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TRIACYLGLYCEROLS IN OILSEED RAPE DURING SEED DEVELOPMENT

GRENVILLE NORTON and JOHN F. HARRIS

Department of Applied Biochemistry and Food Science, University of Nottingham, School of Agriculture, Sutton Bonington, Nr. Loughborough, Leics. LE12 5RD, U.K.

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Key Word Index—*Brassica napus*; Cruciferae; rape; seeds; lipid changes; triacylglycerols; stereospecific analysis; membrane lipids; fatty acids.

Abstract—The lipid content and composition of seeds of oilseed rape (*Brassica napus*) were followed from shortly after anthesis to maturity. Initially lipids, mainly phospho- and glyco-lipid, were a small proportion of the dry matter. With time the lipid content increased, until at maturity it accounted for 45% of the dry matter; 90% of the lipid at maturity was neutral lipid, largely triacylglycerol. Seven molecular species of triacylglycerol were identified throughout development. Three major molecular species having 5,4 and 3 double bonds per molecule, respectively, accounted for 80% of the triacylglycerols. Erucic acid [22:1 (13)] and eicosenoic acid [20:1 (11)] increased while oleic acid decreased in all triacylglycerols with time. At maturity 22:1 (13) accounted for almost 50% of the fatty acids present. Position 2 of the triacylglycerol was absolutely specific for oleic, linoleic and α-linolenic acids in the respective molecular species. 22:1 (13) and to a lesser extent 20:1 (11) occupied positions 1 and 3 of the triacylglycerol as did palmitic and stearic acids when present.

INTRODUCTION

The accumulation of oil and the concomitant changes in fatty acid (FA) composition are well documented for a number of oilseeds [1] including oilseed rape [2, 3] and related species [4]. Few studies, however, have been made to follow the changes in the triacylglycerol (TAG) composition during oilseed development. Exceptions to this include the studies on soyabean [5, 6] and, in much less detail, safflower [7]. In soyabean marked changes in the levels of certain TAG molecular species were observed during seed development. Gurr *et al.* [4] carried out a detailed study on the TAGs in mature seeds of *Crambé abyssinica* but did not investigate changes in the levels of the molecular species throughout seed development.

In recent years much effort has been expended on oilseed breeding programmes to improve the composition and functional properties of the oils. Such work has been especially successful for oilseed rape [8]. Changes in FA composition must also result in changes in the TAG spectrum. For the full implications of such changes to be understood, information on the TAG molecular species in the improved and non-improved seed would be invaluable. The work reported here concerns investigations on the TAGs and the lipids in the developing seeds of a high erucic acid rapeseed variety.

RESULTS

Dry matter and lipid accumulation in the first few weeks after anthesis was extremely slow but during weeks 4–8 inclusive there was a very rapid increase in seed dry wt which was coincidental with high lipid synthetic activity (Table 1). After this time both dry matter and lipid accumulation proceeded at a gradually decreasing rate until the seed attained maturity.

Table 1. Changes in fresh and dry wt and lipid content of developing rapeseed

Weeks after anthesis	Fr. wt (mg per seed)	Dry wt (mg per seed)	Lipid % DM
1	1.51	0.31	4.68
2	3.70	0.59	3.81
3	4.45	0.69	6.48
4	6.49	1.02	8.92
5	7.52	1.34	18.30
6	8.49	2.56	41.22
7	8.50	3.30	44.89
8	9.17	4.45	48.37
9	9.19	4.99	48.48
10	9.04	5.12	46.09
Mature seed	6.07	5.72	43.91

Owing to the small size of the seed, the earliest sampling date which provided a sufficient quantity of material for an accurate and comprehensive lipid analysis was 3 weeks after anthesis (Table 2). At this time storage lipid deposition (oil or neutral lipid) had barely begun and the majority of the seed lipid was metabolic. Lipid only accounted for *ca* 6.5% of the dry matter of the seed at this stage of development. With time there was a very rapid increase in the lipid of the seed and the majority of this was neutral lipid consisting mainly of TAGs. The pattern of storage lipid accumulation was also reflected in the gross FA composition of the lipid.

Seven TAG molecular species were identifiable in the mature seed and at most stages of seed development from

Table 2. Changes in lipid classes in developing rapeseed

Weeks after anthesis	Neutral lipid*	PC	PE	DGG	DDG	Acidic lipid
	(nmol/seed)					
3	11.7	10.4	4.6	—	7.6	24.6
4	57.8	6.4	0.7	3.5	4.5	27.3
5	209.6	23.4	0.4	8.2	13.8	23.0
6	1038.4	44.3	14.2	16.3	22.4	47.5
10	2439.8	24.0	19.1	21.2	32.7	109.4

PC = Phosphatidylcholine; PE = phosphatidylethanolamine; DGG = diacylgalactosylglycerol; DDG = diacyldigalactosylglycerol.
*Includes TAGs and diacylglycerols in addition to other neutral lipids.

week 3 onwards (Table 3). The individual TAG species present in the seed at each sampling date were classified according to the number of double bonds based on the molar proportions of the component saturated, monoenoic, dienoic and trienoic FAs (Fig. 1). The proportions of the individual TAG molecular species and the FA composition of these changed considerably throughout development. At maturity, the major TAG molecular species (TAGs 1, 3 and 5 with 5, 4 and 3 double bonds, respectively) accounted for more than 75% of the TAGs whilst of the minor molecular species (TAGs 2,4,6 and 7 with 4,3,2 and 2 double bonds, respectively) TAG 7 accounted for only 1% of the TAGs. At the earliest sampling date when the mass of neutral lipid was small, 30% of the TAGs was species 7, with TAGs 1,3 and 5 making a total of 60%.

The molecular species with identical mobilities on argentation TLC plates often exhibited considerable variation in FA composition (Figs 1 and 2). This was most pronounced for the minor components (TAGs 2,4,6 and 7) but even the major species showed some differences. These variations were more marked in the early stages of oil deposition. As the seed approached maturity, compositional variations were reduced. The most important FA compositional changes of all TAG molecular species with time were the increase in the preponderance of 22:1(13) and to a lesser extent 20:1(11) with a corresponding reduction in 18:1(9).

Stereospecific analyses of the TAG molecular species at maturity revealed that the FAs occupying position 2 of the TAGs were 18:1(9) (TAGs 5 and 6), 18:2(9, 12) (TAGs 2, 3 and 4) and 18:3(9, 12, 15) (TAG 1) (Table 4). Even in TAG 5 22:1 (13) and 20:1(11) did not occupy position 2 of the TAGs although these two FAs accounted for between 60 and 65% of the FAs in the TAGs. Only TAGs 4 and 6 contained appreciable amounts of the saturated FAs 16:0 and 18:0. These FAs occurred to the same extent in positions 1 and 3 of the respective TAGs.

DISCUSSION

The pattern of oil or TAG and other storage reserves deposition in developing oilseeds is well known [1]. In oilseed rape (Norton, G. and Harris, J. F., unpublished results) and other oilseeds it has been found that the embryo has to achieve a certain developmental stage, namely completion of cell division and organ formation, before storage reserve deposition commences. In this early stage of seed development, which in rape is completed *ca* 2 weeks after anthesis, the lipid content of the seed is low and consists mainly of polar or metabolically active lipid. Only a small amount of neutral lipid is found and its composition is unlike the TAGs found in the mature seed. The embryos in the early stages of development are green, and the FA composition and lipid spectrum resemble those of green tissue [3]. Once the first stage of embryo development is completed, storage reserve deposition in the form of oil and protein and the concomitant expan-

Table 3. Molecular species of triacylglycerols (TAGs) in cotyledons of developing rapeseed

TAGs		Weeks after flowering					
Molecular species		3	4	5	6	8	10
No.	Designation	(mol per 100 mol)					
1	M_2T	13.0	20.5	12.0	16.3	25.1	26.8
2	M_2D	—	5.8	8.0	3.5	—	6.7
3	M_2D	24.8	36.2	32.6	20.7	39.1	29.7
4	$(M/S)_2D$	—	—	8.9	11.3	—	9.8
5	M_3	22.4	22.5	21.8	30.1	27.3	21.5
6	$(M/S)_2M$	9.0	10.6	12.3	7.1	7.5	4.5
7	M_2S	30.8	4.6	4.4	1.0	1.0	1.0

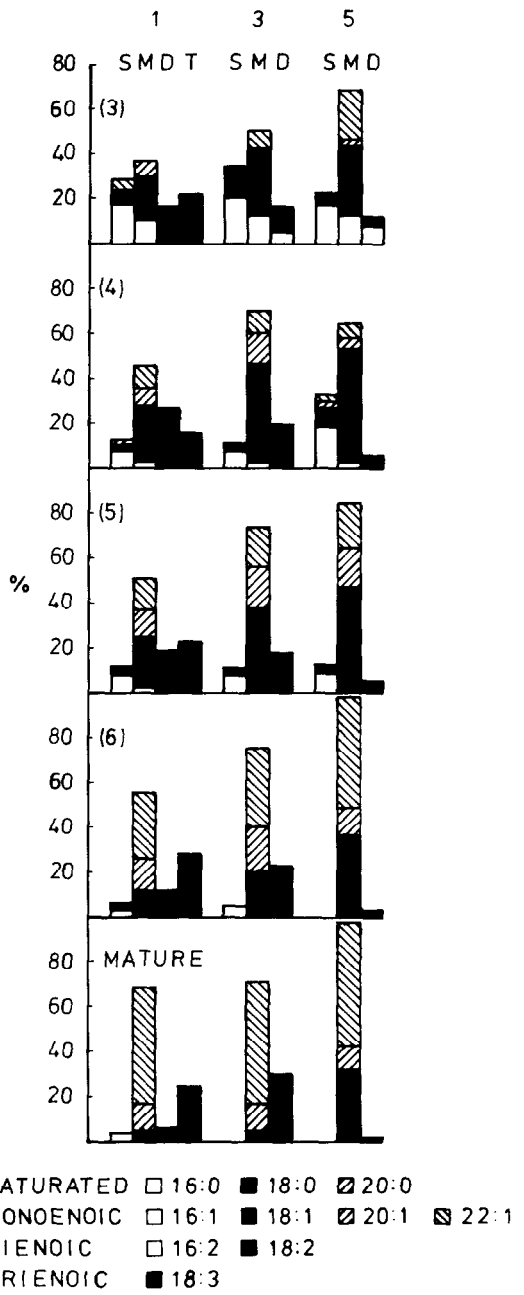


Fig. 1. Fatty acid (FA) composition of the major triacylglycerols (TAGs) in rapeseed. Data expressed as mol FA per 100 mol FA in the TAGs. Each TAG molecular species is identified by the number at the top of the figure (see Table 3 for further details). Seeds were harvested at 3,4,5 and 6 weeks after anthesis (figures in parentheses) and at maturity.

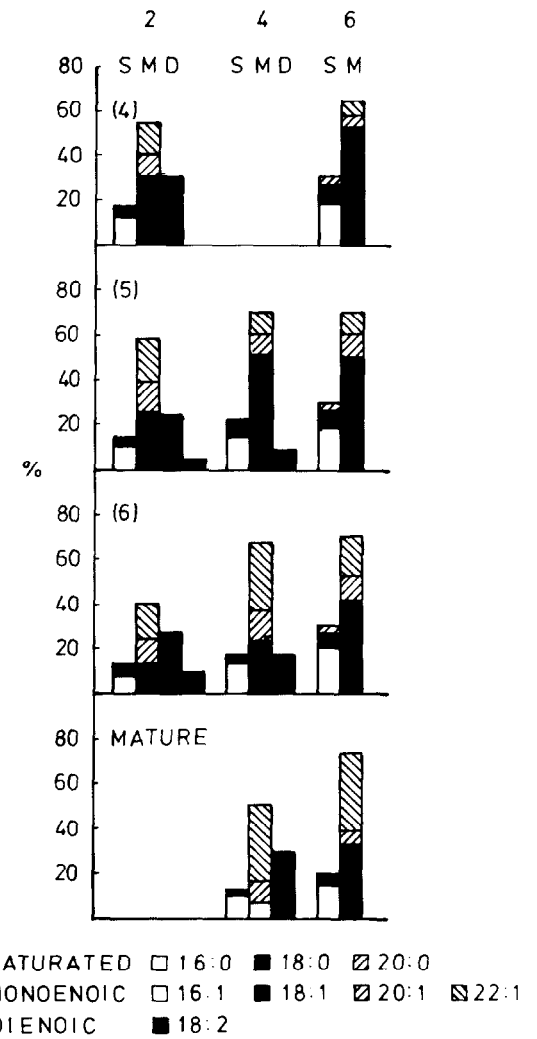


Fig. 2. Fatty acid composition of the minor triacylglycerols in rapeseed. Data expressed as mol FA per 100 mol FA in the TAGs. Each TAG molecular species is identified by the number at the top of the figure (see Table 3 for further details). Seeds were harvested at 4,5 and 6 weeks after anthesis (figures in parentheses) and at maturity.

sion of the cells commences and proceeds very rapidly. The onset of oil deposition (TAG) in this variety of oilseed rape is indicated by the appearance of erucic acid 22:1(13) in the lipid. This acid is the major fatty acid component found largely in the storage TAGs although certain polar lipids in rapeseed have been found to contain trace amounts. 22:1(13) was found to be present in all TAGs with the possible exception of TAG 1, which showed considerable variation in fatty acid composition at all developmental stages of the seed after the onset of oil formation. With time, the proportion of 22:1(13), and to a lesser extent 20:1(11), in the individual TAG species increased to a maximum at week 8. Conversely, the proportion of 18:1(9) decreased to a minimum over the same time period and thereafter remained constant. These changes together with the decrease in the relative content of 16:0 and 18:0 in the TAGs but particularly the major species (TAGs 1,3 and 5) constitute the main compositional changes occurring with time. Thus, the FA composition of the TAG molecular species with identical degrees of unsaturation in immature and mature seeds appears to be quite distinct. Throughout seed development there was a gradual change in the FA composition of certain TAGs to accommodate the increasing 22:1(13) content. With all the molecular species identified, this involved a massive *de novo* synthesis of lipid since such

Table 4. Positional fatty acid composition of the principal molecular species of triacylglycerols in mature seeds of oilseed rape (mol per 100 mol)

Species	Position	16:0	18:0	18:1(9)	18:2(9, 12)	18:3(9, 12, 15)	20:1(11)	22:1(13)	Designation
	1	1.6	1.8	0	0.8	0.2	4.8	22.2	M
1	2	0.7	0.6	1.1	3.9	26.9	0	0	T
	3	1.2	0	0.4	0	0	4.3	28.4	M
	1	0.4	0.1	2.2	0.6	0	6.2	23.5	M
3	2	0	0	2.3	29.7	0	0	0	D
	3	1.2	0.1	0.2	0	0.1	5.4	29.1	M
	1	4.5	1.5	2.2	0.4	0	4.6	19.4	M/S
4	2	0.4	0.3	3.8	28.5	0	0	0	D
	3	4.3	1.6	0.4	0	0	3.9	20.7	M/S
	1	1.1	0.3	2.9	0	0	6.3	22.7	M
5	2	0.4	0	30.0	2.0	0	0	0	M
	3	0.1	0	0.3	0	0	5.5	29.1	M
	1	6.1	2.2	1.9	0	0	3.9	18.2	M/S
6	2	1.0	0.7	29.8	0	0	0.4	0	M
	3	7.2	2.5	0	0.1	0	2.4	15.5	M/S

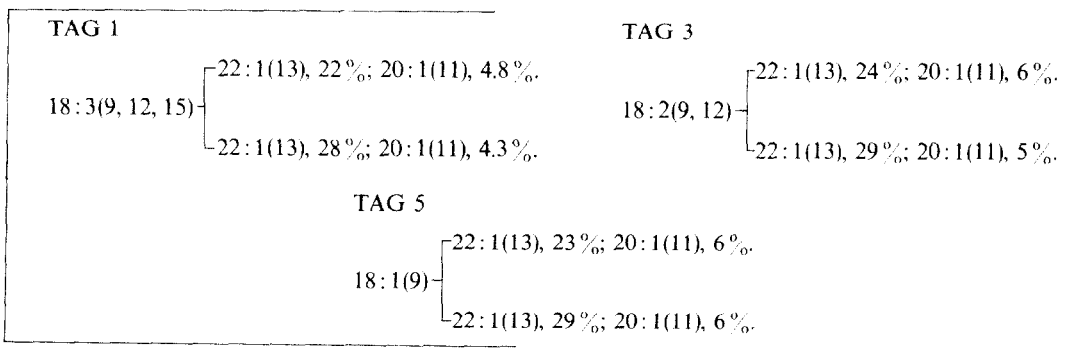
changes could not be explained by the modification of existing TAGs by the replacement of 18:1(9) with 22:1(13) and 20:1(11). It would appear unlikely, therefore, that the TAG molecular species from seeds in the early stages of development and mature seeds, albeit with the same degree of unsaturation and mobility on Ag-TLC plates, can be regarded as being the same species. For the purpose of this discussion it is for convenience only that such TAGs are given the same designation.

The gradual changes in the FA composition of the individual TAG species may be explained as follows. With the onset of storage reserve production, the synthesis of new TAG species containing 22:1(13) and lesser amounts of 20:1(11) is initiated. The FA composition of both the major and minor TAGs is completely different from the extremely small amounts of TAGs with the same designation in the pre-deposition stage. As the rapid synthesis of oil proceeds, the composition of the individual TAG species gradually approaches that of the mature seed. The contribution of the individual TAG species present prior to the deposition of oil to the FA composition of the TAGs with the same designation in the mature seed is small. It must be assumed that the composition of the individual TAG species in the mature seed must approximate that of the seven TAGs whose synthesis is initiated at the onset of storage oil deposition. Such conclusions are in agreement with the earlier work on soyabean TAGs [5].

The contributions of the seven molecular species to the total TAGs were found to vary with time. Similar observations have been made on the TAGs of developing soyabean seeds [5, 6]. TAG 7, the principal species in the very young embryos, only accounted for 1% of the total TAGs in the mature seed. There were also changes in the proportion as well as absolute amounts of other TAGs. TAG 1 accumulated most rapidly in the later stages of reserve oil deposition while the proportion, but not the absolute amount, of TAG 6 decreased over the same period. The reasons for such changes are obscure but the mechanism involved is important. TAG 1 is the most unsaturated and least desirable TAG from a technological viewpoint in rapeseed. Large amounts of a highly unsaturated TAG (D_2T) accumulated in the later stages of soyabean seed development [6]. No 18:3(9, 12, 15) was found in the TAGs of mature safflower seeds [7].

The synthesis of these highly unsaturated TAGs occurs by different routes in rape and soyabean. TAG 1 (M_2T) formation in rapeseed can be accounted for by *de novo* synthesis whereas D_2T formation in soyabean was explained by the large-scale modification and turnover of pre-existing TAGs. The appearance of high amounts of 18:3(9, 12, 15) containing TAGs late on in seed development must also raise speculation as to the origin of this FA and the biosynthesis of the TAGs. Recent work on FA and TAG biosynthesis in oilseeds has been reviewed [1].

The three major TAG molecular species found in mature rapeseed were very similar in terms of fatty acid composition and distribution to those in seeds of *Crambé abyssinica* [4]. In both seed types these three TAGs accounted for over 75% of the total TAGs. The distribution of FAs within the major rapeseed TAGs, and which closely resembled their counterparts in *Crambé*, is presented below:



Four minor species were identified in rapeseed at various stages of seed development whereas only two were reported to be present in mature seeds of *Crambé*. Of the minor TAGs found in mature rapeseed only two were subjected to stereospecific analysis. TAGs 4 and 6 were of similar composition to the respective minor species in *Crambé*. In rapeseed, however, 16:0 and 18:0 were found to be equally distributed between positions 1 and 3 of the TAGs whereas in *Crambé* the saturated FAs were reported to be restricted entirely to position 1 of the TAGs. 20:0 and 22:0 were absent from rapeseed TAGs whereas they were present in *Crambé*.

TAG 4

18:2(9, 12) — 22:1(13), 19%; 20:1(11), 5%; 16:0, 6%. 18:0
18:2(9, 12) — 22:1(13), 21%; 20:1(11), 4%; 16:0, 6%. 18:0

TAG 6

18:1(9) — 22:1(13), 18%; 20:1(11), 4%; 16:0, 8%. 18:0
18:1(9) — 22:1(13), 16%; 20:1(11), 2%; 16:0, 10%. 18:0

The stereospecific analysis revealed that position 2 of the TAGs from rapeseed and *Crambé* was absolutely specific for 18:1(9), 18:2(9, 12) and 18:3(9, 12, 15). It is also remarkable that the TAGs from these diverse species should be so similar particularly when the improvements introduced into the rapeseed variety are taken into consideration.

EXPERIMENTAL

Oilseed rape (*B. napus* L. cv Panter) was grown on the University Farms, School of Agriculture, Sutton Bonington using standard cultural procedures. Seeds for analysis were taken at weekly intervals from shortly after anthesis until maturity.

Lipid extraction. Seeds at all stages of development were killed in boiling *iso*-PrOH and extracted twice by homogenizing in $CHCl_3$–MeOH (2:1). All extracts were combined, evapd to dryness under red. pres. and the residue was dissolved in $CHCl_3$–MeOH prior to carrying out a Folch wash [9]. The $CHCl_3$ phase obtained was evapd to small vol. under red. pres. and finally dried under pure N_2 or Ar. The lipids were redissolved in $CHCl_3$. This soln constituted the crude lipid extract.

Lipid analysis. The crude lipid extract was fractionated into neutral lipids, phosphatidylcholine, phosphatidylethanolamine, diacylgalactosylglycerol, diacyldigalactosylglycerol and acidic lipids by chromatography on DEAE-cellulose [10]. The purity of each fraction was checked by means of TLC. The individual lipids in each fraction were estimated by the hydroxamate procedure [11].

TAGs for molecular species analysis were obtained from the crude lipid extract by means of chromatography on silica gel (Davison). The pure TAGs were subjected to prep. TLC on silica gel G containing 10% $AgNO_3$ (w/w) using 1.5% MeOH in $CHCl_3$ as solvent. The plate was sprayed with 0.1% 2,7-dichlorofluorescein in MeOH. Areas containing TAGs visualized under UV light were scraped off and the lipid was eluted in $CHCl_3$–MeOH (2:1). FA methyl esters of the original lipid classes and TAG molecular species were prepared according to the procedure of ref. [12] and analysed by GC.

GC was carried out on 2 m × 2 mm columns packed with 3% SP2310 and 2% SP2300 using Ar as carrier gas (20 ml/min). Column temps. were 190° initially for 2 min followed by an increase to 220° at 2°/min. Detection was by FID. Quantitative analyses were performed using methyl heptadecanoate as int. standard. Identity of each FA was confirmed by co-chromatography with authentic standards. Further verification was achieved using columns containing 10% PEGA and 10% SP2330. Stereospecific analysis of the TAGs from mature seed was performed by a modified Brockerhoff procedure, and positional fatty acid analysis of the TAGs was determined using pancreatic lipase [13].

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