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Effect of cultivar and environment on chemical composition and geographical traceability of Spanish olive oils

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Abstract

This study aimed to investigate the influence of cultivar and environment on the chemical composition of Arbequina and Empeltre olive oils, and their contribution to geographical identification of olive oils from Aragon. A total of 260 olive oil samples from different cultivars (Arbequina, Empeltre, Royal de Calatayud, Alquezrana, and Royeta de Asque) from the three main oil-producing areas of Aragon, located in northeast Spain, were selected. Fatty acid and sterol composition were analyzed in the course of three crop years (2017, 2018, and 2019). Cultivar was found the main factor influencing the variability of palmitic, palmitoleic, and linolenic fatty acid content, whereas geographic origin was the main contributor to variation in oleic and linoleic fatty acids in Arbequina and Empeltre olive oils. Cultivar also had a significant impact on sterol composition, although the effect of the production area also showed a significant effect on these oils. Crop year showed limited relevance, except for oleic and linoleic fatty acids. The interaction between the environment (e.g., crop year and geographical factors) and the cultivar (Arbequina and Empeltre) exerted a significant influence on oleic/linoleic (O/L) ratio and Δ7-stigmastenol content, particularly in the southeast area of Aragon during the crop year with higher temperatures and drier conditions. Principal component analysis (PCA) and discriminant analysis (DA) confirmed the discriminative potential of the geographic production zone as a factor enabling the differentiation of olive oils from Aragon based on the major fatty acids and sterols.

KEYWORDS
chemical composition, cluster, fatty acids, geographical origin, olive oil, sterols

INTRODUCTION

In Mediterranean countries, the olive tree (*Olea europea* L.) is a traditional, essential crop whose extended history has led to wide genetic variability. In Spain, 668 different genotypes of 1273 accessions from 29 countries have been identified from the World Olive Germplasm Bank of Córdoba, Spain, by EST-SNP markers (Belaj et al., 2022).

Virgin olive oil is an olive juice obtained exclusively by physical processes. Triglycerides are its principal chemical compounds (about 98%), with monounsaturated oleic fatty acid (55%–83%) as its main component. Other minor, but no less significant sterols, phenols, aliphatic and terpenic alcohols, tocopherols, pigments, and volatile compounds make up the remaining 2% of the chemical composition of olive oil. Some of these compounds, such as oleic acid or the phenolic fraction, contribute to the multiple health benefits associated with the consumption of virgin olive oil (EFSA, 2011). Moreover, an olive oil's characteristic fatty acid and sterol composition forms the basis for its authenticity and fingerprint, to avoid its adulteration with other vegetable oils or animal fats of inferior quality or price (EU, 2022). The chemical composition that characterizes and differentiates virgin olive oils is the result of complex biochemical processes that develop in the fruit and its oil. Genotype has therefore been identified as one of the most influential factors in the variability of the chemical composition of olive oils (Uceda et al., 2005). In turn, the biosynthetic pathways are conditioned by other factors that influence the chemical composition of olive oils, namely agronomic, environmental (climatic/geographic), and technological factors (Mousavi et al., 2019; Navas-López et al., 2020; Rallo et al., 2018; Sevim et al., 2022).

Variability in the composition of olive oils due to these or other factors can play a relevant role in their geographical traceability. Geographic origin is inseparable from a region's soil-climate conditions and cultural agronomic practices. Geographical origin is an issue of economic importance in the case of oils protected under designations of origin (PDO) that benefit local and rural economies (EC, 2021).

Spain is the world's leading producer (2020/2021: 46%) and exporter (2020/2021: 41%) of olive oil (IOC, 2022). Aragon, a region located in northeast Spain with two Protected Designations of Origin (Aragon Government, 2009; Aragon Government, 2010) and a third one in process (Aragon Government, 2023a), is the country's sixth-largest virgin olive oil-producing region (2017/2018: 17,363 tons of olive oil) (MAFF, 2023). Olives growing in this region are mainly rainfed (74%) (MAFF, 2023); thus, climatic variables such as water availability or annual temperatures exert a strong influence on oil production. Empeltre and Arbequina are the main cultivars in this region, making up 67% and 21% of the crop, respectively (Aragon Government, 2023b). The rest are autochthonous minority varieties (e.g., Royal de Calatayud, Alquezrana) that are limited to certain geographical areas, to which they confer their own specific identities.

With the aim of contributing toward the geographical traceability of olive oils produced in Aragon (Spain), the present study aims to investigate varietal and geographical differences in their composition. For this purpose, we studied the fatty acid and sterol composition of virgin olive oils from three olive-growing areas over three consecutive harvests.

MATERIALS AND METHODS

Characterization of olive oil samples and geographical areas

Virgin olive oils ($n = 260$) produced in three recognized geographical areas ("northeast," $n = 87$; "west," $n = 74$; "southeast," $n = 99$) of the Aragon region (Spain) (Figure 1) were evaluated. The study was conducted over three consecutive crop seasons (2017, $n = 83$; 2018, $n = 94$; 2019, $n = 83$) to evaluate the effects of interannual variability, which is mainly associated with climatological variables.

Oil samples were supplied from oil mills and cooperatives located in the geographical areas we selected. Fertilization, cultural practices, and technological processes of oil production (two-phases olive oil mills) were the ones habitually applied in the surrounding production areas. In this study, we only used olive oils classified as extra virgin olive oil based on physico-chemical quality (acidity ≤0.8% oleic acid; peroxide value ≤20 meq O_2/kg; $K_{270} \leq 0.22$; $K_{232} \leq 2.50$) according to Annex I of European Commission Regulation (EEC) n° 2568/91 (EEC, 1991) and subsequent amendments.

We carried out a study of varietal and environmental effects on fatty acid and sterol composition of Arbequina ($n = 97$) and Empeltre ($n = 115$) oils from the three selected geographical areas. To characterize the olive oils according to their geographical traceability, we selected, in addition to Arbequina and Empeltre, several autochthonous minority cultivars from Aragon as Alquezrana, Royeta de Asque, and Royal de Calatayud, as well as also coupages of local cultivars from the same three areas.

We selected the geographical areas in view of their important olive growing tradition and because they are culturally and geographically well-defined. Oils produced in the "west" and "southeast" areas can be placed under Protected Designations of Origin (PDO), while those from the "northeast" area are in the process of being recognized as such if they comply with certain requirements (Aragon Government, 2009, 2010, 2023a).

The "northeast" olive-growing area (Figure 1, orange area) comprises 42 municipalities in the province of Huesca. In this region, due to its great diversity of local varieties, a wide range of single-varietal oils is produced. As these are minority varieties, the coupage oils produced in this area are characterized by combining many varieties with small-scale production. The local climate is classified as continental Mediterranean, characterized by warm summers and cool winters (López et al., 2007). The "southeast" olive-growing area includes 77 municipalities in two provinces: Zaragoza and Teruel. The main cultivars grown there are Empeltre (predominant) and Arbequina, traditionally unirrigated. Three climate types can be differentiated in that area: dry steppe, continental Mediterranean, and warm continental sub-Mediterranean (López et al., 2007), with a frequent phenomenon of "thermal inversion" in the winter, marked by episodes of thick fog. The "west" area, located in the province of Zaragoza, comprises 34 municipalities. Empeltre (predominant) and Arbequina are the main cultivars; as in the "southeast" area, they are traditionally dry-farmed. The climate is continental Mediterranean (López et al., 2007).

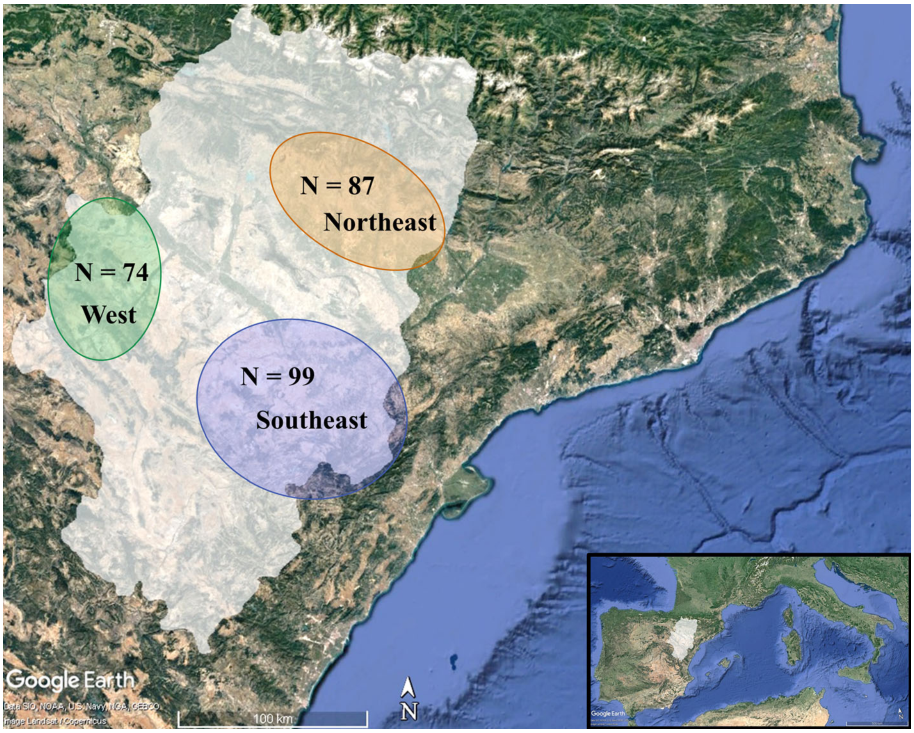

FIGURE 1 Geographical location in Aragon (Northwest Spain) of the three olive-growing areas used in the study.

TABLE 1 Meteorological data of three geographical areas of Aragon, Spain, selected for study during 2017, 2018, and 2019 crop years.

		Northeast (Barbastro[a])			West (Borja[a])			Southeast (Alcañiz[a])		
		2017	2018	2019	2017	2018	2019	2017	2018	2019
Tª annual	Average (°C)	14.4	14.4	14.6	14.7	14.4	14.8	15.6	15.5	16.0
Tª max	Average (°C)	27.6	27.0	27.9	27.5	26.2	28.4	22.8	21.9	23.0
Tª min	Average (°C)	1.5	2.9	2.4	2.3	3.1	2.3	8.9	9.7	9.3
Rainfall	Annual (mm)	473	699	442	332	546	365	223	508	216
Altitude	(m)	341			448			381		

[a] The data refer to the localities Barbastro, Borja, Alcañiz, which are the main cities of the indicated geographical areas.

The climate data recorded during the three crop years under study in the main cities of each selected geographical area are shown in Table 1. Data on monthly temperature (mean, maximum, minimum) and monthly rainfall were taken from the Agro-Climate Information System for Irrigation (SIAR) of the Spanish Ministry of Agriculture, Fisheries and Food (https://eportal.mapa.gob.es), as measured at meteorological stations located in Barbastro (northeast area), Borja (west area), and Alcañiz (southeast area).

Physico-chemical quality parameters

Free acidity, peroxide value, and ultraviolet absorption characteristics (K_{232}, K_{270}, and ΔK) were determined according to methods indicated in European legislation (EEC, 1991). Data not shown.

Fatty acid composition

Fatty acid composition of the oils was determined following the official EU method (EC, 2015). Fatty acid methyl esters (FAME) were prepared by transmethylation at room temperature, adding 0.2 mL of methanolic sodium hydroxide (2 mol/L) to 0.1 mg of oil. FAME analysis was performed by gas chromatography (GC), using an Agilent chromatograph (7890 N, Agilent, Santa Clara, CA, USA) with a capillary column SP-2380 (60 m × 0.25 mm inner diameter × 0.2 μm film thickness; Supelco, Bellefonte, PA, USA). We applied the following operating conditions: helium as a carrier gas (flow: 1.2 mL min^{-1}); oven temperature was 170°C (30 min), increasing by 5°C min^{-1} up to 200°C, and the flame ionization detector (FID) and split/spitless injector temperatures were 260 and 250°C, respectively.

Sterol composition

The oil samples were prepared and subjected to chromatographic analysis to determine their sterol composition, following the guidelines set by the European Commission implementing regulation (EU) No 2019/1604 (EC, 2019). For this purpose, the samples were saponified, purified using a elution mixture of n-hexane/ethyl ether 65:35 (vol/vol) through thin-layer chromatography and derivatized using a silanization reagent (pyridine/hexamethyldisilazane/trimethylchlorosilane in a ratio of 9:3:1, vol/vol/vol).Sterol derivatives (trimethylsilyl ethers) were subsequently analyzed by an Agilent gas chromatograph (GC) (6890, Agilent, Santa Clara, CA, USA) equipped with a split/splitless injector (injection volume: 1 μL; split ratio, 1:50; 285°C), a flame-ionization detector (FID) (300°C), and a CP-Sil 8 CB capillary column (25 m length × 0.25 mm inner diameter × 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). Helium was used as a carrier gas (flow: 1 mL min^{-1}) and the oven temperature was isothermal at 265°C.

Results were expressed as mg/kg oil for total sterol oil content and, for individual sterols, as a percentage (%).

Statistical analysis

All analyses were performed in duplicate, thus utilizing the arithmetic mean of two results for subsequent statistical analysis.

On all the single-varietal olive oils selected for this study ($n = 232$), we performed a descriptive analysis of the variability of principal fatty acids and sterols according to cultivar and crop year. Data were presented as means ± standard deviations, minimum, maximum, and coefficient variation. We then evaluated the effects of the cultivar (Arbequina and Empeltre), crop year (2017, 2018, and 2019), and geographical area (northeast, west, and southeast), along with their interactions, on principal fatty acids ($n = 212$) and sterols ($n = 94$) by applying multivariate factorial analysis of variance (three-way ANOVA; $p < 0.05$). Scheffe post hoc test ($p < 0.05$) was used to determine the existence of significant pairwise differences between the groups.

Principal component analysis (PCA) was performed on Arbequina and Empeltre monovarietal oils ($n = 94$), using the fatty acid and sterol composition, in order to demonstrate the differentiation capacity of geographical production area and to determine chemical composition according to geolocation of a single-variety olive oil.

Geographic traceability was studied using a statistical procedure based on canonical discriminant analysis (DA) applied to fatty acids and sterols of single-varietal and coupage olive oils characteristic of the three geographical areas ($n = 138$). We extracted the most discriminating independent variables among the olive-growing areas by applying the stepwise method, using Wilks' lambda and its chi-square approximation as the exclusion method. Snedecor's F statistic was used as the selection criterion. The model's predictive capability was estimated using the leave-one-out cross-validation method.

Statistical analyses were carried out using IBM SPSS Statistics 24.0 software (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

We studied three consecutive annual crops of the selected olive oils in order to include the season effect, understood as the influence of various abiotic factors such as temperature, humidity, and agronomic practices, among others, depending on the production year. Even with this premise, the variability study of the chemical composition of certain minority local cultivars over the three crop years in this Spanish area, Aragon, proved to be a complex undertaking. Certain data could not be obtained because the sample number was too small. Certain varieties, such as Royeta de Asque or Alquezrana, are only produced in the northeast area. Royal de Calatayud (also known with the synonym "Verdeña" in the northeast zone) is often marketed in the west zone as a coupage oil, although it can likewise be found in the northeast zone as a single-variety oil.

The selected coupage oil samples were only used for the geographical traceability study, as they were considered oils bearing the identity of the production area from which they stemmed. They were not included in the fatty acid and sterol variability studies (descriptive statistics and ANOVA).

Variability in fatty acid composition: Contributing factors

The composition of the principal fatty acids presents in Arbequina, Empeltre, and other single-varietal olive oils from the three olive-growing areas under study, along with their variability (CV%), is shown in Table 2. The acid profile of Arbequina and Empeltre olive oils is in line with that described by other authors for these two varieties (Gracia et al., 2009; Romero et al., 2011). Although they show significant differences in their fatty acid composition (Table 2), both have medium-high palmitic and linoleic and medium oleic content (Uceda et al., 2005). Minority cultivar oils we studied, such as Alquezrana, Royeta de Asque, and Royal de Calatayud, had lower amounts of palmitic and linoleic acid than the aforementioned monovarietals, but higher amounts of oleic acid, which meant much higher O/L ratios, thereby implying that they are nutritionally healthier for the cardiovascular system (Covas et al., 2009).

TABLE 2 Statistical summary of the chemical composition analysis (fatty acids and sterols) of virgin olive oils from the Arbequina, Empeltre, Alquezrna, Royeta de Asque and Royal de Calatayud varieties from three geographical areas of Aragon over three consecutive years ($n = 232$ samples).

		Fatty acid composition											
		16:0 (%)			**16:1 (%)**			**18:0 (%)**			**18:1 (%)**		
Cultivar	**Year**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**
Arbequina ($n = 97$)	2017	13.84 ± 0.96	10.98–15.44	7	1.37 ± 0.17	0.87–1.65	12	1.97 ± 0.14	1.77–2.31	7	71.35 ± 1.61	68.20–75.37	2
	2018	13.15 ± 1.00	10.85–14.89	8	1.16 ± 0.21	0.78–1.62	18	2.01 ± 0.20	1.67–2.43	10	73.48 ± 2.08	69.19–76.88	3
	2019	13.70 ± 1.01	11.33–15.58	7	1.27 ± 0.21	0.87–1.88	17	1.97 ± 0.12	1.72–2.28	6	72.13 ± 2.25	66.48–75.12	3
Empeltre ($n = 115$)	2017	12.56 ± 1.13	10.88–15.05	9	1.09 ± 0.19	0.83–1.52	17	1.94 ± 0.17	1.70–2.34	9	71.59 ± 2.51	68.26–76.30	4
	2018	12.24 ± 1.12	10.56–15.88	9	1.02 ± 0.17	0.76–1.33	17	1.95 ± 0.17	1.68–2.42	9	73.20 ± 1.92	69.40–76.89	3
	2019	12.82 ± 0.91	11.27–15.00	7	1.06 ± 0.16	0.85–1.39	15	1.90 ± 0.15	1.65–2.22	8	72.15 ± 2.05	68.04–75.99	3
Alquezrana ($n = 5$)	2017–2019	11.63 ± 0.57	11.00–12.52	5	0.95 ± 0.08	0.81–1.00	9	2.14 ± 0.21	1.94–2.49	8	77.19 ± 2.10	74.74–79.77	3
Royeta de Asque ($n = 7$)	2017–2019	11.31 ± 0.50	10.69–12.22	4	0.87 ± 0.09	0.78–1.02	11	2.84 ± 0.38	2.45–3.38	13	76.84 ± 1.69	74.56–78.98	2
Royal de Calatayud ($n = 8$)	2017–2019	12.16 ± 0.53	11.23–12.74	4	0.99 ± 0.08	0.90–1.12	9	2.08 ± 0.18	1.87–2.43	9	76.04 ± 1.11	74.42–78.02	1

		Fatty acid composition								
		18:2 (%)			**18:3 (%)**			**O/L ratio**		
Cultivar	**Year**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**
Arbequina ($n = 97$)	2017	9.78 ± 0.85	7.53–11.09	9	0.51 ± 0.04	0.45–0.62	8	7.37 ± 0.80	6.20–9.60	11
	2018	8.48 ± 1.20	7.13–11.48	14	0.51 ± 0.05	0.43–0.67	10	8.85 ± 1.35	6.00–10.60	15
	2019	9.19 ± 1.33	6.78–12.63	14	0.53 ± 0.05	0.42–0.66	10	8.04 ± 1.33	5.30–11.10	17
Empeltre ($n = 115$)	2017	10.90 ± 1.43	8.07–13.41	13	0.69 ± 0.09	0.44–0.83	14	6.72 ± 1.16	5.20–9.50	17
	2018	9.63 ± 1.08	7.91–12.40	11	0.69 ± 0.08	0.58–0.86	11	7.72 ± 1.07	5.70–9.70	14
	2019	10.16 ± 1.49	7.27–12.83	15	0.69 ± 0.08	0.57–0.85	11	7.29 ± 1.31	5.40–10.40	18
Alquezrana ($n = 5$)	2017–2019	6.35 ± 1.47	4.60–8.39	23	0.55 ± 0.06	0.51–0.64	11	12.76 ± 3.26	8.9–17.3	26
Royeta de Asque ($n = 7$)	2017–2019	6.37 ± 1.01	5.29–7.75	16	0.51 ± 0.02	0.48–0.54	5	12.35 ± 2.14	9.6–14.9	17
Royal de Calatayud ($n = 8$)	2017–2019	6.89 ± 0.74	5.67–7.96	11	0.55 ± 0.04	0.51–0.62	8	11.17 ± 1.40	9.4–13.8	13

		Sterol composition								
		Total sterols[a] (mg/kg)			**Campesterol[a] (%)**			**Stigmasterol[a] (%)**		
Cultivar	**Year**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**
Arbequina ($n = 34$)	2017	1355 ± 126	1132–1548	9	3.33 ± 0.17	3.05–3.58	5	0.76 ± 0.09	0.65–0.91	11
	2018	1237 ± 131	1101–1542	11	3.22 ± 0.19	2.89–3.49	6	0.85 ± 0.10	0.66–1.01	12
	2019	1330 ± 164	1112–1671	12	3.30 ± 0.16	3.09–3.61	5	0.91 ± 0.16	0.67–1.27	18
Empeltre ($n = 60$)	2017	1488 ± 141	1263–1795	9	2.94 ± 0.12	2.80–3.41	4	0.68 ± 0.15	0.40–0.95	22
	2018	1490 ± 227	1180–1918	15	2.92 ± 0.07	2.76–3.04	2	1.00 ± 0.35	0.57–1.90	35
	2019	1342 ± 143	1119–1571	11	2.85 ± 0.13	2.66–3.07	5	1.01 ± 0.34	0.60–1.73	34

(Continues)

TABLE 2 (Continued)

		Sterol composition								
		Total sterols[a] (mg/kg)			Campesterol[a] (%)			Stigmasterol[a] (%)		
Cultivar	**Year**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**
Alquezrana (*n* = 4)	2017–2019	1334 ± 151	1116–1457	11	3.15 ± 0.14	3.03–3.34	4	1.27 ± 0.36	0.76–1.53	29
Royeta de Asque (*n* = 5)	2017–2019	1114 ± 59	1028–1191	5	2.61 ± 0.11	2.47–2.77	4	0.61 ± 0.47	0.29–1.43	77
Royal de Calatayud (*n* = 7)	2017–2019	1223 ± 133	1103–1497	11	2.93 ± 0.10	2.75–3.05	3	1.35 ± 0.45	0.67–1.97	33

		Sterol composition								
		β-sitosterol (%)			Δ5-avenasterol (%)			Δ7-stigmastenol[a] (%)		
Cultivar	**Year**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**	**Mean ± SD**	**Min–Max**	**CV%**
Arbequina (*n* = 34)	2017	74.86 ± 2.54	70.32–78.80	3	16.93 ± 2.63	12.80–21.44	16	0.21 ± 0.08	0.12–0.37	36
	2018	73.08 ± 3.29	66.12–77.06	5	18.76 ± 3.32	14.48–25.81	18	0.20 ± 0.07	0.08–0.30	35
	2019	73.58 ± 2.72	68.88–77.87	4	17.64 ± 2.36	14.39–21.44	13	0.17 ± 0.04	0.10–0.26	26
Empeltre (*n* = 60)	2017	83.94 ± 1.35	81.79–86.80	2	7.76 ± 1.32	4.90–10.10	17	**0.66 ± 0.09**	0.44–**0.85**	14
	2018	83.42 ± 1.88	80.05–86.86	2	8.35 ± 1.51	5.38–10.76	18	0.49 ± 0.08	0.36–**0.66**	17
	2019	83.19 ± 2.19	79.69–85.89	3	8.05 ± 1.83	5.69–10.96	23	**0.63 ± 0.10**	0.46–**0.74**	16
Alquezrana (*n* = 4)	2017–2019	76.71 ± 1.12	75.11–77.71	1	14.59 ± 1.39	13.25–16.51	10	0.23 ± 0.09	0.16–0.36	40
Royeta de Asque (*n* = 5)	2017–2019	72.71 ± 2.06	70.95–76.24	3	18.50 ± 1.88	15.21–19.89	10	0.28 ± 0.06	0.21–0.35	21
Royal de Calatayud (*n* = 7)	2017–2019	80.12 ± 1.03	78.79–81.66	1	10.92 ± 1.43	9.62–13.28	13	0.29 ± 0.04	0.24–0.35	16

Note: Values in bold: non-compliant.

Abbreviations: 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; CV, coefficient of variation 16:0, palmitic acid; Max, maximum; Min, minimum; O/L ratio, oleic acid/linoleic acid ratio; SD, standard deviation.

[a] Limits established by the current EU/IOC regulatory for OOV: total sterols ≥1000 mg/kg; campesterol ≤4.0%; stigmasterol ≤ campesterol; Δ7-stigmastenol ≤0.5%.

TABLE 3 Analysis of variance (F-values) by three-way ANOVA with significance levels[†] and comparison of means by crop years (2017, 2018, and 2019), cultivar (Arbequina and Empeltre), and geographical area (northeast, west, and southeast) for the fatty acid composition ($n = 212$ samples).

	Parameters						
Variability source	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**O/L ratio**
Crop year (Y)	4*	10*	0	24***	30***	0	33***
Cultivar (C)	52***	58***	3	14***	6*	173***	2
Geographical area (A)	34***	22***	1	95***	81***	48***	81***
Y x C	1	1	1	0	3*	0	2
Y x A	1	2	3*	2	5***	1	7***
C x A	3*	4*	5**	5**	7**	6**	6**
Y x C x A	1	1	3*	2	6**	0	5**
Crop year							
2017	13.10^{a}	1.20^{a}	1.96	71.51^{a}	10.40^{a}	0.62	7.02^{a}
2018	12.67^{b}	1.09^{b}	1.97	73.33^{b}	9.09^{b}	0.61	8.25^{b}
2019	13.20^{a}	1.15^{ab}	1.94	72.11^{c}	9.79^{c}	0.62	7.57^{c}
Cultivar							
Arbequina	13.54^{a}	1.26^{a}	1.99	72.37^{a}	9.13^{a}	0.52^{a}	8.12
Empeltre	12.51^{b}	1.05^{b}	1.93	72.31^{b}	10.27^{b}	0.69^{b}	7.22
Geographical area							
Northeast	13.45^{a}	1.22^{a}	1.97	72.98^{a}	8.64^{a}	0.54^{a}	8.59^{a}
West	12.31^{b}	1.06^{b}	1.98	74.03^{b}	8.90^{a}	0.55^{a}	8.42^{a}
Southeast	13.22^{a}	1.17^{a}	1.94	70.82^{c}	10.89^{b}	0.70^{b}	6.59^{b}

Note: Different letters values (a–c) indicate significant differences (Scheffe test, $p < 0.05$) for each parameter. Absence of letters: no significant differences between means were found.

Abbreviations: 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; O/L ratio, oleic acid/linoleic acid ratio.

[†] Significance levels: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

In the main cultivars (Arbequina and Empeltre), the fatty acids with the highest variability (CV%) were palmitoleic acid (16:1), followed by linoleic acid (18:2), and linolenic acid (18:3) but, in the minority monovarietals, 18:2 was the most variable fatty acid. On the other hand, oleic acid displayed less variability in all olive oils. The variability results in Empeltre coincide with those obtained in a controlled trial carried out on an experimental farm in the same crop years (Rey-Giménez & Sánchez-Gimeno, 2022a). They also coincide with those shown by Navas-López et al. (2020) in nine varieties, one of them being Arbequina. The high variability in polyunsaturated fatty acids observed in each season could be caused by different harvesting dates for the olives that were used to produce the oils under study (Beltrán et al., 2004).

The varietal factor has been described in many studies as the main contributor to the variability of principal fatty acids in olive oils (Faci et al., 2021; Navas-López et al., 2020). The results obtained in our research indicated that the geographical location of the production area plays a role that is thoroughly relevant, and which is different for each fatty acid. Studies that analyze the environment, identified as location or as a location-crop year combination, together with the cultivar or other factors (Navas-López et al., 2020), corroborate the importance of the location factor and the need to study it further in depth. In our ANOVA study of Arbequina and Empeltre olive oils, we found that the variability of palmitic and linolenic fatty acids (particularly of the latter) was mainly affected by the cultivar, whereas the olive-growing area was a secondary factor (Table 3). The crop year factor was minimally significant for palmitic acid, and its effect was null for linolenic acid. Oleic and linoleic acids displayed different behavior: the location of the production area was the most influential factor in their variability, although the year of production was also important. According to the data in Table 3, the significantly higher linoleic (10.89%) and lower oleic (70.82%) content of the oils from the southeast area could be related to higher drought and temperature stress during oil biosynthesis in that production area (monthly data not shown). These "southeast" oils also had higher linolenic acid content, which, together with their higher amount of linoleic acid, makes them more polyunsaturated (O/L ratio: 6.59%) than the oils from the "west" and "northeast" areas. The oils from the "west" area stood out for their lower palmitic and palmitoleic content and higher oleic acid content. Several authors (Contreras et al., 2023;

Mousavi et al., 2019; Navas-López et al., 2020) have obtained results indicating that temperature exerts a regulatory effect on fatty acid synthesis. High temperatures during olive ripening would lead to a decrease in oleic acid and an increase in linoleic acid, although this effect would depend on the genotype. This hypothesis serves to explain the significant effect we observed in terms of inter-annual variability, mainly due to climatic variables. The oils produced in 2018 had higher oleic but lower palmitic and linoleic acid contents. As a result, these oils from crop year 2018 presented the highest O/L ratio. Thoroughly similar results were observed in a controlled trial on Empeltre carried out during the same period in Alcañiz, a principal town located in the "southeast" area (Rey-Giménez & Sánchez-Gimeno, 2022a).

For all fatty acids, the interaction between cultivar and production area was significant, and in some of them, it was the only significant interaction between factors. This interaction may be due to different local cultural practices according to the variety, as in the case of Empeltre in the southeast area. Empeltre olives are harvested at more advanced stages of ripening because of their dual aptitude (table and oil). This practice gives Empeltre oils their "sweet, almondy" taste as listed in the specifications of the Protected Designation of Origin "Aceite del Bajo Aragón" (Aragon Government, 2009).

Arbequina and Empeltre oils responded in equal measure to annual variation (Y x C) in all fatty acids except for linoleic acid, where the interaction was significant. However, in all acidic compositions, the response according to cultivar varied geographically (C x A). In the oil-producing areas we studied, varying responses were also found in linoleic acid to annual variation (Y x A), as well as in the triple interaction among the factors under consideration (Y x C x A).

Variability of sterol composition: Contributing factors

Over three consecutive crop years, we studied the variability of sterol composition in 110 single-varietal oils produced in the three selected olive-growing areas in Aragon (Table 2). Empeltre oils had the lowest relative content in Δ5-avenasterol, but the highest in β-sitosterol and Δ7-stigmastenol compared to the other single-variety oils. At the opposite extreme were the Arbequina and Royeta de Asque oils, which differed from each other, and also from the other oils, in their campesterol percentages, which were higher in Arbequina but lower in Royeta de Asque. The latter also had very low sterol content.

All oils we analyzed complied with the limits established by European legislation and the IOC trade standard (EEC, 1991; IOC, 2022a, 2022b) regarding purity criteria for sterol composition (data not shown), except for Δ7-stigmastenol. Thirty-three percent of the oils had a Δ7-stigmastenol content higher than 0.5% (EEC, 1991; IOC, 2022a, 2022b), of which 29% were Empeltre single-varietal oils ($n = 60$) and the rest were coupage oils ($n = 28$) in which the Empeltre variety was predominant (individual sample data not shown; average Δ7-stigmastenol values higher than 0.5% in bold, Table 2). High Δ7-stigmastenol values that compromise the authenticity of single-varietal olive oils have been described in Empeltre (Gracia et al., 2009; Rey-Giménez & Sánchez-Gimeno, 2022b; Romero et al., 2011) as well as in other varieties (Manai–Djebali et al., 2012; Sevim et al., 2022). These findings could support the hypothesis that a high Δ7-stigmastenol content is characteristic of certain cultivars.

Considerable differences can be noted in the variability (CV%) of each sterol (Table 2). Stigmasterol, Δ5-avenasterol, and Δ7-stigmastenol exhibited much higher coefficients of variation than campesterol and β-sitosterol. The variability of the two latter sterols even remained minimal in the minority cultivar oils, whose results were not segregated by year as there were not enough samples. These variabilities coincide with those described in previous studies on Empeltre (Rey-Giménez & Sánchez-Gimeno, 2022b), Arbequina, and other cultivars (Navas-López et al., 2020).

ANOVA analysis performed on Empeltre and Arbequina oils showed that cultivar is the main factor contributing to variability in the main sterols (Table 4). This is in agreement with the study by Navas-López et al. (2020) on the variability of β-sitosterol and Δ5-avenasterol. The effect of cultivar was clear and significant for all sterols except for stigmasterol, on which other factors had effects of comparable magnitude. This result reinforces the hypothesis that high Δ7-stigmastenol content is a varietal characteristic, analogous to the high campesterol content of the Cornicabra variety (Rivera del Álamo et al., 2004). The significant differences in Δ7-stigmastenol between the Arbequina (0.19%) and Empeltre (0.59%) varieties are shown in Table 4. Meanwhile, in a study on Arbequina and Coratina from different regions of Argentina, Torres et al. (2022) observed that the growth environment exerted a more pronounced influence than cultivar on the variability of β-sitosterol, stigmasterol, campesterol, and total sterols. In our results, the influence of the growth environment on total sterols was likewise more important than the other factors, or had a similar effect to cultivar on stigmasterol, although the effect was always significant in all sterols. Warmer environments would tend to increase total sterol content according to some authors (Li et al., 2019; Mailer et al., 2010; Torres et al., 2022); in our case, this would be reflected in the "southeast" oils, which had higher sterol concentrations. Low relative humidity and high annual temperatures would tend to increase Δ7-stigmastenol content (Sevim et al., 2022), as would a warmer growing environment (Li et al., 2019).

TABLE 4 Analysis of variance (F-values) by three-way ANOVA with significance levels[†] and comparison of means by crop years (2017, 2018, and 2019), cultivar (Arbequina and Empeltre), and geographical area (northeast, west, and southeast) for the sterol composition ($n = 94$ samples).

	Parameters					
Variability source	**Sterols**[††]	**Campesterol**[††]	**Stigmasterol**[††]	**β-sitosterol**	**Δ5-avenasterol**	**Δ7-stigmastenol**[††]
Crop year (Y)	3	3	8**	3	3	6**
Cultivar (C)	4*	120***	14***	335***	375***	353***
Geographical area (A)	7**	4*	10***	7**	4*	4*
Y x C	3	1	3	4*	3*	7**
Y x A	1	1	1	2	2	1
C x A	6**	0	9***	6**	4*	2
Y x C x A	1	2	2	0	0	3*
Crop year						
2017	1451	3.06	0.70^{a}	81.09	10.66	0.52^{a}
2018	1410	3.01	0.96^{b}	80.17	11.62	0.40^{b}
2019	1325	3.06	0.91^{b}	79.01	12.31	0.43^{b}
Cultivar						
Arbequina	1303^{a}	3.28^{a}	0.82^{a}	73.83^{a}	17.84^{a}	0.19^{a}
Empeltre	1460^{b}	2.91^{b}	0.86^{b}	83.70^{b}	7.95^{b}	**0.59^{b}**
Geographical area						
Northeast	1270^{a}	3.21^{a}	0.97^{a}	75.42^{a}	15.92^{a}	0.26^{a}
West	1357^{b}	2.95^{b}	0.84^{b}	80.69^{b}	10.97^{b}	0.48^{b}
Southeast	1528^{c}	3.01^{b}	0.77^{b}	82.90^{c}	8.96^{c}	**0.55^{c}**

Note: Values in bold: non-compliant.

[†] Significance levels: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Not significant: ns. Different letters values (a–c) indicate significant differences (Scheffe test, $p < 0.05$) for each parameter. Absence of letters: no significant differences between means were found.

[††] Limits established by the current EU/IOC regulatory for OOV: total sterols ≥1000 mg/kg; campesterol ≤4.0%; stigmasterol ≤ campesterol; Δ7-stigmastenol ≤0.5%.

The "southeast" production area exhibited the highest content of this sterol, which may be due to a climate that is drier and warmer than the two other zones under study. This would also corroborate the findings of Sevim et al. (2022). On the other hand, rainfall in 2017 and 2019 was low; however, as opposed to 2019, rainfall in 2017 did not occur during the period of lipogenesis. That could be the reason for the higher Δ7-stigmastenol content of the 2017 oils. Regarding the influence of the environment on sterols, positive results have been reported in other publications, although most of them did not assess other factors simultaneously (Bajoub et al., 2015; Mailer et al., 2010). Significant interactions noted in our results suggest that the intensity of the response of certain individual sterols to the environment (geolocation and year of production) would depend on the cultivar.

Olive oil classification according to geographical origin

Cultivar was one of the factors that exerted the greatest influence (Table 3; Table 4) on most sterols and fatty acids. However, the variability associated with the production area could sufficiently support a differentiated chemical composition of olive oils that would also allow them to be classified according to their geographical origin.

To demonstrate this hypothesis, we carried out a principal component analysis (PCA) according to the geolocation of the oils of the main varieties, Arbequina ($n = 34$) and Empeltre ($n = 60$), basing ourselves on the fatty acid and sterol composition of the single-variety oils obtained during the three consecutive crop years. Figure 2 shows the PCA biplots obtained for the two cultivars. The two principal components explained 65.9% of total variance for Arbequina oils and 70.8% for Empeltre. No grouping by geographical area according to scores was observed in the case of Arbequina, whereas in Empeltre three clusters (northeast, west, and southeast) were clearly differentiated (Figure 2b). This would support the importance of the geographical factor (Table 3 and Table 4), in the Empeltre oils. Therefore, in this scenario, the influence of the geographical factor depends on the cultivar. In the PCA analysis of Empeltre oils, PC1 (53.21%) differentiated the cluster formed by the oils from the southeast zone from the northeast and west zones. Figure 2b thus shows that the oils from the southeast cluster displayed higher

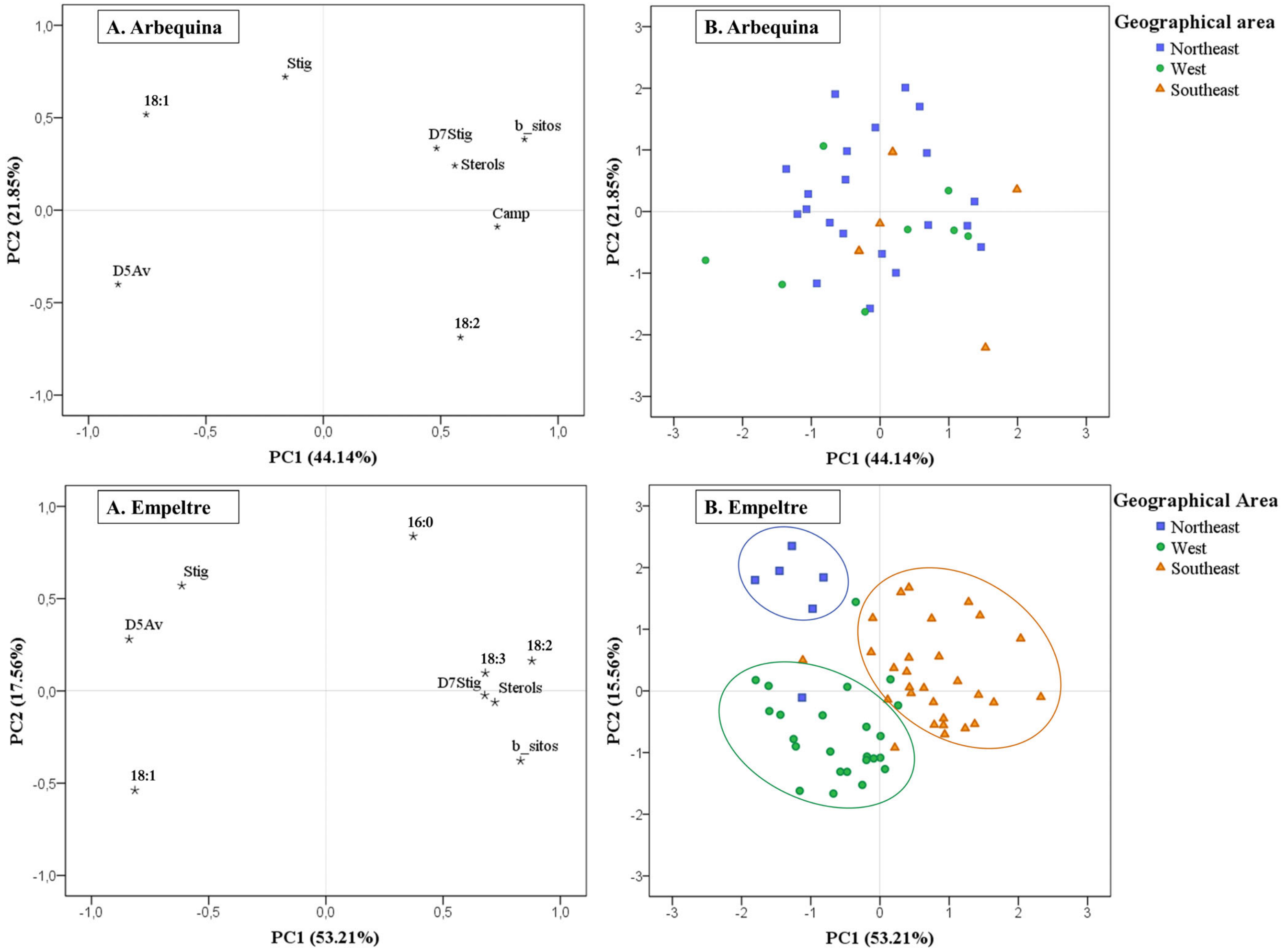

FIGURE 2 Principal component analysis (PCA) loadings (a) and scores (b) biplots for fatty acid and sterols in Arbequina and Empeltre olive oils from three different geographical areas. 16:0, palmitic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; Sterols, total sterols; Camp, campesterol; b_sito, β-sitosterol; D5Av, Δ5-avenasterol; D7Stig, Δ7-stigmastenol; Stig, stigmasterol.

contents of Δ7-stigmastenol (D7Stig), β-sitosterol (b_sito), and total sterols (Sterols), as well as of linoleic (18:2), and linolenic (18:3) fatty acids, but lower contents of Δ5-avenasterol (D5Av), stigmasterol (Stig), and oleic acid (18:1). According to this, Empeltre oils from the southeast part of Aragon would have a higher Δ7-stigmastenol content than Empeltre oils in the other two olive-growing areas under study. Also, they are characterized by a higher content in polyunsaturated fatty acids. Oleic acid correlated negatively with the two main components (PC1, PC2): thus, Empeltre oils from the western cluster would be characterized by higher percentages of oleic acid (18:1). Finally, Empeltre oils from the northeast cluster would be characterized by higher contents of stigmasterol and Δ5-avenasterol.

The olive oils marketed in Aragon are not exclusively Arbequina and Empeltre monovarietal oils, but include other minority varieties as well as coupages made from the characteristic olive production of each region. To demonstrate the differentiating power of the regional factor in olive oils based on their fatty acid and sterol composition, we subjected 138 representative samples of the three geographical areas under study to a classification test using canonical discriminant analysis (DA). Two canonical discriminant functions were obtained with 100% of the total discriminant power (Figure 3). This finding corroborated the differentiation of oils produced in Aragon into the three clusters defined by PCA in Empeltre oils through eight parameters that were finally selected as discriminant variables: palmitic, oleic, linoleic and linolenic fatty acids, the three sterols β-sitosterol, Δ5-avenasterol, and Δ7-stigmastenol, as well as total sterol content. Figure 3, which represents the two canonical functions on the plane, shows the separation of the three geographical areas according to the oils' chemical composition. The classification functions thereby obtained were capable of correctly assigning 91.3% of the samples. Meanwhile, 88.4% of the olive oils we had analyzed were

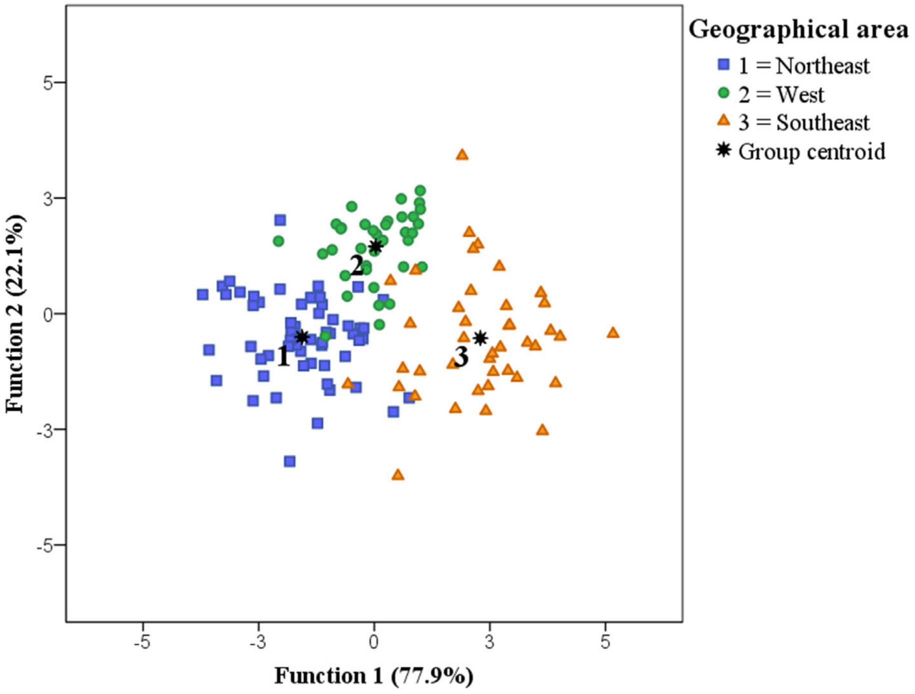

FIGURE 3 Canonical discriminant functions plot obtained to predict the geographical area origin of olive oils.

correctly assigned in the cross-validation procedure (northeast, 91.8%; west, 88.9%; southeast, 82.9%).

CONCLUSION

Cultivar was the factor which exerted the greatest influence upon the variability of chemical composition of olive oils from three oil-producing areas in Aragon. This finding would support the hypothesis of high Δ7-stigmastenol content (greater than 0.5%) obtained in Empeltre oils (29% of the total) as a varietal characteristic. The area of origin mainly affected oleic and linoleic acid content and was significant in all parameters under study. Moreover, significant interaction effects indicated that variability in sterol composition associated with the environment (geolocation and year of production) would depend on the cultivar. PCA analysis showed that the differentiation that the geographic area would bring to the olive oils' chemical composition allows for Empeltre oils to be classified according to their origin but is not sufficient to classify Arbequina oils. This would confirm the importance of environmental influence depending on the cultivar. Our discriminant analysis correctly classified 91.3% of the olive oils from the three studied areas of Aragon, Spain.

AUTHOR CONTRIBUTIONS

Raquel Rey-Giménez a and Ana Cristina Sánchez-Gimeno b conceived and designed the study, Raquel Rey-Giménez a wrote the first draft of the manuscript, Raquel Rey-Giménez a carried out the research, Raquel Rey-Giménez a analyzed the data and Ana Cristina Sánchez-Gimeno b reviewed the manuscript. All authors contributed to and approved the final draft of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

No human or animal subjects were used in the course of the research for this study.

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