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Genotype and Environment Effects on Phytosterol and Tocopherol Contents in Almond Kernel Oil

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Abstract: The phytosterol and tocopherol contents of almond kernel oil were evaluated in three almond cultivars, including the Spanish cultivar 'Marcona' and the French cultivars 'Ferragnès' and 'Ferraduel', grown under two different agro-climatic conditions, Zaragoza (Spain) and Meknès (Morocco). The analysis of variance showed significant effects of the genotype on kernel phytosterol content, Δ^5-Avenasterol, Δ^7-Campesterol, Stigmasterol and on the three tocopherol isomers. The location effect was significant on oil and kernel phytosterol contents, β-sitosterol,Δ^5-avenasterol, Δ^7-stigmastenol, Δ^7-campesterol and α- and γ-tocopherol. The highest value of kernel and oil phytosterol contents were observed at Meknès (1.48 g kg^{-1} and 2.54 g kg^{-1}, respectively), as compared to those obtained at Zaragoza (1.31 g kg^{-1} and 2.54 g kg^{-1}, respectively).The highest values ofβ-sitosterol and Δ^5-avenasterol were obtained at the Zaragoza location (81.93% and 10.55% of total phytosterols, respectively).The highest value of α-tocopherol was observed in the Morocco location (496 mg kg^{-1} oil). These results indicate that under warm climate conditions in Morocco, the almond cultivars tend to accumulate more phytosterol and tocopherol. However, the significance of the genotype × location interaction on the phytosterol and tocopherol contentindicates that the magnitude of variation in these traits mainly depends on the genotype.

Keywords: *Prunus dulcis*; sterols; tocopherol; cultivar; climate; variability

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1. Introduction

Almond [*Prunus dulcis* (Mill.) D.A. Webb] is a major tree nut grown in areas of the Mediterranean climate. Almonds are consumed raw, roasted, blanched, unblanched, and alone or mixed with other foods; in addition, kernel pieces (slices, sticks, dices) are used in different confectioneries. Almonds may also be consumed fresh when the seed is filled but before ripening. Once mature, harvested kernels may be processed into many different kinds of 'turrón' (nougat), marzipan and almond milk [1]. In addition, almond oil is widely utilized in the pharmaceutical and cosmetic industries because of its chemical stability and versatility [2]. Each use requires kernels with a specific composition of fatty acids, proteins, sugars and related phytochemicals [3]. The presence of natural anti-oxidants in almond kernels is determinant of almond quality and health benefit for human body [4,5]. The knowledge of the chemical composition in almond kernels would allow for establishing not only quality criteria, but also consumption criteria, due to the incidence of some compositional parameters on the nutritional and healthy values of almond kernels [6].

Almond is consumed on large scale in the Mediterranean countries [7], including Spain and Morocco, and could be considered as an important complementary dietary source in this region. Considering all the studies on the positive properties of almond kernel consumption, it can be considered a real healthy food [8]. The Spanish cultivar 'Marcona' and the French 'Ferragnès' are two important cultivars planted in commercial almond orchards in many Mediterranean countries, including Morocco and Spain. These cultivars appear to be adapted to the climatic conditions of different growing regions and are highly appreciated by the consumers and growers [9]. 'Marcona', a traditional Spanish cultivar, has been generally associated in Morocco with 'Fournat de Brèznaud', a traditional French cultivar. 'Ferragnès' is normally associated with 'Ferraduel', having been both cultivars obtained in the INRA French breeding program, originating from the cross 'Cristomorto'× 'Aï' [10].

On the other hand, tocopherols and phytosterols are well-known to be of great interest for almond kernel stability and human health [4,5]. Phytosterols belong to the family of triterpenes, and they are present in plants as components of cell membranes, playing an important role in plant function. The present interest in phytosterols is mainly due to the fact that dietary phytosterols inhibit cholesterol absorption, which leads to decreased plasma low-density lipoprotein (LDL) cholesterol levels and thereby a potentially decreased risk of developing cardiovascular diseases [11,12]. Moreover, several studies report that phytosterols might protect against development of colon cancer [13]. Tocopherols are natural monophenols with anti-oxidant activities [14], with several homologues depending on the position and number of methyl groups. Their main biochemical function is believed to be the protection of polyunsaturated fatty acids against peroxidation [15]. A high concentration of tocopherols has also been shown to be very important in the human diet, due to its vitamin E activity [15].

In almond, studies on the effect of environmental conditions on phytosterol content and profiles are scarce. It has been reported that kernel phytosterol content, oil phytosterol content and the concentration of individual phytosterols, except Δ^7-campesterol and clerosterol, depend on the genotype effect [16]. These authors also claimed that the year effect is significant for all individual phytosterol components, except for clerosterol, but not for kernel phytosterol content and oil phytosterol content. Yada et al. [17] reported that there were no significant effects onβ-sitosterol when studying seven cultivars in three different growing conditions in California. However, a significant year effect was observed on the β-sitosterolcontentunder Californian conditions [17].

For tocopherol content, three tocopherol homologues were detected in almond kernel oil. The most important homologue in almond kernel is α-tocopherol, ranging from 200 to 656.7 mg/kg oil. The second isomers of tocopherol detected in almond kernel are γ-tocopherol, which has a strong effect on the protection against oxidation than α-tocopherol, ranging from 2.4 mg/kg oil to 50.2 mg/kg oil. Finally, the δ-tocopherol is the third homologue identified in almond kernel oil with a reduced range of variability fluctuating between 0.1 and 22.0 mg/kg oil [5]. The tocopherol concentration in almond is reported to be affected by the genotype and the environmental conditions [17,18]. Tocopherol concentration in almond kernel oil depends on the genotype, the climatic conditions of the year [18–20] and the environmental conditions of the growing region [17,19]. Several studies point out that the temperatures and drought stress during fruit growth greatly affects the concentration of these three tocopherol homologues in almond kernel oil [19,21].

The available information at present on the biochemical composition and antioxidant effect of the almond kernels is restricted to a reduced number of cultivars, mostly from the country where these cultivars originated or are grown. As a consequence, comparisons among cultivars from different countries are affected not only by the genotype but also by the possible differences related to the climatic conditions of each country and to the different orchards handling management. The present work aims to evaluate the variability of oil, phytosterol and tocopherol contents in the kernel of 'Marcona', 'Ferragnès' and 'Ferraduel' grown under two different Mediterranean environmental conditions.

2. Materials and Methods

2.1. Plant Material

Three of the most importantcultivars grown in the Mediterraneanregionwere included in the present study: 'Marcona' from Spain, and 'Ferragnès' and 'Ferraduel' from France. The present study was carried out in two different locations in Spain and Morocco. The first station was situated in the international almond collection of CITA, Zaragoza, in northern Spain, located at 41°38′50″ N and 0°53′07″ W, at 220 m above sea level. The second station was located in Meknès, in central Morocco, located at 33°53′42″ N and 5°33′17″ W, at 499 m above sea level. In both locations, each cultivar is represented by three contiguous trees, planted in the same experimental plot, and formed in an open vase. At CITA, the orchard was irrigated whereas in Morocco, the plants were conducted under drought conditions.These cultivars were marked and fruits were collected in summer (July and August) during 2014.

2.2. Determination of Oil Content

Nuts were harvested at maturity, when fruit mesocarp was dried and split along the central suture and peduncle abscission was complete [9]. Two replicates of 20 fruits of each genotype were randomly collected. After cracking, seed coats were removed by pouring in warm water. Kernels were dried at room temperature for two days and ground in an electrical grinder. Oil was extracted from 4–5 g of ground almond kernels in a commercial fat-extractor (Selecta, Barcelona, Spain) for 2 h using petroleum ether as solvent and keeping the heating source at 135 °C [22].

2.3. Tocopherol Determination

Tocopherol concentrations were determined in samples of 0.3 g of almond oil as already described [18]. The chromatographic conditions allowed the simultaneous determination of all tocopherol homologues by identifying the different peaks with comparison of retention times with standards and confirmed by their characteristic spectra using an HPLC chromatograph consisted of a Kontron model 360 autosampler (Kontron Instruments, Eching, Germany) connected with a Kontron 440 photodiode array detector, which also confirmed their purity. To quantify all of the isomers, calibration curves were drawn (see Supplementary Material). Standard linearity was verified in each case by analysis of six standards in triplicate, each containing 20–200 mg/kg for α-tocopherol (Sigma-Aldrich, St. Louis, MO, USA), 0.1–8 mg/kg for γ-tocopherol (Sigma-Aldrich, St. Louis, MO, USA), and 0.05–5 mg/kg for δ-tocopherol (Sigma-Aldrich, St. Louis, MO, USA). Detection of δ- and γ-tocopherol was carried out using a FLD SFM25 fluorescence detector under an excitation wavelength of 295 nm and emission of 325 nm. Detection of α-tocopherol was carried out with a Kontron 440 photodiode array detector at a wavelength of 295 nm. Tocopherol compositions were the mean values of three replicates from each sample and were expressed as mg/kg oil.

2.4. Analysis of Phytosterol Content

Phytosterol content was analyzed in two replicates per sample following a previously described procedure for the analysis of free and esterified phytosterols [23]. In short, 200 mg of almond flour was placed in 10-mL propylene tubes and 200 mL of an internal standard solution prepared by dissolving cholesterol (99% purity, reference C8667; Sigma-Aldrich, St. Louis, MO, USA) in hexane–ethanol (3:2) solution at a concentration of 0.1%. Alkaline hydrolysis was performed by adding 2 mL of a solution of potassium hydroxide dissolved in ethanol at a concentration of 2%. Phytosterols were extracted by vertexing with 1 mL hexane and 1.5 mL water. The upper hexane layer was transferred to 2 mL glass vials that were maintained in an oven at 37.5 °C overnight. Fifty microliters of hexane and 50 mL of silylating mixture composed of pyridine:hexamethyldisilazane: trimethylchlorosilane (9:3:1 by volume, Silan-Sterol-1, reference 355650.0922; PanreacQuımica, Barcelona, Spain) were added to the dried pellets and the vials were left at room temperature for 15 min. The

solution was transferred to 2 mL vials containing 200 mL inserts and centrifuged at 2594 g_n for 10 min (Unicen 21; Ortoalresa, Madrid, Spain). The vials were capped and conserved at −20 °C. Gas chromatographic analysis was performed on a gas chromatograph (Clarus 600; Perkin Elmer, Waltham, MA, USA) equipped with a ZB-5 capillary column (i.d. = 0.25 mm, length = 30 m, film thickness = 0.10 mm; Phenomenex, Torrance, CA, USA). Hydrogen was used as carrier gas at a pressure of 125 kPa. The split injector and flame ionization detector were maintained at 320 °C. The oven thermal regime was the following: the initial temperature of 240 °C was increased at 5 °C_min^{-1} to a final temperature of 265 °C and held for 10 min. Total analytical time was 15 min. Peak identification was conducted at the reference laboratory of the Instituto de la Grasa, Seville, Spain. Kernel phytosterol content was expressed as milligrams per kilogram of kernel. Oil phytosterol content, expressed as milligrams per kilogram of kernel oil, was estimated from kernel phytosterol content and kernel oil content using the following formula:

$$\text{Oil phytosterol content} = (\text{kernel phytosterol content} \times 100)/\text{oil content}$$

Such estimation represents the maximum expected phytosterol content in the raw oil, although the actual content will depend on the efficiency of the extraction system. The concentration of individual phytosterols was expressed as a percentage of total free and esterified phytosterols.

2.5. Statistical Analysis

All statistical analyses were performed using SAS programs (SAS Institute, Cary, NC, USA). The analysis of variance was carried out using the PROC GLM procedure. The additive linear model for the statistical analysis was:

$$P_{ij} = \mu + G_i + S_j + (G \times S)_{ij} + \varepsilon_{ij}$$

where P_{ij} is the phenotypic value of the ith genotype at j site, μ is overall mean, G_i is the genotype effect, S_j is the location effect and $(G \times S)_{ij}$ is the genotype and location interaction effect and ε_{ij} is the residual effect. The mean separation was carried out with the LSD test at a $p < 0.05$.

3. Results and Discussion

3.1. Oil Content Variation

The statistical analysis showed that the location effect was significant on oil content (Table 1), confirming the results reported in the literature [24–26]. The highest values of oil content were detected in 'Ferragnès' (59.65% DM) and 'Marcona'(59.52% DM) and the lowest in'Ferraduel' (58.6% DM).The location effect was significant on oil content (Table 2), indicating that environmental and growing conditions are different between the two locations, considering the effect of environmental and climatic conditions on oil content in almond [17]. The highest mean value of oil content was obtained at Zaragoza (60.36% DM) as compared to Meknès (58.14% DM).

Some authors reported that irrigation did not significantly affect almond oil content and fatty acid composition [27,28], but Egea et al. [29] found that kernel oil content was higher in stressed plants than in irrigated ones. Zhu et al. [21] reported that moderate deficit irrigation had no determinant impact on almond kernel lipid content, but severe and extreme deficiencies influenced lipid content. The main differences were temperature and irrigation, as trees were irrigatedin Spain but not in Morocco. The low rainfall and the lack of irrigation at Meknès could explain the low mean value of oil content at this location. However, the significance of the genotype × location interaction (Table 1) indicated that the magnitude of variation in these traits depended on the specific characteristics of each genotype, which behaved differently depending on the specific climatic and environmental conditions of the location. 'Ferraduel' and 'Ferragnès' showed highest values at Zaragoza, whereas'Marcona' showed the highest value at Meknès(Table 2). The present results con-

firmed that the genotype × location interaction hasa great importance in the determination of oil content in almond.

Table 1. Analysis of variance for oil content (%), phytosterol content and different phytosteroland tocopherol components in almond.

	Source of Variation		
Component	**Genotype (G)**	**Location (L)**	**G × L**
Oil content (% DM)	1.31 ns [z]	14.80 **	21.35 **
Oil phytosterol content (g kg^{-1} oil)	0.03 ns	0.4 **	0.04 *
Kernel phytosterol content (g kg^{-1} kernel)	0.02 **	0.08 **	0.05 **
β-sitosterol (% phytosterols)	0.67 ns	7.26 **	16.16 **
Δ^5-Avenasterol (% phytosterols)	2.11 **	2.99 **	3.44
Campesterol (% phytosterols)	0.21 ns	0.07 ns	0.11 ns
$\Delta^{5,24}$-Stigmastadienol (% phytosterols)	0.09 ns	0.18 ns	0.31 ns
Δ^7-Stigmastenol (% phytosterols)	0.28 ns	0.41 *	0.08 ns
Δ^7-Campesterol (% phytosterols)	0.39 *	0.56 *	0.93 **
Stigmasterol (% phytosterols)	1.01 **	0.003 ns	0.13 *
Clerosterol (% phytosterols)	0.05 ns	0.02 ns	0.003 ns
Δ^7-Avenasterol (% phytosterols)	0.56 ns	0.86 *	0.27 ns
δ-tocopherol (mg kg^{-1} oil)	0.84 **	0.36 ns	0.015 **
γ-tocopherol (mg kg^{-1} oil)	165.81 *	77.55 **	235.94 **
α-tocopherol (mg kg^{-1} oil)	6332.19 *	15106.02 **	7696.27 **

[z] ns: non-significant and significant at * $p < 0.05$ or ** at $p < 0.01$, *F-test*.

3.2. Phytosterol Variation

Independently of the location, the highest mean value of phytosterol content (free and esterified phytosterols) in the kernel was detected in 'Marcona' (1.45 g kg^{-1}), followed by 'Ferragnès' (1.42 g kg^{-1}), and finally 'Ferraduel' (1.32 g kg^{-1}). The highest mean value of oil phytosterol content was detected in 'Marcona' (2.42 g kg^{-1}) and the lowest value in 'Ferraduel' (2.26 g kg^{-1}). The statistical analysis showed a significant effect ofthegenotype, the location and the genotype × location interaction for kernel phytosterol content (Table 1). For oil phytosterol content, only the location effect was significant (Table 1).The highest value of kernel and oil phytosterol contents were observed at Meknès (1.48 g kg^{-1} and 2.54 g kg^{-1}, respectively), as compared to the values obtained at Zaragoza (1.31 g kg^{-1} and 2.54 g kg^{-1}, respectively).

The significant effect of the location on kernel and oil phytosterol indicates that the environmental and growing conditions had great effect on the determination of these chemical components in almond. Fernández-Cuesta et al. [16] reported that the year effect was not significant on the determination of kernel phytosterol content when studying the variability of this component in 160 almond genotypes over two consecutive years. Määttäet al. [30] investigated seven oat cultivars grown at three different locations in Sweden and reported significant differences in total phytosterol content among cultivars, but no effect was found for the growing location.

Table 2. Oil content, phytosterol and tocopherol concentrations for each cultivar and location (Meknés in Morocco and Zaragoza in Spain).Values represent the mean ± standard deviation.

Genotype	**Ferragnès**			**Fournat de Bréznaud**			**Marcona**		
Component	**Meknès**	**Zaragoza**	**Mean**	**Meknès**	**Zaragoza**	**Mean**	**Meknès**	**Zaragoza**	**Mean**
Oil content (% DM)	[z] 56.18 ± 0.3 b	62.86 ± 0.03 a	[‡] 59.52 ± 4.5 A	57.3 ± 0.49 b	59.87 ± 1.7 a	58.6 ± 2.39 A	60.92 ± 1.2 a	58.37 ± 1.3 b	59.65 ± 1.91 A
Oil phytosterol content (g kg^{-1} oil)	2.45 ± 0.04 a	2.33 ± 0.16 b	2.39 ± 0.32 A	2.51 ± 0.1 a	2 ± 0.18 b	2.25 ± 0.25 A	2.66 ± 0.49 a	2.18 ± 0.22 b	2.42 ± 0.43 A
Kernel phytosterol content (g kg^{-1} kernel)	1.37 ± 0.01 b	1.46 ± 0.09 a	1.42 ± 0.08 A	1.44 ± 0.07 a	1.19 ± 0.06 b	1.31 ± 0.19 B	1.62 ± 0.22 a	1.27 ± 0.68 b	1.44 ± 0.18 A
Campesterol (% total phytosterols)	2.35 ± 0.36 a	2.62 ± 0.25 a	2.48 ± 0.26 A	2.71 ± 0.14 a	3.14 ± 0.27 a	2.93 ± 0.38 A	2.75 ± 0.04 a	2.54 ± 88.6 a	2.64 ± 0.11 A
Stigmasterol (% total phytosterols)	0.24 ± 0.38 a	0.28 ± 0.16 a	0.26 ± 0.32 B	1.37 ± 0.01 a	0.97 ± 0.78 b	1.17 ± 0.68 A	0.18 ± 0.04 b	0.50 ± 0.14 a	0.34 ± 0.17 B
Δ^7-Campesterol (% total phytosterols)	0.80 ± 0.66 a	0.94± 0.46 a	0.87 ± 0.47 A	0.48 ± 0.01 a	0.12 ± 0.22 a	0.30 ± 0.29 B	0.06 ± 0.73 b	1.57 ± 0.25 a	0.81 ± 0.52 A
Clerosterol (% total phytosterols)	1.04 ± 0.03 a	1.12 ± 0.23 a	1.08 ± 0.18 A	0.84 ± 0.01 a	0.87± 0.08 a	0.85 ± 0.1 A	0.92 ± 0.36 a	1.07± 0.37 a	0.99 ± 0.28 A
β-sitosterol (% total phytosterols)	77.6 ± 0.7 b	83.85 ± 0.42 a	80.75 ± 1.3 A	81.59 ± 0.6 a	80.66 ± 0.01 b	81.13 ± 0.5 A	81.87 ± 1.16 a	81.27 ± 0.24 a	81.57 ± 2.06 A
Δ^5-Avenasterol (% total phytosterols)	8.45 ± 0.11 b	10.23 ± 0.4 a	9.34 ± 1.06 C	8.84 ± 0.02 b	11.18 ± 1.5 a	10.01 ± 1.24 B	11.35 ± 0.62 a	10.23 ± 0.46 b	10.79 ± 0.9 A
$\Delta^{5.24}$-Stigmastadienol (% total phytosterols)	1.97 ± 0.05 a	1.13± 0.09 b	1.55 ± 0.06 A	1.34 ± 0.01 a	1.16 ± 0.18 a	1.25 ± 0.14 A	1.28 ± 0.23 b	1.56 ± 0.86 b	1.42 ± 0.7 A
Δ^7-Stigmastenol (% total phytosterols)	1.32 ± 0.18 a	0.61 ± 0.04 b	0.96 ± 0.16 A	0.60 ± 0.06 a	0.33 ± 0.13 b	0.46 ± 0.22 B	0.63 ± 0.16 a	0.49 ± 0.59 b	0.56 ± 0.37 AB
Δ^7-Avenasterol (% total phytosterols)	2.39 ± 0.46 a	1.32 ± 0.1 b	1.85 ± 0.34 A	1.49 ± 0.05 a	0.99 ± 0.25 b	1.24 ± 0.35 A	1.19 ± 1.2 a	1.16 ± 0.62 a	1.18 ± 0.82 A
δ-tocopherol (mg kg^{-1} oil)	0.22 ± 0.06 b	0.62 ± 0.09 a	0.42 ± 0.27 B	0.66 ± 0.02 a	0.20 ± 0.2 b	0.43 ± 0.43 B	1.39 ± 0.09 a	0.21 ± 0.20 b	0.79 ± 0.06 A
γ-tocopherol (mg kg^{-1} oil)	4.93 ± 0.3 b	21.43 ± 4.1 a	13.18 ± 4.88 A	8.10 ± 0.11 a	4.93 ± 0.32 b	6.51 ± 10.59 B	4.23 ± 0.09 b	11.58 ± 0.09 a	7.90 ± 0.54 AB
α-tocopherol (mg kg^{-1} oil)	505.5 ± 11.9 a	468.1 ± 23.02 b	486.8 ± 39.9 A	428.8 ± 60.5 b	462.8 ± 1.6 b	445.8 ± 57.2 B	555.16 ± 17.5 a	372.73 ± 5.8 b	463.9 ± 96.6 B

[z] Values followed by differentsmall letters in the same line and locationare significantly different at $p < 0.05$. [‡] Values followed by differentcapital letters in the same line and location are significantly different at $p < 0.05$.

In contrast, it has been reported that the variation in kernel phytosterol content of sunflower was mainly attributable to the effect of the location and the interaction of genotype × location [31,32]. Yang et al. [33] compared phytosterol levels in seeds of two *Vaccinium* species, grown at two locations in northern and southern Finland. They found differences within genotypes and explained it by geographic and climatic conditions. In the rye kernels, the high phytosterol content was reported to be responsive to the climate and the high temperature and low rainfall during the month up to harvest. In olive, it has been reported that the phytosterol content in the fruit of some varieties varied significantly among different environments, with a high range of variability in the genotype by environment interaction [34]. Recently, Torres et al. [35] reported that the phytosterol content in olive oil of 'Arbequina' and 'Coratina' varieties was strongly affected by the environmental conditions, being the highest values obtained in the warmer locations than in cooler ones.It is interesting to note the significant effect of the genotype × location interaction on kernel and oil phytosterol contents (Table 1). 'Marcona' and 'Ferraduel' showed the highest values of kernel and oil phytosterol content at Meknès (Table 2), whereas'Ferragnès' showed the highest values at Zaragoza (Table 2).

These results indicate that the magnitude of variation in kernel phytosterol content depends on the specific characteristics of each genotype, which behaved differently depending on the specific climatic and environmental conditions of the location. Fernández-Cuesta et al. [16] reported that the variation in kernel phytosterol in 160 almond cultivars was mainly attributed to the genotype × year interaction, showing that each cultivar behaves differently in different climatic conditions. In sunflower, Fernández-Cuesta et al. [31] reported that the variation in kernel phytosterol content was largely attributable to the effect of the genotype × location interaction. Another study on soybean concluded that phytosterol content in the extracted oil was also influenced by the genotype × planting location interaction [34].

In both locations, the phytosterol fraction of the studied genotypes was mainly made up of β-sitosterol and Δ^5-avenasterol, which together accounted for more than 90% of total phytosterols (Table 2). The highest mean value of β-sitosterol was obtained in 'Ferragnès' (81.57% of total phytosterols) and finally 'Marcona' (80.75% of total phytosterols). For Δ^5-avenasterol, the highest mean value was detected in 'Marcona' (10.79% of total phytosterols) and the lowest value in 'Ferragnès' (9.34% of total phytosterols) (Table 2). The statistical analysis showed that the genotype, the location and the genotype × location interaction had significant effects on Δ^5-avenasterol content (Table 1), whereas the genotype hadno significant effect on β-sitosterol content. Fernández-Cuesta et al. [16] reported a significant effect of genotype on β-sitosterol content. The effect of the location was significant on β-sitosterol and Δ^5-avenasterol (Table 1), the values obtained at Zaragoza being the highest (81.93% and 10.55% of total phytosterols, respectively) (Table 3).Yada et al. [17] reported that there were no significant location effects onβ-sitosterol when studying seven cultivars in three different growing conditions in California. However, a significant year effect was observed on β-sitosterol under Californian [17] and Spanish conditions [16]. All these results indicate that β-sitosterol and Δ^5-avenasterol, the two main phytosterol components, depend on the genotype, but are also largely influenced by the climatic conditions. Thus, these compounds could be considered under polygenic control.

Recently, Font i Forcadaet al. [36] reported that several candidate genes and putative genomic regions were identified in almond and are potentially involved in the control of the expression of different phytosterol components in almond kernel oil. The significance of the genotype × location interaction on β-sitosterol and Δ^5-avenasterol, indicates that the variation in these components depends on the specific characteristics of each genotype, which behaved differently depending on the climatic and environmental conditions of the location.Concerning the minor phytosterol components, the genotype effect was only significant forstigmasterolanΔ^7-campesterol (Table 1).The location effect was significant for Δ^7-stigmastenol and Δ^7-campesterol (Table 1). No location effect was observed for any of the minor phytosterol compounds detected in the present work. The absence of the

location effect observed for campesterol and stigmasterol coincides with those reported by Yada et al. [17].

Table 3. Mean value of chemical component for each location (Meknés in Morocco and Zaragoza in Spain). Values represent the mean ± standard deviation.

Component	Location	
	Meknes [z]	**Zaragoza**
Oil content (% DM)	58.14 ± 2.85 b	60.36 ± 1.90 a
Oil phytosterol content (g kg^{-1} oil)	2.54 ± 0.19 a	2.17 ± 0.24 b
Kernel phytosterol content (g kg^{-1} kernel)	1.48 ± 0.11 a	131 ± 0.19 b
Campesterol (% total phytosterols)	2.60 ± 0.42 a	2.76 ± 0.27 a
Stigmasterol (% total phytosterols)	0.59 ± 0.46 a	0.58 ± 0.52 a
Δ^7-Campesterol (% total phytosterols)	0.44 ± 0.51 b	0.88 ± 0.43 a
Clerosterol (% total phytosterols)	0.93 ± 0.15 a	1.02 ± 0.22 a
β-sitosterol (% total phytosterols)	80.37 ± 1.50 b	81.93 ± 2.15 b
Δ^5-Avenasterol (% total phytosterols)	9.55 ± 1.43 b	10.55 ± 0.85 a
Δ^7-Avenasterol (% total phytosterols)	1.53 ± 0.62 a	1.28 ± 0.14 a
Δ^7-Stigmastenol (% total phytosterols)	0.85 ± 0.45 a	0.47 ± 0.28 b
$\Delta^{5.24}$-Stigmastadienol (% total phytosterols)	1.69 ± 0.24 a	1.15 ± 0.70 a
δ-tocopherol (mg kg^{-1} oil)	0.76 ± 0.65 a	0.34 ± 0.27 a
γ-tocopherol (mg kg^{-1} oil)	5.75 ± 9.85 b	12.64 ± 1.95 a
α-tocopherol (mg kg^{-1} oil)	496.48 ± 90.46 a	434.50 ± 36.43 b

[z] Values followed by different letters in the same line are significantly different at $p < 0.05$.

3.3. Tocopherol Variation

As expected, significant differences were observed for all tocopherol homologues among the three cultivars (Table 1). Independently of the location, the highest mean value of α-tocopherol, the main tocopherol homologue in almond oil, was detected in 'Ferragnès' (487 mg/kg oil) (Table 2). For γ-tocopherol, 'Ferragnès'also showed the highest mean value (13.2 mg/kg oil) and the lowest value was detected in 'Marcona' (6.5 mg/kg oil). The analysis of variance showed a significant effect of the location and the genotype × location interaction on all tocopherol homologues (Table 1), as previously reported [19]. The mean value of α-tocopherol was higher in Morocco (496 mgkg^{-1} oil) as compared to the mean value in Spain (435 mg/kg oil) (Table 3). However, the mean value of γ-tocopherol was higher at Zaragoza (Table 3).

In almond, it has been reported that there is no obvious relationship between almond tocopherol concentrations and the drought stress [21]. The amount of tocopherol concentration, mainly α-tocopherol, was reported to be high in almond kernel oil under warm temperature during fruit development [20,37]. Several studies in sunflower and soybean have reported that high temperature during seed growth significantly increase tocopherol concentration in seed oil [38–40]. Thus, the differences found in the present study could be due to the differences in temperature between the two locations, the Moroccan station being warmer thanthe Spanish one during the nut growth stage as previously reported [41].

However, the genotype × location interaction was significant for all tocopherol homologues (Table 1). In fact, 'Ferraduel' showed higher values of α-tocopherol at Zaragoza, whereas 'Marcona' and 'Ferragnès' showed higher values at Meknès (Table 3). For γ- and δ-tocopherol, 'Ferragnès' and 'Marcona' showed higher values in Spain (Table 3), whereas 'Ferraduel' showed higher values in Morocco (Table 3). These results confirm that the tocopherol concentration in almond oil depends on the genotype [5]. A recent study showed that the heritability estimate of α-tocopherol was low with a value of h^2 = 20.5%, whereas

γ-tocopherol showed higher heritability estimates (h^2 = 60.0%) [42]. Moreover, Font i Forcada et al. [43] reported that the three tocopherol isomers in almond oil are associated with two QTL in LG1 (CPPCT042) and LG4 (PCHGMS55). All these results confirm that tocopherol isomers concentration in almond kernel oil is under polygenic control, with a clear effect of year climatic conditions and environment [19,41].

4. Conclusions

The present study revealed the significant effect of the location and the genotype × location interaction on the kernel and oil phytosterol contentsin almond cultivars, as well as onthe major phytosterol compounds (β-sitosterol and Δ^5-avenasterol). The highest values of kernel and oil phytosterol contents were obtained under Moroccan conditions. Similar results were obtained for α-tocopherol, the major tocopherol homologue in almond oil. These results indicated that under warmer climate and drought conditions, almond cultivars tend to accumulate more phytosterol and tocopherol. However, the significance of the genotype × location interaction on the phytosterol content and compositionindicates that the magnitude of variation in these traits depends on the specific characteristics of each genotype, which behaved differently depending on these climatic and environmental conditions. These results stress the need for evaluating each genotype separately in different environmental conditions to ensure the stability of these components, due to their usefulness for human health, in different environments to determine the best growing conditions in order to obtain the highest values of these chemical components.

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