhydric acid. Saponification may be necessary to eradicate lipids. Lutein in solution obeys Beer–Lambert law: absorbance is directly proportional to quantity. Thus, carotenoids are quantified spectrophotometrically, provided accurate absorption coefficients in desired solvent are available. The separation and dosing lutein by nasturtium petals was done through spectrophotometric method which supposes liposoluble pigment extraction, removal of chlorophylls and of hydroxy–carotenes and then determination of carotenes expressed as lutein spectrophotometrically at a wave length of 445 nm. Solvent effects on absorption are substantial. Tabulated absorption coefficients and ëmax quantities refer to single solvents. HPLC quantification is carried out by means of internal or external calibration, for which quantities of standards are also determined spectrophotometrically as in AOAC. The instability of lutein represented the main inconvenience. In case of lutein diethyl ether solutions without ascorbic acid, environmental temperature conditioned stability of tested solutions. No matter lighting schedules, lutein quantity was constant. Storage schedules at room temperature and with light accelerated process of pigment loss with 10 % compared to similar thermal schedules, but with no light whatsoever. For lutein diethyl ether solutions, stored for 30 days at room temperature is possible only by adding ascorbic acid and only with no light whatsoever. Storing lutein antioxidant in ethylic alcohol at a temperature of 0°C needs no ascorbic acid as stabilizing agent. Low light condition (avoiding direct lighting) was one of main factors for establishes lutein.

The lutein diethyl ether solution storage schedules pointed out determining parameters of lutein stability: a temperature of 0°C and/or presence of ascorbic acid. In lutein diethyl ether solution stored in darkness and at room temperature, ascorbic acid proved to be a stabiliser.

References

1.REZAEI, A. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 5.
2.GE, X.X. XING M.Y. YU, L.F. SHEN, P. Asian. Pac. J. Cancer. Prev., **14**, no. 3, 2013, p. 1911.
3.da COSTA, A. S.V.; HORN, A. H.; BILAL, E.; DE PINHO, A.A.; DONAGEMA, G.K.; SAMPAIO, R.A. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 13.
4.KOSEV, V.I. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 23.
5.TIAMIYU, L.O.; OKOMODA, V.T.; OYENIYI, M.E.; APEREGH, J. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 30.
6.NIKOLOVA, I. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 36.
7.BOVIER, E.R. LEWIS, R.D. HAMMOND, B.R.JR. Nutrients. **5**, no. 3, 2013, p. 750.
8.EED, A.M.; BURGOYNE, A.H. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 45.
9.BÖHM, F. EDGE, R. TRUSCOTT, T.G. Acta. Biochim. Pol., **59**, no. 1, 2012, p. 27.
10.JAHAN, S.; CHOWDHURY, S.F.; MITU, S.A.; SHAHRIAR, M.; BHUIYAN, M.A. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 61.
11.LI, S.Y. FUNG, F.K. FU, Z.J. WONG, D. CHAN, H.H. LO, A.C. Invest. Ophthalmol. Vis. Sci., **53**, no. 10, 2012, p. 5976.
12.KOSEV, V.; ILIEVA, A. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 54.
13.MAOKA, T. TOKUDA, H. SUZUKI, N. KATO, H. ETOH, H. Mar. Drugs., **10**, no. 6, 2012, p. 1391.
14.SAYO, T. SUGIYAMA, Y. INOUE, S. Biosci. Biotechnol. Biochem., **77**, no. 6, 2013, 1282.
15.KOUSHAN, K. RUSOVICI, R. LI, W. FERGUSON, L.R.; CHALAM, K.V. Nutrients. **5**, no. 5, 2013, p. 1823.
16.MIYAZAWA. T. NAKAGAWA, K. KIMURA. F. NAKASHIMA. Y. MARUYAMA, I. HIGUCHI, O. MIYAZAWA. T. J. Oleo. Sci., **62**, no. 10, 2013, 773.
17.HOSSAIN, M.M.; JAHAN, I. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 69.
18.TIAN, Y. KIJLSTRA, A. van der VEEN, R.L. MAKRIDAKI, M. MURRAY, I.J. BERENDSCHOT, T.T. PLoS One. **8**, no. 8, 2013, e73387.
19.GEORGIEVA, N. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 83.
20.WOO, T.T. LI, S.Y. LAI, W.W. WONG, D. LO, A.C. Graefes. Arch. Clin. Exp. Ophthalmol., **251**, no. 1, 2013, p. 41.
21.KUMAR, A.; SENAPATI, B.K. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 90.
22.PONGCHAROEN, S. WARNNISSORN, P. LE[a]TKAJORNSIN, O. LIMPEANCHOB, N. SUTHEERAWATTANANONDA, M. Biol. Res., **46**, no. 1, 2013, p. 39.
23.VASILEVA, V. Banats. J. Biotechnol., **6**, nr. 11, 2015, p. 100.
24.BUTNARIU, M. RODINO, S. PETRACHE, P. NEGOESCU, C. BUTU, M. Dig J Nanomater Bios., **9**, no 2. 2014, p. 745

Manuscript received: 26.11.2015