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Optimization of a Green Methodology to Form Nutritional Rich Streams of Biocarotenoids and Phenolic Compounds from Greek Juice Production Byproduct Streams

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ABSTRACT

Carrot, orange, kiwi, banana, apple and watermelon by-product streams (mainly pulp) derived from 2 different "fresh" juice production agroprocesses from Aegean Islands (Greece), including Lemnos island [(1) by-products of agro process line-1 (PL1) derived from fresh juice contained 30% (v/v) carrot, 30% (v/v) orange, 20% (v/v) apple, 10% (v/v) banana, 5% (v/v) kiwi and 5% (v/v) watermelon (by-products derived from agro food process line-1;PL1) and 2) by-products of agro food process line-2 (PL2) derived from fresh juice of 30% (v/v) orange, 30% (v/v) apple, 20% (v/v) carrot, 10% (v/v) banana, 5% (v/v) kiwi and 5%(v/v) watermelon (PL2)) were analyzed for their nutrient microprofile (total and specific carotenoid content, total phenolic content as well as their antioxidant activity). A green ultrasound assisted extraction process was designed, developed, employed, and optimized. The effect of

a) Different Temperatures (20 °C, 30 °C, 50 °C);

b) Solvents (water, ethanol, 2-propanol, acetone);

c) Extraction Time (0-300min);

d) Treatment of the samples (either after cold storage (stored at -18 °C) or after freezing (4 °C) and after drying at 60 °C for 6h);

e) Solid to liquid ratio (0,02; 0,05; 0,1mg/mL) and

f) Ultrasound power (150W, 200W, 250W) were tested (studying their impact on total carotenoid, phenolic as well as antioxidant content of aforementioned by-products streams). Also, the effect of pH (3 - 8) on chemical stability of extracts examined. The highest total carotenoid and total phenolic as well as antioxidant content achieved were 8.23 ± 1.12(mg/100g), 110.79 ± 3.12 (mg GAE/mL), 243.56 ± 0.45(mmol Fe_2SO_4/mL), respectively in juice agro-processing by-product streams derived from PL1, at optimized conditions (dried samples taken out from freezing conditions, using ethanol:water (60:40% v/v) as a solvent, extracted at 50 °C for 140min, under 250W Ultrasound power with initial solid to liquid concentration of 0.05mg/mL). Identification of carotenoids content, of the stream with the highest antioxidant activity (PL1) showed the presence of β-carotene; a-carotene; lutein; zeaxanthin; β-cryptoxanthin and lycopene at varying concentrations ranking from 0,1512 ± 0,014 (mg/g) to 0,01 (mg/g).

Introduction

Naturally occurring carotenoids, meaning tetra-terpenoids consisted of highly unsaturated isoprene derivatives, are of high significance in different industrial sectors such as food and technology science human nutrition, chemical, pharmaceutical, poultry as well as cosmetics. Lately, carotenoid pigments demand has been increased owning to their diversified market applications, such as coloring agents in food products like margarine, soft drinks, baked products; as precursors of pro - Vitamin A in animal and food feed; as additives in multivitamin preparations as well as antioxidants, aiming at reducing cellular damage (Malik, et al. [1]). Studies indicate the synergistic action of diet micronutrient antioxidants such as carotenoids towards the prevention of chronic diseases development such as cancer (Stahl, et al. [2]) and cardiovascular diseases (recently associated with heart rate variability) (Ying Huang, et al. [3]). Also, the correlation of protein lens oxidation and age modulated possible cataract prevention has been studied in women (a total of 77466) as well as in men (a total of 36644), in USA, through a prospective cohort study regarding carotenoids (lutein, zeaxanthin, a-carotene, b-carotene, lycopene, beta-cryptoxanthin) as well as vitamin-A (and retinol equivalent) intake by Chasan Taber, et al. [4] and Brown, et al. [5]).

Also, the positive effects associated with human intake of a-carotene, β-carotene and β-cryptoxanthin in possible inhibition of antigen specific IgE antibody production, protecting from the development of allergy symptoms (including protection against allergic children rhinitis) as well as from asthma prevalence, have been studied through a semi-quantitative food frequency questionnaire (Rerksuppaphol, et al. [6,7]). The most common methodologies used for carotenoid extraction are those that take advantage of the polarity of solvents. Organic solvents such as hexane, petroleum ether and tetrahydrofuran (non-polar) as well as acetone, ethanol or ethyl-acetate (polar) could be used for the extraction of carotenes (hydrocarbonbased molecules without functional groups) such as b-carotene and xanthophylls (which contain oxygen in the functional group of either aldehydes or ketones or alcohols) [8,9]. Some of them are toxic and expensive and can be used for the extraction of bioactive compounds from fruit by-product and waste streams from juice processing plants [10-12]. The aim of this study was the development and optimization of a green process, namely ultrasound assisted extraction targeting maximization of recovering of bioactive compounds contained in (from Aegean regions) carrot (*Daucus carota L*), orange (*Citrus sinensis L*), kiwi (*Actinidia delicioza*), banana (*Musa paradisiaca L*), apple (*Malus domestica*) and watermelon (*Citrullus lanatus*), fruit by-product streams containing mainly peels and pulp (but also seeds less than 5% w/w), derived from juice processing industry, of medium scale of Northern Aegean. The potential exploitation of agro industrial by-product and waste streams of industrial food sector has been previously underlined [8-11,13-17]. So, within this frame the effect of temperature, sample treatment, solvents, pH on the extraction yield of total carotenoids and total antioxidant activity was studied. Also, identification of carotenoids contained in the extract with the highest antioxidant activity took place. The aim of the above study was the development of an efficient green process leading to the production of a bioactive-rich extract of high antioxidant potency, derived from Greek agro industrial waste and by-product streams of low and or negative cost, that could be used as media for the production of novel Greek originated Miso like food product.

Materials and Methods

Fruit Processing By-Product Streams

Fruit by-product streams of carrot, orange, kiwi, banana, apple and watermelon (containing mainly peels and pulps but also small amounts of seeds and stems) derived from 2 different fresh juice production processes of Aegean Islands:

1) 30% carrot, 30% orange, 20% apple, 10% banana, 5% kiwi and 5% watermelon (FP1) and

2) 30% orange, 30% apple, 20% carrot, 10% banana and 5% kiwi and 5% watermelon (FP2).

Chemicals

Absolute ethanol (pharmaceutical grade), acetone, 2-propanol, acetate, sodium carbonate, solvents for HPLC analysis (acetonitrile, methyl alcohol (usp), dichloromethane), iron chloride hexahydrate, carotenoid standards as well as reagents for the antioxidant measurements, including TPTZ (2,4,6-tris (2-pyridyl)-striazine), iron (¶) chloride, were of analytical grade and purchased from Sigma Aldrich. Methyl tert-butyl ether (MTBE) and methanol (MeOH), both of chromatographic grade, standards of (all-E)-α-carotene (purity: 92%); (all-E)-β-carotene (purity: 97%); (all-E)-lutein (purity: 92%), (all-E)-zeaxanthin (purity: 93%), (all-E)-β-cryptoxanthin (purity: 98%) and citric acid as well as sodium hydroxide were also purchased from Sigma Aldrich.

Ultrasonic-Assisted Extraction Method

By-product streams derived from carrot, orange, kiwi, banana, apple and watermelon were delivered from juice production industries from Aegean islands. The carotenoids of fruits by-products from fresh juice-production lines, were extracted using an ultrasonic bath (20kHz). Samples were stored under cold storage in a novel cylindrical air driven and assisted bubbled lifted laboratory

scale reactor at (-4 °C) and under refrigerating conditions (2 °C, 18% humidity). Also, by-product streams were dried for 6-30h, in air circulating oven at 55-70 °C, depending of the sample, while previously have been passed through from sieve mesh 300mm (air-forced). 1 mg dried samples were mixed with 50ml solvent in a 250ml Erlenmeyer flask. The water in the ultrasonic bath was kept above the level of the solvent in the Erlenmeyer flask. The crude extract that contained the solvent extracted carotenoid was filtered using a Whatman filter paper No. 4 aided by a single stage. The extraction process studied to determine the best conditions. All experiments were performed in shady environment, at ambient conditions. The effect of time (0-300min), treatment (cold storage, refrigerating conditions as well as dehydration) temperature (20 °C, 30 °C, 50 °C), solvent (water, acetone:water (50:50 v/v), 2-propanol and ethanol:water (60:40 v/v)), initial solid to liquid ratio (0,02mg/mL; 0.05mg/mL; 0.1mg/mL) and ultrasound power (150W, 200W, 250W), were studied. All measurements were carried out in triplicate for each sample, and obtained values were averaged.

Carotenoid Content

100μl of each-sample, prepared as described previously, were dissolved in 2ml of hexane dissolved in 0.4M sunflower oil and stirred for 3min. The absorbance was measured using a UV-Vis spectrophotometer (Giorgio Bormac UV 10plus, Italy) at 445nm wavelength. Finally, the absorbance of each sample was compared with a standard curve (R2 = 0.99) to calculate the concentration of carotenoids in micrograms per 100g of crude extract. The standard curve was prepared according to Ying, et al. [18]. All measurements were carried out in triplicate for each sample, and obtained values were averaged.

Total Phenolic Content

Total phenolics were determined according to the colorimetric method of Folin-Ciocalteu previously described [12,14,15]. More specifically, in a 1.5mL Eppendorf tube, 0.78mL of distilled water, 0.02mL of sample and 0.05mL of Folin-Ciocalteu reagent were added and vortexed. After exactly 1min, 0.15mL of aqueous sodium carbonate (Na_2CO_3) 20% (w/v) was added, and the mixture was vortexed and allowed to stand at room temperature in the dark, for 60min. The absorbance was read at 750nm, using a spectrophotometer (Giorgio Bormac Agrolab), and the total polyphenol concentration was calculated from a calibration curve (50-500mg L-1), using gallic acid (GA) as standard. Results were expressed as mg gallic acid equivalents per ml of fruit extracts. All measurements were carried out in triplicate for each sample, and obtained values were averaged.

Ferric Reducing Antioxidant Power

The antioxidant activity was evaluated according to FRAP assay procedure as it has been previously described with slight modifications [7]. A fresh prepared FRAPreagent (25mL acetate buffer, 300mM, pH 3.6 + 2.5mL 10mM TPTZ (2,4,6-tripyridyl-5-triazine) in 40mM HCl mixed with 2.5mL 20 mM $FeCl_3 \cdot 6H_2O$) was made each time prior to analysis. This fresh reagent was heated in water bath at 40 °C for 2min before being transferred (900μL) into tubes containing 100μL of fruit by-product streams extract. The tubes were left in water bath (Giorgio Bormac Agrolab) at 40 °C or 40 minutes. The absorbance was read at 593nm, using a Giorgio Bormac UV spectrophotometer, and the antioxidant activity was calculated from a calibration curve, represented as mmol $FeSO_4$ equivalents/mL (180 - 240mmol $FeSO_4$/mL). All measurements were carried out in triplicate and obtained values were averaged.

High Performance liquid chromatography (HPLC)

Carotenoids were quantified using a high-performance liquid chromatographic system equipped (Shimadzu) with column ZORBAX Eclipse Agilent, XDB-C18. The column was maintained at 30 °C using a heated column. The wavelength of UV detector photodiode (Waters 996) was 450nm. Samples of 10μL injected and analyzed. Standard solutions of reconstituted carotenoids were prepared over a concentration range of 1 to 5mg/L and diluted in solvent B (methanol/MTBE, 20:80). A Waters pump 600 used gradient forming. The mobile phase flow rate was 1.0ml/min. The mobile phases were A: methanol/MTBE/water (70:20:10) and B: methanol/MTBE (20:80). The mobile phase gradient used was as follow: starting at 100% A to 50% A/50% B in 45 minutes followed by 50% A/50% B for 24:58 minutes. The column was re equilibrated between 5 samples for 25 minutes. The rebalancing was necessary to remove residual effects of solvent B. The concentrations of the individual carotenoids (β-carotene, a-carotene, lutein, zeaxanthin, β-cryptoxanthin and lycopene) were calculated based on the retention times and the standard curves of the external reference standards.

Results and Discussion

The Effect of Time, Treatment, and Temperature on Total Carotenoid Extraction from Fresh and Dried Samples

Treatment of samples of foods involves changes in structural integrity which may have negative effects on carotenoid concentration (loss) and positive effects such as increase in bioavailability [19]. The losses of carotenoids most times are attributed to oxidative degradation in samples, mainly stimulated by light exposure of samples containing carotenoids. Table 1 shows total carotenoid yield, at 30 °C and 50 °C extraction temperatures, during ultrasound assisted extraction at 250W ultrasound power, carried out to by-product and waste streams derived from PR1, showed higher total antioxidant activity than that obtained from PR2 (data not shown). The increase in temperature from 30°C to 50°C led to approximately two-fold increase in total carotenoid extraction in fruit by-product streams extracts in all tested samples,

during extraction period. The highest carotenoid extraction yield achieved, at 50 °C, after approximately, 3,33h of extraction, at fresh samples that had been in kept storage at 4oC for 10days. Also, at 50 °C the extraction yield of total carotenoids was higher compared to 30 °C (0.5 ±0,11mg/100g). That it could be attributed to a better release of carotenoids from disturbed matrix of fruit by-product and waste streams at 50 °C. At 50 °C the highest carotenoid yield measured after 3,33h of extraction (6,12±0,01mg/100g sample). Also, at 30 °C the highest carotenoid yield of 2,9 ± 0,02mg/100g, was measured after approximately 200 to 300min of extraction. In both experiments, the highest yields regarding total carotenoid content measured at fresh samples preserved at 4 °C, regarding their freeze storage conditions (after 200 to 300sec extraction) from PL1 samples. In both experiments (Table 1) the extraction yields were almost stable, and no significant increase observed (data not shown), after 200 to 300min, indicating the end of the extraction time process. That might be attributed to the fact that carotenoids and more specifically β-carotene (which is the most predominant carotenoid) exists in the all-trans form. However, during extraction some of the all-trans form might be converted to its different cis-isomers.

Table 1: Total Carotenoid yield (mg/100g) at temperatures of 30 °C and 50 °C of fruit processing samples.

Extraction Time (min)	Treatment of samples at 30 °C		Extraction Time (min)	Treatment of Samples 50 °C	
	Cold Storage	Freezing		Cold Storage	Freezing
60	2,00±0,12	2,2±0,01	20	2,54±0,11	3,34±0,04
100	2,32±0,1	2,45±0,05	60	4,87±0,032	5,23±0,01
160	2,54±0,22	2,67±0,11	100	4,98±0,02	5,86±0,02
200	2,66±0,02	2,9±0,02	140	5,00±0,02	6,01±0,03
260	2,71±0,04	2,9±0,011	160	5,01±0,01	6,05±0,01
300	2,72±0,12	2,9±0,02	200	5,02 ±0,02	6,12±0,01

Rafajlovska, et al. [20] reported that mass transfer processes regarding rhythm of bioactives extraction is positively affected by temperature increase. However, since raw material browning is highly affected by temperature increase the establishment of a procedure, studying the effect of temperature in samples was of high significance. Under that circumstance, the optimum temperature for total carotenoid extraction was 50 °C and the optimal extraction time was 140min. Regarding the outcomes of this study the highest carotenoid extraction observed to fresh samples of Greek Aegean juice agro-processing plants at 50 °C, after either 140-200 and 160-300min, concerning cold or refrigerated preservation and treatment of them. Besides dehydration, also boosted carotenoid extraction, as it can be seen in Table 2. More specifically, samples taken out either from cold storage or freezing where dehydrated in an air-filtered forced horizontal circulating oven, at optimum conditions, for 6-8h at 60 °C. The maximum carotenoid extraction yield observed was 6,53±0,13 mg/100g after 140min of extraction at 50oC, to samples preserved at optimum freezing conditions. On the other hand, the highest carotenoid yield measured to PL1-samples, preserved at optimum cold storage conditions was 5,92±0,04mg/100g. Undoubtedly, treatment of samples before dehydration step as well as dehydration treatment, highly affects the extraction of carotenoids. In both series of experiments studying the effect of temperature at 50 °C extraction, resulted in higher maximum extraction yields of carotenoids extraction, compared to either lower or higher degrees. Also, dehydration of treated samples led to higher carotenoid extraction yields, implying that both optimum conditions of dehydration as well as extraction at 50oC, improved solubility of carotenoids and thus increased measured extraction yields. According to Dutta, et al. [17] heat treatment such as cooking, blanching may help the release of carotenoids bound by proteins, rendering them easily extractable.

Table 2: Total Carotenoid yield (mg/100g) at temperatures of 30 °C and 50 °C from dried*[1] byproduct streams samples*[1],*[2] Treatment of samples at 30 °C.

Extraction Time (min)	Treatment of samples at 30 °C		Extraction Time (min)	Treatment of Samples 50 °C	
	Cold Storage	Freezing		Cold Storage	Freezing
60	2,00±0,12	2,2±0,01	20	2,67±0,11	3,55±0,07
100	2,56±0,01	2,45±0,05	60	4,87±0,032	5,65±0,02
160	2,64±0,13	3,37±0,06	100	4,98±0,02	6,3±0,12
200	3,45±0,02	3,78±0,07	140	5,92±0,04	6,53±0,13
260	3,1±0,05	2,84±0,011	160	5,42±0,03	6,51±0,01
300	3,2±0,12	2,89±0,04	200	5,4 ±0,06	6,32±0,02

Note: *[1]Samples were dehydrated for 6h at 60 °C.

*[2]The results are expressed as mean values of three replicates ± standard deviation

Effect of Solvent, Initial Solid to Liquid Ratio and Ultrasound Power on Total Carotenoid Yield

Solvents tested for carotenoid extraction were water, acetone:water (50:50 v/v), 2-propanol and ethanol:water (60:40 v/v). In all experiments the better solvent system regarding the highest achieved yield of total carotenoid was ethanol: water (60:40 v/v). As it can be seen, in Figure 1 the maximum carotenoid production of 6,53±0,13 (mg/100g), achieved when ethanol: water (60:40) used as extraction solvent, after approximately 140 to 150min of extraction. According to obtained data carotenoid extraction is increasing up to approximately 150min. After that time no further extraction observed. At higher temperatures than 50 °C, using the best solvent-system (data not shown) lower total carotenoid production measured as well as extraction yields were obtained (for instance at 100 °C, maximum carotenoid yield of 2,4±0,01(mg/100g) measured after 30 minutes of extraction step). This finding could be attributed to progressive time and heat dependent carotenoids degradation. However, the loss of carotenes could be partially attributed to isomerization. Unquestionably, the most predominant reaction is oxidative degradation. Calvo, et al. [21] studying the effect of food grade solvents such as ethyl acetate and ethanol in carotenoid extraction from tomato peel powder reported that carotenoid extraction was noticeably higher when the used solvent was ethanol. This study shows and highlight that thoroughly investigation of proper food-grade solvents to extract carotenoids from fruit streams for use as dilution nutritional agent to produce enriched with bioactive compounds novel food production systems is of high academic and scientific not only interest but also significance.

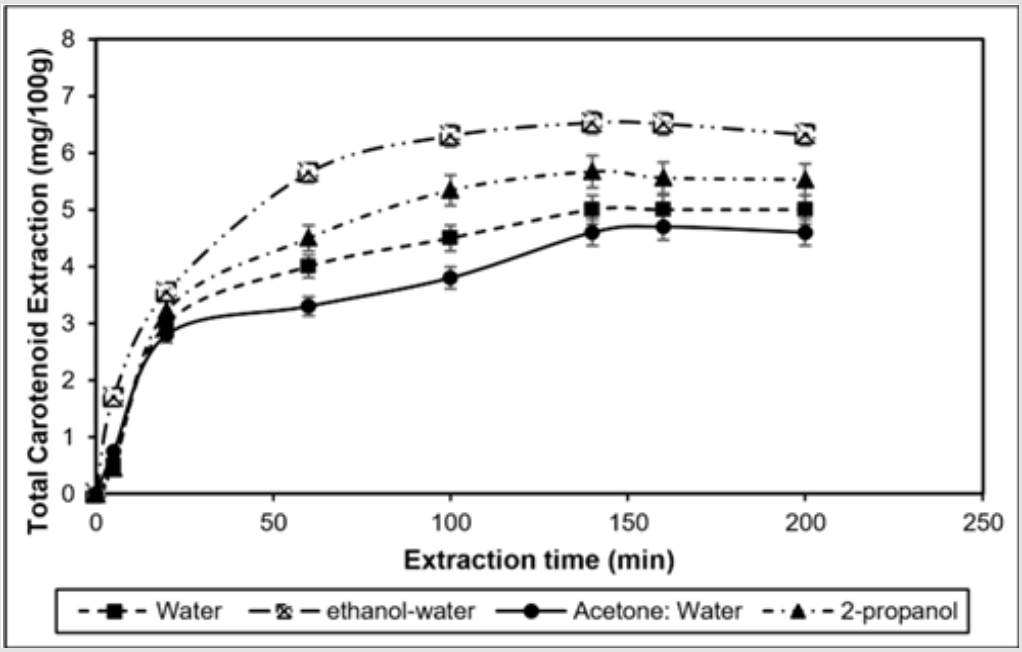

Figure 1: Time dependent extraction of Greek Aegean islands fruit processing line-1 (mg/100g) using different food grade solvent systems.

In this study, according to the displayed data achieved it was shown that optimization of the extraction in fruit by-products and waste streams of fresh juice production agro processes is a multivariant affected aspect directly related to matrix structure as well as time, temperature, ratio and polarity of green food grade solvents used. Also, the effect of solid to liquid ratio (0,02; 0,05; 0,1mg/mL) and ultrasonic power (150W, 200W, 250W) on total carotenoid yield was studied (Table 3). The results showed that the initial solid to liquid ratio as well as ultrasound power affected total carotenoid production measured at the extracts. The highest carotenoid content of 8,23±1,12mg/100g, achieved when the initial solid to liquid ratio was 0.05mg/mL and ultrasonic power was 250W. Another finding from these experiments (Table 3) was the increase of ultrasonic power either from 150W to 200W or 250W resulted in an increase on the extraction of the contained carotenoids in the tested samples of fresh juice by-product streams derived from RP1. It seems that, the increase of ultrasound power led to a subsequent increase to maximum achieved carotenoid production of extracts derived from RP1 fresh juice stream-lined, using aqueous ethanol as food grade solvent. These findings could be attributed to the propagation of ultrasound pressure waves resulting in cavitation forces, generating bubbles which explosively collapsed owning to forcing localized pressure ending up to rupture of plant layers tissues. Thus, improving the release of intracellular substances into the solvent system. In recent years, several studies have studied conventional solvent extraction on carotenoids recovery from different plant food materials and their by-products, such as tomato wastes [22], corn and grapefruit (Ye et al., 2011) [23].

Table 3: Effect of different initial solid to liquid ratios on total carotenoid yield (mg/100g) of fruit by-product stream extracts at 140 to 150min of extraction under different utrasonic powers*[1], *[2].

Initial solid to liquid ratio	Ultrasound Power	Total Carotenoid Content (mg/mL)
0,02	150	6,53±0,16
0,02	200	6,6± 0,08
0,02	250	6,6± 0,13
0,05	150	7,07 ±1,04
0,05	200	7,58 ± 0,78
0,05	250	8,23 ± 1,12
0,1	150	6,7 ± 0,12
0,1	200	7,23 ± 0,12
0,1	250	7,8 ± 0,36

Note:

*[1]Samples were dehydrated for 6h at 60 °C.

*[2]The results are expressed as mean values of three replicates ± standard deviation.

However, no information on the ultrasound-assisted extraction of carotenoids from fresh juice by-product streams of Aegean Greek originated fruits, is available in the literature, apart from that presented previously studying apple bioactives by-product streams extraction [9].The optimum conditions regarding ultrasound assisted extraction regarding PL1fraction were: 250W pressure, ethanol:water (60:40 % v/v) as a solvent system, solid to liquid ratio equal to 0,05 mg/mL (db), 140-150min extraction time, at constant frequency of 20kHz. A key step in the recovery of bioactive compounds such as carotenoids involves a necessary extraction step. Extraction methodologies such as solvent extraction, mechanical expelling, supercritical fluid extraction etc. impose limitations such as use of extra solvent in solvent extraction, low yield in mechanical expelling, large capital in supercritical fluid extraction as well as requirement of aqueous phase in microwave assisted extraction [24]. On the contrary, Ultrasound Assisted Extraction, as it has been employed and optimized in this study offers many advantages such as less time. Furthermore, retention of the quality of the extract and potential extraction of bioactives at relatively low temperatures ranging from 30 to 50oC, offers additional energy requirements.

Bioactives Content in Fruit by-Product Stream Extracts and their Antioxidant Capacity

Comparing total carotenoid content as well as total phenolic content contained in samples of fruit byproduct streams extracts derived from carrot, orange, apple, banana, kiwi and watermelon processing lines; as previous described PL1 and PL2, is obvious (Table 4) that fruit by-product stream extracts derived from Greek Aegean islands fresh juice containing 30% carrot, 30% orange, 20% apple, 10% banana, 5% kiwi and 5% watermelon (PL1) demonstrated higher total carotenoid content compared to fruit by-product streams extracts derived from the production of fresh juice containing 30% orange, 30% apple, 20% carrot, 10% banana , 5% kiwi and 5% watermelon (PL2), (Table 4). These findings are very interesting highlighting the potential of small scale Greek fruit juice by-product and waste extracts, employing the green ultrasound assisted methodology (previously described) using solvents that are considered as food grade and GRAS (as safe), to be used as bioactive rich streams for the production of novel Greek Miso like foods with high nutritional value and possible several positive effects in sustaining good public health while reducing energy requirements aiming at protecting humans, land, air and water resources minimizing emissions and energy requirements. Besides, these extracts derived from costly raw material could possibly form an alternative to chemical preservatives forming a new area of natural preservatives of novel food owning to their relative high antioxidant capacity. In preliminary study bioactive content of extracts, derived from small scale fresh fruit by-products as it can be seen in Table 5 (under the optimized conditions), showed that carrot and orange had the highest total carotenoid and phenolic content as well as total antioxidant capacity of 2,67±0,43 (mg/100g); 2,43± 1,24 (mg/100g) and 54,67±2,45 (mg GAE/mL); 53,78±1,78 (mg GAE/mL); 100,45±3,24 (mmol Fe_2SO_4/mL) and 89,58±2,78 (mmol Fe_2SO_4/mL), respectively followed by apple and banana regarding both total carotenoid and phenolic content as well as antioxidant capacity. The lowest total carotenoid and phenolic content as well as antioxidant capacity measured to kiwi and watermelon extracts.

Table 4: Total Phenolic content, Total Carotenoid content and Antioxidant capacity of fruit byproduct streams extracts under optimum conditions of Utrasound assisted extraction*[1].

	Total Carotenoid Content (mg/100g)	Total Phenolic Content (mg GAE/ mL)	Antioxidant Capacity (mmol Fe_2SO_4/mL)
PL-1	8,23 ± 1,12	110,789 ± 3,12	243,56 ± 0,45
PL-2	6,47 ± 0,68	95,36 ± 1,12	207,34 ± 0,39

Note:

*[1]Dryed samples taken out from freeze conditions, using ethanol:water (60:40 v/v) as a solvent, extracted at 50 °C for 140min, under 250W Ultrasound power with initial solid to liquid concentration of 0.05mg/mL; 140 to 150min at optimized dried conditions.

*[2]Process Line-1: Fruit by-product streams extracts derived from the production of fresh juice containing 30% carrot, 30% orange, 20% apple, 10% banana, 5% kiwi and 5% watermelon (PL1);

*[3]Process Line-2: Fruit by-product streams extracts derived from the production of fresh juice 30% orange, 30% apple, 20% carrot, 10% banana, 5% kiwi and 5% watermelon (PL2).

Study of Stability of Fruit Pomace Extracts

The effect of pH on carotenoid stability of the extracts was studied. Fruit by-product streams derived from Process line-1;PL1, showed the highest carotenoid content as well as the highest antioxidant activity, were subjected to stability analysis regarding their carotenoid content. For that reason, extracts of PL1 were adjusted to pH 8, 7, 6, 5, 4 and 3 using 0.2N HOC(COOH)(CH2COOH)$_2$ or 0.2N NaOH and stored at 4 °C for 5 days. Neutral and slightly basic conditions (pH 6, 7 and 8) reduced total carotenoid content of fruit pomace derived from PR1, by approximately 16,4 %, 23,1 % and 23 %, respectively and acidic conditions (pH 5, 4 and 3) increased the measured total carotenoid content in carrot juice by 10,43 %, 22 %, 18,67 % respectively, after 5 days of storage at 4 °C. These findings indicate that carotenoids in fruit by-product stream, with highest antioxidant activity (RP1), were sensitive to pH. Another interesting finding of this study was that the increase of total carotenoid content (23.1 %) in acidified environment may be due to enhanced solubility of crystallized carotenoids present in the vacuoles of plant material [25] (Figure 2).

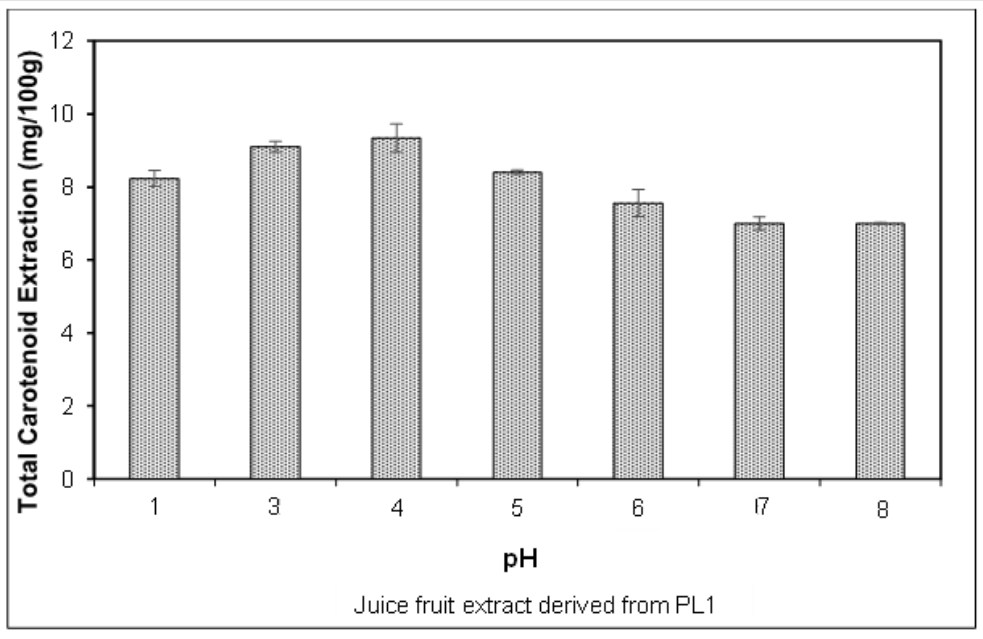

Figure 2: Effect of pH stability on total carotenoid yield (mg/100g) in fruit by-product streams derived from small scale juice producers of Aegean islands, after 5 days of storage at 4 °C.

Formation of a Carotenoid Rich Extract Under Green Assisted Mode for use in Nutrifood Applications: Identified Carotenoids Concentrations

Juice processing by-product stream extracts showed the highest total carotenoid and phenolic as well as antioxidant activity, PL1 (taking under consideration preliminary results (Table 5)) were further HPLC analyzed to identify the major contained sub-carotenoids. Fruit by-product streams extracts derived from the production of fresh juice containing 30% carrot, 30% orange, 20% apple, 10% banana, 5% kiwi and 5% watermelon contained β-carotene, a-carotene, lutein, zeaxanthin, b-cryptoxanthin and lycopene at varying concentrations ranking from 0,1512 ± 0,015 mg/g to 0,01 mg/g, approximately. Sumiashih, et al. [26] studying carotenoids accumulation in citrus species reported that before degreening treatment or maturation of tropical low land citrus fruit, the content of β-carotene was 5.32ug g/FW, while 9 days after precooling treatment with a 24-hour ethylene exposure led to a decrease to 44, 82ug g/FW of β-carotene. Also, in the same research it was found that ethylene treatment led to a 3-times increase of β-cryptoxanthin. In this study β-cryptoxanthin concentration in PL1 stream-line was 0,017± 0,01mg/g, implying that citrus fruits used for the production of fresh juices have been physically maturated at their optimum environmental conditions regarding latitude, altitude as well as other climatical conditions (Table 6).

Table 5: Total Phenolic content, Total Carotenoid content and Antioxidant capacity of fruit byproduct streams extracts*[1].

	Total Carotenoid Content (mg/100g)	Total Phenolic Content (mg GAE/mL)	Antioxidant Capacity (mmol Fe_2SO_4/mL)
carrot	2,67 ± 0,43	54,67± 2,45	100,45± 3,24
orange	2,43 ± 1,24	53,78 ± 1,78	89,58± 2,78
apple	1,12 ± 0,82	45,34± 1,12	85,46 ± 3,63
banana	0,65 ± 0,07	33,67± 1,07	67,35± 1,45
kiwi	0,39 ± 0,03	24,67± 2,56	53,64± 1,63
watermelon	0,68 ± 0,05	30,57± 3,05	57,35 ± 0,56

Note: *[1]at optimized drying and ultrasound extraction conditions.

Table 6: Nutri-carotenoid analysis of fruit by-product extracts streams derived from juice agro processing PL[1]*.

Carotenoid	Carotenoid in Extracts mg/g (dw)	Retention Time (min)
β-carotene	0,1512 ± 0,02	11,3
a-carotene	0,071 ± 0,09	9,5
Lutein	0,043 ± 0,57	4,7
Zeaxanthin	0,022± 0,01	5,1
β-cryptoxanthin	0,017± 0,01	7,5
Lycopene	0,01	15,8

Law, et al. [27] studying carotenoid content evaluating different drying methods, in carrot peels reported that dehumidification drying at 50 °C with RH of 16-21 % was the most effective method as it retained high levels of β-carotene, lutein, and lycopene. Aquino et al., 2018 studying the occurrence and presence of carotenoids in the pulp and peel of 14 different varieties of banana identified the presence of a-carotene, β-carotene and lutein at different ripening stages. Among the tested samples they found out that total carotenoid's concentration ranged from 159.66 to 2553.51μg (100 g MF-1) in 'Caipira' and 'Terrinha', respectively. In this study, total carotenoids concentration was 8.23±1.12mg/100g, in PL-1 (contained Greek Dwarf Cavendish banana variety barks). Also, comparing carotenoids concentration in the ripe and uripe pulp it was reported that in ripe pulp there was a 36% increase in the lutein content in comparison to the unripe pulp and there was 7.3 and 8.5% reduction in α-carotene and β-carotene levels, respectively. To the best of our knowledge, this is the first study that it was studied:

1) Development and optimization of an efficient green methodology to study both carotenoids, phenolics as well as total antioxidant activity of Greek fresh juice by-product streams derived from Greek Aegean islands

2) Development and optimization of each step prior to extraction so as to achieve the highest possible both total and specified carotenoids, total phenolics as well as total antioxidant capacity,

3) Form a safe for human consumption nutritional-super food-rich extract [28-32].

Conclusion

Fruit by-product streams extracts generated by the development of a green-ultrasound assisted extraction, using costless by-product streams led to the production of bioactives rich streams containing both carotenoids and phenolic compounds, that could be possible used toward the production of novel foods such as Greek Miso like products or super food nutritional supplements with possible several positive effects in health and disease preventing. Fruit processing by-product extracts containing β-carotene, αcarotene, β-cryptoxanthin, lutein, lycopene and zeaxanthin, since β-carotene, α-carotene and βcryptoxanthin may convert into vitamin A in the body could be used as a physical sustainable alternative to chemical supplements to prevent Vitamin A deficiency, which is a major public health issue especially in developing countries and in regions where there is little food diversification or in those lacking provitamin-carotenoids-rich foods. Also, juice by-product streams owning to their bioactive compound content could form ideal antioxidant supplements to prevent oxidative damages in food, extending their self-life. This is a very interesting study redirecting usage of costless and unexploited Greek raw material by the development and optimization of a green ultrasound assisted extraction methodology, using GRAS food grade solvents, highlighting bioactives compound content while promoting sustainable development and energy requirements.

Author Contributions

i. **Conceptualization:** Dimou M Charalampia (CMD) and Koutelidakis E. Antonios (KEA);

ii. **Methodology:** CMD and KEA;

iii. **Resources:** CMD, KCC, KEA;

iv. **Writing-Original Draft Preparation:** CMD, CD, STK, KK, KEA;

v. **Supervision:** CMD;

vi. **Project Administration:** CMD;

vii. **Funding Acquisition:** CMD. All authors have read and agreed to the published version of the manuscript.

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The data presented in this study are available within this article.

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Conflicts of Interests

The author(s) confirm that have no conflict of interest.

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