Determination of carotenoids in microalgae using supercritical fluid extraction and chromatography.

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Abstract

A method was developed based on supercritical fluid chromatography for quantitative determination of carotenoids in extracts of *Scenedesmus* sp. By utilizing the low backpressure in supercritical fluid chromatography, a C18 column and a 2-ethyl pyridine column were coupled in series. It was concluded that even minor changes in temperature had a substantial effect on selectivity. A standard mixture of 8 carotenoids and microalgae extracts obtained through supercritical fluid extraction with and without 10% ethanol as a co-solvent were successfully separated. All of the carotenoids were separated within 10 minutes, while the total analysis time was 20 minutes. The method was validated and the carotenoids of microalgae extracts were quantified. Furthermore, the method should be seen as a more rapid and environmentally sustainable alternative to traditional high-performance liquid chromatography methods utilizing organic solvents.

1. Introduction

Microorganisms and plants synthesize carotenoids and thereby giving them their yellow, orange and red color due to carotenoids absorbing visible light. A part from providing color, carotenoids also have anti-oxidative capacity and contribute to dietary vitamin A by conversion to retinol. Fruits and vegetables are the major contributors of carotenoids in human diet and it is suggested that these carotenoids help in the process of preventing diseases such as cardiovascular diseases, cancer and other chronic diseases [1].

Indisputably, awareness of microalgae for biofuel, functional foods or as food additive has increased. Especially carotenoids from microalgae are interesting due to above-mentioned reasons. Carotenoids are currently used as food additives as primarily food colorant but also as an antioxidant [2]. Society prefers products made with additives from a natural origin, which has attracted the industry due to possible social and economical gains. In consequence, the extraction method for obtaining these interesting compounds is extremely important and potentially toxic solvents are not suitable. This is important to take into consideration for both sustainable development and in order to guarantee safe food products.

While green extraction processes are important we should also strive for developing more sustainable analytical methods. An increased demand of *e.g.* carotenoids will also present a need for rapid analysis. It should be noted that carotenoids are sensitive to light, heat, oxygen and acids and standards are often difficult to obtain. These aspects render analysis of carotenoids very difficult [3]. Traditionally high-performance liquid chromatography (HPLC) using either C18 or C30 columns has been the conventional choice for analyzing carotenoids. However, due to the high backpressure of C30 columns, analysis time tend to be rather long [4]. These methods also often use hazardous and non-environmentally friendly mobile phases such as acetonitrile, chloroform or methyl tert-butyl ether [3,5,6]. Thus, applying HPLC to carotenoid analysis is neither a rapid nor a sustainable alternative.

Furthermore, the use of organic solvents goes against the principles of green analytical chemistry. Besides the well-known 12 principles of green chemistry, the three R's are also frequently

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mentioned. The three R's stands for reduce, replace and recycle and mainly focus on organic solvents [7]. Even minor reduction in solvent usage for a HPLC method might result in huge economical gains for a big company performing routine analysis, and the gains would be even greater if organic solvents could be replaced. One good example of replacing organic solvents is the usage of supercritical fluids, more specifically supercritical carbon dioxide ($scCO_2$). Supercritical fluid extraction has been shown to be an efficient technique for extracting carotenoids from microalgae [8].

Supercritical fluid chromatography (SFC) has recently gained a lot of new ground since its rediscovery owed to SFC's versatility in chiral separation. This has improved the general understanding of SFC, and has also led to a rapid development on the instrument side. Although this has recently been the driving force, it should be underlined that the technique allows for environmentally benign analysis. The low viscosity and high diffusivities of $scCO_2$ induce the possibilities of using several columns coupled in series and still maintaining high flow rates, as well as keeping equilibration of columns very short. Therefore, SFC provides unmatched analysis speed compared to traditional HPLC. Hence more rapid routine analysis of carotenoids could be carried out using SFC and also in a sustainable way.

Despite the advantages of SFC, a very few number of authors have investigated the possibilities to separate different carotenoids using SFC. Before packed columns became popular some attention was given to the separation of α and β-carotene isomers using capillary columns [9,10], and also applied to separate carotenoids in carrots and tomato extracts [11]. However, presently packed columns are used exclusively, although very little research has been conducted on applying this technique in order to separate carotenoids. Lesselier et al. showed that a mixture of carotenoids, consisting mainly of carotene isomers, could be separated using a C18 column with a mobile phase consisting of mainly CO_2, a large fraction of acetonitrile and some methanol [12]. Matsubara et al. developed a method using mass spectrometric detection and three coupled monolithic C18 columns to separate acetone extracts of microalgae, *Chlamydomonas reinhardtii* [13]. However, peak resolution was not excellent and no attempt to neither validate the method nor quantify the present carotenoids was made. It can be concluded that no quantitative chromatographic method has been published that fulfills the requirements of being both rapid and environmentally sustainable.

The aim of this paper was to develop a fast, simple and sustainable chromatographic method for determining carotenoids based on SFC separation using a photodiode array (PDA) detector. The method was validated and applied in order to quantify carotenoids in extracts of *Scenedesmus* sp. obtained by SFE. To our best knowledge, this is the first time that a validated method for quantitative analysis of carotenoids using SFC is presented.

2. Material and methods

2.1 Chemicals

Ethanol (99.7%, Solveco, Rosenberg, Sweden) was used as a co-solvent in SFE. Methanol of LC-grade (>99.9%, Honeywell Burdick & Jackson, Seezle, Germany) was used as a co-solvent in SFC. Ultrapure CO_2 was provided by Air Products (Amsterdam, Netherlands). The following standards were acquired: astaxanthin, canthaxanthin, echinenone, lutein, neoxanthin, violaxanthin, zeaxanthin (DHI, Hørsholm, Denmark), which were obtained dissolved in ethanol, and β-carotene (≥95%, Sigma, St Louis, MO,

USA) that was also dissolved in ethanol. A mixture of standards was prepared and the concentration was increased approximately 5 times by partially evaporating the ethanol using gaseous nitrogen for about half an hour.

2.2 Sample preparation and extraction

Carotenoids from microalgae were extracted using SFE. The system consisted of an ISCO 260D syringe-pump (Teledyne Isco, Thousand Oaks, CA) used for pumping liquid CO_2, a Waters 515 HPLC-pump used for pumping ethanol, a HP 5890 gas-chromatography oven (Hewlett-Packard, Wilmington, DE) functioning as oven, a Tescom 26-1700 back-pressure regulator (BPR) (Tescom Europe, Selmsdorf, Germany) and an Eltherm ELTC/3 thermoregulator (Eltherm Elektrowärmetechnik GmbH, Burbach, Germany) was used for heating the lining between the BPR and the collection vessel. The liquid CO_2 pump was cooled by a Neslab RTE7 cooling bath controlled by a Digital One thermoregulator (Thermo Fisher Scientific, Waltham, MA). Throughout this work, any mixture of CO_2 and co-solvent expressed in percentage refers to the volume ratio.

Freeze dried microalgae, *Scenedesmus* sp., was grounded together with liquid nitrogen in a mortar before extraction. One gram of the grounded microalgae was weighted and placed in the extraction vessel and the carotenoids were extracted using a laboratory-built SFE-system (**Fig. 1**). The microalgae was extracted with neat CO_2 (CO_2 density, 830 g L^{-1}) with or without 10% ethanol as a co-solvent, at a pressure of 300 bar, a temperature of 60 °C, a flow of 2 mL min^{-1}, and extraction time was 60 minutes. Chosen pressure and temperature was based on optimized conditions for SFE of *Scenedesmus almeriensis* [14]. Ethanol was introduced at a flow rate of 0.2 mL min^{-1} after the extraction vessel when extracting with neat CO_2, in order to transfer the analytes to the collection vessel after the expansion of the CO_2. The same flow of ethanol was redirected, as shown in **Fig. 1**, in order to be introduced before the extraction vessel when extracting with co-solvent. The extract was collected in a vessel protected from light and placed on ice. Ethanol was added to the collected extract and the final volume was 10 mL.

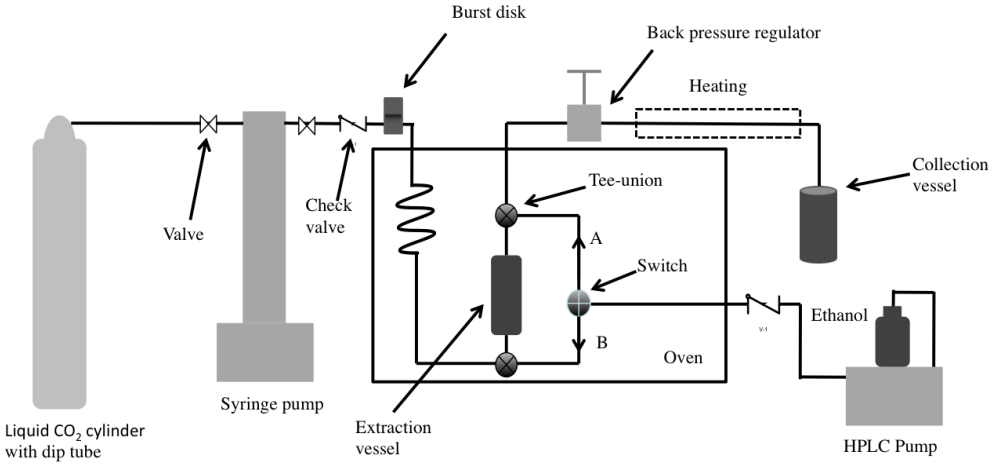

Fig. 1. Schematic of the lab-built SFE apparatus.

2.3 Instrumental and chromatographic conditions

A Thar Investigator semi-preparative SFC (Pittsburgh, PA) was used for carotenoids analysis, consisting of a cooled fluid delivery module with a 6 co-solvent switching valve, a modified Spark Holland Alias autosampler with a 48-vial plate, an analytical-2-prep oven with a 10 column switching valve, an automated backpressure regulator and a Waters 2998 photodiode array detector (Milford, MA). The fluid delivery module was cooled by a Neslab RTE7 cooling bath controlled by a Digital One thermoregulator. The instrument was controlled by SuperChrom (version 6.43) and data was subsequently analyzed with ChromScope (version 2.1), both software were from Thar.

The separation of the carotenoids in the extract was performed using two columns in series, a SunFire C18 (4.6 x 250 mm, 5 µm particle size, 100 Å pore size, Waters) followed by a Viridis SFC silica 2-ethylpyridine (4.6 x 250 mm, 5 µm particle size, 100 Å pore size, Waters) column. Liquid CO_2 and methanol were used as mobile phase. In order to separate the carotenoids in the extract, a gradient of co-solvent was used starting with 10% co-solvent increasing to 17% over 8 min subsequently increasing to 25% over 2 min which was then kept for 5 min. The back-pressure was 120 bar, the temperature was 32 °C, the flow rate was 5 mL min^{-1} and the injection volume was 50 µL. Each peak was quantified at the wavelength of maximum absorption.

3. Results and discussions

3.1 Chromatographic analysis

The separation of carotenoids using a C18 column and a silica-bonded 2-ethylpyridine (2-EP) column coupled in series was optimized by testing both methanol and ethanol as co-solvents, different gradient profiles of co-solvents, pressure, temperature and flow rate. The initial optimization was carried out using a mix of 8 carotenoid standards that are often found in microalgae and included astaxanthin, β-carotene, canthaxanthin, echinenone, lutein, neoxanthin, violaxanthin and zeaxanthin [15]. Many of these carotenoids are isomers of each other and are very similar in structure as shown in **Fig. 2**. Standards are obtained at low concentrations, approximately 1 mg L^{-1}. Thus the ethanol of the standard mix had to be partially evaporated in order to reach detectable concentrations.

In our attempt to separate the standard mix, the initial optimization regarding peak resolution resulted in a gradient method based on CO_2 and methanol for separating a mix of 8 carotenoid standards. The gradient started at 9% methanol, increasing to 17% over 7 min, subsequently increasing to 25% over 2 min and then kept isocratic. Flow was 5 mL min^{-1}, backpressure was 100 bar, and the temperature was 32 °C. All of the 8 carotenoids were separated within 10 min (**Fig. 3**). However, when the method was applied to an extract obtained through SFE in the presence of ethanol as a co-solvent, additional present peaks interfered with separation. The additional peaks were attributed to chlorophylls and unidentified carotenoids based on their characteristic absorbance spectra. Thus, slight modifications were made to the method in an attempt to improve the separation. These adjustments resulted in a less good separation of cantaxathin and astaxanthin, which was although of less importance since cantaxanthin was not present in the SFE extract.

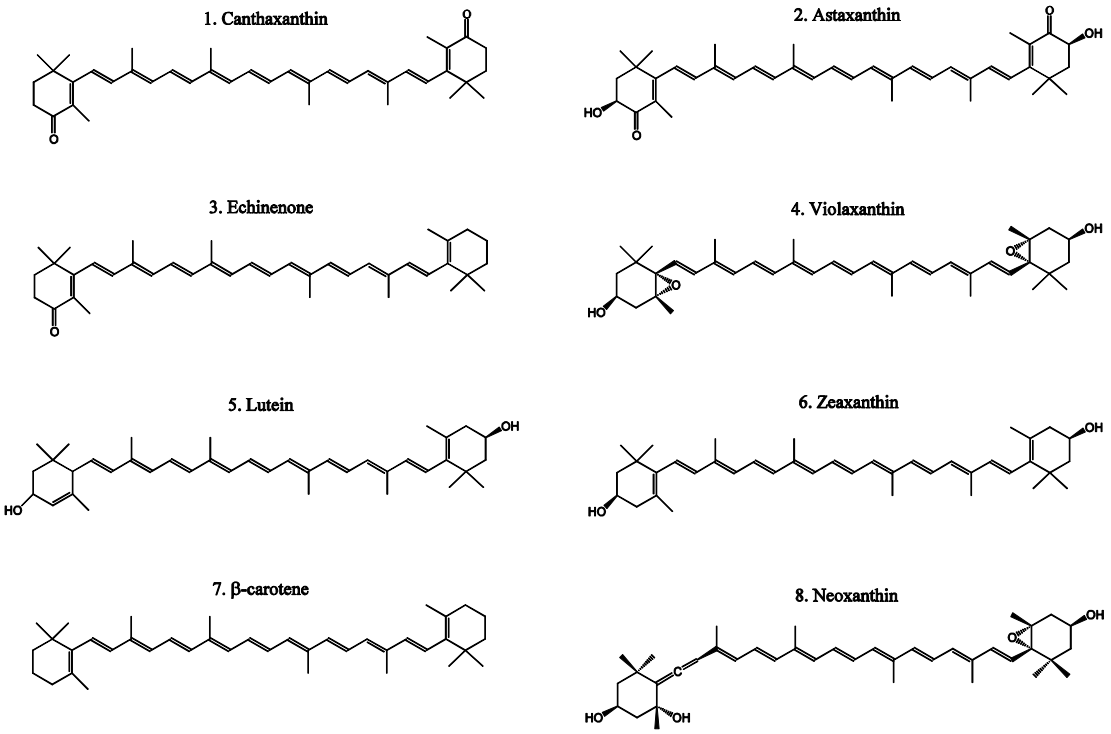

Fig. 2. Carotenoids presented in the order of which they were eluted in SFC.

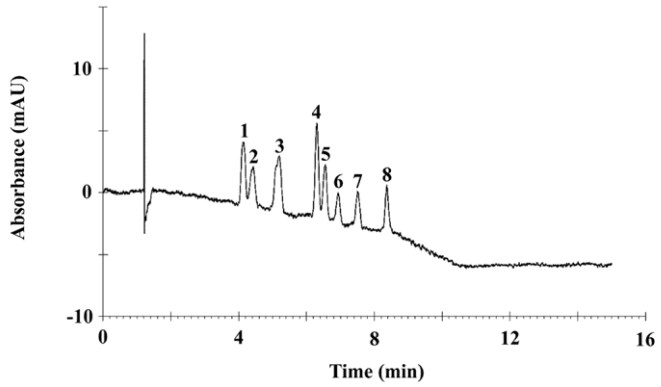

Fig. 3. SFC chromatogram of a standard mixture of cantaxanthin (1), astaxanthin (2), echinenone (3), violaxanthin (4), lutein (5), zeaxanthin (6), β-carotene (7) and neoxanthin (8). Chromatographic conditions are described in section 3.1. Absorbance was measured at 430 nm.

The second optimization step resulted in a final method based on a gradient profile starting at 10% methanol increasing to 17% over 8 min subsequently increasing to 25% over 2 min which was then kept for 5 min. Re-equilibration time was 5 min, resulting in a total analysis time of 20 min. The backpressure was increased to 120 bar and the flow and temperature was kept at 5 mL min^{-1} and 32 °C, respectively. This method was able to separate all the present carotenoids in the extracts of *Scenedesmus* sp. as well as present chlorophyll (**Fig. 4**).

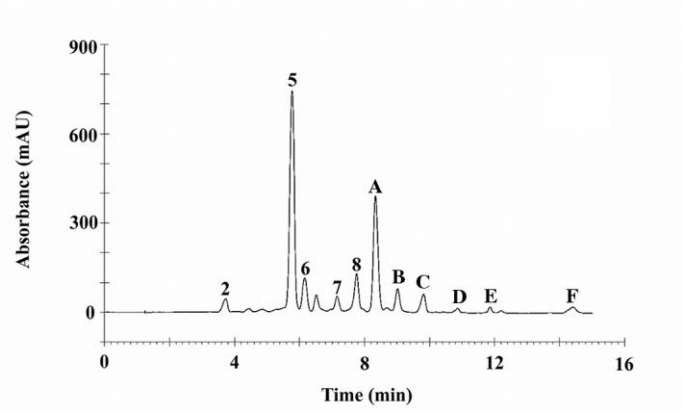

Fig. 4. SFC chromatogram of *Scenedesmus* sp. extract obtained by SFE using ethanol as co-solvent in the extraction process. Identified carotenoids were astaxanthin (2), lutein (5), zeaxanthin (6), β-carotene (7) and neoxanthin (8). Unidentified chlorophylls eluted last (A-F). Chromatographic conditions are described in section 3.1. Absorbance was measured at 430 nm.

Changes in pressure had minor impact on selectivity however substantial effect on the retention factor. We found that even slight changes in temperature had a substantial impact on the selectivity in the SFC separation (**Fig. 5**). This is particularly apparent for β-carotene, which co-eluted with neoxanthin at 28 °C and by increasing the temperature to 40 °C, the retention factor considerably decreased and the β-carotene thereby instead co-eluted with lutein. It was found that 32 °C was the optimal temperature with regards to overall separation of the carotenoids in the extracts, even though the resolution between violaxanthin and lutein was slightly decreased, compared to using a lower temperature. Even a small change of 4 °C, as shown in **Fig. 4**, affected the selectivity in the separation of carotenoids. Therefore temperature may be a very important parameter when optimizing future methods.

In comparison to the study of Matsubara et al. peak shapes, analysis speed and resolution were all substantially improved [13]. It should also be stressed that our results were obtained using fewer columns and a DAD, which is a simpler and lower-cost detector compared to a mass spectrometer.

3.2 Method validation

The chromatographic method used for separating carotenoids in the SFE extract was validated. Identification and purity of the compounds in real samples were determined by comparing retention times and spectra of known standards. The limit of detection (LOD) and the limit of quantification (LOQ) as well as the sensitivity for all the carotenoids were determined (**Table 1**). Determination of the limits were done according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 6.3 and 7.3 based on the standard deviation of the response (n=5) and slope [16]. Standard deviation of the response was based on the manual integrated peaks of the 5 repeatedly injected standards at the lowest concentration of the calibration curve. The LODs for the different carotenoids were between 0.02 and 0.05 mg L^{-1} and the LOQs were between 0.05 and 0.15 mg L^{-1}. Inbaraj and Chen validated a developed method based on HPLC separation using a C30 column, for separating carotenoids extracted from *Chlorella pyrenoidosa* [17].

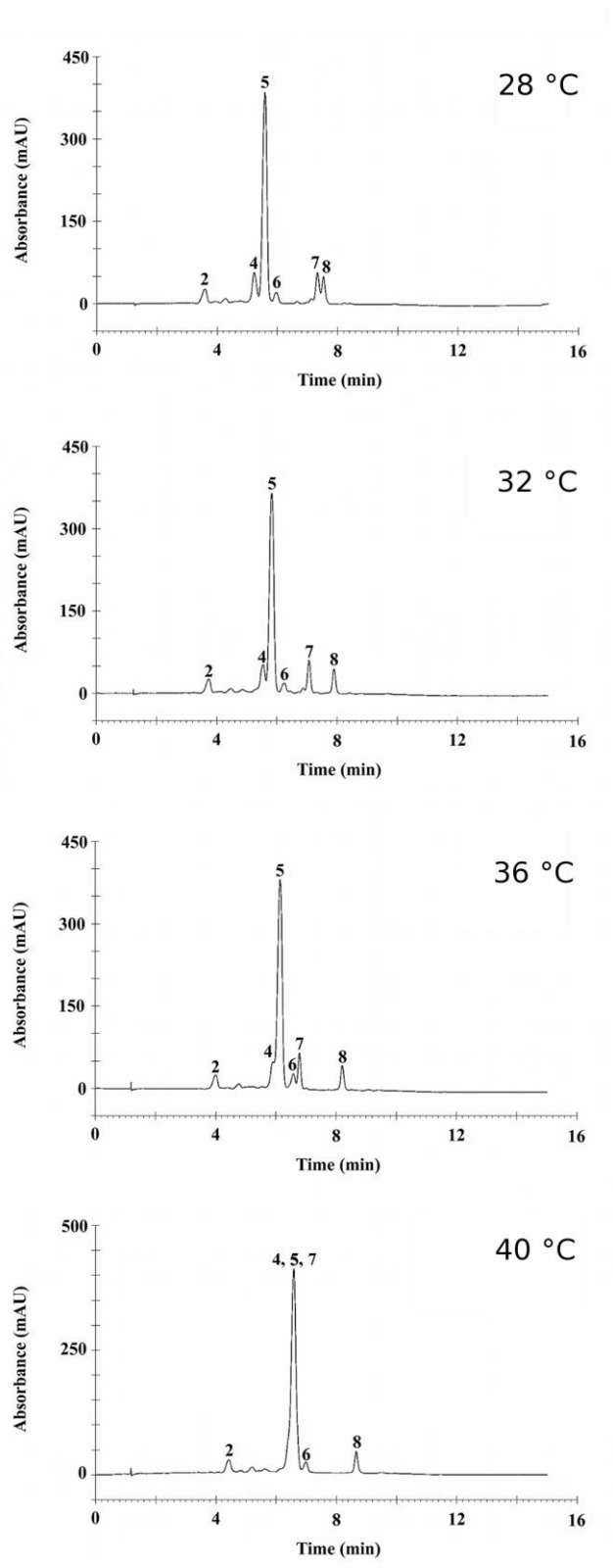

Fig. 5. Chromatograms of *Scenedesmus* sp. extracts obtained by SFE using only neat CO_2 in the extraction process. The chromatograms were obtained at different temperatures using the chromatographic conditions described in section 3.1. Identified carotenoids were astaxanthin (2), violaxanthin (4), lutein (5), zeaxanthin (6), β-carotene (7) and neoxanthin (8). Absorbance was measured at 430 nm.

Table 1. Calibration curves using various carotenoid standards in SFC.

Carotenoid	$\lambda_{max\ 1}$ (nm)	$\lambda_{max\ 2}$ (nm)	$\lambda_{max\ 3}$ (nm)	Calibration range tested (mg L^{-1})	Sensitivity (mAU s L mg^{-1}, 95% CI)	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Astaxanthin	462[1]	-	-	0.137 - 0.686	78.39 ± 2.41	0.9972	0.04	0.13
β-carotene	414	440[1]	466	0.370 - 7.407	64.00 ± 1.28	0.9978	0.02	0.07
Canthaxanthin	460[1]	-	-	0.191 - 0.955	108.0 ± 5.76	0.9916	0.03	0.08
Echinenone	446[1]	-	-	0.204 - 1.032	98.74 ± 3.97	0.9952	0.02	0.05
Lutein	414	434[1]	462	0.194 - 0.968	61.02 ± 1.61	0.9979	0.05	0.14
Neoxanthin	406	427[1]	455	0.185 - 0.927	47.28 ± 2.43	0.9921	0.05	0.15
Violaxanthin	408	430[1]	458	0.188 - 0.938	94.92 ± 3.04	0.9969	0.03	0.08
Zeaxanthin	440[1]	466	-	0.179 - 0.896	44.02 ± 1.30	0.9974	0.05	0.14

[1] Chosen wavelength for quantification.

The authors reported LODs and LOQs between 0.002 and 0.06 mg L^{-1}, 0.007 and 0.18 mg L^{-1} respectively for lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene. Dias et al. reported similar LODs and LOQs for HPLC separation of carotenoids extracted from food matrices [18]. Thus it is apparent that quantification by means of SFC is as good as HPLC in terms of detection limits. Even lower limits should be possible by using an analytical SFC system. Due to wider tubings used in the semi-preparative instrument, we expect that an analytical system would perform even better in terms of both separation and detection limits.

The recovery of the chromatographic method was evaluated by spiking both the microalgae extracts with β-carotene at two levels by adding either 0.45 mg L^{-1} or 0.90 mg L^{-1} and was determined to be 97% and 100% in extract obtained using neat CO_2 and 99% and 103% in the extract using ethanol as co-solvent, respectively. Only β-carotene was chosen due to unavailability of carotenoid standards with high enough concentration to be able to spike an extract. Repeatability and intermediate-precision were calculated based on quantification of β-carotene in both extracts by triplicate injection per day at three different days, however performed by the same operator. The repeatability of quantification in the extracts of neat CO_2 and co-solvent were 0.9% and 2.4% relative standard deviation (RSD), respectively. The intermediate-precision was 2.2% and 6.4% RSD for the extracts of neat CO_2 and co-solvent, respectively. The precision is equivalent to the precision reported for HPLC analysis of β-carotene in tomato extracts [18]. The authors reported 4.0% and 6.8% RSD, respectively.

3.3 Quantification of carotenoids in microalgae

Carotenoids from microalgae *Scenedesmus* sp. were extracted using SFE using either neat CO_2 or CO_2 with ethanol as co-solvent. The extracts dissolved in ethanol were subsequently analyzed using the validated SFC-method (**Table 2**). Astaxanthin, β-carotene, lutein, neoxanthin and zeaxanthin, were identified in both extract, however violaxanthin was somewhat surprisingly only found in the extract using only neat CO_2. Due to the low concentrations of the standards the quantification required substantial dilution, thus increasing the uncertainty of measurement. Considerably higher concentrations of astaxanthin, lutein, neoxanthin and zeaxanthin were obtained using ethanol as co-solvent compared to

Table 2. Quantification of SFE extracts obtained with neat CO_2 and with 10% ethanol as co-solvent using SFC. Extraction conditions were 300 bar, 60 °C, total flow of 2 mL min^{-1}, 60 min extraction time. Values are given within the 95% CI.

	Neat CO_2 (μg g^{-1} freeze dried algae)	CO_2 and EtOH (μg g^{-1} freeze dried algae)
Astaxanthin	43.1 ± 3.1	72.9 ± 11.2
β-carotene	54.7 ± 3.1	59.9 ± 3.1
Lutein	210.5 ± 54.5	436.1 ± 50.5
Neoxanthin	46.9 ± 4.0	670.8 ± 94.4
Violaxanthin	35.0 ± 2.4	ND[1]
Zeaxanthin	20.0 ± 2.6	89.6 ± 26.4

[1] Not detected

neat CO_2. Especially neoxanthin was largely affected by the addition of co-solvent giving 14 times higher yield with ethanol as co-solvent. The extracted β-carotene was not significantly higher using ethanol as co-solvent. By using only CO_2 in SFE, no chlorophylls appeared to be extracted, which the yellow color of the extract indicated. Contrarily, using co-solvent in the extraction process a dark green extract was obtained indicating that chlorophylls were also extracted, further verified by the spectra of some of the unidentified peaks seen in the chromatogram of **Fig. 4**. Therefore, the presented method may also be suitable for quantification of both carotenoids and chlorophylls in less than 15 min. Furthermore, the developed method using the employed instrument would allow a straightforward scale-up to separate and isolate the different carotenoids using preparative chromatography.

4. Conclusions

A new chromatographic method for separation of carotenoids from microalgae using SFC has been developed and validated. By coupling in series a C18 and a 2-EP column, a standard mixture of astaxanthin, β-carotene, canthaxanthin, echinenone, lutein, neoxanthin, violaxanthin and zeaxanthin were separated in less than 10 min. Astaxanthin, β-carotene, lutein, neoxanthin, violaxanthin and zeaxanthin were separated when an adjusted method was applied to real samples obtained from the microalgae *Scenedesmus* sp. by SFE. It was observed that temperature had a major impact on the selectivity of the separation.

Instrumental LODs were between 0.02 and 0.05 mg L^{-1}, and LOQs were between 0.05 and 0.15 mg L^{-1} for the different carotenoids. The repeatability was 0.9% and 2.4% RSD, intermediate-precision was 2.2% and 6.4% RSD of quantification in extracts of SFE using neat CO_2 and co-solvent, respectively.

The developed chromatographic method uses only CO_2 and methanol, and is a sustainable alternative to HPLC separation on C18 or C30 columns using organic solvents.

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