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Fatty acid and phytosterol accumulation during seed development in three oilseed species

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Summary This study was carried out to compare the kinetic accumulation of bioactive lipids during seed development in three oil crops rapeseed, sunflower and woad. Field experiments were conducted under organic conditions during 3 years. After flowering, seeds were collected each 4–5 days until harvest. The three species differed by the quantity and quality of both fatty acids and sterols. Higher levels of phytosterols and fatty acids were reached between 20 and 40 days after flowering (DAF) in sunflower, 40 and 60 DAF in rapeseed and 25 and 45 DAF for woad. The modification of lipid composition during the grain filling depends on species. Knowledge of the composition and accumulation of fatty acids and phytosterols in sunflower, rapeseed and woad seeds would assist in efforts to achieve industrial applications. These seeds may give an interesting source of bioactive lipids.

Keywords Bioaccumulation, fatty acid, oilseed species, phytosterols, seed.

Introduction

The economic and societal interest for quality of oil crop seed composition has grown since the last decade due to the increased number of cardiovascular diseases associated with human fat diet and their fatty acid composition. Indeed, vegetable oils have become greatly important due to nutritional purposes and raw materials of industrial products (Carvalho *et al.*, 2006). At the same time, researches have been intensified on minor oil constituents such as phytosterols, which provide an added value to the crop yield with a double interest in human nutrition, pharmaceuticals or cosmetics (Valerio & Awad, 2011). Unsaturated fatty acids only provided by alimentation are used as precursor molecules for the biosynthesis of other major polyunsaturated fatty acids and are involved in the prevention of cardiovascular diseases (Carvalho *et al.*, 2006; Gillingham *et al.*, 2011). Previous studies have indicated that diets rich in omega-3 fatty acids lower blood pressure significantly in people with hypertension (Holm *et al.*, 2001; Rasmussen *et al.*, 2006). Phytosterols are used for their propriety to reduce

significantly bad cholesterol absorption (Moreau *et al.*, 2002; Casas-Agustench *et al.*, 2013; Silbernagel *et al.*, 2013; Wong 2014) and a protective action against cancers (colon, prostate, lungs) by promoting tumoral cell apoptosis (Woyengo *et al.*, 2009). This increasing attention on phytosterol has prompted the development of functional foods with added plant sterols.

Phytosterol and fatty acids are ubiquitous in plant and play crucial role in cell membrane microstructure and embryogenesis and modify the permeability of membrane in response to environmental stresses (Moreau *et al.*, 2002; Roche *et al.*, 2010a).

Seeds of oil crops contain high contents of these compounds at different composition patterns which provide large possibilities of industrial applications depending on the plant used (Roche *et al.*, 2010a). Among these, rapeseed (*Brassica napus*) has been identified as the species with higher content (Roche *et al.*, 2006). Sunflower (*Helianthus annuus*) seeds are also among the important natural sources of phytosterols (Roche *et al.*, 2010b). No information is available on the seed oil crop *Isatis tinctoria* L., a medicinal herb which was widely cultivated in Europe from the 12th to the 17th century as a source of the indigo dye (Clark *et al.*, 1993). Although lipid contents in sunflower and rapeseed oil were widely genetically improved for a long time (Vear, 2010), woad was mostly selected for pigment content (Angelini *et al.*, 2007). The cultivation of woad was abandoned in the

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In memory of Andrée Bouniols who left us in 2008

19th century when synthetic dyes were developed (Spartaro *et al.*, 2007). But the increasing interest in natural products from a renewable source has encouraged growers to reintroduce indigo-producing crops into the European agriculture (Sales *et al.*, 2006). Several studies have examined the accumulation of FA (Carvalho *et al.*, 2006; Baud & Lepiniec, 2010; Nguyen *et al.*, 2015) or sterols in seeds of oil species (Herchi *et al.*, 2009; Roche *et al.*, 2010a; Merah *et al.*, 2012).

Different ratios combining fatty acid and phytosterol patterns represent new seed quality traits particularly interesting for food and nonfood industries. To determine the time point corresponding to the best qualitative and quantitative yield of phytosterols and fatty acids, this study aimed to compare the kinetic accumulation of fatty acids and phytosterols during seed ripening in two commonly used oil crops rapeseed and sunflower, and in woad seeds (*Isatis tinctoria* L.).

Materials and methods

Plant material

Accumulations of phytosterols and fatty acids during seed development were studied with the sunflower hybrid variety Santiago II (NK-Syngenta seed), widely grown in south-west France in the period 2000–2006. Woad seeds were collected from CAPA Institute (Coopérative Agricole des Plaines d'Ariège) and rapeseed cultivar Brutor (Ringot Cie, France) from agricultural college of Auzerville, near Toulouse (south-west of France).

Field trials

Field experiments were carried out in south-western France at INRA, Toulouse (31), in 2006. The site had deep limestone clay–silt soil with high water-holding capacity (Cabelguenne *et al.*, 1999). Fatty acid accumulation was also followed in the years 2002 and 2005 only on sunflower. Sunflower plants were sown at 7.1×10^4 plants ha^{-1} on 15 April in 2002 and in 2005, and on 26 April in 2006, as conventional sowing date of south-western France. Woad and rapeseed plants were planted at 3.0×10^4 plants ha^{-1} on 15 and 5 September 2006, respectively.

Seed water content (SWC in % of seed dry matter) was measured on each sample as an indicator of stage of physiological maturity to make possible comparison between the different oil crops. Ten per cent was the SDM selected to reach this condition.

The main phenological stages were noticed weekly until flowering. After flowering, plants were collected each 4–5 days until harvest. Seeds were dried at 0% of moisture before analysing. Woad seeds were extracted from their pericarp before grounding. Although harvest

was realised at different days after flowering according to oil crops (53, 59 and 64 DAF for woad, rapeseed and sunflower, respectively), seeds were harvested at 10% of SWC, which corresponded to seed maturity.

Biochemical analyses

Data are expressed as percentage of total fatty acids (mean values) for major fatty acids, as weight percentage of seed dry matter for crude oil, and as mg per 100 g of dry matter for phytosterols. Fatty acid data were also expressed in mg per 100 g of dry matter. Seeds were dried at 80 °C for 48 h to make sure to have 0% humidity.

Crude oil was extracted from dried ground seeds using an automatic Soxhlet extractor (accelerated solvent extractor ASE-200; Dionex) equipped with 11 mL cells, analytical balance and Dionex vials for the collection of extracts (40 mL; P/N 49465), using cyclohexane as solvent. Oil extract was concentrated in a rotary evaporator (Janke & Kunkel IKA-Werke) by distillation at reduced pressure and 40 °C until the solvent was totally removed. Mean seed oil content was determined gravimetrically using three different samples, and it was replicated three times.

The small-scale sample extraction method (Roche *et al.*, 2010a) was used to extract and determine sterol content of sunflower, rapeseed and woad seeds. Ground seed samples (1.3 g) were added to each tube containing 100 µg of cholestanol (dihydrocholesterol; Aldrich Chemicals Co., Lyon, France), used as an internal standard. Saponification was performed by adding ethanolic KOH (1 M) (TITRINORM, Prolabo) for 60 min at 75 °C. One millilitre of distilled water was added to the samples, and the unsaponifiable fraction was extracted from the saponified lipids with 6 mL of *iso*-hexane (Merck). Sterols were silylated by *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide (MSHFBA; Macherey-Nagel, Hoerdts, France) mixed with 1-methylimidazole (Sigma, Lyon, France). One microlitre of sterol trimethylsilyl ether derivatives was injected into a Perkin-Elmer GC equipped with a CP-SIL 5CB 30 m column (i.d.: 0.25 mm), and an on-column injector. Detection was performed with a flame ionisation detector (FID).

Fatty acids were determined by a method based on the oil solubility in *tert*-butylmethyl ether (TBME; ME0552, Scharlau) and the transformation of fatty acids to their methyl esters (norm ISO 5509: 1990). Whole seeds (3 g) were ground with 5 mL of TBME. A sample of 2 mL was then filtered using 13-mm-diameter GHP filters. A volume of 50 µL of 0.2 M trimethylsulphonium hydroxide in methanol (Macherey-Nagel) was mixed to 100 µL of the filtrate according to the norm NF EN ISO 12966-3. One microlitre of this mix was injected using an automatic sampler into a GC-3800

chromatograph (Varian, Les Ulis, France), with FID. The GC was equipped with a CP Select CB 50 m capillary column (ID: 0.25 mm). The initial oven temperature was held at 185 °C for 40 min, increased at a rate of 15 °C min⁻¹ to 250 °C and then held there for 10 min. The injector and detector temperatures were set at 250 °C. The fatty acid methyl ester (FAME) proportions were identified by comparison with the retention times of a known standard mixture of FAME (Fatty Acid Methyl Ester Mix of rapeseed oil; Supelco, USA), used as an external standard. To evaluate the combination of seed quality traits in the three oil crop seeds (sunflower, rapeseed and woad) for food industrial purposes and to make sure the results would be comparable, data at harvest were expressed as concentration of the SDM, which was around 10% of the SDM to make sure to harvest the seeds at the same physiological stage.

Results

Rapeseed contained the highest concentration of phytosterols (1.8-fold more) as well as total monounsaturated fatty acids (2.4-fold more) compared to sunflower (Table 1). Woad seeds contained the weakest concentrations of phytosterols. In sunflower seeds, MUFA are only represented by oleic acid (C18:1), whereas in rapeseed, it regroups palmitoleic (C16:1), oleic and eicosenoic acids (C20:1). In woad seeds, four components of this category were represented (C16:1, C18:1, C20:1 and C22:1), and the content reached 47%.

Concerning the polyunsaturated fatty acids (PUFA), sunflower seeds contained the highest concentration with 2.0 and 1.4 times more PUFA than rapeseed and woad, respectively (Table 1). In this category, rapeseed and sunflower oils were mainly composed of linoleic acid (C18:2), whereas woad and rapeseed held the highest concentration of C18:3, which represents the main ω -3 unsaturated fatty acid in plants. Nevertheless, rapeseed showed nearly two times more linoleic acid than woad (Table 1).

Palmitic (C16:0) and stearic (C18:0) acids mainly represent the saturated fatty acids (SFA), and sunflower seeds contained the highest concentration compared to rapeseed and woad (Table 1). Behenic acid (C22:0) was also represented as the highest concentration in sunflower seeds, whereas woad seeds contained the highest arachidic acid concentration (C20:0).

Sterol composition was also different between the three species (Table 1). Although there was contrasted level of contents, β -sitosterol remained the main sterol with more than 50% of total sterol content whatever the seed was. Similar to total sterols, the content of β -sitosterol in rapeseed was the highest, with 192.2 mg per 100 g of SDM. The same result was obtained for

Table 1 Fatty acid and sterol compositions of rapeseed, sunflower and woad seeds at harvest

Compound	Species		
	Sunflower	Rapeseed	Woad
SFA			
Cis Palmitic C16:0	5.73 ± 0.00	5.01 ± 0.01	5.07 ± 0.00
Cis Stearic C18:0	5.81 ± 0.03	1.70 ± 0.00	2.35 ± 0.01
Cis Arachidic C20:0	0.35 ± 0.01	0.45 ± 0.01	1.68 ± 0.00
Cis Behenic C22:0	0.84 ± 0.05	0.23 ± 0.01	0.48 ± 0.00
Total SFA	12.72 ± 0.04	7.37 ± 0.03	9.58 ± 0.02
MUFA			
Cis Palmitoleic	/	0.28 ± 0.00	0.18 ± 0.00
C16:1n9c			
Cis Oleic C18:1n9c	27.70 ± 0.07	61.77 ± 0.04	16.51 ± 0.02
Cis 9 Eicosenoic	/	0.95 ± 0.01	10.01 ± 0.01
C20:1n 9			
Cis Erucic C22:1n9	/	<0.1	20.30 ± 0.01
Total MUFA	27.70 ± 0.07	62.99 ± 0.07	47.01 ± 0.06
PUFA			
Linoleic C18:2n6c	59.52 ± 0.06	21.05 ± 0.03	12.40 ± 0.02
Linolenic C18:3n3	/	8.59 ± 0.02	31.00 ± 0.04
Total PUFA	59.58 ± 0.05	29.64 ± 0.02	43.41 ± 0.03
Oil content (%)	41.01 ± 0.08	44.05 ± 0.05	16.09 ± 0.03
β Sitosterol	98.02 ± 0.22	192.21 ± 0.31	68.25 ± 0.12
Campesterol	7.93 ± 0.05	131.56 ± 0.14	20.32 ± 0.07
Brassicasterol	Trace	36.73 ± 0.09	3.37 ± 0.04
Δ 5 Avenasterol	9.42 ± 0.05	10.62 ± 0.03	14.82 ± 0.12
Δ 7 Stigmastenol	26.43 ± 0.11	Trace	Trace
Stigmasterol	12.71 ± 0.06	4.91 ± 0.01	3.41 ± 0.02
Δ 7 Avenasterol	12.67 ± 0.05	Trace	Trace
Total phytosterols	209.52 ± 0.32	386.46 ± 0.39	114.11 ± 0.32

The values of SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids) are expressed in % of the DM, and sterols are given in mg.100 g⁻¹ DM. The total amount of phytosterols represents only the desmethylsterols.

campesterol content with a high proportion of this compound (131.5 mg per 100 g) compared to both the others (7.9 and 20.3 mg per 100 g for sunflower and woad, respectively). Once again, rapeseed contained a high level of brassicasterol (36.7 mg per 100 g of SDM), which is a specific compound to *Brassicaceae* species, whereas it is absent in sunflower seeds. Woad seeds contained brassicasterol at a lower level, 10-fold less than that in rapeseed. The opposite situation was observed concerning Δ 7-stigmastenol and Δ 7-avenasterol that were present only in sunflower seeds compared to rapeseed and woad ones. Highest stigmasterol content was also noticed in sunflower compared to rapeseed and woad, whereas Δ 5-avenasterol highest content was recorded in woad seeds compared to the others.

During ripening of sunflower seeds, C18:1 accumulation (expressed in % of total fatty acid content) increased until 20–25 DAF to reach a maximum of

50% at 75% of SDM (Fig. 1a) at a rate of 2.6% per day. Following this phase, a twofold decrease was observed, reaching around 25% from 25 to 35 DAF. Linoleic acid (C18:2) content showed the opposite variation (a decrease phase until 20–25 DAF followed by an increase stabilising from 35 DAF), followed by an increase rate of 1.3% per day, leading to 60% of total fatty acids at harvest (Fig. 1b). Stearic acid content (C18:0), which is under the regulation of the well-known $\Delta 9$ -desaturase to produce C18:1, followed globally the same evolution as C18:1 reaching a maximum slightly earlier (20 DAF) (Fig. 1c) with a rate of -0.16% per day to contribute to the accumulation of C18:2. Palmitic acid (C16:0) content decreased at a rate of -1.7% per day from 5 DAF until 20 DAF and stabilised when C18:1 level was maximum (Fig. 1d).

In rapeseed, C18:1 content increased from 15 DAF to 40 DAF and stabilised until harvest to reach 60% (Fig. 2a). Linoleic acid content decreased as much as oleic acid increased. The C18:0 and C16:0 both presented a peak at 25 DAF with, respectively, a maximum of 8% and 13%, followed by a decrease until 40 DAF to reach 2% and 4% at harvest (Fig. 2b). Linolenic acid (C18:3) was regularly synthesised from 20 DAF to reach a maximum of 8%.

During woad seed ripening, C18:1 content decreased dramatically from 17 DAF to 24 DAF (55% to 20% of total fatty acid content) to stabilise until harvest at 15% (Fig. 3a). Linoleic acid content varied weakly (from 20% to 12%). Starting to accumulate at 15 DAF, C20:1 content stopped at a maximum of 10% at 30 DAF, whereas C22:1 continued to increase until more than 20% at harvest. Woad seeds presented a continued increase of C18:3 from 15 DAF to reach the highest content at harvest (more than 30%) compared to rapeseed and sunflower (Fig. 3a). The accumulation pattern of saturated fatty acid contents in woad seeds followed the one of rapeseed with a maximum at 25 DAF and a final content of 2% and 15% for C18:0 and C16:0, respectively (Fig. 3c).

Comparing the three species, rapeseed was the one containing the best level of total phytosterol content (around 390 mg per 100 g of SDM) with an increase occurring all along the development to reach 9.4 mg day^{-1} (for 100 g of SDM). In sunflower seeds, total phytosterol content increased progressively at a rate of approximately 7.8 mg day^{-1} (for 100 g of SDM) until 40 DAF, followed by a plateau until maturity at around 210 mg per 100 g of SDM (corresponding to 46% less than that in rapeseed). Woad is the species whose seeds contained the lowest content at maturity (around 115 mg per 100 g of SDM), due in part to the slight decrease observed from 37 DAF until maturity. The rate of the accumulation during the first phase (from 15 to 37 DAF) was around 3.9 mg day^{-1} (for 100 g of SDM).

Discussion

In sunflower seeds, the opposite correlation between C18:1 and C18:2 acids may be explained by the biochemical relation already demonstrated to be steady in different genotypes and conditions (Roche *et al.*, 2006) that involve the $\Delta 12$ -desaturase enzyme responsible for the conversion of C18:1 into C18:2 (Baud & Lepiniec, 2010). Our results are in accordance with those reported by Harris *et al.* (1978) showing a peak of C18:1 concomitantly to a minimum of C18:2 at 15 DAF, which was however earlier than our report and may be due to the cultivars studied and/or environmental factors. Moreover, C18:1 and C18:2 reach different levels compared to our results at harvest (15% less of C18:1 and 15% more of C18:2).

The C18:1 and C18:2 profiles of rapeseed are in accordance with the one of a low-erucic acid ZS9 rapeseed cultivar described by Hu *et al.* (2009). Although fatty acid accumulation in rapeseed revealed a different profile compared with sunflower seeds, whatever the fatty acid, the correlation between C18:1 and C18:2 was also observed with a decrease in C18:2 content until 40 DAF, followed by a phase of stabilising until harvest.

C18:3 was detected in rapeseed since 15 DAF to reach a maximum at 40 DAF of almost 10%. In an opposite way compared to sunflower and rapeseed seed profile, C18:1 content is very low through the seed desiccation, whereas C18:2 one is slightly increased. This result shows that the relevant highly significant correlation between C18:1 and C18:2 contents established for rapeseed and sunflower does not exist in woad seeds. This could be explained by the presence of the unsaturated fatty acids (C20:1 and C22:1) representing 65% of the MUFA that modifies C18:1 metabolic requirements in the seed (Fig. 3b).

However, saturated fatty acids (C16:0 and C18:0) accumulate in the same way as in rapeseed and woad seeds (maximum at 25 DAF and a plateau until harvest), whereas their profiles are different in sunflower seeds. It may be suggested that the similar kinetic patterns of saturated fatty acids in rapeseed and woad could be related to the presence of the supplementary step in the unsaturated fatty acid metabolic pathway leading to the accumulation of the C18:3 (catalysed by the $\Delta 15$ desaturase), similar to that suggested in *Arabidopsis* leaves (Browse *et al.*, 1986). Although woad and rapeseed species both belong to *Brassicaceae* family, erucic acid (C22:1) is less present in rapeseed as a consequence of breeding programmes that aimed to eliminate this component responsible for myocardial lipodosis and heart lesions in test animals (Charlton *et al.*, 1975; Food Standards Australia New Zealand, 2003; Abbadi & Leckband, 2011), whereas woad crop was not bred for fatty acid profile modification.

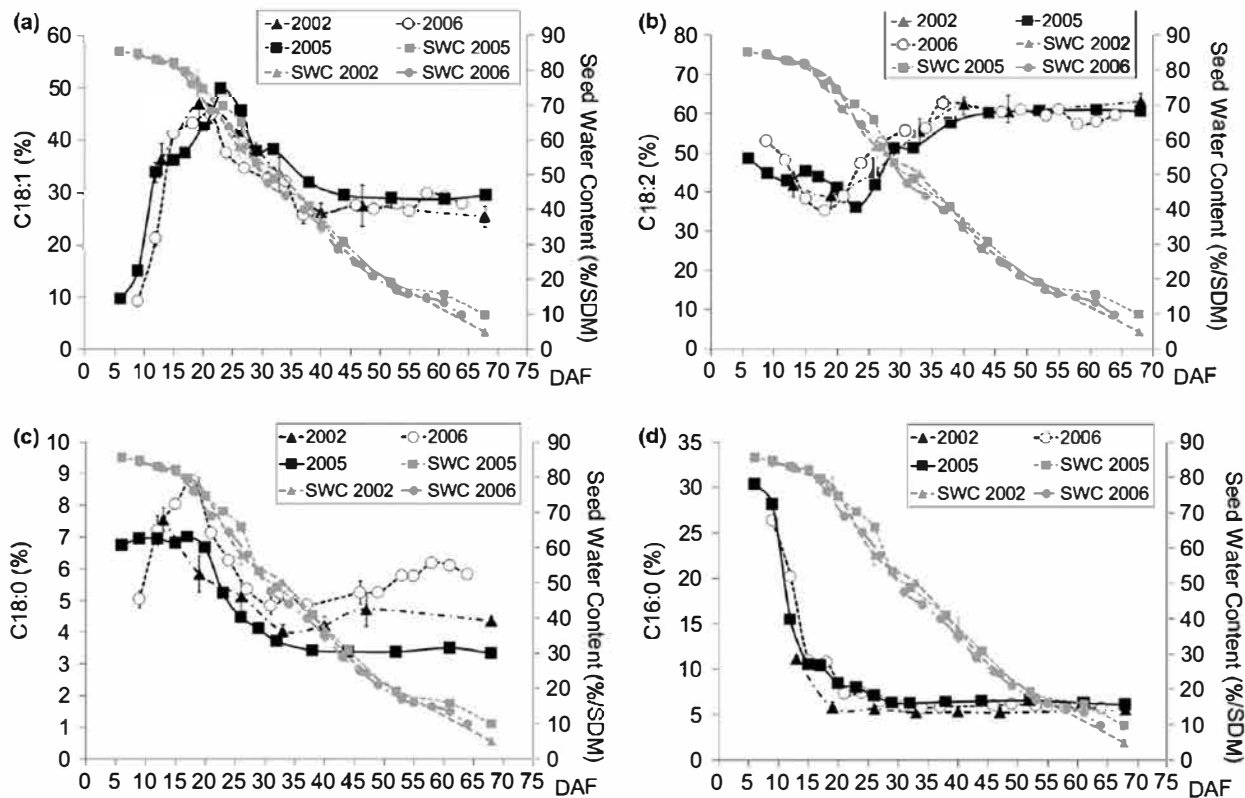


Figure 1 Accumulation of unsaturated fatty acids (a) oleic acid (C18:1) and (b) linoleic acid (C18:2), and saturated fatty acids (c) stearic acid (C18:0) and (d) palmitic acid (C16:0) during 3 years (2002, 2005, 2006) of sunflower seeds during seed ripening. DAF: days after flowering.

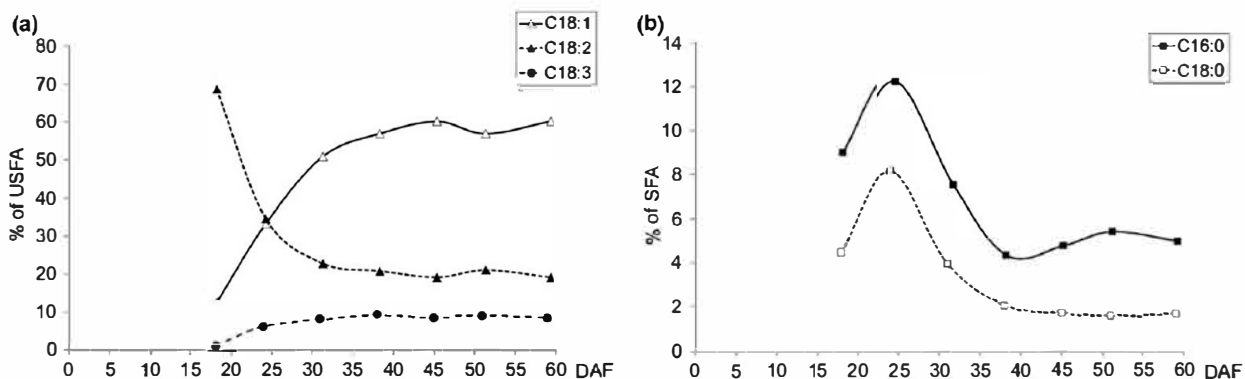


Figure 2 Accumulation of unsaturated fatty acids (UFA) (a) oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3), and saturated fatty acids (SFA) (b) stearic acid (C18:0) and palmitic acid (C16:0) during 2006 in rapeseed during seed ripening.

Total phytosterols accumulate at three different patterns according to the oilseeds (Fig. 4). Total phytosterol content of sunflower seeds reached a maximum at 40 DAF followed by a slight decrease, and total sterol content in rapeseed increased continuously until harvest, reaching the highest level of total phytosterol content among the three oilseeds (360 mg per 100 g of SDM). The levels of sterols in woad seeds exhibit only modest

increases during the early stages of development, but increase rapidly from 30 to 40 DAF. After this point, as the seed starts to desiccate (between 35 and 40 DAF corresponding to 35% of seed water content), the rate of sterol accumulation decreases until harvest to reach the weakest rate of 110 mg per 100 g of SDM compared to sunflower (3.3-fold less) and rapeseed (1.7-fold less). The accumulation of total sterols during the maturity of the

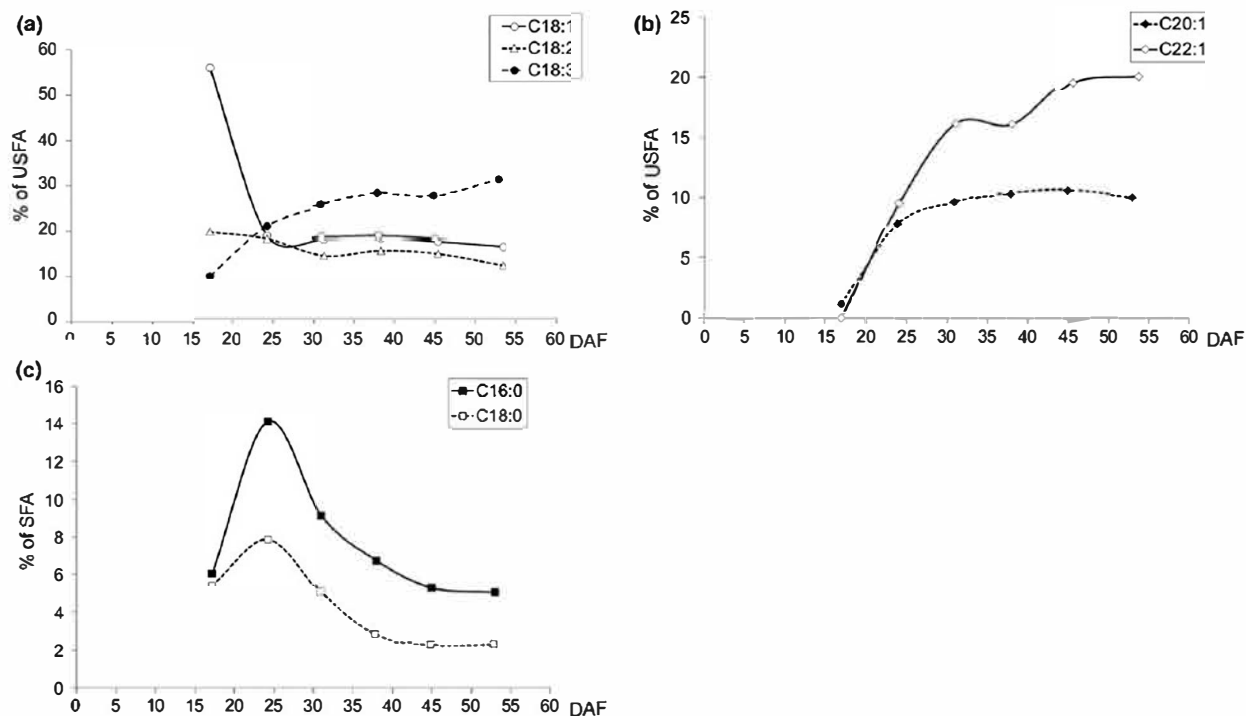


Figure 3 Accumulation of unsaturated fatty acids (UFA) (a) with 18C: oleic (C18:1), linoleic (C18:2) and linolenic acids (C18:3); (b) with 20 and 22 C: eicosenoic (C20:1) and erucic acids (C22:1); and (c) saturated fatty acids (SFA): stearic (C18:0) and palmitic acids (C16:0) during 2006 in woad seeds during seed ripening.

three oilseeds presented variations alternated with maximums and minimums of sterol levels. This intermediate decrease in sterol levels may be explained by the fact that, at some precise moments of seed ripening, the plant led to conversion of existing sterols into steroidal hormones (brassinosteroids) and vitamins that regulate growth and development of immature tissues (Jang *et al.*, 2000; Herchi *et al.*, 2009).

Among phytosterols present in oilseeds, β -sitosterol with 192.2 mg per 100 g of SDM is known to

have anticancer properties (Woyengo *et al.*, 2009). $\Delta 5$ -Avenasterol was reported in the literature to be associated with antioxidant effects (Herchi *et al.*, 2009). It can be suggested to breeders to focus their efforts on total sterol content by increasing the most abundant of them or component with antioxidant activities as $\Delta 5$ -avenasterol in seeds of oil plant. Roche *et al.* (2006, 2010a) highlighted the increase of total sterol content as well as the modification of their composition. The late sowing may help to raise the content of sterols in seeds.

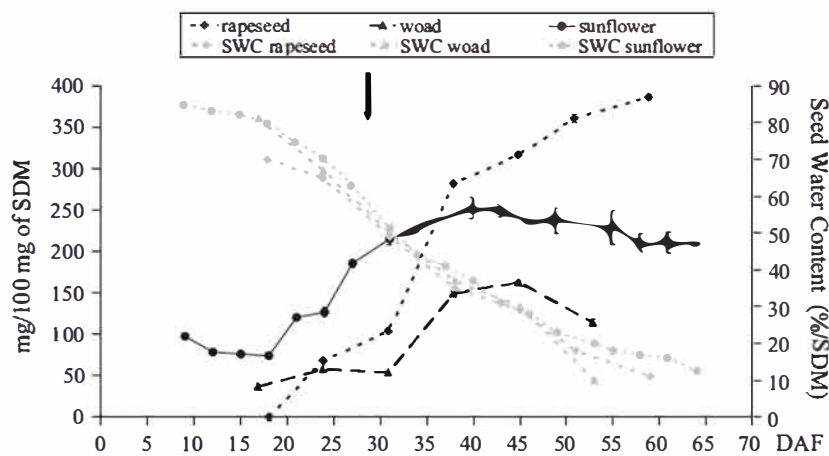


Figure 4 Total phytosterol contents of sunflower, rapeseed and woad seeds during seed ripening. SDM: seed dry matter, DAF: days after flowering.

Conclusion

Improved knowledge of the composition and accumulation of sunflower, rapeseed and woad seeds would assist in efforts to achieve industrial application of these plants. Particularly, these seeds may give a good source of lipid-soluble bioactives, for food and non-food industrial uses of oilseeds that have increased as a result of the development of new 'green-chemistry' molecules, including fatty acids and phytosterols. Therefore, in this study, we compared the accumulation of phytosterols and fatty acids in sunflower, rapeseed (largely cultivated crops) and woad that could help to modulate the oil composition in order to obtain desirable compositions for industrial purposes. Moreover, the description of the accumulation of phytosterols in the studied oilseeds could help breeders to understand the possibilities to modify this accumulation in order to satisfy industrial demands.

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