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Changes in Lipid Composition and Antioxidant Capacity of Bitter Orange (*Citrus aurantium.* L) and Mandarin (*Citrus reticulata.* Blanco) Oilseeds on Different Stages of Maturity

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Abstract Oilseeds from bitter orange and mandarin and its antioxidant activities were investigated in this study. The effects of harvesting times (D1: green color, D2 yellow color, D3: orange color) on the extraction yield of oilseed were studied. The maximum yield of 44% was achieved at D3 (bitter orange). The chemical composition of the oilseed was analyzed by gas chromatography (GC). The main methyl esters were linoleic acid (C18:2, 32–42%), palmitic acid (C16:0, 22–26%)), stearic acid (C18:0, 9.01%) and linolenic acid (C18:3, 4–10%). The antioxidant activity of oilseed was assessed by means of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical-scavenging assay and β-carotene bleaching test. Both methods demonstrated notable antioxidant activities of bitter orange and mandarin oilseeds, which is nearly comparable to the references ascorbic acid and butylated hydroxytoluene (BHT). The antioxidant activities of the oilseeds were also found to be harvesting time-dependent.

Keywords Autoxidation · Lipid chemistry · Lipid analysis · Fats and oils · Chromatography · Oilseeds · Extraction · Processing technology

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Introduction

The *Citrus* genus is very diverse and consists of numerous species such as *Citrus sinensis* (orange), *Citrus aurantium* (bitter orange), *Citrus reticulata* (mandarin), *Citrus limon* (lemon) and *Citrus paradisi* (grapefruit). Thus, *Citrus* fruits are of great economic importance because of their varied uses. In addition, *Citrus* fruits are one of the major fruit crops and about 80% of the harvest is used by the juice industry, which leads to the generation of large quantities of waste (55% of the weight products) and a serious environmental problem for disposal.

Therefore, a new commercial industry utilizing the waste by-products could emerge within the juice industry. The essential oils of *citrus* fruits relative to the skin and leaves have a very characteristic and pleasant odor. However, the importance of *Citrus* fruit is not only due to its essential oils but also because of the importance of its seeds that contain different components that can be used for value-added production of a number of products. According to El-Adawy et al. [1], *Citrus* seeds are rich in oil and protein and also serve as a good source of K, Ca, Na, Fe and Mg. They are also rich in limonoids and their glucoside derivative [2].

The fatty acids (FA) of *Citrus* oilseed have been examined for promoting health and preventing disease due to its high polyunsaturated fatty acid (PUFA) contents. PUFA are considered desirable compounds in the human diet because of their effect in reducing the incidence of cardiovascular disease [3] and cancer [4]. Similarly, the potential beneficial aspect of lipids should also be investigated. Consequently, *Citrus* seeds are of great importance because of interest in newer source of edible oils [5]. However no work has been reported on fatty acid composition and antioxidant activity of *Citrus* oilseed from

different stages of maturity. Thus, in this investigation, the lipid composition of seeds of two species of Tunisian *Citrus* fruits with reference to their oil contents, total fatty acids, lipid classes and their antioxidant activity at different stages of maturity were determined.

Materials and Methods

Materials

Fruits of *Citrus* of two species (*Citrus reticulata* and *Citrus aurantium*) were evaluated in this study. Samples were collected at three harvesting period (D1: green color, D2 yellow color, D3: orange color), in 2008, from Menzel Bouzelfa in the North East of Tunisia (latitude 36°42′13.17″; longitude 10°29′46.93″). These *Citrus* fruits were used for juice extraction and the seeds of every species of *Citrus* were separated from the waste product. The seeds were washed and dried at ambient temperature in the dark until used.

Total Lipid Extraction

One gram of seeds was ground in a china mortar into a mixture of 15 ml chloroform–methanol (2:1, v/v). After centrifugation at 2,000 rpm for 15 min, two phases were obtained and the chloroform layer (lower phase) containing the lipids was dried under a stream of nitrogen and the residue was then dissolved in 0.5 ml of toluene-ethanol (4:1, v/v) for further analysis.

Oilseed Extraction

Three samples of *Citrus* seeds were finely ground with a blade-carbide grinding (IKA-WERK, Type: A: 10). This ground material (20 g) was extracted in a Soxhlet-extractor with ethyl ether (100 ml) for 8 h. The extract was then filtered and after evaporation of the solvent under reduced pressure and temperature, the oil content was determined.

Separation of Lipid Classes by Thin Layer Chromatography

Lipids classes were separated by thin layer chromatography (TLC) using plates of 20 cm × 20 cm × 0.25 mm covered with silica gel (G60, Merk, described by Mangold [6]) using a developing system composed of petroleum ether-diethyl ether-acetic acid (70:30:0.4, v/v/v). Lipid spots were detected after a brief exposure of plates to iodine vapors saturating in tightly closed vat [7].

Fatty Acid Analysis

Fatty acids were converted to their corresponding methyl esters according to the method described by Cecchi et al. [8]. An aliquot (200 µl) of the solution was evaporated, and then 2 ml of hexane, a known quantity of methyl heptadecanoate as an internal standard and 0.5 ml of sodium methoxide (1%) were added. After stirring for 1 min, methyl esters were then washed with 1.5 ml distilled water. The solvent from the upper phase was removed under vacuum.

Fatty acid analysis was achieved by gas chromatography using an HP6890 gas chromatograph (Agilent Palo Alto, CA) equipped with a flame ionization detector and a capillary column (HP Innowax; 30 m × 0.25 mm × 0.25 µm id) with a stationary phase made of polyethylene glycol. Analyses were performed by using an oven temperature of 150 °C for 1 min, followed by an increase from 150 to 200 °C at a rate of 15 °C/min, and then from 200 to 225 °C at a rate of 2 °C/min and finally held there for an additional 2 min period. Nitrogen was used as the carrier gas at a flow rate of 1.6 mL/min; injection temperature, 250 °C; and detector temperature, 275 °C [7].

Determination of Antioxidant Activity

The oil obtained was subjected to screening for its possible antioxidant activity. The oil was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay and the β-carotene bleaching test. All the data were the averages of triplicate determinations of three tests.

The DPPH free radical-scavenging activity of oil was measured using the method described by Gorinstein et al. [8]. A 0.1-mM solution of DPPH in methanol was prepared. An aliquot of 0.2 mL of sample was added to 2.8 mL of this solution and kept in the dark for 30 min. The absorbance was immediately measured at 517 nm. The ability to scavenge the DPPH radical was calculated with the following equation:

$$\text{Inhibition percentage} = (I\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control, A_1 is the absorbance in the presence of sample.

The β-carotene bleaching assay was also carried out to determine the antioxidant activity of the samples [9, 10]. Briefly, 2 ml of β-carotene solution (0.2 mg/ml chloroform), prepared from the β-carotene powder (95%), was pipetted into a round-bottom flask containing 20 µg of linoleic acid and 200 µl of Tween 40. Chloroform was removed under a stream of nitrogen gas. Oxygenated water (100 ml) was added to the emulsion and vigorously mixed, with a vortex-type mixer for 5 min, to form a stable

emulsion. An aliquot (5 ml) of the β-carotene/linoleic acid emulsion was transferred to different test tubes, each containing 200 µl of the extract and then mixed again using the vortex type mixer for 1 min. The absorbance was immediately measured at 470 nm, the tubes were placed in a water bath at 50 °C and the absorbance was measured at the end of 2 h. Distilled water, instead of the sample extract, was used as the control sample. The antioxidant activity (%) of the juice was evaluated in terms of the bleaching of the β-carotene using the following formula:

$$\text{Inhibition percentage} = (\text{I}\ \%) = [(A_t - C_t)/(A_0 - C_t)] \times 100$$

where A_t and C_t are the absorbance measured for the test sample and control, respectively, after incubation for 50 min, and C_0 is the absorbance values for the control measured at zero time during the incubation.

Statistical Analyses

All data are reported as means ± standard deviation of three samples. Statistical analysis was performed with STATISTICA. Differences were tested for significance by using the ANOVA procedure, using a significance level of $p < 0.05$.

Results and Discussion

According to Traveset et al. [11], *Rutaceae* fruit's color can be adopted as a visual ripening criterion. In fact, bitter orange and mandarin ellipsoidal berries turn from green (D1), to pale yellow (D2) and to orange (D3). In this paper we aimed to study evolution of lipid composition of *Citrus* seeds and their relative antioxidant activity.

Oilseed Yields

The evolution of oil yields from bitter orange and mandarin seeds during three harvesting periods is reported in Table. 1. An increase of oil content was observed from 36% dry matter weight (DW) at D1 to 44% (DW) at D3 in bitter orange seeds. During mandarin seed development, the oil yields increased progressively from 18% to a maximum of 37% DW at D3. However, the highest seed yield was obtained during the third harvest period.

In fact, species and harvest time have significant effects on the oilseed yield. This latter was compared to some oleaginous seeds such as *Brassica napus* (39.7%) [12] and *Helianthus annuus* (45%) [13].

Vietnamese *Citrus* ripe fruit yield varied from 40–46% in mandarin seeds and 50–60 to 60–65% in bitter orange seeds [13]. No reported literature was found concerning the variation of oil yield of seeds during maturity. However, the oil yield evolution of *Citrus* seeds was similar to that of oleaginous fruits and seeds. For example, Chahed et al. [14] indicated that there is a slow increase of oil yields of *Pistacia vera* seeds during the first stages of maturity. Then, a rapid increase was observed to obtain a maximum of 36.79% at ripeness with slight decrease at final stage due to lipase activation in the over ripened seed.

Fatty acid profiles of the seed samples revealed a small variation for most of the fatty acids examined. Linoleic acid was the predominant fatty acid, while oleic acid accounted for second highest percentage (Table 1).

In *Citrus* seeds and at three harvested times, saturated fatty acids (SFA) varied from 33.08 to 26.94% of TFA in bitter orange and 24.33–26.25% in mandarin. Palmitic acid (C16:0) contributed 22 to 26% of TFA. Other representative SFA was stearic acid (C18:0), which was the highest in bitter orange at 6.89% of TFA in green fruit.

Monounsaturated fatty acid (MUFA) proportions were mainly represented by oleic acid in green bitter orange with 23.07% of TFA, but it increased significantly during all the harvesting periods to reach 28.94% of TFA in bitter orange fruit. For mandarin seeds, MUFA percentage varied from 26.78 to 31.4% (Table 1).

The other MUFA, palmitoleic (C16:1n-7) accounted for a lower percentage during three harvested times and this *Citrus* seed had the highest percentage of polyunsaturated fatty acids (42.83–44.04% in bitter orange; 44.25–46.95% in mandarin). Linoleic acid was the predominant fatty acid in bitter orange (34.99%) and mandarin (42.37%). These seeds may be useful in production of good quality oils. Oleic acid is important for cooking and salad oils [15] whereas a high level of palmitic acid is required for the production of margarine, shortening, and other fat products [16].

The evolution TFA content from *Citrus* seeds during three harvesting periods is reported in Table 2. In fact, a rapid increase of TFA rates from 58.2 mg/g DMW and 9.8 mg/g DW at D1 to 119.3 mg/g DW and 121.85 mg/g DW at D3 in bitter orange and mandarin, respectively, was observed during the first phase. No reports in the literature were found concerning the variation of TFA content of *Citrus* seeds during ripeness. However, the TFA content evolution of bitter orange and mandarin seeds was different to that of oleaginous fruits and seeds. For example, Chahed et al. [14] indicated that there is a slow increase in total fatty acid contents of *Pistacia vera* seeds during the first stages of maturity. Then, a rapid increase was observed a maximum of 36.79% (i.e. 367.99 mg/g DMW) at ripeness with slight decrease at the final stage due to lipase activation when in the overripe seed.

Table 2 summarizes the variations in fatty acid compositions of *Citrus* seeds with harvested times. These results

Table 1 Changes in oil content (% of dry weight), fatty acids (% of total fatty acids) and intact lipid classes (% of total lipids) of bitter orange and mandarin oilseeds at different stages of maturity

	Bitter orange			Mandarin		
	D1	D2	D3	D1	D2	D3
Oil content	36 ± 2.3^{Ca}	41 ± 3.2^{Ba}	44 ± 1 .5^{Aa}	18 ± 0.85^{Cb}	29 ± 1.01^{Bb}	37 ± 1.15^{Ab}
Palmitic acid (C16:0)	26.19 ± 0.28	24.85 ± 0.99	24.18 ± 0.74	22.60 ± 1.4	24.50 ± 1.5	22.27 ± 1.2
Palmitoleic acid (C16:1)	0.35 ± 0.03	0.17 ± 0.09	0.05 ± 0.01	0.17 ± 0.13	0.70 ± 0.63	0.12 ± 0.05
Stearic acid (C18:0)	6.89 ± 0.13	2.26 ± 0.3	2.76 ± 0.49	1.73 ± 0.3	1.75 ± 1.2	2.41 ± 0.78
Oleic acid (C18:1)	23.70 ± 0.07	29.40 ± 1.5	28.94 ± 0.59	31.23 ± 1.13	25.08 ± 1.5	29.23 ± 1.4
Linoleic acid (C18:2)	32.48 ± 0.35	33.48 ± 0.33	34.99 ± 0.19	38.79 ± 0.9	42.37 ± 2.28	41.51 ± 0.84
Linolenic acid (C18:3)	10.35 ± 0.17	9.80 ± 0.62	9.05 ± 0.33	5.46 ± 0.05	5.58 ± 0.74	4.44 ± 0.04
Saturated fatty acids (SFA)	33.08 ± 0.41	27.61 ± 1.9	26.94 ± 1.53	24.33 ± 1.70	26.25 ± 2.70	24.39 ± 1.98
Monounsaturated fatty acids (MUFA)	24.05 ± 0.1	29.57 ± 1.59	28.99 ± 0.60	31.4 ± 0.60	26.78 ± 2.13	29.35 ± 1.45
Polyunsaturated fatty acids (PUFA)	42.83 ± 0.52	43.28 ± 0.95	44.04 ± 0.52	44.25 ± 1.03	47.95 ± 3.01	46.95 ± 0.88
Polar lipids (PL)	38.84 ± 1.17	19.48 ± 1.28	6.21 ± 1.3	54.33 ± 0.51	6.04 ± 1.37	3.22 ± 0.86
Free fatty acids (FFA)	2.9 ± 1.34	16.48 ± 0.99	11.66 ± 0.85	2.29 ± 1.1	1.91 ± 1.2	2.13 ± 1.5
Diacylglycerols (DAGs)	24.32 ± 0.13	24.1 ± 0.21	8.27 ± 0.53	40.59 ± 0.44	31.11 ± 0.74	13.23 ± 1.12
Triacylglycerols (TAGs)	33.88 ± 0.93	31.82 ± 1.2	73.84 ± 0.9	2.77 ± 1.3	60.92 ± 1.9	81.4 ± 1.29

D1, D2, D3 indicate three harvest period (D1: *green color*, D2: *yellow color*, D3: *orange color*). Values given are the means of three replicates ± standard deviation. "Means followed by a different letter (Ca, Aa, Bc, etc.) are significantly different. The data marked with different capital letters, for stage and small letters for species, share significant at $p < 0.05$ (Duncan test)"

Table 2 Changes in fatty acid compositions (mg/g DW) of bitter orange and mandarin oilseeds on different stages of maturity

	Bitter orange			Mandarin		
	D1	D2	D3	D1	D2	D3
TFA contents	58.26 ± 2.3^{Ca}	65.63 ± 3.2^{Ba}	118.53 ± 1.25^{Aa}	9.9 ± 1.1^{Cb}	54.8 ± 2.15^{Bb}	120.68 ± 2.75^{Aa}
Palmitic acid (C16:0)	15.27 ± 1.17	16.50 ± 1.28	29.25 ± 1.3	2.40 ± 0.51	12.14 ± 1.37	26.57 ± 0.86
Palmitoleic acid (C16:1)	0.20 ± 0.0 3	0.15 ± 0.10	0.07 ± 0.03	0.061 ± 0.04	0.07 ± 0.04	0.31 ± 0.12
Stearic acid (C18:0)	4.0 ± 0.34	1.58 ± 0.25	3.54 ± 0.12	0.18 ± 0.15	1.35 ± 0.68	2.38 ± 0.30
Oleic acid (C18:1)	13.81 ± 0.93	18.66 ± 0.02	33.85 ± 0.43	2.57 ± 0.65	16.03 ± 2.90	40.98 ± 1.29
Linoleic acid (C18:2)	18.9 ± 0.96	21.74 ± 0.61	41.33 ± 0.60	4.19 ± 0.13	22.75 ± 3.97	45.02 ± 1.05
Linolenic acid (C18:3)	6.02 ± 0.26	6.71 ± 1.67	10.46 ± 0.05	0.46 ± 0.18	2.43 ± 0.43	6.39 ± 0.76

D1, D2, D3 indicate three harvest period (D1: *green color*, D2: *yellow color*, D3: *orange color*). Values given are the means of three replicates ± standard deviation. "Means followed by a different letter (Ca, Aa, Bc, etc.) are significantly different. The data marked with different capital letters, for stage and small letters for species, share significant at $p < 0.05$ (Duncan test)"

show that linoleic acid is the major fatty acid of studied seeds representing 18.9–41.33 mg/g DW in bitter orange and 4.19–45.02 mg/g DW in mandarin. It is well known that dietary fats, rich in linoleic acid, prevent cardiovascular disorders such as coronary heart diseases, atherosclerosis and high blood pressure. Also, it was reported that the nutritional value of linoleic acid is because of its metabolism at the tissue levels, which produces the long-chain polyunsaturated fatty acids and prostaglandins [17]. Besides linoleic acid, the main fatty acids detected in bitter orange seeds were palmitic (C16:0 = 15.27–29.25 mg/g DW), and oleic (C18:1 = 13.81–33.85 mg/g DW). For mandarin, the same fatty acids were identified but their contents were different. In fact, palmitic and oleic acid contents of 2.40 and 2.57 mg/g DMW, respectively at D1 were lower than those of bitter orange. Other fatty acids present in small amounts in the seeds of *Citrus* were palmitoleic (C16:1 = 0.07–0.2 mg/g DW in bitter orange and 0.06–0.31 mg/g DMW in mandarin) and linolenic (C18:3 = 6.02–10.46 mg/g DW in bitter orange to 0.46–6.39 mg/g DMW in mandarin) acids. The source of this variability in fatty acid composition may be genetic (i.e. species).

The ratio of saturated fatty acids to unsaturated fatty acids (SFA/PUFA) decreased during fruit maturation to reach 0.36 and 0.31 in fully ripe fruit of bitter orange and mandarin. Similar results were also found in ripe coriander

and niger seeds with a ratio of 0.379 and 0.370, respectively [18]. However, the level of unsaturation/saturation (2.2%) was not different during cherry laurel fruit maturation [19]. Interest in the PUFA, as health-promoting nutrients has expanded in recent years. A growing literature illustrates the benefits of PUFA in alleviating cardiovascular, inflammatory, heart diseases, atherosclerosis, autoimmune disorder, diabetes and other diseases [20].

Table 1 shows the proportions of unhydrolyzed intact lipid classes (LC) in bitter orange oilseed during maturity were mainly TAG (33–74%). In addition, lipid classes varied with the harvesting stage. In fact, at D1 intact lipid classes were mainly PL (38.84%). This proportion decreased progressively until 6.21% at the final stage (D3). Free fatty acids (FFA) accounted for 2.9% at D1 and increased to 11.66% at D3 while, diacyl-glycerols (24.32%) at D1 decreased to 8.27% at D3. Nevertheless, LC profile of *Citrus aurantium* seeds was characterized by exceptionally high levels of FFA (3–16% of total lipid). Widely distributed in food, the PL have both pro- and antioxidant effects [21]. Even though they are used as food emulsifiers worldwide and at the same time have a very positive image; their use in functional foods is still limited. Considering the amount of clinical data, there is no doubt that PL will become a standard ingredient for this rapidly expanding category of food [22]. As shown in Table 1, PL from bitter orange seeds account for 40% (D1) of the total lipids (TL).

At the first harvesting time (D1), mandarin seeds are primarily composed of PL (54.33%) and DAG (40.59%), but also contain TAG (2.77%) and FFA (2.29%). In orange fruits seeds (D3), PL and DAG percentages decreased to reach 3.22 and 13.23%, respectively. In contrast, TAG rate increased to reach 81.40%.

Antioxidant Activity of Oilseed

The antioxidant capacity of oilseed bitter orange and mandarin was assessed with the DPPH radical-scavenging assay and β-carotene bleaching test, in comparison with known antioxidants ascorbic acid and BHT (Fig. 1). The oilseed was assayed over a range of dilutions. Ascorbic acid inhibited 80% of the DPPH radicals. It can be observed that oilseed from bitter orange (84.5% at D2) and mandarin (79.9% at D2) exhibited notable DPPH radical-scavenging activity, with an efficacy similar than that of the reference ascorbic acid (80%). This result showed that bitter orange and mandarin oilseeds scavenged DPPH radicals better than that of oilseed from *Opuntia dillenii* (11.43%) [23].

The β-carotene bleaching test is a convenient test used to measure the ability of a compound or a mixture to inhibit oxidation of β-carotene. BHT inhibited carotene bleaching by 85% compared to the control. The results were consistent with the data obtained from the DPPH test. Oilseed from bitter orange (74.06% at D2) and mandarin (73% at D2) have good activity when compared to BHT (84.5%). The antioxidant activity of the extracts of almond, Brazil nut, hazelnut, pecan, pine nut, pistachio and walnut oils have been studied with the β-carotene bleaching test [24]. The results showed that the pecan oil extracted by chloroform/methanol exhibited the highest inhibition ratio (72.2 ± 1.2%), followed by the walnut oil (70.0 ± 1.4%) and then the Brazil nut oil (62.5 ± 1.1%). The inhibition ratio of oilseed from studied *Citrus* fruits obtained in the present work was higher than all of them. Consumption of foods rich in natural antioxidants has been reported as being protective against certain types of cancer and may also reduce the risk of cardiovascular and cerebrovascular

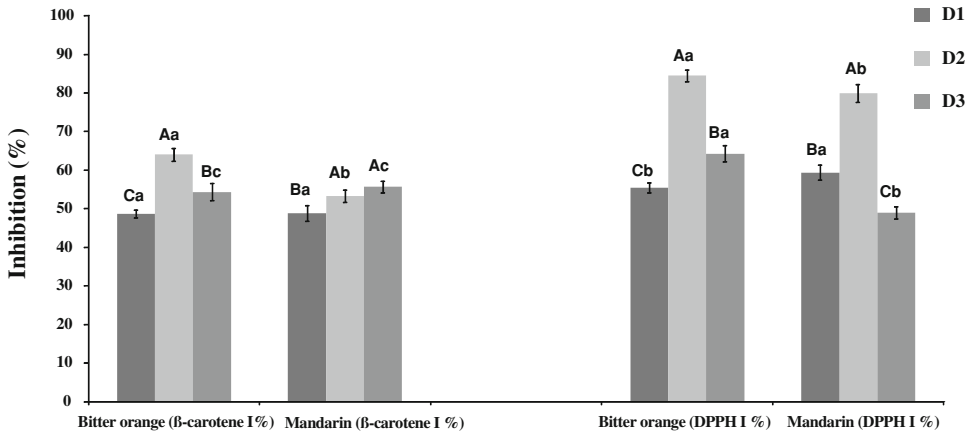

Fig. 1 Antioxidant activity of oilseed from bitter orange and mandarin on different stages of maturity assessed by the β-carotene bleaching and DPPH radical scavenging assay. D1, D2, D3 indicate three harvest period (D1: *green color*, D2: *yellow color*, D3: *orange color*. Values given are the means of three replicates ± standard deviation. "Means followed by a different letter (Ca, Aa, Bc, etc.) are significantly different. The data marked with different capital letters, for stage and small letters for specie, share significant at $p < 0.05$ (Duncan test)"

events [24]. Currently, no information is available on the chemical composition and antioxidant activity of oilseed from bitter orange and mandarin in the literature. In the present paper, to study variation of oilseed from these *Citrus* fruits lasting three harvest times demonstrated marked antioxidant activity in the DPPH radical-scavenging assay and β-carotene bleaching test.

Conclusions

Currently, no information is available on the evolution of chemical composition and antioxidant activity of oilseed from bitter orange and mandarin during maturation. In the present study, the three dates (D1, D2 and D3) were used for the recovery of oilseed from Tunisian *Citrus* fruits on different stages of maturity, and the oilseed demonstrated marked antioxidant activity in the DPPH radical-scavenging assay and β-carotene bleaching test. Based on the obtained results, we can conclude that the oilseeds from yellow bitter orange and mandarin (D2) may play potential roles as health-promoting agents with antioxidant activity in human diets, as well as providing valuable natural antioxidants for the pharmaceutical industry.

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