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Lipid maturity trend in crops as characterized by α -linolenic acid decay and by NIRS study

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Abstract: The present work investigates the trend and characteristics of lipids, and considers the feasibility of Near Infrared spectroscopic (NIRS) prediction, in twelve crops (borage, chia, false flax, flax, galega, hemp, niger, perilla, quinoa, ravizzone, safflower and sunflower). A total of 143 samples, harvested at different growth stages, were collected and processed as freeze-dried or oven-dried specimens. As plants grow, a proportional increase in linoleic, stearic, oleic, palmitic, arachidic and palmitoleic acid and a relative decrease in the total lipids and in other four fatty acids (FAs) were observed, but only the α -linolenic acid (ALA) decreased significantly. The ALA mobilization over a 20 day term was almost -15% of its mean value. In order to express the dynamics of the lipid trend, two maturity indices were calculated: a quantitative indices, i.e. total lipid maturity indices (TLMI) and a qualitative indices, i.e. fatty acid maturity indices (FAMI). Both indices were calculated as the sum of the standardized deviates of the total lipid content and of the twelve FA percentage contents, respectively, which were weighted with +1 or -1 on the basis of whether the regression coefficient increased or decreased over time, respectively. As a result, the twelve species were more differentiated in FAMI (6 levels, with borage being the most mature and sunflower the least mature) than in TLMI (3 levels, with false flax being the most mature and ravizzone, hemp and quinoa being the least mature). Application of the maturity indices formulae to the results of published experiments has shown that it can be a meaningful and simple way of interpreting the experimental effects concerning the anabolism of lipids in the pre-reproductive phase of the crops. Overall, the prediction of the lipid composition of the crops, by means of NIRS, has proved to be equally efficient for the two preparation methods (ratio performance deviation, RPD =2.11 for the freeze-dried samples and 2.03 for the oven dried ones, respectively). However, although FAMI was not well predicted in the oven-dried samples (R-square in cross-validation 1-VR =0.51), it was predicted slightly better in the freeze-dried samples (0.68). The species (0.90 and 0.92), the days after seeding (1-VR =0.78 and 0.73) and the ALA content (0.77 and 0.76) were predicted well in both kinds of samples. It has been concluded that the lipid trend maturity may be summarized by means of simple indices in any kind of experiment that attempts to modify the ontogeny of crops. The driver FA is ALA, whose prediction may be obtained through a rapid NIRS examination of conventional crop samples.

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1. Introduction

Lipids constitute a minor part of the overall crop, although they have frequently been characterized because of the qualitative implications in ruminant feeding (Waghorn and Clark, 2004; Vandewalle et al., 2010). Fatty acid composition and content of forage species are highly variable mainly due to genetic (Palladino et al., 2009), seasonal (Dewhurst et al., 2001) and/or maturity (Khan et al., 2012) differences and also due to difference in nutrient application (Witkowska et al., 2008). The utilization of long

chain fatty acids (FAs) is very poor in ruminants: only a small amount (less than 2%) of ingested α -linolenic acid (ALA) is constitute and ejected in milk (Couvreur et al., 2006). ALA utilization efficiency in ruminants may depend on the plant species as well as plant growth stage. Boufaïed et al. (2003a) compared the hydrogenation of ALA and linoleic acid (LA) in timothy and observed a linear increase of the stearic acid, appearing during the biohydrogenation process, according to the maturity stage of the crop.

Little is known about the maturity trend of total lipid (TL) and its components, which can be considered as the FA percentage. According to Sticklen (2013) about 94-95% per dry matter (DM) of TL crop is in the form of triacylglycerol (TAG): in the non-oil crops TAG could be potentially increased in the vegetative residues, such as corn stover, rice and wheat straws by genetic overexpression. A report from Zhang et al. (2009) showed a dramatic acceleration of lipid maturity after overexpression of the acetyltransferase (DGAT) in *Arabidopsis*: in fact the ALA percentage was reduced to 35% vs. 67% in the wild control, but the lipid synthesis rose from 1.5 to around 25% in transgenic. Attempts to promote extra ether extracts in crops have shown possible side effects. In fact, according to Araujo et al. (2012), the yield and lipids of maize hybrid silages are negatively correlated ($r = -0.71$).

The management of dual-crops, as forage sources during the vegetative stage, has recently been emphasized by Bell et al. (2015). The regrow of both oil and non-oil crops can to be harvested as mature grain, thus increasing the net economic gains by 25–75%.

The aim of the present work was to describe and model lipid maturity trend during the growth cycle, in a varied contest involving oil and non-oil crops. Near infrared spectroscopy (NIRS) is a rapid nondestructive method that is capable of providing reliable predictions of the chemical and nutritive parameters (Foley et al., 1998; Tassone et al., 2014). The possibility of predicting the FA composition, and the FA maturity indices, using samples obtained either through laborious preparation procedures, such as the freeze-drying, or through the more

conventional oven-dried preparation process, has been examined in this paper.

2. Materials and Methods

Several sets of forage crop field data, collected in various studies from 2002 to 2013 in the Western Po Valley near Cuneo, Italy (Longitude: 7° 11' 0" E - Latitude: 44° 41' 0" N - Altitude: 1200 m a.s.l.), were used in this experiment: borage (*Borago officinalis* L.; Peiretti et al., 2004), galega (*Galega officinalis* L.; Peiretti and Gai, 2006), false flax (*Camelina sativa* L.; Peiretti and Meineri, 2007), sunflower (*Helianthus annuus* L.; Peiretti and Meineri, 2010), flax (*Linum usitatissimum* L.; Peiretti and Meineri, 2008), chia (*Salvia hispanica* L.; Peiretti and Gai, 2009), safflower (*Carthamus tinctorius* L.; Peiretti, 2009), hemp (*Cannabis sativa* L.; Peiretti, 2009), perilla (*Perilla frutescens* L.; Peiretti, 2011), ravizzone (*Brassica campestris* L. var. *Oleifera*; Peiretti et al., 2012), quinoa (*Chenopodium quinoa* Willd.; Peiretti et al., 2013), and niger (*Guizotia abyssinica*; Peiretti et al., 2015).

In total, 143 samples were collected at progressive morphological stages in order to provide a large variability (Table 1). Fresh crops were immediately chopped after harvesting. An aliquot of each chopped plant, at each growth stage, was oven-dried in a forced-draft oven at 65°C to a constant weight, air-equilibrated, ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen and analyzed by means of NIRS. A second aliquot was immediately frozen, freeze-dried, ground in a Cyclotec mill and analyzed by means of NIRS. The freeze-dried samples were also analyzed for their TL content and FA profile, as reported in Peiretti et al. (2013).

Table 1. Species, botanic family, number of samples and of stages, harvest range in days after seeding at the first stage (D_{first stage}), and the last stage (D_{last stage})

Name	Species	Botanic family	Sample (n)	Stage (n)	D _{first stage}	D _{last stage}
Borage	<i>Borago officinalis</i>	<i>Boraginaceae</i>	9	5	35	75
False flax	<i>Camelina sativa</i>	<i>Brassicaceae</i>	12	4	42	70
Chia	<i>Salvia hispanica</i>	<i>Lamiaceae</i>	11	4	42	76
Hemp	<i>Cannabis sativa</i>	<i>Cannabaceae</i>	12	4	45	65
Safflower	<i>Carthamus tinctorius</i>	<i>Asteraceae</i>	15	5	36	62
Galega	<i>Galega officinalis</i>	<i>Fabaceae</i>	9	3	48	70
Sunflower	<i>Helianthus annuus</i>	<i>Asteraceae</i>	15	5	36	62
Flax	<i>Linum usitatissimum</i>	<i>Linaceae</i>	15	6	45	99
Perilla	<i>Perilla frutescens</i>	<i>Lamiaceae</i>	11	4	42	62
Quinoa	<i>Chenopodium quinoa</i>	<i>Chenopodiaceae</i>	14	5	49	76
Ravizzone	<i>Brassica campestris</i>	<i>Brassicaceae</i>	12	4	49	70
Niger	<i>Guizotia abyssinica</i>	<i>Asteraceae</i>	8	4	68	87

D_{first stage} = days after seeding at the first stage; D_{last stage} = days after seeding at the last stage.

Table 2. Reclassification percentage obtained from discriminant analysis. Total error 0.6%

Species (Family)	1	2	3	4	5	6	7	8	9	10	11	12
Borage (<i>Boraginaceae</i>)	100	0	0	0	0	0	0	0	0	0	0	0
False flax (<i>Brassicaceae</i>)	0	100	0	0	0	0	0	0	0	0	0	0
Chia (<i>Lamiaceae</i>)	0	0	100	0	0	0	0	0	0	0	0	0
Hemp (<i>Cannabaceae</i>)	0	0	0	100	0	0	0	0	0	0	0	0
Safflower (<i>Asteraceae</i>)	0	0	0	0	100	0	0	0	0	0	0	0
Galega (<i>Fabaceae</i>)	0	0	0	0	0	100	0	0	0	0	0	0
Sunflower (<i>Asteraceae</i>)	0	0	0	0	0	0	100	0	0	0	0	0
Flax (<i>Linaceae</i>)	0	0	0	0	0	0	0	100	0	0	0	0
Perilla (<i>Lamiaceae</i>)	0	0	0	0	18	0	0	0	82	0	0	0
Quinoa (<i>Chenopodiaceae</i>)	0	0	0	0	0	0	0	0	0	100	0	0
Ravizzone (<i>Brassicaceae</i>)	0	0	0	0	0	0	0	0	0	0	100	0
Niger (<i>Asteraceae</i>)	0	0	0	0	0	7	0	0	0	0	0	100

Vibrational spectroscopy: Thawed freeze-dried or oven-dried samples were scanned in the 714 to 3333 nm range by means of an FT-NIR Spectrum IdentiCheck FTNIR System (Perkin-Elmer, Beaconsfield, England). The spectra were elaborated without pre-treatment (2751 absorbance points) with WinISI II vers.1.04 software (Infrasoft International, Port Matilda, PA, USA). To obtain the optimized equations for each considered variable, the modified partial least squares (MPLS) method, which admits one passage for elimination of the outliers ($t > 2.0$), and a cross-validation test were utilized.

The ability in estimating the dependent chemical variables was testified by the R-square in cross-validation (1-VR), the standard error in cross-validation (SECV) and the average prediction response, defined as the ratio performance deviation ($RPD = SD / SECV$). The RPD values of the two preparations were compared using a non-parametric Friedman paired test (StatBox V6.5 software - Grimmer Logiciels, Paris). In this test, the observed Kruskal-Wallis H value is distributed as chi ($df = 1$) and, being one-sided, the P -value is compared at the significance limit: $\alpha = 0.05$. As far as the different efficiency of the predictions of each constituent, obtained from the two preparations, is concerned, a z -score was calculated from the Fisher transformation according to Preacher (2002), adopting a two-sided $\alpha < 0.05$ limit.

Statistical analyses: A discriminant analysis procedure, developed by PROC DISCRIM from SAS (SAS/STAT® 9.2. SAS Inst. Inc., Cary, NC), based on the FA composition and TL content, was used to assess the features of the species, through a

calculation of the reclassification table. The total lipid maturity indices (TLMI) and the fatty acid maturity indices (FAMI) in the whole body of the species was calculated using a multivariate cluster hierarchical analysis (CHA), included in StatBox V6.5.

A univariate GLM model was used to test the species (S) and the ontogenic factor, which were resumed as linear regression coefficients of FA on the days after seeding (D):

$$Y_{ijk} = M + S_i + b_i * D_{ij} + E_{ijkl} \quad [1]$$

where: Y = Variable of the ij^{th} species of the k^{th} replicate; M = common average; S = Effect of the i^{th} Species; b_i = common linear regression of the crop type effect on D (seeding-harvesting interval, in days); E_{ijk} = Error term.

The relative importance of the species over the whole variation was expressed as the difference from the R-square of the full model, minus the R-square of a sub-model containing only the regression coefficient.

In order to appreciate the FA trends, the regression coefficients were evaluated by the ratio to the ALA, because of its dominant role in the temporal transformations. But what about lipid trend maturity? Two criteria were thus envisaged to define the maturity of the lipids during ontogeny. The first criterion was the fitting of D, that is, the age of the crops at harvest, to the TL and to the FA percentages; such a prediction has the purpose of assessing whether any factor modifies (delaying or accelerating) the potential growth. For this purpose, each species was modelled in a separate stepwise forward multiple time-regression procedure.

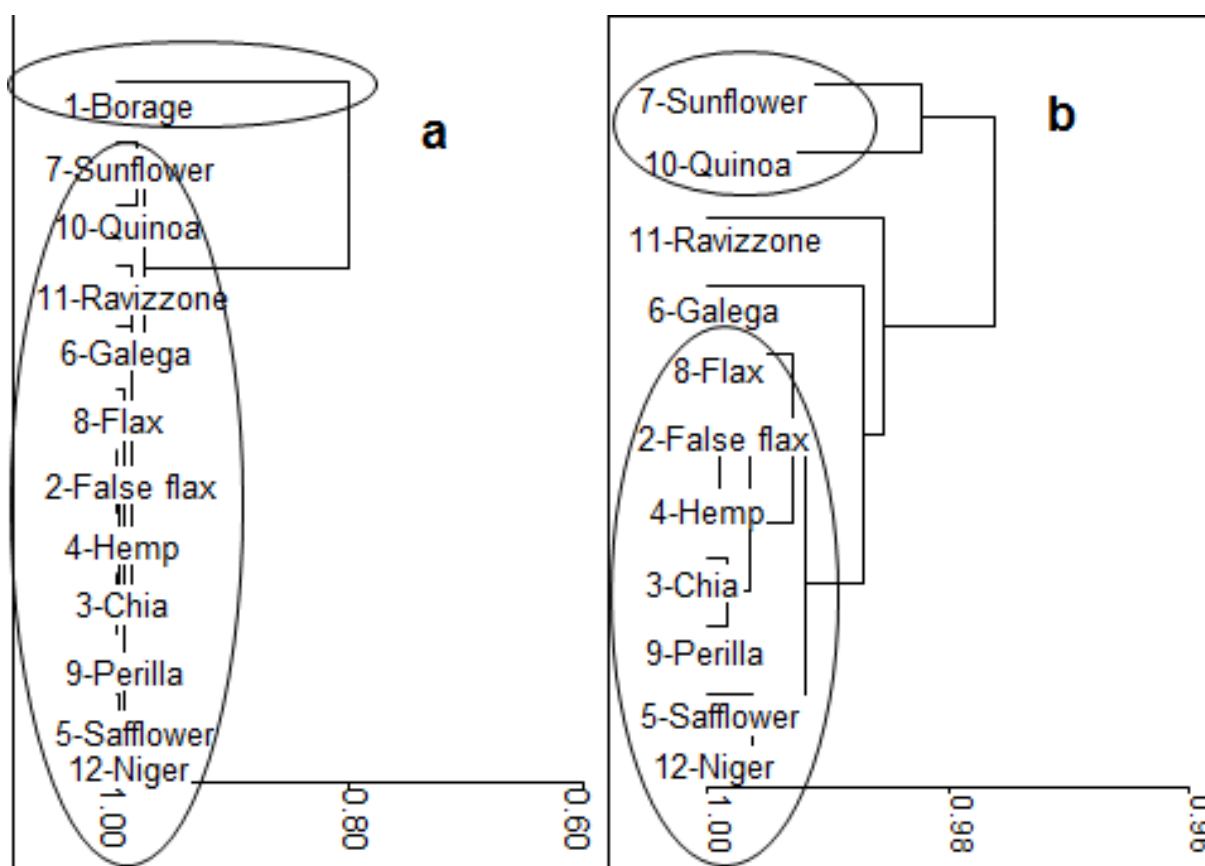


Figure 1. Cluster hierarchical analysis on the average fatty acid composition of twelve crops (a) and excluding borage (b).

The second criterion was a system based on the relative TL and on the composition of the FAs; this procedure consisted of first normalizing the single FAs to their mean ($FA-FA_{mean}$) and standardizing; two equations were then determined for the TL maturity indices (TLMI) and the fatty acids maturity indices (FAMI). FAMI was expressed by the summative equations of each normalized component weighed with +1 or -1, according to whether the average regression of that component was positive or negative on D. Finally, the resulting FAMI sums were normalized and standardized to the unity mean and standard deviation. Low (or negative) values in fact mean a low lipid maturity status of the plant, and high values mean a high mobile status in the lipid trend of the green organs, during the anabolic evolution.

The indices were in fact time-dependent, thus the least squares means of the species were estimated in the GLM model at a time constant basis, using D as a covariate. A final study regarding the mutual connections between the FAs, days after seeding, TLMI and FAMI in the whole body of the species was envisaged using a multivariate CHA model on

the full data-set. Examples of the applications of the maturity indices formulae to the results of two published experiments (Boufaied et al., 2003b), considering the trend of the FAs or experimental treatments in grass and legumes, are also presented in the discussion section.

3. Results and discussion

The FA composition appeared to be very highly specific. In fact, an almost perfect discrimination of the twelve species was obtained with an error (in perilla) limited to 0.6% (Table 2). When the FA composition (Table 3) was examined, it emerged that only five FAs out of twelve were present in each species (ALA, LA, palmitic, stearic, and oleic acid). Borage is a very different crop from the rest of studied crops, because it has a very low level of ALA, which is balanced by a very high content of γ -linolenic (GLA) and stearidonic acid. The cluster analysis that can be observed in Figure 1 highlights this singularity, where it can also be observed that only two other crops (sunflower and quinoa) are neighbors when borage is excluded.

Table 3. Mean total lipid and fatty acid values for the twelve species during growth cycle

	Borage	False flax	Chia	Hemp	Safflower	Galega	Sunflower	Flax	Perilla	Quinoa	Ravizzone	Niger	Min	Max
Total lipid	17.4	12.6	16.3	22.1	15.8	15.5	15.9	16.2	18.1	21.4	22.1	12.1	10	33
Lauric	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	4.6	0.0	0	13
Myristic	37.1	0.0	0.0	0.8	0.0	4.3	2.1	4.0	0.0	6.9	3.5	0.0	0	75
Palmitic	146.2	121.9	111.3	121.1	103.7	115.8	110.8	99.6	92.5	128.5	96.6	110.5	76	214
Palmitoleic	0.0	0.0	0.0	2.3	0.0	0.0	0.0	2.6	3.3	0.0	3.2	0.0	0	8
Stearic	30.7	18.4	23.0	15.1	9.7	25.2	10.1	56.8	13.4	18.7	34.8	17.7	8	128
Vaccenic	0.0	0.0	0.0	4.6	3.3	0.0	3.1	0.0	2.9	0.0	23.3	3.5	0	52
Oleic	67.0	36.9	22.6	22.7	15.5	14.8	18.1	5.0	18.1	81.5	57.8	25.7	4	212
Linoleic	177.4	135.3	112.4	139.9	178.4	82.9	181.1	147.1	124.2	160.1	137.3	179.4	79	274
γ -Linolenic	102.9	0.0	0.0	0.0	0.0	0.0	12.0	0.0	10.8	5.0	0.0	37.6	0	160
α -Linolenic	273.7	527.5	579.3	500.6	547.0	628.6	510.1	501.8	530.8	404.8	424.2	488.4	132	656
Stearidonic	165.0	0.0	0.0	6.4	0.0	0.0	78.0	21.7	0.0	47.0	0.0	0.0	0	241
Arachidic	0.0	0.0	8.1	0.0	0.0	0.0	0.0	10.5	0.0	0.0	51.0	0.0	0	62

Some systematic and characteristic changes in the TL and FA percentage trends have been observed throughout the growing phases of the twelve crop species (Table 4). TL is a component that declines during plant growth: the daily linear decrease in TL in the whole data set was equal to -0.127 g/kg DM ($P < 0.0001$), that is, a 15% decrease in the average lipid content every 20 days.

As shown in Table 3, the ALA content in the twelve crops varied to a great extent, and ranged from 132 to 656 g/kg total FA. It is important to notice that the trend of the FAs was driven by the ALA decay, which amounted to -3.647 g/kg total FA per day; this

notable decrease was able to compensate for the relative increase in all the other FAs.

This increase was statistically significant for another eight FAs, which are here ordered according to their decreasing regression R^2 values: stearic (0.31), palmitic (0.08), oleic (0.08), LA (0.05), GLA (0.03), arachidic (0.02), palmitoleic (0.02), and vaccenic acid (0.01). Insignificant decreases were observed for lauric, myristic, and stearidonic acid (Table 4). As far as the balancing of the relative amount of FAs over time is concerned, the main ALA substitutions were in favor of LA (25%), stearic (20%), oleic (16%), and palmitic acid (12%).

Table 4. Trend of the total lipid, fatty acid and fatty acid maturity indices (FAMI) expressed by means of the common linear regression of the dependent variables on the days after seeding, and reference to the α -linolenic acid content

	Weight	R ² tot	R ² species	r ² b	VC	RMSE	M-Mean	P	b	sb	P	b/ b ALA	D20 /M%
								species					
Total lipid	-1	0.54	0.45	0.09	19	3.33	17.27	<.0001	-0.127	0.03	<.0001	-	-15%
Lauric	0	0.45	0.45	0.00	297	1.57	0.53	<.0001	0.000	0.012	0.9996	0%	0%
Myristic	0	0.65	0.65	0.00	159	6.80	4.28	<.0001	-0.023	0.053	0.6663	-1%	-11%
Palmitic	1	0.63	0.56	0.08	10	11.56	112.39	<.0001	0.430	0.089	<.0001	12%	8%
Palmitoleic	1	0.67	0.66	0.02	102	1.02	0.99	<.0001	0.022	0.008	0.0054	1%	45%
Stearic	1	0.77	0.46	0.31	39	9.05	22.90	<.0001	0.712	0.070	<.0001	20%	62%
Vaccenic	1	0.69	0.68	0.01	129	4.43	3.43	<.0001	0.082	0.034	0.0183	2%	48%
Oleic	1	0.57	0.50	0.08	70	22.05	31.58	<.0001	0.597	0.170	0.0006	16%	38%
Linoleic	1	0.72	0.67	0.05	13	19.15	148.05	<.0001	0.917	0.148	<.0001	25%	12%
γ -Linolenic	1	0.92	0.89	0.03	72	8.08	11.16	<.0001	0.184	0.062	0.0038	5%	33%
α -Linolenic	-1	0.87	0.65	0.22	7	36.51	494.51	<.0001	-3.647	0.282	<.0001	-100%	-15%
Stearidonic	0	0.94	0.93	0.00	45	11.75	25.98	<.0001	-0.097	0.091	0.2898	-3%	-7%
Arachidic	1	0.96	0.94	0.02	51	3.08	6.01	<.0001	0.106	0.024	<.0001	3%	35%
FAMI		0.86	0.64	0.22	40	0.4	1.0	<.0001	0.036	0.003	<.0001	-	70%

FAMI = fatty acid maturity indices.

Table 5. Prediction of the days after seeding by means of a forward regression in the twelve species

Species (Family)		Coefficient	Standard Error	Probability	R ² forward	R ² total
Borage (<i>Boraginaceae</i>)	Intercept	147.9	89.3	0.1488		0.86
	Linoleic	55.0	12.3	0.0042	0.73	
	γ -Linolenic	-52.2	21.2	0.0486	0.14	
False flax (<i>Brassicaceae</i>)	Intercept	53.3	66.3	0.4453		0.95
	Linoleic	52.0	10.0	0.0008	0.76	
	Oleic	-76.9	16.1	0.0014	0.14	
	Stearic	67.7	27.9	0.0416	0.04	
Chia (<i>Lamiaceae</i>)	Intercept	2586.5	649.4	0.0053		0.99
	Arachidic	372.6	137.5	0.0302	0.96	
	α -Linolenic	-26.1	6.0	0.0034	0.01	
	Palmitic	-72.9	22.6	0.0144	0.02	
Hemp (<i>Cannabaceae</i>)	Intercept	1539.7	162.7	<.0001		0.79
	Palmitic	-81.9	13.4	0.0001	0.79	
Safflower (<i>Asteraceae</i>)	Intercept	1756.6	210.3	<.0001		0.91
	α -Linolenic	-17.1	3.3	0.0003	0.62	
	Vaccenic	-243.3	47.9	0.0004	0.21	
	Oleic	145.0	43.0	0.0063	0.09	
Galega (<i>Fabaceae</i>)	Intercept	211.7	67.5	0.035		0.98
	Lauric	2119.0	110.8	<.0001	0.67	
	Myristic	-605.9	87.3	0.0023	0.28	
	Total lipid	159.7	34.7	0.010	0.03	
Sunflower (<i>Asteraceae</i>)	Intercept	-215.8	133.1	0.131		0.76
	Palmitic	91.4	17.1	0.0002	0.70	
	Linoleic	-16.9	9.4	0.098	0.06	
Flax (<i>Linaceae</i>)	Intercept	2609.2	35.5	<.0001		0.98
	α -Linolenic	-35.4	0.4	<.0001	0.94	
	Stearidonic	-59.7	1.9	<.0001	0.02	
	Oleic	970.4	21.5	<.0001	0.02	
Perilla (<i>Lamiaceae</i>)	Intercept	412.0	76.6	0.0007		0.85
	Stearic	189.1	44.5	0.0028	0.56	
	Total lipid	-73.5	19.1	0.0048	0.29	
Quinoa (<i>Chenopodiaceae</i>)	Intercept	158.4	114.8	0.1976		0.89
	γ -Linolenic	115.0	24.1	0.0007	0.68	
	Stearic	85.8	19.5	0.0013	0.13	
	Stearidonic	53.5	19.9	0.0229	0.08	
Ravizzone (<i>Brassicaceae</i>)	Intercept	1411.0	123.5	<.0001		0.98
	Stearic	-24.0	11.9	0.1002	0.88	
	α -Linolenic	-21.2	1.9	0.0001	0.05	
	Oleic	-15.9	2.1	0.0007	0.04	
Niger (<i>Asteraceae</i>)	Intercept	1480.9	138.9	<.0001		0.81
	α -Linolenic	-14.6	2.8	0.0021	0.81	
All species without borage	Intercept	876.7	133.9	<.0001		0.68
	α -Linolenic	-7.0	1.3	<.0001	0.32	
	Total lipid	-65.7	15.4	<.0001	0.14	
	Stearic	27.5	4.4	<.0001	0.09	
	γ -Linolenic	34.7	6.8	<.0001	0.04	
	Stearidonic	-8.8	2.7	0.0011	0.04	
	Palmitic	16.3	4.3	0.0002	0.04	

A close fit of the days after seeding was obtained in the forward regression ($R^2 > 0.76$) for each species (Table 5). Three FAs were selected as being the most important: ALA (with a frequency of 17%), stearic (14%), and oleic acid (14%). After excluding the borage crop, the fit of the days after seeding in the pooled calculation with eleven species reached an R^2 of 0.68. The most relevant variables were ALA (0.32)

and TL (0.14). In the final study regarding the relationships between the variables, several clusters appeared (Figure 2). ALA was the most easily distinguished variable, which was opposed to all the other FAs, to days after seeding and to TL.

A first cluster joined the days after seeding and the stearic acid, as a result of the overall maximum positive correlation. A second cluster connected the

FAMI, palmitoleic, vaccenic, and arachidic acid. A third cluster concerned GLA, palmitic, myristic, and stearidonic acid. The other FAs appeared as singularities in the whole context.

The trend of the FAs across the species has been highlighted by the two proposed maturity indices (Table 6). Six grades were observed for FAMI (a-f).

The most mature FA type pertained to the borage (FAMI =2.80a), while the least mature type was that of the sunflower (0.11f). The TLMI values were graduated in three bands (a, b, c), from the most mature (false flax: TLMI =2.16a) to the least mature (ravizzone:-0.65c, quinoa -0.58c, and hemp -0.48c). The two indices were almost independent, as can be seen in Figure 3, where four clusters can be noted:

borage vs. niger - perilla vs. hemp - quinoa - ravizzone vs. other crops.

Overall, the prediction of the lipid crop composition by means of vibrational spectroscopy was equally efficient (Table 7) for the two kinds of samples (average RPD =2.11 for the freeze-dried samples and 2.03 for the oven-dried ones). However, the FAMI was not well predicted for the oven-dried samples (1-VR =0.51), but slightly better predicted for the freeze-dried ones (0.68). The days after seeding were predicted well for both kinds of samples (1-VR =0.78 and 0.73, respectively), as were the species (1-VR =0.90 and 0.92, respectively) and the ALA (1-VR =0.77 and 0.76, respectively).

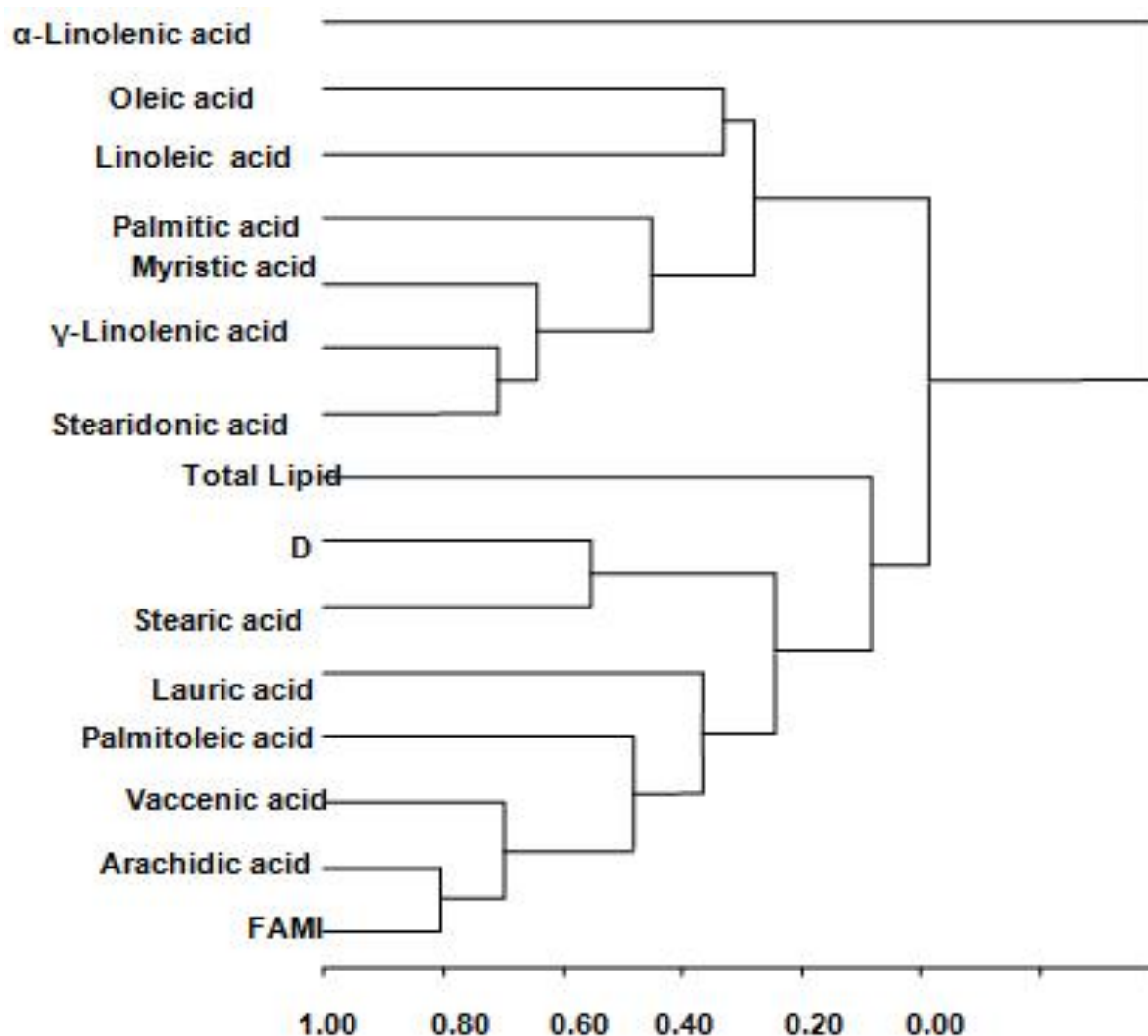


Figure 2. Average cluster hierarchical analysis of the fatty acid composition, the total lipid, the days after seeding (D), and the fatty acid maturity indices (FAMI).

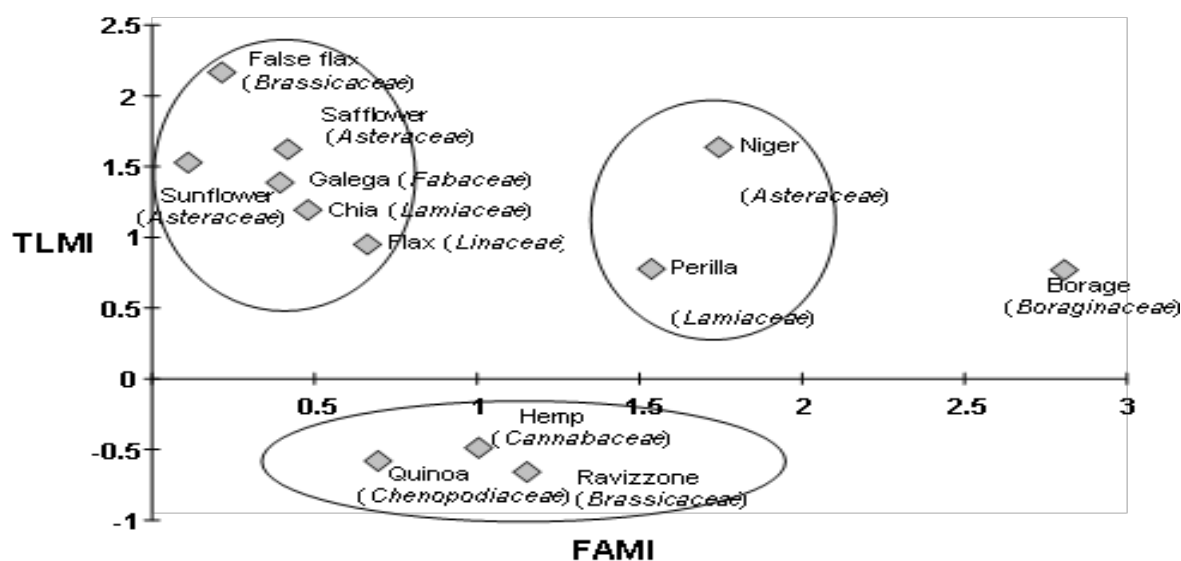


Figure 3. Plot of the twelve species on the fatty acid maturity indices (FAMI, X axis), the total lipids maturity indices (TLMI, Y axis), and clustering; data from Table 6.

Table 6. Ranking of the twelve species according to their decreasing fatty acid maturity indices (FAMI), with the total lipid maturity indices (TLMI), from the most mature (a) to the least mature (f)

No.	Species (Family)	FAMI	TLMI
1	Borage (<i>Boraginaceae</i>)	2.80a	0.76b
2	Niger (<i>Asteraceae</i>)	1.74b	1.63ab
3	Perilla (<i>Lamiaceae</i>)	1.53b	0.77b
4	Ravizzone (<i>Brassicaceae</i>)	1.15c	-0.65c
5	Hemp (<i>Cannabaceae</i>)	1.00cd	-0.48c
6	Quinoa (<i>Chenopodiaceae</i>)	0.69de	-0.58c
7	Flax (<i>Linaceae</i>)	0.66de	0.95b
8	Chia (<i>Lamiaceae</i>)	0.48de	1.19b
9	Safflower (<i>Asteraceae</i>)	0.41e	1.62ab
10	Galega (<i>Fabaceae</i>)	0.39ef	1.38b
11	False flax (<i>Brassicaceae</i>)	0.21ef	2.16a
12	Sunflower (<i>Asteraceae</i>)	0.11f	1.52ab

abcdef in columns: a>b>c>d>e>f. (P<0.05)

Vibrational spectroscopy was able to discriminate the species, with high RPD values, that is, 3.2 and 3.6 for the freeze- and oven-dried samples, respectively (Table 7). The days after seeding was also be predicted reliably: RPD =2.1 and 1.9 for the freeze- and oven-dried samples, respectively.

The TL content was not perceived, with an RPD of 1.3 for both of the samples, and this result is directly related to the poor prediction of the TLMI (1.5 and 1.3, respectively). Conversely, good performances (RPD >2.0) were obtained in both

samples for some FAs, in particular for ALA, stearidonic, and oleic acid. A lower prediction ability (RPD <2.0) was observed for GLA, myristic and lauric acid. Because of the non-uniform responses of the different FAs to NIR radiation, the maturity indices was not predicted well, with 1–VR values of 0.68 and 0.51 for the freeze- and oven-dried samples, respectively.

4. Discussion

Lipids in plant can be grouped into storage and structural compounds. These latter are present in protective surface layers and in various membranes (McDonald et al., 1995).

A diminution in ALA may be explicated by a dilution factor. During growth, leaves are in negative allometry, whereas other organs with fewer chloroplasts, such as stems and petiole, are positively modified. A decline in the ether extract concentration with advancing maturity was described by Gervais and St-Pierre (1979) in orchardgrass and smooth brome grass. The leaves proportion in the plant, usually expressed as the leaf-to-stem ratio, normally decreases in grasses during growth cycle; the stems have reduced lipid concentrations from half to one-third of the FA concentration in comparison with FA content of leaves (Jarrige et al., 1995). Thus, the drop in the leaf-to-stem ratio with the aging plants will partially explain the decrease in total FAs content. The declining FA concentrations with increasing maturity was shown by several studies.

Table 7. Effect of the sample preparation on the calibration and cross-validation performance for the factor design, the fatty acids (FA), the fatty acid maturity indices (FAMI) and the total lipid maturity indices (TLMI) from freeze-dried and oven-dried samples

Variables	Mean	SD	Freeze dried		Oven-dried	
			1-VR	RPD	1-VR	RPD
Species	6.38	3.47	0.90	3.2	0.92	3.6
Days after seeding	57.7	12.8	0.78	2.1	0.73	1.9
Total lipids	17.27	4.68	0.43	1.3	0.39	1.3
Lauric	0.53	2.02	0.49	1.4	0.35	1.2
Myristic	4.28	10.97	0.52	1.4	0.32	1.2
Palmitic	112.39	18.29	0.77a	2.1	0.56b	1.5
Palmitoleic	0.99	1.71	0.82a	2.4	0.61b	1.6
Stearic	22.90	18.04	0.71	1.9	0.78	2.1
Vaccenic	3.43	7.62	0.73	1.9	0.82	2.4
Oleic	31.58	32.25	0.90	3.2	0.90	3.1
Linoleic	148.05	34.82	0.78a	2.1	0.61b	1.6
γ -Linolenic	11.16	26.76	0.65a	1.7	0.40b	1.3
α -Linolenic	494.51	96.48	0.77	2.1	0.76	2.1
Stearidonic	25.98	45.68	0.84	2.5	0.86	2.7
Arachidic	6.01	14.50	0.67b	1.7	0.90a	3.2
Average	67.62	24.14	0.71	2.11A	0.66	2.03A
FAMI	0.85	0.82	0.68a	1.8	0.51b	1.4
TLMI	0.77	1.45	0.54	1.5	0.43	1.3

^{A A} Paired Friedman test: *Prob* = 1; a>b (P<0.05) two sided r Fisher test.

a>b (P=0.00228); 1-VR = R-square in cross-validation mode; RPD = relative prediction deviation in cross-validation = standard deviation / standard error in cross-validation. RPD, according to Williams (1987).

Barta (1975) studied six grass crops and reported a reductions greater than 30% with the aging plants. The involution of the total FA has been confirmed by Boufaïed et al. (2003b) in timothy, with advancing maturity. The authors observed that four of the FAs (namely ALA, LA, palmitic, and palmitoleic acid) diminished, while the contents of another four FAs (lauric, myristic, stearic, and oleic acid) were stable throughout growth.

In a three cuts experiment with *Lolium perenne* the lipid profile was more different in the first than in the following cuts (Palladino et al., 2009). William et al (2005) confirmed the borage singularity in a framework of 13 crops, as being the poorest in ALA contents; however they could confirm the prevalent relative decrease of ALA at three consecutive stages in only 4 of the 13 studied crops; in 9 crops significant interactions between plant species and stage occurred.

According Khan et al (2012) in maize silage ALA content appeared the most characterizing FA as being opposed on the first principal component to all the other while in the grass silages, ALA was opposed to the other FAs only on the second principal component; silages of less mature grass had a high content of ALA, which appeared strictly related with the chemical composition ($R^2=0.73$).

Plot of daily diurnal variation in orchardgrass and meadow fescue from Gregorini et al. (2008) lighted a major shift decreasing in ALA, that was however recovered by night, while oleic acid increased in the noon.

The incidence on the lipid profile of the time in the year, however confounded with growth stage effects, were highlighted in a meta-analysis performed by Glasser et al. (2013); a minimum content for ALA was recorded in June, however it can be inferred that ALA was more stable over months than the other four studied FAs; furthermore a cluster hierarchical analysis can show that ALA is completely separated from the other FAs.

Table 8. Application of the two maturity indices to recalculate the original data from Boufaïed et al. (2003b)

Name (Species)	Season	TLMI	FAMI
Orchardgrass (<i>Dactylis glomerata</i>)	Spring	3.36	0.37
Orchardgrass (<i>Dactylis glomerata</i>)	Summer	-0.37	-0.45
Timothy (<i>Phleum pratense</i>)	Spring	3.42	0.91
Timothy (<i>Phleum pratense</i>)	Summer	-1.08	-0.06
Alfalfa (<i>Medicago sativa</i>)	Spring	1.84	2.13
Alfalfa (<i>Medicago sativa</i>)	Summer	1.57	2.34
Red clover (<i>Trifolium pratense</i>)	Spring	0.52	1.25
Red clover (<i>Trifolium pratense</i>)	Summer	-1.27	1.49

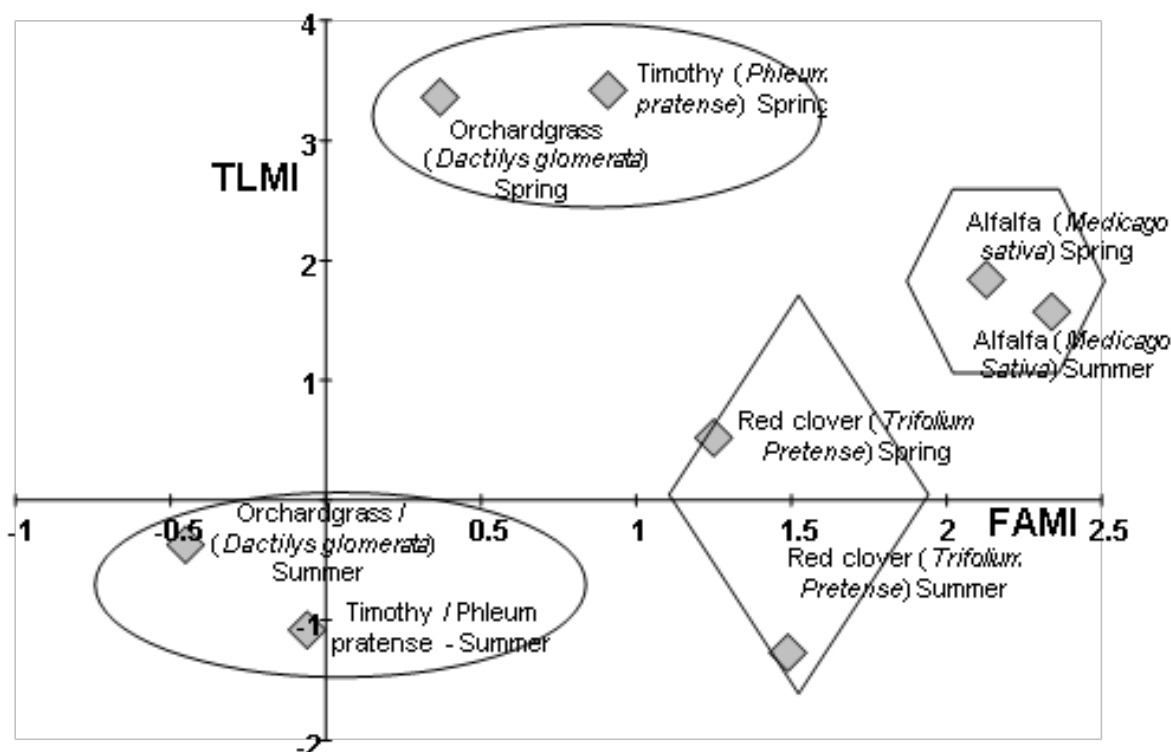


Figure 4. Application of the maturity indices to data obtained from Boufaïed et al. (2003b): plot of the fatty acid maturity indices (FAMI, X axis) and of the total lipid maturity indices (TLMI, Y axis) of four forage species for spring growth and summer regrowth, as reported in Table 8, and clustering.

The results of the application of the two maturity indices to average data obtained from Boufaïed et al. (2003b), recalculated from the original “Experiment 3: Growth Period and species”, are given in Table 8, related to Figure 4. As regard the two species of grasses considered (orchardgrass and timothy), lipid maturity increases during the spring season, whereas its regrowth appears minimal at summer harvesting. Nevertheless, lipid maturity appears more increased in the two legume species (alfalfa and red clover) than in the two grasses, regardless of the harvesting season, which apparently has no effect (Figure 4).

A further application of the FA maturity indices to different data, again taken from Boufaïed et al. (2003b) and recalculated from the original “Experiment 1. FA concentrations of timothy at four growth stages and two nitrogen fertilization applications” is reported in Figure 5. Two features appear relevant: a positive regression of the FAMI (Y axis) on the growth stage (X axis, 1-4), in agreement with the equation elaborated in this paper, and a clear effect of the nitrogen fertilization, which - as expected - delays the lipid maturity to a great extent.

The effects of NIRS features on FA prediction were studied by Foster et al. (2006) in eleven forage species (grasses, legumes, and forbs) collected at three growth stages, thus constituting as extensive a data-set as the present one: on average, the ALA represented 62% of the total FA, whose content ranged from 6.7 to 38.7 g/kg DM. The specimens had been frozen immediately after harvesting and freeze-drying. A very high NIRS performance level was achieved: the RPD was >3.0 for all the FAs, except for lauric (2.6) and miristic acid (2.9).

According to Boufaïed et al. (2003b), an oven-drying treatment can reduce the FA content by 20-30% compared to a freeze-drying treatment. In the present paper, FA determination was only carried out on freeze-dried specimens, but the spectra from oven-dried samples showed equivalent FA prediction performances. A close correlation between the FAs in the two treatments could explain this result. In fact, Boufaïed et al. (2003b) revealed differences related to species, growth stage and nitrogen fertilization, which were still evident in both types of analyzed specimens.

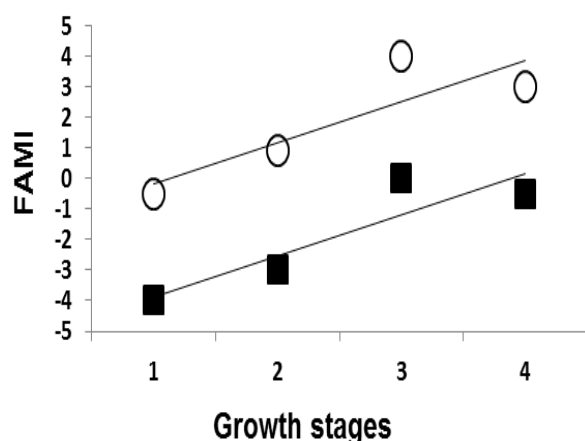


Figure 5. Application of the maturity indices to data (exp. 1) obtained from Boufaïed et al. (2003b), recalculated from the original fatty acid concentrations of timothy at four growth stages with zero nitrogen (○) or with 120 nitrogen (■) fertilization applications.

A cheap, reliable and fast methods to predict the ALA content in dried, ground and stored clover and grass samples has been developed by Vandewalle et al. (2010). Variations in the ALA content were observed between and within most species: Italian ryegrass had the lowest content on average, while timothy showed the highest one. In general, the clovers presented more variations in the ALA content, and white clover showed a higher content than red clover.

In order to identify the optimal approach to prepare samples prior to FA analysis, different sample preparation methods have been studied by Arvidsson (2009). They found satisfactory the most commonly used method for handling samples before analysis, *i.e.* freeze-drying and grinding treatments. However, they reported that heat drying samples at 60°C was good, or even better in some cases.

In dried wheat, examined at three different growth stages, NIR spectroscopy was able to give a very accurate classification of the validation set samples (Gatius et al., 2004). In the present work, the NIRS method has again been shown to be reliable in discriminating the species in freeze-dried or oven-dried samples (RPD =3.2 and 3.6) as well as in back-predicting the days after seeding (RPD =2.1 and 1.9). In each case, with the freeze-dried or with the oven-dried samples, the RPD threshold of 2.0 has been exceeded in six FAs out of twelve, thus this rapid method could gain credibility.

5. Conclusion

It has been suggested that the lipid maturity trend of green crops may be resumed by means of simple indices in any kind of experiment that attempts to modify the ontogeny of crops, as well as in studies targeted on the management of single or dual-purpose crop systems. The total lipid in the crops were diminishing. But, absolutely, is the α -linolenic acid which rules the FA maturity trend by a modulated decay. The twelve crops were more differentiated in FAMI (6 levels, with borage being the most mature and sunflower the least mature) than in TLMI (3 levels, with false flax being the most mature and ravizzone, hemp and quinoa being the least mature). Application of the lipid maturity indices formulae to the results of published experiments has shown that this can be a meaningful and simple way of interpreting the experimental effects concerned with the anabolism of lipids during the pre-reproductive phases of the crops.

List of Abbreviations

ALA: α -Linolenic acid; DM: Dry matter; FA: Fatty acid; FAMI: Fatty acid maturity indices ; LA: Linoleic acid; NIRS: Near Infrared Spectroscopy; TAG: Triacylglycerol; TL: Total lipids; TLMI: Total lipids maturity indices; RPD: Ratio performance deviation.

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Competing Interests

The authors declare that there is no potential conflict of interest.

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