

RECOVERY OF CAROTENOIDS FROM AGROINDUSTRIAL BY-PRODUCTS USING CLEAN EXTRACTION TECHNIQUES: SUPERCRITICAL FLUID EXTRACTION AND ULTRASOUND ASSISTED EXTRACTION

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Abstract. One of the main reasons for unsuccessful recovery of carotenoids from agroindustrial by-products is the absence of effective extraction procedures. In this work, lutein was obtained from a mixture of agroindustrial wastes produced in tropical conditions (lettuce, cabbage, ayuama) using an optimized supercritical fluid extraction method (SFE-CO₂). This study analyzed the effect of a range of temperatures (40-70 °C) and pressures (300-600 bar) on lutein yield using response surface methodology (RSM). With mathematical model, optimal conditions were predicted to 70°C and 600 bar to obtain maximum lutein yield. Validation of the model was achieved, obtaining a SFE-CO₂ extract with total yield of 7,34±0,11 g/g material, lutein yield of 1,6±0,15 µg/g material and ORAC value of 2,08±0,06 mmol TE/100 g material, which had lower yield but higher selectivity compared to ultrasound assisted extraction (UAE).

Keywords : Carotenoids, lutein, agroindustrial wastes, supercritical fluid extraction, ultrasound assisted extraction

1. Introduction

Due to increasing of food production, disposal of by-products represents a growing problem since the plant material is usually prone to microbial spoilage, thus limiting further exploitation. On the other hand, costs of drying, storage and shipment of by-products are economically limiting factors for use in applications with low added value. The problem of disposing by-products is further aggravated by legal restrictions. Thus, efficient, inexpensive and environmentally sound utilization of these materials is becoming more important especially since profitability and jobs may suffer [1].

By-products of plant food processing represent a major disposal problem for the industry concerned, but they are also promising sources of compounds which may be used because of their favorable technological or nutritional properties. Furthermore, consumers are increasingly aware of diet related health problems, therefore demanding natural ingredients which are expected to be safe and health-promoting [2]. For instance, the use of artificial dyes is a common practice in the modern food industry, but there is growing concern about their actual or potential effect on human health. This concern has led to an increasing interest and utilization of natural products as alternative food colorants [3].

Carotenoids are one of the major groups of natural pigments that find widespread utilization in the food industry. The addition of carotenoids to food serves two purposes: 1) impartation of color and 2) nutritional value, since its role as bioactive compounds. Specially, Lutein is one of the most important carotenoids and is widely found in foods and also in human serum. Lutein, together with zeaxanthin, imparts color to the macula lutea, the spot in the human retina that allows the appreciation of fine details, and it is believed to have an important role as an antioxidant. Moreover, during the past few years, lutein has attracted increasing attention with regard to its application to human health. In a number of studies, lutein has been found to play a role in

the prevention of age-related macular degeneration (AMD), cataracts, amelioration of the first stages of atherosclerosis and even some types of cancer [4].

However, key steps in the production of lutein for food industry are related to: 1) the exploration of “unlimited” sources with low cost, 2) the development of extraction techniques, which can minimize alteration of the carotenoids during the extraction process and 3) the use of GRAS (Generally Recognized As Safe) materials for extraction in order to guarantee food application.

Thus, the present study has been conducted to evaluate the feasibility of lutein extraction from agroindustrial wastes produced under tropical conditions using Supercritical Fluid Extraction and Ultrasound Assisted Extraction.

2. Materials and Methods

2.1 Reagents and chemicals

Carbon dioxide, 99.9% pure, was acquired from Indura- Cryogas (Medellín, Colombia). Acetonitrile and ethanol high-performance liquid chromatography (HPLC) grade were obtained from Merck (Darmstadt, Germany). Trolox, fluorescein, 2,2'-azobis(2-methylpropionamide), dihydrochloride (AAPH) and the other reagents were all of analytical grade from Sigma (St. Louis, MO, USA). Reference standard of lutein was purchased from ChromaDex Inc. (Irvine, CA, USA).

2.2 Raw material

Agroindustrial wastes were collected in Public Market of the city (Central Mayorista de Antioquia, Medellín, Colombia). A preliminary study showed the by-products with high production throughout the year. It was selected the largest volume wastes and became a screening to assess the presence of lutein. The residues were dried directly in a freeze-dryer (Eyela, Tokyo, Japan) for 24 h and then ground with a blade mill (Ika works Inc, Wilmington, NC, USA), sieved and collected in a 24-80 mesh.

2.3 Extraction

Supercritical fluid extraction with carbon dioxide (SFE-CO₂) was performed using a SFT-150 (Supercritical Fluid Technologies, Newark, DE, USA). Approximately 20 g of powder from the lyophilized material was placed in the extractor vessel (capacity 100 ml) fitted with a plug of polypropylene wool at the top and bottom of the vessel to provide a continuous flow of CO₂. Each experiment had 10 min of soaking time (static extraction) to promote greater contact between the particles and the CO₂, followed by 120 min of dynamic extraction at flow rate of 1.8 g/min. This study analyzed the effect of a range of temperatures (40-70 °C) and pressures (300-600 bar) on lutein yield. The supercritical CO₂ flowing through the fixed bed in the extraction vessel was expanded into a collection vial tube, where the extracted sample and the CO₂ solvent were easily separated. To minimize the decomposition and oxidation of the extracted compounds, all samples were collected in brown bottles to prevent UV-activated degradation. Total yield was calculated as the ratio of the total mass of extract to the initial mass of raw material (dry basis), while lutein yield corresponds to µg of solute extracted per gram of raw material (dry basis).

Ultrasound assisted extraction (UAE) was performed using a multi-frequency ultrasonic bath (Scientz, Ningbo City, China). Conditions for optimal extraction were adjusted previously (Data not shown). Extraction was conducted at frequency 25 kHz, potency 300 W, temperature 40°C, time 60 min, ratio solute/solvent 1 % (w/v).

2.4 HPLC analysis of lutein

Separation was performed on an Agilent 1200 HPLC apparatus (Agilent Techniques, Santa Clara, CA) equipped with a reverse phase YMC J'Sphere ODS column (250 mm x 4.6 mm, 5 µm particle size) (Waters Co., Milford, MA, USA) with acetone (A) and water (B) as eluents. 75% A was maintained during first 30 min; a linear gradient was utilized for going from 75% A until 90% A in 5 min, and was then held isocratically for 15 min. The solvent flow was set at 1.0 mL/min, with detection performed at 452 nm. Peak identification was achieved by comparing their retention time to that of injected standards and by taking their

absorption spectra during elution from the column. Lutein was quantitated by comparing with standard of known concentration.

2.5 Antioxidant activity

Antioxidant activity was determined using the ORAC assay proposed by Ou [5] and slightly modified by us [6]. Extracts obtained from SFE-CO₂ and ultrasound extraction were diluted in ethanol and then adjusted at final dilution with 10 mM phosphate buffer at pH 7.4. Trolox (0–200 μM) was used as the standard. A mixture of the fluorescent probe fluorescein (150 μL of a 1 μM solution) and extract (25 μL) was preincubated for 30 min at 37 °C. Then, 25 μL of a 250 mM AAPH solution in phosphate buffer was added. Fluorescence intensity was measured every 2 min during 120 min with excitation and emission wavelengths set at 485 and 520 nm, respectively. The results were reported as micromoles of Trolox equivalents per gram of extract (mmol TE/100 g of extract).

2.6 Experimental design and statistical analysis

The influence of pressure and temperature in the recovery of lutein was measured using a response surface method (RSM). The experimental design was proposed to take into account quadratic responses and interactions. For this purpose a central composite design was proposed according to experimental plan shown in Table 1. A full second-order polynomial model of the design was used to evaluate the total yield and lutein yield (response variables, Y) as a function of independent variables (x) and their interactions (Eq. (1)).

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_j x_j + \sum \beta_{ii} x_i^2 + \sum \beta_{jj} x_j^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Where Y is the response, β_0 is the constant coefficient, β_i β_j are the linear coefficients, β_{ii} , β_{jj} are the quadratic coefficients, β_{ij} is the linear-by-linear interaction coefficient, and x_i , x_j are the coded values of independent variables. Experimental design, data analysis, and quadratic model building were conducted using the software Statgraphics Centurion (Stat Points Technology Inc., Minneapolis, MN, USA). Two replicates at the center of the design in each block were used to allow for the estimation of a pure error sum of squares. Differences between variables were tested for significance using the one-way ANOVA analysis procedure using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). Significances were accepted at P<0.05.

Table 1. Central composite experimental design for SC-CO₂ extraction of lutein from agroindustrial wastes and results obtained

Run	Block	Factor values		Response	
		Temperature (°C)	Pressure (bar)	Total yield (g/g)	Lutein (μg/g)
1	1	40	300	3,90	0,18
2	1	70	300	5,60	0,48
3	1	55	600	6,35	1,30
4	1	55	300	4,91	0,26
5	1	55	450	5,80	1,08
6	1	70	450	6,70	1,30
7	1	55	450	5,60	1,12
8	1	40	600	5,69	1,00
9	1	70	600	7,55	1,90
10	1	40	450	5,00	0,60
11	2	40	300	4,00	0,20
12	2	70	300	5,50	0,50
13	2	55	600	6,20	1,25
14	2	55	300	4,80	0,25
15	2	55	450	5,75	1,00
16	2	70	450	6,60	1,20
17	2	55	450	5,70	1,20
18	2	40	600	5,60	0,98
19	2	70	600	7,60	2,00
20	2	40	450	4,90	0,65

3. Results and Discussion

In the Figure 1 it can be seen the chemical profile of several agroindustrial wastes produced under tropical conditions. Presence of lutein was revealed in lettuce, cabbage and auyama. Other studies have reported that vegetables belonging to the *Brassicaceae* family, like cabbage, are an important dietary source of carotenoids, especially lutein [7]. Lettuce and other green leafy vegetables that are eaten raw can bring high content in dietary carotenoids, especially lutein and zeaxanthin [8]. Meanwhile, auyama, is a kind of pumpkin that contains different carotenoids depending to variety [9].

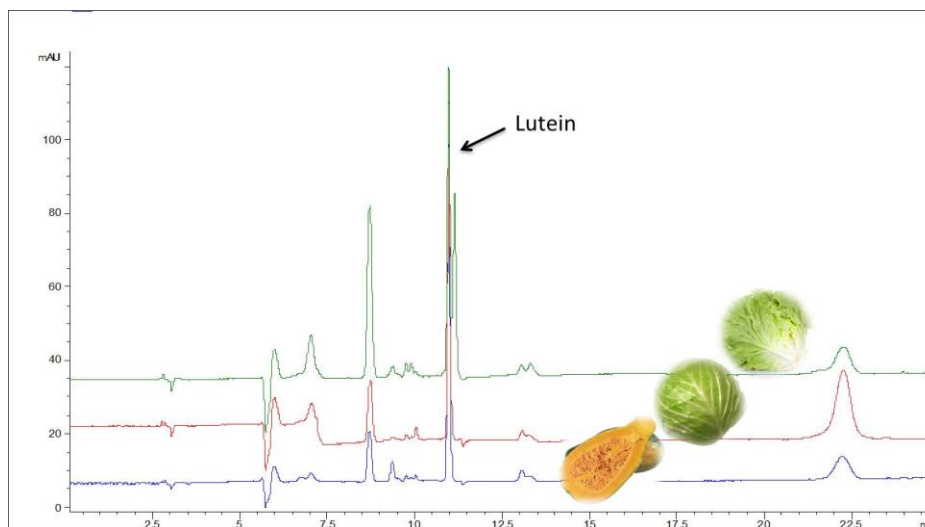


Figure 1. HPLC profile of carotenoids from agroindustrial wastes showing presence of lutein in lettuce (*Lactuca sativa* var. Batavia), cabbage (*Brassica oleracea* var. capitata) and auyama (*Cucurbita maxima*).

Table 1 summarizes the total yields and extraction yields of lutein in the mixture of selected agroindustrial wastes (cabbage, lettuce and auyama) under SFE-CO₂ extraction conditions. The total extraction yield increased with both temperature and pressure. The highest total yield was obtained from the highest pressure of 600 bar and a temperature of 70 °C; the lowest total yield, at 40 °C and 300 bar. The highest lutein yield (microgram lutein per gram of raw material) was obtained at 55°C and 600 bar.

In our study, the response surface methodology (RSM) statistical design and analysis procedure, which is able to provide information on the importance and the statistical significance of individual factors, was used to investigate the effects of temperature and pressure on both the total yield and lutein yield. Results are shown in Figure 2.

Eq. (2) expresses an empirical relationship between the total yield and the test variables in terms of the independent variables, temperature and pressure.

$$\text{Total yield} = 1,04762 - 0,00884921 * \text{Temperature} + 0,00896587 * \text{Pressure} + 0,00045873 * \text{Temperature}^2 + 0,0000366667 * \text{Temperature} * \text{Pressure} - 0,00000585714 * \text{Pressure}^2 \quad (2)$$

A second-order polynomial model was fitted to the experimental data for total yield. The statistics (R²) of the model was 0.9897, which indicates that the model adequately represented the observed relationships among the selected extraction parameters, accounting for 98,97% of the variation in the data.

For lutein yield, Eq. (3) expresses an empirical relationship between the total yield and the test variables in terms of the independent variables, temperature and pressure.

$$\text{Lutein} = -1,28341 - 0,017119 * \text{Temperature} + 0,00609683 * \text{Pressure} + 0,0000460317 * \text{Temperature}^2 + 0,0000733333 * \text{Temperature} * \text{Pressure} - 0,00000720635 * \text{Pressure}^2 \quad (3)$$

Also, a second-order polynomial model was fitted to the experimental data for the lutein extracted. The statistics (R^2) of the model was 0.9721, which indicates that the model adequately represented the observed relationships among the selected extraction parameters, accounting for 97,21% of the variation in the data.

The significance of each coefficient determined by F-value and P-value showed that the temperature, pressure, and the quadratic term for pressure are significant model terms for total yield, while for lutein yield the linear interaction between temperature and pressure was also significant.

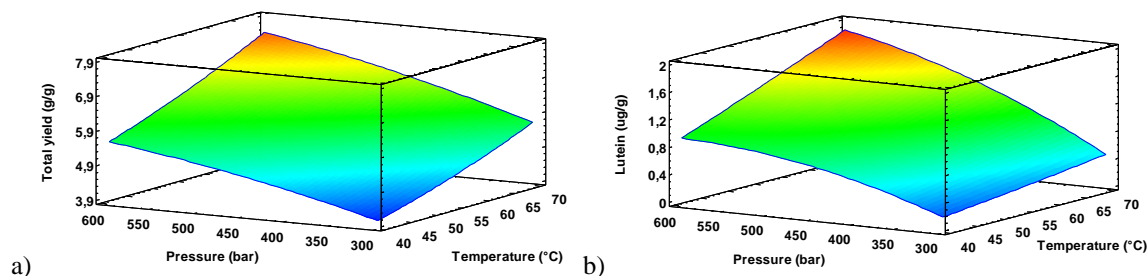


Figure 2. 3D response surface plots showing effects of temperature and pressure on a) total yield, b) lutein yield

Supercritical fluid extraction recovery and selectivity depend greatly on the equilibrium between fluid density and solute vapor pressure. Generally, an increase in pressure would increase the fluid density, consequently increasing the solubility. An increase in temperature would reduce the fluid density, which decreases the solubility. However, an increase in temperature would also increase the solute vapor pressure, which would increase the solubility. Commonly, temperatures under 40 °C are recommended for classical extraction of carotenoids due to the thermal degradation that often occurs, while the use of temperatures up to 70 °C has also been found to increase the yields of carotenoids in a previous study with SFE-CO₂ [10]. Therefore, the actual values of temperature and pressure finally chosen must take into consideration carotenoids' thermal degradation and the balance of density and vapor pressure for solute solubility.

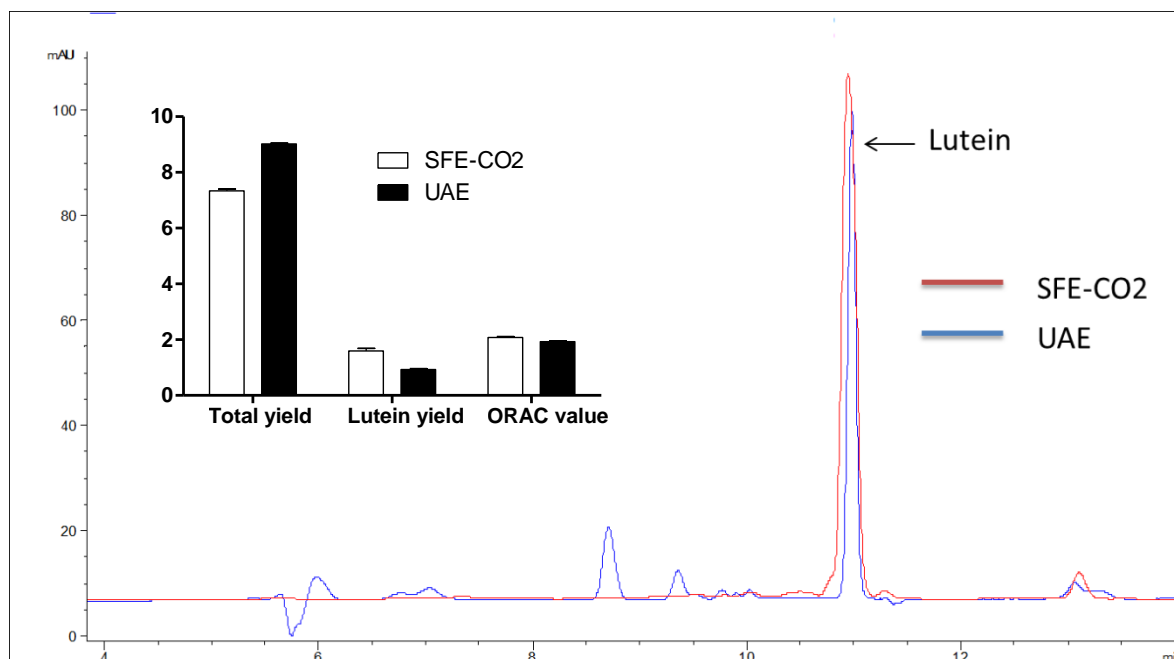


Figure 3. Comparison of extracts obtained by SFE-CO₂ and UAE. Main figure shows a typical chromatogram of extracts. Inset figure displays comparison between techniques according to total yield (g extracted/g material), lutein yield (µg extracted/g material) and ORAC value (mmol TE/100 g extract).

The ranges of temperature and pressure in the present study were between 40 and 70 °C and 300–600 bar, respectively. Here, it was seen that pressure is the most significant term with a positive effect ($P < 0.0001$), while temperature was important, but at 600 bar, the effect of increasing temperature on the amount of carotenoids extracted was not significant. Results obtained in the present study are in accordance with others yet reported for carotenoids and lutein using SFE-CO₂ [11]. In fact, Shi et al, found a high yield in the extraction of lutein from pumpkin as were higher temperatures (70 °C), however, only evaluated pressures up to 350 bar [12].

Under optimal conditions for both SFE-CO₂ (600 bar and 70°C) and UAE (as described in 2.3), it was conducted an extraction experiment in order to compare the techniques and to confirm performance of the prediction model. Extracts obtained were characterized account total yield, lutein yield and antioxidant activity. Results are shown in Figure 3.

In general, SFE-CO₂ can produce a purer extract compared to UAE due to selectively of extraction as can be seen for chromatographic profile and lutein yield. On the other hand, UAE achieved higher total yield, probably as a result of simultaneous extraction of other components, which could also have antioxidant activity, since the ORAC value is maintained although less lutein extracted with UAE. The results presented here for the antioxidant activity of the extracts are related to those reported in the literature. Hayes et al [13] found a ORAC value of 3,715 mmol TE/100g of pure lutein, which is not far to the findings in the present study (2,08±0,06) with extracts from agroindustrial wastes.

4. Conclusions

In conclusion, this study demonstrates that SFE technique with CO₂ can be utilized for a value added scheme by recovering antioxidant-rich extracts from agroindustrial wastes. The exploitation of these by-products could increase the value for the agroindustry in tropical countries, which commonly manage with difficulties the disposal of its high-volume wastes. The added value of using a GRAS solvent as CO₂ demonstrates the potential value of the obtained extracts on different markets (food, pharma and cosmetics).

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