Valorisation of oils extracted from four Tunisian citrus species seeds

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Considered for a long time as debris, citrus seeds and their components deserve further investigation and valorisation. Relatively few studies have yet examined the oil extracted from citrus seeds. The objective of this work is to identify the constituents of citrus seed oils in order to valorise them, notably for the least exploited species; the bigarade orange. Four citrus species were studied: Lemon, sweet orange (Maltese), Grapefruit and Bigarade (bitter Orange). Different parameters were measured: oil content, fatty acids composition, the percentage and the different compounds of the unsaponifiable fraction. The analysis of total lipid showed a high content of citrus seed oil (44.56%). This value is higher than those obtained in some industrial plants such as cotton and maize seeds. Hence, *Citrus* seeds are considered oleaginous. The fatty acid profile showed that citrus seed oil contains eight fatty acids including three major acids: linoleic (C18: 2cis ω6), oleic (C18: 1) and palmitic (C16: 0) acids. Results also showed that *Citrus* oils are highly unsaturated (71.58%), therefore of very good quality. This study showed the richness of these oils in ω 6 (C18: 2cis ω6) and ω3 (C18: 3 ω3). Bigarade species revealed a high rate of the unsaponifiable fraction and a high number of bands compared to other species.

Keywords: *Citrus* seeds. Total lipids Fatty acids. Unsaponifiable fraction.

INTRODUCTION

In addition to proteins and carbohydrates, lipids are among the three main classes of the food products. Indeed, dietary lipids are essential for health to cover energy requirements and to provide the essential fatty acids and fat-soluble vitamins for the human body [1]. Vegetable oils are considered as a precious material of important utility in the agri-food, pharmaceuticals, cosmetology and natural base insecticides [2]. Around the world, they are commonly extracted from five oleaginous plants: olive, palm, soybean, soya, and sunflower [3]. However, oleaginous crops have encountered problems in recent years as a result of increasing demand due to the population over-increase [4]. Thus, finding new vegetable oil resources, which are not yet or insufficiently exploited, is of worldwide interest.

Citrus are one of the most important fruits in the world [5]. They are cultivated in over 130 countries [6]. Several studies have been conducted on the different parts of citrus proving their richness in essential oils and flavonoids [7], meanwhile, only few have addressed seed constituents. Aware of the promising commercial use of citrus seed oil, several researchers have investigated the composition of this resource in several citrus species [8]. In turn, while the composition of seed oils of orange, lemon and grapefruit have been the subject of some studies [9]. Oils of bitter orange seeds have barely been investigated. Peel and seeds

are the major waste component after processing of citrus [10]. These seeds, which have long been considered as waste and with short life spans [11], could be an interesting alternative. Thus, it is important to develop the research in order to improve the management of the large quantities of fruit seed waste produced by the food industry [12]. Undoubtedly, marketing and valorisation of oil seeds requires the recognition of their composition.
The objective of this work is to evaluate the oil content and study the lipid profile (the fatty acid composition and the unsaponifiable fraction) of seed oils of four citrus species cultivated in Tunisia.

EXPERIMENTAL PART

PLANT MATERIAL

The plant material consists of different citrus seeds: lemon (*Citrus limonum*), sweet orange or Maltese (*Citrus siensis*), grapefruit (*Citrus paradisi*) and bitter orange or bigarade (*Citrus aurantium*).
Fruits of autochthonous cultivars, cultivated in an experimental parcel, were provided by the National Agricultural Research Institute of Tunisia. Citrus seeds were removed in the laboratory and kept at room temperature until use.

EXTRACTION OF TOTAL LIPIDS

About 80 g of citrus seeds were dried at 60°C and milled into fine powder. The lipid contents were extracted by an organic solvent (hexane) for 4h using Soxhlet method. Three replications of 20 g each were used for lipid extraction [13].
The oil content (OC) is expressed by the percentage of seed dry matter according to the following formula

$$OC = (m1 / m2) \times 100$$

Where:
$m1$ = mass of extracted oil (g),
$m2$ = mass of the dry matter (g).
The extracted oils were stored in the refrigerator at a temperature of - 4°C until use [13].

ANALYSIS OF TOTAL FATTY ACIDS

An aliquot of 0.1 g of the extracted oil were converted to methyl esters of fatty acids using 0.2 mL of 2 N methanolic solution of potassium hydroxide [16]. A volume of 2 mL of heptane was added to this solution and then vortexed. After standing, 1 µL of the upper phase was injected in the column of gas chromatography (GC Agilent Technologies, 7890A GC system, Wilmington, USA) to analyse the fatty acids. The column used is Agilent J & W GC columns (HP-INNOWAX) with 30 m long, 0.25 mm diameter. The initial temperature of the oven is 180°C (1 min) and then programmed to reach a final temperature of 240°C at 4°C / min for 15 min, then held at 240°C during the rest of analysis period (2 min). The total duration of the analysis was 18 min. The detector and the injector temperature was 300°C.
One µl of fatty acids methyl-esters is injected into the column via a micro-syringe. The fatty acid profile is provided by data processing software "Agilent Technologies Chem Stati". The identification of the peaks was made by comparing retention times of fatty acid methyl esters with a standard chromatogram of C4 - C24 fatty acid methyl-ester mixture (Supelco, USA).

EXTRACTION OF THE UNSAPONIFIABLE FRACTION

To separate the unsaponifiable fraction from the citrus oil, the total lipids underwent a saponification reaction. The extraction was determined according to the method of [14], using 5 g of oil mixed in 50 mL of an alcoholic solution of potassium hydroxide (12 N). After boiling and cooling, the mixture was decanted four times using 50 mL petroleum ether, concentrated in a rotary flash evaporator at 40°C, then recuperated in chloroform solution and stored.
The unsaponifiable content (USC) is determined by the following formula:

$$USC = (m / M) \times 100$$

Where:
m: mass of unsaponifiable fraction,
M: mass of the oil sample.

IDENTIFICATION OF UNSAPONIFIABLE FRACTION COMPOUNDS BY TLC (THIN LAYER CHROMATOGRAPHY)

The identification of unsaponifiable fraction compounds by TLC was determined according to [15]. The different compounds of the unsaponifiable fraction migrate at different rates due to the attraction differences in the stationary phase (silica gel: Merck Kisel gel 60, 20/20 dimension and 250 µm thickness) and the solubility differences in the mobile phase (mixture of hexane / diethyl ether 6/4).
The unsaponifiable fraction was diluted in 400 µL of chloroform, and then 100 µL of the mixture was placed at two centimetres from the lower edge. The migration time was approximately 45 min. Migration was stopped when the solvent front reached 2 cm from the top edge of the plate.
The revelation was made by sputtering of 2′,7′-Dichlorofluorescein under UV light at 254 nm. The fractions identification was revealed by reference to standard chromatographic solution.

STATISTICAL ANALYSIS

The analysis of the ANOVA variance was performed with the SAS 2002 software, version 9. The averages comparison was performed by the LSD test (the Smallest Significant Difference). The values followed by the same letter are not significantly different, at the 5% probability level.

RESULTS AND DISCUSSION

OIL CONTENT OF CITRUS SEEDS

The results showed that citrus seeds provide high oil content with an average equal to 44.56%. A high significant difference (p <0.0001) was recorded between species. Grapefruit seeds showed the best yield (54.95%) followed by Maltese orange seeds and bitter orange with very similar oil contents, respectively 44.6% and 42.7% (Fig. 1). The lowest oil content was observed in lemon seeds (36.02%). According to [16] orange seeds contain 30 to 40% lipid, and those of grapefruit contain 29 to 37%. Citrus seeds are a potential source for nutrients and oils such as linoleic and oleic acid, tocopherols, vitamin E and sterols [17, 18]

The citrus seeds oil content obtained in our work is higher (36.02 to 54.95%) than those reported in the literature. A study carried out on Tunisian citrus seeds (C. *siensis*, C.*aurantuim* and C. *bergamia*) showed that the oil content varied between 26.1 and 36.1% [19]. Compared to other studies conducted in Iran, Pakistan, Egypt and Nigeria, the percentage of Tunisian citrus seed oil is on average much higher. Iran seeds (C.*limon* and C.*siensis*) contain about 33.4 to 41.9% of oil [14], those of Pakistan (C. *limetta*, C. *paradisi*, C. *sinensis* and C. *reticulata*) about 27.0 to 36.5% [20] whereas Egyptian seeds (C. *siensis*, C. *reticula*, C. *limonia* and C. *aurantium*) contain about 40.2 to 45.5% [21]. Similarly, the oil content of Nigerian citrus seeds (C. *sinensis*, C. *paradisi*, C. *aurantium*, C. *reticulata*, C. *aurantifolia*) and *tangelo* (hybrid of C. *paradisi* and C. *reticulata*) varies between 24.3% and 41.1%. This difference can be explained by the inter-specific variability and the ecological conditions [22]

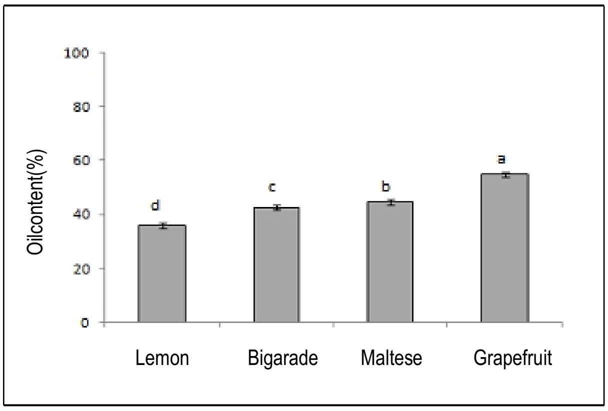

Figure 1 - V ariation of oil content seeds (%) of the different citrus species.

It is important to note that the oil content of citrus seeds studied in this work is much higher (36.02 to 54.95%) than three classic oleaginous seeds: cotton (15.0 to 24.0%), soybean (17.0 - 21.0%) and olive (20 - 25%) [23].

FATTY ACID COMPOSITION

The chromatograms presented in Figure 2 revealed eight major fatty acids. These acids, in order of appearance, are the following: C16:0 (palmitic acid); C16:1 (palmitoleic acid); C18:0 (stearic acid); C18:1 (oleic acid); C18:2 cis ω6 (linoleic acid); C18:3 ω3 (linolenic acid), C20:0 (arachidonic acid); C20:1 (gadoleic acid). The fatty acids composition is like

Table I - Composition of fatty acids (%) and the retention time (min) of the different citrus species.

		Content of fatty acids in %				
	Retention time	Lemon	Maltese	Grapefruit	Bigarade	Fatty Acids
1	7.85	21.40	27.57	28.31	26.99	C16:0
2	8.17	-	0.74	-	0.21	C16:1
3	10.9	-	4.19	-	5.90	C18:0
4	11.4	33.33	25.65	24.62	22.25	C18:1 cis
5	12.5	34.30	38.88	41.36	34.95	C18:2ω6 cis
6	12.65	0.15	-	-	-	C18:2trans
7	13.23	10.37	4.61	5.51	9.28	C18:3 ω3
8	14.58	0.43	0.34	0.19	0.39	C20:0
9	14.98	0.11	-	-	-	C20:1

Table II - Palmitic, oleic, linoleic contents and the total amount expressed in % of the different citrus species.

	Species			
Fatty acids	Lemon	Maltese	Grapefruit	Bigarade
palmitic C16:0	21.40	27.57	28.31	26.99
oleic C18:1 cis	33.33	25.65	24.62	22.25
linoleic C18:2ω6 cis	34.30	38.88	41.36	34.95
Total	89.03	90.1	94.29	84.19

those reported in the literature [4].

The quantitative study of fatty acids (Tab. I) showed the presence of linoleic acid (C18:2 cis ω6), precursor of fatty acids of the omega-6 family, as the major fatty acid in the different studied species of citrus. The highest percentage of this fatty acid was observed in grapefruit (41.36%), followed by Maltese orange (38.88%), bigarade (34.95%) and lemon (34.45%). Linoleic acid also was shown [24] as the most abundant acid, which varied from 40.0% to 46.1%

The fatty acids analysed, revealed that palmitic (C16:0), oleic (C18:1) and linoleic (C18:2 cis ω6) acids represented 94.29% in grapefruit, 90.10% in Maltese orange, 89.03% in lemon and 84.19% in bitter orange (Tab. II). This lipid profile was like that of Maltese orange studied by [25] with 89.86% total percentage. With these fatty acid proportions, oils have good semi-drying properties and could be used as excellent

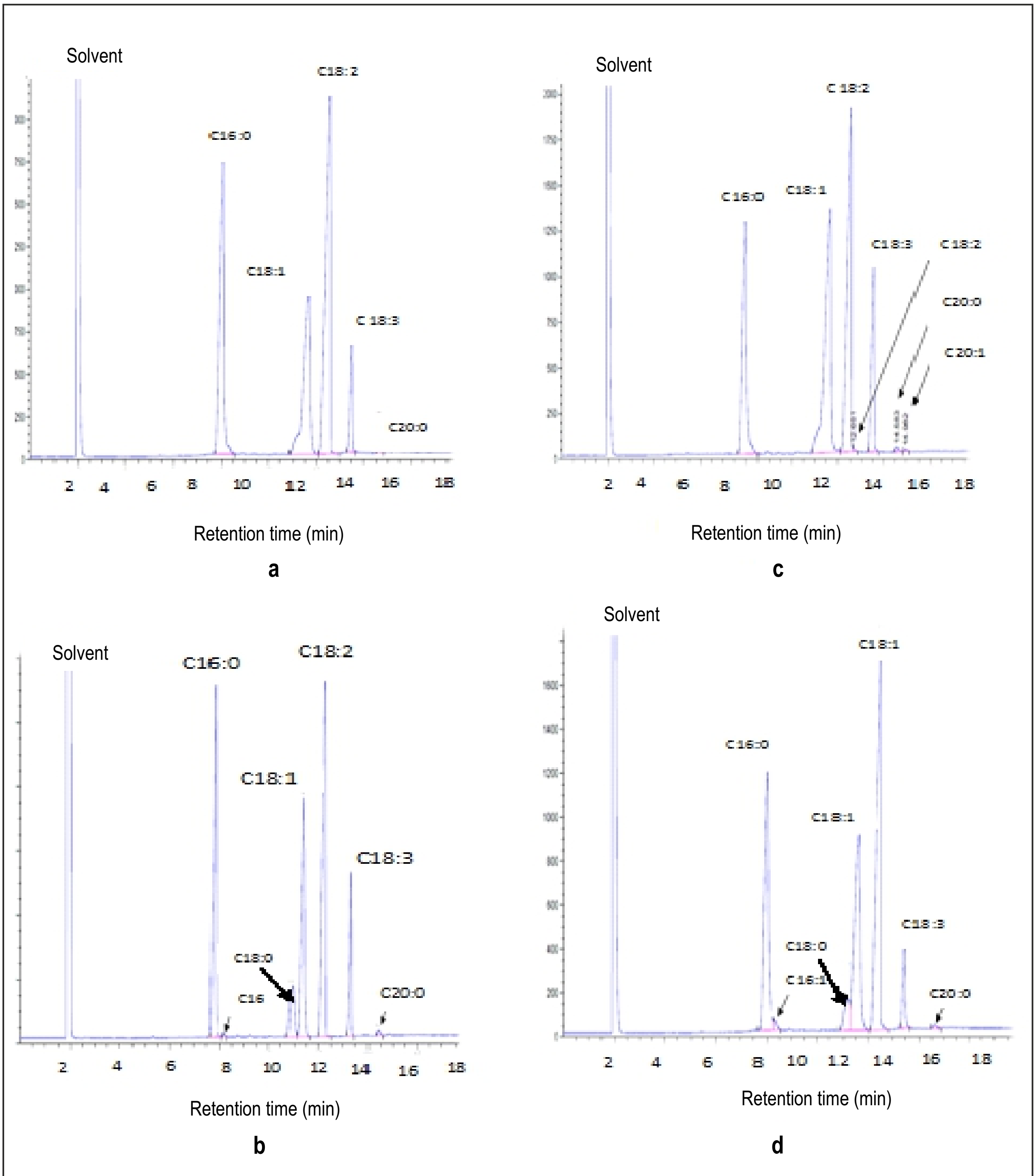

Figure 2 - Fatty acid chromatogram of oils seeds of Bigarade (a), Grapefruit (b), Lemon (c) and Maltese Orange (d) analyzed by gas chromatography (Palmitic acid : C16:0, palmitoleic acid: C16:1, stearic acid: C18:0, oleic acid: C18:1, linoleic acid: C18:2 cis ω6, linoleic acid: C18:2 trans, alpha linolenic acid: C18:3 ω3, arachidonic acid: C20:0, gadoleic acid: C20:1)

edible cooking oil, salad oil or in the margarine manufacture [4].

This work showed, only in lemon, the presence under traces of the trans form of linoleic acid (C18:2 trans). According to [26], consuming fatty acids under this form in excess increased the risk of cardiovascular disease.

Linolenic acid (C18:3 ω3), precursor of fatty acids of the omega-3 family, is present in all citrus seeds. However, its content was higher in lemon (10.37%) and bigarade (9.284%) than in grapefruit (5.51%) and Maltese orange (4.617%). These results are in concordance with those reported by [4], where lemon is also the species which had the highest amount of linolenic acid (9.6%) with a nutritional value that elects it a source of essential fatty acid C18:3 ω3.

SATURATED AND UNSATURATED FATTY ACIDS (SFA, USFA)

Figure 2 showed the presence of five unsaturated fatty acids represented by C16:1, C18:1, C18:2 cis ω6, C18:3 ω3 and C20:1, while the saturated fatty acids are three represented by C16:0, C18:0 and C20:0.

Lipid profile of the different citrus species (Fig. 3) showed the dominance of USFA (71.58%). Hence, Citrus oil is highly unsaturated and therefore of very good quality. Statistical results showed a high significant difference ($p < 0.0001$). The richest species in USFA are respectively Maltese orange (78.73%), lemon (78.23%) and grapefruit (71.49%). However, the species with the lowest percentage of USFA was the bigarade (66.70%). Our results agree with other studies carried out on Tunisian citrus seed oils (56.5 to 75.0%) [17] and Egyptian ones (65 to 69%) [4]. However, these values are higher than those recorded in Pakistan's citrus seed oils (53.7 - 66.0%) [27]. Thus, a high percentage of unsaturated fatty acids is a characteristic of good edible oil [6]. This oil is of very good quality and have important therapeutic virtues. An unsaturated oil can allow a reduction of cardiovascular diseases and certain chronic diseases: Parkinson and Alzheimer [28]. The citrus seed oil can be used in human consumption. However, this oil is slightly bitter due to the presence of limonoid bitter principals and must be refined to improve its taste [16].

MONOUNSATURATED AND POLYUNSATURATED FATTY ACIDS (MUFA, PUFA)

The MUFA and PUFA expressed in percent of the total fatty acids are shown in Figure 4. Monounsaturated showed a high significant difference ($p < 0.0001$) while for the polyunsaturated fatty acids the difference was not significant ($p < 0.1003$). The percentage of the UFA of all citrus oils is significantly higher than that of MUFA contrary to virgin olive oil with 72% of monounsaturated fatty acids (MUFA) and 14% of polyunsaturated fatty acids (PUFA) [29].

The highest percentage of PUFA was recorded in grapefruit and lemon respectively (46.87% and 44.82%). In bigarade species, the PUFA content was twice that of the MUFA (44.23 and 22.46%). The presence of mono and polyunsaturated fatty acids in large quantities in a diet is interesting. In fact, by consuming enough amounts of unsaturated oils, several digestive and hepato-biliary pathologies, and osteoporosis can be prevented or reduced [30].

Our results revealed that in citrus oil, the two main PUFAs are linolenic acid (C18:3 ω3) and linoleic acid (C18:2 cis ω6) and they represent 41.36% of grapefruit seeds oil, 38.88% of Maltese orange oil, 34.95% of bigarade oil and 34.45% for lemon oil. It should be noted that levels of essential fatty acids linoleic (18:2, cis ω6) and linolenic (18:3, ω3) of citrus oils were largely sufficient to prevent an acid fatty deficiency for people who use these oils as a main fat supplement in their diet [31].

THE PERCENTAGE AND THE DIFFERENT COMPOUNDS OF THE UNSAPONIFIABLE FRACTION

The statistical analysis showed a high significant difference ($p < 0.0001$). The unsaponifiable content, expressed in (%) of the total lipid content (Fig. 5), was

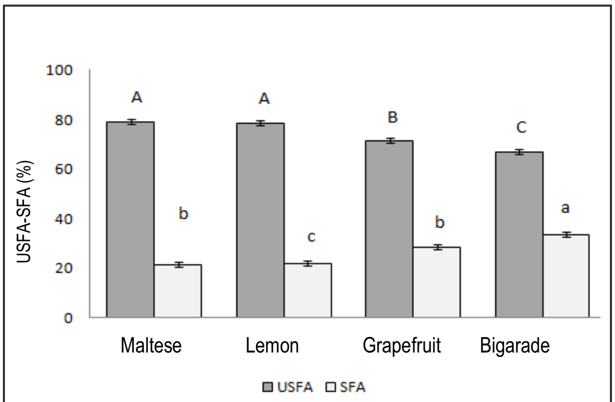

Figure 3 - The percentage of saturated (SFA) and unsaturated fatty acids (USFA) of the seeds oils of the different citrus species.

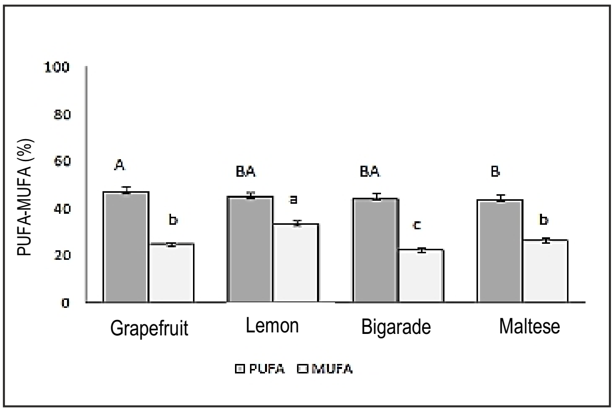

Figure 4 - The percentage of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) of the seeds oils of the different citrus species.

relatively high and analogous in bitter orange (2.6%) and grapefruit (2.33%). These values are like that of olive oil (0.7 to 2.5%) [23]. The seed oils of Maltese orange and lemon showed low levels of unsaponifiable fraction equal to 0.2%, slightly lower than those of citrus seeds studied by [20] (0.5 - 0.7%), [22] (0.9 - 1.3%) and [21] (0.3 - 0.5%), than those of other species; such as cottonseed (0.5 to 1.5%), olive (0.7 to 2.5%) and corn (0.5 to 2.8%) [23].

Qualitatively, the non-glyceridic fraction analysed by thin layer chromatography (TLC) showed six main bands (Fig. 6): sterols (F1), aliphatic alcohols (F2), triterpene (F3) and two unknown fractions (F4 and F5), probably corresponds to fat-soluble vitamins (tocopherols, alpha tocopherols …) and carotenoids, finally the last band corresponds to hydrocarbons (F6). Results of [12] showed that the orange seed oils are rich in total carotenoids (19.01 mg/kg), α-tocopherol (135.65 mg/kg) and phytosterols (1304.2 mg/kg). According to [24] the tocopherol composition of citrus seed oils is α-Tocopherol, followed by γ-tocopherol, while β-tocopherol and δ-tocopherol were not detected, a high value of tocopherol can be effective in applied heat treatment. The qualitative phytochemical screening of Citrus seeds oil showed that alkaloids and steroid are absent in all the Citrus seeds, while tannins, cardiac glycosides, saponins, flavonoids and terpenoid are present [2].

Figure 6 showed a similarity of unsaponifiable profile, between the bigarade and grapefruit with the presence of six bands and between the lemon and the Maltese orange with only five bands (absence of F4).

Bigarade with a relatively high percentage of the unsaponifiable fraction compared to other species and a high number of bands may be considered the best species. In fact, the unsaponifiable fraction has excellent pharmacological and cosmetological properties when used as anti-aging creams and skin treatment [32]. Unsaponifiable compounds are also recommended in the diet [33] and medicine, for their anticancer activities [34] and/or anti-inflammatory [35]. The sterol fraction has beneficial effects to reduce colon cancer, the risk of cardiovascular diseases and hypercholesterolemia [36]. Similarly, the carotenoids of this fraction, precursors, of the trans β-carotene (provitamin A) is converted into antioxidant vitamin A during intestinal absorption [37].

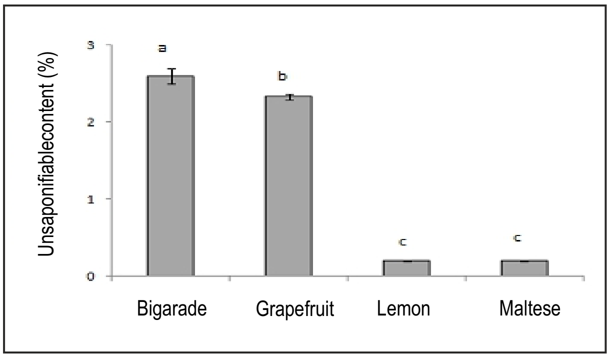

Figure 5 - Unsaponifiable content in (%) of the seeds oils of the different citrus species.

CONCLUSIONS

Analysis of the lipid content of citrus seeds revealed a significant percentage of oil higher than those obtained in industrial plants such as cotton and even in the olive seeds. The oleaginous characteristic of these seeds can play an important role in cosmetics, perfumery, phyto-therapy and agri-food.

Citrus seed oil is rich in oleic (C18:1) and linoleic (C18:2 cis ω6) fatty acids and it is characterized by a predominance of unsaturated fatty acids (USFA) with a high

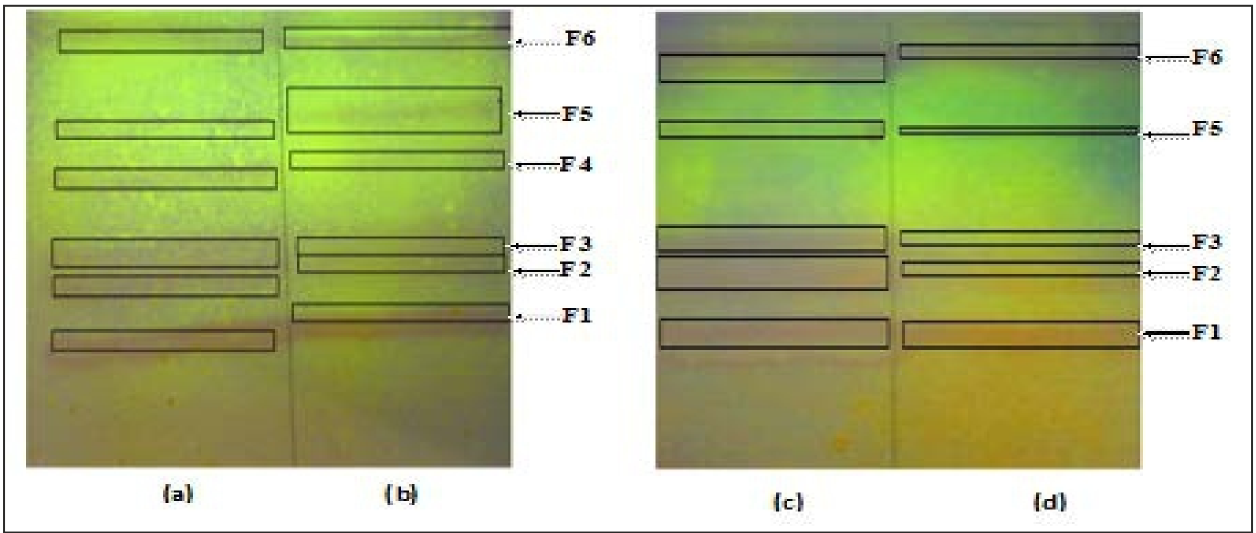

Figure 6 - TLC plate of the seed oil of Bigarade (a), Grapefruit (b), Lemon (c) and Maltese Orange (d).
TLC: thin layer chromatography, F1: Sterols, F2: Triterpene alcohols, F3: Aliphatic alcohols, F4 and F5: Fat -soluble vitamins and carotenoids, F6: Hydrocarbons.

content ranging from 65.61% to 78.73%.

In addition, the comparative study of these species showed that grapefruit followed by Maltese orange recorded a high content of linoleic acid (C18:2 cis ω6). However, for linolenic acid (C18:3 ω3), precursor of fatty acids of the omega-3 family, its content is higher in lemon and bigarade. Therefore, it is advisable to use grapefruit or Maltese orange oils in diets to compensate a lack of omega-6 and lemon oils and bigarade in the case of omega-3 deficiency. The importance of bitter orange oil is illustrated by the fact that the PUFA content is twice that of the MUFAs (44.23 and 22.46%). The study of the unsaponifiable fraction also showed that this fraction was quite high in the bigarade and the grapefruit. Thus, the oil of bitter orange could be of considerable importance.

In this study, we demonstrated the importance of citrus oils. It would be important to further identify the different constituents of citrus oils and to enhance the value of its seeds, considered for a long time as waste, especially for the least exploited species: the bigarade.

Acknowledgments

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