Ontogenic variations in free and esterified fatty acids during somatic embryogenesis of flax *(Linum usitatissimum* L.)

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Abbreviations: EC – embryogenic *calli*; EFA – esterified fatty acids; FA – fatty acids; FAME – fatty acid methyl ester; FFA – free fatty acid; GC – gas chromatography; GC-MS – gas chromatography and mass spectrometry; HS – hypocotyl segments; HSC – hypocotyl segments with incipient *calli* formation; LCFA – long-chain fatty acid; MCFA – medium-chain fatty acids; NEC – non-embryogenic *calli*; PCA – principal component analysis; PUFA – polyunsaturated fatty acid; VLCFA – very long-chain fatty acid; SE – somatic embryo.

Summary

In vitro cultures of flax (*Linum usitatissimum* L.) were established on MS medium and four samplings were made during the 7 weeks of culture. The samples varied from the original

hypocotyl segments (HS) at t_0 and segments with incipient *calli* formation (HSC) after 2 weeks (t_2), to embryogenic *calli* (EC), non-embryogenic *calli* (NEC) and somatic embryos (SE) collected after 5 (t_5) and 7 weeks (t_7). The respective free fatty acids (FFA) and esterified fatty acids (EFA) were extracted and analyzed, as methyl esters, by GC and GC-MS, and the data were submitted to ANOVA and PCA statistical analysis. The main FFA in all samples were 16:0, 18:0, 18:1, 18:2 and 18:3. EFA fractions were more diverse containing some less common FA. The development of SE was associated with a decrease in neutral lipids. Both the total FFA and the total EFA, as fractions of total lipids, increased with the dedifferentiation and the early *calli* formation leveling off thereafter (t_5 to t_7). FA variations related to dedifferentiation of hypocotyl tissues and growth of non-embryogenic *calli*, somatic embryogenesis, and development of somatic embryos were observed. Predominant or tissue-specific FA were also found. The value of 18:3/18:2 and 18:0/18:3 ratios as potencial indicators of SE autotrophy and *calli* embryogenic capacity is discussed.

Introduction

Lipids are essential constituents of all organisms and cells. Representing only ca. 6 % of plant biomass, they play major structural, functional, metabolic and regulatory roles (1,2). As triglycerides (TAGs), they constitute an efficient form of carbon and energy storage in 80 % of plant species (3). While membrane lipids are under a strict metabolic control, with a highly conservative fatty acid composition (16 and 18 carbon fatty acids, up to 3 double bonds), the quantity and quality of TAGs vary extremely, playing an important role in germination and plant fitness (4). Changes in lipid content and/or composition are visible not only during plant development – from germination to senescence – but also as a response to stress or challenging conditions, namely, chilling (5-7) and freezing temperatures (8, 9), osmotic stress (10), water deficit (11, 12), high temperatures (13) and light regimes (14). Variations in lipids were also described in *in vitro* cultures, during developmental processes such as somatic embryogenesis (15-18) and shoot morphogenesis (19), as well as in induced cell suspensions (20) or *callus* differentiation (21). It has been argued that the differences in lipid contents and composition, particularly with respect to the acyl profile, between *calli* cultures and parent plant tissues is a reflection of the nutrition mode due to differentiation (21). The potencial sources of lipid variation – *calli* autotrophic differentiation stage, *calli* morphogenic capacity, tissue development stage – make plant tissue culture a suitable technique to study lipid biochemistry in oil-producing plants (21).

Flax (*Linum usitatissimum* L.) is an ancient cultivated oil species still with an important impact in world economy (22). Traditionally cultivated for its main products – fibre and seed oil – this species has gained a new interest in the emergent market of functional food due to its high content in fatty acids, mainly α-linolenic acid, and lignan oligomers (22).

Tissue culture of flax has been carried out for 3 decades (23) and somatic embryogenesis was already obtained, directly from immature zygotic embryos (24) and indirectly from hypocotyl segments derived *calli* (25, 26). However, either the characterization of the fatty acid profile of the induced *in vitro* cultures or their potencial ontogenic variations associated with this developmental process were not reported yet. In this work, a somatic embryogenesis flax system was used as a model to study tissue specific differences and ontogenic variations in the free fatty acid (FFA) and esterified fatty acid (EFA) fractions.

Materials and Methods

Plant material and culturing conditions

One-week old flax seedlings germinated on hormone free MS basal medium (27) were used to obtain the primary explants. Hypocotyl segments of the sub-cotyledonary region were inoculated on embryogenic medium (25). The growth conditions and the sampling procedure were as previously described (18). During the growth period, samples were taken at time zero (t_0), after 2 (t_2), 5 (t_5) and 7 (t_7) weeks comprising a total of 5 types of tissues: hypocotyl segments (HS) at t_0; hypocotyl segments with incipient *callus* formation (HSC) obtained at t_2; and non-embryogenic *calli* (NEC), embryogenic *calli* (EC) and somatic embryos (SE) at t_5 and t_7. All samples were collected in duplicate and were composed of several cultures (18). The plant material was stored at –70 °C and then freeze-dried before lipid extraction and analysis.

Lipids extraction

Freeze-dried powdered material was extracted for total lipids as previously described (18). Neutral (N) and acid (A) lipid fractions were obtained from known amounts of total lipid extracts by a serial *n*-hexane:propanol (76:56 v/v) partitions at pH 12 and 3, respectively. After evaporation of the solvent in a rotary evaporator and under nitrogen flow, fractions were redissolved in a fixed volume of *n*-hexane.

Analysis of the fatty acid fractions

For GC quantitative analysis of the acyl fraction of the esterified fatty acids (EFA) present in the N fraction, a known amount of 5-α-cholestane (SIGMA standard) was added to each sample as an internal standard. The N fractions were transesterified according to Mason and Waller (28). At least 3 analyses were made per replicate. FAMEs were identified with a mixture of authentic reference esters standards (SIGMA) and by the regression equation of retention time over the FAMEs' number of carbon atoms ($P << 0.001$). The addition of the internal standard to the standard mixture followed by the same derivatization procedure and GC analysis program (6-7 analyses) allowed to estimate a correction expression that accounted for differences in FAMEs concerning both the detector response factor and split discrimination.

For GC quantitative analysis of the free fatty acids (FFA), known amounts of the A fractions were subjected to methylation following the procedure previously described (29, 30) with some modifications. After evaporation of the solvent, 100 μl of BF_3 in 20 % of metanol (MERCK) and 200 μl of *n*-hexane were added. The derivatization mixture reacted for 2 min. at 90 °C. A known amount of 5-α-cholestane was also used as internal standard and at least 3 analyses were made per replicate. FAMEs were identified with authentic reference FFA standards (SIGMA) and by the regression equations ($P < 0.001$) as in the case of EFA standard mixture. The quantification method was similar to that used for the EFA.

All the FAMEs analyses were performed with a GC instrument (Perkin Elmer 8600) equipped with a split-splitless injector at 300 °C, a flame ionization detector at 320 °C and a SGE BP20 column (25 m long x 0.22 mm ID x 0.25 μm film thickness of polyethyleneglycol). The set temperature program was from 100 °C to 240 °C at a rate of 5 °C/min. followed by an isothermal for 5 min. The identification of peaks was confirmed by GC-MS (Perkin Elmer 8500), equipped with a 30 m long x 0.25 mm ID DB5 column (0.25 μm film thickness of 5 % phenyl dimethyl siloxane) connected to a Finnigan MAT ion trap detector (ITD) operating in Electron Impact (EI) mode at 70 eV. Split-splitless injector, interface transfer line and ion source temperatures were 300 °C, 260 °C and 220 °C, respectively. The oven temperature program was as above described. H_2 and He were used as carrier gases in GC and GC-MS, respectively, with a column head pressure of 12.5 PSI.

Statistical analyses

Mean differences in the contents of N and A fractions, FFA and EFA contents between samples were analysed running the analysis of variance test (ANOVA). The assumption of

homocedasticity was met to all variables, recurring to square root transformation when necessary (Bartlett's test with $P > 0.05$). *Post-hoc* comparisons were made performing the HSD Tukey test. All the statistics were performed with the software "Statistica 4.1" from StatSoft.

Principal component analysis (PCA) was applied not only to the individual FFA and EFA data but also to constructed variables derived from original data. A maximum of three factors and a minimum of 75 % of the variability explained were fixed. The PCA were performed with "ADE-4" (31).

Results and Discussion

Changes in the neutral and acid lipid fractions

The contents of the N and A fractions of the total lipid extracts of all the *in vitro* flax samples are represented in Table 1.

The highest contents in both lipid fractions were observed in primary explants (HS), decreasing progressively with the dedifferentiation of the hypocotyl tissues and the induction and growth of *callus*. Somatic embryos showed intermediate contents between *calli* and HS and showed a significant decrease of the N fraction with growth. No significant differences were detected between embryogenic (EC) and non-embryogenic *calli* (NEC) concerning the content of both lipid fractions. Although the variation of the A fraction content had shown a trend similar to that of the N fraction, the N/A ratio, amplifying specific differences, was significantly lower in ECt_5 than in $NECt_5$ (Fig. 1) mainly due to a smaller proportion of N fraction (Table 1). These observations support the hypothesis that the morphogenic process in EC may function as an extra sink of neutral lipids.

Table 1 – Contents of neutral and acid lipid fractions as a percentage of plant biomass in HS, HSC, NEC, EC and SE of flax sampled during an experimental period of 7 weeks.

Plant material	Neutral fraction (%)				Acid fraction (%)			
	Sampling time							
	t_0	t_2	t_5	t_7	t_0	t_2	t_5	t_7
HS	15.4 a				6.04 a			
HSC		4.98 bd				1.73 bd		
NEC			2.70 be	1.03 e			0.90 bc	0.44 c
EC			1.67 ce	1.25 ce			1.19 bc	0.53 c
SE			6.36 d	2.83 bc			2.81 d	1.54 bd

For each experimental variable, values followed by the same letter were not statistically different ($P > 0.05$).

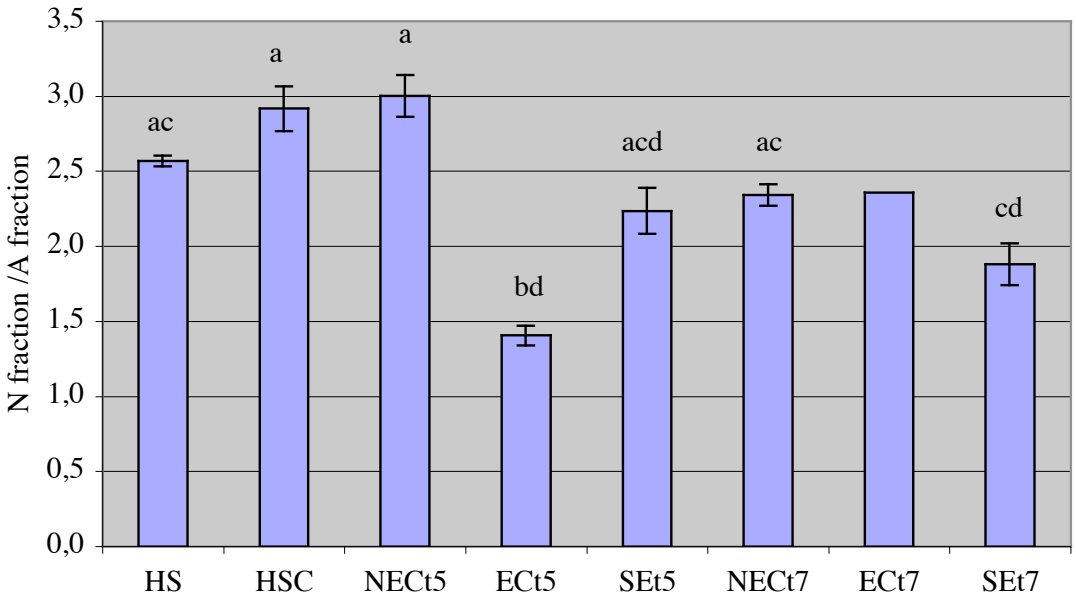

Figure 1 – N/A fractions ratio in the different samples. The SD is represented on the top of each bar. Bars with the same letter represent ratios statistically similar.

Changes in FFA contents and composition

The free fatty acids (FFA) identified and quantified in plant tissue samples are described in Table 2. Table 3 shows the values obtained from total or parcial sums of coherent groups of FFA and ratios between specific FFA.

Table 2 – Contents of the individual FFA in the acid fractions obtained from the different plant tissues and structures (HS, HSC, NEC, EC and SE) sampled during the experiment (t_0, t_2, t_5 and t_7).

FFA	RT (min.)	Samples (sampling time)							
		HS (t_0)	HSC (t_2)	NEC (t_5)	EC (t_5)	SE (t_5)	NEC (t_7)	EC (t_7)	SE (t_7)
14:0	9.81	1.56 (0.308)	1.08 (0.154)	1.17 (0.085)	0.54 (0.07)	1.20 (0.093)	1.31 (0.234)	0.63 (0.013)	1.44 (0.604)
15:0	11.68	0.92 (0.194)	0.55 (0.110)	0.85 (0.154)	0.52 (0.009)	0.57 (0.067)	0.99 (0.235)	0.71 (0.005)	0.48 (0.001)
16:0	13.55	10.8 (1.47)	15.6 (2.58)	34.7 (11.88)	31.9 (3.69)	23.7 (2.53)	32.9 (5.24)	31.0 (4.15)	46.0 (13.03)
16:1	13.87	1.14 (0.638)	0.97 (0.569)	0.77 (0.169)	1.03 (0.074)	0.97 (0.051)	0.91 (0.047)	1.18 (0.092)	2.75 (1.267)
17:0	15.37	0.86 (0.187)	0.56 (0.046)	0.72 (0.141)	0.49 (0.077)	1.66 (0.024)	0.94 (0.104)	0.72 (0.062)	0.77 (0.219)
18:0	17.15	5.60 (0.675)	3.86 (0.198)	6.18 (1.412)	4.19 (0.530)	6.47 (1.426)	5.72 (1.444)	3.80 (0.463)	5.43 (0.136)
18:1	17.42	1.64 (0.418)	3.03 (0.515)	6.73 (2.437)	6.03 (0.293)	3.08 (0.407)	3.20 (0.608)	2.97 (0.288)	2.27 (0.159)
18:2	18.21	4.68 (0.765)	18.1 (3.45)	51.5 (20.66)	61.5 (7.11)	32.5 (3.94)	45.2 (4.78)	52.2 (8.72)	52.1 (3.04)
18:3	19.31	3.83 (0.368)	13.5 (1.04)	26.8 (10.19)	35.6 (3.37)	29.5 (3.04)	30.4 (2.92)	36.3 (5.39)	63.2 (0.76)
20:0	20.81	0.37 (-)	0.31 (-)	0 (-)	0.56 (0.030)	0.58 (0.035)	0.30 (0.028)	0.34 (0.004)	0.17 (-)
20:1	21.45	0.98 (-)	0.92 (0.282)	1.01 (0.062)	0.36 (0.054)	1.12 (0.058)	0.76 (0.234)	0.48 (0.058)	0.35 (0.047)
22:0	23.98	0.80 (-)	0.95 (-)	2.05 (-)	0.9 (0.161)	0.93 (0.170)	1.59 (0.060)	1.63 (0.195)	1.31 (0.075)
23:0	26.74	1.19 (-)	1.66 (0.565)	1.97 (0.143)	1.83 (0.017)	1.43 (0.124)	2.29 (0.126)	1.82 (0.005)	1.3 (0.088)
24:0	27.04	1.76 (0.386)	2.49 (1.416)	1.31 (0.222)	0.74 (0.027)	1.42 (0.066)	0.82 (0.143)	0.56 (0.040)	0.55 (0.224)

For each compound, its retention time (RT) for the GC setting conditions, the mean value (mg/g total lipids) and the respective standard error (SE) are shown; (-) due to technical limitations in the quantification of one of the replicates, the error associated to the mean (SE) was not estimated.

Table 3 – Sums of the individual contents of FFA (total and parcial) and ratios between individual or groups of FFA present in the acid fractions obtained from the different plant tissues and structures (HS, HSC, NEC, EC and SE) sampled during the experiment (t_0, t_2, t_5 and t_7).

Variables	Samples (sampling time)							
	HS (t_0)	HSC (t_2)	NEC (t_5)	EC (t_5)	SE (t_5)	NEC (t_7)	EC (t_7)	SE (t_7)
Total FFA (T)	35 (2.1)	63 (14.6)	135 (67.7)	146 (21.6)	105 (16.4)	127 (22.8)	134 (26.4)	178 (16.3)
Unsaturated FFA (U)	12 (0.8)	36 (8.3)	87 (47.4)	104 (15.2)	67 (10.4)	80 (12.1)	93 (20.1)	121 (3.7)
Saturated FFA (S)	23 (2.9)	26 (6.3)	48 (20.3)	42 (6.4)	38 (6.0)	47 (10.7)	41 (6.2)	57 (20.0)
PUFAs	8.5 (1.60)	32 (6.4)	78 (43.6)	97 (14.8)	62 (9.9)	76 (10.9)	88 (20.0)	115 (5.4)
VLCFAs	3.4 (1.81)	5.7 (2.31)	5.3 (1.02)	4.4 (0.28)	5.5 (0.19)	5.8 (0.76)	4.8 (0.43)	3.6 (0.28)
16:0+18:1	13 (2.7)	19 (4.4)	41 (20.2)	38 (5.6)	27 (4.2)	36 (8.3)	34 (6.2)	48 (18.2)
PUFAs/T	0.25 (0.061)	0.50 (0.015)	0.57 (0.037)	0.66 (0.003)	0.59 (0.002)	0.60 (0.021)	0.66 (0.020)	0.65 (0.090)
U/S	0.52 (0.102)	1.4 (0.01)	1.8 (0.24)	2.5 (0.02)	1.8 (0.003)	1.7 (0.14)	2.2 (0.15)	2.3 (0.85)
U/T	0.34 (0.044)	0.58 (0.002)	0.64 (0.032)	0.71 (0.002)	0.64 (0.0003)	0.63 (0.018)	0.69 (0.014)	0.68 (0.083)
VLCFAs/T	0.10 (0.059)	0.089 (0.0161)	0.043 (0.0140)	0.030 (0.0025)	0.053 (0.0064)	0.045 (0.0022)	0.037 (0.010)	0.020 (0.0003)
16:0/T	0.31 (0.041)	0.25 (0.001)	0.26 (0.005)	0.22 (0.004)	0.23 (0.001)	0.26 (0.012)	0.23 (0.002)	0.26 (0.080)
18:0/T	0.16 (0.018)	0.062 (0.010)	0.048 (0.0094)	0.029 (0.0009)	0.061 (0.0097)	0.044 (0.0081)	0.028 (0.0007)	0.031 (0.0017)
18:1/T	0.047 (0.014)	0.048 (0.0004)	0.050 (0.0006)	0.041 (0.0034)	0.029 (0.0009)	0.025 (0.0023)	0.022 (0.0013)	0.013 (0.0024)
18:2/T	0.14 (0.040)	0.29 (0.012)	0.38 (0.029)	0.42 (0.007)	0.31 (0.005)	0.36 (0.011)	0.39 (0.016)	0.30 (0.051)
18:3/T	0.11 (0.022)	0.22 (0.027)	0.20 (0.008)	0.24 (0.003)	0.28 (0.003)	0.24 (0.011)	0.27 (0.004)	0.36 (0.039)
18:0/18:3	1.5 (0.45)	0.29 (0.011)	0.25 (0.058)	0.12 (0.005)	0.22 (0.037)	0.19 (0.042)	0.10 (0.004)	0.086 (0.0045)
18:3/18:2	0.83 (0.080)	0.76 (0.125)	0.53 (0.019)	0.58 (0.017)	0.91 (0.023)	0.67 (0.010)	0.70 (0.019)	1.2 (0.08)

For each independent variable the mean value and the standard deviation (SD) are shown. In the case of sums the values are expressed in mg/g of total lipids. FFA – free fatty acids ; PUFAs – polyunsaturated fatty acids; VLCFA – very long chain fatty acids.

The FFA content in the total lipids increased with the dedifferentiation of the primary flax explants and the initial phases of *callus* development. However, differences associated with *calli* embryogenic capacity or with time in culture were not detected. The variation in total

FFA (T) is mainly explained by the variation of unsaturated FFA (U), compounds which accounted for most of the total content ($U = 0.75T - 10.9$; $r^2 = 0.98$, $P < 0.001$). However, different tissues showed different proportions of unsaturated FFA. The primary explants (HS) contained the lowest unsaturated FFA (U) content as well as the lowest U/T ratio. The values of U and polyunsaturated fatty acids (PUFAs) in SEt_7 were much higher than in SEt_5 although no differences were detected in the saturated FFA (S) content between these samples (Table 3). The free PUFAs (18:2 and 18:3) were the most abundant unsaturated FFA accounting for 90-96 % of these compounds in *calli* and somatic embryos, and 72 and 87 % in HS and HSC, respectively. The values obtained for the variables U/S, U/T and PUFAs/T are shown in Table 3. The unsaturation level increased very significantly with the initial phases of dedifferentiation and *calli* development (HS to HSC) leveling off in posterior sampling times. Significant differences in the U/S, U/T and PUFAs/T associated with *calli* morphogenic capacity were found. Either at t_5 or at t_7, NEC had lower values of these ratios than EC.

Contrarily to what occurred with the total FFA and long-chain FFA, the VLCFA, generally associated with the cuticle, did not suffer relevant oscillations during the experiment.

Accordingly to what has been described for other *calli* cultures (32), our results are consistent with a cellular FFA pool mainly composed by 16:0, 18:0, 18:1 and PUFAs (18:2 and 18:3). All the other FFA were present in low amounts (0.3-2.0 mg/g total lipids) and showed minor fluctuations during the embryogenic process. Tissue-specific differences and changes associated with the embryogenic process were detected. The contents of medium-chain fatty acids (MCFA) (14:0 and 15:0) in EC were lower than in NEC. Their maximum levels were recorded in HS. From the long-chain fatty acids (LCFA) group, the maximum level of 18:1 was registered in actively growing *calli* (t_5) and that of 18:2 in *calli*, irrespective of time in culture, and in SE at t_7, while the maximum levels of 16:0 and 18:3 were recorded in SEt_7. No significant variations in 18:0 contents were observed. Although rarely described in plant tissues, free 17:0, in small amounts, was found in all samples. The presence of this compound together with 15:0 in germinating seeds of flax had already been reported (33). The presence of these uncommon fatty acids were attributed, by these authors, to an α-oxidation system. The primary explants (HS) had the highest proportion of saturated FFA, specially 16:0, the main compound in this tissue.

A marked increase of 16:0 and PUFAs, specially 18:2, the most abundant fatty in mithocondrial membranes (34), was observed with the dedifferentiation process and early *calli* formation. The development of somatic embryos, was also accompained by an increase of those compounds, particularly of 18:3, the most abundant fatty acid in thylacoidal membranes (34) and characteristic of photosynthetic tissues (17). This increase in the level of

18:3 was also observed during somatic embryogenesis from carrot cell cultures (16). On the basis of these variation patterns and with the attempt to bring about biochemical indicators of *calli* embryogenic capacity and *calli* growth phase, as well as of somatic embryo development status, two variables were also calculated – 18:0/18:3 and 18:3/18:2 (Table 3). The 18:0/18:3 ratio decreased markedly with the explant dedifferentiation and *calli* initiation and was significantly lower in EC than in NEC. On the other hand, this ratio decreased significantly from SEt_5 to SEt_7. Such variations in the18:0/18:3 ratio translate important FFA differences associated with *calli* embryogenic process and ontogenic variations during the development of somatic embryos in culture, probably related to thylacoid differentiation and chloroplast maturation. On the contrary, the ratio 18:3/18:2 didn't change significantly during the first stages of the culture establishment, presenting the lowest values in *calli* tissues, specially in t_5, and the highest in SEt_7. The differential increment in 18:2 in relation to 18:3 in non-organized actively growing tissues (*calli*), together with the net accumulation of 18:2, suggests an active membrane formation, in particular those of mitochondria. This mithocondria biogenesis is probably associated with the high demands for carbon and energy during the phase of most intensive growth. The higher proportion in 18:3 in relation to 18:2 in differentiated green tissues, with emphasis to SEt_7, corroborates the idea of thylacoid differentiation and, eventually, could be a measure of the photosynthetic potencial of SE grown in *in vitro* conditions.

Principal Component Analysis (PCA): specific patterns of FFA variation

All the elements of Table 2 were included in the PCA, with the exception of 18:0 which did not vary significantly. Three factors were chosen – f1, f2 e f3 – explaining 79 % of total variability. The multicolinearity was relatively low ($r < 0,8$) being 16:0 positively correlated with 18:2 and 18:3 and negatively with 24:0. Figures 2 and 3 represent the distribution of samples on the factorial planes f1-f2 and f1-f3, respectively, with the correspondent correlation circles. The first two factors explained a total of 63 % of the variability and the factorial plane generated by them, f1-f2, organized the samples in three main groups: 1) HS, HSC and SEt_5; 2) all types of *calli*; and 3) SEt_7 (Fig. 2). This distribution pattern revealed a clear separation between types of tissues – *calli* vs. differentiated tissues – and between somatic embryos in two different developmental stages. It was evident that SE in an earlier developmental stage resembled more primary explants, in terms of FFA composition, than the more developed ones. It was also possible to constitute in *calli* group two coherent sub-groups, based on their embryogenic capacity. The overall distribution of the variables in this

factorial plane allowed to distinguish two different pathways: one related to hypocotyl tissues dedifferentiation and *calli* tissue formation, and a second related to the development of SE in culture (arrows in Fig. 2).

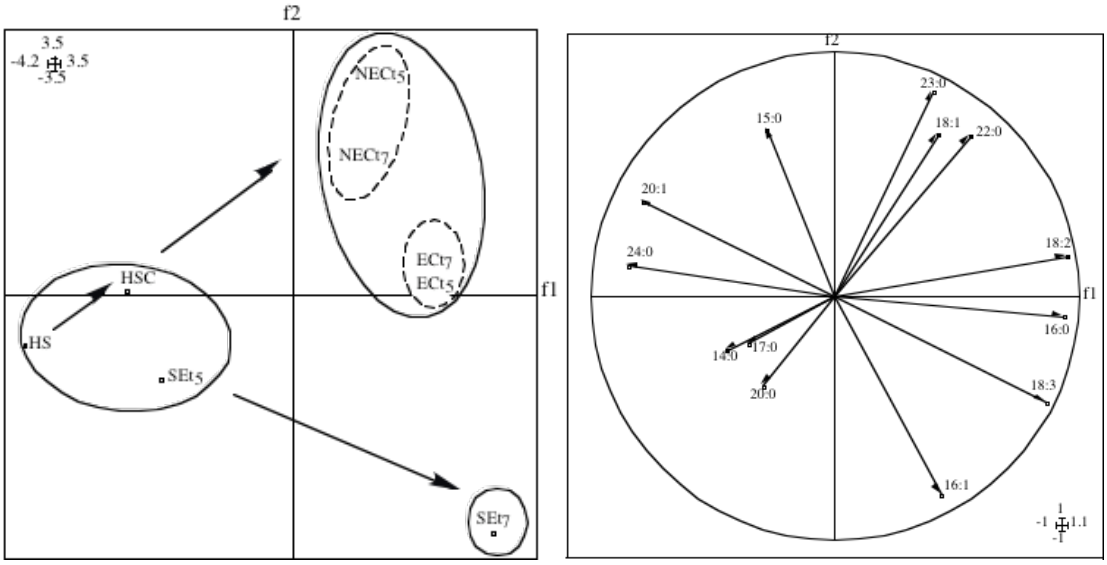

Figure 2 – Distribution of the independent variables (samples) on the f1-f2 factorial map obtained with the FFA, and respective correlation circle.

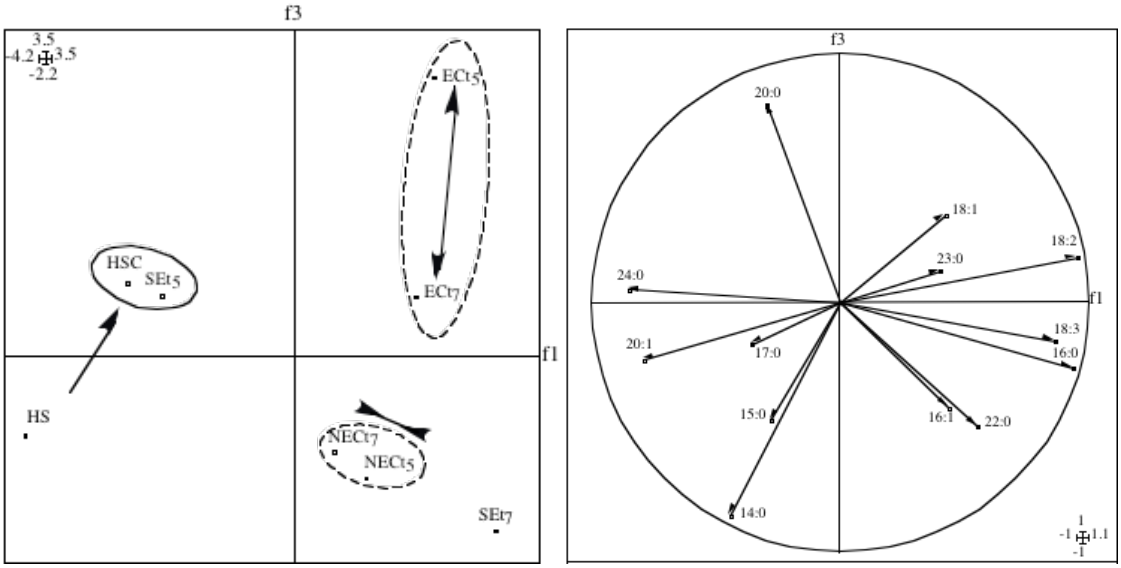

Figure 3 – Distribution of the independent variables (samples) on the f1-f3 factorial map obtained with the FFA, and respective correlation circle.

Analysing the correlation circle from Figure 2 – which shows the relative contribution of the individual FFA to the generation of the axis - it is possible to understand the underlying FFA variations responsible for that distribution pattern. It was deduced that the segregation between *calli* and differentiated tissues (with the exception of SEt_7) was explained by an increment of the main FFA (16:0, 18:2 and 18:3) with the calogenic process. SEt_7, that

experienced similar changes, were separated from *calli* because they had specifically high contents in 16:1 and 18:3 and lower content in the FFA that contributed to +f2, namely 18:1. EC were separated from NEC for having higher contents of 16:1, 18:2 and 18:3 and lower contents of 15:0, 20:1 and 24:0 (Table 2 and Fig. 2). The isolation of SEt_7 in relation to SEt_5 was mainly due to a very significant increment of 16:0, 16:1 and PUFAs and, similarly to what was observed between EC and NEC, a decrease in the VLCFA 20:1 and 24:0 during their development (Table 2 and Fig. 2).

In the plane f1-f3 (Fig. 3), the reordination of the variables resulted basically in a better separation of NEC from EC, but here, the embryogenic *calli* segregated on the basis of time in culture. From the observation of the correlation circle from Figure 3, we can see that f3 was generated essentially by 14:0 and 20:0. Just like what happened with the f2 axis, the f3 separated NEC from EC on the basis of a MCFA. A higher content in 14:0 in NEC justified its separation from EC, and a higher content in 20:0, the separation of ECt_5 from ECt_7 (Table 3 and Fig. 3).

The same multivariate analysis was applied to the Table 3 elements. In this case, some variables were excluded due to high redundancy ($r > 0.990$) with other variables – PUFAs, U/S and PUFAs/T – or to the lack of significant variation – S and VLCFAs. In this PCA two factors were extracted, explaining 90 % of total variability. The overall distribution of the independent variables (Fig. 4) was not fundamentally different from those obtained with FFA (Figs. 2 and 3). A metabolic change associated with calli formation and ontogenic development of SE seems to be evident, but here, the temporal pathway of dedifferentiation, induction and growth of calli in culture became more apparent. The longer distance between HS and HSC reveals that the process of explant dedifferentiation more than a morphological transition corresponds to a metabolic active phase, preparatory for the high demanding calli growth phase. Although no obvious separation of calli due to its embryogenic capacity was observed from this factorial map (dashed ellipses), the PCA result highlighted that EC are richer in unsaturated FFA, and NEC presented higher proportions of 16:0 and 18:0 and higher 18:0/18:3 ratio (Table 3 and correlation circle from Fig.4).

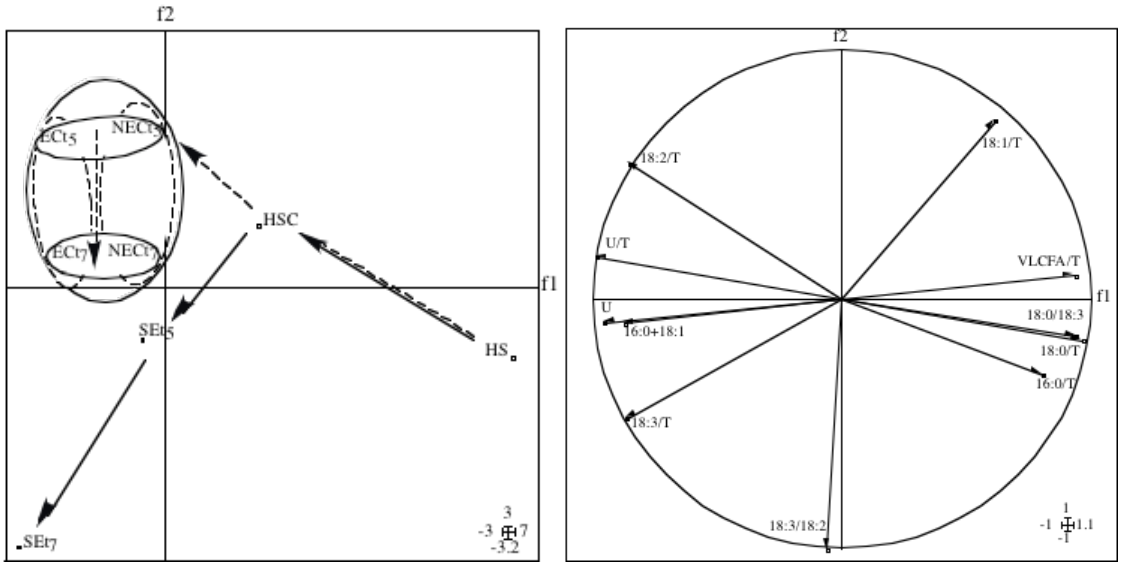

Figure 4 – Distribution of the independent variables (samples) on the f1-f2 factorial map obtained with the elements of Table 3, and respective correlation circle.

The correlation circle (Fig. 4) showed that the most important variable for the generation of f2 axis was the 18:3/18:2 ratio and that the relative proportions of saturated FFA and VLCFA were positively correlated with f1 axis while the unsaturated ones were negatively correlated. That means that the FFA ontogenic variations associated with the process of somatic embryogenesis, presented two phases: a first one, during the dedifferentiation of the tissues (from HS to HSC), characterized by a significant increase in the proportions of unsaturated LCFA and a decrease in VLCFA/T and a second one, coincident with the development of SE, characterized by a specific higher increment of the proportion of 18:3, leading to an increase in the 18:3/18:2 ratio. Changes associated with the early growth of *calli* (from HSC to ECt_5) maintained the trend registered during the dedifferentiation phase but, in addition, it was characterized by an increment in the proportion of 18:2. Further growth (from ECt_5 to ECt_7) resulted in alterations similar to those experienced by the developing SE – an increase in 18:3/T and 18:3/18:2 ratio and a decrease in 18:1/T. These results showed that 18:3/18:2 ratio allowed to separate both SE in different developmental stages and *calli* in different growth phases. Given the importance of 18:3 in thylacoidal membranes this result may reflect a change in the mode of nutrition of SE – from heterotrophic, more dependent on *calli*, to autotrophic or mixotrophic – and possibly, a trend to mixotrophy in *calli* in the stationary phase.

Changes in EFA content and composition

The results obtained from the analysis of the esterified fatty acid (EFA) fractions are shown in Tables 4 and 5. With the exception of 15:0, all the fatty acids from the FFA fractions were found in EFA fractions. These include 12:0 and 22:1 which were absent in the FFA fractions. Although the esterified 16:0, 18:0, 18:1 and PUFAs still constitute important compounds in all tissues with a pattern of variation similar to that found in FFA, esterified MCFA were present in high amounts in some tissues. The same was true for the esterified VLCFA which revealed a tissue-specific distribution.

The specific content of the total EFA increased very significantly with dedifferentiation through the more active *calli* growth phase (t_5) where the highest values were reached, independently of *calli* embryogenic capacity. However, unlike to what was observed for FFA, where the highest increment was detected during *calli* early growth phase (from HSC to *calli* t_5), the higher EFA increase was observed during dedifferentation (from HS to HSC). Moreover, the EFA content in NEC decreased about 50 % from t_5 to t_7.

Although the total unsaturated fatty acids accounted for most of the EFA content ($U = 0.72\ T - 14.8$; $r^2 = 0.95$, $P < 0.001$), in *calli* and SE, the proportion of PUFAs in the total unsaturated EFA were smaller than that recorded for the unsaturated FFA – 83-88 % against the 90-96 %. The proportion of PUFAs found in the unsaturated EFA fraction of HS was as low as 38 %, almost half of the proportion obtained in the unsaturated FFA fraction of the same tissues (72 %). These results revealed that the EFA fraction is not only richer in unsaturated FA other than PUFAs but also that the production of PUFA esters in the early phases of the explant dedifferentiation (from HS to HSC) is very active, probably to cope with the high growth pressure. With a smaller amplitude of variation, the saturated EFA (S) contents roughly paralleled the total EFA (T) contents (Table 5). The lowest U/S and U/T ratios occurred in HS followed by SEt_5 and HSC. The highest ratios occurred in SE_7 in contrast to what was observed with the FFA fractions (Tables 3 and 5).

The profile of distribution of the main EFA in SEt_5 resembled more that of $NECt_5$ than that of SEt_7 (Fig. 5). The relative ontogenic variation of 18:2 and 18:3 in SE indicates that, as observed with FFA, the 18:3/18:2 ratio in the EFA fraction could also be used as a biochemical tool in the evaluation of the SE developmental phase and possibly of their photoautotrophic capacities in *in vitro* culture conditions.

The EFA fractions presented a higher content of total VLCFA and a higher proportion of the monounsaturated ones than the FFA fractions. In contrast with FFA fractions, where the VLCFA stood at levels of 4-5 mg/g of total lipids, in all the samples, the content and composition of VLCFA in the EFA fractions changed significantly according to the type of sample. The total content of these compounds in EC was significantly higher than in NEC

(Fig. 5). However, like in the FFA fractions, $NECt_5$ had levels of 20:1 and 24:0 higher than EC and lacked 20:0. Saturated esterified VLCFA were absent in HS and were represented only by 20:0 in SEt_5 (Tables 4 and 5).

Table 4 – Contents of the individual EFA in the neutral fractions obtained from the different plant tissues and structures (HS, HSC, NEC, EC and SE) sampled during the experiment (t_0, t_2, t_5 and t_7).

EFA	RT (min.)	Samples (sampling time)							
		HS (t_0)	HSC (t_2)	NEC (t_5)	EC (t_5)	SE (t_5)	NEC (t_7)	EC (t_7)	SE (t_7)
12:0	6.14	25 (2.7)	14 (1.6)	17 (4.1)	4.5 (0.39)	17 (2.3)	nd	nd	3.2 (-)
14:0	9.37	4.1 (0.52)	3.0 (0.25)	nd	1.7 (-)	nd	1.4 (0.11)	2.2 (-)	1.4 (0.26)
16:0	13.09	12 (2.3)	34 (2.2)	43 (4.8)	42 (2.0)	21 (1.6)	23 (2.4)	39 (0.6)	18 (5.9)
16:1	13.54	3.1 (0.37)	nd	nd	3.9 (0.11)	5.6 (-)	2.3 (0.24)	4.7 (0.03)	6.6 (2.47)
17:0	14.94	nd	nd	nd	nd	8.3 (-)	nd	nd	nd
18:0	16.67	5.7 (1.61)	8.3 (0.69)	11 (1.4)	8.8 (0.27)	11 (0.9)	4.9 (1.06)	6.8 (0.190	4.5 (0.99)
18:1	16.93	4.3 (0.71)	12 (1.4)	15 (2.7)	11 (0.5)	nd	4.4 (0.31)	7.1 (0.69)	2.2 (0.71)
18:2	17.72	6.4 (0.16)	58 (5.5)	77 (12.7)	74 (4.3)	31 (4.8)	36 (3.6)	65 (0.2)	34 (14.3)
18:3	18.79	4.0 (0.29)	33 (1.6)	49 (6.9)	52 (3.6)	22 (2.4)	28 (3.2)	51 (1.8)	37 (18.2)
20:0	20.02	nd	4.0 (-)	nd	4.1 (0.48)	8.7 (-)	2.1 (0.20)	4.9 (0.53)	2.1 (-)
20:1	20.87	4.5 (2.09)	3.9 (0.52)	5.4 (0.81)	2.4 (0.54)	4.5 (1.22)	0.99 (0.003)	2.0 (-)	1.2 (0.58)
22:0	23.42	nd	nd	nd	4.8 (-)	nd	nd	nd	nd
22:1	23.81	6.9 (-)	5.3 (-)	nd	9.1 (-)	11 (0.62)	2.1 (0.98)	5.2 (-)	2.8 (-)
23:0	24.72	nd	6.9 (3.12)	nd	5.6 (2.89)	nd	2.6 (0.29)	7.8 (2.97)	2.8 (-)
24:0	26.17	nd	6.3 (0.53)	11 (1.6)	6.6 (0.16)	nd	5.3 (0.32)	8.9 (1.27)	4.9 (-)

For each compound, its retention time (RT) for the GC setting conditions, the mean value (mg/g total lipids) and the respective standard error (SE) are shown; (-) due to technical limitations in the quantification of one of the replicates the error associated to the mean (SE) was not estimated; nd – not detected.

Table 5 – Sums of the individual contents of EFA (total and parcial) and ratios between individual or groups of EFA present in the neutral fractions obtained from the different plant tissues and structures (HS, HSC, NEC, EC and SE) sampled during the experiment (t_0, t_2, t_5 and t_7).

Variables	Samples (sampling time)							
	HS (t_0)	HSC (t_2)	NEC (t_5)	EC (t_5)	SE (t_5)	NEC (t_7)	EC (t_7)	SE (t_7)
Total EFA (T)	73 (2.6)	183 (8.5)	228 (49.5)	222 (7.0)	127 (0.9)	113 (16.5)	199 (2.8)	113 (66.9)
Unsaturated EFA (U)	26 (0.2)	109 (9.0)	146 (32.8)	148 (3.5)	72 (13.3)	74 (11.0)	131 (3.0)	83 (53.2)
Saturated EFA (S)	47 (2.4)	74 (0.5)	82 (16.8)	74 (3.5)	56 (14.2)	39 (5.4)	68 (5.7)	30 (13.7)
PUFAs	10 (0.6)	91 (10.1)	125 (27.8)	126 (11.2)	53 (10.2)	64 (9.6)	116 (2.9)	71 (45.9)
VLCFAs	8.0 (1.96)	22 (9.4)	17 (3.4)	26 (7.4)	20 (7.0)	13 (1.7)	25 (1.7)	7.3 (9.4)
Saturated VLCFAs (SV)	0.0 (0.0)	15 (6.4)	11 (2.2)	19 (0.3)	4.4 (6.17)	10 (0.3)	22 (6.8)	4.7 (6.58)
PUFAs/T	0.14 (0.004)	0.49 (0.032)	0.55 (0.002)	0.57 (0.033)	0.42 (0.083)	0.57 (0.002)	0.58 (0.006)	0.62 (0.039)
U/S	0.55 (0.023)	1.5 (0.13)	1.8 (0.04)	2.0 (0.05)	1.4 (0.58)	1.9 (0.02)	1.9 (0.21)	2.6 (0.58)
U/T	0.36 (0.010)	0.59 (0.021)	0.64 (0.005)	0.67 (0.005)	0.56 (0.109)	0.65 (0.002)	0.66 (0.024)	0.72 (0.046)
SV/T	0.0 (0.0)	0.083 (0.0389)	0.049 (0.0009)	0.084 (0.0038)	0.034 (0.0482)	0.09 (0.0102)	0.11 (0.032)	0.029 (0.0411)
16:0/T	0.17 (0.039)	0.18 (0.009)	0.19 (0.011)	0.19 (0.007)	0.16 (0.019)	0.20 (0.010)	0.19 (0.001)	0.17 (0.026)
18:0/T	0.078 (0.0283)	0.045 (0.0032)	0.050 (0.0026)	0.040 (0.0004)	0.079 (0.0090)	0.043 (0.0070)	0.034 (0.0018)	0.043 (0.0013)
18:1/T	0.059 (0.0117)	0.063 (0.0075)	0.065 (0.0029)	0.051 (0.0048)	0.0 (0.0)	0.039 (0.0018)	0.036 (0.0048)	0.020 (0.0032)
18:2/T	0.088 (0.0004)	0.31 (0.028)	0.34 (0.006)	0.33 (0.017)	0.24 (0.055)	0.32 (0.002)	0.33 (0.003)	0.30 (0.001)
18:3/T	0.055 (0.0036)	0.18 (0.004)	0.21 (0.003)	0.23 (0.016)	0.17 (0.028)	0.25 (0.004)	0.25 (0.009)	0.32 (0.040)
18:3/18:2	0.63 (0.041)	0.58 (0.040)	0.64 (0.021)	0.70 (0.012)	0.71 (0.043)	0.77 (0.018)	0.78 (0.035)	1.04 (0.13)

For each independent variable the mean value and the standard deviation (SD) are shown. In the case of sums the values are expressed in mg/g of total lipids; EFA – esterified fatty acid; PUFAs – polyunsaturated fatty acids; VLCFA – very long chain fatty acids.

The major EFA were the same LCFA described for the FFA fraction, with the exception of 18:1 which was absent in SEt_5 (Table 4). The esterified 12:0 was present in high levels in HS, HSC, $NECt_5$ and SEt_5 being the most abundant EFA in HS (Fig. 5). The levels of 22:1,

identified only in EFA fractions, were particularly important in ECt_5 and SEt_5. The esterified 22:0 was present only in ECt_5 while the esterified 17:0 was present only in SEt_5. These results suggest the existence of a esterification mecanism operating for the removal of 17:0. The high accumulation of 20:0, 20:1 and 22:1 and the absence of 18:1 in SEt_5 suggests that in younger SE, the18:0 elongation stops in 20:0 being the esterified 20:1 and 22:1 attributed to 18:1 elongation as referred for other oleaginous species (35).

During germination, prior to initiation of photosynthesis, nearly all seed plants rely exclusively on reserves stored in seeds for both energy and carbon (4). For plants that emerge and grow for a time in low light conditions, seed reserves play also a crucial role during that growth period. Similarly, in *in vitro* conditions, during germination and growth of SE from EC, SE should accumulate reserves for their early heterotrophic growth. The lower content of esterified 12:0 in EC comparatively to NEC, together with its accumulation in SEt_5 suggests a mobilization of this compound from embryogenic *calli* to the emerging SE where, by β-oxidation, it could provide a source of carbon for growth in the absence of photosynthesis, as in other systems (36).

From the bulk of this preliminary results the flax somatic embryogenesis model seems to have a good potencial to study the importance of the α-oxidation system in the SE, the metabolism of MCFA in *calli* and the putative role of some VLCFA in somatic embryogenesis, namely, 20:1 which is present in $NECt_5$ at higher contents than in ECt_5, and EFA 22:1 which is present in EC and absent in NEC.

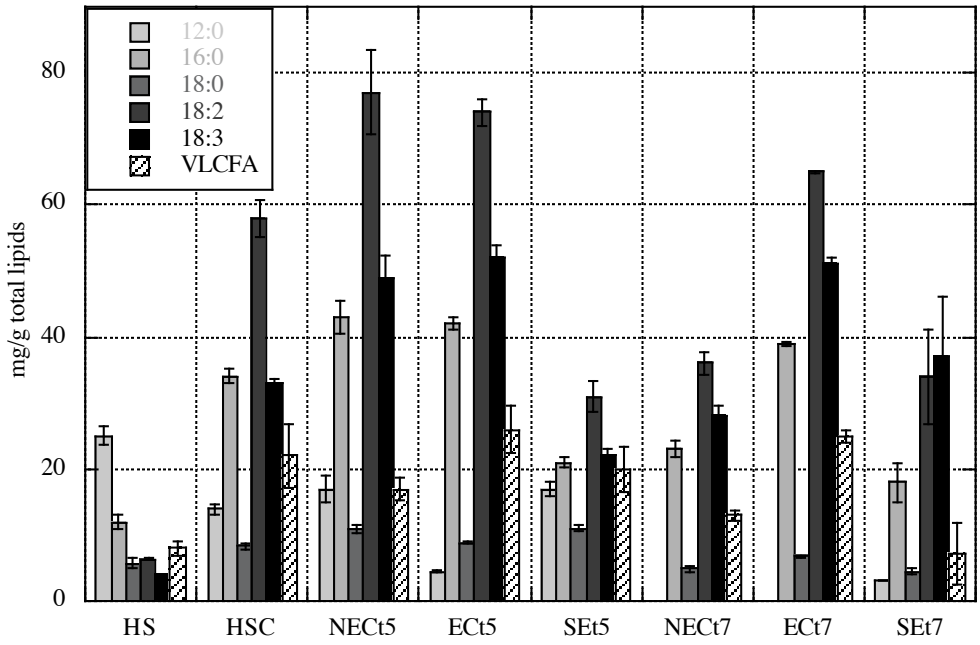

Figure 5 – Major EFA and total esterified VLCFA in all the samples. The standard deviation (SD) is represented on the top of each bar.

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