Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 2, April - June 2014, p. 745 - 755

DETERMINATION AND QUANTIFICATION OF MAIZE ZEAXANTHIN STABILITY

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Zeaxanthin was intended for use in foods and beverages as a colouring and as a nutritional supplement. The main goal of this article was the study of the carotenoid antioxidants by spectrophotometric methods, in order to identify and determine maize zeaxanthin by using known extinction coefficients or experimentally calculated from calibration curves. Zeaxanthin concentration in acetone, by means of the extinction coefficient of 2340, was 1.4 times higher than the one obtained from the calibration curve ($n = 8$, $r^2 = 1.000$). The carotenoid levels in maize presented a wide range, with the highest levels of zeaxanthin being 16.3 mg kg^{-1}. In order to establish ethanol efficiency in zeaxanthin conditioning, ethanol extracts were tested for their stability. The study of the selected conditions for zeaxanthin storage highlighted the determining parameters of zeaxanthin stability, meaning the temperature of 0°C. Qualitatively, zeaxanthin can be identified based on its characteristic spectrum intervals of carbon–carbon single bond (1156 cm^{-1}) and from the carbon–carbon double bond stretch vibrations (1657 cm^{-1}) of its molecule backbone (zeaxanthin characteristic bands).

(Received April 9, 2014; Accepted May 28, 2014)

Keywords: Carotenoids; zeaxanthin; Zea mays; Stability test; Spectrophotometric analysis

1. Introduction

From a chemical point of view, carotenoids are polyisoprenic compounds made up of isoprene units [1]. There are approximately 600 compounds in the carotenoid group, which can be classified into carotenes and xanthophylls [2].

Carotenoids prevent the damage caused by excessive irradiation activating directly the triplet chlorophyll (^{3}Chl) or the singlet oxygen (^{1}O2), compounds formed during photodynamic reactions [3], through a direct contact mechanism of carotenoids with photosensitive substances (chlorophyll) [1, 4, 5]. These compounds can also form intermediary metabolites that stimulate or inhibit plant development [6, 7], being important for their activity in photo-tropes and photoaxes [8].

The zeaxanthin belongs to xanthophylls group and has no provitamin A activity [9-12]. It is localised in cytoplasmic lipid globules [4], absorbs light in the blue area [13] and protects the photosynthetic apparatus from oxidation [14].

When the cells are not subjected to light excess, zeaxanthin globules concentrate around the nucleus [15]. This shows that zeaxanthin serves as a physico-chemical protection barrier, preventing the damage caused by free radicals to the lipids of the functional membranes, and to the

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DNA [16]. Another mechanism ensures the conversion into water of molecular oxygen, resulted from the photosynthesis, by including it in the plastoquinone desaturation reaction where the electron transfer and formation of zeaxanthin molecule take place [17]. The other mechanisms include the involvement of already synthesised zeaxanthin molecules that achieve their photo-protection function by reducing photon excess [18] functioning as antioxidants against oxygen reactive species and forming zeaxanthin esters that serve as carbon and energy sources [19]. The main biological functions of the zeaxanthin in the body include protection of cell components, including that of polyenic fatty acids, from the process of oxidation; formation of immune response; and regulation of gene triggering [5].

The zeaxanthin mobility ensures its ability to penetrate different structures of the body [20] and to mediate the generalised positive effect on numerous structures and functions [10].

The mechanism of the antioxidant action of zeaxanthin is similar to that of other carotenoids and is based on the absorption of reactive oxygen species along the oxidative chain [5]. Zeaxanthin inhibits peroxyl radicals in microsome systems and in unilamellar vesicles. It inactivates singlet oxygen [21] and inhibits photo-oxidation in unilamellar liposomes. As an antioxidant, it is superior to other types of antioxidants used to prevent oxidation of polyenic fatty acids with formation of peroxides and degradation of cell membranes [22]. The superiority of the antioxidant effect of zeaxanthin in the prevention of lipid oxidation is independent of the nature of the pro–oxidant factor [23]. In organic solutions, zeaxanthin is a trap for the singlet oxygen and an inhibitor of peroxyl radicals that result from lipid oxidation [24, 25].

2. Material and methods

2.1 Tissue extraction

In order to prepare a homogeneous, representative sample for analysis and to facilitate the extraction, the samples have been cut into small pieces or minced. Extraction followed without delay, because tissue disruption releases catabolic enzymes, (e.g., lipoxygenase) which biodegrade zeaxanthin through oxidation, and acids that promote *trans–cis* isomerization. Actually, sample maceration, homogenization, and extraction with an organic solvent have been carried out simultaneously. Maize kernels were studied in order to establish their carotenoid profile. Zeaxanthin distribution in maize was quantified by extraction in different solvents. The extraction was realised by direct saponification with a solution of 30% KOH in ethanol. PG Instruments, UV–VIS spectrophotometer and UV WIN 5.05 software were used to measure extracts absorbance (to determine total content and reference solutions concentration) [26].

Analytical purity solvents were used for extraction and ascorbic acid was added to solvents. The chemicals used were: hexane, acetonitrile, and acetone UV; chloroform, spectrophotometric grade methanol, L–Ascorbic acid, Sigma–Aldrich GmbH. (Germany); Absolute ethanol (USP–grade ethyl alcohol, 64–17–5) purchased from Warner–Graham Co. (Cockeysville, Maryland); Standard zeaxanthin, Product No. 14681, CAS 144–68–3 from Fluka, Buchs, Switzerland.

2.2 Extraction and saponification

In order to obtain carotenoid extract (zeaxanthin), 50.00 g of the maize sample was subjected to solvent extraction, by addition of 0.2g ascorbic acid (antioxidant). The extraction was repeated several times with additional fresh volumes of solvents until the material was exhausted [27]. Joined extracts were void concentrated at 35°C in a refrigerated vapour trap, (Model Savant SpeedVac RVT400), until soft consistency. Subsequently, the extract was subjected to saponification with 30 mL of solution of KOH (15%) in ethanol (96%), in darkness, at room temperature, for a period of 16 h.

The non-saponifiable fraction was extracted with petroleum ether and washed several times with a concentrated solution of sodium chloride and further rinsed with water until complete removal of soaps and alkali. From the reaction mixture, an aliquot of 1 mL was drawn every 1h

and analysed by HPLC to determine saponification completion (indicated by complete disappearance of zeaxanthin ester peaks) [28]. After saponification, the reaction mixture was neutralized with 10–30% aqueous acetic acid and distilled water was added to this reaction mixture while the temperature was increased to 70°C stirring continuously for 15 - 30 min. The mixture was centrifuged 150 min. at 12500 RPM in a tubular centrifuge, with continuous replenishment of distilled water, 2–3 cycles, until clear supernatant. The precipitate was collected and washed with warm distilled water 2–3 times to remove impurities and finally dried under vacuum for 3 hours, resulting a fine crystalline powder. Each experiment was conducted in triplicates [29].

2.3 Chemical Tests

The zeaxanthin extract was dissolved in ethyl acetate and subjected to spectrophotometric analysis [30, 31].

Quantification of total zeaxanthin was done spectrophotometrically, based on equation (1)

$$\text{zeaxanthin } (\mu g/g) = A \cdot V \cdot 104 / A \cdot m \quad (1)$$

where: A – absorbance; V – total extracts volume (mL); extinction coefficient of E1% of 2540 zeaxanthin in the solvents; m – sample mass (g).

2.4 Stabilising zeaxanthin ethanol extracts

Upon initiation of the present study, the selection of zeaxanthin extraction solvent was taken in consideration for further evaluations [27]. Experiments were carried out on the stability of ethanol zeaxanthin extracts [31] and of those supplemented with ascorbic acid.

2.5 HPLC Analyses

Carotenoids analytic methods are classified by chromatographic technique such as open-column (OCC) and high–performance liquid chromatography (HPLC) methods. The method used in the present study was the state-of the art procedure for each step performed along the experimental process. Chromatographic steps for carotenoid determination were performed with Instrument: LC HP1090, Hewlett-Packard; Detector: DAD, Wave length: 440 ± 4 nm; Injection volume: 10 µL; Column C18, 3.0 × 250 mm, particle size 5 µg, 250 mm. times. 4.6 mm particles; Flow rate: 1.5 mL/min; Mobile phase: A: acetone–water (75:25, v/v); B: acetone–methanol (75:25, v/v); Gradient: from 0 to 25% B in 10 min, from 25 to 100% B in 35 min, 100% B in 45 min and 0% B in 65 min Post time: 15 min. A HPLC method was developed for the separation and quantification of zeaxanthin. The selected experimental conditions represented a binary gradient system based on acetonitrile–containing solvents and a non-endcapped C18–RP–column was used for chromatography of the experimental extracts. Pigments were detected by monitoring absorbance at 440 nm and identified by their on–line recorded absorption spectra at flow–rate 3 mL/min and outlet pressure 15MPa. On–line coupling is suitable for the differentiation and identification of carotenoids, especially the similar carotenoids (lutein/zeaxanthin) [21, 32, 33].

2.6 Spectral analysis

The absorption spectra mid–IR (MIR) was recorded using a FTIR spectrophotometer (IR–Prestige, Shimadzu Europa GmbH, Duisburg, Germany) equipped with a DLATGS detector. Spectral resolution was 4 cm^{-1} and for each spectrum were gathered 128 recordings. The samples were analysed using an ATR accessory with 10 consecutive reflexions (Pike Technology, Madison, USA). The spectrometer was interfaced to a cooled charge-coupled device camera that is controlled by a Window based (Microsoft, Redmond, WA) custom software package.

2.7 Statistical analysis

The values were expressed as mean ± S.D. Statistical significance was evaluated by Students–"t" test at 5% level of significance ($p < 0.05$). The tests were performed using Sigma-Stat software (Jandel Scientific Software, San Rafael, CA).

3. Results and discussion

3.1 Extraction of zeaxanthin carotenoid

Zeaxanthin extractability reaches acceptable values from a biotechnological point of view (up to 85%) only with self–clavation of alkaline mixture. It was used a mixture of NaOH with methanol to prevent zeaxanthin degradation through saponification, and organic acids (citric, acetic, formic) 2N to achieve acid hydrolysis. By this method, small amounts (only 3.4%–19.4%) of the zeaxanthin accumulated in the maize sample were extracted. To monitor the efficiency of the tested methods, it was used the notion of zeaxanthin extractability.

3.2 Determination of Zeaxanthin Content (Wt %) by HPLC in maize sample

The analyses were performed on a separation system using a version of ChemStation software installed on the data station. The chromatographic separation was performed on a reverse-phase column (C18, particle size 5 μg, 250 mm x 4.6 mm). The eluent was a ternary gradient of methanol/water/acetone (M: W: A) at 1.0 mL/min., with initial composition of M: W: A (0:25:75, v/v/v). An initial linear gradient was applied for 15 minutes and yielded a composition of M: W: A (20:5:75, v/v/v). This composition was held for 15 minutes, followed by another linear gradient for 5 minutes to yield a composition of M:W:A (0:0:100, v/v/v) and held for 5 min. Another linear gradient was applied for 5 minutes to initial conditions and held for 15 minutes before next injection. The compounds were detected photometrically (between 400–600 nm) on a photodiode array detector using an injection volume of 20 μL.

Zeaxanthin content was measured in reference to a calibration curve generated from a purchased authentic sample. Trans–zeaxanthin obtained from Fluka was dissolved in 90% acetone/10% acetone containing 6% glacial acetic acid. This stock solution was diluted in acetone and run on a spectrophotometer at 452 nm. This extinction coefficient was used with an E1% of 2540 to calculate the concentration of stock solution. The stock solution was then diluted with acetone to generate a 5-point external calibration curve covering concentrations ranging from 1.0–8.0 μg/mL with a linear fit. 9–cis–zeaxanthin was quantified using the trans–zeaxanthin calibration curve, assuming a response factor of 1:1. A system check sample (zeaxanthin 20%) was run on the day of analysis at a level between 25.0–45.0 μg /mL.

The results were corrected only if check sample was not within 5% of the expected value. The carotenoid levels had a wide range in the maize sample, with the highest levels of zeaxanthin being 16.3 mg kg−1. The regression of hue angle versus zeaxanthin was described by the equation: hue angle = 81.32 + 3.70 x exp(−0.21 x zeaxanthin)+12.49 x exp(−0.25 x zeaxanthin), r^2 of 0.9896.

The most efficient method to break down cell wall proved to be the use of chlorhydric acid (Table 1). In acetone, the highest values of the zeaxanthin concentration were obtained under acid hydrolysis of the cell wall (4.89 μg /mL).

Table 1. Amount of zeaxanthin (μg/mL) extracted in acetone from the maize sample while using different cell wall damage techniques

Extraction method	Zeaxanthin (μg/mL)*
Frozen biomass homogenization	3.47±0.01
Ultrasound	3.19±0.96
Self-clavation	3.17±0.03
Mortar mechanical homogenisation	3.51±0.06
Homogenizer mechanical homogenization	3.12±0.21
Acid hydrolysis, HCl [0.1 M]	4.89±0.42
Acid hydrolysis, HCl [1.0 M]	3.73±0.66
Acid hydrolysis, HCl [2.0 M]	2.71±0.98
*x± S.D, $\overline{X}$ –independent triplicates.	

Lower zeaxanthin extract recovery may be explained by the profound damage of the cell wall that allowed zeaxanthin molecules to degrade by temperature effect (2.71 and 3.73 μg/mL in HCl 1N and 2N, respectively).

Separation and quantification of zeaxanthin was realised through the spectrophotometric method which presumes extraction of liposoluble pigments, removal of chlorophylls and of hydroxy–carotenes, and subsequently spectrophotometric determination of carotenes expressed as zeaxanthin (λ–452 nm). In the far UV spectrum, the λmax were obtained at 445 and 452 nm that are the characteristic absorption peaks of chemical structures such as β–carotene and β–carotene–3, 3'–diol (zeaxanthin).

The visible spectrum of zeaxanthin (a derivative of β−carotene) resembles to that of β−carotene (Figure 1). This dihydroxy carotenoid is reflected by its behaviour in HPLC (RF ~0.19).

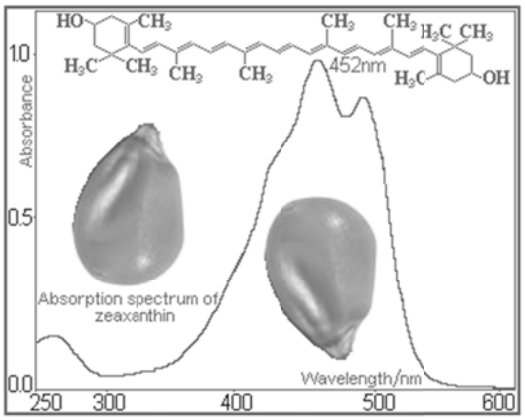

Fig 1. Zeaxanthin–UV–VIS spectrum, absorption spectrum of zeaxanthin solubilised in ethanol; λ_{max}=448 nm

The presence and non–allylic position of the hydroxyl groups was shown by its positive response to acetylation with acetic anhydride and negative response to methylation with acidified methanol, respectively.

3.3 Calibration curve

The calibration curve (Figure 2) was recorded for the concentrations of standard zeaxanthin (purity > 98%), in the interval 1.0–8.0 μg/mL (452 nm, n = 8; r^2 = 1.000). The calibration curve was obtained for the recorded absorbance 452 nm, with its known extinction

coefficient of E1% of 2540. The concentration values used were 1.0–8.0 µg/mL (n = 7) and the correlation coefficient concentration/absorbance was $r^2 = 0.9878$.

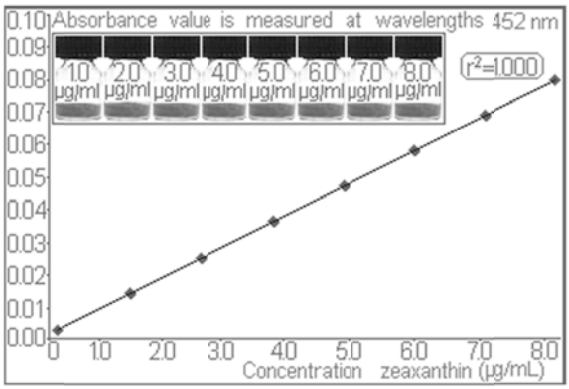

Fig 2. Calibration curve for standard zeaxanthin in acetone

In order to compare the two methods, an arbitrary concentration of 5 µg/ mL was selected, corresponding to absorbance of 1.443 (Figure 2). The calibration curve was designed for the concentrations of zeaxanthin in ethanol within 0.5 to 4.0 µg/mL range (n = 7) and the correlation coefficient being $r^2 = 0.9896$ for measurements performed at 445 nm. The parameters (correlation coefficient $r^2 > 0.99$ and position of the curve close to 0) allowed us to use this curve and the corresponding equation with a higher degree of accuracy. Extraction techniques of zeaxanthin in ethanol were used, and zeaxanthin content was calculated according to calibration curve for standard solutions of pure zeaxanthin in ethanol. Solubilisation of zeaxanthin was realised at intervals of 0, 1, 12, 24 and 48 h, respectively (Figure 3). Curve linearity was within experimental concentration range (0–10 µg/mL).

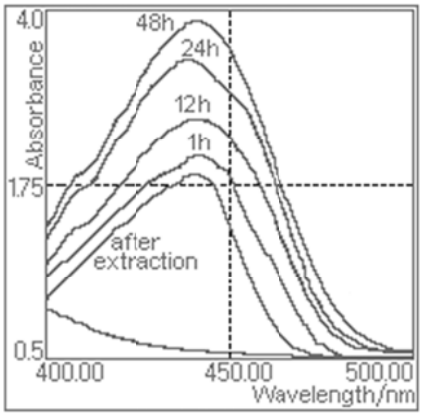

Fig 3. Assessment of zeaxanthin solubilisation at time intervals of 0, 1, 12, 24 and 48 h – absorption spectra

3.4 Spectrum prints specific to the antioxidant zeaxanthin

Figure 4 shows the spectrum of the maize sample within the interval 400–4000 cm^{-1}.

Two zeaxanthin characteristic peaks shown in Figure 4 originate from rocking motions of the C–C single bond stretch vibrations (1156 cm^{-1}) and from the carbon–carbon double bond stretch vibrations (1657 cm^{-1}). The intensities of the stretching bond vibrations C=C at 1657cm^{-1} in samples showed that the lowest intensity corresponds to zeaxanthin. Deformation vibrations of the C–H bonds of the carbon atoms with a single bond had the lowest intensity. The results are in

accordance with the known composition of maize fatty acids that contains saturated fatty acids leading to medium–low intensities of the vibrations from the double–bonded carbon atoms.

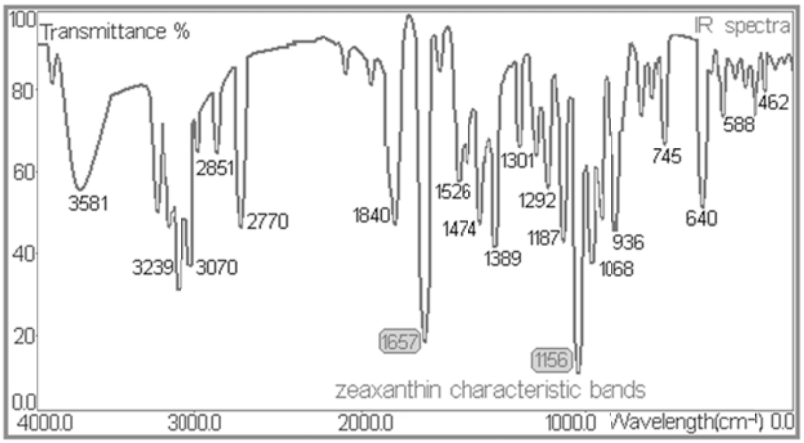

Fig 4. Raman spectrum of the maize sample within the interval 400–4000 cm^{-1}

The stretching vibration intensities of the bond C=C and the deformation of the bond =C–H of the double-bonded carbon atom are higher. In addition, there are several new bands in the spectrum at 1526, 1187, 1125 and 1006cm^{-1}, attributed to the carotenoid content of maize. Analysing the band intensity of the carotenoid content, a qualitative assessments concerning zeaxanthin was obtained. For instance, using the bands within 1657–1156cm^{-1} range as marker bands (attributed to the stretching vibrations of the bonds C=C and C–C, respectively) it was obvious that maize samples have a high content of zeaxanthin. For zeaxanthin, it was identified the characteristic spectral area containing useful molecular structural information within the spectral intervals of 640–1301 cm^{-1} (1156) and 1526–3070 cm^{-1} (1657). In the domain of high wave numbers, it can be seen clearly the marker bands: the band at 2851 cm^{-1}, due to the stretching vibrations –CH2, as well as the band at 3010 cm^{-1} due to the stretching vibration =C–H. The bands within the interval 904–1153 cm^{-1} may be attributed to the stretching vibrations C–O and C–O stretching, while the bands within the interval 1474–1199 cm^{-1} to vibrations of groups such as OC–H, C–C–H and C–O–H. In summary, spectroscopy is a viable, quick, non-destructive method analysis for the assessment of quality and authenticity of zeaxanthin (lipid antioxidants).

3.5 Conditions selection for the stabilisation of zeaxanthin ethanol extracts

The factors that may influence the zeaxanthin pigments stability are light and high temperature (i.e., pure crystalline zeaxanthin degrades under the influence of atmospheric oxygen and light). The optimal storage conditions for the carotenoid extract in acetone were the freezing conditions. Addition of ascorbate to the zeaxanthin extract lowered the carotenoid oxidation process. The zeaxanthin concentration diminished by 50% in 30 days. This shows the importance of establishing the storage conditions of zeaxanthin extract, which depends not only on environmental conditions, but also on the nature of the solvent utilized. To solubilise zeaxanthin in ethanol, it was dissolved 10 mg of zeaxanthin in 0.5 mL of chloroform, and then the matrix solution of zeaxanthin was set up by ethanol. In stabilisation experiments, were used samples of 100 mL ethanol solution each with a concentration of 50 μg/mL of zeaxanthin. These samples were stored under the following conditions: room temperature, darkness (1); room temperature, daylight (2); temperature of 0°C, darkness (3); room temperature, darkness, with an antioxidant (4); room temperature, daylight, with an antioxidant (5); temperature of 0°C with an antioxidant, darkness (6). The quantitative test was repeated at day one, at 7 days, and 30 days subsequent to extraction. Room temperature oscillated between 18 and 22°C. Samples exposed to daylight were kept away from direct sunlight and from artificial light. Ascorbic acid was added to concentrations of 1.0–100 mg zeaxanthin.

Practically, regardless of conditions, zeaxanthin content during the first storage days did not change substantially (49.56 and 50.80 μg/mL) (Table 2).

Table 2. Stability test of the zeaxanthin ethanol extract

Conditions	Zeaxanthin extract concentration (μg/mL)			
	Right after extraction	After 1 day of storage	After 7 days of storage	After 30 days of storage
1	49.86±0.34	50.80±1.94	50.05±0.10	33.85±0.12
2	49.86±0.34	49.56±2.65	48.89±0.07	28.86±1.15
3	49.86±0.34	50.70±2.90	49.76±1.14	49.83±0.43
4	49.86±0.34	49.80±1.38	50.17±0.11	48.76±1.32
5	49.86±0.34	49.61±1.59	50.01±0.12	35.58±1.61
6	49.86±0.34	49.65±0.50	50.02±0.07	50.01±0.56
*x± S.D, $\overline{X}$ –independent triplicates.				

At 30 days of storage, zeaxanthin concentration decreased by 42% under light conditions and by 32% under darkness. After 30 days, the zeaxanthin content, supplemented with ascorbic acid, decreased by 29% when kept under light and room temperature. In ethanol solutions with ascorbic acid, zeaxanthin kept under dark conditions did not changed and was not affected by thermal factor. Zeaxanthin content in both samples remained at the initial level at 0 and 20°C.

4. Discussions

In corn, xanthophylls are mostly found in the horny endosperm. In order to break the cell wall and obtain the experimental compounds, alkaline and acid hydrolysis methods were applied. Alkaline hydrolysis can cause irreversible degradation of zeaxanthin [34]. The value of extractability was calculated according to the formula (2),

$$\text{Extractability}(\%) = \text{Free Zeaxanthin}(\mu g/ml) \Big/ \text{Total Zeaxanthin}(\mu g/ml) \cdot 100 \quad (2)$$

where “free zeaxanthin” is the amount of maize sample zeaxanthin in acetone following 1h of stirring, and “total zeaxanthin” is the amount of maize zeaxanthin obtained by acetone extraction under identical conditions from the same sample. The selection of solvents for zeaxanthin recovery, dose, and extraction, confirmed the efficiency of ethanol, the zeaxanthin extractability obtained being 93–95%.

The total zeaxanthin was determined after saponification of extracts. Carotene isomers were obtained by iodine isomerization. The extraction of zeaxanthin and geometrical isomers was carried out in a homogenisator with acetone/water (9:1, v/v) that led to small extraction volumes without further concentration steps during preparation. Complete release of zeaxanthin esters in free or non–esterified form was helpful to effectively isolate and concentrate zeaxanthin to a desired extent. To verify the type and position of substituents in xanthophylls were performed the appropriate chemical reactions [35].

Zeaxanthin content is considered as a sum of all zeaxanthin isomers. To verify the geometric configuration, it was carried out iodine–catalyzed isomerization.

Their physical properties, especially the wavelength shift in the UV/Vis spectrum allowed for their detection by HPLC (UV detector). The visible spectrum of β–carotene (zeaxanthin) derivative resembled that of β–carotene. Its dihydroxy carotenoid characteristic was reflected in its behavior on open–column and HPLC methods (RF is around 0.19). Temperature regulation is recommended to maintain everyday reproducibility. Variations in column temperature resulted in substantial fluctuation of the carotenoid retention times [33]. The presence and non-allylic position of these groups were shown by the positive response to acetylation with acetic anhydride and

negative response to methylation with acidified methanol, respectively. Partial acetylation would yield two acetylated products, which corresponds to the acetylation of only one of the hydroxyl groups. Complete acetylation yields one product with both hydroxyls acetylated. These conditions were used in the validation part of evaluation step. Zeaxanthin is a carotenoid that belongs to a class of natural fat–soluble pigments found in maize. The same principle described above can be used for the "estimation" of the zeaxanthin content of food colorant extracts, pharmaceuticals, food, biological samples, or chromatographic fractions.

In addition to maxim absorption of zeaxanthin, the "shape" of its spectra provides important information to be used for identification of purified zeaxanthin extracts or pure standard. Maximum values of absorption have been specified for most solvents [36]. The choice of extracting solvents is based on a guideline set by the U.S. Dept. of Health and Human Services, FDA. The draft guideline recommends acceptable amounts of residual solvents in pharmaceuticals, for the safety of patients as well as the use of less toxic solvents in the manufacture of drug substances and dosage forms (solvents should be limited in food products because of their inherent toxicity).

Zeaxanthin was identified on the basis of UV–visible absorbance/acceptance criteria, as described in United States Pharmacopeia, 2010. The amount of zeaxanthin was theoretically calculated afterwards, from the initial absorbance of 1.443 and from the extinction coefficient E1% of 2540 according to the below equation (3) and determined the concentration of 6.8 μg/mL zeaxanthin.

$$\text{zeaxanthin } (\mu g/mL) = \text{Absorbance} \bullet 10.000/2540 \quad (3)$$

The calculated zeaxanthin concentration in acetone (according to absorbance and using extinction coefficient) was 1.4 times higher than zeaxanthin concentration in acetone calculated from calibration curve.

Consequently, zeaxanthin concentration results (via extraction and solubilisation in acetone and use of extinction coefficient method) were higher than those obtained under similar conditions, as calculated from calibration curve.

The stability of the extract of zeaxanthin was measured for different temperature conditions. The extract was protected from light and stored in sealed airtight containers. The zeaxanthin remained stable after 30 days of storage in a cool place, protected from light and oxygen.

Stability testing indicated that in order to maintain stability it is necessary the addition of antioxidants such as ascorbic acid (sodium ascorbate).

5. Conclusions

The most efficient method of cell wall disintegration was addition of chlorohydric acid. Saponification may be necessary to remove lipids. Zeaxanthin in solution obeyed the Beer–Lambert law: its absorbance was directly proportional to its concentration. Consequently, carotenoids quantified spectrophotometrically, provided accurate absorption coefficients in the experimental solvents tested.

The separation and dosing of zeaxanthin was achieved by spectrophotometric method which involves liposoluble pigment extraction, chlorophylls and hydroxy–carotenes removal and spectrophotometric determination of carotenes expressed as zeaxanthin at a wave length of 452 nm.

HPLC quantification was carried out by means of internal or external calibration, for which the standard concentrations were also determined spectrophotometrically according to AOAC. Carotenoid levels had a wide range in the maize sample, the highest levels of zeaxanthin being 16.3 mg kg^{-1}. Spectroscopy was applied for the assessment of maize quality, by identifying its characteristic spectrum intervals of C–C single bond stretch vibrations (1156 cm^{-1}) and from the C=C double bond stretch vibrations (1657 cm^{-1}) of the molecule backbone (zeaxanthin characteristic bands). The main problem in the analysis of zeaxanthin was the instability and it had to be taken precautionary measures, to avoid shifts and quantitative deficiency. For zeaxanthin

ethanol solutions with no ascorbic acid, environmental temperature conditioned the stability of the tested solutions. Regardless of the lighting conditions, zeaxanthin concentration was constant.

Storage conditions at room temperature and with light accelerated the process of pigment loss with 10% compared to similar thermal conditions, but with no light whatsoever. When ethanolic zeaxanthin solutions were stored without ascorbate, the temperature played an essential role on their stability. Regardless of illumination conditions, only at 0°C zeaxanthin concentration remained the same. For ethanolic zeaxanthin solutions, a storage interval of 30 days at room temperature was achievable only after ascorbate addition under darkness conditions. Storage of the antioxidant zeaxanthin in ethanol at 0°C did not require ascorbate supplementation as a stabilizer. Ethanolic extracts of zeaxanthin should not be preserved in the presence of light. Therefore, avoiding direct light resulted in a stable zeaxanthin preparation.

This conclusion was also confirmed by identical conditions of storage of samples supplemented with ascorbate, where a reduction in zeaxanthin concentration was observed, too. The supplemented ascorbate played only a reduced role in the oxidative process.

Acknowledgements

This work has been financed by UEFISCDI, research contract no PN-II-PT-PCCA 106/2012.

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