Extraction and Analysis of the Major Carotenoids of Agro-Industrial Waste Materials Using Sequential Extraction Techniques and High Performance Liquid Chromatography

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ABSTRACT

The objective of the present study was to develop sequential extraction procedures for the major carotenoids – beta-carotene and lycopene from agro-industrial waste materials – tomato skin, tangerine and orange peels using the ultrasound-assisted extraction and the supercritical fluid extraction techniques. A rapid, effective and selective high performance liquid chromatographic method for quantitative determination of beta-carotene and lycopene in organic extracts solutions was developed and validated with respect to robustness, specificity, linearity-range, accuracy, precision, limit of detection (LOD) and quantitation (LOQ) as well. The effect of the operating pressure, the temperature, the extraction time, the flow rate of supercritical fluid, the sample size, the ultrasound power and the solvent nature used was investigated. The optimal conditions for extraction were found. The LOD and the LOQ are 0.0081µg/mL and 0.00405 µg/mL for beta-carotene, 0.034 µg/mL and 0.0085 µg/mL for lycopene, respectively. No interference was observed. The content of beta-carotene per 1 g of dried agro-industrial waste material varies 8.39 – 12.75 µg (tomato skin), 25.65 – 32.18 µg (tangerine peel), 41.66 – 59.16 µg (orange peel) and the content of lycopene – 165.11 – 179.56 µg (tomato skin), 11.12 – 17.91 µg (tangerine peel), 8.37 – 10.65 µg (orange peel).

Keywords: ultrasound-assisted extraction, supercritical fluid extraction, carotenoids, high performance liquid chromatography, analytical method validation

INTRODUCTION

Due to consumer concerns for food safety and strict government regulations, the consumption of synthetic colorants is decreasing and the demand for natural colorants is growing. Carotenoids are one of the most important natural food colorants and have attracted by many researchers, because of their commercially desirable properties, such as their natural origin, wide distribution, structural diversity, very important biological function, null toxicity and high versatility. Carotenoids provide both lipo- and hydro-soluble colorants and have provitamin A activity. More than 700 natural carotenoids have been identified and the number is still increasing every year [1-2]. These compounds can be biosynthesized in the plant, by algae, yeast, fungi and photosynthetic bacteria and contain 40 carbon atoms. Carotenoids are classified into carotenes (e.g., α-carotene, α-carotene, lycopene) and xanthophylls (e.g., β-cryptoxanthin, lutein, zeaxanthin, canthaxanthin) [3-4].

Carotenoids have been credited with beneficial effects on human health - enhancement of the immune response. They show antioxidant activities, which may prevent degenerative diseases, such as cardiovascular, dermatological, renal, pulmonary diseases, the oxidative damages that are specific to ageing phenomena, cataract

and macular degeneration, toxic liver damage, metabolic syndrome, sepsis, autoimmune disorders, diabetes and several types of cancer, especially, prostate and digestive-tract tumors [5-8]. Also, it was determined that the antimutagenity is, in the main, associated with hydrocarbon carotenoids fractions (α-, β-carotene, lycopene) and with xanthophylls (lutein, β-cryptoxanthin) and their supplementation can increase CD4 counts in HIV-infected patients. In addition to this, it was shown by studies of Bureau and Bushway (1986) that retinol and several carotenoids (β-carotene, β-cryptoxanthin, zeaxanthin, lutein, capsorubin, capsanthin, lycopene and capsanthol) were involved in the cytoprotective injury of gastric mucosa. Carotenoids confer to the tissues a yellow, orange or red colour. They are widely used as colorants in food and added directly many food products such as butter, popcorn, salad dressings and beverages or indirectly via animal up take in, for example, chicken and fish as described by Britton (1992), Birtigh et al. (1995) and Vega et al. (1996). Studies have shown that β-carotene's provitamin A activity or antioxidant properties prevent diseases, such as arthrosclerosis and multiple sclerosis. Moreover, in recent studies, β-carotene has been proposed to decrease cell proliferation and induce apoptosis of various cancer cell lines by inhibiting Ca^{2+}/calmodulin-dependent protein kinase IV. In another study, β-carotene has been reported to have anticancer stem cell actions on neuroblastoma, and this anticancer action is enhanced by retinoic acid receptor β. β-Carotene protects the skin from harmful effects of UV light by its prevention of reactive oxygen species formation and anti-inflammatory properties [9-10].

Carotenoids are consumable in pharmaceutical, perfumery and cosmetic industry as well. Nowadays, the importance is increasing due to a more extensive use of natural compounds in the food, cosmetic and pharmaceutical industries, following the European Union (EU)/ Food and Drug Administration (FDA) directives in favor of natural rather than synthetic compounds [11-13]. In recent years, some synthetic colorants are consider to be responsible for allergic and intolerance reactions. They have been banned in many countries because of their toxicity and cancerogenic activity. Scientists have determined a link between artificial food coloring and cancer. Other studies have linked artificial food coloring with brain tumors, attention deficit hyperactivity disorder and other disruptive behavior, especially, in children. None of these additives have any beneficial or nutritional value to the human body. The concern is so great that the FDA has ordered warning labels be placed on foods containing artificial dyes and coloring [14-19]. Therefore, to replace synthetic food colorants by natural ones is the very actual problem.

The most important natural sources of carotenoids are fruits, vegetables and their agro-industrial waste materials. Tomato skin, orange and tangerine peels are a potential cheap source of carotenoids, especially, β-carotene (beta-carotene), lycopene, β-cryptoxanthin, violaxanthin and lutein.

Before making a carotenoids extract you first need to consider the chemical and physical properties of the substance you want to extract from the vegetal material. Most extractions are based on solubility of the chemicals of interest. The distribution to a variety of materials and fluids of the biologically active compounds made it impossible to adopt a generally–available extraction method that can be applied universally as a standard technique. This is due to the various structures of biologically active compounds. Even those belonging to the same class differ by the chain length, by the specific functional groups or configuration, by different solubility in solvents, etc. Carotenoids are soluble in non-polar solvents, including edible fats and oils. Basically, carotenoids are lip soluble; they are usually extracted from the plant sources with organic solvents (chloroform, hexane, acetone, petroleum ether, etc.). The plant material can contain the large quantity of water; water-miscible organic solvents such as ethylic alcohol are also used. One of the inconveniences is the elimination of the residual solvents to obtain a safe extract. This can be avoided by using food grade solvents such as ethylic alcohol, found that the yield of each carotene from plant material was perceptibly higher with extraction performed with ethylic alcohol than with that using ethyl acetate.

The method of extraction is selected according to the nature of the plant material, the ease of extraction solvent, the amount and properties of extracted compounds (solubility, thermolability), etc. The choice of solvent is determined by the nature of the active ingredients, the physico–chemical properties of the solvent, the selectivity of the solvent. Various methods have been developed in order to recover carotenoids from being destroyed by the process of separate. Thermolabile bio-substances including carotenoids are cold extracted by leaching or agitation. For the hot extraction are used several methods, i.e., the reflux extraction, solid–liquid or liquid–liquid extraction. The best results are obtained by the ultra-sonication of the material extracted with solvents – the ultrasound-assisted extraction (UAE) and the supercritical fluid extraction (SFE) [20-21].

There are some interesting studies in developing extraction and assay methods for carotenoids by different analytical techniques. Traditional separation methods for carotenoids employ open column and thin-layer chromatography (TLC) [22]. The development of high-performance liquid chromatographic (HPLC) methods has contributed recently to advances in the analysis of carotenoids. HPLC methods use isocratic and gradient mobile phases in either reversed-phase or normal-phase mode. Coupling a photodiode array detector to the HPLC allows for a continuous collection of spectrophotometric data during the analysis [23-29]. Much effort has been devoted to developing HPLC–mass spectrometry (HPLC-MS) methods with mainly atmospheric pressure ionization interfaces (APCI) or electrospray ionization interfaces (ESI) [30]. Also, capillary electrophoresis (CE) [31], high
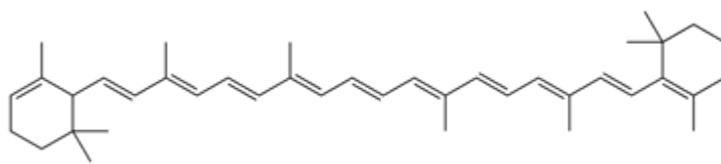
Figure 1. Chemical structure of beta-carotene
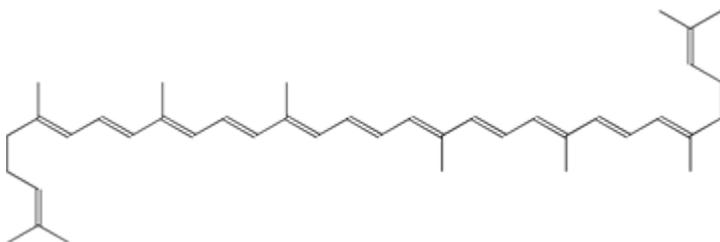
Figure 2. Chemical structure of lycopene

performance thin layer chromatography (HPTLC), ultraviolet-visible (UV-Vis), infrared (IR) and Raman spectrometry have been employed as an analytical technique to identify and quantify low levels and various forms of carotenoids [21, 32-34].

The objective of the present study was to develop and validate extraction procedures of carotenoids from agro-industrial waste materials – tomato skin, tangerine and orange peels using the ultrasound-assisted extraction and the supercritical fluid extraction techniques and a rapid, effective and selective HPLC method for quantitative determination of beta-carotene and lycopene in organic solvent extract solutions.

Beta-carotene - β-carotene - $C_{40}H_{56}$, (molecular weight - 536.9) is a strongly colored red-orange pigment abundant in plants and fruits. Beta-carotene is red or reddish-brown to violet-brown crystals or crystalline powder, soluble in carbon disulfide, in benzene and chloroform, sparingly soluble in ether, in solvent hexane and vegetable oils, practically insoluble in alcohol, insoluble in water, acids and in alkalis. Beta-carotene consists predominantly of all-*trans*-beta-carotene and minor quantities of other *cis*-isomers [35].

Lycopene - $C_{40}H_{56}$ (molecular weight - 536.9) is a natural constituent of red fruits, vegetables, certain algae and fungi. Tomatoes and tomato-based products are the major sources of lycopene in the human diet. In analogy to other carotenoids, lycopene occurs in various geometrical configurations. Lycopene present in fresh tomatoes consists predominantly of all-*trans*-lycopene. The synthetic lycopene contains approximately 70% of all-*trans*-lycopene, up to 23% of 5-*cis*-lycopene, and minor quantities of other *cis*-isomers. Lycopene is a mixture of geometrical isomers of lycopene - all-E-lycopene, 5Z –lycopene. Lycopene is insoluble in water and nearly insoluble in ethanol, but is freely soluble in chloroform and tetrahydrofuran, sparingly soluble in ether, hexane and vegetable oils [35].

EXPERIMENTAL

Sample Materials

Ripe tangerine (Citrus Unshiu), orange and tomato were bought in the local agrarian market of Georgia. The peel and skin were manually removed from the selected fruit/vegetable and dried in laboratory room under the controlled conditions (the temperature - 20-25⁰C and the relative humidity - 30-60 %) and protected from direct sun light.

Reagent and Chemicals

The certified analytical standards of beta-carotene and lycopene, the HPLC grade acetonitrile, methanol, n-hexane, chloroform and 1,2-dichloromethane were purchased from Sigma-Aldrich.

Instrumentation

An eco-friendly laboratory supercritical fluid extraction system (500 mL) was used for sample extraction. The extraction was carried out in a dynamic system. The extraction temperature was monitored by a thermocouple
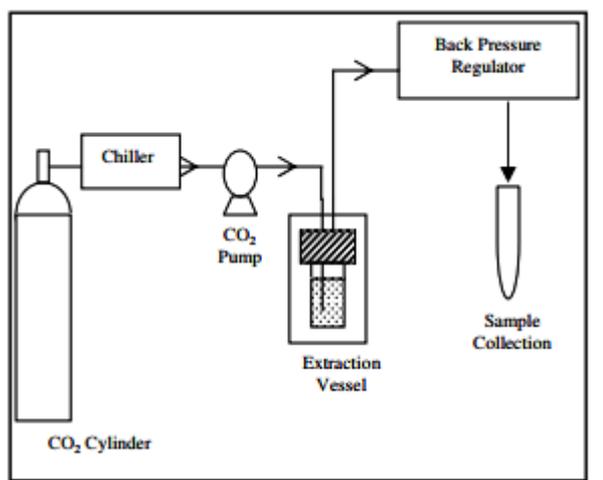
Figure 3. The scheme of supercritical fluid extraction system

immersed at extraction vessel and the pressure was controlled by a back pressure regulator. The previously weighed sample was loaded in a stainless steel basket of an extractor. The carbon dioxide (CO_2) was compressed and chilled at -5^0C. The flow rate of CO_2 was controlled by a pump. Liquefied CO_2 was pumped continuously into the vessel at the specific extraction conditions.

Elmasonic P 300 H ultrasonic bath (Elma Schmidbauer, Germany) was used for ultrasound-assisted extraction. The HF-frequency was 37 and 80 kHz, the temperature was controlled at 15 ± 2^oC during ultrasonication.

The HPLC grade water was prepared using Milli Q Advantage A10 purification system (Merk-Millipore, France).

The chromatographic analysis was performed using an Agilent 1260 Infinity system (AG Technologies, USA). The output signal was monitored and processed using Chemstation software. Analytical balance CPA 232S Sartorius (Germany) was used for standard and sample preparation. All the measuring equipment was appropriately calibrated and qualified.

Chromatographic System and Conditions

The HPLC method was developed using a column - RP-18 endcapped Lichrocart 4 x250 mm, 5 µm (Merck-Millipore) with the binary gradient elution program of mobile phases (MP) A (a mixture of methanol, acetonitrile, dichloromethane and n-hexane 475:475:25:25 v/v) and B (a mixture of methanol and dichloromethane 70:30 v/v); The gradient program: 0-12 min 100 % MP A and 0 % MP B (isocratic), then 12-16 min MP A was changed from 100 % to 0 % (linear gradient), then 16-18 min from 0 % to 100 % (linear gradient) and finally, 18-20 min MP A 100 % and MP B 0 % (isocratic); The flow rate of elution was 1.5 mL/min; The UV detector wavelength was 455 nm for beta-carotene and 272 nm for lycopene; The injected volume was 50 µL; The column temperature was maintained at 40 °C. Mobile phase B was used as a diluent and a blank solution.

Preparation of Beta-Carotene Standard Solution

10 mg of standard of beta-carotene was weighed and transferred to 100 mL volumetric flask, dissolved in 30 mL dichloromethane and diluted to volume with methanol, mixed well. The obtained solution was filtered through 0.45 µm polyvinylidene fluoride (PVDF) microporous membrane filter, discarding the first 5 mL of the filtrate and transferred 5 mL of this solution to 200 mL volumetric flask, diluted to volume with diluent, mixed well (2.5 µg/mL).

Preparation of Lycopene Standard Solution

5 mg of standard of lycopene was weighed and transferred to 200 mL volumetric flask, then dissolved in 100 mL chloroform with ultrasonication and periodically mixed, diluted to volume with the same diluent, mixed well. The obtained solution was filtered through 0.45 µm PVDF microporous membrane filter, discarding the first 5 mL of the filtrate (Stock solution). 1 mL of this solution was transferred to 25 mL volumetric flask and diluted to volume with diluent, mixed well (1.0 µg/mL).

Preparation of System Suitability Check Solution

0.5 mL of standard solution of beta-carotene and 2.5 mL standard stock solution of lycopene were transferred to 100 mL volumetric flask, diluted to volume with diluent, and mixed well.

The sample and standard solutions were prepared and stored in dark glassware under refrigeration to prevent any degradation by heat, air and light.

Calculation Formulae

The concentration of beta-carotene and lycopene - Cu, μg/mL in extract sample solution was calculated by the following formula: $Cu = Au \cdot W_1 \cdot D_1 \cdot P \cdot 1000 / As$. where Au - Peak area of beta-carotene/ lycopene obtained with the extract sample solution; As - Peak area of beta-carotene/ lycopene obtained with the standard solution; W_1 – Weight of beta-carotene/ lycopene standard, mg; D_1 - Dilution factor; P - Purity of standard, %.

The content of beta-carotene and lycopene – X, μg in 1 g of the dried sample (waste material) was calculated by the formula: $X = Cu \cdot V \cdot D_2 / W_2$ where Cu - the determined concentration of beta-carotene/ lycopene in the sample extract solution, μg/mL; V – The volume of extract, mL; D_2 - Dilution factor; W_2 – Weight of dried sample, g.

Method Validation

The developed method was validated with respect to robustness - standard solution stability and filter compatibility test, system suitability test, specificity, linearity-range, accuracy, precision, limit of detection (LOD) and quantitation (LOQ) according to ICH guideline and statistical assessment was performed using Microsoft Excel 2010. The statistical analysis and the evaluation of uncertainty of analytical procedure were performed using Microsoft Excel 2010 according to NATA, ISO, EUROLAB guidelines [36-41].

RESULTS AND DISCUSSION

Sequential Supercritical Fluid Extraction

Sequential extraction procedure requires a correct extraction sequence of target substances. The effect of the operating pressure and the temperature, extraction time, the flow rate of the SC-CO_2, the sample size and the nature of solvent used was investigated to develop the sequential extraction procedure and establish their optimal parameters.

The effect of pressure on the extraction of carotenoids was investigated by carrying out the experiments at pressures from 100 to 250 atm. The flow rate of SC-CO_2 was kept constant at 2 mL/min throughout the extraction. The low temperature - 40^0C was selected because of avoiding thermal degradation of target analytes.

To investigate the effect of temperature on the extraction the experiment was carried out at the different temperatures - 40, 50, 60^0 C; The extraction pressure and the flow rate of SC-CO_2 were kept constant at 100 atm and 2 mL/min, respectively.

The effect of the flow rate of SC-CO_2 on the extraction was investigated at the different flow rates – from 1 mL/min to 5 mL/min. The optimal operating temperature and pressure were established in the previous experiments. Also, the effect of the sample size (agro-industrial waste material) ranging 10 - 50 g was investigated on the extraction of target analytes.

Hence, the two-step extraction procedure for the supercritical fluid extraction of carotenoids from dried samples was developed. The optimal parameters are for the extraction step I: the sample size – 40-45 g, the extraction pressure - 150 atm, the extraction temperature - 40^0C, the dynamic extraction time – 30 min, the flow rate of SC-CO_2 - 2 mL/min and the extraction pressure - 100 atm; for the extraction step II: the extraction temperature (40^0C), the dynamic extraction time – 60 min, the flow rate of SC-CO_2 - 2 mL/min. The obtained collorless residue indicated complete extraction of target compound. In the experiment acetone (7 %) was used as co-solvent in the second step of SFE.

The results of experiment show that the effect of pressure on the recovery of carotenoids at a constant temperature is a function of amount of SC-CO_2. The solubility of target analytes increased with increase of pressure. At a constant temperature, ingreasing the pressure increases the density of SC-CO_2. The solvent strenght increases with the density and increases the solubility of carotenoids. The carotenoids content decreases at the higher extraction temperature at a constant pressure, because the temperature reduces the density of SC-CO_2. The recovery of carotenoids increases with increase of the extraction time at a constant temperature as well. Also, the effect of sample size on the carotenoids recovery is a function of the extraction time. The less the sample size is, the more is the extraction time and the more the flow rate is, the less is the extraction time.

Sequential Ultrasound-Assisted Extraction

The results of UAE procedure indicate that the effects of the extraction time and ultrasound power are significant for both analytes. The effect of the extraction time on the extraction of carotenoids was investigated by carrying out the experiments at 30, 45, 60 and 90 min. It was observed that the recovery of carotenoids increased exponentially in 5-10 minutes, then increased gradually in 25 minutes and then became constant during extraction. Most of carotenoids extracted during the 2/3 of total extraction time (60 min), then ultrasound degradation and isomerization leads to the reduction of the amount of carotenoids due to the side effect of ultrasonication. Thermal effect plays an important role in UAE. At comparatively low ultrasonic power (37 kHz), thermal effect can be ignored because the heat produced by ultrasound may be completely diffused. The high ultrasonic power (80 kHz) causes thermal effect and isomerization of thermally sensitive target substances.

Hence, the three-step extraction procedure for ultrasound-assisted extraction of carotenoids from dried samples was developed. The ultrasonic bath was set at 37 kHz; The optimal parameters are for the extraction step I: the sample size – 10 g, the extraction time – 30 min, acetone was used as a solvent, for the extraction step II: the extraction time – 15 min, acetone was used as a solvent, for the extraction step III: the extraction time – 15 min, ethyl acetate was used as a solvent. The obtained organic extracts were transferred to dark glassware and stored within 5 days. For HPLC analysis the extracts were transferred to a dark volumetric flask and diluted to volume with diluent, mixed well. The sample extract solutions were filtered through 0.45 µm PVDF microporous membrane filter, discarding the first 5 mL of the filtrate.

Optimization of Chromatographic System Conditions

The final chromatographic conditions were determined by optimizing the system operational parameters: the wavelength for detection, the gradient program of mobile phase, the composition of mobile phase, the flow rate, the nature of stationary phase and the injection volume. The system suitability parameters: theoretical plates, tailing factor, peak purity were checked.

Uncertainty Evaluation of the HPLC Method

In order to obtain an estimate of the uncertainty associated with the measurement result the following tasks were to be performed: to specify the measurand; to identify the sources of uncertainty; to calculate the uncertainty components associated with each potential source of uncertainty identified; to calculate the standard uncertainty applying the appropriate coverage factor; to give an expanded uncertainty (U). The following sources of uncertainty were identified: the repeatability of measurement (n=6), the measuring equipment and glassware - analytical balance, HPLC, pipette, flask, etc. The A type of uncertainty - U_A (the repeatability of measurement) and the B type of uncertainty – U_B (the analytical procedure) were estimated, separately. The expanded uncertainty value of the method is 4.35 % for beta-carotene and 4.31 % for lycopene.

Method Validation

Specificity

The specificity of an analytical method is the ability to assess the analyte in the presence of components that may be expected to be present. The specificity was checked by injecting the standard solution, the system suitability check solution, the background control – blank and the sample extract solution. It has been shown that there is no interference from the blank at the retention time of an analyte's peak. The retention time for principal peaks on the chromatogram obtained with sample extract solution corresponds to that of the respective peaks in the chromatogram obtained with the standard solution. The both principal peaks are pure. Purity factor (999.5 for beta-carotene and 999.7 for lycopene) was more than purity threshold (990.0). (**Figures 4**, **5**, **6**, and **7**) shows the chromatogram obtained with the standard solution of beta-carotene, the standard solution of lycopene, the system suitability check solution and the blank, respectively.
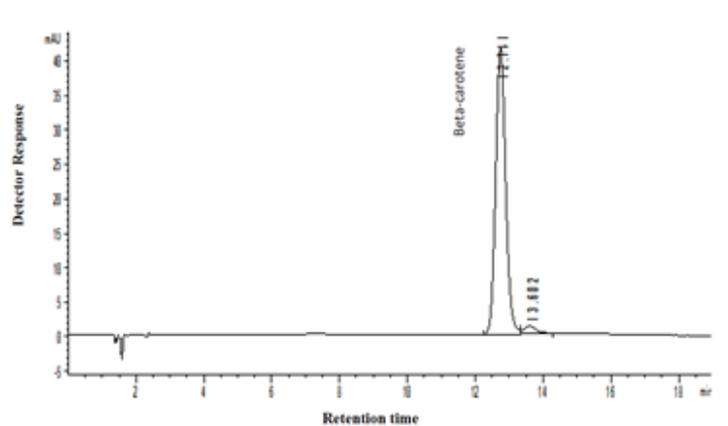
Figure 4. Chromatogram of the standard solution of beta-carotene
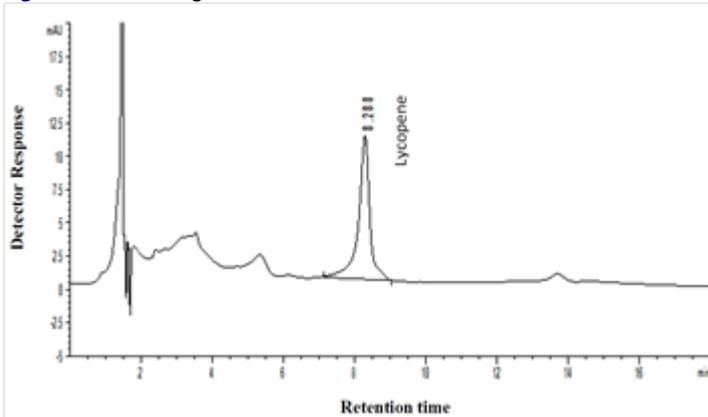
Figure 5. Chromatogram of the standard solution of lycopene
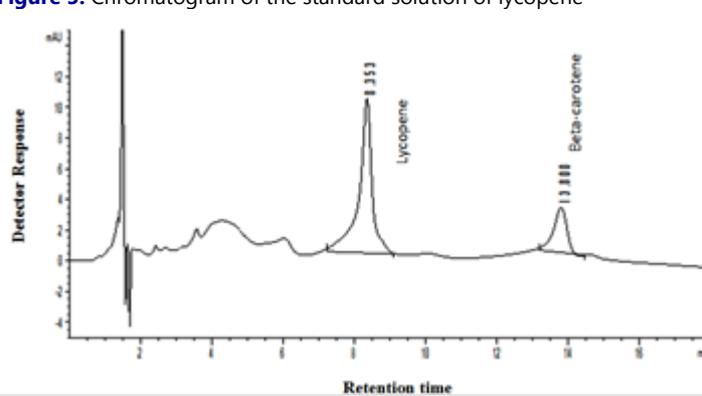
Figure 6. Chromatogram of the system suitability check solution
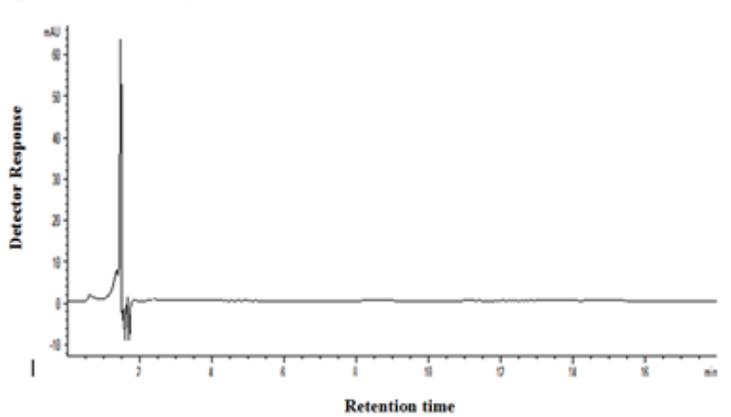
Figure 7. Chromatogram of the blank

Table 1. The linear regression data for lycopene

Level	Concentration, µg/mL	Average peak area	RSD of peak areas, % (n=3)
I	18.75980	8781.0	0.099
II	3.75196	1758.0	0.647
III	0.84419	336.5	0.889
IV	0.75039	294.7	0.678
V	0.13644	53.1	0.713
VI	0.06822	26.1	0.442
VII	0.03411	12.7	1.200
Correlation coefficient (r)		0.99995	
Square of correlation coefficient (r^2)		0.99990	

Table 2. The linear regression data for beta-carotene

Level	Concentration, µg/mL	Average peak area	RSD of peak areas, % (n=3)
I	6.65800	2571.6	0.509
II	2.43622	924.6	0.087
III	1.29932	541.8	0.287
IV	0.12993	91.6	0.315
V	0.06497	65.5	0.895
VI	0.01624	11.6	0.499
VII	0.00812	5.6	1.025
Correlation coefficient (r)		0.99962	
Square of correlation coefficient (r^2)		0.99924	
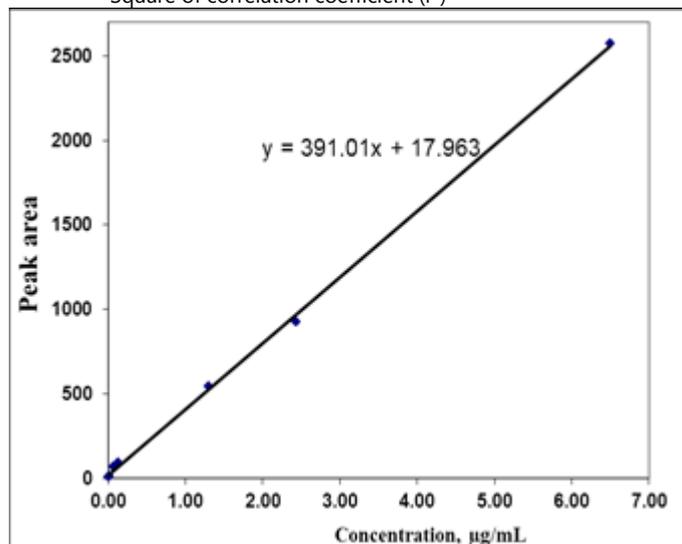
Figure 8. The linear response function for beta-carotene

Linearity and range

The response function of an analytical method is its ability to elicit results that are directly or by a well-defined mathematical transformation, proportional to the concentration of the analyte in a sample within a given range. From the standard stock solution working solutions were prepared at seven different concentration levels ranging from 0.34 µg/mL to 18.76 µg/mL for lycopene, from 0.081 µg/mL to 6.497 µg/mL for beta-carotene. Three replicate injections (n=3) were performed at each concentration level. The linearity was checked by the correlation coefficient (acceptance criteria: >0.990), the square of correlation coefficient (acceptance criteria: >0.98), the relative standard deviation (RSD, %) of peak areas (acceptance criteria: <1.5 %) at all the concentration levels, the RSD, % of retention times (acceptance criteria: <1.0 %). The calibration curve was constructed by plotting the response area against the corresponding concentration of the injected solutions. A value closer to unit of the correlation coefficient indicates a good linearity. The calibration plot and the corresponding statistic parameters of the regression for each analyte are shown in **Tables 1** and **2**. (**Figures 8** and **9**) shows the linearity plot.
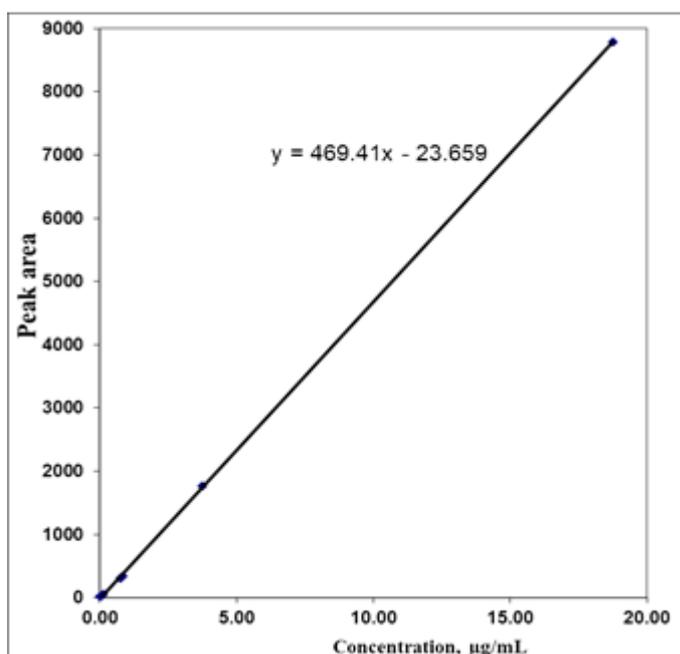
Figure 9. The linear response function for lycopene

Table 3. The LOQ and LOD for beta-carotene

Parameter	Value
LOQ, µg /mL	0.0081
LOD, µg /mL	0.00405
RSD of peak areas, % for LOQ (n=6)	8.206
RSD of peak areas, % for LOD (n=6)	12.318
s/N for LOQ	12.7
s/N for LOD	4.1

Table 4. The LOQ and LOD for lycopene

Parameter	Value
LOQ, µg /mL	0.034
LOD, µg /mL	0.0085
RSD of peak areas, % for LOQ (n=6)	5.432
RSD of peak areas, % for LOD (n=6)	24.092
s/N for LOQ	12.2
s/N for LOD	3.3

Limit of quantitation (LOQ) and limit of detection (LOD)

The LOD is the smallest quantity of the target substance that can be detected but not accurately quantified in the sample and the LOQ of method is the lowest amount of the target substance, which can be quantitatively determined under the experimental conditions prescribed with included inside the acceptance limits over the concentration range investigated. The signal-to-noise ratio (S/N) was adopted for the determination of the LOQ. This is estimated to be ten times the S/N ratio; the LOQ is estimated to be three times of s/N ratio (acceptance criteria). The LOQ was achieved by injecting a series of stepwise diluted solutions and precision was established at the specific determined level. The RSD, % of peak area values should not be more than 10 % (acceptance criteria). The determined LOQ and LOD for target analytes are presented in **Tables 3** and **4**. The LOQ of the method for beta-carotene was estimated to be equal to 0.0081µg/mL and 0.00405 µg/mL could be considered as the LOD; for lycopene - 0.034 µg/mL and 0.0085 µg/mL, respectively, according to the acceptance criteria.

Table 5. The results of system suitability test

Parameter	Acceptance Criteria	Result
Tailing factor	<2.0	For beta-carotene: 0.76-0.89; For lycopene: 1.11-1.22
Column efficiency	>2000 theoretical plates	For beta-carotene: 8851-9426; For lycopene: 5016-5348
Resolution factor	>7.5	9.52-9.75
RSD, % of peak areas (n=6)	< 2.0 %	For beta-carotene: 1.642 %; For lycopene: 1.040 %
RSD, % of the retention times (n=6)	< 1.0 %	For beta-carotene: 0.682 % For lycopene: 0.046 %

Table 6. The results of recovery for beta-carotene

Beta-carotene			
The concentration of beta-carotene	Spiked sample solution Average peak area (n=3)	Extract sample solution Average peak area (n=3)	Rec, %
80%	311.5	217.6	109.9
100%	363.1	272.0	106.6
120%	415.3	326.4	104.0
The average Rec, %			106.8
RSD, % of the percentage recovery			2.745

Table 7. The results of recovery for lycopene

Lycopene			
The concentration of lycopene	Spiked sample solution Average peak area (n=3)	Extract sample solution Average peak area (n=3)	Rec, %
80%	1367.1	1098.8	107.1
100%	1626.6	1373.5	101.1
120%	1888.5	1648.2	101.4
The average Rec, %			101.4
RSD, % of the percentage recovery			5.521

System suitability test

The system suitability test parameters were measured to verify the chromatographic system performance. System suitability was checked by six replicate injections (n=6) of the standard solution of the both analytes. The main parameters including the RSD, % of peak area values (acceptance criteria: <2.0 %), the RSD, % of the retention time values (acceptance criteria: <1.0 %), the peak tailing factor (the USP coefficient of the peak symmetry) (acceptance criteria: < 2.0), the column efficiency - the number of theoretical plates (acceptance criteria: >2000) and the resolution factor (acceptance criteria: >7.5) were measured. The results are summarized in **Table 5**.

Accuracy

The accuracy of the method was assessed by comparing the analyte amount determined versus the known amount spiked at three different concentration levels (80, 100, 120 % of standard solution concentration) with three replicate injections (n=3). The sample solutions were spiked with standard solution at 0.055 μg/mL and 0.06 μg/mL concentrations of beta-carotene and lycopene, respectively. The accuracy is expressed as the percentage of standard compound recovered from a spiked solution (extract sample solution + standard) with a corresponding RSD, %. The average recovery should be within 85.0 –115.0 % and the RSD, % of the percentage recovery should be <6.0 % for each concentration level of spiked sample solution (acceptance criteria). The recovery - Rec, % for each concentration level of spiked solution was calculated by the following formula: *Rec, %= (Au$_1$ – Au$_2$) · 100/ As*. Where, Au$_1$ – the peak area of each carotenoid obtained with the spiked sample solution (endogenous added carotenoid standard), Au$_2$ - the peak area of each carotenoid obtained with the sample solution (endogenous added carotenoid standard) and As – the peak area of each carotenoid obtained with the standard solution. The results of the recovery are given in **Tables 6** and **7** which is well within the usually accepted limits indicating the accuracy of the method.

Table 8. The precision results for beta-carotene

Solution #	Extract sample solution			
	Repeatability (intra-day)		Intermediate precision (inter-day)	
	Peak area	Concentration, μg/mL	Peak area	Concentration, μg/mL
1	438.1	1.148	486.0	1.230
2	429.4	1.125	469.8	1.189
3	429.1	1.125	469.6	1.188
4	427.5	1.120	469.1	1.187
5	427.0	1.119	468.6	1.186
6	426.3	1.117	467.6	1.183
Average	429.6	1.126	471.8	1.194
RSD (n=6)	1.013	1.012	1.486	1.486
F-test	0.46			

Table 9. The precision results for lycopene

Solution #	Extract sample solution			
	Repeatability (intra-day)		Intermediate precision (inter-day)	
	Peak area	Concentration, μg/mL	Peak area	Concentration, μg/mL
1	1848.7	4.991	2097.1	4.925
2	1834.3	4.952	2076.1	4.876
3	1835.1	4.954	2077.3	4.878
4	1823.6	4.923	2066.0	4.852
5	1830.2	4.941	2071.9	4.866
6	1817.0	4.905	2060.1	4.838
Average	1831.5	4.944	2074.8	4.872
RSD (n=6)	0.594	0.594	0.613	0.613
F-test	0.94			

Precision

The precision of an analytical method is the degree of agreement among the individual determination results obtained, when the method is repeated with multiple samples from the same homogeneous sample mix. This analytical parameter was estimated by measuring repeatability (intra-day precision) and time-dependent intermediate precision (inter-day) on six individual determinations of each analyte in the extract sample solution at the same concentration. The intermediate precision (inter-day) was carried out in a different day. The repeatability was checked by the RSD, % of determined concentrations (μg/mL) for six individual determinations of each target compound which should not be more than 3.0 %; The intermediate precision was checked by the F-test which should not be more than 5.05 (acceptance criteria). The results obtained with the precision study are given in **Tables 8** and **9**. The RSD, % of determined concentrations (μg/mL) for six individual determinations of each carotenoid and the F-test were within than acceptance criteria which indicate that this method has a good precision.

Standard solution stability

The standard solution stability was studied initially, after 24 and 48 hours, 3, 5, 7 days against freshly prepared standard solution. This parameter was checked using two standard solutions and calculated the percentage bias between peak areas of standard solutions stored under refrigeration in dark glassware and freshly prepared which should not be more than 3.0 % (acceptance criteria). The bias in terms of peak area between two standard solutions should be within 0.98-1.02 (acceptance criteria). The standard solution of beta-carotene is stable for the period up to 5 days prepared in refrigerator and dark glassware (2.12 % after 5 days) and the standard solution of lycopene – 48 hours (2.87 %).

Filter compatibility test

The PVDF membrane filter compatibility was evaluated using standard solution and by calculating the percentage bias between peak areas obtained with standard solutions filtered and non-filtered of both analytes
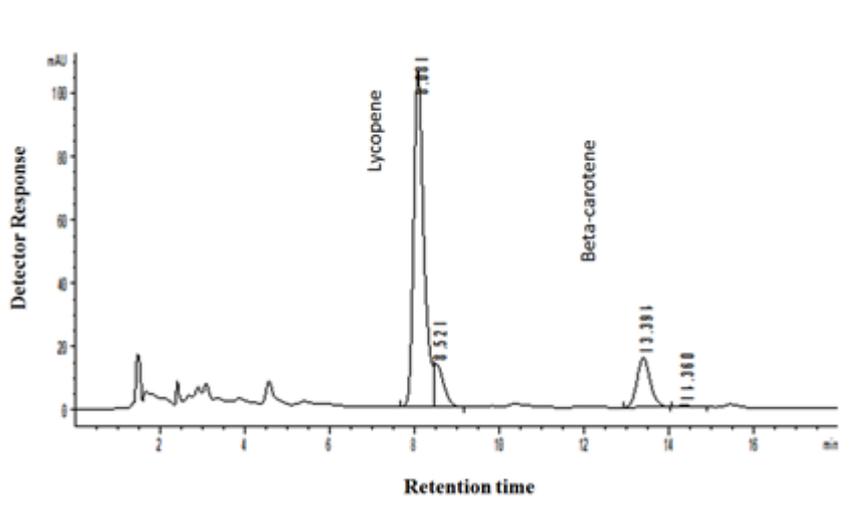
Figure 10. Chromatogram of the extract sample solution

Table 10. The content of beta-carotene and lycopene in dried agro-industrial waste material

Samples	The content of carotenoids, μg/g			
	Beta-carotene (±U=4.35 %)		Lycopene (±U=4.31 %)	
	Extraction technique			
	UAE	SFE	UAE	SFE
Tomato skin	9.06 - 10.67	8.39 - 12.75	165.11 – 173.79	165.76 - 179.56
Tangerine peel	26.77 - 31.64	25.65 – 32.18	12.25 – 15.96	11.12 – 17.91
Orange peel	42.01 - 54.65	41.66 – 59.16	8.37 - 9.89	9.65 - 10.65

which should not be more than 0.5 % (acceptance criteria). The result is 0.21 % for beta-carotene and 0.19 % for lycopene, which gives the confidence that adsorption of target compound does not occur on the used filter.

Estimation of beta-carotene and lycopene content in dried samples

The organic extract solutions prepared using the developed supercritical fluid and sequential ultrasonic assisted extraction procedures and were analyzed using the validated HPLC method. (**Figure 10**) shows the chromatogram obtained with the extract sample solution.

The content of each carotenoid – beta-carotene and lycopene in μg per g of tomato/tangerine/orange dried waste materials was calculated as well. The results are given in **Table 10**.

CONCLUSION

Hence, the developed sequential extraction techniques of carotenoids using the ultrasound-assisted extraction and the supercritical fluid extraction are simple, effective, eco-friendly separation procedures, which provide high quality of target compounds and can be used to develop a standard technological process for utilization of agro-industrial waste materials - tomato skin, tangerine and orange peels. Also, the developed and validated HPLC method for quantitative determination of the major carotenoids namely beta-carotene and lycopene is rapid, effective and selective analytical procedure which can be successfully used by scientific and quality control laboratories.

ACKNOWLEDGEMENTS

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