Oil Phytosterols concentration in sunflower presents a dilution response with oil weight per grain

Running title: Oil phytosterols concentration in sunflower

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30 **Conflict of interest**

31 The authors declare that they have no conflicts of interest.

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35 **Abstract**

36 Phytosterols are plant sterols that present several benefits for human health. In crop
37 species, such as sunflower, oil phytosterols concentration shows genetic and environmental
38 variation. The reasons that cause such varied concentration of phytosterols in the oil are
39 unknown. The aim of this work was to investigate the relationships between phytosterols
40 concentration and composition and grain oil content in sunflower. Two sets of samples, with
41 lines and hybrids grown in different environments in Argentina and Spain, were used to
42 establish and validate the relationships between phytosterols and grain oil content. Oil
43 phytosterols concentration decreased in both types of genotypes, lines and hybrids, when oil
44 content per grain increased. A dilution like relationship accounted for 82% of the variability in
45 phytosterols concentration among genotypes and environments. Phytosterols concentration
46 rapidly increased with oil contents lower than 11.4 mg per grain. This relationship was
47 observed for total phytosterols and each one of the phytosterols analyzed. With low oil per
48 grain, phytosterols composition varied, with higher proportions of β -sitosterol and
49 Δ^7 -stigmastenol. The relationships established in this work could serve to design breeding
50 and crop management strategies.

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52 Keywords

53 *Helianthus annuus*, phytosterols composition, oil quality

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Introduction

Phytosterols are one of the most important bioactive compounds in vegetable oils. These compounds have a very important role in human health, with anti-inflammatory (Valerio and Awad, 2011), antipyretic and antidiabetic properties (Brufau, et al., 2008), and a demonstrated effect in preventing atherosclerosis and reducing the incidence of cardiovascular diseases (Hansel, et al., 2011). However, decreasing the total amount of cholesterol in blood, and primarily low-density lipoprotein (LDL) is the best characterized and scientifically proven phytosterols function. A meta-analysis of 41 trials showed that intake of 2g of plant sterols/day reduced LDL by 10% (Schwartz, et al., 2008). More than nine phytosterols are presented in vegetable oils (Fernández-Cuesta, et al., 2014), but it is known that β -sitosterol and campesterol are the most common phytosterols in plants oils and the best allies for reduce cholesterol levels (Brufau, et al., 2008). Therefore, identifying, characterizing and quantifying their variability in sunflower will contribute to answer social and industrial demands. In turn, knowing the factors that affect the accumulation of phytosterols and their relationship with other constituents of the grain is important to design breeding and crop management strategies.

It has been shown that crop genetics may influence phytosterols concentration in sunflower oil. (Roche, et al., 2010). Nolasco, et al. (2010) observed variations in these compounds between 3513 and 4936 mg/kg oil in hybrids from different environments. In the same species, Velasco et al. (2013) reported a variation from 2954 to 3810 mg/kg kernel in 22 commercial hybrids grown in six environments. Roche, et al. (2010) found differences of up to 1180 mg of phytosterols/kg seed due to changes in sowing date in sunflower.

The environmental effects vary according to the species. In soybean and sunflower seeds higher levels of total phytosterols were observed as a result of higher temperatures during flowering and ripening (Roche, et al., 2006, Vlahakis and Hazebroek, 2000), while no temperature effect has been reported in canola seeds (Hamama, et al., 2003). Different authors have reported that water stress during seed development enhance phytosterols

accumulation in sunflower seeds (Anastasi, et al., 2010, Roche, et al., 2006). Roche, et al. (2006) consider that the physiological role of sterols in plant tissue protection and their influence on the regulation of membrane permeability and fluidity could explain the highest level of phytosterols in sunflower oil under stress conditions environmental (warm temperature and severe water deficit). Solar radiation intercepted by plants represents the energy source supporting their growth and metabolism. Previous studies have shown the influence of radiation on grain quality traits in sunflower. An increase in intercepted radiation during grain filling increased the amount of tocopherols and oil in sunflower grains (Izquierdo, et al., 2011). As the oil increased more than tocopherols, these compounds had lower concentration in the oil (González Belo, et al., 2017). Therefore, there is a dilution-like response between the tocopherols concentration in sunflower oil and the amount of oil in the grain (Nolasco, et al., 2004). It has not been studied whether a dilution-like response to oil content per grain is associated with phytosterols content and composition. The aim of this work was to investigate the relationships between phytosterols concentration and composition and grain oil content in sunflower.

Materials and Methods

Source of material and experiments set up

Two data sets were used in this study. The first one was originated from two experiments grown at the Unidad Integrada Balcarce (37°S, 58°W, Balcarce, Argentina), on two years, 2012 and 2014 (Exp A and B, respectively, Table 1). In Exp A, three genotypes were sown: Macon (Syngenta), Olisun 2 and HS05 (Advanta Seeds SAIC). The Macon and HS05 genotypes were also sown in Exp B. These genotypes were selected as representative of each type of hybrids (traditional, high oleic and high stearic-high oleic). The sowing dates were Oct-30 and Oct-22 for Exp A and B, respectively. The experiments were carried out with a randomized complete block split plots design with three replications.

Genotypes were assigned to the main plots and treatments to modify weight per grain and oil content were assigned to the subplots. Treatments were i) removal of 75% or 80% of the leaves, ii) removal of 50% of the grains and iii) control. These treatments allowed to obtain variability in oil concentration and oil per grain concordant with the results previously reported in the literature (Andrade and Ferreiro, 1996, Dosio, et al., 2000, Izquierdo, et al., 2009). Further details about Exp A and B are presented in González Belo, et al. (2018).

The second data set consisted of five lines grown in different environments. Three of the lines were selected for a modified phytosterols profile. They were GF5-1 and GF5-2, with increased proportion of campesterol, and GF5-7, with increased proportion of Δ^7 -stigmasterol. They are F5:6 lines developed by recombination from selected germplasm populations reported by Fernández-Cuesta, et al. (2014). The other two lines were the maintainer line HA89, with conventional phytosterols content and profile, and line IASP-18, with increased levels of total phytosterols in the oil (Fernández-Cuesta, et al., 2014). The five lines were grown in pots under open air conditions in 2015 (E1), 2016 (E2), and 2017 (E4) and in the field in 2015 (E3), 2016 (E5), and 2017 (E6), in all cases in Córdoba (Spain). The plants were arranged following a randomized complete block design with three replications of 16 plants each.

The plant material selected ensured genetic variability, as the genotypes used presented differences for grain quality traits such as fatty acid and phytosterol profiles. The experiment performed in Argentina and Spain contributed to obtain environmental variability. The mean temperature and incident solar radiation, which is intercepted by plants to support grain filling, for each environment are presented in Table 1. Temperature was measured with a data logger and incident solar radiation was measured daily with a pyranometer (LI-200SB, LI-COR, Lincoln, NE, USA). Genotypes included lines and hybrids with different genetic backgrounds, and also with modifications in the fatty acid or the phytosterol profile. All hybrids from Exp A and B and lines from environments E2 to E5 were used to fit the dilution curves. Lines from environments E1 and E6 were used to validate the relationships.

Quantification of oil content per grain

For every plot of each experiment, weight per grain and grain oil concentration were determined. Weight per grain was calculated as the quotient between the weight of the whole sample and the grain number. Oil content was measured according to Robertson and Morrison (1979). Samples were dried in an oven at 60°C for 18 h and then placed in desiccators. When samples reached 22–24°C, their oil concentration (%) was determined using a nuclear magnetic resonance (NMR; Spinlock S.R.L., Córdoba, Argentina). The NMR was calibrated and validated using quantitative oil extraction samples for each type of hybrid (traditional, high oleic and high stearic-high oleic). Samples of 10g were measured in duplicate and values were averaged. Oil per grain calculated as (weight of grains x oil concentration)/100.

Quantification of total and individual phytosterols concentration in the oil

Phytosterols content and composition were analysed in duplicate by GLC following the method reported by Fernández-Cuesta, et al. (2012). In short, around 10 g of kernels were dried in an oven at 60 °C for 18 h and ground in a laboratory mill. Two hundred mg of the resulting flour were weighted and placed in 10-ml propylene tubes. Two hundred µL of internal standard solution, prepared by dissolving cholesterol (99% purity, reference C8667, Sigma-Aldrich, St. Louis, MO, USA) in hexane-ethanol (3:2) at a concentration of 0.1%, were added. Alkaline hydrolysis was performed by adding 2 mL of a solution of potassium hydroxide dissolved in ethanol at a concentration of 2% and maintaining the tubes in a water bath at 80 °C for 15 min. After this process, the unsaponifiable was extracted by vortexing with 1 mL hexane and 1.5 mL water. The upper hexane layer, containing the phytosterols, was transferred to 2-mL glass vials that were maintained in an oven at 37.5 °C for 16 h. Fifty µL of hexane and 50 µL of silylating mixture (Silan-Sterol-1, reference 355650.0922, Panreac Química, Barcelona, Spain) were added to the dried pellets. After 15 min at room temperature, the solution was transferred to 2-mL vials containing 200 µL inserts and

centrifuged at 4,000 rpm for 10 min (Unicen 21, Ortoalresa, Madrid, Spain). The supernatant was transferred to another vial that was capped and used for GLC analysis. GLC was performed on a Perkin Elmer Clarus 600 GC equipped with a ZB-5 capillary column (id = 0.25 mm, length = 30 m, film thickness = 0.10 μ m; Phenomenex, Torrance, CA, USA). Carrier gas was hydrogen, at a pressure of 125 KPa. Split injector and flame ionization detector were maintained at 320 °C. The oven was set at 240 °C with a ramping rate of 5 °C min⁻¹ to final temperature of 265 °C, maintained for 15 min. Phytosterol content was expressed as mg kg⁻¹ grain and mg kg⁻¹ oil, calculated on the basis of the oil content of the grains. The concentration of individual phytosterols was expressed as percentage of total phytosterols. β -sitosterol, campesterol, stigmasterol, Δ^7 -stigmastenol and Δ^7 -avenasterol were analyzed individually, whereas other phytosterols (e.i. Δ^7 -campesterol, Δ^5 -avenasterol, $\Delta^{5,24}$ -stigmastadienol) were considered as minor phytosterols (CODEX STAN, 2005).

Statistical analysis

Statistical analyses were carried out with the R statistical package (R CORE TEAM, 2012). Exponential decay regressions were used to determine the relationships between phytosterols concentration and other seed traits, which can be represented as $y = y_0 + a \cdot \exp(-b \cdot x)$, being y the μ g of total phytosterols g oil⁻¹, x is the oil content per grain and y₀, a and b are the model fitting parameters. To analyze the relationship between phytosterols and oil contents per grain, an exponential growth regression was used, which can be represented as $z = c \cdot \exp(d \cdot x)$, being z the μ g of total phytosterols per grain, x is the oil content per grain and c and d are the model fitting parameters.

Results and Discussion

The variability in oil per grain and oil phytosterols concentration of the data set used for establishing the relationships was 6.0-34.5 mg and 3157-20024 μ g/g oil, respectively (variations up to 575% and 634% for oil per grain and oil phytosterols, respectively)

(Table 2). The ranges of these variables of the data set used for validation were 5.3-28.3 mg and 3989-14872 $\mu\text{g/g}$ oil for oil content and phytosterols concentration, respectively.

A dilution relationship between oil phytosterols concentration and oil content per grain was observed in both lines and hybrids (Fig. 1a). Oil content per grain explained 77 and 46% of the variability in oil phytosterols concentration for lines and hybrids, respectively ($p < 0.0001$). In all cases, an inverse relationship between both variables was observed. Since both curves fit did not differ statistically ($p > 0.052$), a single curve was adjusted for both, lines and hybrids. This unique relationship accounted for 82% of both genetic and environmental variability in oil phytosterols concentration (Fig. 1a). Similar association was reported by Velasco, et al. (2013), who suggested that germplasm with low seed yield or low oil content, might be associated with higher oil phytosterols content, while evaluated 22 hybrids in six locations in southern Spain. Environmental conditions for high yield and oil also produce high phytosterols content per grain.

A dilution relationship was also observed for oil tocopherols concentration in sunflower (González Belo, et al., 2017, Nolasco, et al., 2004) and other oilseed species, such as soybean and canola (Izquierdo, et al., 2011). According to the adjusted equation in this work, the total phytosterols concentration was shown to increase with decreasing oil content for grains with an oil content of 11.4 mg or below ($p < 0.0001$). These values were slightly different for lines and hybrids (10.1 vs. 17.6 mg), due to differences in the oil phytosterols concentration range explored. Despite these differences, the grain oil content break point for phytosterols is lower than the break point for the relationship between oil tocopherols concentration and oil per grain in sunflower (22 mg per grain for hybrids of Exp. A and B (González Belo, et al., 2017) and 23 mg, (Nolasco, et al., 2004)). Consequently, the concentration of phytosterols in the oil would be more constant than the one of tocopherols, but in small grain with a low oil content the concentrations of both compounds rapidly increase. In the case of phytosterols this increase was from 4562 to 20024 $\mu\text{g/g}$. Oil phytosterols concentration tended to stabilize for oil contents per grain higher than 11.4 mg.

The amount of phytosterols per grain increased with an increase in oil weight per grain ($p < 0.0001$, Fig. 2). A unique exponential relationship accounted for 67% of the variability in phytosterols weight per grain of all genotypes. When oil weight per grain (mg oil/grain) increased, phytosterols weight per grain (μg phytosterols/grain) also increased but in general in a lower proportion than oil per grain, mainly at low oil weight per grain contents. These results indicate a non-stoichiometric relationship between oil and phytosterols accumulation, being both compounds synthesized during grain filling (González Belo, et al., 2018). Phytosterols are synthesized in the cytoplasm cell from the isoprenoid via, being the main precursor Acetyl-CoA and involving more than 25 reactions catalyzed with several enzymes (Merah, et al., 2012). The accumulation of oil in higher proportion than phytosterols produces the lower oil phytosterols concentration originating the dilution response. The stoichiometric oil-phytosterols accumulation would only occur at high oil weight per grain, where oil phytosterols concentration remained steady.

The relationship between oil phytosterols concentration and oil content per grain was validated with data of all lines from environments E1 and E6, which presented a similar range of oil per grain and phytosterols concentration than those used to establish the relationship (Table 2). The equation was able to predict the overall trends of oil phytosterols concentration (Fig. 1b). The line of predicted vs. observed values of oil phytosterols concentration did not differ from 1:1 relation ($p > 0.0589$, data not shown).

The relationship between each one of the phytosterols concentration in the oil and oil content per grain was also evaluated. Oil content per grain significantly explained the variation of oil concentration of each one of them Fig. 3). Despite the difference in magnitude of each phytosterol concentration in the oil, oil weight per grain accounted for between 65.9 and 77.7% of the variability in oil phytosterols concentrations ($p < 0.0001$). In the case of campesterol, genotypes GF5-1 and GF5-2 presented higher concentrations of this phytosterol than the other genotypes in all the oil weight per grain range. This was expected, as these genotypes were selected for high campesterol content. Hence, these genotypes

were excluded of the analysis of these phytosterols. All fitted functions properly predicted independent validation set values (insets in Fig. 3). β -sitosterol and campesterol showed the greatest variation in their concentrations, while Δ^7 -stigmastenol presented a more gradual decrease even at high oil contents per grain. This result is particularly interesting because this phytosterol is found in high proportion in sunflower, varying from 11.7% to 17.1% of total phytosterols (Velasco, et al., 2013). In our data set, the concentration of this phytosterol varied from 288.7 up to 3438.0 $\mu\text{g/g}$ oil (i.e. between 7.6 and 17.2% of the total phytosterols, respectively).

Although all phytosterols presented a dilution- relationship with oil weight per grain, phytosterols composition was not constant (Fig. 4). The proportion of β -sitosterol and Δ^7 -stigmastenol decreased at the expense of campesterol, stigmasterol and minor phytosterols. The concentration of β -sitosterol, the most abundant phytosterols, stabilized from 11.4 mg grain, while the proportion of Δ^7 -stigmastenol stabilized at higher oil contents per grain. The concentration of Δ^7 -avenasterol remained nearly steady, independently of the oil weight per grain. It is important to notice that β -sitosterol and campesterol are the best allies to reduce cholesterol levels because of a chemical structure that resembles that of cholesterol. Accordingly, they compete with cholesterol for transporter-mediated absorption in the intestine (Brufau, et al., 2008). Since β -sitosterol, campesterol and stigmasterol became almost steady at high oil per grain, oils with high concentrations of these phytosterols can be obtained by selecting grain batches with oil per grain lower than 11.4 mg.

Nowadays, the prediction models for sunflower crop predict phenology, grain yield (e.g. grain number and weight) and only some grain quality variables (e.g. grain oil concentration, oleic and linoleic proportion and oil tocopherols concentration), but do not predict oil phytosterols concentration (Pereyra-Irujo and Aguirrezábal, 2007, Villalobos, et al., 1996). Hence, the relationships established in this work could be incorporated into crop simulation models to improve the prediction of quality. It is interesting that, as all adjusted

relationships accounted for by environmental and genetic variability (except for lines GF5–1 and GF5–2 for campesterol) it would not be necessary to re-calibrate the equations for each genotype as usually occur for other quality traits (e.g. grain weight and oil content; Villalobos et al., 1996). Also, these models include environmental variations. Consequently, these relationships could be used to predict oil phytosterols concentration and composition of sunflower seeds produced in different environments.

For a sunflower genotype, de la Vega, et al. (2001) reported variations in grain weight and oil content due to different environments in Argentina, with variations in oil content per grain from 13.5 to 32.6 mg. That range of variation was similar to the range observed in hybrids from Exp A and B and, according to the relationships from Fig. 1 and 3, implies variations in oil phytosterols concentration and oil phytosterols composition. In the same sense, data set from the Argentine National Trial Network of Commercial Sunflower Hybrids (INTA-ASAGIR) show that low values of oil content per grain were obtained in the northern environments of Argentina (ASAGIR, 2018). Therefore, in this area sunflower grains would present high tocopherol (Nolasco, et al., 2004) and phytosterols concentration in the oil.

High heritability for the most abundant phytosterols content and concentration in sunflower were reported by Velasco, et al. (2013). These authors suggest then that selecting for these compounds would be feasible in this species. In previous work, we evidenced that the environment where the crop is developed, also play a key role in determining final phytosterols concentration. Environmental conditions for high yield and oil also produce high phytosterols content per grain, but according to the relationship established in this work, those environments would be associated with low oil phytosterols concentration. (González Belo, et al., 2018). So, even when selecting genotypes with modified content and/or composition of phytosterols it would be necessary to evaluate the effect of the environment to select the best location to sow each genotype in order to maximize final grain quality. For the industry, this knowledge could serve to classify batches of grains with oils concentrated

in phytosterols for the purpose of offering sunflower oil for human consumption with added value with the benefit of reducing cholesterol.

Conclusions

In this work, the variability in oil phytosterols concentration was associated to the variability in oil weight per grain. These compounds presented a dilution response with the content of oil per grain. Also, oil per grain accounted for the variations in phytosterols composition. For breeding programs the relationship could be used to identify superior genotypes in phytosterols concentration as proposed for tocopherols by (Aguirrezábal, et al., 2015) and as occurred with high campesterol genotypes in this work. These results could serve to design breeding and crop management strategies.

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388 **Tables**

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390 **Table 1. Mean temperature and incident solar radiation during grain filling for each environment**

Environments	Mean temperature (°C)	Incident solar radiation (MJ/m ²)
Exp A	22.0 ± 0.20	20.6 ± 0.36
Exp B	22.0 ± 0.16	20.1 ± 0.45
E1	24.0 ± 0.40	23.1 ± 0.49
E2	20.7 ± 0.52	20.0 ± 0.90
E3	25.8 ± 0.48	23.4 ± 0.53
E4	23.8 ± 0.61	24.1 ± 0.76
E5	26.6 ± 0.48	25.4 ± 0.40
E6	25.6 ± 0.56	25.9 ± 0.57

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Table 2. Range of oil weight per grain and oil phytosterols concentration of each genotype and environment. Environments with an asterisk were used for validation

Type	Genotypes	Environments	Oil Content Per Grain (mg)	Oil Phytosterols Concentration (µg/g of oil)
Hybrids	Macon	Exp A	19.5-31.6	3218-4171
		Exp B	24.0-34.5	3157-4213
	Olisun 2	Exp A	18.9-29.5	3626-4355
	HS05	Exp A	12.4-19.6	3546-5139
		Exp B	14.7-20.9	3419-4664
Lines	GF5-1	E1*	26.2-28.3	4570-5339
		E2	14.8-22.5	4846-5425
		E5	26.5-27.9	4121-4630
		E6*	17.8-22.6	3989-4261
	GF5-2	E1*	10.7-14.8	5310-6007
		E2	7.7-17.1	4678-6583
		E3	13.8-19.7	4270-4990
		E5	16.5-17.8	4178-4297
		E6*	13.8-16.2	4443-4750
	GF5-7	E1*	7.9-9.2	6500-7788
		E2	10.6-11.7	5822-5955
		E3	10.4-11.4	4563-4932
		E4	9.5-10.9	5648-6647
		E5	11.6-15.1	5060-5439
		E6*	6.9-7.3	7078-7567
	HA89	E1*	9.4-10.7	8805-9847
		E4	8.0-11.3	8298-9439
		E5	12.0-12.7	6603-6988
		E6*	8.3-10.0	6223-7443
	IASP-18	E1*	5.3-7.5	13536-14872
		E4	6.8-9.3	10643-11673
		E5	6.0-7.5	15551-20024
		E6*	6.3-6.7	11398-11826

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396

397 Figure legends

398

399 Fig. 1 Oil phytosterols concentration ($\mu\text{g/g}$ oil) as a function of oil per grain (mg) for (a) lines and hybrids
400 used to establish the relationship and (b) independent data from environments E1 and E6 used
401 as validation data set. In both graphs the line represents the adjusted equation of data points
402 from plot a.
403

404 Fig. 2 Phytosterols content per grain (μg) in relation to oil content per grain (mg) for lines and hybrids
405 used to establish the dilution relationship. The line represents the adjusted equation of data
406 points. IASP-18 is a genotype with increased total phytosterols content and it was not consider
407 in the adjustment.
408

409 Fig. 2 Oil phytosterols concentration ($\mu\text{g/g}$ oil) in relation to oil per grain for β -sitosterol (a), campesterol
410 (b), stigmasterol (c), Δ^7 -stigmasterol (d) and Δ^7 -avenasterol (e). Solid lines represent the
411 adjusted regressions. For campesterol, filled diamonds and empty triangles correspond to
412 modified lines 1 and 2 with high content of that phytosterol. The inset graphs show independent
413 data from environments E1 and E6 used as validation data set for each phytosterol.
414

415 Fig. 3 Proportion of each phytosterol in the oil as a function of oil weight per grain.
416