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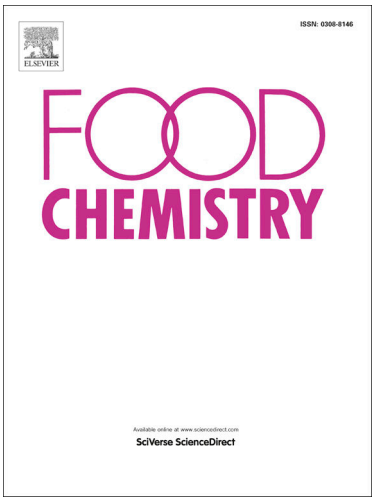

Analytical Methods

A fast and simple method for determination of β-carotene in commercial fruit juice by cloud point extraction-cold column trapping combined with UV–Vis spectrophotometry

Mehdi Safdarian, Payman Hashemi, Alireza Ghiasvand

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A fast and simple method for determination of β-carotene in commercial fruit juice by cloud point extraction-cold column trapping combined with UV–Vis spectrophotometry

Mehdi Safdarian [a,b], Payman Hashemi [a*], Alireza Ghiasvand [a,c]

[a] Department of Chemistry, Faculty of Science, Lorestan University, Khoramabad, Iran

[b] Nanotechnology Research Centre, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[C]Australian Centre for Research on Separation Science (ACROSS), School of Natural Sciences, University of Tasmania, Hobart, Tasmania 7001, Australia

ABSTRACT

Cloud point extraction with cold column trapping (CPE-CCT) was used for the rapid preconcentration and UV-Vis spectroscopy of beta-carotene in fruit juice samples. A central composite design was employed to optimize parameters such as pH, incubation time, cloud point temperature and surfactant concentration. A detection limit of 0.01 mg/L of beta-carotene ($3S_B/m$), a coefficient of determination of 0.998 and a linear range of 0.04-10 mg/L were obtained. The CPE-CCT method was confirmed in comparison with the corresponding direct HPLC standard method. A simple, portable and cost-effective device was also utilized. Owing to eliminating centrifugation, the conditions of CPE-CCT were more moderate and its sample handling easier compared to conventional CPE.

Keywords: Triton X-100, Cloud point extraction, cold column trapping, multivariate optimization, beta-carotene

1. Introduction

Beta-carotene, known as provitamin A, is the most common carotenoid in fruits with the highest vitamin A activity (Stinco, Pumilia, Giuffrida, Dugo, Meléndez-Martínez, & Vicario, 2019). Beta-carotene as a natural fat-soluble compound in many flowers and fruits and a strong antioxidant and scavenger of singlet oxygen, beta-carotene contributes to the majority of yellow, red and orange colorations (Marx, Schieber, & Carle, 2000; Sricharoen, Limchoowong, Techawongstien, & Chanthai, 2016). Because of the features mentioned above that could confer them with a preventive role in cardiovascular disease and cancer, measuring the amount of beta-carotene in commercial fruit juices (e.g., carrot, mango, orange, and apple) and quality control of this products is very important (Sakaew, Sricharoen, Limchoowong, & Chanthai, 2018). Beta-carotene is sensitive to degradation and especially to oxidation due to its substantial number of double bonds. The beta-carotene lose is mainly observed during fruit processing, i.e., juice or puree production. In fact, the loss of tissue integrity, temperature increase and exposure to oxygen and light during thermal treatment drastically increase the rates of destruction reactions (Pénicaud, Achir, Dhuique-Mayer, Dornier, & Bohuon, 2011).

The methods proposed to determine beta-carotene include HPLC (Brabcova, Hlavackova, Satinsky, & Solich, 2013; Syamila, Gedi, Briars, Ayed, & Gray, 2019), ultra-performance liquid chromatography (Delpino-Rius, Eras, Marsol-Vall, Vilaro, Balcells, & Canela-Garayoa, 2014), liquid chromatography-mass spectrometry (Carail, Fabiano-Tixier, Meullemiestre, Chemat, & Caris-Veyrat, 2015; Vinas, Bravo-Bravo, Lopez-Garcia, & Hernandez-Cordoba, 2013), fluorometry (Liu, Liu, Liu, Wang, Xu, & Wang, 2016), near-infrared spectroscopy (Rungpichayapichet, Mahayothee, Khuwijitjaru, Nagle, & Müller, 2015) and thin layer chromatography-densitometry (Starek, Guja, Dąbrowska, & Krzek, 2014). UV-Vis spectroscopy

was, however, found to be the best method in terms of cost-effectiveness, availability, versatility, simplicity and speed (Abadi, Ashraf, Chamsaz, & Shemirani, 2012; Biswas, Sahoo, & Chatli, 2011).

UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes (Upstone, 2013). In this method, determining the analyte concentration in the unknown sample is very simple and fast. Measuring the analytes in the complex samples is, however, difficult and sample preparation, extraction and pre-concentration steps are required before measurement. Although the amount of Beta-carotene in commercial fruit juices may be higher than the detection limit of a UV/Vis spectroscopy method, however, the complexity of the matrix in these samples does not allow direct measurement of it. Methods of extraction that are used before the final spectrophotometric determination of analytes therefore include solvent extraction and partitioning (Zheng, Zhu, Wu, Yan, Meng, & Song, 2015), dispersive liquid-liquid microextraction (Sereshti, Ahmadvand, & Asgari, 2014), and solid phase extraction (Ma & Lin, 2004; Mehraban & Farzaneh, 2006).

The growing interest in the use of CPE as a preconcentration method lies in its procedures being efficient, fast and simple while promoting the enrichment factors and not using large amounts of toxic organic solvents (Campillo, Marín, Viñas, Garrido, Fenoll, & Hernández-Córdoba, 2019; Castor, Portugal, Ferrer, Hinojosa-Reyes, Guzmán-Mar, Hernández-Ramírez, et al., 2016). An aqueous solution of a non-ionic surfactant turns into a cloud during CPE at a narrow temperature range, above which the solution is separated into a small-volume phase rich in the surfactant and another phase poor in the surfactant whose concentration approaches the critical micellar concentration (Watanabe & Tanaka, 1978). There are many theories to explain the

presence of cloud point. However, it is still not completely resolved (Koshy, Saiyad, & Rakshit, 1996).

Given that phase separation in a conventional CPE is performed at high temperatures (N Pourreza & Elhami, 2009) or a prolonged incubation (N. Pourreza, Rastegarzadeh, & Larki, 2011), this method is not used for separating thermally-unstable analytes (Chen & Huang, 1998; Syamila, Gedi, Briars, Ayed, & Gray, 2019). A thorough review of literature suggested CPE has not been applied yet to extracting beta-carotene.

CPE can be integrated into online systems to take advantage of both methods. Numerous studies have been conducted on online CPE or CPE automation (Fang, Du, & Huie, 2001; Gil, Gásquez, Olsina, Martinez, & Cerutti, 2008; Li, Hu, & Jiang, 2006; Paleologos, Vlessidis, Karayannis, & Evmiridis, 2003; Silva & Roldan, 2009). A semi-automated CPE coupled to a cold column trapping (CCT) system developed to control the temperature of a packed mini-column through an online CPE was used to determine phenazopyridine (Nazari Serenjeh, Hashemi, Safdarian, & Kheirollahi, 2013) and curcumin (Rahimi, Hashemi, & Nazari, 2014). The developed flow system accelerated the phase separation, decreased the surfactant concentration required and the extraction temperature and the incubation duration and obviated the need for centrifugation in CPE. The successful use of this method in previous studies has led us to consider the possibility of using it to the extraction and preconcentration of thermally-unstable analytes such as beta-carotene. In addition, the use of triton X-100 surfactant was considered to compare it with the traditional CPE methods.

Overall, the presented study pioneered the application of a semi-automated CPE-CCT before the UV-Vis spectroscopy of beta-carotene while taking advantage of CPE-CCT and the simplicity of UV-Vis spectroscopy for the rapid preconcentration and determination of beta-carotene in diverse

commercially-available fruit juices. This study also aimed at accelerating the method by eliminating the centrifugation step and turning it into a semi-automated technique through coupling CPE to a flow system. A multivariate response surface methodology was employed to optimize the factors affecting the efficiency of extraction.

2. Experimental

2.1. Reagents and materials

Beta-carotene was procured from Merck in Germany, and 1 g/l of its prepared in acetone was diluted using a 0.02-M ammonium acetate buffer to develop working standards for beta-carotene. Triton X-100 as a non-ionic surfactant, ethanol, Chromabond octadecylsilane (C18) adsorbent, hydrochloric acid, sodium hydroxide, acetone and other chemicals were analytically graded and procured from Merck. The solution obtained by dissolving an appropriate amount of Triton X-100 in water was utilized without purification. A sodium hydroxide solution and a 1-M hydrochloric acid were used to adjust pH and double-distilled water was employed throughout the process.

2.2. Apparatus and analysis

UV-Vis spectroscopy was performed using the single-beam Shimadzu UV-Vis spectrometer UVmini-1240 equipped with a pair of 350-µl quartz microcells (Model Q124) made by ES Quartz in Spain for the determination of absorbance.

Two Shimadzu LC-10AD reciprocating pumps, a column oven (CT10-10AC), an in-line degasser (DGU-14A), a UV-Vis detector (SPD-10A) and a high-pressure manual injection valve equipped with a 20-µl injection loop (Rheodyne) were used to perform HPLC. Data acquisition and processing were conducted in Class VP 6.1. The compounds were separated on a 25 cm×4.6 mm i.d. RP-18 HPLC Column (Shim-Pack CLC-C18) equipped with a 1-cm guard column and packed

with 5-µm particles. The mobile phase with a flow rate of 0.8 ml/min was delivered as a mixture of 20% tetrahydrofuran and 80% acetonitrile from separate pumps. The column temperature and the detector wavelength were respectively adjusted at 40 degrees Celsius and 456 nm. The samples were pumped using an RP-1000 peristaltic pump made by Eyela World and distributed by Tokyo Rikakikai Co., Japan. Doubled-distilled water was prepared using a WSC/4D water still provided by Hamilton Economy Water Stills.

According to Fig. 1, the CPE procedure was automated and integrated with CCT using a slightly-modified homemade portable device comprising 2 parts. The CPE reactor or the upper cell was made through drilling a column 10 mm in diameter and 100 mm in length in a 14×40×120 mm Plexiglass block. Eight heating elements surrounded the CPE cell. A 5×10 mm polypropylene cartridge as the lower column (CCT) packed with the C18 adsorbent (Shen, Hu, Huang, Yin, Chen, & Yao, 2009) was fitted into the Plexiglass block. To control the temperature, the column block was inserted between two plates of thermal electric coolers. A temperature sensor was also mounted in a hole next to the column in the lower section of the Plexiglass block. Two aluminum heatsinks, each of which was connected to an electric fan, were attached to the opposite sides of the CCT column. Moreover, a unit comprising an interface device with properties of an LCD was programmed and mounted for controlling the column temperature.

(Fig. 1)

2.3. Procedure of CPE-CCT

According to the method proposed, a mixture of one ml of a 2% (v/v) Triton X-100 solution and four ml of the aqueous sample with pH 8 was transferred to the CPE column as per Fig. 1 and kept at 70 degrees Celsius for three minutes. Meanwhile, the “Go to min” key of the control panel was pushed to adjust the temperature of the CCT column at the minimum of zero degree Celsius. The

extraction phase was then trapped by allowing the cloudy solution to flow into the cooled CCT column. Afterwards, the CCT column temperature was raised to thirty five degrees Celsius and the rich phase of the surfactant was eluted using 0.4 ml of ethanol. In the next step, the eluated phase was transferred to a quartz microcell for absorbance measurement at 456 nm. Fig. 2 shows the stages of CPE-CCT.

(Fig. 2)

2.4. Sampling and pretreating the samples

Two commercial orange juices (from Merikhi and Sporta brands) and one apple juice (from Paki brand), available in 120 mL aluminum packagings, were purchased from a local market in Khoramabad (Iran) and fridged at 4±2 °C before being analyzed. They were used for beta-carotene extraction without any pretreatments and/or dilution. The CPE-CCT procedure began by adding an appropriate amount of ammonium acetate to individual samples and then adjusting their pH with HCl or NaOH solutions. Whereas for the purpose of direct HPLC determination, 15 ml of individual samples was centrifuged at 8000 rpm for 15 min before injection. The calculated recovery was reported for the method developed.

3. Results and discussion

The beta-carotene absorption spectrum showed a maximum absorbance intensity at about 456 nm, which was used for quantitative measurements (Fig. S1 in electronic supplementary information, ESI). As discussed earlier, the proposed device comprised 2 parts. The upper part (CPE cell) performs the analyte extraction independently of the phase separation in the CCT part. To operationally optimize the CCT part, the conditions of an ordinary CPE of beta-carotene obtained from some preliminary experiments were applied to the CPE cell. These preliminary condtions

included pH 7, the surfactant concentration=8% (v/v), temperature=80 ˚C and incubation duration=15 minutes.

3.1. CCT optimization

Initially, the adsorption and desorption temperatures of the CCT column under the preliminary conditions of the CPE column were investigated. Research suggests the of extraction is significantly affected by the CCT column temperature as well as the type and mass of its adsorbent. Fifty mg of the C18 adsorbent and 0.4 cc of ethanol as an eluent were used to investigate the CCT column temperature in the adsorption and desorption steps.

According to Fig. 3a, a temperature decrease in the adsorption step increased the trapped amount of the surfactant-rich phase containing the analyte in the CCT. Decreasing the temperature from ten to five degrees Celsius significantly increases the recovery by increasing the surfactant phase viscosity (melting point of the Triton X-100= 6 °C) and its better entrapment into the CCT unit. Because the hot solution constantly enters the column, a zero temperature was applied in the following experiments to maximize the amount of the surfactant-rich phase trapped in the column.

Increasing the CCT unit temperature at the elution stage was also found to increase the recovery. The permissible temperature for the elution step depended on the type of solvent used. Increasing the temperature helped decrease the surfactant-rich phase in viscosity and complete the column elution process. On the other hand, higher evaporation of the solvent occurred at higher temperatures. According to Fig. 3b, increasing the temperature from 30 to 35 degrees Celsius while using ethanol as the eluent raised the recovery by approximately ten percent. Column desorption was performed at 35 °C in the following experiments so as to prevent solvent evaporation at higher temperatures.

The solvent volume should be selected by taking account of the CCT elution and the desorption of the phase rich in the surfactant from the column. This solvent along with the column elution are necessary for diluting the surfactant-rich phase for injection into the HPLC or delivering the appropriate volume for UV-Vis measurements. The effect of ethanol volumes of 100-500 µl was therefore investigated on the analyte elution from the CCT and the efficiency of extraction. Fig. 3c shows an optimal ethanol volume of 400 µl given the increase in the recovery with the eluent volumes of below 400 µl and the constant recovery for the volumes exceeding 400. Table S1 summarizes the optimal conditions of the CCT column.

(Fig. 3)

3.2. Optimizing the CPE process using a central composite design (CCD)

A CCD was adopted as a multivariate optimization technique to optimize the parameters of the CPE cell (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). A CCD is defined on the basis of the fit of polynomial equations to experimental data as an indicator of the behavior of datasets to make statistical inferences. Given the effects of different variables on the CPE column operation, a CCD is applicable to optimization the parameters of a CPE column (Khani, Sheykhi, & Bagherzade, 2019; Kolachi, Kazi, Khan, Wadhwa, Baig, Afridi, et al., 2011; Oliveira Gde, de Oliveira, da Conceicao, & Leles, 2016). According to this method and the data of table S2, all the factors, including incubation duration (ID), pH, CPE temperature (Temp) and the surfactant concentration (CS), were evaluated at 5 levels. The experimental error was measured by incorporating 7 replicates of the center point into the design. The extraction recovery denoted as E% was shown in table S3 for 31 experiments simulated in Minitab.

Fig. 4 shows the relationships of the parameters and beta-carotene recovery as 3D diagrams. As seen in Fig. 4a shows a positive relationship between the recovery and pH at a fixed temperature. Given beta-Carotene as a non-polar compound, its solubility and separation are higher in a neutral pH. On the other hand, the lower pH create an electrostatic repulsion between the micelles and avoid them from phase separation and aggregation (Koshy, Saiyad, & Rakshit, 1996). Fig. 4b shows a negative relationship between beta-carotene extraction and the surfactant concentration. With the increase of surfactant concentration from 2% to 10%, the recovery has approximately decreased 3 times. This event may be due to the reduced efficiency of the CCT column in trapping larger amounts of surfactant-rich phase. As a result, some analyte with surfactant phase passes through the CCT column, which reduces the percentage of beta-carotene extraction. According to Fig. 4c, the extraction was maximized at a surfactant concentration of 2% V/V and the lowest temperature (70 °C). It is to be noted that optimum temperature for the use of Triton X 100 in beta-carotene extraction, compares well with the literature CP value of 67 °C (Valaulikar & Manohar, 1985). According to the last row of table S3, beta-carotene extraction being optimized at milder conditions, i.e. a lower temperature and surfactant concentration, constitutes the main advantage of the CPE-CCD system, which inserted the cloudy solution obtained into the CCT cell for the phase separation rather than performing the commonly-used centrifugation in conventional CPE methods. Due to the fact that the cell of the CCT column is designed to be narrow and long, it allows uniform and fast heat to reach all parts of the solution and, as a result, only 3 minutes is enough to produce a cloudy solution.

(Fig. 4)

The CCD method yielded an optimized recovery of 85%, which was not acceptable. Investigating the effect of adding more adsorbent to the CCT cell on the recovery showed that

raising the C18 amount to 90 mg results in an analyte recovery efficiency of over 99% as per Fig. S2.

3.3. Performance evaluation

The performance of CPE-CCT was analyzed in terms of accuracy, linearity, the detection limit and the quantitation limit in optimal conditions, i.e. pH 8, the surfactant concentration=2% (v/v), incubation temperature=70 °C and incubation duration=3 minutes. Table 1 shows the analytical features of the optimized CPE-CCT. Six replicated analyses in optimized conditions from 6 independent standard preparations yielded a 100.2% recovery with an inter-day relative standard deviation of 1.5%. Intermediate precision was calculated as 1.94% by performing six experiments in three consecutive days (inter-day relative standard deviation).

The quantitation ($10S_B/m$) and detection ($3S_B/m$) limits (Sereshti, Ahmadvand, & Asgari, 2014) were respectively obtained as 0.04 mg/L and 0.01 mg/L, with m denoting the slope of the calibration curve and S_B the standard deviation of the blank sample. For 0.05-10 mg/L of beta-carotene, the calibration curve was linear and R^2=0.998. The enrichment factor, defined as the ratio of the slopes of the calibration curves after and before the enrichment, was calculated to be 9.68. The pre-concentration factor, defined as the volume ratio of the beta-carotene solution before and after the pre-concentration, was also obtained as 10.

Table S4 compares the study extraction technique with of the methods reported in literature on the beta-carotene determination and extraction, suggesting the application of centrifugation and organic or toxic extraction solvents such as tetrahydrofuran or chloroform in all the other methods. As a fast extraction and separation method, CPE-CCT uses an environmentally-friendly surfactant as the extraction solvent rather than a toxic solvent while eliminating centrifugation. The detection limit of this method is also lower and its intermediate precision better compared to those of the other methods cited (Karnjanawipagul, Nittayanuntawech, Rojsanga, & Suntornsuk, 2010;

Sereshti, Ahmadvand, & Asgari, 2014; Sricharoen, Limchoowong, Techawongstien, & Chanthai, 2016).

(Table 1)

3.3.1 Analysis of real samples

The technique proposed was applied to 3 commercial fruit juice samples of orange and apple purchased from a local market to confirm the method reliability and applicability. An HPLC was taken as the reference to measure the accuracy of the spectroscopy of beta-carotene (the analytical performance of HPLC method investigated but not reported). Table 2 shows the consistency between the results obtained from the proposed CPE-CCT coupled with UV-Vis spectroscopy and the corresponding results of a direct HPLC as the standard method. The positive bias in measuring beta-carotene in the juice samples was associated with the disturbing effect of their excipients and/or other carotenoids such as xanthophylls and lycopene. Statistical analysis was used for comparison of the proposed and direct HPLC methods and no significant difference between the two methods was observed at 95% confidence level. According to Fig. S3, the small peaks observed around the beta-carotene peak in the direct chromatogram of sample 3 using the HPLC suggested the existence of certain matrix compounds with absorbance at 456 nm. The recovery obtained as 99.6-111.3% using the study method showed negligible matrix effect on the results.

(Table 2)

4. Conclusions

The present findings suggested the proposed CPE-CCT is efficient and fast in extracting beta-carotene from real samples without requiring dilution or pretreatment. Compared to conventional CPE, the proposed system eliminated centrifugation and decreased the temperature and incubation duration, which is essential for the extraction of thermally-unstable analytes. CPE-CCT coupled

with UV-Vis spectroscopy was found simple, sensitive, environmentally-friendly, rapid and cost-effective with less sample handling required for determining beta-carotene in a juice sample. Given the simple extraction procedure and short spectrophotometric analysis, the method is recommended for the screening and fast quality control of beta-carotene contents in fruit juices.

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Figures captions:

Fig. 1. A schematic diagram of the main components of a CPE-CCT device

Fig. 2. The stages of the CPE-CCT procedure

Fig. 3. Optimizing the parameters of CCT; (a) adsorption temperature, (b) desorption temperature for eluent volume=0.4 ml, pH 8, incubation duration=15 minutes, surfactant concentration=8% (v/v), CPE temperature=80 °C; (c) effects of the volume of ethanol as the eluent for pH=8, column desorption temperature=35 °C, CPE temperature=80 °C, incubation duration=15 minutes, column adsorption temperature=0 °C and surfactant concentration=8% (v/v) (n=3).

Fig. 4. The response surface diagrams of the extraction recovery (E%) versus (a) pH and temperature, (b) pH and surfactant concentration, (c) surfactant concentration and temperature

CRediT author statement

Mehdi Safdarian: Methodology, Software, Writing- Original draft preparation,

Payman Hashemi: Conceptualization, Writing- Reviewing and Editing, Supervision

Ali Reza Ghiasvand: Conceptualization

Table 1. Analytical characteristics of CCT-CPE for the UV-Vis spectrophotometric determination of β-carotene under the optimized conditions.

Analytical parameter	Analytical feature

Linear range (µg mL^{-1})	0.04-10
Correlation coefficient (R^2)	0.998
LOD (µg mL^{-1}, n=20)	0.01
LOQ (µg mL^{-1}, n=20)	0.04
Relative standard deviation (%) for:	
Intra-day analysis (n = 6)	1.50
Inter-day analysis (n = 3)	1.94
Enrichment factor (EF)	9.68
Preconcentration factor (PF)	10.00

Table 2. β-Carotene content and recovery of fruit juice samples using the CCT-CPE method (n = 3) under the optimized conditions.

Sample	Brand		Added, µg mL^{-1}	Found, µg mL^{-1}	Recovery, %	P-Value
S1:	Merikhi	Direct, HPLC	-	0.246±0.002	-	
Orange juice		Method	-	0.268±0.023	108.9	0.06
		Spiked	1.00	1.300±0.040	104.3	
S2:	Sporta	Direct, HPLC	-	0.667±0.010	-	
Orange juice		Method	-	0.678±0.012	101.6	0.28
		Spiked	1.00	1.660±0.051	99.6	
S3:	Paki	Direct, HPLC	-	0.450±0.006	-	
Apple juice		Method	-	0.501±0.046	111.3	0.14
		Spiked	1.00	1.580±0.080	109.0	

Highlights

- Cloud point extraction coupled to a cold column trapping system eliminates the centrifugation step
- The method is faster than ordinary cloud point extraction and uses a more moderate extraction conditions
- The method is appropriate for quantitation of thermally unstable compounds such as β-carotene
- The method is semi-automated and is simply coupled to UV-Vis Spectrophotometry