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## Comparative analysis of fatty acids, tocopherols and phytosterols content in sunflower cultivars (*Helianthus annuus*) from a three-year multi-local study

Análisis comparativo del contenido en ácidos grasos, tocoferoles y fitoesteroles en cultivares de girasol (*Helianthus annuus*) en un estudio trianual y multilocal

Ayerdi Gotor A<sup>1,5\*</sup>, M Berger<sup>1</sup>, F Labalette<sup>2</sup>, S Centis<sup>3</sup>, J Daydé<sup>1</sup>, A Calmon<sup>1,4</sup>

**Abstract.** Sunflower oil is naturally rich in both unsaturated fatty acids and numerous minor components, with high levels of  $\alpha$ -tocopherol and phytosterols that could play an important role in plant protection. These micronutriments, tocopherols and phytosterols, also exhibit health benefits for humans including reducing both cardiovascular diseases and aging disorders.  $\alpha$ -Tocopherol represents more than 95% of total sunflower tocopherol and has the greatest vitaminic activity. Furthermore, this oil contains high amounts of  $\beta$ -sitosterol which is a promising health beneficial phytosterol. The objective of this study was to compare the content and composition of fatty acids, tocopherols and phytosterols in sunflower oil between different cultivars and locations in a three-year experiment, and to estimate the relationships between these minor components, fatty acids and climatic conditions. Results revealed that content and composition of these molecules are mainly determined by plant genotype. Nevertheless, the environmental conditions seemed to strongly influence the tocopherol content. The results suggest possible improvement in sunflower breeding for minor components.

**Keywords:** Sunflower oil; Fatty acids; Tocopherols; Phytosterols; Genotype x environment interaction; Temperature.

**Resumen.** El aceite de girasol es naturalmente rico en ácidos grasos no saturados y en numerosos componentes menores. Posee altos niveles de  $\alpha$ -tocoferol y fitoesteroles que podrían desempeñar un papel importante en la protección de las plantas. Estos micronutrientes, tocoferoles y fitoesteroles, también presentan beneficios para la salud de los seres humanos, reduciendo el riesgo de padecer enfermedades cardiovasculares y trastornos del envejecimiento. El  $\alpha$ -tocoferol representa más del 95% de los tocoferoles del girasol y tiene la mayor actividad vitamínica. Además, este aceite contiene altas cantidades de  $\beta$ -sitosterol que es un prometedor fitoesterol benéfico para la salud. El objetivo de este estudio fue comparar el contenido y la composición de ácidos grasos, tocoferoles y fitoesteroles en el aceite de girasol entre los diferentes cultivares y sitios en un experimento de tres años, y evaluar las relaciones entre estos componentes minoritarios, los ácidos grasos y las condiciones climáticas. Los resultados revelaron que el contenido y la composición de estas moléculas principalmente están determinados por el genotipo de la planta. Sin embargo, las condiciones ambientales parecen influir mucho en el contenido de tocoferoles. Los resultados sugieren una posible mejora genética del girasol con respecto a sus componentes menores.

**Palabras clave:** Aceite de girasol; Ácidos grasos; Tocopheroles; Fitoesteroles; Interacción genotipo x ambiente; Temperatura.

<sup>1</sup> Université de Toulouse; Institut National Polytechnique de Toulouse; Ecole d'Ingénieurs de PURPAN; Département Sciences Agronomiques et Agroalimentaires; Laboratoire d'Agrophysiologie; 75 voie du TOEC, BP 57611, F-31076 Toulouse Cedex 3, France.

<sup>2</sup> ONIDOL; 12 avenue George V, F-75008 PARIS, France.

<sup>3</sup> ASEDIS-SO; 39 chemin Virebent, F-31200 TOULOUSE, France.

<sup>4</sup> INRA; UMR 1010 CAI; F-31030 Toulouse Cedex 4, France.

<sup>5</sup> Institut Polytechnique LaSalle Beauvais; BP 30313, F-60026 Beauvais Cedex, France. Tel: + 33 (0)3 44 06 25 49 - Fax: +33(0)3 44 06 25 26 (Permanent address).

Address Correspondence to: Alicia Ayerdi Gotor, Institut Polytechnique LaSalle Beauvais BP 30313, 60026 BEAUVAIS CEDEX, France. Tel : + 33 (0)3 44 06 25 49 - Fax :

+33(0)3 44 06 25 26, e-mail: alicia.ayerdi-gotor@lasalle-beauvais.fr

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## INTRODUCTION

Sunflower world production represents up to 20% of the total world production of oil seeds (FAO, 2013). France was the 9<sup>th</sup> world sunflower producer in 2003 and 2004 with 2 million hectares; this surface has increased up to 2.5 millions in 2011 (France was 14<sup>th</sup> in the sunflower producers ranking) (FAO, 2013). Sunflower oil is rich in natural micronutrients, and various studies have shown the health benefit properties of polyunsaturated fatty acids (PUFA), tocopherols and phytosterols. The ingestion of PUFAs can have beneficial effects on human health such as the reduction of fatal coronary heart disease by decreasing the plasma low density lipoproteins (LDL)-cholesterol (Ruiz-Gutiérrez et al., 1997; Zock & Katan, 1998). Vitamin E isomers and specially  $\alpha$ -tocopherol are able to reduce the risk of cardiovascular disease and exhibits anti-carcinogenic activities (Bramley et al., 2000; Beardsell et al., 2002). This can be achieved through prevention of polyunsaturated fatty acids oxidation and free radicals scavenging (Niki, 2004). Since it is retained by the liver by means of a specific transfer protein,  $\alpha$ -tocopherol is considered to be the most biologically active form (Packer et al., 2001). Sterols inhibit cholesterol absorption in humans by limiting micellar solubility of cholesterol, leading to a reduction of cholesterol absorption (Kritchevsky, 1997; Thompson & Grundy, 2005; von Bergmann et al., 2005).

Since Seiler (1983), where the factors affecting the variability of fatty acids in sunflower were studied, other studies like Lagravère et al. (2004) have confirmed that genotypes and environmental growing conditions affect fatty acid composition. Various works on the variation of tocopherols and phytosterols have been implemented for different vegetal species studying the genotype and environmental effects; however, most of them have focused only in one minor component: Almonor et al. (1998) reported that  $\gamma$ -tocopherol content diminished in soybeans with reduced temperatures; Goffman and Becker (2002) found significant variations between genotypes and locations for the tocopherol content and composition in rapeseed; Määttä et al. (1999) described a significant difference for the phytosterol content in 7 oat cultivars or for 6 of rye (Zangenberg, 2004), Vlahakis and Hazebroek (2000) found differences in total phytosterol contents among canola, sunflower and soybean crops and also within each crop. Regarding sunflower, Demurin et al. (1996) and Velasco et al. (2002), have observed that genetic and different environmental conditions lead to variation of tocopherols content. However, in these studies only one year of cultivation was considered. Results concerning simultaneous variations in the content and composition of several families of oil components have been rarely addressed in the field. Fatty acids and tocopherols have also been studied in sunflower by Baydar and Erbas (2005), who found a positive correlation between total tocopherol and oleic acid contents. In soybean oil, Dolde et

al. (1999) found a positive correlation between linoleic acid and tocopherol levels. A study including the three families (fatty acids, phytosterols and tocopherols) was also conducted by El-Shami et al. (2001) in rapeseed oil, but no correlations between these components were treated.

Regarding the composition of sunflower oil in health promoting molecules (Bramley et al., 2000; Vlahakis & Hazebroek, 2000; Holliday & Phillips, 2001; Velasco et al., 2002; Lagravère et al., 2004) and the importance of sunflower production in France, the objectives of our study were to (1) investigate the variability and (2) understand the relationship of these three families of components (PUFA, tocopherols and phytosterols) in sunflower oil. Our comparative study was carried out in four different commercial varieties grown in different locations in France during three consecutive years. At the same time, climatic parameters were determined in order to take into account their effects. To the best of our knowledge, this is the first time that a study comparing different varieties and over three years has been performed to study the variability in sunflower of tocopherol and phytosterol contents, in a multilocal study over a three-year period.

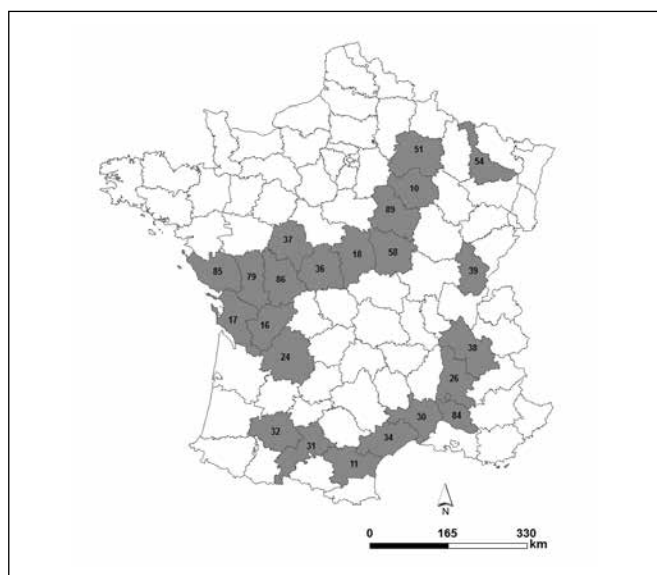
## MATERIALS AND METHODS

**Sunflower samples.** The study was carried out during three years: 2002, 2003, 2004. Four commercial sunflower (*Helianthus annuus* L.) hybrids [Allstar RM (Euralis Semences), Aurasol (High Oleic, Monsanto Dekalb), Melody (Syngenta Seeds) and Prodisol (Monsanto Dekalb)] were grown in clay-chalky, deep enough (1 m) soils at different locations all over France (Fig. 1, Table 1). Each location is represented by a code number assigned by the French authorities, also called a French department.

In each location, four blocks of around 30 m<sup>2</sup> for each variety were sown between April the 1<sup>st</sup> and 20<sup>th</sup>. As recommended (Metropolitan Oleaginous Interprofessional Technical Center - CETIOM, France), the seedling rate was 65000 seeds/ha with 0.6 m between rows and 0.25 m between plants. Main plot treatments were: two tillages in Autumn and March-April; weeding using trifluralin, and at pre-seedling and flurochloridone at post-seedling, metaldehyde (7 kg/ha) to control slugs; fungicidal treatments were made when necessary with a mix of flusilazole and carbendazime (0.8 L/ha); and two fertilizations applied: one before spring tillage (60 kg P/ha and 60 kg K/ha) and the other one during the seedling morphological developmental stage (50 kg N/ha with 1.2 kg B/ha). Thus, 97 samples (each one a bulk of the four blocks) were obtained for further analysis (Table 1).

**Meteorological dataset.** Measurements were taken in six meteorological stations all over the country: Carcassonne (43° 13' N, 2° 21' E; 11<sup>th</sup> Department), Montignac (45° 47' N, 0° 28' E; 17<sup>th</sup> Department), Auch (43° 39' N, 0° 35' E;

32<sup>nd</sup> Department), Montaud (45° 16' N, 5° 34' E; 38<sup>th</sup> Department), Fours (46° 49' N, 3° 43' E; 58<sup>th</sup> Department) and Blagnac (43° 38' N, 1° 24' E; 31<sup>st</sup> Department). These stations were selected due to their proximity to a greater number of experimental sites (fields), as well as for representing the 6 major climatic zones in France. This allowed us to consider a larger variability of weather conditions in France, all over the locations of the study during the three years. Four parameters were considered: rainfall (mm per day), maximum temperature (°C per day,  $T_{MAX}$ ), minimum temperature (°C per day,  $T_{MIN}$ ) and radiation (kW/m<sup>2</sup>) for the period from April 1<sup>st</sup> to September 30<sup>th</sup>. Figure 2 sums-up the meteorological



**Fig. 1.** Map of France showing the locations (in grey) where the study took place in the CETIOM (Centre technique interprofessionnel des Oleagineux Metropolitans) farms' network in 2002-2004.

**Fig. 1.** Mapa de Francia que muestra los lugares (en gris) en los que se llevó a cabo el estudio en la red de fincas del CETIOM (Centro Técnico Interprofesional de los Oleaginosos Metropolitans) en el período 2002-2004.

dataset for six locations that represented the six main regions where sunflower is cultivated in France. That allows us to estimate the climate differences in these areas, which could influence minor components content and composition.

**Sample Preparation.** For each sample, 40 g of akenes were freeze-dried (HETO CT 110, Saint Herblain, France) and stored at -18 °C.

**Reagents and standards.** For oil extraction, hexane (mixture of isomers) was obtained from Prolabo-Subra (France), and for analysis, solvents such as hexane, methanol, ethanol, acetone and diethyl ether at HPLC grade from SDS (France) were used. Sodium hydroxide was purchased from Prolabo-Subra (France). All fatty acid standards [palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and arachidic (C22:0) acids (99% minimum purity)], all sterol standards ( $\beta$ -sitosterol, stigmasterol and campesterol), and betulin were purchased from Sigma (Paris, France). The four  $\alpha$ -,  $\beta$ -,  $\delta$ - and  $\gamma$ -tocopherol standards (99% minimum purity) were purchased in a Chromadex kit (USA). Boron trifluoride (BF<sub>3</sub>) was obtained from Aldrich (France). The trimethyl silyl ether (TMS) derivatives of all sterols were prepared using 1-methyl imidazol and N-methyl-N (trimethylsilyl)-heptafluorobutyramide reagent (Sigma, France).

**Oil extraction.** Sunflower dry-frozen grains were ground with a sample mill (KnifeTec 1095; Foss Tecator AB, Sweden) during three periods of 10 s. Around 18 g were carefully weighed in a cellulose cartridge (Whatman  $\phi$  = 30 mm, h = 100 mm) covered with backcombed cotton (Lohmann & Rauscher). Full cartridges were placed in a six soxhlet ramp for extraction (four hours) with hexane, following the reference method (AFNOR, 1998). Oil was recovered after a low pressure evaporation of the extraction solvent with a rotavapor (HS 40 Huber, Bioblock Scientific, Heildolph) and frozen (-20 °C) to minimize oxydative reactions before analysis.

**Fatty acid analysis.** Fifty mg of oil were weighed in a glass tube with a refrigerated system. As internal standard, 1 mL of arachidic acid was added and the extracts were saponified

**Table 1.** Relation of cultivars and planting location for each year. Each location is represented by the department French code number.  
**Tabla 1.** Relación de los cultivares y la ubicación de siembra de cada año. Cada localidad está representada por el número de código de los departamentos franceses.

Cultivars	Three years multilocal study		
	2002	2003	2004
	Locations in France		
Allstar RM	11-16-17-34-36-37-38-79	11-16-17-26-32-34-36-37-38-58-85-86-89	18-26-31-32-37-39-51-54-84
Aurasol	11-16-17-32-34-36-37-38-79	89	17-18-26-30-31-32-51
Melody	11-16-38	11-16-17-26-32-34-36-38-58-85-86	24-31-38-58-79-84
Prodisol	11-16-17-34-36-37-38-79	11-16-17-26-32-34-36-37-38-58-85-86-89	10-16-18-26-31-32-39-54-84

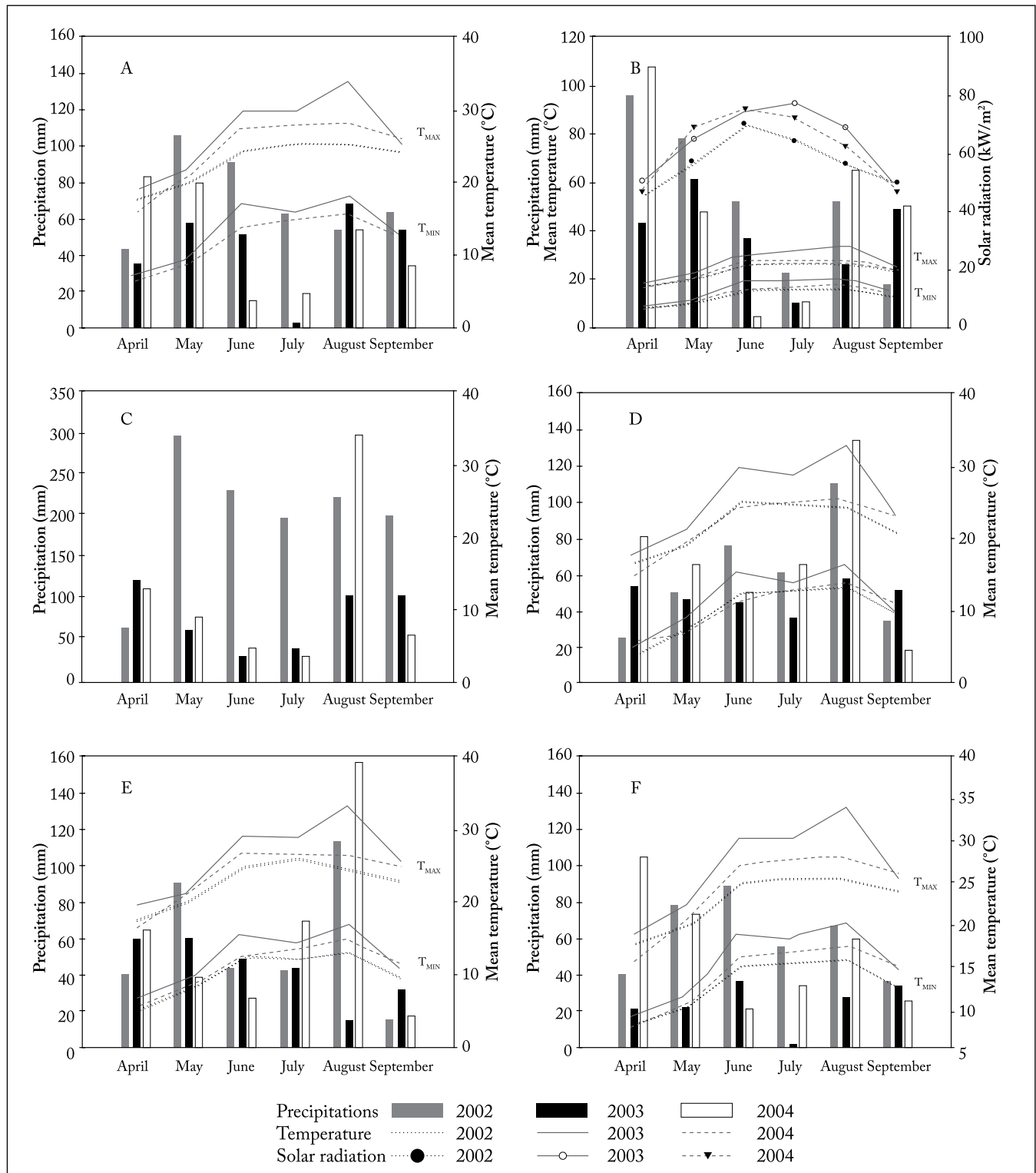


Fig. 2. Climatic conditions: rain Precipitation (sum of daily rainfall in a month, mm); Temperature: mean of maximal ( $T_{MAX}$ ) and minimal ( $T_{MIN}$ ) daily temperatures of each month ( $^{\circ}C$ ) and sum of daily solar radiation for each month for 6 locations: A: Auch (32); B: Carcassonne (11); C: Montaud (38); D: Fours (38); E: Montignac (16) and F: Blagnac (31) and for the three study years.

Fig. 2. Condiciones climáticas: precipitaciones (suma de las lluvias diarias en un mes, mm), Temperatura: media diaria de máximas ( $T_{MAX}$ ) y mínimas ( $T_{MIN}$ ) de cada mes ( $^{\circ}C$ ) y la suma de la radiación solar diaria para cada mes en 6 lugares: A: Auch (32); B: Carcassonne (11); C: Montaud (38); D: Fours (38); E: Montignac (16) y F: Blagnac (31) para cada uno de los tres años de estudio.

by adding 1 mL of 0.5 M methanolic NaOH, and heated to 65 °C for 30 min. Then, 2.5 mL of methanolic BF<sub>3</sub> (12% v/v) were added, and the mixture was heated for exactly 3 min at 65 °C, followed by a rapid refrigeration and the addition of 5 mL of ultrapure water and 10 mL of hexane (HPLC grade). After vigorous shaking and settle, the organic phase was recovered and analyzed immediately by gas chromatography.

Analyses were carried out with a Fisons 8000 series gas chromatograph (MMFC 800 Multi-function controller, EL 980 electrometer Unit, Italy) with an autosampler. Splitless injections were used with 0.5 µL sample volumes at an inlet temperature of 200 °C. Nitrogen carrier gas pressure was 200 kPa.

The fatty acids were separated using a Phenomenex fused silica capillary ZB-FFAP column (30 m x 0.25 mm x 0.25 µm, Paris, France). The oven temperature was programmed to (1) begin at 185 °C, (2) hold for 15 min; (3) increase to 200 °C at 5 °C/min and (4) remain constant for 7 min at 200 °C. The flame ionization detector (FID) was operated at 200 °C with hydrogen pressure at 100 kPa and air flow at 60 kPa. The major sunflower oil fatty acids were determined by comparison of retention times of the fatty acids methylated standards. The calibration results were based on the amount of arachidic acid detected in the sample.

**Table 2.** Calculation parameters used to determine the real concentration of the solution with an UV-Spectrophotometer.

**Tabla 2.** Parámetros de cálculo utilizados para determinar la concentración real de la solución con un espectrofotómetro UV.

Tocopherol	Wave length (nm)	Division factor
α-tocopherol	292	0.0076
β-tocopherol	296	0.0089
δ-tocopherol	298	0.0091
γ-tocopherol	298	0.0087

$$\text{Solution concentration } (\mu\text{g/ml}) = \frac{\text{Read absorbance value (mm)}}{\text{Division factor}}$$

**Tocopherols analysis.** Following the simplified international norm (ISO, 1997), a methanolic solution of each standard (0.1 mg/mL) was prepared. Solutions were measured with a UV lamp spectrophotometer (Hitachi U-1100 photometer) as it is shown in Table 2. The external calibration was carried out with dilutions of the stock solution with hexane and propan-2-ol (norm modification) to have a range of concentrations between 5 and 40 µg/mL of each form. For each sample, 1g of oil was carefully weighed (Sartorius Analytical balance Precisa 205 A, Italy), and adjusted up to 25 mL with hexane. The mixture was directly injected in a high performance liquid chromatography (HPLC) system (SpectraPhysics, Thermo Separation Products, USA). The complete separation of all native tocopherols was achieved with a normal phase LiChrosorb Si60 column (250 cm x 4 mm x 5 µm, CIL

Cluzeau, France). The mobile phase was a mixture of hexane/isopropanol (99.7:0.3 v/v) at 1 mL/min flow rate. Detection was performed with a fluorescence detector (excitation wavelength = 298 nm and emission wavelength = 344 nm; Waters 2475 multi λ). The concentrations of the tocopherol homologs were calculated from the peak area.

**Sterols extraction and CPG analysis.** Sterols analysis procedure is an adaptation of the norm (AFNOR, 1999). The oil (250 mg) was dissolved in ethanol containing 1 mL of a freshly prepared solution of betulin (1mg/mL acetone) as internal standard. Saponification was achieved by adding 5 mL of 5 % (w/v) ethanolic KOH and heating at the boiling point, and kept up for 15 minutes in a refrigeration system. Saponification was stopped with 5 mL of ethanol. The mixture (5 mL) was placed in a glass column fill up with 10 g of aluminum oxide (Neuter, Akt. 1; 50-200 µm; pH 7.5; ICN Prolabo/Subra France) dampened with ethanol. After elution a new balloon was placed to recover the unsaponifiable matter washed with 5 mL of ethanol and 30 mL of diethyl ether. Unsaponifiable matter was concentrated in the balloon by evaporation under pressure of solvents and recovered with 2 mL of diethyl oxide. Then the mixture was evaporated to dryness with nitrogen (N<sub>2</sub>, Air liquide, France). Before injection, TMS derivatives were prepared by dissolving the samples of the unsaponifiable fraction into 100-µL of 1-methyl imidazol and N-methyl-N (trimethylsilyl)-heptafluorobutyramide (5:95 v/v), and heating the mixture for 15 minutes to 105 °C in an oil bath. The saponification procedure was duplicated for each oil extract. Analyses were performed by a Fisons gas chromatograph (GC 8000 series MMFC 800 Multi-function controller, Italy) with an autosampler. Splitless injections were made with 1 µL sample volumes. Separations were achieved with a Phenomenex fused silica capillary ZB-5 (30 m x 0.25 mm x 0.25 µm, Paris, France). Nitrogen carrier gas pressure was 130 kPa. The injector was at 320 °C and the FID was 320 °C. The initial temperature of the column was 240 °C and increased to 320 °C at 4 °C/min, and held for 10 minutes until it decreased to initial conditions. Identification of sterols was based on the retention time of standards referred to above and with the relative times to betulin given in the norm. The calibration results were based on the integrated areas and adjusted according to the amount of betulin detected in the samples.

**Method validation and quality control.** Samples have been analysed by the ITERG reference laboratory (Paris, France). At the same time, one control sample was analyzed daily with each analytical series and for each kind of analysis (fatty acids, tocopherols, sterols) to determinate ageing changes in columns.

**Statistical analysis.** The data were statistically analyzed using Statistica V7 (StatSoft, Tulsa, USA). A three-way-ANOVA with three crossed factors (genotype, year) and one imbricated

factor (location) with second order interaction was performed to determine the relationships between fatty acids, total tocopherol, total phytosterol contents, hybrids, planting location and year of study. It was also used to calculate bilateral correlation between variables (Pearson correlation coefficients).

## RESULTS

**Meteorological dataset.** The six meteorological locations showed significant differences for the rainfalls; mean maximal and minimal temperatures ( $p < 0.01$ ) between the 3 years (Table 3). Significant differences in temperature were also observed between locations. In 2003, the plants were exposed to extreme growth and maturation conditions (highest temperatures and lowest rainfall) compared with 2002 (coldest and high precipitations) and 2004 (middle conditions).

**Oil and fatty acid content.** The sunflower seeds yielded around 50% oil by soxhlet extraction (Table 4). However in

2003, we observed significant differences between oil yields: Melody in the 16<sup>th</sup> location: 0.38 g/g of dry matter (DM) *vs.* Prodisol in the 26<sup>th</sup> location: 0.61 g/g DM. Nevertheless, in the ANOVA analysis, neither the effect of year and genotypes, nor their interactions were significant as shown in Table 7. Concerning fatty acid (FA) composition (Table 4), three cultivars (Allstar RM, Melody and Prodisol) had high linoleic acid content, moderate oleic acid content and traces of linolenic acid content, whereas Aurasol presented a high level in oleic acid and moderate linoleic acid content. Palmitic acid was the only major saturated acid (4–6%) either in high oleic or in high linoleic cultivars. There was a significant difference in fatty acid composition (percentage) both between varieties within a year ( $p < 0.001$ ) and between the years for the four cultivars ( $p < 0.001$ ). The genotype  $\times$  year (G  $\times$  Y) interaction was not significant for fatty acid composition. In 2002, with a colder and a more rainy summer, 3 varieties (Allstar RM, Aurasol and Prodisol) showed higher content in oleic acid compared to 2003 and 2004.

**Table 3.** Mean values for each year for the 6 locations except for solar radiation that is analyzed only in Carcassonne.

**Tabla 3.** Valores medios anuales de los 6 lugares a excepción de la radiación solar que se analiza sólo en Carcassonne.

Year	Precipitation (mm)	T <sub>MAX</sub> (°C)	T <sub>MIN</sub> (°C)	Solar Radiation (kW/m <sup>2</sup> )
2002	60.25 a	22.82 a	11.57 a	57.06 a
2003	39.48 b	26.39 b	13.51 c	64.12 b
2004	55.33 a	23.96 a	12.32 b	62.16 b

Values in the same column with the same letter are not statistically different at the 5% significance level.

**Table 4.** Oil and fatty acid amount for each sunflower hybrid and each year. The average over all the locations in a year was given by mean value  $\pm$  SD.

**Tabla 4.** Cantidad de aceite y de ácidos grasos para cada híbrido de girasol por año. La media de todos los lugares en un año es dada por el valor promedio  $\pm$  DE.

Cultivars	Year	Oil content (g/g DM)	Fatty acid composition (%)				
			16:0	18:0	18:1	18:2	18:3
Allstar RM	2002	0.49 $\pm$ 0.05	4.3 $\pm$ 0.4	1.8 $\pm$ 0.3	40.2 $\pm$ 3.2	53.7 $\pm$ 3.1	0.01 $\pm$ 0.02
	2003	0.51 $\pm$ 0.04	6.7 $\pm$ 0.3	2.1 $\pm$ 0.2	31.8 $\pm$ 1.9	59.3 $\pm$ 1.9	0.04 $\pm$ 0.01
	2004	0.52 $\pm$ 0.04	6.7 $\pm$ 0.3	1.8 $\pm$ 0.3	25.6 $\pm$ 3.0	65.8 $\pm$ 2.9	0.07 $\pm$ 0.02
Aurasol	2002	0.50 $\pm$ 0.03	2.0 $\pm$ 0.3	0.9 $\pm$ 0.2	81.5 $\pm$ 2.3	15.6 $\pm$ 2.2	0.01 $\pm$ 0.01
	2003	0.45	4.2 $\pm$ 1.1	1.3 $\pm$ 0.9	70.6 $\pm$ 8.4	24.0 $\pm$ 3.1	0.00
	2004	0.52 $\pm$ 0.04	4.4 $\pm$ 0.5	1.0 $\pm$ 0.4	70.8 $\pm$ 3.6	23.8 $\pm$ 3.4	0.06 $\pm$ 0.02
Melody	2002	0.48 $\pm$ 0.04	3.4 $\pm$ 0.6	2.9 $\pm$ 0.5	33.0 $\pm$ 4.6	60.8 $\pm$ 4.4	0.00
	2003	0.47 $\pm$ 0.04	6.7 $\pm$ 0.3	1.7 $\pm$ 0.2	33.8 $\pm$ 2.2	57.8 $\pm$ 2.1	0.04 $\pm$ 0.01
	2004	0.49 $\pm$ 0.03	6.1 $\pm$ 0.6	1.7 $\pm$ 0.5	31.9 $\pm$ 4.5	60.2 $\pm$ 4.4	0.03 $\pm$ 0.02
Prodisol	2002	0.49 $\pm$ 0.04	2.6 $\pm$ 0.4	2.0 $\pm$ 0.3	43.4 $\pm$ 2.9	52.9 $\pm$ 2.8	0.03 $\pm$ 0.02
	2003	0.52 $\pm$ 0.05	6.1 $\pm$ 0.3	2.2 $\pm$ 0.2	34.7 $\pm$ 1.9	56.9 $\pm$ 1.9	0.03 $\pm$ 0.01
	2004	0.50 $\pm$ 0.03	6.1 $\pm$ 0.4	1.8 $\pm$ 0.3	29.4 $\pm$ 3.0	62.8 $\pm$ 2.9	0.07 $\pm$ 0.02

**Tocopherol composition and content.**  $\alpha$ -tocopherol represented the greatest proportion of total tocopherols, ranging from 88.5 to 97.9%, whereas  $\beta$ -tocopherol was less expressed (1.6 – 9.1%). We observed only traces of  $\gamma$ -tocopherol and  $\delta$ -tocopherol. The tocopherol content in the seed oil varied between 303.8 mg/kg oil (Allstar in 2003, 85<sup>th</sup>

location) and 1187.9 mg/kg oil (Prodisol in 2004, 54<sup>th</sup> location) (Table 5).

We observed an important decrease of tocopherol content in 2003 vs. 2002 (around 40%), which was probably caused by the high temperatures during the 2003 summer. ANOVA (Table 7) showed that both genetic background and years (explained

**Table 5.** Tocopherol amount for each sunflower hybrid and each year. The average over all the locations in a year is given by the mean value  $\pm$  SD.

**Tabla 5.** Cantidad de tocoferol para cada híbrido de girasol por año. La media de todos los lugares fue dada por el valor promedio  $\pm$  DE para cada año.

Cultivar	Year	Tocopherol content (mg/kg oil)				Total-T <sup>a</sup>
		$\alpha$ -T	$\beta$ -T	$\gamma$ -T	$\delta$ -T	
Allstar RM	2002	781.5 $\pm$ 51.5	47.0 $\pm$ 6.9	8.9 $\pm$ 2.2	4.6 $\pm$ 1.6	842.0 $\pm$ 57.4
	2003	461.5 $\pm$ 83.1	20.5 $\pm$ 3.8	2.9 $\pm$ 1.0	0.3 $\pm$ 0.3	485.1 $\pm$ 84.9
	2004	644.5 $\pm$ 37.3	19.1 $\pm$ 5.1	2.0 $\pm$ 1.9	0.3 $\pm$ 0.4	665.9 $\pm$ 39.9
Aurasol	2002	909.7 $\pm$ 51.3	66.8 $\pm$ 5.1	8.2 $\pm$ 2.8	4.0 $\pm$ 1.1	988.6 $\pm$ 53.4
	2003	520.3 $\pm$ 14.7	24.4 $\pm$ 5.3	1.0 $\pm$ 0.8	0.00	545.7 $\pm$ 19.9
	2004	713.5 $\pm$ 75.1	23.9 $\pm$ 3.6	1.9 $\pm$ 1.7	0.3 $\pm$ 0.3	739.5 $\pm$ 77.5
Melody	2002	771.1 $\pm$ 13.9	40.3 $\pm$ 6.0	3.2 $\pm$ 0.9	2.1 $\pm$ 0.5	816.6 $\pm$ 19.0
	2003	496.2 $\pm$ 55.4	20.7 $\pm$ 2.5	2.6 $\pm$ 0.9	0.00	519.5 $\pm$ 56.7
	2004	637.0 $\pm$ 66.7	17.4 $\pm$ 3.8	1.3 $\pm$ 0.8	2.6 $\pm$ 4.0	658.2 $\pm$ 70.5
Prodisol	2002	916.1 $\pm$ 61.3	84.5 $\pm$ 9.2	11.4 $\pm$ 2.7	6.6 $\pm$ 1.9	1018.4 $\pm$ 70.4
	2003	559.4 $\pm$ 79.7	39.5 $\pm$ 9.3	3.0 $\pm$ 1.6	0.6 $\pm$ 0.6	602.5 $\pm$ 87.1
	2004	864.2 $\pm$ 113.9	43.4 $\pm$ 8.2	4.1 $\pm$ 2.6	1.6 $\pm$ 0.7	913.4 $\pm$ 121.5

<sup>a</sup>. Total tocopherol calculated as the sum of all 4 isomers

**Table 6.** Phytosterol amount for each sunflower hybrid and year. The average over all locations in a year is given by the mean value  $\pm$  SD.

**Tabla 6.** Cantidad de fitoesterol de cada híbrido de girasol por año. La media de todos los lugares fue dada por el valor promedio  $\pm$  DE para cada año.

Cultivars	Year	Phytosterol (g/100 g oil)			Total-P <sup>a</sup>
		Campesterol	Stigmasterol	$\beta$ -Sitosterol	
Allstar RM	2002	37.4 $\pm$ 4.4	41.8 $\pm$ 6.9	231.3 $\pm$ 20.2	440.6 $\pm$ 37.0
	2003	36.9 $\pm$ 3.5	34.1 $\pm$ 4.4	212.2 $\pm$ 19.2	360.0 $\pm$ 36.7
	2004	39.2 $\pm$ 9.4	35.0 $\pm$ 7.8	198.8 $\pm$ 26.5	350.4 $\pm$ 56.5
Aurasol	2002	35.5 $\pm$ 3.4	38.6 $\pm$ 3.7	202.4 $\pm$ 22.4	388.8 $\pm$ 29.9
	2003	41.1 $\pm$ 11.4	42.1 $\pm$ 9.4	236.6 $\pm$ 52.5	404.2 $\pm$ 94.2
	2004	32.3 $\pm$ 7.7	30.0 $\pm$ 8.8	193.4 $\pm$ 34.0	306.8 $\pm$ 54.8
Melody	2002	35.9 $\pm$ 1.6	35.9 $\pm$ 0.2	188.5 $\pm$ 7.7	370.2 $\pm$ 22.52
	2003	36.2 $\pm$ 4.1	30.3 $\pm$ 4.2	198.1 $\pm$ 20.1	338.8 $\pm$ 34.8
	2004	30.8 $\pm$ 3.2	21.5 $\pm$ 3.7	193.2 $\pm$ 24.8	299.1 $\pm$ 34.33
Prodisol	2002	44.9 $\pm$ 2,6	39.5 $\pm$ 1.8	229.5 $\pm$ 10.7	467.7 $\pm$ 22.6
	2003	36.5 $\pm$ 3,9	31.2 $\pm$ 6.6	195.4 $\pm$ 21.8	337.4 $\pm$ 44.3
	2004	45.4 $\pm$ 13.5	34.7 $\pm$ 11.1	227.5 $\pm$ 40.0	358.9 $\pm$ 68.9

<sup>a</sup>. Total phytosterol calculated as the sum of campesterol, stigmasterol,  $\beta$ -sitosterol and  $\Delta^7$ -campesterol,  $\Delta^5$ -avenasterol,  $\Delta^7$ -stigmasterol and  $\Delta^7$ -avenasterol, data not shown.



**Table 7.** Mean squares of combined analysis of variance for oil content (g/g DM) and Stearic (16:0), Palmitic (18:0), Oleic (18:1) and Linoleic (18:2) acid concentrations (%); for  $\alpha$ -,  $\beta$ -,  $\gamma$ - and total tocopherol contents (mg/kg oil) and for campesterol, stigmaterol,  $\beta$ -sitosterol and total phytosterol contents (g/kg oil). The factors were cultivars ( $n = 4$ , namely Allstar RM, Aurasol, Melody and Prodisol), years ( $n = 3$ , 2002 to 2004) and locations (experimental culture locations in France).

**Tabla 7.** Cuadrados medios del análisis de varianza combinado para la cantidad de aceite (g/g MS) y la concentración de ácidos grasos (%): esteárico (16:00), palmítico (18:0), oleico (18:1) y linoleico (18:2); la cantidad de tocoferoles totales y de  $\alpha$ -,  $\beta$ -,  $\gamma$ - tocoferol (mg/kg de aceite), y de la cantidad total en fitoesteroles y de campesterol, estigmaterol y  $\beta$ -sitosterol (g/kg de aceite). Los factores estudiados fueron los cultivares ( $n = 4$ , es decir, Allstar RM, Aurasol, Melody y Prodisol), los años ( $n = 3$ , 2002 a 2004) y la ubicación (localización de las parcelas en Francia).

Compound		Genotype (G)	Year (Y)	G × A	Location (Year)
Fatty acids	16:0	0.000932***	0.003857***	0.000072	0.000079
	18:0	0.000123	0.000063	0.000060	0.000043
	18:1	0.244696***	0.044974***	0.003497	0.005092
	18:2	0.21016***	0.02594	0.0029	0.00425
Tocopherols	$\alpha$ -T	103654***	342795***	11653*	5997
	$\beta$ -T	3499***	6580***	221***	43
	$\gamma$ -T	17***	172***	11***	7***
	Total-T	149043***	448121***	14148*	6669
Phytosterols	Camp.	134**	0	71*	57**
	Stig.	188**	287**	57	41
	$\beta$ -Sit.	1765	6	1088	528
	Total-P	8260*	30079***	3262	1891
Oil yield	0.0021	0.0026	0.0003	0.0022**	
D.F. <sup>a</sup>	3	2	6	36	

<sup>a</sup>. Degree of freedom

\*, \*\*, \*\*\* significant at  $p=0.05$ ,  $p=0.01$  and  $p=0.001$

mainly by climatic conditions during grains filling) significantly affect each tocopherol and total tocopherol content in the oil ( $p<0.001$ ). No statistical significance for  $\alpha$ -tocopherol or total tocopherol content between locations was observed. The correlations between tocopherol isomers were generally positive and highly significant (Table 8). The  $\alpha$ -tocopherol content was closely related to the total tocopherol content ( $r^2 = 0.99$ ;  $p<0.05$ ). Moreover,  $\beta$ -tocopherol and total tocopherol contents were also positively correlated ( $r^2 = 0.83$ ;  $p<0.05$ ).

**Phytosterol composition and content.** The most abundant sterol in all cultivars tested was  $\beta$ -sitosterol (around 60%), followed by campesterol stigmaterol,  $\Delta 7$ -campesterol and  $\Delta 5$ -avenasterol (Table 6).  $\Delta 7$ -stigmaterol and  $\Delta 7$ -avenasterol were also detected and quantified but at very low levels. Total phytosterol content in oils tested ranged between 228.9 mg/100 g oil (Aurasol in 2004 in the 26<sup>th</sup> location) and 501.6 mg/100 g oil (Prodisol in 2002 in the 11<sup>th</sup> location). The total phytosterol content was significantly higher in 2002 than in 2003 or 2004, but these variations were less important than those observed for tocopherol content. Differences in phytosterol content between years 2002 and 2003 ranged from +4% to -28% among (or: depending on varieties) varieties.

ANOVA (Table 7) showed that the year affect total phytosterol content in oil ( $p<0.001$ ). Genotype affects also campesterol and stigmaterol contents ( $p<0.01$ ) but not  $\beta$ -sitosterol content. Location (Year) effect was only significant for campesterol content, but not for the total phytosterol content.

Significant and positive correlations between phytosterol components (Table 8) were observed: (1) between  $\beta$ -sitosterol and total phytosterol content ( $r^2 = 0.78$ ;  $p<0.05$ ); (2) between campesterol and  $\beta$ -sitosterol ( $r^2 = 0.67$ ;  $p<0.05$ ), and (3) between campesterol and stigmaterol ( $r^2 = 0.69$ ;  $p<0.05$ ).

**Relationships between fatty acid, tocopherol and phytosterol composition.** The total tocopherol content was slightly but significantly correlated with oleic acid ( $r^2 = +0.40$ ;  $p<0.01$ ), and negatively correlated with linoleic acid ( $r^2 = -0.36$ ;  $p<0.01$ ) (Table 8). Total tocopherol content (as well as  $\alpha$ -tocopherol and  $\beta$ -tocopherol contents) was negatively correlated with palmitic acid ( $r^2 = -0.70$ ;  $p<0.01$ ). Generally, the individual phytosterols and total phytosterol content were not significantly correlated with fatty acid content. This was the case for  $\beta$ -sitosterol, the main sterol in sunflower oil, which did not correlate with any fatty acid. Finally, total tocopherols and total phytosterols were positively correlated ( $r^2 = 0.47$ ;  $p<0.01$ ).

**Table 8.** Significant Pearson correlation coefficients between individual and total tocopherols, individual and total phytosterols and individual and total fatty acid contents in oil from four hybrids grown in different locations during 2002, 2003 and 2004 summers.**Tabla 8.** Coeficientes de correlación de Pearson significativos entre tocoferoles individuales y totales, fitoesteroles individuales y totales, y contenido de ácidos grasos individuales y totales del aceite de cuatro híbridos cultivados en diferentes lugares en 2002, 2003 y 2004.

Component	Correlation Coefficient	Component	Correlation Coefficient
<b>Tocopherols - Tocopherols</b>		<b>Tocopherol - Phytosterol</b>	
$\alpha$ -tocopherol - $\beta$ -tocopherol	+ 0.78 **	$\alpha$ -tocopherol - Campesterol	+ 0.26 *
$\alpha$ -tocopherol - $\gamma$ -tocopherol	+ 0.56 **	$\alpha$ -tocopherol - $\beta$ -sitosterol	+ 0.27 **
$\alpha$ -tocopherol - $\delta$ -tocopherol	+ 0.66 **	$\alpha$ -tocopherol - Total phytosterol	+ 0.44 **
$\alpha$ -tocopherol - Total tocopherol	+ 0.99 **	$\beta$ -tocopherol - Campesterol	+ 0.30 **
$\beta$ -tocopherol - $\gamma$ -tocopherol	+ 0.78 **	$\beta$ -tocopherol - Stigmasterol	+ 0.43 **
$\beta$ -tocopherol - $\delta$ -tocopherol	+ 0.75 **	$\beta$ -tocopherol - $\beta$ -sitosterol	+ 0.25 *
$\beta$ -tocopherol - Total tocopherol	+ 0.83 **	$\beta$ -tocopherol - Total phytosterol	+ 0.57 **
$\gamma$ -tocopherol - $\delta$ -tocopherol	+ 0.79 **	$\gamma$ -tocopherol - Campesterol	+ 0.32 **
$\gamma$ -tocopherol - Total tocopherol	+ 0.61 **	$\gamma$ -tocopherol - Stigmasterol	+ 0.49 **
$\delta$ -tocopherol - Total tocopherol	+ 0.70 **	$\gamma$ -tocopherol - $\beta$ -sitosterol	+ 0.30 **
		$\gamma$ -tocopherol - Total phytosterol	+ 0.63 **
		$\delta$ -tocopherol - Stigmasterol	+ 0.33 **
<b>Phytosterol - Phytosterol</b>		$\delta$ -tocopherol - Total phytosterol	+ 0.51 **
Campesterol - Stigmasterol	+ 0.69 **	Total tocopherol - Campesterol	+0.27**
Campesterol - $\beta$ -sitosterol	+ 0.67 **	Total tocopherol - $\beta$ -sitosterol	+ 0.27 **
Campesterol - Total phytosterol	+ 0.68 **	Total tocopherol - Total phytosterol	+ 0.47 **
Stigmasterol - $\beta$ -sitosterol	+ 0.23 *		
Stigmasterol - Total phytosterol	+ 0.54 **		
$\beta$ -sitosterol - Total phytosterol	+ 0.78 **	<b>Tocopherols - Fatty acids</b>	
		$\alpha$ -tocopherol - Palmitic	- 0.68 **
<b>Fatty acids - Fatty acids</b>		$\alpha$ -tocopherol - Stearic	- 0.22 *
Palmitic - Oleic	- 0.70 **	$\alpha$ -tocopherol - Oleic	+ 0.40 **
Palmitic - Linoleic	+ 0.66 **	$\alpha$ -tocopherol - Linoleic	- 0.35 **
Palmitic - Linolenic	+ 0.36 **	$\beta$ -tocopherol - Palmitic	- 0.71 **
Stearic - Oleic	- 0.52 **	$\beta$ -tocopherol - Oleic	+ 0.36 **
Stearic - Linoleic	+ 0.51 **	$\beta$ -tocopherol - Linoleic	- 0.31 **
Oleic - Linoleic	- 0.99 **	$\gamma$ -tocopherol - Palmitic	- 0.63 **
Oleic - Linolenic	- 0.28 **	$\gamma$ -tocopherol - Oleic	+ 0.28 **
Linoleic - Linolenic	+ 0.27 **	$\gamma$ -tocopherol - Linoleic	- 0.24 *
		$\gamma$ -tocopherol - Linolenic	- 0.24 *
		$\delta$ -tocopherol - Palmitic	- 0.57 **
<b>Phytosterols - Fatty acids</b>		$\delta$ -tocopherol - Oleic	+ 0.24 *
Campesterol - Stearic	+ 0.20 *	Total tocopherol - Palmitic	- 0.70 **
Campesterol - Linolenic	- 0.22 *	Total tocopherol - Stearic	- 0.21 *
Stigmasterol - Palmitic	- 0.26 *	Total tocopherol - Oleic	+ 0.40 **
Total Phytosterol - Palmitic	- 0.33 **	Total tocopherol - Linoleic	- 0.36 **

Correlation coefficient significant at  $p = 0.05$  (\*) and  $p = 0.01$  (\*\*) level, respectively.

**Relationships between components composition and meteorological conditions.** Correlation between minor components and the meteorological parameters were made to have a first sight of the influence of temperature and rainfall during seed growth. The cumulative temperatures in August showed a negative correlation with the total tocopherol content ( $r^2 = -0.69$ ;  $p < 0.05$ ). This negative correlation was also observed for each tocopherol isomer ( $r^2 = -0.60$ ;  $p < 0.05$ ) and for the total phytosterol content ( $r^2 = -0.38$ ;  $p < 0.05$ ). The same tendency was also found for the cumulative temperatures in June, July and September. On the contrary, there was a positive correlation between the sum of precipitations in August and the total tocopherol and total phytosterol contents (Fig. 3). Campesterol and stigmasterol contents were positively correlated to the sum of precipitation in June ( $r^2 = +0.70$ ;  $p < 0.05$ ).

## DISCUSSION

Sunflower oil yield is generally influenced by temperature during seed development. Thus, high temperatures during seed maturation may have a negative effect in oil synthesis (Baydar & Erbas, 2005; Ayerdi Gotor et al., 2006).

Alpha-tocopherol was the dominant isomer detected in this study, similar to the results obtained by Dolde et al. (1999) and Velasco et al. (2002). They only detected traces of  $\delta$ -tocopherol in the sunflower materials analyzed. Our tocopherol total content was similar to the values reported by Nolasco et al. (2004) (500 to 1200 mg/kg oil). However, the maximum measured value was lower than the one found by Velasco et al. (2002) (562-1872 mg/kg oil). Despite this, Velasco et al. (2002) also found a similar correlation between tocopherols homologues.

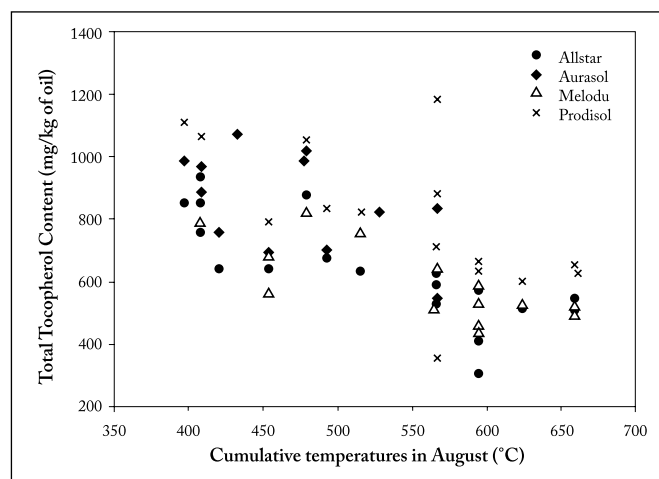


Fig. 3. Correlation between cumulative temperatures of August and total tocopherol content.

Fig. 3. Correlación entre la temperatura acumulada de agosto y el contenido total de tocoferoles.

This study clearly showed that the variation of total tocopherol content is dependent on genotype and cultivation year. The annual effect was mainly influenced by the meteorological conditions such as temperature. Results obtained show that the tocopherol content in sunflower oil is highly correlated to temperature. This is in agreement with results of Almonor et al. (1998) on soybean oil and Ayerdi Gotor et al. (2006) on sunflower oil. Both studies showed a reduction in total tocopherol content when plants were exposed to high temperatures during grain filling. However, Britz and Kremer (2002) reported that total tocopherol content in soybean oil remains constant despite differences in growth temperatures. The reduction in tocopherol content during a hot year could be explained by the role of these molecules in the plant. Tocopherols, particularly  $\alpha$ -tocopherol protects the plants against heat stress by scavenging singlet oxygen and lipid peroxil radicals leading to decreased (detected) amounts of tocopherols (Munné-Bosch, 2005). Previous studies concerning tocopherol variation in sunflower and in other plant species (Peterson & Qureshi, 1993; Goffman & Becker, 2002; Marwede et al., 2004; Fernández-Cuesta et al., 2012) show that genotype and environment either individually or their interaction affect the content and composition in seeds.

The range found on phytosterol content is close to the one reported by Vlahakis and Hazebroek (210 to 454 g/100 g oil) and others authors (Vlahakis & Hazebroek, 2000; Ayerdi Gotor et al., 2008; Fernández-Cuesta et al., 2012). As mentioned previously, the climate differed between years; 2002 was cold and wet, while 2003 was excessively warm and dry. The data presented indicate that low temperatures together with precipitation correlate with an increase in the total phytosterol content. These results are in agreement with those found by Zangenberg et al. (2004) in rye (*Secale cereale* L.) grains. On the contrary, Vlahakis and Hazebroek (2000) showed that the total phytosterol levels in greenhouse grown soybean seed increase under higher temperatures under controlled conditions. However, Hamama et al. (2003) did not show significant differences concerning phytosterol content between different genotypes of canola (*Brassica napus* L.), indicating that environmental conditions may affect the various species in a different manner.

The positive correlation observed between campesterol, stigmasterol and  $\beta$ -sitosterol indicate that the amount of different phytosterols could be simultaneously increased. In the literature, little is known concerning correlations between phytosterols contents. Fernandez-Cuesta et al. (2012) found a negative correlation between campesterol and stigmasterol, and between stigmaterol and  $\beta$ -sitosterol, but a positive correlation between campesterol and  $\beta$ -sitosterol. In canola oil, the same order of slight positive correlations between phytosterol components were observed (Hamama et al., 2003). In contrast, Hamama et al. (2003) found negative correlations between both campesterol and  $\beta$ -sitosterol

content and campesterol and  $\Delta 5$ -avenasterol contents. In this study, the only significant positive correlation was between brassicasterol and total phytosterol contents ( $r^2 = +0.40$ ;  $p < 1\%$ ).

Similar to the results of our study, Baydar and Erbas (2005) reported significant correlations between tocopherols and oleic acid content ( $r^2 = +0.79$ ) as well as tocopherols and linoleic acid content ( $r^2 = -0.82$ ).

The negative correlation between  $\beta$ -sitosterol and campesterol or stigmasterol could be explained due to the different pathways of biosynthesis of  $\beta$ -sitosterol and the others phytosterols (Schaller, 2003). Indeed, Schaller (2004) identified in (1) *Arabidopsis* the  $\Delta 24$ -isomerase-reductase which produces  $\beta$ -sitosterol and campesterol, and (2) higher plants a  $\Delta 22$ -desaturase implicated in the transformation of  $\beta$ -sitosterol to stigmasterol and campesterol to brassicasterol (Vlahakis & Hazebroek, 2000). Such enzymes were species, genotype and growth-temperature dependent (Garcés et al., 1992; Almonor et al., 1998). These previous results could contribute to explain the absence of correlation between  $\beta$ -sitosterol and stigmasterol, and the high correlation between campesterol and stigmasterol observed herein.

The correlation between total tocopherols and total phytosterols for sunflower oil in this study are in agreement with earlier results obtained on 12 cultivars from the same planting location ( $r^2 = +0.69$ ,  $p < 0.05$ ) (Vlahakis & Hazebroek, 2000).

The relationships between fatty acids, tocopherols and phytosterols and the climatic conditions during plant growth found in this study cannot be compared with data obtained by El-Shami et al. (2001) in rapeseed oil. In their study, correlations were obtained with values from only one genotype and one cultivation year, study which did not reflect any environmental influence.

However, this study indicated that both genotype and the growing conditions had a stronger influence on tocopherol composition and content than on phytosterol ones. Moreover, the contents of tocopherol, oleic acid percentage and phytosterol contents were positively correlated. Thus, it would be possible to increase tocopherol and phytosterol contents including high oleic acid content simultaneously through a breeding program.

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