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Identification of Adulterants in Extra Virgin Olive Oil Using HS-SPME-GC-MS and Multivariate Data Analysis

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Food fraud, such as the adulteration of extra virgin olive oil (EVOO), is found to cause substantial negative impacts on both the economy and the health of consumers. This work was aimed at evaluating 19 EVOO samples commercialized in Foz do Iguaçu, a frontier city located around the triple border of Brazil, Argentina, and Paraguay. To detect the presence of adulteration in EVOO samples, the present study employed gas chromatography coupled to flame ionization detector (GC-FID) and headspace solid phase microextraction with gas chromatography coupled to mass spectrometry (HS-SPME-GC-MS) in order to evaluate fatty acids composition and volatile organic compounds (VOCs), respectively. The quantitative results obtained from the analysis of fatty acids composition showed that 32% of the EVOO samples were adulterated, presumably with soy oil, due to the high levels of linoleic, linolenic, and myristic acids present in them. Principal component analysis (PCA) conducted using the complete chromatographic aroma profiles obtained from the VOCs helped distinguish authentic EVOO samples from adulterated ones and the country of origin of the samples. The following aromatic compounds were first described, as possible adulterant markers: 3,3-dimethylheptanoic acid, propyl pentanoate, methyl cyclohexanecarboxylate, ethyl cyclohexanecarboxylate, 2-phenylethyl acetate, 6,10,14-trimethylpentadecan-2-one, and 1,2-dimethoxy-4-methylbenzene.

Keywords: fatty acid profile, volatile organic compounds, aroma, adulteration, PCA

Introduction

Over the past few years, there has been a disturbingly steady rise in food fraud worldwide; food fraud is a collective term that encompasses the deliberate and intentional substitution, addition, alteration or adulteration of food, food ingredients or food packaging for the purposes intended.[1] Giving false or misleading information about food products for economic gains also constitutes food fraud.[1] Food fraud is one of the major threats to public health and can exert considerable effects, in varying degrees, on national security, as well as on the society, economy, politics, and particularly on the health of the consumers.[2]

Virgin olive oil is extracted from the fruits of *Olea europaea* L. through mechanical processes such as pressure or centrifugation.[3] There have been reports in the literature regarding the adulteration of extra virgin olive oil (EVOO) by substituting the oil partially or totally with low-cost oils, adding lower quality olive oil referred to as "lampante olive oil" or concealing the real place of origin of the olive oil.[4-7] Numerous studies[8-10] that have sought to investigate and identify EVOO adulteration have reported to have detected the presence of virgin olive oil, soybean oil, corn oil, sunflower oil and rapeseed oil employed mainly as EVOO adulterants. The adulteration of EVOO with other vegetable oils is a direct infringement of the rights

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of the consumer, and in some cases, leads to potentially harmful effects on the health of consumers; for instance, the adulteration of EVOO with soybean oil poses serious health risks to consumers that are allergic to soy protein.[8,11]

EVOOs are oils of excellent nutritional quality that are rich in natural antioxidants and which bring several benefits to human health.[12] EVOO composition is characterized by the presence of high content of mono unsaturated fatty acids in which oleic acid (C18:1n-9, 55-83%) constitutes the main fatty acid which is responsible for the physiochemical and some nutritional properties found in EVOO.[7] Other acids present in relatively smaller quantities in EVOO include polyunsaturated fatty acids (linoleic acid: C18:2n-6, 2.5-21.0%, and alpha linolenic acid: C18:3n-3, < 1.0%) and saturated fatty acids. For the oil to be classified as EVOO, the content of these monounsaturated and polyunsaturated fatty acids must meet the tolerance range recommended by the legislation.[13-17] To detect possible adulteration in EVOOs, previous studies reported in the literature[4,8,18] have employed chromatography coupled to flame ionization detector for the analysis of fatty acid methyl esters (FAMEs) present in these oils.

During the extraction process, the enzymes present in olives are released and they undergo specific reactions with the compounds present in the olives, leading to the generation of several final products which are responsible for the aroma and flavor of olive oil.[19,20] Volatile compounds are produced from the oxidation of the fatty acids present in olive oil; these compounds essentially contribute to the sensory profile of extra virgin olive oil.[7]

The non-destructive headspace solid phase microextraction technique is used to evaluate the volatile composition of EVOOs; this solvent-free sampling technique reduces the steps of extraction, cleaning and concentration to a single step and produces relatively higher quality results compared to traditional techniques.[21,22] Solid phase microextraction combined with gas chromatography and mass spectrometry (SPME-GC-MS) has been increasingly employed for analyzing the following: (*i*) the authenticity of EVOOs, where the technique allows one to distinguish the geographical origin of the oil samples; (*ii*) traceability, cultivars; (*iii*) ripening; (*iv*) storaging, and presence of adulterants.[20,23-29]

The triple border region formed by the cities of Foz do Iguaçu (Brazil), Ciudad del Este (Paraguay), and Puerto Iguazu (Argentina) substantially facilitates the mobility of consumers across the borders of these countries, and this helps to promote trade in these regions, leading to mutual considerable profitability, since the products available in their markets and trade fairs are marketed and consumed by the population of the three countries that share the triple border. Argentina is globally known for its large-scale production of EVOO.[4] The fact that EVOO is a high quality product with high added value and of high demand in the region, highly demanded by the local population and, particularly, by the tourists that flock into the region, the product has become considerably targeted by fraudsters and ill-intended traders that use fraudulent practices to adulterate the oil for economic gains and to maximize their profits. Taking the above considerations into account, the present work was aimed at evaluating the presence of adulteration in EVOOs commercialized in the triple border region through the identification of the sources of vegetable oils and compounds used as adulterants in the product.

Experimental

Sampling

Vegetable oils were purchased from the local markets in Foz do Iguaçu (Brazil), Puerto Iguazu (Argentina), and Ciudad del Este (Paraguay) in 2018 and 2019. The EVOO samples had labels that indicated that they were made from the following countries: Argentina (S1-S9), Chile (S10-S11), Spain (S12-S14), and Portugal (S15-S19). In addition, other vegetable and animal oils, including sunflower oil (SF), fish oil (FO) capsules, corn oil (CO), rice oil (RO), soy oil (SO), coconut oil (CCO), frying oil (FY) and yard oil (YA), were investigated as possible adulterants. The samples were fractionated and kept under refrigeration at –20 °C, protected from light until the time of analysis.

Titratable acidity

The acidity content (free fatty acids) of the EVOO samples (1.0 g) was measured by titration with standardized sodium hydroxide (NaOH, 0.010 mol L^{-1}) where phenolphthalein was employed as indicator according to International Olive Council (2017); the results obtained from this analysis were expressed in percentage oleic acid (m/m).[30-32]

Composition of fatty acids

FAMEs were obtained by weighing 0.025 ± 0.001 g oil in a conical tube followed by derivation based on the method reported by Hartman and Lago,[33] which was adapted by Santos *et al.*[34] and described by Führ *et al*.[35] An amount of 4 mL of 0.5 mol L^{-1} sodium hydroxide (NaOH, Neon, São Paulo, Brazil) in methanol (MeOH, Sal-R, São Paulo, Brazil) were added into the tube. The mixture was then subjected to ultrasound bath for 6 min, and this was

followed by the addition of 5 mL of ammonium chloride (NH_4Cl, Synth, São Paulo, Brazil) and sulfuric acid (H_2SO_4, Biotec, Paraná, Brazil) solution in MeOH, in the ratio 1:1.5:30 (m/v/v), respectively. The mixture was subjected to ultrasound bath again for 6 min, and the phases were separated by the addition of 4 mL sodium chloride (NaCl, Dinâmica, São Paulo, Brazil) (saturated solution), followed by vortex agitation for 30 s. An amount of 2 mL of iso-octane (Sigma-Aldrich, St. Louis, USA) was then added to the mixture, and the mixture was stirred again for 30 s and kept at –4 °C for 24 h for phase separation. The organic phase was collected and kept at –20 °C for chromatographic analyses. The analyses were performed in triplicate.

The analysis of the FAMEs was conducted using gas chromatography coupled to flame detector (GC-FID) (gas chromatograph model TR-1310, from Thermo Scientific, Milan, Italy), equipped with TR-FAME column (120 m length, 0.25 mm internal diameter, and 0.25 µm film, Thermo Scientific, Pennsylvania, USA) under the chromatographic conditions described in detail in our previous work.[35] The FAMEs were identified based on a comparison of the retention times of the components of the samples relative to the standard FAME solution (≥ 99%, Supelco® 37 Component FAME Mix, Sigma-Aldrich, St. Louis, USA). The results obtained were expressed as percentage of relative area (%).

Volatile organic compounds

The analysis of volatile compounds was conducted based on the methods described by Fernandez *et al*.[23] and Peršurić *et al*.[36] Volatile organic compounds (VOCs) were analyzed in the 19 EVOO samples investigated. An amount of approximately 5.00 g of each of the EVOO samples was placed in 20 mL vials and heated in silicone bath (60 °C) for 20 min under agitation (750 rpm). After that, the triple phase 50/30 µm fiber (divinylbenzene/carboxen/polydimethylsiloxane) (Supelco, Bellefonte, PA, USA), was exposed to the headspace above the sample (3 cm) for 50 min at 60 °C and was then immediately inserted into the gas chromatograph (GC) injector at 250 °C.

The volatile compounds were separated and identified by gas chomatography using a chromatography system TRACE 1300 gas chromatograph (Thermo Scientfic, Milan, Italia) coupled to quadrupole mass analyzer ISQ single quadrupole MS (Thermo Scientific, Milan, Italia). The chromatographic analysis was conducted using the TR-WAX column (30 m × 0.25 mm × 0.25 µm) (Thermo Scientific, Pennsylvania, USA). The temperature gradient of the chromatographic separation process was programmed as follows: 40 °C (8 min), 1 °C min^{-1} to 120 °C, 10 °C min^{-1} to 200 °C, 60 °C min^{-1} to 250 °C (2 min) with total run of 99 min. For the separation and identification of the volatile compounds, the injection was performed in the splitless mode using helium gas as carrier gas at a constant flow rate of 1.0 mL min^{-1}. The mass spectrometer was operated via electronic impact with ionization energy of 70 eV. The line of transfer was kept at a temperature of 250 °C. The ThermoXcalibur software version 2.2 (Thermo Scientfic, Massachusetts, USA) was used for both data acquisition in the full SCAN mode, with a mass range of 30-400 and data treatment using NIST library mass spectrum. The extraction procedure involved the application of SPME, followed by GC-MS in duplicate.

Statistical analysis

The statistical analysis was performed using the R software version 4.2.1, "Funny-Looking Kid", 2022 (The R Foundation for Statistical Computing). For graphical interface, was employed the RStudio 2022.07.1+554 "Spotted Wakerobin" and R Commander version 2.7-2 released for Windows.[37,38] Principal component analysis (PCA), as well as hierarchical clustering on principal components (HCPC) analysis and description of categories were performed using the FactoMineR package.[39] The statistical analysis plot was constructed using factoextra Package 1.0.7. The data and imaging processing was performed using x86_64-w64-mingw32/x64 (64-bit) platform, notebook Dell Inspiron 13-7348-B20, Intel(R) Core™ i5-5200U processor, CPU @ 2.20GHz 2.20 GHz, and 8 Gb RAM DDR3L.

Results and Discussion

Composition of fatty acids

The main composition of fatty acids in the EVOO samples (Table 1) was evaluated based on the stipulated legislation.[13,16]

The fatty acid found in the highest concentration in the EVOO samples was oleic acid (C18:1n-9c); this acid constituted approximately 67.5 to 78.3% of the fatty acid composition of the authentic EVOO samples (samples S1-S3 and S10-S19). Interestingly, the analysis of S4 showed that the sample contained between 51.3 and 51.8% of oleic acid, while samples S5-S9 recorded even lower concentrations of the acid, with values ranging from 22.7 to 36.5%, which is extremely below the threshold allowed by the legislation on olive oil (55-83%).[15,40] In addition, samples S5-S9 exhibited fatty acids content above the legally stipulated thresholds for linolenic acid (C18:2n-6c, 3.5-21%), linoleic acid (C18:3n-3 ≤ 1), myristic acid (C14 ≤ 0.05%) and behenic acid (C22:0 ≤

0.2), and below the legally stipulated theshold for palmitoleic acid (C16:1, 0.3-3.5%)[13,16] (Table 1). The samples S4-S9 (Table 1) were found to contain adulterated EVOOs, which corresponded to 32% of the samples investigated in this study.

To evaluate any possible sources of adulteration, the fatty acids profiles of the adulterated samples were compared with the fatty acid composition of other oils. Figure 1 shows the results obtained from the PCA of the fatty acids of the EVOO samples and those of the samples of other oils of vegetable and animal origin. Samples S4-S9, which corresponded to the adulterated samples, are grouped under cluster 3, along with the samples of sunflower oil (SF), corn oil (CO), rice oil (RO), soybean oil (SO) and frying oil (FY) (Figures 1a and 1b). Chromatograms are presented in Supplementary Information (SI) section (Figure S1). The samples were grouped under cluster 1 due to the amounts of linoleic acid, linolenic acid, and myistic acid in them (Figure 1b). The presence of elevated quantities of linoleic acid (48-59%) and linolenic acid (3.5-11%) is indicative of fraudulent practices using soybean oil; this phenomenon was observed in samples S5-S9.[41] In sample S4, a partial addition of vegetable oil was hypothesized, since the sample recorded intermediary concentration levels of oleic acid, linoleic acid and linolenic acid, which corresponded to 51, 33, and 0.72%, respectively.

The samples of coconut oil (CCO), fish oil (FO) and yard oil (YA) tended to form a distinctive group that clearly reflected their peculiar characteristics and that differentiated it from the rest of the oils investigated (Figures 1a and 1b); this behavior can be attributed to the fact that these samples (CCO, FO, and YO) have different fatty acids composition. Samples S1-S3 and S10-S19 were grouped under cluster 5 (Figure 1b) due to the higher levels of oleic acid and lower levels of myristic acid present in these samples (C14 ≤ 0.05%).

Samples S4, S6 and S9 (Table 1) exhibited levels of titratable acidity above 0.8%, which is the legally stipulated level, and this made them clearly differ from the rest of the EVOO samples.[13,40]

Volatile organic compounds

The HS-SPME-GC-MS technique was applied in order

Table 1. Main fatty acids composition of the EVOO samples investigated in this study

Sample	Fatty acids composition (relative area) / %										
	C14:0	C16:0	C16:1	C18:0	C18:1n-9c	C18:2n-6c	C18:3n-3 (LNA)	C20:1	C22:0	C24:0	Free acidity / (g 100 g^{-1})
Quality parameters[a]	≤ 0.05	7.5-20.0	0.3-3.5	0.5-5.0	55-83	3.5-21	≤ 1	≤ 0.4	≤ 0.2	≤ 0.2	≤ 0.8
Argentina											
S1	0.02 ± 0.001	13.91 ± 0.212	1.31 ± 0.054	2.24 ± 0.063	67.54 ± 0.381	13.39 ± 0.230	1.14 ± 0.015	0.23 ± 0.013	0.09 ± 0.006	0.05 ± 0.002	0.82 ± 0.001
S2	n.d	13.59 ± 0.045	1.13 ± 0.060	2.04 ± 0.034	73.71 ± 0.619	8.27 ± 0.187	0.94 ± 0.019	0.21 ± 0.011	0.12 ± 0.004	0.05 ± 0.009	0.23 ± 0.030
S3	n.d	13.88 ± 0.089	1.21 ± 0.012	1.77 ± 0.013	70.94 ± 0.013	10.61 ± 0.050	0.95 ± 0.012	0.25 ± 0.005	0.08 ± 0.008	n.d	0.30 ± 0.030
S4	0.05 ± 0.007	9.09 ± 0.107	0.70 ± 0.044	3.29 ± 0.059	51.27 ± 1.449	33.85 ± 1.467	0.79 ± 0.124	0.18 ± 0.010	0.46 ± 0.010	0.17 ± 0.008	1.00 ± 0,004
S5	0.08 ± 0.007	11.74 ± 0.107	0.36 ± 0.030	3.93 ± 0.257	27.13 ± 0.740	51.02 ± 0.820	3.66 ± 0.166	0.16 ± 0.021	0.30 ± 0.033	0.12 ± 0.009	0.63 ± 0.012
S6	0.08 ± 0.015	10.48 ± 0.061	0.19 ± 0.026	4.23 ± 0.087	27.49 ± 0.275	51.53 ± 0.216	3.64 ± 0.049	0.19 ± 0.014	0.38 ± 0.016	0.15 ± 0.016	0.80 ± 0.017
S7	0.07 ± 0.005	9.95 ± 0.171	0.16 ± 0.010	4.10 ± 0.155	26.25 ± 0.339	52.68 ± 0.395	4.96 ± 0.060	0.17 ± 0.006	0.34 ± 0.026	0.13 ± 0.009	0.69 ± 0.001
S8	0.07 ± 0.003	11.78 ± 0.137	0.28 ± 0.006	4.28 ± 0.043	28.54 ± 0.326	48.83 ± 0.287	3.75 ± 0.046	0.21 ± 0.007	0.35 ± 0.011	0.14 ± 0.009	0.69 ± 0.011
S9	0.16 ± 0.172	9.56 ± 0.129	0.45 ± 0.006	3.38 ± 0.014	36.62 ± 0.425	46.63 ± 0.209	2.27 ± 0.035	0.15 ± 0.003	0.38 ± 0.014	0.13 ± 0.003	1.30 ± 0.020
Chile											
S10	n.d	10.38 ± 0.113	0.53 ± 0.016	1.90 ± 0.037	79.80 ± 0.803	6.02 ± 0.592	0.91 ± 0.068	0.24 ± 0.003	0.10 ± 0.012	n.d	0.28 ± 0.029
S11	n.d	10.81 ± 0.045	0.63 ± 0.020	1.86 ± 0.031	78.27 ± 0.363	6.89 ± 0.369	0.94 ± 0.048	0.24 ± 0.004	0.11 ± 0.007	n.d	0.35 ± 0.032
Spain											
S12	n.d	10.70 ± 0.183	0.78 ± 0.030	2.86 ± 0.058	78.09 ± 1.234	6.00 ± 0.725	0.95 ± 0.034	0.20 ± 0.041	n.d	n.d	0.19 ± 0.001
S13	0.02 ± 0.001	14.15 ± 0.354	1.40 ± 0.087	2.03 ± 0.028	69.90 ± 0.498	10.95 ± 0.042	0.92 ± 0.010	0.21 ± 0.006	0.09 ± 0.002	n.d	0.33 ± 0.051
S14	n.d	11.38 ± 0.690	0.87 ± 0.056	2.78 ± 0.034	75.94 ± 0.823	7.40 ± 0.667	1.09 ± 0.160	0.20 ± 0.027	n.d	n.d	0.17 ± 0.010
Portugal											
S15	0.03 ± 0.002	9.98 ± 0.256	0.68 ± 0.019	2.76 ± 0.045	74.74 ± 0.451	10.04 ± 0.429	1.09 ± 0.009	0.27 ± 0.018	0.10 ± 0.003	0.05 ± 0.001	0.32 ± 0.400
S16	n.d	13.00 ± 0.041	1.02 ± 0.018	2.66 ± 0.127	72.33 ± 0.170	9.27 ± 0.018	1.06 ± 0.020	0.24 ± 0.004	n.d	0.06 ± 0.005	0.42 ± 0.061
S17	n.d	11.51 ± 1.25	0.90 ± 0.041	2.70 ± 0.070	77.18 ± 0.189	6.27 ± 0.118	0.92 ± 0.014	0.19 ± 0.004	0.08 ± 0.007	n.d	0.31 ± 0.005
S18	n.d	10.90 ± 0.017	0.79 ± 0.020	2.49 ± 0.071	78.14 ± 0.194	6.23 ± 0.056	0.91 ± 0.006	0.20 ± 0.007	0.09 ± 0.003	n.d	0.24 ± 0.003
S19	0.07 ± 0.078	13.93 ± 0.175	1.13 ± 0.034	2.18 ± 0.035	70.53 ± 0.638	10.31 ± 0.522	1.05 ± 0.053	0.21 ± 0.002	0.10 ± 0.002	0.05 ± 0.008	0.27 ± 0.001

[a]In accordance with the stipulated legislation.[16,40] Values expressed as mean ± standard deviation of three replicates. n.d: non-detectable; LNA: locked nucleic acid.

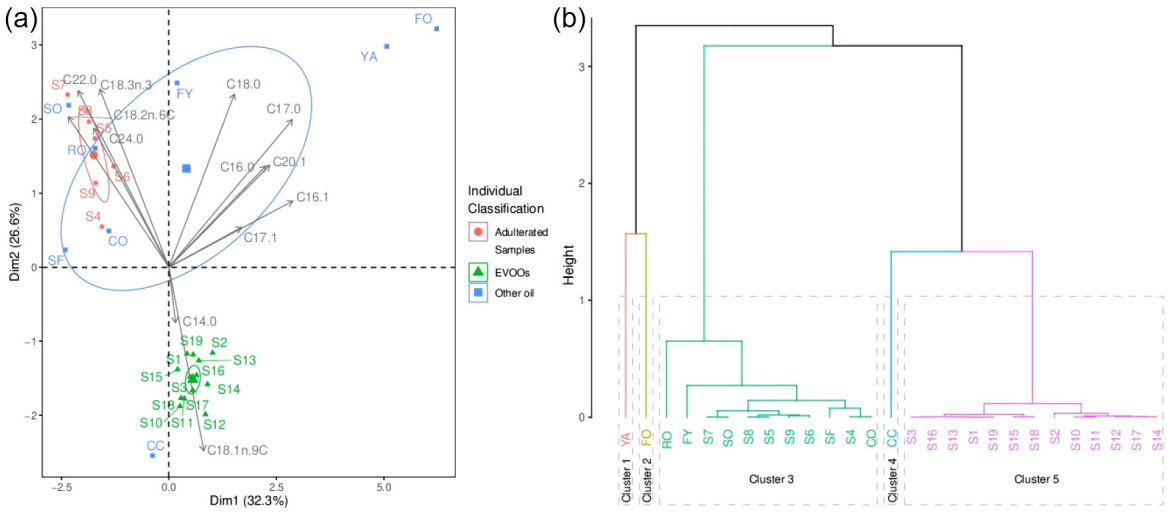

Figure 1. Multivariate data analysis of EVOO samples and other oils of animal and vegetable origin. Biplot of individual and variable factor map (PCA) (a) variables are shown as gray arrows (→). Hierarchical clustering analysis on principal components (b). The classification made for the individual elements revealed 5 clusters.

to detect the presence of VOCs in both the authentic EVOO samples and the adulterated samples. Chromatograms are presented in SI section (Figure S2). In total, 92 compounds were identified (Table S1, SI section); the compounds detected were classified as follows: organic acids (17), alcohols (14), esters (14), aldehydes (13), other organic compounds (12), ketones (6), phenols (4), hydrocarbons (4), nitrogen compounds (3), ethers (2), benzoic acid derivatives (2), and sulfur compounds (1) (Figure 2). As can be noted, the classes of VOCs identified and their degree of predominance in the samples are clearly in line with other studies reported in the literature[23,42-45] which pointed out the prevalence of organic acids, aldehydes, alcohols, esters, ketones, and other organic compounds in olive oil. Occurrence of organic compounds described in Figure 2 does not directly correlates with the actual percentage by mass of the compounds in the sample.

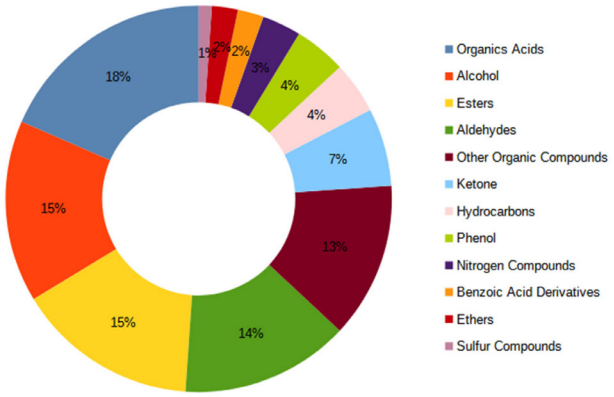

Figure 2. Degree of predominance/occurrence (in %) of volatile organic compounds identified according to their classification.

Markers of origin

The VOCs were also evaluated through the application of PCA. Two particular individuals made substantial contributions to the model construction, accounting for a significant portion (47.5%). In order to mitigate the influence of outliers, these specific samples (S8 and S9) were excluded from the analysis. Furthermore, the samples were simulated using variables (compounds) in a range of occurrence between 5 and 100%. Compounds present in only one sample (5%) were excluded from the PCA. The results obtained from the PCA are shown in Figure 3.

The PCA conducted in this study allowed us to differentiate the samples according to the country of origin. Samples from Argentina consisted of adulterated and authentic EVOO samples. Owing to the geographical proximity of the two regions, samples S2 and S3 from Mendozza exhibited characteristics similar to the Chilean samples (S11 and S12). The EVOO samples from Portugal and Spain were grouped under other cluster in the PCA (Figure 3).

The following compounds were detected in the EVOO samples: hexyl acetate, (*Z*)-pent-2-en-1-ol, (*E*)-hex-3-en-1-ol, (*E*)-hex-2-en-1-ol, (3*E*,5*E*)-octa-3,5-dien-2-one, methylsulfinylmethane, (*E*)-4-oxohex-2-enal, methyl benzoate, methyl 2-hydroxybenzoate, methylsulfonylmethane, and (*E*)-hex-2-enoic acid.

Cluster 3 consisted of the samples S2, S3, S10, S11, S12, S13, S14, S15 and S18. In these samples, no signs of adulteration were observed. The cluster was characterized by high levels of the variables methyl benzoate, (*Z*)-pent-2-en-1-ol, and pent-1-en-3-ol, and low values of the variables hexanal, 3-hydroxy-2-methylpyran-4-one,

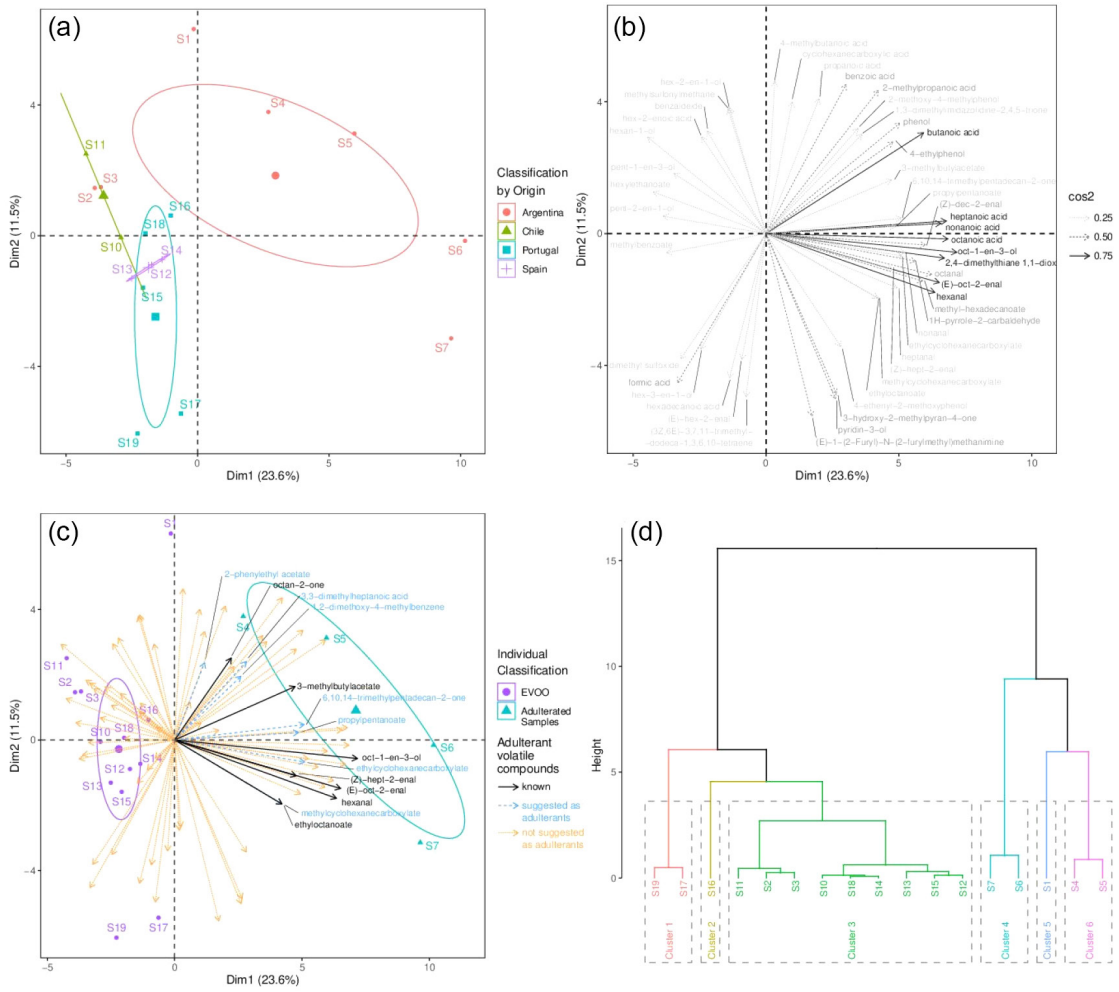

Figure 3. Multivariate data analysis of VOCs in EVOO samples. Individual factor map (PCA) with confidence ellipses around the origin (a); individual elements are colored according to their category for the qualitative variable origin. Variable factor map (PCA) of the VOCs in EVOO samples (b); variables with $\cos^2 \geq 0.25$ are labeled (tagged). Biplot of individual and variable factor map (PCA) with confidence ellipses around the EVOO samples results obtained from the fatty acids analysis (c); individual elements are colored according to their category for the qualitative variable results, individual elements previously reported and suggested as adulterants are labeled (tagged). Hierarchical clustering on principal components (HCPC) with ascending hierarchical classification of individual elements (d); the classification made for individual elements revealed 6 clusters.

butanoic acid, pyridin-3-ol, 2-methylpropanoic acid, phenol, (*E*)-1-(2-furyl)-*N*-(2-furylmethyl)methanimine, benzoic acid, and methyl hexadecanoate. Pent-1-en-3-ol was detected in samples from Argentina, specifically from the Mendozza region (S2 and S3), and from Santiago, Chile (S10 and S11)-these two regions are geographically very close to each other.

The (*E*)-hex-2-enal, (*E*)-hex-2-en-1-ol and hexan-1-ol were identified in the samples from Argentina, Chile, Spain and Portugal; these compounds are described as typical EVOO markers.[9,46-51]

Some compounds considered to be typically characteristic of EVOO were also found to be present in adulterated samples. The compounds (*E*)-hex-2-en-1-ol, (3*E*,5*E*)-octa-3,5-dien-2-one, and (*E*)-hex-2-enoic acid were detected in sample S5 (adulterated), which was found to be constituted by a mixture of soybean oil and EVOO (Table 1). The 2-methoxy-4-methylphenol was found in EVOO samples S1, S2 and in adulterated samples from Argentina, as well as in S19 from Portugal.

Cluster 1 consisted of individual samples such as S17 and S19 from Portugal. The samples grouped under this cluster did not exhibit any signs of adulteration; the most predominant compounds recorded in these samples included the following variables: (3*Z*,6*E*)-3,7,11-trimethyldodeca-1,3,6,10-tetraene, (*E*)-1-(2-furyl)-*N*-(2-furylmethyl) methanimine, pyridin-3-ol, hexadecanoic acid, 3-hydroxy-2-methylpyran-4-one, 2-ethylhexanoic acid, (1*R*,4*R*)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one and (*E*)-hex-2-enal. Cluster 3 consisted of individual samples including S01 and S19, which recorded high values for the variables dodecanoic acid, 6-methylhept-5-en-2-ol, 6-methylhept-5-en-2-one, 2,6-dimethoxy-4-methylphenol, cyclohexanecarboxylic acid, 3-methylbutanoic acid,

methylsulfonylmethane, 2-methylpropanoic acid, hexan-1-ol, butanoic acid, (*E*)-hex-2-en-1-ol, (2*S*,3*S*)-butane-2,3-diol, acetic acid, benzaldehyde and pentylcyclopropane.

Markers of adulteration

In the context of this sampling, twenty-five (25) VOCs investigated and identified as 3,3-dimethylheptanoic acid, hept-6-enoic acid, oct-1-en-3-ol, cycloheptanol, 3-methylbutyl acetate, propylpentanoate, ethyl hexanoate, 3-methylbutyl butanoate, ethyl 2-hydroxypropanoate, methyl cyclohexanecarboxylate, ethyl cyclohexanecarboxylate, ethyl octanoate, ethyl decanoate, 2-phenylethyl acetate, (*Z*)-hept-2-enal, (*E*)-oct-2-enal, (2*E*,4*E*)-deca-2,4-dienal, octan-2-one, (3-hydroxy-2,2,4-trimethylpentyl)2-methylpropanoate, heptan-2-one,4-methylphenol, 3-(hydroxymethyl)nonan-2-one, 2-methoxy-4-propylphenol, 6,10,14-trimethylpentadecan-2-one, and 1,2-dimethoxy-4-methylbenzene were found to be present only in adulterated samples S4-S9 from Argentina. The VOCs consisted of 10 esters, 4 ketones, 3 aldehydes, 2 organics acids, 2 alcohols, 2 phenols, 1 ether, and 1 other organic compound. Also, higher levels of octanoic acid were recorded in the adulterated samples.

Clusters 4 and 5 were constituted by samples that exhibited signs of adulteration. Cluster 4 consisted of individual samples including S4 and S5; this group was characterized by the presence of high levels of variables like 2-methoxy-4-methylphenol, 4-ethylphenol, propane-1,2,3-triol, benzoic acid, phenol, and 2-methoxyphenol, along with the following compounds which exhibited strong evidence of adulterating characteristics: 1,2-dimethoxy-4-methylbenzene, octan-2-one, 3,3-dimethylheptanoic acid, acetic acid, and 2-phenylethyl acetate. Cluster 5 consisted of individual samples including S7 and S8; this group exhibited high levels of variables like octanal, heptanoic acid, (*Z*)-dec-2-enal, methyl hexadecanoate, heptanal, and hexanal, along with the following compounds with adulterating characteristics: (*E*)-oct-2-enal, oct-1-en-3-ol and 2,4-dimethylthiane 1,1-dioxide. It is worth noting that out of the total of 25 VOCs found only in the adulterated samples, 10 compounds (hept-6-enoic acid, cycloheptanol, ethyl hexanoate, 3-methylbutyl butanoate, ethyl 2-hydroxypropanoate, ethyl decanoate, (2*E*,4*E*)-deca-2,4-dienal, 3-(hydroxymethyl)nonan-2-one, 4-methylphenol, 2-methoxy-4-propylphenol) were present only in one sample; considering that they were only present in 5% of the samples, these compounds were removed from the statistical analysis (Table S2).

Of the remaining 15 VOCs (3,3-dimethylheptanoic acid, oct-1-en-3-ol, 3-methylbutyl acetate, propyl pentanoate, methyl cyclohexanecarboxylate, ethyl cyclohexanecarboxylate, ethyl octanoate, 2-phenylethyl acetate, (*Z*)-hept-2-enal, (*E*)-oct-2-enal, (3-hydroxy-2,2,4-trimethylpentyl) 2-methylpropanoate, heptan-2-one, octan-2-one, 6,10,14-trimethylpentadecan-2-one, 1,2-dimethoxy-4-methylbenzene) which were found to be present in two or more adulterated samples, the volatile compound 2-phenylethyl acetate was only found in adulterated samples S4, S8 and S9. The 2-phenylethyl acetate is a volatile ester which is considered an aromatic/flavoring agent; this aromatic agent, which displays a flowery pink odor and a colorless oily liquid appearance, is commonly applied in order to provide flavor and aroma to foods and beverages.[52] There is evidence that this volatile ester was intentionally added during the adulteration of the oil.

Among the VOCs identified in the adulterated samples, (2*E*,4*E*)-deca-2,4-dienal, which is derived from vegetable oils as *per* their natural oxidation, was found in sample S5. As previously pointed out by Guillen and Goicoechea,[47] when EVOO is adulterated with other oils, such as sunflower oil, soybean oil and corn oil, it usually has its highest compositional percentages proportional to the oxidation levels of the oil added in it.

Studies reported in the literature[9,43,49,51,53-57] have already detected the presence of 3-methylbutyl acetate, heptan-2-one, octan-2-one, and oct-1-en-3-ol in adulterated EVOO samples; these VOCs were found in EVOO mixed with other oils such as olive oil, corn oil, soybean oil, sunflower oil, peanut oil, hazelnut oil, rapeseed oil, and safflower oil.

Esters, which are the predominant class of adulterant VOCs, are commonly formed during the refining stage of common oils, especially during deodorization, carried out at high temperatures. It should be noted that since high temperatures are not applied during the extraction and production process of EVOO, one would not expect to see a conceivably striking presence of esters in authentic EVOOs. In line with the observations of Kamikata *et al.*[10] Navratilova *et al.*[58] and others, considering that the adulterated EVOO samples investigated in the present study exhibited considerable amounts of esters, one can conclude that the samples were adulterated with refined oils.

The volatile compound (3-hydroxy-2,2,4-trimethylpentyl)2-methylpropanoate was found in only one sample (S6) with adulterating characteristics; there are no previous reports in the literature regarding the presence of this VOC in EVOO samples. This compound (3-hydroxy-2,2,4-trimethylpentyl)2-methylpropanoate has been detected in green tea, grapes, melon strains, and in the volatile composition of soybeans; thus, taking these observations into account, it appears that this compound

detected in sample S6 may have originated from soybean oil added in the EVOO sample.[59-62] Known for its mild odor, (3-hydroxy-2,2,4-trimethylpentyl)2-methylpropanoate can be considered a marker of EVOO adulteration, provided it is accompanied by other characteristic adulterating VOCs.

The VOC hexanal is a product derived from the oxidation of linoleic acid via lipoxygenase (LOX); considering that this VOC was found in all the adulterated EVOO samples (S4-S9), this clearly reflects its relationship with linoleic acid and EVOO adulteration with soybean oil. Hexanal is a flavoring/aromatic agent which is associated with the following notes: green, grass, fat, fresh oil, and green apple; thus, when the EVOO sample is adulterated with soybean oil, the aroma will not be drastically affected, and this helps conceal the adulteration.[23,63-65] Hexanal was identified in 3 EVOO samples from Portugal (S16, S17 and S19); this finding is line with the studies reported by Kiritsakis,[63] and Kalua *et al*.[65] which pointed out the existence of characteristic hexanal in oils of European origin.

Among the VOCs identified in the adulterated samples, the aldehydes (*Z*)-hept-2-enal, (*E*)-oct-2-enal and (2*E*,4*E*)-deca-2,4-dienal are considered flavoring/aromatic agents and reflect the presence of rancid and oxidized oils which are derived from the oxidation of unsaturated fatty acids; this observation is in agreement with the findings of Kanavouras *et al*.[64] Cecchi *et al*.[28] and Kalua *et al*.[65]

The compound ethyloctanoate found only in samples S7 and S9 is a flavoring/aromatic agent which is characterized by a fruity, floral, vintage and sweet odor. As reported by Vichi *et al*.[66] ethyloctanoate was identified in post-fermentation olives; thus, the presence of this compound may be associated with the fermentation of the olives used for the production of the EVOO sample.

The following VOCs were identified only in adulterated EVOO samples: 3,3-dimethyl heptanoic acid, propyl pentanoate, methyl cyclohexanecarboxylate, ethyl cyclohexanecarboxylate, 6,10,14-trimethyl pentadecan-2-one, and 1,2-dimethoxy-4-methyl benzene; these VOCs are flavoring/aromatic agents with fruity odor that are employed in the food industry along with 2-phenylethyl acetate, previously mentioned. Considering that the aforementioned VOCs have not yet been reported in the literature as possible markers of adulteration, they can be employed as markers of adulteration in parallel with other VOCs that are widely known to exhibit the distinctive characteristics that help distinguish authentic EVOOs from adulterated EVOOs (Table S2, SI section).

Finally, further studies need to be carried out in order to trace and identify other possible volatile compounds that are exclusively derived from vegetable oils, such as soybean oil, corn oil, and sunflower oil, so as to effectively evaluate their presence in adulterated EVOOs. Similarly, additional studies are also required in order to discover other aromatic compounds that are possibly added to camouflage the adulteration of EVOO with other oils of lower quality and commercial value.

Conclusions

The present study investigated the presence of adulteration in EVOO commercialized in the region around the triple border of Argentina, Brazil and Paraguay; the presence of adulteration was evaluated through the analysis of acidity, fatty acids profile, and VOCs. Out of the 19 EVOO samples investigated, 6 samples were found to have been adulterated with vegetable oil of lower commercial value. The authentic EVOO samples from Argentina, Chile, Spain and Portugal exhibited VOCs that were typically characteristic of EVOOs of high nutritional value.

Regarding the analysis of acidity, only four of the six adulterated EVOO samples exhibited content levels that were not in accordance with the limits stipulated in the legislation on olive oil; this result points to the outstanding analytical efficiency and suitability of the GC-FID technique when applied for the detection of adulteration in EVOO. For the analysis of volatile compounds found in only the adulterated samples, the results obtained showed that only seven VOCs (3,3-dimethyl heptanoic acid, propyl pentanoate, methyl cyclohexanecarboxylate, ethyl cyclohexanecarboxylate, 6,10,14-trimethyl pentadecan-2-one, 1,2-dimethoxy-4-methyl benzene, and 2-phenylethyl acetate) can be used as markers of adulteration.

The PCA analysis of the fatty acids present in the EVOO samples allowed us to effectively determine the sources of adulteration, where soybean oil was found as one of the major sources. The combined application of the analytical SPME-GC-MS data and PCA helped categorize the adulterated and non-adulterated samples and enabled us to distinguish the samples according to their country of origin.

Supplementary Information

Supplementary data, volatile organic compounds in the EVOO samples investigated (Table S1) and chromatograms (Figures S1 and S2), is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Marcela Boroski was responsible for conceptualization and project administration; Martha Belen Ramirez Cabrera and Leticia Maria Simião Santos for investigation; Luana Estefani Knaul for writing and data analysis; Priscila Maria Manzini Ramos for investigation and data analysis; André Luis Rüdiger and Marcelo Nepomoceno Kapp for performing the statistical analysis; Aline Theodoro Toci for evaluating the development of the SPME-GC method and review.

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