SUPPLEMENTARY MATERIAL

Chemical characterization of old cabbage (*Brassica oleracea* L. var. *acephala*) seed oil by liquid chromatography and different spectroscopic detection systems

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Abstract

We report an extensive chemical characterization of various components, namely fatty acids, triacylglycerols, tocopherols, carotenoids and polyphenols contained in the oil extracted from old cabbage (*Brassica oleracea* L. var. *acephala*) by cold-pressing of the seeds. Analyses were performed by gas chromatography combined with flame ionization detection and mass spectrometry, high performance liquid chromatography with photodiode array, fluorescence and mass spectrometry detection. We observed that 94% of the total fatty acids contained in the oil were unsaturated and were constituted primarily by erucic acid (Er, more than 50%) followed by linoleic (L), linolenic (Ln) and oleic (O) acids accounting for approximately 10% each. The most abundant triacylglycerols (TAGs > 13%) were represented by ErGL, ErErL and ErErO. Among tocopherols, γ -tocopherol accounted for over 70% of the total content. 13 carotenoids and 15 polyphenols were identified and measured. The present work is the first report providing a thorough chemical characterization of the oil extracted from old cabbage seeds by cold pressing.

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Keywords: *Brassica oleracea*, fatty acids, triacylglycerols, carotenoids, tocopherols, polyphenols, HPLC-PDA-ESI-MS.

2.2.1. Analysis of the fatty acid content

GC-MS analyses for identification purposes were carried out on a GCMS-QP2010 system (Shimadzu, Milan, Italy) equipped with a split-splitless injector, an AOC-20i autosampler, and a Shimadzu GCMS-2010 mass spectrometer (Shimadzu, Milan, Italy). MS parameters in all applications were as follows: mass range 40-400 amu, scan speed: 2000 amu/s, ion source temperature: 200°C, interface temperature: 250°C. The *GCMSsolution* software (Ver. 2.71 Shimadzu, Milan, Italy) was used for data collection and handling; identification was further achieved through library using the "FAMES Fatty Acid Methyl Esters: Mass Spectral Database" (Wiley) with the simultaneous use of Linear Retention Indices calculated by the injection of a FAMES solution. GC-FID analyses for quantification purposes were carried out on a GC-2010 system (Shimadzu, Milan, Italy) equipped with a split-splitless injector (280 °C), an AOC-20i autosampler, and a FID detector. A Supelcowax-10 column was employed (30 m × 0.25 mm I.D., 0.25 µm *dp*, Sigma-Aldrich/Supelco, Bellefonte, USA) and operated under the following programmed temperature: 50 °C to 280 °C at 3.0 °C/min. The injection volume was 0.2 µL with a split ratio of 10:1. Helium was used as the carrier at a constant linear velocity of 30 cm/s.

Individual fatty acid methyl esters are reported as percentage of total FAMES. Area correction was performed to correct the FID response by means of theoretical relative response factors (TRF) (Ackman, 2007). Reliability of TRF was previously checked by means of standard mixtures analysis.

2.2.2. Analysis of the triacylglycerol content

NARP-HPLC-APCI-MS analyses were performed on a Shimadzu Prominence LC-20A system (Shimadzu, Milan, Italy), consisting of a CBM-20A controller, two LC-20AD dual-plunger parallel-flow pumps, a DGU-20A5 degasser, a SIL-20A autosampler and a Shimadzu LCMS-2010 mass spectrometer (Shimadzu, Milan, Italy) equipped with an APCI source operated in the positive ionization mode. The Shimadzu LCMSsolution software (Ver. 3.60.361) was used for data collection and handling. Chromatographic separation was achieved on an Ascentis Express C18 column (150 × 4.6 mm I.D., 2.7 µm *dp*, Sigma-Aldrich/Supelco, Bellefonte, USA); 5 µL of the sample were injected. A linear gradient of increasing IPA (B) percentages in ACN (A) was run, at a mobile phase flow rate of 1 mL/min: 0 min, 0% B; 50 min, 70% B (hold for 4 min); 54 min, 0% B.

70 MS parameters for full scan analysis were as follows: 250-1100 m/z mass range, scan speed: 4000
71 amu/s, nebulizing gas (N_2) flow rate: 2.0 L/min; event time: 0.25 s; detector voltage: 1.5 kV;
72 interface temperature: 450 °C; CDL temperature: 250 °C; heat block temperature: 200 °C.

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74 2.2.3. Analysis of the tocopherol content

75 Tocopherol quantification was carried out by using five different concentrations of each component,
76 in the range between 5 and 0.005 mg/L, prepared by diluting a stock solution of about 100 mg/L,
77 using Hex as a solvent (α -tocopherol, $y=337097x-15482$; $R^2=0.9988$; γ -tocopherol, $y=481282x-$
78 4784.3 ; $R^2 = 0.9997$; δ -tocopherol, $y=264858x+62755$, $R^2=0.9983$). The analyses were carried out
79 by using a Shimadzu HPLC system (Kyoto, Japan) equipped with a LC-10AD Vp high pressure
80 isocratic pump, an SCL-10A Vp controller, and an RF-10 AXL fluorescence detector (programmed
81 for excitation at 290 nm and emission at 330 nm). Data acquisition was performed using the
82 LCsolution software (Ver. 1.12). The analyses were performed in triplicate, at room temperature
83 (25 °C) using two serially coupled Ascentis silica columns (100 $\times$ 1.0 mm I.D., 3.0 μm dp , Sigma-
84 Aldrich/Supelco, Bellefonte, USA). The mobile phase consisted of a of Hex and IPA (99:1 v/v) with
85 a flow-rate of 50 $\mu L/min$, and the injection volume was 2 μL .

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87 2.2.4. Analysis of the carotenoid content

88 Carotenoids were extracted from the seed oil sample by liquid-phase distribution (LPD) between
89 DMF and Hex, according to the methodology reported by Minguez-Mosquera et al. (1992), with
90 some modifications. Briefly, 25 g of oil were dissolved in 150 mL of DMF and treated with 5
91 successive 50 mL portions of Hex in a decanting funnel. The polar components and the
92 xanthophylls were retained in the DMF phase. The hexane phase contained lipids and carotenes.
93 The DMF phase was treated with a 2% Na_2SO_4 solution at 0 °C and extracted two times with a 100
94 mL mixture of Hex/ethyl ether (1:1 v/v). The aqueous phase was discarded, eliminating the more
95 polar components. The organic phase was evaporated to dryness using a rotary evaporator at 30°C.
96 The dry residue was dissolved in 2 mL of MeOH/MTBE (1:1, v/v) and analyzed by RP-HPLC-
97 PDA-MS. The hexane phases were combined, concentrated, filtered through a 0.45 μm syringe
98 filter, and reconstituted in 1.0 mL of Hex for spectrophotometric measurement. The obtained
99 solution only contained *trans* β -carotene pigment together with a minor content of a *cis* isomer, as it
100 was also previously determined by HPLC analysis of the hexane phase.

101 *Spectrophotometric analysis*

102 The total carotenes were analysed by a Shimadzu UV-2600 UV-Vis spectrophotometer (Shimadzu,
103 Milan, Italy). The absorbance (A_λ) of hexane phase containing carotenes was measured at the
104 absorbance maximum, at 450 nm. Quantification was carried out using the following equation:
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$$106 \quad c = \frac{A_\lambda}{\varepsilon \cdot l} \cdot \frac{V \cdot D_F}{m}$$

107 where c is the concentration, ε is the mass absorption coefficient ($\text{g} \cdot \text{cm}^{-2}$), l is the light path-length
108 (1 cm), V is the sample volume (mL), D_F is the dilution factor, m is the sample weight (g).

109 *RP-HPLC-PDA-MS analysis*

110 The HPLC analysis of carotenoids were performed on a Shimadzu Prominence LC-20A (Shimadzu,
111 Milan, Italy), consisting of a CBM-20A controller, two LC-20AD dual-plunger parallel-flow pumps,
112 a DGU-20 A₅ degasser and an SPD-M20A photodiode array detector (2.5 μ L detector flow cell
113 volume). The data were processed with the software LCMSsolution (Ver. 1.12). The LC system was
114 coupled to an LCMS-2010 mass spectrometer through an APCI source (Shimadzu, Kyoto, Japan)
115 operated in positive and negative ionization mode. Separations were performed on a YMC C30
116 column (250 $\times$ 4.6 mm I.D., 3.0 μ m d_p , YMC Europe GmbH, Dinslaken, Germany); the mobile
117 phase was a gradient of MeOH/MTBE/H₂O (90:7:3, v/v/v; eluent A) and MeOH/MTBE/H₂O, (8:90:2,
118 v/v/v; eluent B), as follows: 0 min 0% B; 60 min 60% B, 70 min 100% B. The flow rate was 1
119 mL/min and the injection volume was 20 μ L. The UV–Vis spectra were acquired in the range of
120 250–700 nm, while the chromatograms for quantification were extracted at 450 nm (sampling
121 frequency: 1.5625 Hz; time constant: 0.64 s). The MS was set as follows: Scan, both APCI positive
122 (+) and negative (-), detector Voltage: 1.60 kV in negative mode, 1.35 kV; interface temperature:
123 450 °C; CDL temperature: 300 °C; Block heater temperature: 300 °C; Nebulizing gas flow (N₂): 2.5
124 L/min; full scan range: 350-1200 m/z; event time: 600 ms. Samples were analysed in triplicate.

125 Carotenoid quantification was carried out from the calibration curve attained using lutein reference
126 material at six concentration levels in the range between 1 and 200 mg/L ($y=2233x-42.22$;
127 $R^2=0.9962$).

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129 2.2.5. Analysis of the polyphenolic content

130 Roughly 3.01 g of the seed oil were weighed in a centrifuge tube, treated with 6 mL of a
131 MeOH/H₂O (8:2, v/v) mixture, stirred and centrifugated for 10 minutes at 3000 rpm. After the first
132 separation in two phases, the aqueous-phase (the lower one), containing polyphenols, was collected
133 in a flask, while the remaining oil was subsequently re-treated with further four portions of 6 mL of
134 MeOH/H₂O (8:2, v/v) mixture. The five obtained aqueous portions, containing polyphenols, were

135 combined and evaporated to dryness at 30 °C. The extract was reconstituted in 1 mL of ACN and
136 re-extracted 3 times with 1 mL of Hex, and then centrifugated for 10 minutes at 3000 rpm. The
137 lower phase (ACN) was analyzed by HPLC analysis. The obtained extract (3.2 mg) was solubilised
138 in 100 µL of an ACN/H₂O (1:1, v/v) for the three analyses.

139 The analyses were carried out on a Shimadzu Prominence LC-20A (Shimadzu, Milan, Italy),
140 equipped of a binary LC-20AB pump, a CBM-20A controller, a SPD-M20A diode array detector, a
141 DGU-20A5 degasser and a manual injector with a loop of 2 µL. For data collection and handling an
142 LCMSsolution (Ver. 5.53) was used. The LC system was coupled to a Shimadzu LCMS-2020
143 Shimadzu, Kyoto, Japan), mass spectrometer through an ESI source operated in the negative
144 ionization mode. The Shimadzu LabSsolution software (version 5.53 SP2, Milan, Italy) was used
145 for data collection and handling.

146 Chromatographic separation was achieved on an Ascentis Express C18 column (150 × 4.6 mm I.D.,
147 2.7 µm *dp*, Sigma-Aldrich/Supelco, Bellefonte, USA) using as mobile phase a gradient of H₂O
148 (0.1% of HCOOH) and ACN as follows: 0 min, 0% B; 5 min, 0% B; 30 min, 20% B; 60 min, 100%
149 B; 65 min, 100% B; 70 min, 0% B. The flow rate of 1 mL/min (splitted to 0.2 mL/min prior to ESI-
150 MS analysis), and the injection volume was 2 µL. Data were acquired using a PDA in the range of
151 wavelength from 190 to 400 nm while the chromatograms were extracted at 280 nm (sampling
152 frequency: 1.5625 Hz; time constant: 0.64 s). MS parameters for full scan analysis using ESI in
153 negative mode were: mass range from 100 to 800 *m/z*; nebulizing gas (N₂) flow rate: 1.5 L/min;
154 event time: 1 s; desolvation line (DL) temperature: 250°C; heat block temperature: 300°C.

169 **Figure S1.** Picture of the old cabbage (*Brassica oleracea* L. var. *acephala*)

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173 **Table S1.** FAs identified by GC-MS analysis along with their peak area ratio percentage
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CN:DB ^a	Area%±SD
C16:0	3.11±0.024
C16:1n9	0.03±0.003
C16:1n7	0.10±0.006
C16:1n5	0.01±0.001
C16:2n4	0.01±0.001
C17:0	0.02±0.001
C16:3n3	0.06±0.001
C18:0	0.89±0.003
C18:1n9	10.46±0.047
C18:1n7	0.83±0.025
C18:2n6	11.36±0.072
C18:3n3	10.16±0.119
C20:0	0.62±0.003
C20:1n11	6.08±0.015
C20:1n9	1.57±0.012
C20:2n6	0.57±0.003
C22:0	0.61±0.004
C22:1n9	50.57±0.241
C22:2n6	0.81±0.004
C21:3n3	0.26±0.030
C24:0	0.36±0.015
C24:1n9	1.50±0.009

^a CN, carbon number; DB, double bond.

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191 **Table S2:** Identified TAGs by NARP-HPLC/APCI-MS analysis

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#	NDB	t _R	TAGs	PN	Area % (n=3)	S.D.	CV%
1	6	32.06	LLL	42	0.16	0.03	18.01
2	5	32.79	PLLn	42	0.33	0.04	13.32
3	5	34.83	OLL	44	0.26	0.03	12.53
4	7	34.97	ErLnLn	44	0.57	0.09	15.06
5	5	35.13	OOLn	44	0.37	0.03	8.76
6	4	35.32	PLL	44	0.45	0.04	8.79
7	3	36.18	PPLn	44	0.51	0.08	16.45
8	6	37.34	ErLLn	46	1.35	0.14	10.50
9	4	37.54	OOL	46	1.47	0.11	7.64
10	4	38.09	GPLn	46	1.32	0.03	2.05
11	4	38.09	SOLn	46	*	*	*
12	2	38.68	PPL	46	0.92	0.10	10.80
13	5	39.59	LL	48	1.12	0.08	6.86
14	5	39.92	ErOLn	48	3.96	0.14	3.48
15	3	40.33	OOO	48	0.55	0.08	13.85
16	4	40.47	ErPLn	48	5.24	0.34	6.58
17	2	40.78	OPO	48	0.54	0.05	9.57
18	5	41.72	ErEsL	50	1.17	0.08	7.01
19	5	42.14	ErGLn	50	7.53	0.23	3.11
20	4	42.14	ErOL	50	*	*	*
21	4	42.14	GGL	50	*	*	*
22	3	42.36	GOO	50	0.99	0.14	13.70
23	3	42.73	ErPL	50	6.99	0.16	2.31
24	2	43.00	GPO	50	0.88	0.05	5.63
25	3	43.85	ErSL	52	0.72	0.08	10.96
26	4	44.24	ErGL	52	14.21	0.69	4.86
27	5	44.24	NrGLn	52	*	*	*
28	3	44.60	ErOO	52	3.97	0.17	4.40
29	3	44.60	GGO	52	*	*	*
30	3	45.06	ErSL	52	1.30	0.16	12.40
31	2	45.18	ErPO	52	5.24	0.02	0.43
32	2	45.36	GSO	52	0.24	0.03	10.75
33	4	46.33	ErErL	54	13.20	0.31	2.35
34	3	46.63	ErGO	54	5.33	0.08	1.53
35	2	47.16	ErAL	54	0.64	0.02	3.68
36	2	47.33	ErSO	54	1.46	0.09	5.99
37	2	47.62	BOO	54	0.11	0.01	9.38
38	4	48.37	NrErL	56	0.75	0.07	9.06
39	3	48.59	ErErO	56	13.06	0.10	0.78
40	3	49.16	BErL	56	0.75	0.01	1.98
41	2	49.35	ErAO	56	0.59	0.03	5.76
42	3	50.49	NrErO	58	0.88	0.07	8.50
43	3	51.13	LiErL	58	0.34	0.01	2.16
44	2	51.29	NrAO	58	0.53	0.06	11.74
45	2	51.29	ErBO	58	*	*	*

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197   **Abbreviations:**

198   **P:** Palmitic acid (C16:0); **S:** Stearic acid (C18:0); **O:** Oleic acid (C18:1); **L:** Linoleic acid (C18:2);

199   **Ln:** Linolenic acid (C18:3); **A:** Arachidic acid (C20:0); **G:** Gadoleic acid (C20:1); **B:** Behenic acid

200   (C22:0); **Es:** Eicosadienoic acid (C20:2); **Er:** Erucic acid (C22:1); **Li:** Lignoceric acid (C24:0); **Nr:**

201   Nervonic acid (C24:1).

202   * : TAGs coeluting with the previous one

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