Extra-virgin olive oil and cheap vegetable oils: distinction and detection of adulteration as determined by GC and chemometrics

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***Abstract*— Refined oils including corn, sunflower, soybean and palm oils as well as low-quality olive oil such as refined lampante and pomace-olive oils are commonly used for extra-virgin olive oil (EVOO) adulteration. Indeed, (K_{270}) could be used as a parameter for the detection of EVOO fraud for each type of the studied refined oils, 10 % olive, 4 % pomace-olive, 10 % palm, 5 % corn, and 2 % soybean oils. Moreover, the adulteration could also be detected by the increase of the trans-fatty acid contents with 10 % pomace-olive, 3 % soybean, 3 % sunflower, 2 % corn, and 10 % palm oils. Actually, stigmasta-3,5-diene content is one of the most effective means of detecting refined oils in EVOO at low levels: 2 % olive, 0.4 % pomace-olive, 1 % palm, 0.2 % soybean, 0.5 % sunflower, and 0.1 % corn oils. Finally, the application of linear discriminant analysis could represent an alternative and innovative tool for faster and cheaper evaluation of EVOO adulteration.**

***Keywords*— Extra-virgin olive oil adulteration . Refined oils . trans-Fatty acids . Stigmasta-3,5-diene . Linear discriminant analysis**

I. Introduction

Oils are generally obtained from seeds or fruits either by crushing or by solvent extraction using a suitable solvent. In the world market, there is a growing demand for extra-virgin olive oils (EVOOs) thanks to their high nutritional properties, remarkable antioxidant properties and superior organoleptic characteristics (Owen et al. 2000; Jafari et al. 2009; Gargouri et al. 2013). EVOO can be considered as a natural fruit juice since it is obtained from the fruit of the olive tree just by mechanical or other physical operations under conditions that do not lead to oil alterations (IOOC 2013; Ammar et al. 2014a). In Tunisia, olive tree populations play a major socio-economic role as they consist of more than 30 % of agricultural groves (1.7 million ha) and account for more than 4 % of the olive oil produced in the world. Moreover, Tunisia, with a production around 170,000 tons a year, is the fourth largest producer and exporter of olive oil in the world. In Tunisia, there are more than 50 different olive oil cultivars such as Chemlali, Chétoui, Meski, Chemchali, Oueslati, and Zalmati, etc. In fact, Chemlali is the main cultivated variety, covering 60 % of the olive growing surface, spread from the northeast of the country to the extreme south (Gargouri et al. 2013; Ammar et al. 2014b; Gargouri et al. 2014).

In the world market, EVOO is characterized by one of the highest economic value in comparison with other vegetable oils thanks to its well-known nutritional and sensory qualities. Adulteration with refined vegetable oils is a major issue in the EVOO market. The detection of fraud is important for the protection of the consumers' health and wealth. EVOO, a premium food product, whose price is relatively high, is a target for adulteration with low price/quality oils. Refined oils including corn, sunflower, soybean and palm oils as well as low-quality olive oil such as refined lampante and pomace-olive oils are commonly used for EVOO adulteration by fraudulent practices (Jafari et al. 2009; Jabeur et al. 2014). These oils make up the most commonly consumed edible oils in the Tunisian market.

In comparison to EVOO, vegetable refined oils are characterized by stigmasta-3,5-diene. The origin of this hydrocarbon, which has been studied by Cert et al. (1994), is the dehydration of β-sitosterol during the refining of edible oils and fats. The detection of such hydrocarbon is among the most effective techniques to verify the presence of refined oil in virgin olive oil and crude vegetable oils. Many vegetable oils are refined through steps, namely bleaching and deodorization, which include treatment with acid bleaching earths and steam at high temperature, respectively. These processes dehydrate the sterols present in the oil to form a series of steroidal hydrocarbons or sterenes (Cert et al. 1994). In fact, the dehydration of the β-sitosterol, major component of plant sterols, gives 24-ethylcholesta-3,5-diene (stigmasta-3,5-diene) and less quantities of positional isomers (stigmasta-2,4-diene and stigmasta-2,5-diene). Other sterenes, such as 24-methylcholesta-3,5-diene (campesta-3,5-diene) and 24-ethylcholesta-3,5,22-triene (stigmasta-3,5,22-triene) are the dehydration products of campesterol and stigmasterol, respec-

tively. Moreover, trans-fatty acids (TFAs) can be found in considerable amounts in refined vegetable oils during the deodorization process (Ackman and Mag 1998; Ceriani and Meirelles 2007). Their presence can be used as a criterion to differentiate "pressed", "centrifuged" or solvent extraction oils from refined oils. Indeed, refined vegetable oils may already contain TFA at higher levels than those permitted for EVOO. During the refining process, compounds with conjugated double bonds can be formed from both unsaturated fatty acids linked to glycerol and unsaturated free fatty acids. The oxidation of polyunsaturated fatty acids (e.g. linoleic and linolenic acids) by the action of bleaching earth facilitates the formation of conjugated double bonds (dienes and trienes) (Vasvazova et al. 1998). Conjugated trienes formed during bleaching present a triple absorption band at 270 nm, 266 nm and 274 nm. The presence of these compounds in refined vegetable oils can be detected by the increase in ultraviolet absorption at 270 nm (K_{270}) and the increase in the specific extinction variation (ΔK) (Aparicio 2003; Zribi et al. 2013; Zribi et al. 2014; Jabeur et al. 2015). Thus, K_{270} and ΔK can be markers for the detection of EVOO adulterated with refined oils.

The identification and the quantification of the olive oil percentage in a blend is an analytical challenge. Indeed, chemometric tools such as linear discriminant analysis (LDA) and others have been used for the construction of models capable of verifying and recognising the percentage of olive oil in a binary blend (Kalua et al. 2005; Longobardi et al. 2012; Jabeur et al. 2014).

The aim of this study is to detect the adulteration of EVOO by lower cost refined oils. Consequently, various blends of EVOO and low-quality olive, soybean, corn, sunflower or palm oils were prepared and analyzed for K_{270}, ΔK, TFAs, and especially stigmasta-3,5-diene. The adulteration percentages ranged from 0.1 to 10 % in order to determine a threshold of detection. The International Olive Oil Council has included in its Regulations a limit of 0.05 ppm in the detection of stigmasta-3,5-diene in EVOOs (IOOC 2013).

II. Materials and Methods

Chemicals, Reagents and Standards

Ethanol ($\geq$ 99.9 %) and n-heptane (99.0 %) were obtained from Riedel-de Haën (Steinheim, Germany). Cyclohexane (99.5 %), n-hexane (99.0 %) and diethyl ether ($\geq$ 99.7 %) were purchased from Merck KGaA (Darmstadt, Germany). Potassium iodide ($\geq$ 99.0 %) was purchased from Chem-Lab (Zedelgem, Belgium) and sodium hydroxide ($\geq$ 99.0 %) was supplied by Scharlau Chemie S.A (Barcelona, Spain). Acetic acid (100.0 %) and chloroform ($\geq$ 99.1 %) were obtained from AnalaR NORMAPUR (La Chapelle-sur-Erdre cedex, France). Cholesta-3,5-diene ($\geq$ 99.0 %), n-nonacosane ($\geq$ 99.0 %) and fatty acids methyl esters (FAMEs) multi-standards ($\geq$ 99.0 %) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Vegetable Oils

Four different EVOO samples were obtained from the Tunisian Chemlali olive variety whose cultivar was harvested from Sfax region (southern Tunisia) during two crop seasons (2011/2012 and 2012/2013) (n = 4; two different samples for each crop season).

The ripening degree was the same for the four Chemlali olive samples (maturation indices were 4.5). The olive samples were collected at the beginning of December from orchards in the same neighborhood carried out with the same cultural practices. The olive ripening index (RI) was determined according to the method developed by Boskou (1996), on the basis of the evaluation of the olive skin and pulp colors. The RI values ranged from 0 (100 % intense green skin) to 7 (100 % purple flesh and black skin).

The refined corn and sunflower oils purchased from the local market in Tunisia. However, refined olive, pomace-olive, soybean and palm oils were obtained from the National Oil Office of Sfax. All refined oil samples were kept in dark glass bottles and stored at $-20°C$ and they were used as adulterants.

For this investigation, different mixtures of EVOO with the aforementioned refined oils (at the levels of 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, 5 and 10 % w/w) were prepared for each type of adulterant using an A & D instrument Ltd HR-200-EC analytical balance (resolution: 0.1 mg). Both Chemlali EVOOs and adulterants used for the mixing process were randomly selected. These mixtures were analyzed immediately after preparation.

III. Determination of Quality Indices

Free acidity (FA) was determined according to the International Norm ISO 660 (2009) "Determination of acid value and acidity", peroxide value (PV) was determined according to the International Norm ISO 3690 (2001) "Determination of peroxide value", and the specific extinction coefficients at 232 and 270 nm (K232 and K270) as well as the specific extinction variation (ΔK) were determined according to the IOOC (2010) "Spectrophotometric investigation in the ultraviolet". Free fatty acid (FFA) given as a percentage of the major fatty acid (% of oleic acid for the olive oils, % of linoleic acid for the seeds oils and % of palmitic acid for the refined palm oil) was determined by the titration of a solution of oil dissolved in ethanol/diethyl ether (1:1, v/v) with the aqueous solution of soda (0.1 N). PV, expressed in milliequivalents of active oxygen per kilogram of oil (meq O2/kg of oil), was determined as follows: a mixture of oil and chloroform/acetic acid (2:3, v/v) was left to react with a saturated solution of potassium iodide in the dark; the free iodine was then titrated with a sodium thiosulfate solution (0.01 N). K_{232}, K_{270} and ΔK were calculated from absorptions at 232, 270, 266 and 274 nm, respectively, with a spectrophotometer (UNICO, SQ-2800, USA), using a 10-mm quartz cuvette after the suitable dilution of samples within the sensitivity range with pure cyclohexane as a blank. These values were normalized for specified dilutions using Lambert's Law.

IV. Determination of trans-Fatty Acids (TFAs) Composition

The methyl esters for the determination of the TFAs by the use of capillary column gas chromatography (GC) were prepared according to a standard protocol (vigorous shaking of the solution of oil in n-heptane (0.1 g in 2 mL) with 0.2 mL of 2 N methanolic potassium hydroxide) (IOOC 2001a; IOOC 2001b).

FAMEs were separated and quantified using an Agilent model 6890N GC instrument (Palo Alto, CA, USA) equipped with a flame ionization detector (FID). A highly polar capillary column (length = 50 m, i.d. = 0.25 mm, and film thickness = 0.20 μm) of CP-Sil 88 (Varian, USA) coated with a 100 $\%$ cyanopropyl polysiloxane stationary phase was used to separate the FAMEs. The analysis conditions were as follow: the initial column temperature was settled at $165°C$ for 25 min, then raised at a gradient of $5°C$/min to $195°C$; the temperature of the injector and detector was set at $250°C$; helium was used as the carrier gas at a flow rate of 1 mL/min and 1:100 split ratio; the injection volume was 1 μL.

trans-FAMEs were identified through a comparison of their retention times versus pure standards analyzed under the same conditions. They were quantified according to their percentage area, obtained by the integration of the peaks. The results were expressed as the percentages of individual fatty acids in the lipid fraction. The major TFA isomers considered in this study included trans-octadecenoic (C18:1,9t), trans-trans-octadecadienoic (C18:2,9t,12t), cis-trans- and trans-cis-octadecadienoic [(C18:2,9c,12t); (C18:2,9t,12c)], and trans-cis-trans-, cis-cis-trans-, cis-trans-cis-, and trans-cis-cis-octadecatrienoic [(C18:3,9t,12c,15t); (C18:3,9c,12c,15t); (C18:3,9c,12t,15c); (C18:3,9t,12c,15c)] acids expressed as percentages of FAMEs.

V. Determination of Stigmasta-3,5-diene in Vegetable Oils

The stigmasta-3,5-dienes in vegetable oils contained low concentrations of these hydrocarbons, particularly in virgin olive oils and crude pomace-olive oil. Stigmasta-3,5-diene analysis was carried out according to the IOOC (2001c). Briefly described, vegetable oil samples (20 g) were spiked with 1 mL of internal standard (20 mg cholesta-3,5-diene/mL n-hexane) and saponified with 75 mL of a 10 $\%$ alcoholic potassium solution by gentle boiling for 30 min. After saponification, the mixture was transferred by the use of 100 mL water into a separating funnel and extracted with 100 mL of n-hexane. The mixture was vigorously shaken for 30 s and left to separate. The lower aqueous phase was transferred into a second separating funnel and extracted again with 100 mL of n-hexane. The combined n-hexane extracts were washed with 100 mL volumes of ethanol/water (1/1) until neutral pH. Generally, three washings were required to reach neutral pH. The extract was dried over anhydrous sodium sulfate and evaporated. With the aid of 2 $\times$ 1 mL portions of n-hexane, the residue was transferred on a silica gel column (15 g silica, column 45 $\times$ 1.5 cm internal diameter) and the chromatographic elution was conducted with n-hexane at a flow rate of 1 mL/min. The first 30 mL fraction thus obtained was discarded and the following 40 mL fraction containing the stigmasta-3,5-dienes was collected and evaporated. After evaporation, the residue was dissolved in 0.2 mL of n-hexane, transferred in a 2 mL vial and stored in the refrigerator at $4°C$ until analysis within 7 days of preparation.

VI. Determination of Sterene in Refined Vegetable Oils

The determination of sterenes (campestadienes and stigmastadienes), hydrocarbons originated originating from sterols during refining or desterolising treatments was applied to vegetable oils. The standards may be used to detect refined oils and admixtures of these oils with Chemlali EVOO. The procedure may also be used to quantify stigmastadienes in oils in which they occur at a greater concentration than 4.0 mg/kg (IOOC 2001d).

The oil sample (1 $\pm$ 0.01 g) was mixed with 1 mL of a standard solution of cholesta-3,5-diene (200 ppm) and the solution taken to the chromatographic silica gel column with the aid of the two 1 mL portions of hexane. The fractionation was started with n-hexane at a flow rate of 1 mL/min. The first 30 mL fraction was discarded and the remaining 40 mL, which was supposed to contain the hydrocarbon fraction of interest, was collected and evaporated at $30°C$ under reduced pressure. The residue was dissolved in 0.2 mL of n-hexane and subjected to analysis by GC.

Gas Chromatographic Conditions

Gas chromatographic separations were performed by an HP 7890 series gas chromatograph (Pudong, Shanghai, China) equipped with a FID. The column used was a capillary HP-5 (5 $\%$ phenyl; 95 $\%$ dimethylpolysiloxane). A temperature program with injection at $235°C$ which was held for 6 min increased from $2°C/min$ to $285°C$. It continued the oven heating at a speed from $15°C$/min to $320°C$ with 3 min hold. The temperatures of injector were $300°C$ and detector set at $320°C$, respectively. Helium was the carrier gas, with a flow through the column of 1 mL/min and 1:15 Split ratio, and the injection volume was 10 μL. The quantification and identification of stigmasta-3,5-diene were performed according to the relative retention time to the internal standard (cholesta-3,5-diene) reported in the IOOC (2001c) and IOOC (2001d). The gas chromatographic system should be checked by injecting a mixture of the stock solution of cholesta-3,5-diene and n-nonacosane solution. The internal standard peak has to appear before the n-nonacosane; if this does not occur, two measures can be taken to bring down the initial oven temperature or to replace the GC-column by a less polar one.

VII. Statistical Analysis

The results were expressed as mean of four measurements for the analytical determination. LDA was applied to discriminate Chemlali EVOO from those adulterated with

different percentages of refined olive, pomace-olive, soybean, sunflower, palm, and corn oils (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, 5, and 10 %) according to all the investigated parameters. LDA plots were performed using SPSS statistics 17.0 for Windows (SPSS Inc., 2008).

VIII. Results and Discussion

Characteristics of Chemlali EVOO and Refined Oils

Table 1 presents the characteristics of samples from Chemlali EVOO, refined olive oil (ROO), refined soybean oil (RSbO), refined pomace-olive oil (RPOO), refined sunflower oil (RSfO), refined palm oil (RPmO), and refined corn oil (RCoO). The studied parameters such as acidity, peroxide value (PV), specific extinction coefficients at 232 nm and 270 nm (K_{232} and K_{270}) and specific extinction variation (ΔK) show high differences between EVOO and refined vegetable oil samples.

Table 1. Quality characteristics of Chemlali extra-virgin olive oil and refined vegetable oil samples

	EVOO	ROO	RPOO	RSbO	RSfO	RCoO	RPmO
FFA(%)	0.33	0.09	0.16	0.10	0.12	0.10	0.17
PV (meq O2/kg)	11.25	2.84	4.11	1.85	3.70	2.77	3.68
K_{232}	2.12	2.84	4.21	3.82	3.27	3.44	2.97
K_{270}	0.162	0.720	1.450	1.870	0.580	1.082	0.564
ΔK	-0.002	0.064	0.143	0.145	0.054	0.102	0.028

According to studied chemical parameters, Chemlali olive oil samples belong to EVOO category, as the mean values were within the legal limits for free fatty acid (FFA $\leq$ 0.8 %), peroxide value (PV $\leq$ 20 meq/kg), specific extinction of conjugated dienes at 232 nm (K232 $\leq$ 2.50), specific extinction of conjugated trienes at 270 nm (K270 $\leq$ 0.22) and variation of the specific extinction between 266 nm and 274 nm ($\Delta K \leq 0.01$).

The objective of the refining process of lampante olive oil, pomace-olive oil, or other vegetable seed oils is to provide oils that meet the expectations of consumers and industrial users. EVOO has a high resistance to oxidative deterioration due not only to the triacylglycerol composition which is low in polyunsaturated fatty acids, but also to a group of phenolic antioxidants composed mainly of polyphenols and tocopherols. Polyphenols are of greater importance to virgin olive oil stability compared to refined oils that are eliminated or drastically reduced during the refining process (Velasco and Dobarganes 2002).

The refined oils contain a lower percentage of FFAs because these compounds are reduced during alkaline or physical refining (Young 1994; Korver and Zevenbergen 1997). Alkaline refining follows a neutralization stage to eliminate FFAs, whereas in physical refining, these compounds are removed by a steam distillation step. Another parameter for measuring the oil quality is peroxides which are indicators of oxidation and rancidity in oils. Actually, they are chemical compounds composed mainly of O-O bonds (GuzmÃ¡n et al. 2011). Yet, peroxides and hydroperoxides are unstable on heating at high temperatures and readily decompose to form mainly mixtures of volatile aldehyde compounds. Besides, peroxides are degraded and transformed during refining (bleaching and deodorization) steps. A reduction of the PV is generally observed during bleaching as the hydroperoxides react in the presence of bleaching earth to yield secondary oxidation products (Jahouach et al. 2006). In general, the peroxide values of refined vegetable oils are lower than those found in EVOO.

UV spectrophotometry is a good index to measure oxidative alterations. The determination of the specific absorption coefficients in the ultraviolet region is needed for estimating the oxidation stage of oil. The absorption at specified wavelengths at 232 nm and 270 nm is related to the formation of conjugated dienes and trienes in olive oil due to oxidation or refining processes (Kiritsakis et al. 2002).

EVOO is considered resistant to oxidation in comparison with other vegetable oils due to its low content of polyunsaturated fatty acids and the presence of natural antioxidants. Moreover, it has a moderate conjugated dienes at K_{232} and low conjugated trienes at K_{270}.

The high values of K_{232} and K_{270} in refined oils indicate the presence of diene and triene conjugated systems. A major task of the refining step is the destruction of the possibly existing hydroperoxides in order to improve the stability of the refined product. Conjugated dienes and trienes are formed by protonation and radical mechanism (DGF 2001). The spectrophtometric constants are not reliable quality indices for freshly refined oils because these parameters are influenced by the method with which the processing is achieved (Morchio et al. 1989). Besides, in a previous study about refined oils having different oxidative levels and different amounts of antioxidants and metals (iron and copper), Gomes et al. (2008) found that the overall oxidation level was the main factor affecting the resistance to oxidation.

Sometimes, the determination of K_{270} alone is insufficient to distinguish between virgin oil of poor quality and a refined olive oil or blended one. The determination of ΔK by the accurate measurement of trienic peak is effective since trienes are formed during the bleaching step.

In EVOO, ΔK is far smaller than 0.01 (is near zero), while this spectrophotometric constant is much higher to zero in refined vegetable oils owing to the conjugated trienes formed during the bleaching process performed with the activated bleaching earth (Kaynak et al. 2004).

trans-Fatty Acids Percentage and Stigmasta-3,5-diene Content in EVOO and Refined Vegetable Oils

The experimental results of TFAs percentage and stigmasta-3,5-diene content in EVOO, refined olive, pomace-olive, soybean, corn, sunflower, and palm oils are summarized in Table 2. TFAs are generally defined as unsaturated fatty acids that contain non-conjugated carbon-carbon double bounds in the trans configuration. These compounds which can be found in refined oils and heated oils, are used to differentiate genuine olive oils, or "pressed" oils from refined oils. EVOO, which was not subjected to the industrial process of refining, has,

Table 2. trans-Fatty acids and stigmasta-3,5-diene of Chemlali extra-virgin olive oil and refined vegetable oil samples

	EVOO	ROO	RPOO	RSbO	RSfO	RCoO	RPmO
TC18:1(%)	0.008	0.052	0.056	0.043	0.052	0.054	0.038
Σ(TC18:2+TC18:3)(%)	0.017	0.174	0.285	0.662	0.733	1.740	0.440
Stigmasta-3,5-diene (ppm)	0.007	4.250	12.540	37.020	16.400	72.650	7.050

according to IOOC standards, a 0.05 % maximum acceptable value of trans isomer of octadecanoic acid (TC18:1). Furthermore, the sum of octadecadienoic and octadecatrienoic TFA isomers (TC18:2 + TC18:3) should not exceed 0.05 %.

Refined vegetable oils contain TFAs at levels higher than those permitted for EVOO. TFAs can be formed in considerable amounts during the deodorization process of vegetable oils. Such process has been shown to induce geometrical isomerization of linoleic and linolenic acids (Ackman and Mag 1998; Ceriani and Meirelles 2007). The formation process is influenced mainly by temperature, and relatively less by time. The isomerization rate depends on the number of double bonds (Hou et al. 2012).

As regards stigmasta-3,5-dienes, they are formed by the acid catalyzed sterol dehydration reaction during the bleaching process (Ackman and Mag 1998; Zschau 2001), or during the deodorization process, enhanced by high temperatures (Kim and Nawar 1991). Vegetable refined oils are characterized principally by stigmasta-3,5-diene, which is a reliable indicator for the presence of refined oil in crude oils as reported by León-Camacho et al. (2004).

Compared to olive oil, other vegetable oils with high β-sitosterol content, such as corn and sunflower oils, yield, after refining, higher amounts of stigmasta-3,5-diene reaching 72.65 ppm in the case of RCoO. Our experimental values (Table 4) are in accordance with those reported by Homberg and Bielefeld (1989). In the same vein, similar findings were also seen in the study of Piironen et al. (2000) demonstrating that a variety of factors, such as bleaching temperature or the amount of earth and its activity affect the reaction of stigmasta-3,5-diene formation. In addition, the formation of stigmasta-3,5-diene from β-sitosterol and therefore stigmasta-3,5-diene contents are strongly dependent on the temperature used during the physical refining. Indeed, León-Camacho et al. (2004), has shown that the formation of stigmasta-3,5-diene in refined vegetable oils depends on different variables namely time, temperature, flow of stripping gas and thickness of oil layer.

In Chemlali EVOO, stigmasta-3,5-diene is almost absent (0.007 mg/kg) regardless of the olive's origin and production processes, provided that the usual treatments and temperatures below $100°C$ are applied (Gordon and Firman 2001). For virgin olive oils sold as non-refined, a limit of 0.05 mg/kg of stigmasta-3,5-diene is applied, adopted by the IOOC and by the European Union (EU).

Identification of EVOO Adulteration with Other Refined Low-Cost Oils

To avoid oil adulteration, anti-fraud controls require that specific tests be performed to assess the purity of EVOOs. This research study is meant to detect the adulteration of EVOO by lower or cheaper quality oils. Consequently, various blends of EVOO and refined oils including corn, sunflower, soybean and palm oils as well as refined low-quality olive oil such as refined olive and pomace-olive oils were prepared and analyzed for spectrophotometric constants K_{270}, ΔK, TFA percentages and stigmasta-3,5-diene content. The adulteration percentages ranged from 0.1 to 10 % in order to determine a threshold of detection.

Use of K_{270} and ΔK for the Detection of Fraud

The UV specific extinction determination (K_{270}) and variation of specific extinction (ΔK) of EVOO spiked with 0.1-10 % (w/w) quantities of refined olive, pomace-olive, soybean, sunflower, corn and palm oils are summarized in Table 3.

Taking into account the results presented in Table 3, it is concluded that the analysis of K_{270} and ΔK does not produce satisfactory results with regard to the level of adulteration in this Research study. The results show that the values of these two parameters increase with the increase in the concentration of refined vegetable oils. The maximum permitted values of K_{270} and ΔK for EVOOs are 0.22 and 0.01 respectively (IOOC standards). According to the results presented in Tables 3 and 5, the determination of K_{270} can be used as a parameter for the fraud detection of Chemlali EVOO with 10 % refined olive (0.241 $\geq$ 0.22), 4 % pomace-olive (0.233 0.22), 10 % palm (0.231 $\geq$ 0.22), 5 % corn (0.224 $\geq$ 0.22), and 2 % soybean (0.225 $\geq$ 0.22) oils. However, K_{270} is not an effective parameter for the detection of the adulteration of EVOO with refined sunflower oil even after the addition of 10 % (K_{270} = 0.214 $\leq$ 0.22).

Similarly, ΔK could be used as a parameter for the detection of EVOO fraud with 10 % pomace-olive (0.012 $\geq$ 0.01), 10 % corn (0.011 $\geq$ 0.01) and 10 % soybean (0.013 $\geq$ 0.01) oils. ΔK is not an effective parameter for the detection of the EVOO adulteration with the following vegetable oils: olive, sunflower and palm oils, up to the level of 10 %.

Based on the data and the abovementioned observations, the following conclusions can be drawn. Although these two parameters in the examined refined oils are different from those of Chemlali EVOOs, K_{270} and ΔK could not be satisfactorily used as discriminatory parameters between the EVOO and the blended olive oil.

Use of the trans-Fatty Acids for the Fraud Detection

TFA isomers are found in refined edible oils due to the high temperatures used during the deodorization procedure and their presence can be used to differentiate virgin olive oils from refined oils.

In order to evaluate the possibility of detecting refined vegetable oils in EVOO, the adulteration with refined low-cost oils, binary mixtures containing a percentage from 99.9 to 90.0 % of EVOO with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, and 10 % (w/w) of refined edible oils were prepared. The trans-fatty isomers from oleic acid (TC18:1), linoleic and linolenic acids (TC18:2 + TC18:3) contents of

the adulterated EVOO mixed with 0.1-10 % (w/w) quantities of refined vegetable oils were summarized in Table 4. TFAs increase with the increase in the adulteration percentage of refined vegetable oils (from 0.1 to 10 %). The content of these isomeric acids could be used as a parameter for the detection of EVOO fraud with the following refined vegetable oils: 10 % pomace-olive (0.053 % $\leq$ 0.05 %), 3 % soybean oil (0.055 % $\leq$ 0.05 %), 3 % sunflower (0.054 % $\leq$ 0.05 %), 2 % corn (0.059 % $\leq$ 0.05 %), and 10 % palm (0.058 % $\leq$ 0.05 %) oils (Tables 4 and 5). However, the established limit for the TFAs is not satisfactory for detecting percentages lower than or equal to 10 % of the refined olive oil in EVOO. In fact, the TFAs percentage has shown low values within the limit of the extra-virgin category even after the addition of 10 % of ROO in the Chemlali EVOO (Σ (TC18:2 + TC18:3) = 0.047 % $\leq$ 0.05 % maximum limit permitted by IOOC (2013)).

Use of the Stigmasta-3,5-diene for the Fraud Detection

The formation of stigmasta-3,5-diene can be ascribed to the degradation of the oxidation products of β-sitosterol during the refining of edible oils and fats. This hydrocarbon is useful to detect the presence of refined oil in EVOO because such a hydrocarbon originates from the refining process.

The comparison between the Chemlali EVOO and the studied refined vegetable oils reveals that there are high differences relating to stigmasta-3,5-diene content as shown in Table 2. At this level, stigmasta-3,5-diene was adopted for the quantitative determination of the levels of adulterant in EVOO.

Experimentally, its quantification is performed by means of an analytical methodology comprising the previous purification of stigmasta-3,5-diene by preparative column chromatography followed by its analysis using GC. The result of Table 4 profile of sterenes is almost decisive in clarifying the contamination of EVOO with some refined cheaper vegetable oils. The use of stigmasta-3,5-diene proved to be more effective in detecting even low levels of adulteration of Chemlali EVOO with most of the refined vegetable oils under study. According to the data on the fraudulent mixtures presented in Tables 4 and 5 and Figure 1, the determination of stigmasta-3,5-diene can be used as a parameter for the detection of EVOO fraud with each one of studied refined oils: 2 % olive (0.081 ppm $\geq$ 0.05 ppm), 0.4 % pomace-olive (0.062 ppm $\geq$ 0.05 ppm), 1 % palm (0.063 ppm $\geq$ 0.05 ppm), 0.2 % soybean (0.069 ppm $\geq$ 0.05 ppm), 0.5 % sunflower (0.062 ppm $\geq$ 0.05 ppm) and 0.1 % corn (0.065 ppm $\geq$ 0.05 ppm) oils. In this context, an investigation was carried out to evaluate the use of GC coupled with mass spectrometry (GC-MS) to quantify the sterene compounds of EVOO, low-grade olive oils, refined seed oils as well as their blends. Therefore, this technique is sensitive to low levels of stigmasta-3,5-diene and the mixtures of much less than 5 % of refined oils can be detected (Crews et al. 2014).

Chemometric Analysis

LDA is probably the most frequently used supervised pattern recognition method. In principle, LDA determines linear discriminant functions that maximize the ratio of between-class variance and minimize the ratio of within-class variance. It should be noted that LDA selects a direction that achieves maximum separation among the given classes. Because the data structure analysis gave a good sample characterization, a classification model was built. LDA was applied to find a predictive classification model able to differentiate the pure EVOO and the adulterated olive oils.

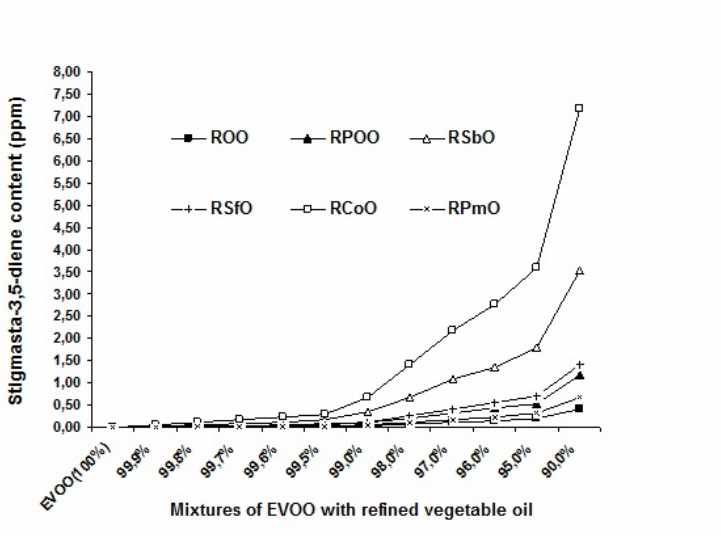

Fig. 1. Stigmata-3,5-diene content of mixtures of extra-virgin olive oil (EVOO) with refined olive, pomace-olive, soybean, sunflower, corn and palm oils. Each value represents the mean of four determinations of two successive crop seasons (n=4; standard deviation $\leq$ 0.001 %) (■): ROO; (▲): RPOO; (△): RSbO; (+): RSfO; (□): RCoO and (X): RPmO

First, it is difficult to discriminate the adulterated EVOO with 0.1 % of RSfO (4) or RPmO (7) and the pure EVOO (1), whereas those adulterated with other vegetables oils (RSbO (2), RCoO (3), ROO (5), or RPOO (6)) are clearly separated at the same level (Fig. 2a). Second, the separation was clearly observed as shown in Fig. 2b when the percentage of adulteration increases. All samples were correctly discriminated using the two functions. Indeed, the plot of the discriminant functions (figure 2) obtained by LDA shows a clear discrimination between the Chemlali EVOO (1) and the adulterated EVOOs only mixed with 0.2 % of RSbO (2), RCoO (3), RSfO (4), ROO (5), RPOO (6), or RPmO (7).

The application of LDA after feature selection was sufficient to differentiate Chemlali EVOO and all adulterated EVOOs. The success was 100 % in classification and close to 100 % in prediction.

Compared to classical methods, this new approach of LDA application could represent an alternative and innovative tool for a faster and cheaper evaluation of EVOO adulteration (Jabeur et al. 2014).

IX. Conclusions

The present study aims to explain and compare analytical methods (conjugated dienes, conjugated trienes, TFAs, and stigmasta-3,5-diene) used to detect and quantify the possible

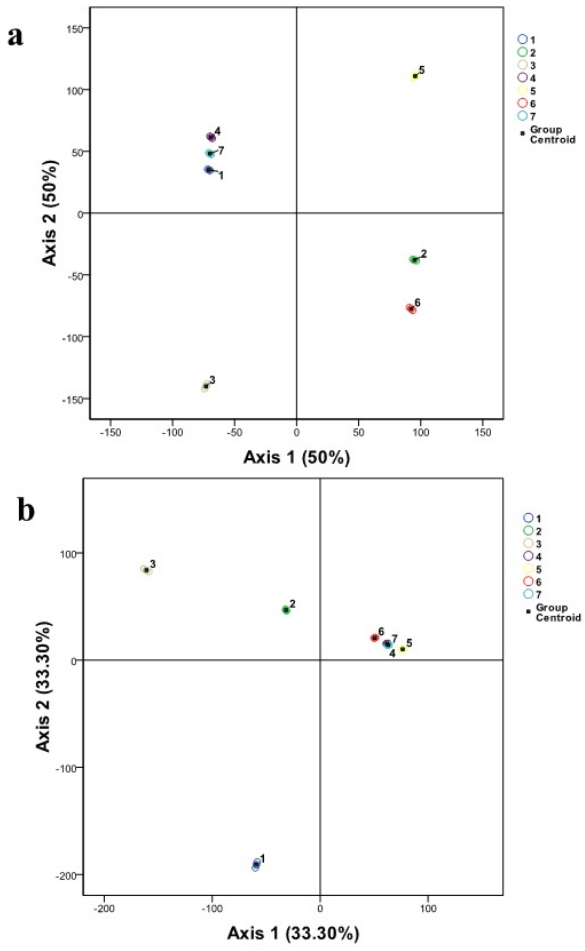

Fig. 2. LDA score plot of pure extra-virgin olive oil (EVOO) and adulterated EVOO at the same level based on all of the analyses performed with four determinations: (a) EVOO + 0.1 % of refined oil; (b) EVOO + 0.2 % of refined oil; (1) EVOO; (2) EVOO + % of soybean oil; (3) EVOO + % of corn oil; (4) EVOO + % of sunflower oil; (5) EVOO + % of refined olive oil; (6) EVOO + % of refined pomace-olive oil; (7) EVOO + % of refined palm oil

adulterations of EVOO by refined vegetable oils. In fact, K270 and ΔK are complementary parameters that indicate the presence of some of the adulteration types up to relatively high threshold. A GC analysis for TFA composition permits the detection of a wider range of adulteration with a lower range of thresholds (from 2 % to 10 % except for refined olive oil). However, the stigmasta-3,5-diene determination remains the best way to detect and quantify all types of adulterations with a threshold as low as 0.1 % in case of RCoO. Although this technique is the most effective, it is the longest and most expensive compared to those mentioned above.

To the best of our knowledge, these techniques are the most used in fraud detections. Therefore, this study has the advantage that many industrials operating in the field of olive oil could use them in order to avoid adulteration and protect the quality and purity of EVOO.

X. Acknowledgements

The authors would like to acknowledge the support of the Ministère de l'Enseignement Supèrieur et de la Recherche Scientifique and the Ministère de l'Agriculture (ONH Laboratory-Sfax), Tunisia, to this research work. They are also grateful to Mrs. Leila Mahfoudhi, teacher of English at the Sfax Faculty of Science, for having proofread the manuscript.

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