Studies on changes in lipid profiles of new varieties of rape during seed maturation

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1. INTRODUCTION

Oilseed crops are considered as strategic sources for edible oils and accordingly, extensive work has been carried out on oilseed composition particularly during maturation (1-25). Rapeseed oil crop has also received the interest of many investigators (25-31) during seed development. In addition, the influence of temperature during seed development on fatty acid composition of oilseed rape was studied in one low-linolenic acid and one conventional cultivar (26).

Newly developed varieties of rapeseed (Brassica napus) were locally cultivated in Experimental Agricultural Station, National Research Centre, however chemical constitution of their lipids, have not been hitherto determined. Thus, the main objective of this work was to follow compositional changes of triacylglycerol, fatty acids, sterols, sterol esters, sterylglycosides and tocopherol in these new varieties.

It was also planned to interpret changes in lipids components in maturing seeds, in the frame of the probable lipid conversion reactions that may occur during maturation process. High-efficiency HPLC and GLC methods of analysis are also adopted to help detect the different lipid components even they are in minute quantities.

2. MATERIALS AND METHODS

2.1. Materials

Three varieties of rapeseeds (Brassica napus) were cultivated in the Experimental Agricultural Station of the National Research Centre of Egypt (season 1997-1998).

These varieties, namely, semu DNK 65/84, 53 and semu DNK 201/85 are designated VA, VB and VC respectively and they are genetically different. Seeds were cultivated at 15-11-1997 and the seeds were collected at 65, 80 and 95 days after flowering (DAF).

2.2. Methods

A representative sample of seeds (100g) was extracted with chloroform-methanol (2:1 by volume) in a warning Blender. The extract was dried over anhydrous sodium sulphate and the solvent was...
removed from the filtrate using a rotary evaporator under reduced pressure at 40°C.

The lipid patterns, namely, fatty acids (FA), triacylglycerol (TAG), tocopherols (T), whole oil sterols and sterol lipids, namely, free sterols (FS), acylated sterols (AS), free sterylglycosides (FSG) and acylated sterylglycosides (ASG) were determined. All HPLC and GLC data recorded in the tables were the mean of two concordant replicates. In addition the methods were calibrated using authentic compounds to obtain higher accuracy.

A. Fatty Acid Pattern

The oil was converted into methyl esters, via transesterification, with 5% methanolic hydrogen chloride (18, 32). Transesterification reaction was monitored with the help of TLC using silica gel G plates and n-hexane:diethyl ether:acetic acid (80:20:1 by Volume) as a developing solvent. Hewlett Packard-HP 5890-A gas chromatograph was employed for the analysis of the mixed methyl esters under the following operating conditions: column, DB-23 (0.32 mm x 30 m); temperature programming, 150-230°C, 3°C/min; injector, 230°C; detector, FID at 240°C; carrier gas, Helium at flow rate of 1.3 ml/min. and split ratio, 100:1. Calibration was made using standard fatty acid methyl esters. The results were recorded by an electronic integrator as peak area per cent.

B. Triacylglycerol Profile

HPLC instrument (Toyo-Soda-CCPM) was employed for the determination of triacylglycerol (TAG) profiles of the rapeseed oils. A 10 µl solution of oil in chloroform (300 mg/ml) was injected onto the column, ODS capcel Pak, C18 (4.4 x 100 mm). Gradient elution with acetonitrile : dichloromethane (starting from 90:10 to 35:65 v/v) in 150 minute was conducted. FID detector (with moving band, Tracer 945) was attached to the instrument µl. The carbon number assignment for the separated peaks was determined using HPLC chromatogram of soybean oil taken as reference containing 29 TAG starting with trilinolein and terminating with tristearin (33,34). The elution sequence was the same as that reported by El-Hamdy and Perkins (35).

C. Tocopherol Pattern

Direct determination of tocopherols (T) in oil was accomplished using Toyo-Soda-CCPm HPLC instrument. An oil sample of 10 gram was solubilized in n-hexane to make 10% solution and 10 µl was injected onto the silica column (YMC-A-012, 6.0 x 150 mm). Isocratic elution was conducted using n-hexane: isopropyl alcohol (100: 0.5, by volume) as mobile phase, at a flow rate of 1-2 ml/min. Hitachi-650-10S fluorescence detector was used. Spectral absorption was set at excitation and emission wave lengths of 295 and 325 nm respectively. The conditions were optimized to elute delta-T after 10 minutes. The results were automatically recorder as peak area percentages by electronic integrator. From the peak area and the corresponding weight of each individual T in the standard mixture, the weight of each individual T in the oil (ppm) can be calculated (18,33).

D. Whole Sterol Profile

The prepared unsaponifiable portion of the oil (18,36), was subjected to preparative TLC on silica gel G plates (0.5 mm thickness) using chloroform/ diethylether/acetic acid (95/4/1 by volume) as developing solvent. The sterol zone was located with the help of standard beta-sitosterol applied alongside the sample prior to development. The scrapped zone was thoroughly extracted with diethyl ether and the solvent was distilled off from the filtered solution. Hewlett Packard-HP 5890A gas chromatograph, was employed for analysis using the following operating conditions: column DB-17 (0.32 mm x 15 m, 0.25 µm coating) at 250°C; detector, FID at 260°C; injection 250°C; carrier gas, Helium (8,6 ml/min) and split ratio, 35:1. Standard sterols mixture containing known weights of available standard sterols, was used for identification and quantitation.

E. Sterol Patterns of Free and Acylated Sterols

The subsequent free sterol (FS) and acylated sterol (AS) isolations, their derivatization into 9-nthroylnitriles (S-9-AN) and HPLC determination, were followed according to El-Mallah et al. (33,34). FS and AS were isolated from the oil by preparative TLC using n-hexane / diethylether / formic acid (70/30/1 v/v/v) as developing solvent. The FS (Rf = 0.16) and AS (Rf =0.63) were scrapped off and extracted with chloroform. Only AS were deacylated via mild alkaline hydrolysis (0.5 N KOH in isopropyl alcohol). The obtained FS as well as the original FS were separately derivatized into their sterol S-9-AN derivatives according to the same method mentioned above.

HPLC of S-9-AN was conducted under the following conditions: reversed phase column, ODS-1250Y, Senshu Pak (4.6 x 250 mm); detector, fluorescence; excitation and emission wave lengths set at 360 and 460 nm; isocratic elution using acetonitrile / dichloromethane (75/25, v/v) at flow rate 1 ml/min.
F. Sterylglycoside Profile of Free and Acylated Sterols

The procedure of separation from the oil, derivatization into sterlylglycoside 1-anthroylnitrile (SG-1-AN) and HPLC analysis were carried out according to El-Mallah et al. (33,34).

The free sterylglycoside (FSG) and the acylated sterlylglycoside (ASG) were separated from the oil by injecting the oil solution into silica gel cartridge (Sep-Pak, Waters) and were eluted with chloroform: methanol (1:1 v/v). The mixed compounds were subsequently fractionated into pure FSG and ASG with the help of preparative TLC. Chloroform / methanol / formic acid mixture (90/15/1, v/v/v) was used as developing solvent. Only ASG was subjected to alkaline hydrolysis (0.5N KOH in isopropanol) to obtain FSG. The original and obtained FSG were derivatized separately, into SG-1-AN according to the same method mentioned above.

HPLC instrument, equipped with UV detector (Toso, UV 8000) and ODS Wakosil-5,C 18 (6.4 mm x 250 mm was used for SG-1-AN analysis. Gradient elution with acetonitrile/dichloromethane (from 50/50 to 68/32 v/v) was used. Absorption was measured at 254 nm. It is noteworthy to mention that this method is sensitive to 0.5 nanogram of SG.

3. RESULTS AND DISCUSSIONS

3.1. Fatty Acid Pattern

The changes in fatty acid profiles in maturing rapeseed, belonging to the three varieties are shown in Table I. Generally the unsaturated fatty acids showed marked variation during maturation process. Thus, oleic acid isomer (n-9) was increasing, however linoleic and linolenic acids were decreasing as maturation proceeded.

Palmitic and stearic acids were the main saturated fatty acid constituents. It can be noticed that palmitic acid was decreasing by maturation whereas stearic acid decreased at mid maturation and then it was kept constant at full maturation stage in VA and VB whereas it slightly decreased in VC. Other fatty acids, namely, C22:0 and C24:1 were detected in reasonable amounts at the different maturation stages. On the other hand, C22:5, C22:1, C24:0 and C24:1 fatty acids were detected in minor amounts at the different stages of maturation.

It is suggested that palmitic acid as well as linoleic and linolenic acids can be converted into oleic acid via enzymatic transformations including addition of C2 units (37,38,39).

3.2. Triacylglycerol Pattern

From the data recorded in Tables II, III and IV it can be seen that the TAG species, as determined by HPLC, showed marked variations during maturation of the different varieties of rapeseed.

From the data recorded in Table I it can be seen that the major TAGs of the first variety (VA) were XLO, LLO, XOO, LOO, LOP, OOO and OOP where other TAGs were present in comparatively lower amounts, namely, XLL, XXO, LLL, XLP, LLP, XLS, LOS and OOS. Also, it was observed that XOO, LOO and OOO exhibit an increase in quantity as maturation proceeded, whereas XLO, LLO and LOP showed a decrease as maturation proceeded. LOS and OOP exhibited their minima, however OOS showed its maximum value at mid-maturation stage.

In the second variety (VB), LLO, XOO, LOO and OOO generally increased as maturation proceeded.

### Table I

<table>
<thead>
<tr>
<th>Days after flowering (DAF)</th>
<th>Content (%)</th>
<th>Fatty acid composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>16:1</td>
</tr>
<tr>
<td>VA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>98.7</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>98.9</td>
</tr>
<tr>
<td>VB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>98.9</td>
</tr>
<tr>
<td>VC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>98.8</td>
</tr>
</tbody>
</table>
On the other side, XLO and LOP decreased as maturation was progressing. On the other side OOP decreased and then it was kept nearly constant at mid (80 DAF) and full maturation stage (95 DAF).

Concerning changes in the third variety (VC), XOO, LOO and OOO showed an increase in their amounts as maturation was progressing. XLO, LLO and LOP showed marked decrease only at mid maturation stage (80 DAF) and then it was nearly kept constant at full maturation stage (95 DAF).

Generally, there are certain TAGs, namely, XOO, LOO and OOO that showed marked increase during seed maturation in the three varieties of rapeseed. On the contrary, other TAGs showed general decrease, as maturation was progressing, such as XLO and LOP.

### 3.3. Tocopherol Patterns

Changes in the total amounts of tocopherols and the tocopherol composition are illustrated in Table V. Total tocopherols was markedly increasing during maturation stages in the three varieties however, VB showed a comparatively higher tocopherol content at different maturation stages than the other two varieties. Concerning the changes in the composition of tocopherol patterns, alpha-tocopherol was gradually decreasing as maturation proceeded while gamma-tocopherol was gradually increasing at mid-maturation stage whereas it markedly increased at full maturation stage.

Meanwhile, beta-tocopherol was detected in small amounts but it disappeared at full maturation.
stage in VA and VC varieties. Whereas in VB variety, it was detected only at early maturation stage (65 DAF). On the other side, delta-tocopherol appeared only as minor component at full maturation stage in both VB and VC varieties.

It is clear that tocopherols acquire the oil more oxidative stability particularly for those oils enriched in linolenic acid (40). However, it was reported that there is no direct biochemical link between the synthesis of tocopherol and the storage lipids (41, 42).

3.4. Sterols Pattern

The characteristic sterol for *Brassica napus*, namely, brassicasterol showed some marked variation during maturation process in the three varieties (Table VI). Thus it showed a maximum value at mid maturation stage in VA and at full maturation stage in both VB and VC varieties.

Campesterol exhibited higher values at early stage of maturation in VB and VC and it decreased as maturation was progressing, where it reached its maximum value at full maturation stage in VA.

An unknown sterol, probably identified as lophenol, showed higher value at full maturation stage in VA and VB and at mid maturation stage in VC. Lophenol seems to be one of the intermediates in the sterol biosynthesis.
5-stigmasterol showed generally gradual decrease as maturation was progressing in VA whereas it showed no regular change in the other two varieties, VB and VC.

Beta-sitosterol showed a decrease during maturation in VA, whereas no marked variation in the same sterol was observed in VB. In VC, beta-sitosterol was nearly constant at early and mid-maturation stages but it decreased in quantity at full maturation stage.

Isofucosterol generally decreased as maturation proceeded in VB and VC, however it increased gradually in VA.

From the above mentioned results, it can be noticed that there are marked variations in the whole sterols patterns in the three varieties during maturation process and the varietal changes can also be presented as follows:

– Lower brassicasterol level in VA at full maturation and higher brassicasterol in both VB and VC. However VC exhibited comparatively lower brassicasterol at early maturation stage.
– Campesterol showed a maximum value only in VA but at full maturation stage.
– Isofucosterol reached its optimum value at early maturation stage only in VB.

– An unknown sterol was proved to be lophenol by mass spectral analysis (43) and it showed its maximum value at mid maturation stage only in VC.

### 3.5. Sterol Lipids

Sterol Lipids, included four sterol groups, glycosidic and non-glycosidic in the form of free and acylated forms, namely, free and acylated sterols and sterylglycosides.

### 3.6. Free and Acylated Sterols

In VA, isoheuosterol, brassicasterol, campe / stigmasterol and beta-sitosterol were detected in the free and acylated sterol fractions showing marked variation during maturation (Table VII). In free sterol (FS) fraction, isoheuosterol, was not detected at early stage of maturation and showed very slight variation during maturation, whereas brassicasterol and the unseperable pair of campe/stigmasterol increased as maturation proceeded. However, beta-sitosterol decreased as maturation proceeded showing an opposite behavior to brassicasterol and campe / stigmasterol.

#### Table VII

<table>
<thead>
<tr>
<th>Days after flowering (DAF)</th>
<th>State of sterol</th>
<th>Composition of FS and AS sterols (%) Content (mg/100g oil)</th>
<th>Isofuco-Sterol</th>
<th>Brassica-Sterol</th>
<th>β Unknown Sterol</th>
<th>Campe/stigma-Sterol</th>
<th>β-Sito-Sterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Free</td>
<td></td>
<td>510</td>
<td></td>
<td>13.5</td>
<td>29.1</td>
<td>57.4</td>
</tr>
<tr>
<td>80</td>
<td>Ester</td>
<td>320</td>
<td>4.0</td>
<td>5.6</td>
<td>36.6</td>
<td>29.5</td>
<td>53.8</td>
</tr>
<tr>
<td>95</td>
<td>Ester</td>
<td>300</td>
<td>5.1</td>
<td>6.7</td>
<td>34.9</td>
<td>31.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>
<pre><code>                                  |                 |                                                              |                |                |                  |                     |              |
</code></pre>
<p>| 65                        | Free            |                                                              | 350            | 6.0            | 6.3              | 35.5                | 50.0         |
| 80                        | Ester           | 330                                                          | 1.0            | 1.5            | 15.6             | 32.1                | 50.8         |
| 95                        | Ester           | 310                                                          |                | 6.6            | 36.6             | 34.0                | 46.0         |
|                 |                                                              |                |                |                  |                     |              |
| 65                        | Free            |                                                              | 330            | 5.7            | 6.6              | 36.2                | 51.5         |
| 80                        | Ester           | 280                                                          | 4.5            | 17.5           | —                | 38.8                | 52.0         |
| 95                        | Ester           | 310                                                          | 4.7            | 6.2            | 35.1             | 26.5                | 52.0         |
|                 |                                                              |                |                |                  |                     |              |
| 65                        | Free            |                                                              | 340            | 4.3            | 7.0              | 34.0                | 53.0         |
| 80                        | Ester           | 330                                                          | 4.7            | 6.2            | 35.1             | 26.5                | 52.0         |
| 95                        | Ester           | 370                                                          | 4.3            | 7.0            | 34.0             | 53.0                | 50.5         |</p>

FS: Free sterol; AS: Acylated sterol.

* Unknown sterol that may stand for cholesterol.
In acylated fraction (AS), isofucosterol and brassicasterol were detected in somewhat lower values whereas campe/stigmasterol and \( \beta \)-sitosterol were found in much higher levels. Isofucosterol, and brassicasterol were generally increased as maturation was progressing, whereas campe/stigmasterol and \( \beta \)-sitosterol were generally decreased by maturation.

In FS fraction of VB isofucosterol, brassicasterol and campe/stigmasterol showed gradual increase by maturation, however, \( \beta \)-sitosterol behaved in an opposite manner. On the other side, isofucosterol, and brassicasterol in the AS fraction were at their comparatively lower values at early maturation stage and they generally increased by maturation. Campe/stigmasterol and \( \beta \)-sitosterol were present at their higher levels at this stage and generally decreased as maturation was progressing.

In VC, the FS fraction contained no isofucosterol at early and mid maturation stage, however it appeared only at full maturation stage. Brassicasterol showed a very marked variation during maturation thus it increased as maturation was progressing. However campe/stigmasterol increased slightly at mid maturation and then it decreased to reach its minimum value at full maturation stage. On the other side, \( \beta \)-sitosterol behaved in an opposite manner to brassicasterol, thus it decreased as maturation was progressing.

In the FS fraction, isofucosterol showed no marked variation, however brassicasterol increased as maturation proceeded. Campe/stigmasterol decreased markedly at mid maturation stage and then slightly decreased at full maturation stage, whereas \( \beta \)-sitosterol reached its maximum value at mid-maturation stage (80 DAF).

Other unknown component of sterol, which may stand for cholesterol, appeared only as minor component in VA and VC at full maturation stage in the FS and AS fractions.

### 3.7. Free and Acylated Sterylglycoside

In VA isofucosterylglycoside, brassicasterylglycoside, campe/stigmasterylglycoside and \( \beta \)-sitosterylglycoside were detected in the free and acylated sterylglycoside fractions showing marked variation during maturation (Table VIII). In free sterylglycoside (FSG), isofucosterylglycoside (isofuco-SG) and avena-SG were detected only at full maturation stage, whereas brassica-SG and campe/stigma-SG showed marked variations during maturation, thus brassica-SG showed its maximum value at mid maturation stage then it decreased at full maturation stage.

<table>
<thead>
<tr>
<th>Days after flowering (DAF)</th>
<th>SG fraction</th>
<th>Content ppm</th>
<th>Composition of FSG and ASG sterols (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Avena-SG</td>
</tr>
<tr>
<td>VA</td>
<td>FSG</td>
<td>65</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>ASG</td>
<td>80</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASG</td>
<td>44</td>
</tr>
<tr>
<td>VB</td>
<td>FSG</td>
<td>65</td>
<td>980</td>
</tr>
<tr>
<td></td>
<td>ASG</td>
<td>80</td>
<td>2230</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASG</td>
<td>44</td>
</tr>
<tr>
<td>VC</td>
<td>FSG</td>
<td>65</td>
<td>950</td>
</tr>
<tr>
<td></td>
<td>ASG</td>
<td>80</td>
<td>2240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASG</td>
<td>22</td>
</tr>
</tbody>
</table>

SG: Sterylglycoside; FSG: Free sterylglycoside; ASG: Acylated sterylglycoside.
some unique variations were observed in one variety differences in compositional changes of some lipids, 4. CONCLUSION opposite manner. progressing whereas beta-sito-SG behaved in an stigma-SG decreased as maturation was increasing whereas isofuco-SG at early and mid maturation stages in VB. In addition, LSS was present in higher quantity in VA at the early maturation stage than in the other two varieties. Glycerides containing XOO, LOO and OOO showed gradual increase in the three varieties as maturation proceeds however glycerides containing linoleic and linolenic acid decrease. Therefore, it can be concluded that these changes may be due to biochemical conversions into oleic acid via enzymatic hydrogenation (37,38).

With reference to changes in tocopherol composition of the three varieties, marked variation in tocopherol constituents were observed. In the three varieties, namely, VA, VB and VC alpha-tocopherol decreased clearly by maturation. Beta-tocopherol decreased at mid-maturation stage in VA and VC and disappeared at full maturation stage; the tocopherol appeared at early maturation stage in VB. Gamma-tocopherol increased markedly in the three varieties, in an opposite manner with alpha-tocopherol, as maturation was progressing. On the other side, delta-tocopherol was detected in minor quantity only at full maturation stage in the three maturing seeds.

It can be concluded that alpha-tocopherol may be possibly converted into gamma-tocopherol during maturation via S-adenosyl methionine (SAM) responsible for removing one methyl group (38,44,45).

With reference to changes in sterols of the four sterol lipids, certain varietal changes occurred in the free and acylated sterols (FS and AS) of the three rapeseed varieties. Isofucosterol in FS fraction was not detected in early maturation stage in VA, however it appeared only at full maturation stage of the maturing VC. In acylated sterol fraction (AS) of VA, isofucosterol showed regular increase as maturation was progressing. An unknown sterol was detected in both FS and AS fractions only at full maturation stage in VA and VC varieties. However it did not appear during any maturation stage in VB. This sterol may stand for cholesterol. On the other hand, betasitosterol of the FS fraction in VB was significantly lower at full maturation stage compared to the other two varieties.

Concerning changes in sterols of acylated sterylglucosides (ASG), brassica-SG showed its rather than the others. These variations may be due to certain genetically differences.

Concerning the changes in certain TAG molecular species in VB, LLO increased as maturation was progressing, however it decreased generally in the other two varieties. On the other side, LOS and OOP in VA decreased at the mid maturation period then increased whereas they decreased as maturation was progressing in the other two varieties. POP decreased as maturation was proceeding in the three varieties whereas it was present in larger quantities at the early and mid maturation stages in VB. In addition, LSS was present in higher quantity in VA at the early maturation stage than in the other two varieties. Glycerides containing XOO, LOO and OOO showed gradual increase in the three varieties as maturation proceeds however glycerides containing linoleic and linolenic acid decrease. Therefore, it can be concluded that these changes may be due to biochemical conversions into oleic acid via enzymatic hydrogenation (37,38).

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Concerning changes in sterols of acylated sterylglucosides (ASG), brassica-SG showed its
maximum value at full maturation in VA, however it reached a minimum value at full maturation stage in the other two varieties. Unlike the other two varieties, VB showed a maximum value of campe-SG/ stigma-SG in the ASG fraction at mid maturation stage, in VA and VC it generally decreased as maturation was proceeding.

Marked variation of sterols in maturing seeds manifested themselves only in the whole sterols profile of the three rape varieties. Accordingly, specific variations can be observed in each of the three varieties.

Generally, these characteristic varietal changes in certain lipid components in one variety may help, to some extent, to distinguish one variety from the other at any maturation stage. From the biochemical approaches mentioned above, it can be noticed that conversion-interconversion processes may occur at different rates in one variety than the other.

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