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Analytical Methods

Application of carbon dioxide in subcritical state (LCO_2) for extraction/fractionation of carotenoids from red paprika

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ABSTRACT

Carbon dioxide, as a liquid (LCO_2) in subcritical state, was applied for extraction of carotenoids from ground paprika. The increasing polarity of LCO_2 with the decrease of its temperature enabled fractionation of pigments according to their increasing polarity. The main constituents of the fractions of +6 °C and −6 °C were triacylglycerols (TAGs) including a small concentration of β-carotene. At −16 °C, more polar pigments (capsorubin, capsanthin, zeaxanthin) and their fatty acid (FA) esters were extracted. The pre-concentration ratio of carotenoids in the fraction at -16 °C was 17.2 with respect to Fraction at +6 °C.

In the FA composition (GC) of the fraction at −16 °C, (11.9%) prevailed lauric acid ($C_{12:0}$) and also myristic ($C_{14:0}$, 8.2%) and palmitic ($C_{16:0}$, 18.0%) acids. Linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids amounted to 19.4% and 3.1%, respectively. In contrast to the fraction at −16 °C, in the hexane/ethyl ether paprika extracts, unsaturated linoleic ($C_{18:2}$, 59.4%) and linolenic ($C_{18:3}$, 5.1%) acids prevailed.

1. Introduction

The aim of this work was to take advantage of specific properties of carbon dioxide in the subcritical (liquid) state (LCO_2) for the extraction and fractionation of carotenoids from natural sources.

Carotenoids are organic pigments that are naturally occurring in plants and some other photosynthetic organisms, e.g. some types of fungi and some bacteria. In plants, carotenoids are synthesized both in leaves and in fruits. The main carotenoids of leaves and other green vegetables are lutein, β-carotene, violaxanthin and neoxantin (Britton, 1991).

With respect to humans, carotenoids have important health-promoting properties; for example, α, β, γ carotene and β-cryptoxanthin are known as pro-vitamin A. More recently, protective effects of carotenoids against serious disorders, such as cancer, heart disease and degenerative eye disease, have been recognised and have stimulated intensive research into the role of carotenoids as antioxidants and regulators of the immune response system (Krinsky & Rock, 1998; Shekelle et al. 1981; Stahl & Sies, 2003; Tapiero, Townsend, & Tew, 2004). The ability of carotenoids to quench singlet oxygen is related to the conjugated double – bond system, and the maximum protection is given by those having nine or more double bonds (see Fig. 1).

The health-promoting properties of carotenoids present in natural plant sources depend, however, on their bioavailability. Components of vegetables and fruits, such as dietary fibre, may affect carotenoid concentrations in human plasma (Olson, 1994; Parker, 1977).

Thus differences in the plasma carotenoid responses to vegetable and fruit intake may be determined by the varying food forms in which the carotenoids are consumed, e.g. vegetable juice versus raw or cooked vegetables.

Therefore, it may be expected that carotenoids in natural edible oils could be easily absorbed. Such concentrates, encapsulated or not, could be considered as food supplements for direct use or for the fortification of meals prepared in a kitchen.

In such a case, however, a question arises of how to pre-concentrate and transfer carotenoids present in their natural matrices to a new matrix or a natural edible oil.

So far, the concentrates of carotenoids are mainly prepared by extraction (with hexane) of ground red paprika. The commercial product, known as "oleoresin", consists of red pigments dissolved in triacylglycerols (TAGs) present naturally in red paprika. The imperfection of such "oleoresin" is caused by the fact that the last traces of solvent must be removed from the extract by evaporation at relatively high temperatures, at which carotenoids sensitive to elevated temperatures are partially isomerised or decomposed (Drescher M. & Weidner E., 2002; Minguez-Mosquera & Jarén-Galan, 1995; Wilska-Jeszka, 2002).

The carotenoids from paprika were also successfully extracted by supercritical carbon dioxide $SFCO_2$ (Jarén-Galán, Nienaber, & Schwartz 1999). By increasing the pressure of $SFCO_2$ from 2000 to 7000 psi (14 MPa to 49.2 MPa) the authors were able to

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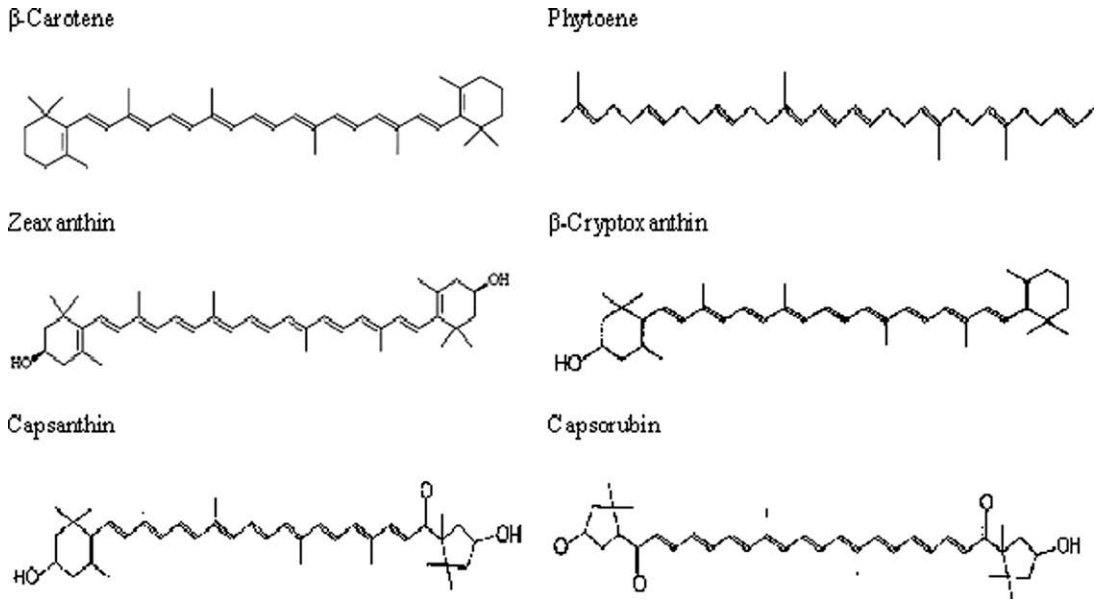

Fig. 1. The structures of some carotenoids identified in red paprika extracts.

fractionate and pre-concentrate paprika pigments according to their increasing polarity. The $SFCO_2$, however, requires expensive equipment, able to withstand such a high pressures and an adequate high pressure CO_2 pumping system. In operation, the $SFCO_2$ extraction system also demands much of energy.

An alternative, less expensive, method of pre-concentration of carotenoids is the use of carbon dioxide in its liquid (subcritical) state (LCO_2) as the extracting solvent. LCO_2 reveals useful physical and practical properties. The "food grade" CO_2 is available commercially in cylinders, usually at prices comparable to the price of mineral water. In the cylinders, at usual pressures of 5.6 MPa and room temperature, the CO_2 is in its liquefied state (LCO_2) and may be used as a solvent for extraction on condition that it is drawn from the bottom of the cylinder.

At room temperature the polarity of LCO_2 is comparable to the polarity of hexane but, as opposed to hexane, the LCO_2 is easily soluble in water. Such a property allows the use of LCO_2 for extraction of relatively wet materials.

The most exiting properties of LCO_2 are its solvating properties. The polarity of LCO_2 depends on its density, which in turn depends on its temperature. By decreasing the temperature of LCO_2, its polarity (as a function of density) increases. Thus the LCO_2 may potentially be applied as a solvent for extraction, pre-concentration and fractionation of carotenoids from their natural sources.

The preliminary attempts at using LCO_2 for fractional extraction of carotenoids from ground red paprika are described herein.

2. Materials and methods

2.1. Materials and treatments

Commercial ground red paprika was supplied, free of charge, by Kamis (Kamis Przyprawy, Warsaw, Poland). β-Carotene was purchased from Merck (Merck, Darmstadt Germany). The solvents for HPLC: acetonitrile (gradient grade), 2-propanol and hexane (HPLC tested) (Baker, Holland) were supplied by Witko (Witko Lodz Poland). The Soxhlet extraction using hexane/ethyl ether 1:1 (v/v) was applied (as reference) for isolation of extractable matter from commercial ground paprika. Carbon dioxide "Food grade" for LCO_2 extraction was supplied by Linde (Linde, Warsaw, Poland). Unsaponifiable matters (UM) were isolated by saponification of extracted lipids and of Fraction IV (−16 °C) overnight at room temperature using 0.5 N NaOH in methanol. The UM were extracted from soap solutions three times with hexane/ethyl ether 1:1 (v/v). The solvents were removed on a rotary evaporator at 40 °C. Precisely 0.1% solutions in toluene (1:1v/v) were prepared for the HPLC analysis. The LC 1050 (Hewlett Packard, USA) equipped with quaternary pump, diode array detector (DAD) controlled by HP ChemStation software was used for the analysis of extracted fractions.

The composition of FA methyl esters (FAME) was determined using GC, Agilent 6890 N, split (50:1 injection) and Rtx 2330 100 m; 0.25mm ID; d_f 0.2 μm (Restek Corp. USA). The composition of unsaponifiable matter was determined by GC, "cool on column" injection and a Rtx TG 50 high temperature column 30 m; 0.25 mm ID and d_f 0.1μm (Restek Corp. USA).

2.2. Extraction system

The block diagram of the extraction system of our own design is shown in Fig. 2. Its operation is as follows: at room temperature, the LCO_2 is drawn by the factory installed (siphon tube) which extends to the bottom of the cylinder. The LCO_2 passes through a "shut off" valve [3] equipped with manometer [2] indicating pressure of the CO_2 in the cylinder, three way purge valve [4] and four way "tee" union [5] to which is attached another pressure gauge, indicating the pressure in the installation. Next, the stream of LCO_2 passes through the heat exchanger [6] in which it is chilled to the required temperature controlled by the set up of temperature controllers [14a,b]. The temperature controllers start or shut off the pump of the cooling medium (ethanol) drawn from the cooling system [13]. The LCO_2, chilled to the desired temperature (all the time at pressure equal to that in the CO_2 cylinder), then passes through the extracted material packed into the extraction column [7] which is also chilled to the desired temperature controlled by the temperature controller [14b]. The internal volume of the extraction column is 345 cm^3.

Two modes of extraction can be used, namely, "batch" extraction or "flow-through" extraction. In the latter case, if necessary, additional solvent ("moderator"), for example ethyl ether or etha-

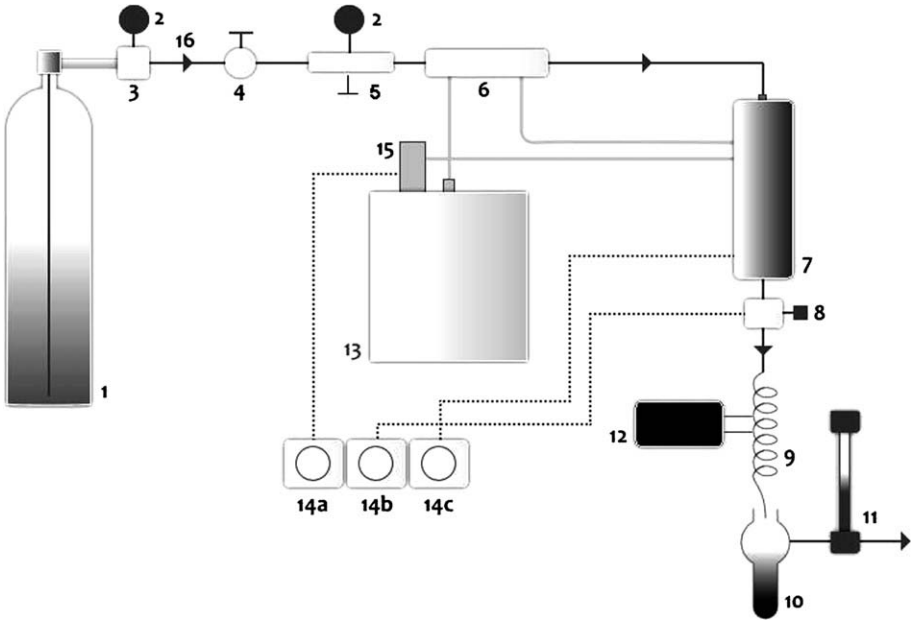

Fig. 2. Block diagram of the laboratory extraction system for the use of *LCO₂* as extraction medium. (Abbreviations: (1). cylinder with compressed (5.6 MPa) CO_2; (2). manometer; (3). "shut off" valve; (4). 3. way purge valve (5). four way "tee" union; (6). heat exchanger; (7). extraction column; (8). precision needle valve; (9). heated stainless steel 0.5mm ID capillary; (10). fraction collector; (11). rate meter; (12). temperature controller; (13). cooling system; (14a). flow of cooling medium on/off; (14b) and c temperature controllers (15). cooling medium pump controlled by 14a).

nol, may be injected with a constant (or programmed) rate to the stream of LCO_2 with the use of an HPLC pump connected to the four way "tee" union [5].

After saturation with extracted compounds, the LCO_2 is decompressed through the controlled orifice assembly [8] composed of a precision high pressure needle valve connected in series with the constant restrictor (stainless steel capillary) [9]. The orifice and capillary are heated in order to supply heat adequate for evaporation of LCO_2. After decompression, the mist of extracted compounds is deposited by centrifugal force on the walls of the glass collector [10]. The rate meter at the end of line enables measurement of volumetric flow rate of gaseous CO_2 at room temperature and atmospheric pressure. In the case of the described extraction system, no recycling of CO_2 was designed.

2.3. Extraction of commercial ground red paprika with LCO_2

160 g of commercial ground red paprika were loaded onto the extraction column and flushed with gaseous CO_2. Then, the column was cooled down to the initial temperature +6 °C, the orifice (needle valve) [8] closed and the extraction column filled up with LCO_2. Next, the needle valve (heated to 45 °C) was slowly opened and the flow rate, as measured by rate meter, was adjusted to 9.8 litres/min (19.40 g/min) of gaseous LCO_2 (atmospheric pressure, room temperature). The stream of the extract, which deposited on the walls of the collector, yellow at the beginning, later turning to the red colour (at lower temperatures), was observed.

After 120 min of extraction, 5.1 g of Fraction I (F + 6 °C) were collected and the temperature of the column was lowered to −6 °C, the collector replaced and the process was continued up to the moment when the visible stream of the extract deposited on the walls decreased substantially [Fraction II (F−6 °C), 3.1 g]. Fractions III (F − 13 °C) (1.8 g) and IV (F − 16 °C) were collected at −13 °C and below -16 °C. In order to speed up rate of extraction at −16 °C the flow rate of LCO_2 was increased four times.

Some difficulties were encountered by freezing of the orifice with increased CO_2 flow rate, which caused unstable conditions of the extraction. A remote control of the temperature of the orifice, working as a function of the flow rate, would substantially improve operation of the extraction system.

Alternatively, in other experiments with the help of an HPLC pump, ethyl ether was continuously injected into the stream of LCO_2 at the rate of 0.5 ml/min. Deeply red extract was collected almost instantaneously. The ethyl ether, being very volatile, was not condensed, because even last traces of the solvent were almost instantaneously flushed out from the extract in a strong stream of gaseous CO_2.

2.4. HPLC/DAD analysis of extracted fractions

The main reason for application of HPLC in the analysis of Fractions I–IV was to check whether the fractionation of extractable components takes place.

Separation of carotenoids and accompanying substances of Fractions I–IV, and "on line" recording of UV–Vis spectra in different detection channels, required application of a mobile phase "transparent" in UV–Vis. In turn, the complex composition of extracted fractions demanded a gradient elution technique and efficient HPLC column. Therefore, a relatively selective mobile phase, sufficiently transparent in the UV, and an elution programme were elaborated. The mobile phase consisted of solvent A (acetonitrile, gradient grade) and solvent B (propan-2ol/hexane, 22/13v/v). The elution was carried out as follows: initially 100% A during 3 min; next a linear gradient to reach 30% A and 70% B over 40; next hold. The HPLC column was a Synergi™ Hydro-RP (Phenomenex Inc., Torrance, California) 25 cm, ID 4.5 mm, dp 4μm, polar end-capped C18, injection loop 5 μl. During separation the flow rate of the mobile phase was constant (0.9 ml/min).

The detection was carried out in two parallel channels, namely Channel I: 460 nm; BW 80 nm for the detection of most carotenoids and Channel II; 215 nm, BW 5 nm, in which TAGs were detected by absorbance of the carbonyl group of ester bonds. Below 220 nm, the absorbance of −C═O strongly increases with decrease of the wavelength.

3. Results and discussion

3.1. Results of extraction

Bearing in mind that extraction was carried out at a constant flow rate and a constant LCO_2 pressure of 5.6 MPa (CO_2 cylinder pressure) and assuming that, after decompression, the density of CO_2 gas at room temperature and atmospheric pressure was 1.98 g/dm^3, it was possible (on the basis of indications of the flow rate meter) to calculate the ratio of mass of CO_2 used for extraction of one gramme of extracted matter. In total, the LCO_2 extraction yielded 15.8 g of extract, which amounted to 9.9% with respect to the mass of the substrate. For comparison, the Soxhlet extraction with hexane/ethyl ether (1:1 v/v) yielded 11.8% of extractable matter. Other results of measurements and calculations are presented in Table 1. The rate of extraction decreased as the experiment progressed, indicated by the increasing ratio of mass of CO_2/1 g of the extracted material.

Table 1
Yield of extraction at different extraction temperatures.

Fraction	Temp. of extraction (°C)	Mass of fractions g and % of total extract	Mass ratio CO_2 (kG)/extract (g)	Ratio of B/A as in Fig. 3	Composition of fraction as in Figs. 6 and 7
F(+6 °C)	+6	5.1 (32.3%)	0.67	0.105	Mainly TAG's
F(-6 °C)	−6	3.1 (19.6%)	1.4	0.179	Mainly TAG's but extraction of carotenoid FA esters begins
F(−13 °C)	−13	1.8 (11.4%)	1.6	1.89	Extraction of TAGs and carotenoid FA esters
F(−16 °C)	below −16	5.8 (36%)	Data not available[a]	1.81	
Total Recovery		15.8/160 g 9.9%			
Recovery by Soxhlet extraction (as reference)		11, 8%		1.50	TAGs and carotenoids without fractionation

[a] Data not available because of unstable conditions of extraction at high flow rates of LCO – freezing of the orifice.

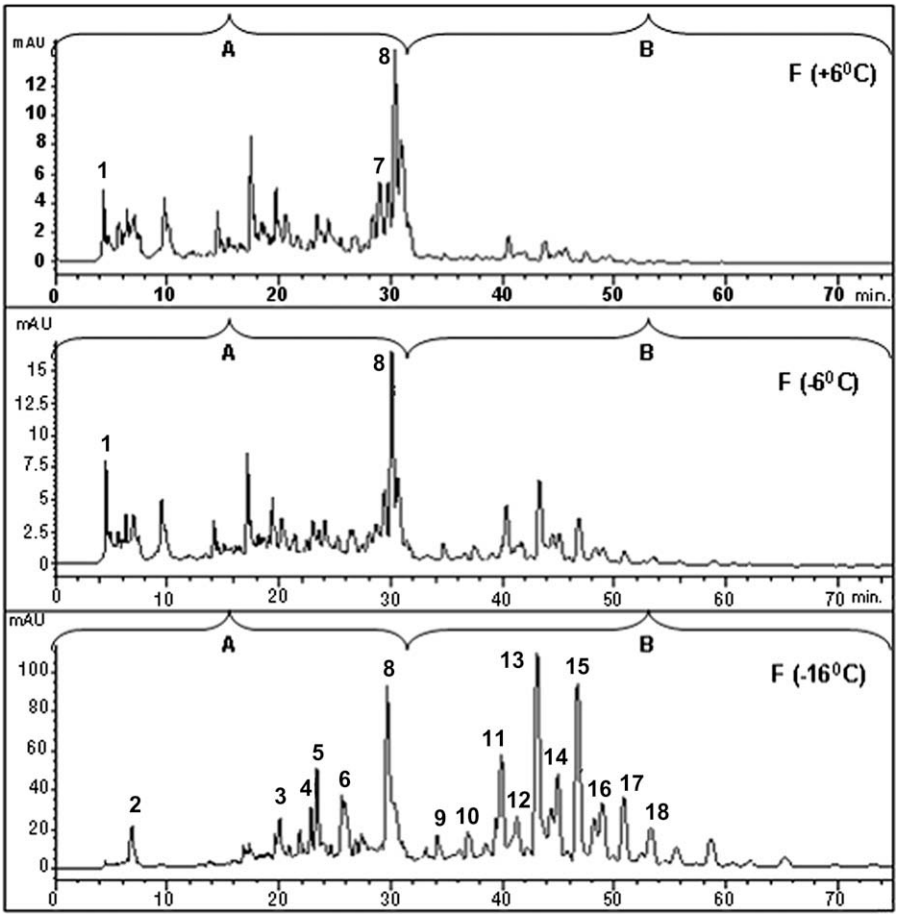

Fig. 3. Chromatograms at 460 nm, BW 60 nm, of fractions. (Identification: 1-phytofluene; 2-zeaxanthin; 3-capsanthin;" 4-ester capsorubin ?" 5-β-cryptoxanthin; 6-ester zeaxanthin; 7-ξ-carotene; 8–β-carotene; "9,10-NI"; 1-ester capsorubin; "12-NI"; "13, 14,15 -ester capsanthin"; "16,17–ester zeaxanthin", 18-ester lutein. NI-Non Identified. Conditions: The HPLC column Synergi^{TM} Hydro-RP (Phenomenex Inc., Torrance, California) 25 cm, ID 4,5 mm, dp 4μm, polar end-capped C18, in loop 5 μl; flow rate of the mobile phase 0.9 ml/min. Peaks as indicated in Fig. 3 were identified only on the basis of DAD spectra (with the exception of standard of β-carotene- positive RT identification) and their comparison with carotenoids spectra published by other authors (Lipid Bank Carotenoid; Breithaupt & Schwack, 2000; Collera-Zúñiga et al., 2005; Lam & Deinzer, 1987).

3.2. Selectivity of extraction of carotenoids and TAGs at different temperatures of extraction

Unique solvating properties of LCO_2 result from the fact that, with decrease of temperature, the density of LCO_2 increases. For example, at temperatures of 6.11 °C and −15.56 °C, the corresponding densities of LCO_2 are: 0.874 g/cm^3 and 0.9829 g/cm^3, respectively (Hyatt, 1984; Lide, 2004; Masturah, Harcharan, & Masitah, 2001). The increase of density is associated with the increase of polarity of LCO_2.

Variable solvating properties of LCO_2 with respect to carotenoids are demonstrated on chromatograms recorded at 460 nm BW80 of F(+6 °C), F(−6 °C) and F(−16 °C) (Fig. 3).

Peaks in Fig. 3 were identified only on the basis of DAD spectra (with the exception of standard β-carotene- positive RT identification) and their comparison with carotenoid spectra published by

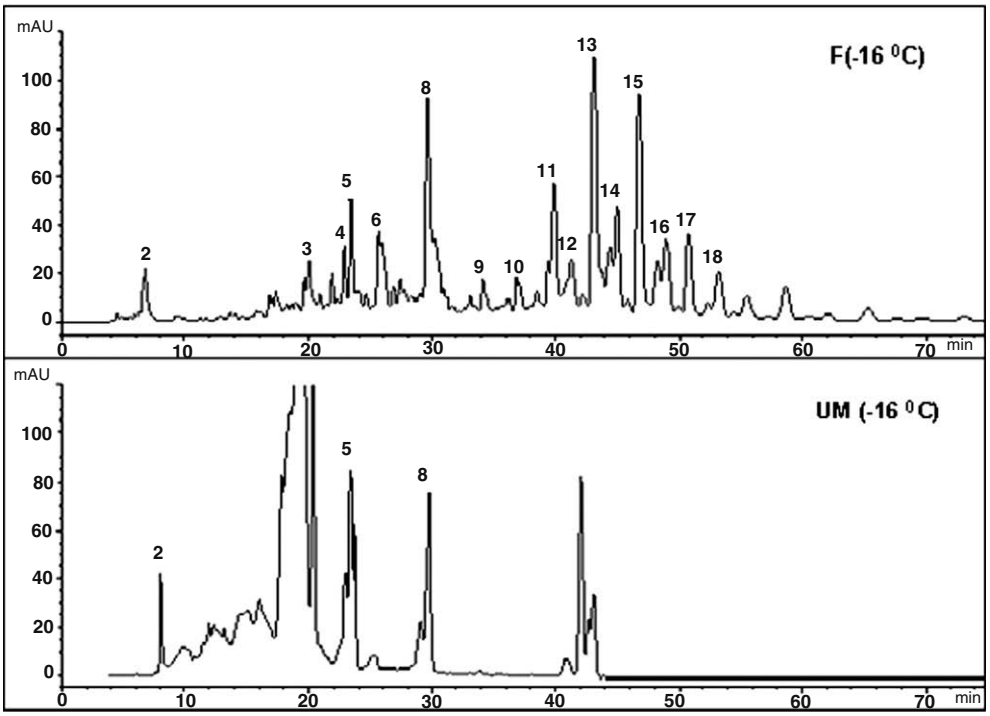

Fig. 4. Chromatograms at 460 nm, BW 60 nm, of F(-16 °C) and of UM (−16 °C. Identification: F(−16 °C) as in Fig. 3; UM: 2-zeaxanthin; 5-β-cryptoxanthin; 8-β-carotene.

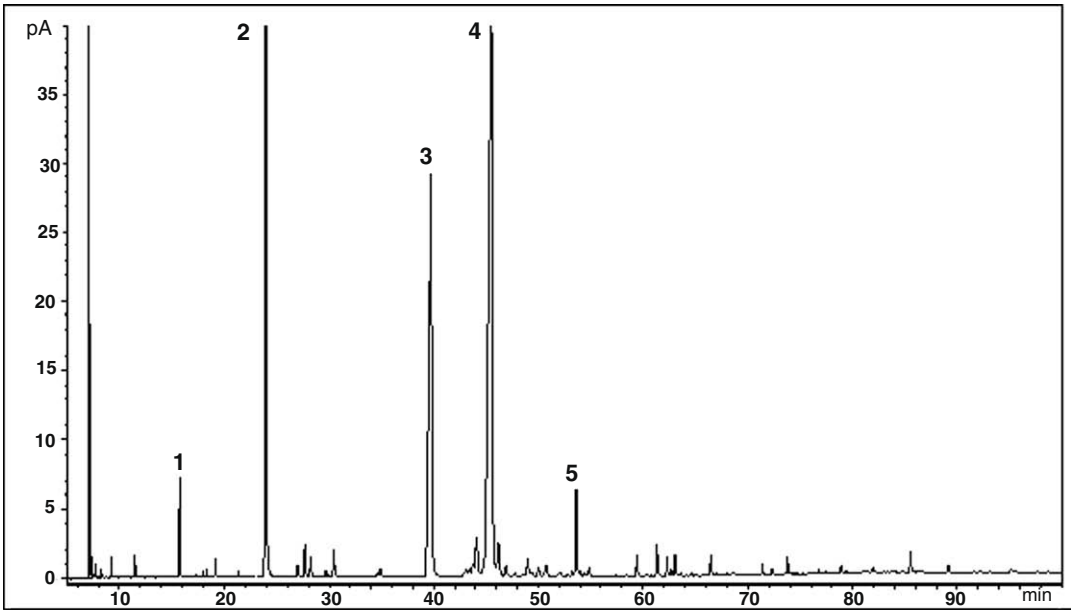

Fig. 5. Separation of FA composition of UM of F(-16 °C) by GC. Capilar Column Rtx 2330 (Restek Corp., USA) "split/splitless" injection; sample 0.015% in toluene, in 1 μl, column temperature: (Ramp I) initial temp 155 °C, time 45 min, next gradient at the rate 1.5 °C/min, final temp. 210 °C, time 50 min; hydrogen flow rate constant, 0.8 ml/min. Identification: 1-$C_{14:0}$; 2-$C_{16:0}$; 3-$C_{18:0}$; 4-$C_{18:1}$; 5-$C_{18:2}$.

other authors (Lipid Bank Carotenoid; Breithaupt & Schwack, 2000; Collera-Zúñiga, Jiménez, & Gordillo, 2005; Lam & Deinzer, 1987).

Despite the concentrations and sample sizes, in each case being precisely the same, increased numbers of peaks and intensities [12mAU – F(+6 °C) and almost 100 mAU- F(−16 °C), respectively] appeared in accordance with the decrease of the temperature of extraction.

The selectivity of extraction may be expressed semi-quantitatively by calculation of the ratio of the sum of integrated peak areas (B) with RT greater than β-carotene (excluding β-carotene) to the sum of integrated peak areas (A) with RT smaller than that for β-carotene (including its area) (Table 1). For the F(−16 °C) the value B/A (1.81) is 17.2 times greater than that for the F(+6 °C) (0.105), indicating that fractionation of carotenoids took place during extraction.

Table 2
FA composition of F(−16 °C) and Soxhlet extract of ground paprika.

Fatty acid	F(−16 °C) % (area)	Soxhlet extract % (area)
Lauric $C_{12:0}$	11.9	1.5
Myristic $C_{14:0}$	8.2	3.6
Palmitic $C_{16:0}$	18.0	13.5
Oleopalmitic $C_{16:1}$	1.3	0.4
Not identified	5.8	Traces
Stearic $C_{18:0}$	4.5	2.4
Oleic $C_{18:1\ 9\ c}$	9.0	11.0
Octadecenoic $C_{18:1\ 11\ c}$	0.4	1.1
Linoleic $C_{18:2\ (n-6)}$ (LA)	19.4	59.4
Eicosanoic $C_{20:0}$	0.4	0.4
Linolenic $C_{18:3\ (n-3)\ (ALA)}$	3.1	5.1
Behenic $C_{20:0}$	0.9	0.3
Others	Below 0.3 each	Below 0.2 each

3.3. Chromatographic analysis of unsaponifiable Matter (UM) isolated from F(−16 °C)

In order to estimate what kind of components are extracted, in particular at −16 °C, the sample of F(−16 °C) was saponified; UM were extracted from the soaps and separated under the same chromatographic conditions. The chromatograms of F(−16 °C) and of isolated UM are shown in Fig. 4.

It follows from Fig. 4 that most of the peaks of F(−16 °C) detectable at 460 nm, BW 80, are mono or di-esters of xanthophylls because, after saponification of F(−16 °C), they did not appear on the chromatogram of UM.

Despite the absence of mono or di-esters, the composition of UM is still rather complex, as estimated with the more efficient GC, "cool on column" injection and more efficient capillary column (Fig. 5). Presumably, some of the carotenoids were changed during saponification or isomerised at higher temperatures of the GC column.

3.4. Composition of fatty acids of F(−16 °C) and of the Soxhlet extract of commercial ground red paprika

The solution of soaps obtained during saponification of the F(−16 °C) was acidified; FA were extracted with hexane and converted to FAME using 14% BF_3/methanol solution according to the AOAC Method No 969.33. A similar procedure of preparation of FAME was applied to the matter extracted from commercial ground paprika in a Soxhlet extractor with a mixture of hexane/ethyl ether 1:1 (v/v). Comparison of FA composition (by GC) of F(−16 °C) and that of the Soxhlet extract is presented in Table 2.

Despite the fact that the fraction F(−16 °C) is only enriched in carotenoid FA esters, its FA composition is substantially different from that of the Soxhlet extract (Table 2). In the fraction F(−16 °C) rather saturated short chain FA ($C_{12:0}$ to $C_{16:0}$) are pre-

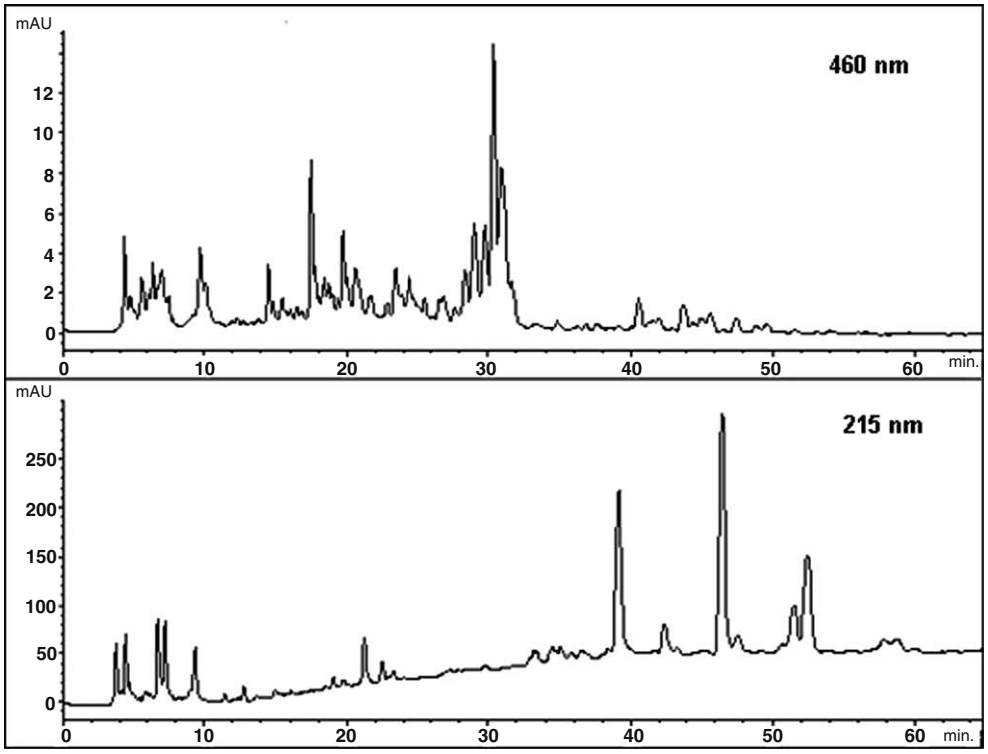

Fig. 6. Comparison of chromatograms and absorbancies of (F + 6 °C) recorded at 460 nm BW (carotenoids) and at 215 nm, BW 5 nm (TAGs) (separation conditions as in Fig. 3).

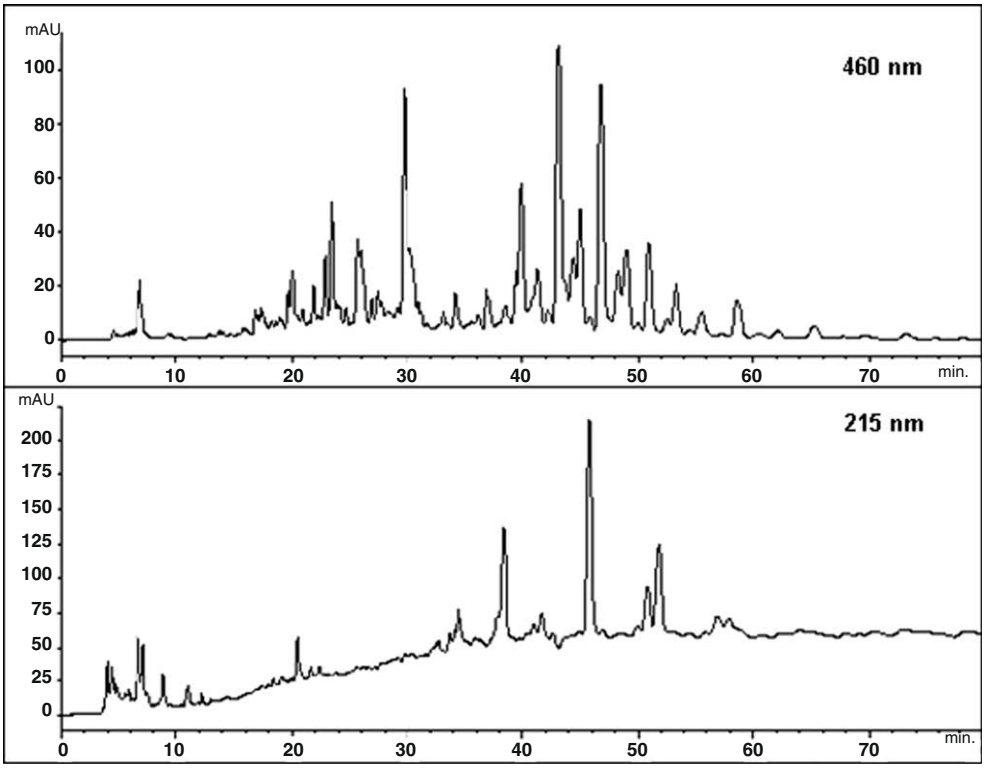

Fig. 7. Comparison of chromatograms and absorbancies of (F – 16 °C) recorded at 460 nm BW (carotenoids) and at 215 nm, BW 5 nm (TAGs) (separation conditions as in Fig. 3).

dominant while LA and ALA are present in TAGs of the Soxhlet extract. The above data indicate that xanthophylls, present in the commercial paprika, are esterified mainly with short chain FA. LA and ALA are present mainly in TAGs.

3.5. Fractionation of carotenoids and TAG's

The extraction of ground paprika with LCO_2 also allows preconcentration of carotenoids. This is shown in Figs. 6 and 7 in which there are overlaid chromatograms recorded simultaneously at 460 nm BW 80 (carotenoids) and 215 nm BW 5 (TAGs) of F(+6 °C) and F(−16 °C), respectively.

In order to compare chromatograms recorded in different detection channels, the response factors of DAD with respect to carotenoids and TAG should be taken into account.

It is known that carotenoids absorb mostly in the visible region (430–510 nm) with some exceptions; for example, phytoen and phytofluen absorb at λ_{max} 286 nm and 367 nm, respectively (Rodriquez-Amaya, 2001). The absorbance coefficient $A^{1\%}$/cm of carotenoids (absorbance at given wavelength of a 1% solution in 1 cm light-path spectrophotometer cuvette) (Britton, 1991; Britton, 1995) is relatively high and extends from 2620 for β-carotene (ethanol) at λ max 450 nm up to 2800 for α-carotene (at λ max 444 nm – petroleum ether) (Rodriquez-Amaya, 2001).

The TAGs absorb UV only below 220 nm. The absorbance coefficient ($A^{1\%}$/ cm), originating from absorbance of the carbonyl group of the ester bond is rather weak. At 215 nm $A^{1\%}$/cm does not exceed 50mAU (Stolyhwo, 2003).

Consequently, it may be estimated that, at λ 460 nm, a given mass of carotenoids is able to generate a DAD response at least 50 times greater than that of the same mass of TAGs at 215 nm, BW 5 nm.

As results from comparison of Figs. 6 and 7, bearing in mind response factors, the concentration of carotenoids in F(+6 °C) with respect to TAGs is much smaller than in the F(−16 °C), which confirms the selective properties of LCO_2 at different temperatures.

4. Conclusions

The LCO_2 at different temperatures may be used for pre-concentration and /or fractionation of carotenoids. Almost 51.9% of extractable matter, containing mainly TAGs with only a small content of non-esterified carotenoids (as non-polar hydrocarbons), is extracted at higher temperatures (+6 °C and −6 °C), leaving non extracted carotenoid FA mono and di-esters in the substrate.

At lower temperatures (−16 °C) more efficient extraction of carotenoid FA esters takes place. Since the recovery (11.8%) of extractable matter, using Soxlet extraction, was higher than when using LCO_2 (9.9%) it may be expected that, at temperatures much lower than −16 °C, the most polar carotenoid fraction could be extracted.

The remote control of temperature of the orifice, as a function of the flow rate of decompressed LCO_2, is highly desirable in order to improve operation of the extraction system. The recycling of CO_2 should substantially reduce the cost of extraction.

Extraction /fractionation with LCO_2 does not produce any waste material, does not cause any harm to the environment and is a good example of a "green technology". Products extracted with "food grade" CO_2 are safe with respect to human health.

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