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ARTICLE

Characterization and Quantification of the Aliphatic Hydrocarbon Fraction during Linseed Development (*Linum usitatissimum* L.)

Wahid Herchi,*,† Saoussem Harrabi,† Sophie Rochut,§ Sadok Boukhchina,† Habib Kallel,† and Claude Pepe§

†Laboratoire de Biochimie des lipides, Département de Biologie, Faculté des sciences de Tunis, 2092 ELmanar, Tunisia, and §LADIR, UPMC Case courrier 49, 4 Place Jussieu, 75005 Paris, France

Changes in hydrocarbon composition were investigated during maturation of three varieties of linseed (H52, O116, and P129) cultivated in Tunisia. The hydrocarbon fraction of the three linseed oil samples was found to contain mainly *n*-alkanes and squalene. The greatest decrease of these components occurred between 7 and 21 days after flowering (DAF); thereafter, the changes were slight. At 7 DAF, P129 had a significantly higher level of squalene (27.24 mg/100 g of oil) than H52 (3.36 mg/100 g of oil), but from this date until 21 DAF squalene decreased much more actively in P129, resulting in similar levels in H52 (0.57 mg/100 g of oil) and P129 (0.52 mg/100 g of oil) at full maturity. In three varieties of linseed, 13 *n*-alkanes were detected ranging from C_{22} to C_{34} carbon atoms. The *n*-alkane composition of linseed oil was influenced by the ripening stage of seeds. At 7 DAF, C29 was the most predominant hydrocarbon (19.84 mg/100 g of total oil), followed by C_{27} (11.82 mg/100 g) and C_{25} (11.28 mg/100 g). C_{29} exhibited the most significant decrease from 7 to 21 DAF, as a result C_{27} being the most significant *n*-alkane component for the remainder of the period. At full maturity, the content of total *n*-alkanes in three varieties of linseed ranged from 4.0 to 4.26 mg/100 g of oil.

KEYWORDS: Aliphatic hydrocarbons; linseed; *n*-alkanes; squalene; GC-MS

INTRODUCTION

Hydrocarbons are minor components of the unsaponifiable fraction of oils and fats. The hydrocarbon profile can be used to assess the authenticity of vegetable oils (*1*). Squalene, a terpenoid hydrocarbon, is an important constituent of the unsaponifiable matter of numerous edible vegetable oils, particularly olive and pumpkin oils (*2*). It was demonstrated that squalene had diverse biological functions, such as preventing cell deterioration, an antisenescence effect, enhancing immunity, and a sexual function (*3*). Squalene is normally used in its natural or hydrogenated form as a moisturizing or emollient agent in cosmetic preparations. In higher plants, *n*-alkanes are mainly involved in the synthesis of epicuticular wax layer. Their physiological function within the plant cells is poorly understood (*4*). The *n*-alkanes are thought to be endogenous to a plant; they are formed as a result of the decarboxylation of long-chain fatty acids (*5*). The α-oxidation system was described to participate in the synthesis of even-numbered *n*-alkanes (*6*). The *n*-alkanes in seeds may act as energy storage components (*7*). Recently, *n*-alkanes were used as markers for the determination of diet intake, food selection, and digestibility in ruminant species (*8*). The crude vegetable oils contain elements of the *n*-alkane series from C10 to C35, the odd-numbered compounds being the most abundant (*9*). In addition, low amounts of other hydrocarbons have been detected, such as *n*-alkenes (*2*). The *n*-alkane profile can be used to distinguish between different olive varieties (*10*).

The composition of vegetable oils can be influenced by genetic factors, ripening grade of fruits, or climatic conditions. Information on the influence of ripening stage of fruits on *n*-alkane composition is scarce. There seem to be no previous studies on changes in the *n*-alkane composition during linseed maturation. Cunha et al. (*7*) studied the ontogenic variations in the content and distribution patterns of total *n*-alkanes during the induction and expression of somatic embryogenesis from linseed hypocotyl explants. They showed many differences between the different organs, but the resulting oils were quite similar. The aim of the present study is to carry out a qualitative and quantitative characterization of the hydrocarbon fraction of three varieties of linseed (H52, O116, and P129) cultivated in Tunisia. Changes occurring in hydrocarbon composition during linseed development were also investigated.

MATERIALS AND METHODS

Reagents and Standard. Methanol and *n*-hexane, solvents of GC analysis, were purchased from Panreac Quimica SA (Barcelona, Spain). Chloroform and petroleum ether were purchased from Fisher Scientific SA (Spain). Ethanol was purchased from Sientific Limited (Northampton, U.K.). Hydrocarbon standards were acquired from Sigma Aldrich

*Author to whom correspondence should be addressed (e-mail wahid1bio@yahoo.fr).

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Table 1. Harvest Dates, Days after Flowering, Seed Color, and State of Maturity of Linseed during Maturation

harvest	harvest date	days after flowering	state of maturity, seed color	BBCH scale
1	April 28, 2007	7	unripe, fully green	10−71% of capsules were formed
2	May 5, 2007	14	unripe, fully green	50−75% of capsules were formed
3	May 12, 2007	21	unripe, fully green	79, the end of capsule formation
4	May 19, 2007	28	unripe, green-brown	81, the end of the growth of capsules
5	May 26, 2007	35	half-ripe, green-brown	83, capsules were green
6	June 2, 2007	42	half-ripe, green-brown	85, capsules turn yellow
7	June 9, 2007	49	half-ripe, majority brown	87, capsules were brown-yellow
8	June 16, 2007	56	fully ripe, brown	89, capsules were brown and sec

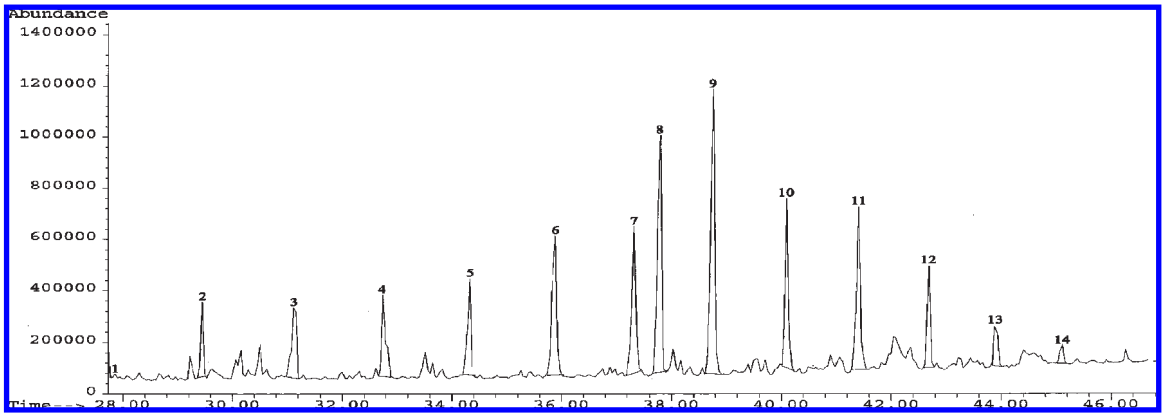

Figure 1. GC-MS chromatogram of unaltered hydrocarbons: (1) C_{22}, (2) C_{23}, (3) C_{24}, (4) C_{25}, (5) C_{26}, (6) C_{27}, (7) C_{28}, (8) squalene, (9) C_{29}, (10) C_{30}, (11) C_{31}, (12) C_{32}, (13) C_{33}, (14) C_{34}.

(Madrid, Spain). TLC silica plates (silica gel 60G F254, 20 × 20 cm, 0.25 mm thickness), potassium hydroxide pellets, and anhydrous sodium sulfate were obtained from Merck (Darmstadt, Germany).

Plant Material. The three varieties of linseed (H52, O116, and P129) were obtained from INRAT (Institut National Recherche Agronomie Tunis, northern Tunisia). The three varieties of linseed (*Linum usitatissimum* L.) were grown in restricted zones (15 m × 3 m) on the agronomy farm of INRAT from the middle of November 2006 until the end of June 2007. The harvest period was stretched from 7 days after flowering (DAF) to 56 DAF, the period required for complete maturity (Table**1**). Moisture and seed weights were determined by weighing 100 seeds before and after drying to constant weight in a vacuum oven at 60 °C.

Lipid Extraction. The total lipids were extracted according to the method of Folch et al. (*11*) as modified by Bligh and Dyer (*12*). In fact, this method has been used by numerous authors for the extraction of lipids from vegetable matrices, Msaada et al. (*13*), Nasri et al. (*14*), Yoshida et al. (*15*), and Harrabi et al. (*16*). In addition, immature seeds presented a high percentage of water.

Seeds (2.5 g) were washed with boiling water for 5 min to inactivate the phospholipases (*17*) and then crushed in a mortar with a mixture of $CHCl_3$/MeOH (2:1, v/v). The water of fixation was added, and the homogenate was centrifuged at 3000*g* for 15 min. The chloroformic phase containing the total lipids was kept and dried in a rotary evaporator at 40 °C.

Saponification. Unsaponifiable lipids were determined by saponifying 5 g of lipid extracts with 50 mL of ethanolic KOH 12% (w/v) and heating at 60 °C during 1.5 h. After cooling, 50 mL of H_2O was added and the unsaponifiable matter was extracted four times with 50 mL of petroleum ether. The combined ether extract was washed with 50 mL of EtOH/H_2O (1:1). The ether extracted was dried over anhydrous Na_2SO_4 and evaporated. The dry residues were dissolved in chloroform for TLC analysis.

Thin-Layer Chromatography (TLC). The unsaponifiable matter was separated into subfractions on preparative silica gel thin-layer plates (silica gel 60G F254), using one-dimensional TLC with hexane/Et_2O (9:1 v/v) as the developing solvent. The unsaponifiable matter (4 mg in 100 μL of $CHCl_3$) was applied on the silica gel plates in 3 cm bands. To correctly identify the hydrocarbon bands, a reference sample of purified hydrocarbon was applied on the left and right sides of the TLC plates. After development, the plate was sprayed with 2′,7′-dichlorofluorescein and viewed under UV light. On the basis of the reference spots, the hydrocarbon bands were identified. The bands corresponding to hydrocarbons was scraped off separately, and each fraction was extracted three times with $CHCl_3/Et_2O$ (1:1), filtered to remove the residual silica, dried in a rotary evaporator, and stored at −10 °C for further analysis.

Gas Chromatography−Flame Ionization Detection (GC-FID). Although the GC-MS detector has higher sensitivity compared to the GC-FID one (*18*, *19*), the latter is more easily amenable to quantification with fewer standards. Thus, the quantification of hydrocarbons was performed using the GC-FID apparatus. The GC system used was a Thermo Finnigan Trace GC (Hewlett-Packard, Avondale, PA) equipped with a split−splitless injector, a FID detector, and a Varian Vf-SMS column (length = 30 m, i.d. = 0.25 mm, film thickness = 0.25 μm). The initial column temperature was 80 °C and programmed to increase at a rate of 5 °C/min to 320 °C and then held for 10 min. The injector and detector temperatures were 300 and 350 °C, respectively. Helium was used as carrier gas giving a column flow of 1 mL/min. Compounds were semiquantified by directly comparing their respective total ion chromatogram peak area with that of an internal standard (hexadecane). There was a difference in the retention time and response factor of the hydrocarbons with chain length > C_{26}; the *n*-alkane series from C_{26} to C_{34} was underestimated.

GC-MS Analysis. GC-MS analysis was performed on a Hewlett-Packard 5890 A series II GC equipped with a capillary column HP-5MS (length = 30 m, i.d. = 0.22 mm, film thickness = 0.22 mm) and coupled to a 5989A mass spectrometer with Mass Laboratory data system. Helium was used as carrier gas at 1 mL/min. The injector temperature was 300 °C, and samples were injected at the same conditions reported above for GC analyses. The oven temperature was pogrammed from 80 to 320 °C at 10 °C/min. Manual injection of 1 μL of the solution of hydrocarbons was performed in the split mode. Mass spectra were recorded at an electron energy of 70 eV, and the ion source temperature was 250 °C. Qualitative analysis was based on a comparison between the relative retention times, the mass spectra, and the corresponding data in the literature. All hydrocarbons were identified in comparison with data from the literature, spectra of pure reference standards, and entries in the Wiley 275 HP Chemstation library. A typical GC-MS chromatogram of unaltered hydrocarbons is shown in **Figure 1**.

Statistical Analysis. Statistical analysis was performed by using the Proc ANOVA in SAS (software version 8). Duncan's multiple-range test was used. For each oil sample, three determinations have been done.

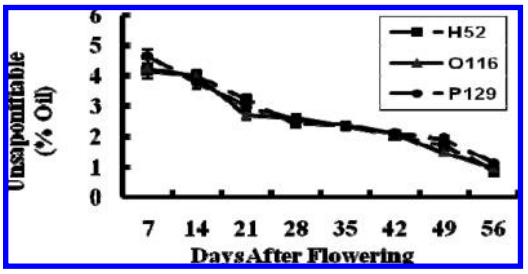

Figure 2. Evolution of unsaponifiable content (in percent of oil) during maturation of three varieties. (Vertical bars indicate standard Ecart of the means.)

RESULTS AND DISCUSSION

Unsaponifiable Matter Content of Maturing Linseed. The unsaponifiable fraction is made up of minor constituents, which may vary both qualitatively and quantitatively depending on genetic factor, climatic conditions, extraction and refining procedures, and storage conditions (*20*). During linseed development, the total amount of unsaponifiables followed a similar decreasing trend in three varieties (**Figure 2**). Thus, the highest level (4.14−4.66% of total oil) of unsaponifiables was detected at 7 days after flowering (DAF). This result may be due to the decline of the amount of phytosterols, which are the most abundant compounds of the unsaponifiable fraction (*21*) during linseed development. In fact, in immature seeds phytosterols are converted to brassinosteroids, which are steroidal hormones that regulate the growth and development of immature tissues (*22*). At full maturity, the three varieties H52, O116, and P129 had unsaponifiable contents of 0.87 ± 0.02, 0.97 ± 0.04, and 1.12 ± 0.01% of the total oil, respectively. In other varieties of linseed, the unsaponifiable amount ranged from 1.28 to 1.67% (*23*, *24*). The total unsaponifiable content of olive oils ranged from 1 to 2% of the total lipid composition. These minor lipids greatly influence the organoleptic quality and stability of the oil (*25*). The effectiveness of unsaponifiable matters in retarding oil deterioration has been demonstrated by many investigators (*26*).

Hydrocarbon Profiles of Three Varieties of Linseed at Maturity. GC-MS was used to identify the various components of the hydrocarbon fraction of linseed oils. **Table 2** lists the identified compounds and their levels in three varieties of linseed, at full maturity. The hydrocarbon fraction of the three linseed oil samples was found to contain *n*-alkanes and squalene. This result is in contrast with the results of Tuberoso et al. (*27*), who indicated that squalene was absent in linseed oil. Differences in variety and in method of analysis were likely the reason for the observed results. The presence of squalene in different vegetable oils (*2*) could be linked to its role as a precursor of phytosterols. The *n*-alkenes were not detected in the hydrocarbon fraction of three varieties of linseed. Moreda et al. (*2*) reported that some unsaturated hydrocarbons such as pentadecene and octadecene appear during the refining process. In three varieties of linseed, 13 *n*-alkanes were detected, ranging from C_{22} to C_{34} carbon atoms. According to Cunha et al. (*7*), hydrocarbons could be regrouped into three groups: short-chain-length compounds, medium-chain-length compounds (C_{22}−C_{26}), and long-chain-length compounds (C_{27}−C_{31}). Linseed oil samples contained only medium- and long-chain-length compounds. No high-chain-length *n*-alkanes (C_{40}−C_{50}), characterizing a mineral origin (*28*), were detected. The variety O116 had a significantly ($p < 0.05$) lower level of total hydrocarbon content than H52 and P129. At full maturity, the content of total *n*-alkanes in three varieties of linseed ranged from 4.0 ± 0.07 to 4.26 ± 0.12 mg/100 g of oil. Virgin olive oil, sunflower oil, and sesame oil had *n*-alkane contents of 9.90, 7.27, and 5.22 mg/100 g of oil, respectively (*29*). The *n*-alkane profile of P129 was characterized by the predominance of C_{27}, C_{25}, and C_{29}, whereas, in the oil obtained from the O116 variety, C_{27}, C_{31} C_{25}, and C_{23} have the main levels. The highest abundance *n*-alkanes in the H52 variety were C_{23}, C_{27}, C_{24}, and C_{29}. A comparison between three varieties showed that there was a significant difference ($p < 0.05$) in their squalene contents. O116 and H52 varieties had the lowest level (0.29 ± 0.07 mg/100 g of oil) and the highest level of squalene (0.57 ± 0.08 mg/100 g of oil), respectively. Linseed oil had a considerably lower level of squalene compared to olive oil, which had 599 mg/100 g of oil (*27*). Considering the fact that squalene is the precursor of sterols, we suggested that this difference in its amount could be explained by the lower synthesis of sterols by olive seeds. In fact, linseed oil had a much higher amount of sterols (699 mg/100 g of oil) (*30*) than did the olive oil (221 mg/100 g of oil) (*31*). Among the three varieties of linseed, O116 has the lowest squalene level, which suggested that it produces more sterols than the other two varieties.

Table 2. Concentration[a] of Aliphatic Hydrocarbons in Three Varieties of Linseed Oil, at Full Maturity

	concentration (mg/100 g of oil)		
carbon	H52	O116	P129
nC_{22}	0.36 ± 0.06 a	0.30 ± 0.05 b	0.28 ± 0.08 b
nC_{23}	0.50 ± 0.03 a	0.35 ± 0.07 b	0.34 ± 0.05 b
nC_{24}	0.40 ± 0.07 a	0.31 ± 0.04 b	0.33 ± 0.02 b
nC_{25}	0.36 ± 0.04 a	0.37 ± 0.02 a	0.57 ± 0.06 b
nC_{26}	0.28 ± 0.05 a	0.29 ± 0.03 a	0.24 ± 0.05 a
nC_{27}	0.44 ± 0.05 a	0.39 ± 0.02 a	0.61 ± 0.10 b
nC_{28}	0.35 ± 0.06 a	0.26 ± 0.05 b	0.29 ± 0.03 b
nC_{29}	0.39 ± 0.04 a	0.29 ± 0.05 b	0.52 ± 0.07 c
nC_{30}	0.30 ± 0.02 a	0.33 ± 0.04 a	0.34 ± 0.05 a
nC_{31}	0.22 ± 0.05 a	0.38 ± 0.06 b	0.37 ± 0.04 b
nC_{32}	0.15 ± 0.04 a	0.23 ± 0.07 b	0.15 ± 0.02 a
nC_{33}	0.17 ± 0.06 a	0.26 ± 0.02 b	0.15 ± 0.04 a
nC_{34}	0.17 ± 0.04 a	0.23 ± 0.04 b	0.07 ± 0.01 c
squalene	0.57 ± 0.08 a	0.29 ± 0.05 b	0.52 ± 0.09 a
total	4.68 ± 0.13 a	4.29 ± 0.09 b	4.78 ± 0.11 a

[a] Each value is the mean ± standard deviation (SD) of a triplicate analyses performed on different samples. Means with different letters (a−c) within a row are significantly different at $p \leq 0.05$.

Changes in Aliphatic Hydrocarbon Composition of Maturing Linseed. Aliphatic hydrocarbons are synthesized in the epidermal cells from saturated long-chain fatty acids (*32*). Changes in the total aliphatic hydrocarbons content (mg/100 g of oil) in developing linseed is shown in **Figure 3**. The concentration of total aliphatic hydrocarbons experienced a drastic decrease from 7 to 14 DAF. This decrease paralleled the observed decrease in unsaponifiable matter. At 7 DAF, P129 had a significantly ($p < 0.05$) higher level of total aliphatic hydrocarbons than H52 and O116, but from this date until 21 DAF this level decreased much more actively in P129, resulting in similar levels in H52 and P129 at full maturity. In the early stages, aliphatic hydrocarbons were present in higher levels, which suggested that they play an important role during this period.

Changes in medium- and long-chain *n*-alkane contents during maturation of the P129 variety are presented in **Figure 4**. At 7 DAF, P129 had a significantly ($p < 0.05$) higher level of total long-chain *n*-alkanes than medium-chain alkanes, but from 14 until 21 DAF the first fraction decreased much more actively, resulting in similar levels for the remainder of the period.

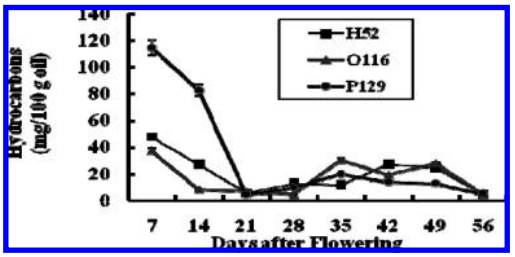

Figure 3. Evolution of aliphatic hydrocarbons (mg/100 g of oil) in developing linseed. (Vertical bars indicate standard Ecart of the means.)

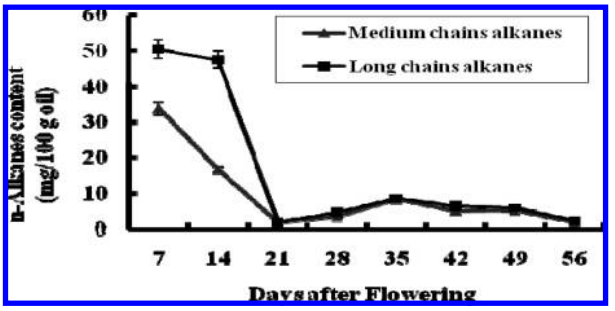

Figure 4. Changes in medium- and long-chain *n*-alkane concentrations during maturation of P129 variety. (Vertical bars indicate standard Ecart of the means.)

This result suggested that the biosynthesis of *n*-alkanes in linseed occurred before 7 DAF, whereas their catabolism occurred between 7 and 21 DAF. The *n*-alkane composition of linseed oil in the mature seed was established at about 21 DAF. Longer chain length hydrocarbons were mainly located in the epicuticular waxes and synthesized in epidermal cells. The decrease of long-chain *n*-alkanes could be required by cells for the maintenance of water balance (*33*) and cell structure (*34*). The biosynthesis and transient accumulation of medium chains remain unexplained (*7*).

Changes in *n*-Alkane Composition during Linseed Development. Differences in the concentrations of the major *n*-alkane components during linseed development (variety P129) are evident in **Figure 5**. The greatest decrease of these components occurred between 7 and 21 DAF; thereafter, the changes were slight. At 7 DAF, C_{29} was the most predominant hydrocarbon (19.84 mg/100 g of total oil), followed by C_{27} (11.82 mg/100 g) and C_{25} (11.28 mg/100 g). C_{29} exhibited the most significant decrease from 7 to 21 DAF; as a result, the levels of C_{27} were higher than those of C_{29} for the remainder of the period.

The major *n*-alkane compounds could be degraded to their corresponding fatty acids or to chain-shortened secondary metabolites, which might contribute to the synthesis of other compounds. In seeds, a lipid is stored mainly as triacylglycerols; thus, the decline of *n*-alkane content could be a reflection of the higher synthesis of those compounds during linseed development. In fact, it has been reported that the amount of triacylglycerols, which are the assembly of glycerol-3-phosphate with fatty acids, increased during seed development (*35*). During ripening of olives (Leccino sample) the *n*-alkane content also shows a decrease, especially for components with fewer than 30 carbons (*36*).

Changes in Squalene Content during Linseed Development. Squalene is a key intermediate in the phytosterol biosynthetic pathway. Squalene accumulation was known to modify the bilayer structure of membranes and to increase cell membrane permeability as well as to interfere with other membrane-associated processes (*37*). **Figure 6** shows changes in the abundance of squalene (expressed as mg/100 g of oil) during linseed maturation. During linseed development the highest level of squalene was

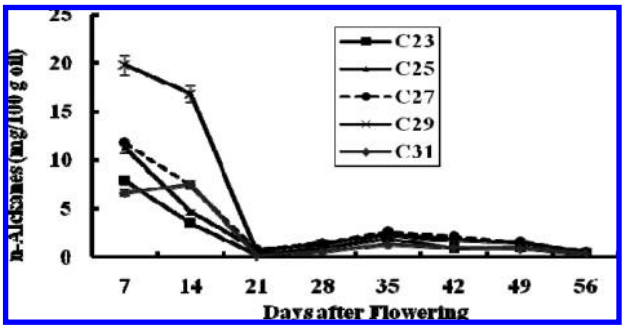

Figure 5. Changes in major *n*-alkane concentrations during maturation of P129 variety. (Vertical bars indicate standard Ecart of the means.)

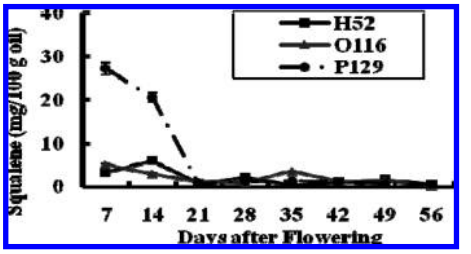

Figure 6. Changes in squalene content (mg/100 g of oil) during linseed maturation. (Vertical bars indicate standard Ecart of the means.)

detected at 7 DAF.The greatest decrease in squalene content occurred from 7 to 21 DAF. At 7 DAF, P129 had a significantly ($p < 0.05$) higher level of squalene than H52 and O116, but from this date until 21 DAF squalene decreased much more actively in P129, resulting in similar levels in H52 and P129 at full maturity. Given that squalene is the precursor of phytosterols and that the immature seed cells need those compounds for the construction of their membranes, it is reasonable to expect that the decline of squalene content was a reflection of the higher synthesis of phytosterols during the early stages of linseed development. A number of factors regulate squalene accumulation in seeds, including the abundances and the activities of squalene synthase and squalene epoxidase. In fact, the squalene synthase could catalyze the reductive condensation of two molecules of farnesyl diphosphate to squalene in the presence of NADPH and Mg^{2+}, via the intermediate presqualene diphosphate, whereas squalene epoxsidase catalyzes the conversion of squalene to 2,3(*S*)-epoxysqualene (*38*).

The results of our investigation provide useful information on the hydrocarbon composition of linseed oil, which contained *n*-alkanes (C_{22}−C_{34}) and squalene. The high-chain-length *n*-alkane (C_{40}−C_{50}), characterizing a mineral origin (*28*), and *n*-alkenes were absent in the hydrocarbon fraction of three varieties of linseed. Determination of the aliphatic hydrocarbon profile of vegetable oils has been used as a marker to reveal contamination with mineral oil residues, which could be diffused in vegetable oils. The most rapid change in the hydrocarbon composition of linseed oil is complete at about 21 DAF. The *n*-alkane composition of linseed oil was influenced by the ripening stage of seeds.

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