Method Article

Pigments' analysis of Citrus juicing making by-products by LC-MS/MS and LC-DAD

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A B S T R A C T

Citrus fruits Cold Press Essential Oils (CPEOs) constitute a low-cost by-product produced during the juice making process that are composed by a volatile and a non-volatile fraction. Their non-volatile fractions are rich in valuable secondary metabolites, such as carotenoids, coumarins, psoralens and flavonoids [1,2]. Study herein concerns the development of a quantitative method for their carotenoids analyses using the LC-MS/MS and LC-DAD methodology.

- CPEOs carotenoids content was characterized quantitatively.
- Natural carotenoids β-cryptoxanthin, lutein and zeaxanthin were determined as the most abundant high added value molecules.

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A R T I C L E   I N F O

Method name: Analysis of Citrus by-product by LC-MS/MS and LC-DAD
Keywords: Mass spectrometry, Triple quadrupole, Carotenoids, Cold press essential oil, Citrus fruits juicing, β-cryptoxanthin, Mandarin, (Citrus reticulata), Orange (Citrus sinensis (L.)), Lemon (Citrus limon (L.))
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**Specifications table**

| Subject area | Chemistry |
|--------------|-----------|
| More specific subject area | CPEOs Carotenoid Content Characterization |
| Name of your method | Analysis of Citrus by-product by LC-MS/MS and LC-DAD |
| Name and reference of original method | Saponification procedure: D.J. Hart, K.J. Scott, Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK, Food Chemistry. 54 (1995) 101–111. https://doi.org/10.1016/0308-8146(95)92669-B |
| LC–MS/MS Analysis: S. Rivera, F. Vilaró, R. Canela, Determination of carotenoids by liquid chromatography/mass spectrometry: effect of several dopants, Analytical and Bioanalytical Chemistry. 400 (2011) 1339–1346. https://doi.org/10.1007/s00216-011-4825-6. |
| Resource availability | N.A. |

**Method details**

Citrus Cold-Pressed Essential Oils (CPEOs) are low economic value by-products of the juice making industry, consisting of a volatile and a non-volatile fraction [3]. In a recent study, we published the development of a novel methodology for the separation of CPEOs’ non-volatile fractions under reduced pressure and temperatures below 30°C as azeotrope mixture with isopropanol [4]. The evaluation of the azeotropic procedure efficiency was carried out through the determination of their carotenoids recoveries using the presented herein method, specially developed for the determination of carotenoids content into the non-volatile fractions of orange, tangerine and lemon CPEOs using LC-MS/MS and HPLC-DAD instruments.

**Reagents - standards**

All solvents used for the extractions and separations were of analytical grade. Specifically, ethanol (EtOH) was purchased from Acros Organics, methanol (MeOH), hexane and petroleum ether were provided by Carlo Erba, while isopropanol (IPA) and methyl-tert-butyl-ether (MTBE) were obtained from Fisher Chemicals. The HPLC grade solvents acetonitrile (ACN), chloroform and dichloromethane (DCM) were also obtained from Fisher Chemicals. Water, acetonitrile and methanol used for the LC-MS/MS determinations were obtained from J.T. Baker as LC-MS grade solvents. Finally, dimethyl sulfoxide (DMSO), potassium hydroxide (KOH), ethyl acetate (EtOAc) and diethyl ether (Et2O) were purchased from Sigma-Aldrich. Silica gel 60 (230–400 mesh) was obtained from Merck.

Zeaxanthin (≥98%), lutein (≥95%), and β-cryptoxanthin (≥97%) standards were purchased from Extra Synthese; (E/Z) phytoene (≥95%) and α-carotene (≥95%) standards were obtained from CaroteNature. Astaxanthin (≥95%), lycopene (≥95%), α-carotene (≥95%) and fucoxanthin (≥95%) standards were provided by Sigma-Aldrich. These carotenoid standards were selected as the most significant carotenoids in different matrices, such as plant material, animal feed, aquafeed and various tissues.

**Preparation of standard stock solutions**

For each investigated carotenoid a standard stock solution was prepared by diluting the analyte into dimethyl sulfoxide, chloroform or in a methanol-chloroform mixture. In particular, fucoxanthin was diluted in dimethyl sulfoxide at 1,000 μg/mL concentration, zeaxanthin and lutein in chloroform-methanol mixture at 27.5 and 38 μg/mL respectively and astaxanthin, β-cryptoxanthin, α-carotene, β-carotene and phytoene in chloroform at 96, 970, 490, 90 and 1,225 μg/mL respectively. The problem of lycopene’s low solubility that constitutes a serious drawback for its stock solution preparation, was circumvented by diluting lycopene in chloroform and subsequent filtration through a PTFE syringe filter (pore size: 0.45 μm). The solution absorbance was measured at 484.5 nm (ε=164 × 103 L/mol × cm in chloroform) [5] leading to the determination of lycopene concentration as 6.33 μg/mL through the application of the Beer–Lambert law. To avoid carotenoids decomposition, all stock
solutions were stored in freezer at −20°C, in the absence of light and protective coating of argon gas. The stock solutions of all analytes were diluted in methanol to achieve final concentrations ranging from 0.01 μg/mL to 1.00 μg/mL, except for concentrations ranging from 0.026 μg/mL to 0.390 μg/mL.

**Saponification procedure**

Prior to chromatographic analysis, the non-volatile fractions of CPEOs containing carotenoids were saponified using a modified procedure of method developed by Hart & Scott (1995) [6]. Briefly, 200 mg of non-volatile residues were diluted in 10 mL of a petroleum ether-diethyl ether mixture (1:1) and an equal volume of 10% potassium hydroxide solution in methanol was added. The mixture was stirred overnight in darkness at room temperature and then poured into a separation funnel containing 10 mL of water. The aqueous-alcoholic phase was separated and backwashed twice with 2 × 10 mL of a petroleum ether-diethyl ether (1:1) mixture. The organic phases were combined, washed with brine (3 × 20 mL) and evaporated to dryness under reduced pressure to afford the saponified carotenoids extract [7] as a light red slurry for the lemon CPEO to reddish-brown slurries for orange and tangerine CPEOs.

**LC–MS/MS analysis**

The detection and quantitation of carotenoids contained into the non-volatile fractions was performed using an Accela Ultra High-Performance Liquid Chromatography system, coupled with a TSQ Quantum Access triple quadrupole mass spectrometer that operates in selected reaction monitoring mode and is equipped with an autosampler (Thermo Fischer Scientific, Waltham, MA, USA) and a Nitrogen Generator (Peak Scientific). The LC operation parameters were those of literature [8] with some modifications.

The separation of carotenoids was performed on a 120 EC-C18 reverse phase column with 2.1 × 100 mm internal diameter and 1.9 μm particle size (Infinity Lab Poroshell-Agilent). For column protection a 120 EC-C18 guard column was used with internal diameter 2.1 × 5 mm and particle size 2.7 μm obtained from the same provider. Mobile phase was consisted of acetonitrile (A), water (B) and MeOH (C), which were degassed at 25°C for 10 min. The injection volume for each sample was 10 μL and the tray and column temperatures were set at 10 and 42°C, respectively. The applied gradient elution conditions for mobile phases (A), (B) and (C) were as follows: 0.0–0.5 min, 62% A, 10% B, 28% C (flow rate: 0.45 mL/min); 0.5–2.0 min, 62→75% A, 10→0% B, 28→25% C (flow rate: 0.45 mL/min); 2.0–5.0 min, 75% A, 0% B, 25% C (flow rate: 0.45 mL/min); 5.0–7.0 min, 75% A, 0% B, 25% C (flow rate: 0.45→0.60 mL/min); 7.0–8.0 min, 75→100% A, 0% B, 25→0% C (flow rate: 0.60 mL/min); 8.0–12.0 min, 100% A, 0% B, 0% C (flow rate: 0.60 mL/min); 12.0–14.0 min, 100→75% A, 0% B, 0→25% C (flow rate: 0.60 mL/min); 14.0–16.0 min, 75% A, 0% B, 25% C (flow rate: 0.60→0.45 mL/min); 16.0–18.0 min, 75→62% A, 0→10% B, 25→28% C (flow rate: 0.45 mL/min); 18.0–24.0 min, 62% A, 10% B, 28% C (flow rate: 0.45 mL/min) for the re-equilibration of the column between injections.

The ion source used was Atmospheric-Pressure Chemical Ionization (APCI), operated in two complementary polarity modes (positive and negative). The determination of molecular ion transitions for the target analytes was achieved by direct infusion in full scan mode (mass range: 150–1500 m/z) of their standard solutions for 2 μg/mL concentration. A Selected Reaction Monitoring (SRM) mode was used to confirm the presence of analytes, while the ion source and vacuum parameters of the mass spectrometer were optimized to be applicable for all analytes. A nitrogen generator (Peak Scientific) provided the nitrogen that was utilized as stealth and auxiliary gas with the initial pressures set at 30 and 5 Arb, respectively. The capillary and vaporizer temperatures were regulated at 340 and 450°C respectively and the collision pressure of the argon gas was adjusted at 1.5 mTorr. All selected ion transitions are included in Table 1.

The linearity of the analytical method was evaluated using calibration curves in order to determine the respective correlation coefficients, slopes and intercept values. The linear equations, squared correlation coefficients, Limits of Detection (LOD) and Limits of Quantification (LOQ) are presented in Table 2.
Table 1
Monitoring of ion transitions (parent→products), polarities and retention times for each analyte.

| Carotenoid              | Parent & Product Ions | Polarity | Retention Time (min) |
|------------------------|-----------------------|----------|----------------------|
| Fucoxanthin            | 658.797 > 641.205 / 657.694 | Negative | 1.23                 |
| Astaxanthin            | 597.057 > 118.834 / 146.836 | Positive | 1.90                 |
| Zeaxanthin             | 569.055 > 118.865 / 551.119 | Positive | 2.37                 |
| Lutein                 | 568.885 > 118.938 / 139.145 | Positive | 2.37                 |
| β-Cryptoxanthin        | 553.032 > 120.660 / 144.754 | Positive | 6.30                 |
| Lycopene               | 537.083 > 156.830 / 248.643 | Positive | 9.55                 |
| α-Carotene             | 537.207 > 176.649 / 536.453 | Positive | 14.46                |
| β-Carotene             | 536.961 > 118.820 / 176.714 | Positive | 15.23                |
| Phytocene              | 545.041 > 94.978 / 108.981 | Positive | 17.84; 18.43         |

Table 2
Calibration curves, correlation coefficients (R2), detection and quantification limits for the LC analyses of compounds extracted, identified and quantified from CPEOs.

| Carotenoid              | Linear equations | R²       | LOD(ppm) | LOQ(ppm) |
|------------------------|------------------|----------|----------|----------|
| Fucoxanthin            | y = - 17817.2+4404190x | 0.9999  | 0.047    | 0.142    |
| Astaxanthin            | y = 162650+10238800x  | 0.9995  | 0.172    | 0.522    |
| β-Cryptoxanthin        | y = -2616.07+40714.9x | 0.9996  | 0.138    | 0.418    |
| Lycopene               | y = -4003.79+166207x  | 0.9996  | 0.016    | 0.047    |
| α-Carotene             | y = -30039.5+972849x  | 0.9999  | 0.073    | 0.220    |
| β-Carotene             | y = -216151+205275x   | 0.9995  | 0.135    | 0.411    |
| Phytocene              | y = -18076.6+1310840x | 0.9997  | 0.043    | 0.131    |
| Lutein                 | y = -0.935207+24.2383x | 0.9994  | 0.065    | 0.197    |
| Zeaxanthin             | y = -1.06364+60.1119x | 0.9983  | 0.080    | 0.242    |

Table 3
Carotenoids content in CPEOs non-volatiles fractions before (bs) and after saponification (as). Results are expressed as mg of carotenoid/100g CPEO.

| Carotenoid              | Orange      | Tangerine   | Lemon      |
|------------------------|-------------|-------------|------------|
|                        | b.s. | a.s. | b.s. | a.s. | b.s. | a.s. |
| Fucoxanthin            | nf  | nf  | nf  | nf  | nf  | nf  |
| Astaxanthin            | nf  | nf  | nf  | nf  | nf  | nf  |
| β-Cryptoxanthin        | 2.6±0.2 | 2.6±0.2 | 2.6±0.2 | 2.6±0.2 | 2.6±0.2 | 2.6±0.2 |
| Lycopene               | tr  | tr  | tr  | tr  | tr  | tr  |
| α-Carotene             | 0.30±0.003 | 0.30±0.003 | 0.30±0.003 | 0.30±0.003 | 0.30±0.003 | 0.30±0.003 |
| β-Carotene             | tr  | tr  | tr  | tr  | tr  | tr  |
| Phytocene              | nf  | 0.22±0.07 | nf  | 0.60±0.03 | nf  | 0.20±0.03 |
| Lutein                 | tr  | 12±2 | tr  | 21±1 | tr  | 36±0.01 |
| Zeaxanthin             | nf  | 4.5±0.7 | nf  | 4.5±0.7 | nf  | tr  |

nf: not found, tr: traces.

LC/DAD analysis

The developed and applied herein LC-MS/MS method was not capable to allow the complete separation of zeaxanthin and lutein molecules, since the structures of these two carotenoid isomers are almost identical leading to comparable retention times and the formation of identical ions, with starting point of their fragmentation the removal of a H₂O molecule. Their separation was finally succeeded with the application of a specific HPLC/DAD analytical method applied on a Hewlett Packard, series 1100 instrument, equipped with binary pump system, degasser and diode array detector (DAD, Hewlett Packard, series 1050). The separation was carried out on a Nucleosil 100-5 C18 column, of internal diameter 4.6 × 250mm and 5μm particle size with a guard column of the same material. The mobile phase was acetonitrile:MTBE 70:30 under isocratic conditions for 35 min with
flow rate adjusted at 1.0 mL/min. The column temperature was maintained at 25°C and the sample was injected manually with a 20 μL volume loop. Detection was achieved at a wavelength of 450 nm. Under these conditions, it was feasible to fully separate the molecule of lutein from zeaxanthin, based on their retention times which were respectively 19.8 and 23.0 min. The method linearity was evaluated using calibration curves in order to determine the respective correlation coefficients, slopes and intercept values. The lutein and zeaxanthin’s equations, squared correlation coefficients, Limits of Detection (LOD) and Limits of Quantification (LOQ) are presented in Table 2.

The results of the analyses of orange, tangerine, and lemon CPEOs depicted in Table 3.

CRediT author statement

Eleni D. Myrtsi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization Sofia D. Kouloucheri: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Writing – reviewing and editing, Visualization Epameinondas Evergetis: Writing – original draft, Writing – reviewing and editing Serkos A. Haroutounian: Resources, Supervision, Project administration, Writing – reviewing and editing

Supplementary material and/or additional information
Supplementary material associated with this article can be found, in the online version, at E. D. Myrtsi, S. D. Kouloucheri, E. Evergetis, S. A. Haroutounian, Agro-industrial co-products upcycling: Recovery of carotenoids and fine chemicals from Citrus sp. juice industry co-products, Industrial Crops and Products, 186 (2022), 115190, https://doi.org/10.1016/j.indcrop.2022.115190

Additional information Zotero was used to create the citations list. LCQuan 2.7.0.20 software (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to create the standard calibration curves and the statistical functions of Microsoft Office 365 was used to utilize standard deviations for the LOD, LOQ assessments. One-way analysis of variance (ANOVA) was used to test whether there were significant differences between the mean values of different samples.

Declaration of Competing Interests

Please tick the appropriate statement below (please do not delete either statement) and declare any financial interests/personal relationships which may affect your work in the box below.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Please declare any financial interests/personal relationships which may be considered as potential competing interests here.

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References

[1] G. Dugo, L. Mondello, Citrus Oils: Composition, Advanced Analytical Techniques, Contaminants, and Biological Activity, CRC Press, Boca Raton, FL, 2011.
[2] E.D. Myrtsi, A. Angelis, S.D. Kouloucheri, S. Mitakou, S.A. Haroutounian, Retrieval of high added value natural bioactive coumarins from mandarin juice-making industrial byproduct, Molecules 26 (2021) 7527, doi:10.3390/molecules26247527.
[3] V.N. Kapsaki-Kanelli, E. Evergetis, A. Michaelakis, D.P. Papachristos, E.D. Myrtsi, S.D. Kouloucheri, S.A. Haroutounian, Gold® pressed essential oil: an essay on the volatile fragment from citrus juice industry by-products chemistry and bioactivity, Biomed. Res. Int. (2017) (2017) 1–8, doi:10.1155/2017/2761461.
[4] E.D. Myrtsi, S.D. Kouloucheri, E. Evergetis, S.A. Haroutounian, Agro-industrial co-products upcycling: Recovery of carotenoids and fine chemicals from Citrus sp. juice industry co-products, Ind. Crops Prod. 186 (2022) 115190, doi:10.1016/j.indcrop.2022.115190.
[5] M. Takehara, M. Nishimura, T. Kuwa, Y. Inoue, C. Kitamura, T. Kumagai, M. Honda, Characterization and thermal isomerization of (all-E)-lycopene, J. Agric. Food Chem. 62 (2014) 264–269, doi:10.1021/jf404497k.
[6] D.J. Hart, K.J. Scott, Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK, Food Chem. 54 (1995) 101–111, doi:10.1016/0308-8146(95)92669-B.
[7] R.E. Kopec, J.L. Cooperstone, M.J. Cichon, S.J. Schwartz, Analysis methods of carotenoids, in: Z. Xu, L.R. Howard (Eds.), Analysis of Antioxidant-Rich Phytochemicals, Eds., Wiley-Blackwell, Oxford, UK, 2012, pp. 105–148, doi:10.1002/9781118229378.ch4.
[8] S. Rivera, F. Vilaró, R. Canela, Determination of carotenoids by liquid chromatography/mass spectrometry: effect of several dopants, Anal. Bioanal.Chem. 400 (2011) 1339–1346, doi:10.1007/s00216-011-4825-6.