Evaluation of the Efficacy of Various Green Extraction Methods for High Valorisation of Vegetal Antioxidant Sources

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This work presents the efficacy of various green extraction methods used to ensure the optimal amount of antioxidant compounds extracted from vegetal material. The new developed extraction procedures are easy-to-use and have a low implementation cost providing health-safe and high-quality extracts (products). The complete characterization of the obtained extracts was performed by a HPLC method (quantification of antioxidant compounds) and the ORAC method (efficacy assessment). These methodologies have high potential in by-products industry, where managing the residues is an important step in increasing the technological process economic efficiency and mitigation of environmental impact and, moreover, can by further extended to other types of raw materials.

Keywords: β-carotene, green extraction, HPLC-PDA, antioxidant capacity

Due to various constraints of the traditional extraction methods for high valued antioxidant compounds to be used in food and nutraceutical industries, a great importance is given to the development of safe extraction procedures in order to ensure a safe and high-quality extracts or products.

Carotenoids represent a class of fat soluble compounds also known as natural pigments that provide the unique yellow, orange and reddish colours to leaves, fruits, and vegetables [1]. Some of the most well-known carotenoids occurring in food are β-carotene, lutein, zeaxanthin and lycopene and are thought to play an important role in human health [2,3]. Since humans are not able of de novo carotenoids synthesis they need to include in their daily diet carotenoids from fruits and vegetables. Well documented studies have found that diets rich in fresh fruits and vegetables are associated with reduced risks of different diseases. Beta-carotene represents the most important source of provitamin A. Its health benefits are coming from the antioxidant properties, UV skin protection, and capability of mediating cell to cell communication and from its role of immune system stimulator. Moreover, there are information proving the decrease of the risk for lung and breast cancer, cataract and age-related macular degeneration. Carotenoids play a beneficial role in cardiovascular and retinal diseases and also decrease the rate of incidence for certain cancers of the stomach, pancreas, skin and especially the prostate [4]. Due to their hydrophobic properties carotenoids present some issues related to their bioavailability such as solubilisation problems. The use of fat-soluble media might be a solution since result in extracts that are more available for transportation and absorption at tissue levels. Some studies have demonstrated that bioavailability of carotenoids in human body may be enhanced by addition of fats and oils to their daily diet [5-7]. Their hydrophobicity is affecting equally the extraction from vegetal material, their removal from vegetable matrix requiring the cell-wall disruption through physical or chemical mechanisms.

The extraction methods for this class of compounds varied over the years considering the type of matrix from which they were extracted and the impact of the solvents used on the environment. Although it started from the classic Soxhlet extraction that utilizes toxic and hazardous organic solvents like hexane, tetrahydrofuran, and/or acetone, in the recent years the modern techniques such as extraction assisted by enzymes, supercritical fluid extraction, microwave or ultrasound assisted extraction (MAE / UAE) using friendly environmental solvents are more economical in terms of safety, time and budget [7]. In the attempt to provide a better bioavailable formulation for carotenoids the use of oil [8-11] as extraction solvent might be a viable alternative. Among the analytical methods applied to assess the carotenoid content in various types of samples, liquid-chromatography (LC) coupled with various detectors (e.g. PDA, mass spectrometry) [12-14], Raman and Fourier Transform Infrared spectroscopy (FTIR), Nuclear Magnetic Resonance spectroscopy (NMR) and even matrix assisted laser desorption ionization-time of flight (MALDI-TOF) [15,16] were performed.

In the current work the authors propose new alternative extraction methods for carotenoids from vegetal material based on cavitation phenomena and using a food grade solvent, sunflower oil. The chemical characterization of obtained extracts was performed by an improved HPLC method which allows the identification and quantitation of carotenoids in a single run of 15 min. The antioxidant capacity of formulated extracts was assessed using ORAC assay in micellar systems. The results obtained using our extraction methods were compared with those from literature data obtained in similar conditions. The stability of the obtained extracts was appropriate according to assessment performed with respect to β-carotene concentration and extracts antioxidant capacity. A survey of the literature data regarding carotenoid compounds identified in various extracts of carrots showed that β-carotene is the major compound found in all forms of extraction (using conventional organic solvents or alternative solvents for extraction), the exception being the yellow carrots specie where lutein is the main carotenoid component. Therefore, considering the carrot specie commonly available on the market, we have chosen β-carotene as the main target compound for carrot extracts characterization.

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Experimental part

Chemicals and reagents

Beta-carotene standard (≥ 97%, purity), butylated hydroxytoluene (≥ 99%, purity) and sodium tetraborate decahydrate (99.5-105.0%) were purchased from Sigma Chemical Co.

2,22 -Azobis(2-methylpropionamidine) dihydrochloride (AAPH) along with sodium dodecyl sulfate (SDS) from Fluka were used during experiments. Acetonitrile, tetrahydrofuran and methanol (LiChrosolv), dichloromethane and 2-Propanol (Chromasolv) were of HPLC grade. Deionized water was obtained using a Milli-Q water purification system, Elix 3 (Millipore Co.,USA).

Standard and samples preparation

The stock solution was prepared by dissolving the carotenoid standard in tetrahydrofuran at a concentration level of 1 mg mL^{-1}. Further the working standard solutions were prepared by diluting the stocks according to the calibration levels in a mixture of methanol: dichloromethane (1:1, v/v) and analysed in triplicate via the developed HPLC method. Carrot (Daucus carota) samples were purchased from a Romanian market. Samples were washed with tap water to remove dirt and other possible interferences and dried in ambient conditions. Further, the samples were blended in a knife mill Grindomix GM 200 (Retsch GmbH, Haan, Germany) for approximately 1 minute on high speed to ensure homogenisation and to obtain a representative sample for carotenoid analysis. The obtained material, as a puree, was transferred to 50 mL recipients and used further for extraction and analysis as fresh (FS) or dried in a Memmert UFB 400 device at 30° C (to avoid analytes degradation) followed by extraction and analysis (DS).

All procedures were performed using amber glassware in reduced light where possible, to avoid loss of the pigments through degradation. The edible oil (100 % refined sunflower oil UNISOL) was commercially available, supplied by supermarkets; it was further used for extraction without any preparation as *green solvent* for carotenoid extraction due to its nonpolar properties.

Sample extraction

The carrot samples either in fresh or dried form were used to investigate the effect of moisture on the extractability of β-carotene prior to optimization of the extraction conditions. The ratio between the sample and the oil, was set to 1:5 (m: m) after some trials, and the mixtures were submitted to extraction procedures based on cavitation phenomena, ultrasounds and microwaves, as follow:

a)MAE was performed using an input microwave power of 800W and 60 s extraction time followed by homogenization for 120 min under continuous agitation.

b)Ultrasound extraction was performed for an hour and a half into an ultrasonic bath operating at a frequency of 35 kHz and ultrasound power (RMS) of 85 W, filled with water and ice for temperature control necessary to prevent the degradation of carotenoids.

The mixtures were characterized in the stability studies, with or without the addition of a preservative, butylated hydroxytoluene (BHA) at the concentration of 3 % in the solutions. All manipulations were carried out under dim light and extracts were always manipulated using amber-coloured glass within the shortest time possible. All samples were further analysed using HPLC based on the method reported by Aruna et al. [12] modified according to our laboratory conditions and validated in terms of linearity, precision, and accuracy so the data obtained can fit for the intended purpose.

Due to the well-known antioxidant propriety of carotenoids based on their capability to quench the reactive species of oxygen and nitrogen, the antioxidant capacity of the extracts obtained were assessed using a new ORAC protocol developed in our laboratory, based on micellar solutions. The result is obtained by calculating the area under the fluorescence curve, and it is expressed as equivalent of micromoles of Trolox per g of vegetal material used for extraction since, prior to sample assessment, the proper calibration of modified ORAC protocol is performed using Trolox compound as reference.

Usually, when the fluorescent probe is fluorescein (excitation at 490 nm, emission at 514 nm), the working conditions involve the use of phosphate buffer at *p*H 7.40; millimolar concentration of AAPH as radical initiator (using thermal generation of free radicals) and nanomolar concentration of fluorescein are used in the measuring cell. The micelles are obtained by mixing the following compounds, SDS, sodium tetraborate decahydrate, isopropanol and phosphate buffer in a combining ratio of 1:1:1:7. The Trolox concentration normally is 1 $\mu molL^{-1}$. The ORAC method is frequently used to evaluate the antioxidant capacity of water-soluble antioxidants.

Instrumentation

The HPLC method was optimized using an HPLC Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with LC-20AD SP solvent delivery system, a LC 20AC autosampler, CTO-20AC Column Oven thermostat, DGU-20A5-Degasser and an SPD-M20A Diode Array Detector. Samples were injected onto a Kromasil C18 (250 x 4.6 mm, 5μm) column set at a temperature of 25^0 C during the analysis. Mobile phases consisted of acetonitrile (solvent A) and methanol: dichloromethane (50:50, v/v) (solvent B) and the flow was maintained at 0.8 mL/min. Isocratic elution was employed, using a mobile phase ratio of 40:60 (v/v) solvent A to solvent B, and the injection volume was set to 20 μL. For the optimization of the mobile phase composition, different ternary mixtures of methanol (MeOH), acetonitrile (ACN) and dichloromethane (CH_2Cl_2) were examined and a composition of 40:60 (v/v) solvent A to solvent B provided an efficient separation within the shortest time possible. Because of the CH_2Cl_2 water immiscibility an intermediate mobile phase consisting of MeOH: ACN (60:40, v/v) was used for column washing. The flow rate of 0.8 mL/min was found to be optimum from a studied range of 0.5-1 mL min^{-1} (data not shown) as a compromise between optimum retention time, baseline stability and noise, and low consumption of reagents. The UV-Vis absorbance of the peaks were recorded between 200 and 800 nm using a PDA detector and monitored at 456 nm for β-carotene.

Stability studies

The stability studies were performed during a determined period of time, under the influence of main environmental factors affecting extract stability, namely temperature and light by assessing the concentration of the active compound evolution and extracts efficacy evolution. The experimental setup followed the scheme: day 1, day 7, day 21, day 45, then at the end of every month (totally 5 months) for samples kept at ambient temperature and darkness and at a temperature of 4^0 C (in the refrigerator).

Results and discussions

As previously mentioned, the β-carotene analysis was performed by HPLC-PAD method able to identify the compound of interest by comparing the retention time and the spectral data collected with PDA detector with those of the corresponding standard. The specific retention time for β-carotene was 12.03±0.18 min and the calibration curve performed on a linearity range of 0.0001 to 0.050 mg mL^{-1} with a correlation coefficient of 0.998 provided a detection limit of 0.00013 mg mL^{-1} and a limit of quantification of 0.00045 mg mL^{-1}. The results proved that the method is accurate allowing repeated quantitative determinations on the same day (repeatability) and on different days (intermediate precision) with a relative standard deviation (RSD%) of 1.31% (repeatability) and 3.65% (intermediate precision) calculated for peak area.

The *eco-friendly* solvent chosen for the extraction experiments, sunflower oil, is free of carotenoids (fig. 1), our data being confirmed by literature reports [16,17]. The extraction methods were compared with data reported in the literature for carrot samples [11, 18]. The carotenoid composition, expressed as µg/g of fresh or dry weight (FS or DS) according to the sample form, for two carrot extracts obtained by cavitation-based extraction procedures is presented in table 1. These results indicate that the form of the sample is important and can impact the extraction efficiency. Data show that dry basis carrot extracts have the highest level of β-carotene (ten times more) compared to fresh basis carrot extracts. Concentrations are given as the mean data of three assays.

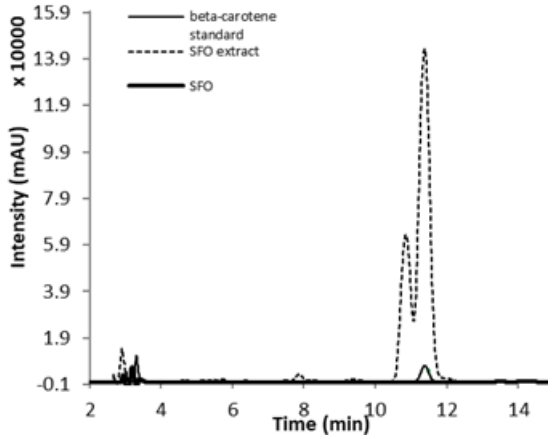

Fig. 1.HPLC chromatogram of a sunflower oil (SFO) compared with those of carotene standard and a carrot sample (SFO extract); PDA detection at 456 nm

The carotenoid content in fresh carrot samples was consistent with other literature data [7, 19, 20] where values ranging from 46 to 193 µg/g FW β-carotene were reported. Sun et al. [7] reported the β-carotene content of dry carrots ranging from 333.76 - 899.97 µg/g when canola oil was introduced as a co-solvent in supercritical carbon dioxide extraction, which is substantially similar to our values when using sunflower oil as solvent. The author also reported

Table 1

β-CAROTENE CONTENT (µg/g) OF CARROTS IN SUNFLOWER OIL EXTRACTS USING CAVITATION EXTRACTION METHODS

Extraction procedure	β-carotene (DS)	β-carotene (FS)
Microwave	898.7	92 .05
Sonication	768.1	68.65

smaller amounts of carotenes in fresh basis samples and pointed out the importance of using samples in dried form in the extraction processes since water can negatively influence the extraction of nonpolar compounds such as carotene by decreasing the diffusion phenomenon between the vegetable matrix and solvent for extraction.

We also have performed tests regarding variation of different functional parameters of MAE system on dry basis carrot samples (e.g. microwave power, operating mode, working time) and the results are presented in table 2. Based on the amount of β-carotene that could be extracted from carrots it was noted that by using continuous microwave radiation and prolonging the time of irradiation an enhancement of the bioactive component concentration can be obtained. Also, the microwave power had a significant effect on the carotene yield since the highest value (800W) gave results comparable with lower microwave powers and longer time for extraction in terms of analyte concentration.

These results demonstrated the potential of using sunflower oil as alternative solvent in extracting carotenoids from vegetables. In addition, MAE had a positive influence on carotenoids extraction since this phenomenon involves an increase in the contact between the solvent and the cell contents which enhances the mass transfer of targeted compounds.

Table 3 presents the results of the stability studies, expressed as concentration of β-carotene, determined over a period of 5 months in carrot extracts using MAE and UAE methods.

Within the first 30 days fluctuations in the concentration of β-carotene occurred probably due to simultaneous processes that occur, isomerization and degradation of the compound which may lead to increases and drops of the amount of carotene. After that period, significant losses of around 30- 50% of the initial concentration occurred in the extracts stored at room temperature while the samples obtained by MAE showed a less pronounced drop of the concentration and rather a stabilization trend.

In terms of percentage, β-carotene is degraded in proportion of 26% when kept in a refrigerator and using BHA, and around 50% upon storage at room temperature. In conclusion the addition of BHA may improve the extraction of β-carotene and have a significant contribution for the conservation of analyte in a long period of time. Storage of the samples at room temperature lead to the most pronounced decrease of β-carotene concentration when more than half of the initial content of β-carotene

Set MW power (W)	Time (min)	Irradiation mode	β-carotene content (mg/100g DS)
800	1	continuous	89.87
160	3	continuous	33.05
160	10	continuous	63.22
160	7	intermittent	35.41
320	4	intermittent	14.95
320	9	intermittent	19.36

Table 2
COMPARISON BETWEEN DIFFERENT CONDITIONS OF MAE ON CARROTS

Time (days)	Storage conditions							
	MAE				UAE			
	R		RT		R		RT	
	0% BHA	3% BHA	0% BHA	3% BHA	0% BHA	3% BHA	0% BHA	3% BHA
1	849.0	898.7	849.0	898.7	676.9	768.1	676.9	768.1
7	667.9	716.9	637.7	703.6	536.5	593.9	555.0	587.4
21	608.3	776.3	607.3	699.5	557.4	658.0	419.0	565.3
45	601.8	664.8	568.2	550.7	528.7	587.1	413.9	526.8
60	591.8	643.2	451.2	519.4	483.1	537.0	353.9	410.8
90	590.2	616.2	448.3	476.3	453.3	472.1	323.7	406.2
120	581.7	593.7	365.6	455.1	431.9	450.0	288.2	361.1
150	515.6	588.4	319.9	435.5	397.9	377.5	283.4	350.0

Table 3
STABILITY AS β-CAROTENE CONTENT (mg/g DS) OF CARROT EXTRACTS, WITH AND WITHOUT THE ADDITION OF BHA

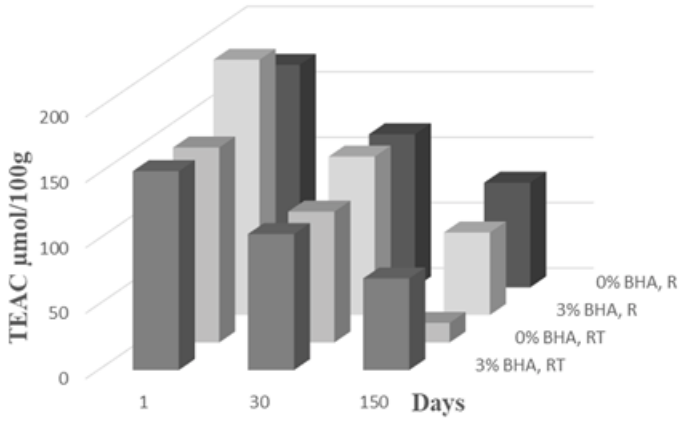

Fig. 2. The antioxidant capacity test results for carrot sunflower oil extracts obtained using MAE, expressed as µmol/100g TEAC

was lost through degradation processes. The efficacy as antioxidants of the vegetable oil enriched with carotenoids as biologically active compounds support the idea of using MAE extraction for safe and efficient formulation of nutraceutical supplements. The result obtained from antioxidant capacity determination tests are presented as figure 2. The ORAC values were significantly higher for extracts containing BHA compared to those obtained without the addition of a preservative. The antioxidant capacity of carrot extracts dropped significantly after five months among the samples preserved at room temperature, whereas in the other cases when stored in refrigerator, the extracts showed the highest ORAC and carotenoid content values.

The ORAC values obtained for our samples were consistent with data reported for the lipophilic-ORAC tests by USDA on selected foods, expressed ìmol of Trolox equivalents per 100 grams (µmolTE/100 g).

Conclusions

Different extraction methods based on cavitation phenomena to obtain antioxidant compounds from raw vegetal material was investigated in this study. The effect of the sample moisture on the extractability of β-carotene from carrots prior to extraction was tested along with the optimization of the conditions to yield a higher content in the bioactive compound. Two innovative extraction methods based on microwave and ultrasound assistance were evaluated using a HPLC method which provides reliable results, together with a modified ORAC protocol for lyophilic compounds, based on micellar solutions, to assess the antioxidant capacity. The data generated through MAE/UAE in sunflower oil could be helpful for industries that will be forced in the future to apply innovatory and environmental-friendly extraction processes in order to improve the energy consumption, the risks on human health and to succeed in economic terms.

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