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Changes in the content of γ-linolenic C18 : 3 (n-6) and stearidonic C18 : 4 (n-3) acids in developing seeds of viper's bugloss *Echium vulgare* L.★

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Changes in the composition of fatty acids (FA) were determined in lipid extracts isolated from developing ovaries containing ovules and developing seeds of *Echium vulgare* L. The samples were collected successively over 20 days beginning with the first day after flowering. The contents of the n-6 FA family members, i.e., γ-linolenic (GLA) ($C_{18:3}$) and linoleic (LA) ($C_{18:2}$) acids changed in a parallel manner and reached the maximum of 13.9% and 24%, respectively, on the 12th day, after which they fell systematically down to 8.6% and 18.2%, respectively, on the 20th day after flowering. Starting with day 13, the content of α-linolenic acid (ALA) ($C_{18:3}$ n-3) begins to grow intensively, from 24.2% to 39.3% on the 20th day after flowering. The increase in the content of stearidonic acid (SDA) ($C_{18:4}$ n-3), up to 10.5% on the 20th day after flowering, occurred steadily as the seeds developed, and was independent of the changes in the content of GLA and LA. The pattern of changes in the content of SDA, GLA, LA and ALA during the development of seeds, and the occurrence of SDA in the seed oil of other plants, demonstrate that the biosynthesis of SDA in the seeds is critically dependent on the presence of ALA. The above condition indicates that SDA biosynthesis in the seeds of *Echium vulgare* follows the scheme LA → simultaneous, competitive, action of Δ^6 and Δ^{15} desaturases, leading to the formation of GLA and ALA, respectively, and then ALA (Δ^6 des) → SDA. The biosynthesis according to the scheme: GLA (Δ^{15} des) → SDA is highly unlikely.

Keywords: *Echium vulgare* L., *Boraginaceae,* viper's bugloss, seeds, lipids, octadecatetraenoic acid, stearidonic acid, γ-linolenic acid

INTRODUCTION

In contrast to common edible oils such as rapeseed or soybean, the seed oil of the *Echium* L. genus contains unique fatty acids (FA), that is γ-linolenic acid $C_{18:3}$ n-6 (GLA) and stearidonic acid $C_{18:4}$ n-3 (SDA). Exactly the same FA, GLA and SDA, are produced in the human body through desaturation (–2H) of appropriate precursors, that is of linoleic acid $C_{18:2}$ n-6 (LA) and α-linolenic acid $C_{18:3}$ n-3 (ALA), respectively. The concentrations of GLA and SDA in tissues of the human body are marginal, since both GLA and SDA, as intermediate products, are metabolized very quickly through the alternating reactions of elongation and desaturation into the very important, long chain polyunsaturated fatty acids (LC PUFA), such as dihomo-γ-linolenic acid $C_{20:3}$ (DGLA) and arachidonic acid $C_{20:4}$ (AA) of the n-6 family, and also of the n-3 family, i.e., eicosapentaenoic acid $C_{20:5}$ (EPA) and docosahexaenoic acid $C_{20:6}$ (DHA).

★This paper is dedicated to Professor Tadeusz Chojnacki from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw on the occasion of the 50th anniversary of his scientific activity and 75th birthday.

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Abbreviations: ALA, linolenic acid $C_{18:3}$ (n-3); AA, arachidonic acid $C_{20:4}$ (n-6); DHA, dokosahexaenoic acid $C_{22:6}$ (n-3); EPA, eikosapentaenoic acid $C_{20:5}$ (n-3); FA, fatty acids; FAME, fatty acid methyl esters; GLA, γ-linolenic acid $C_{18:3}$ (n-6); LC PUFA, long chain polyunsaturated fatty acids; SDA, stearidonic acid $C_{18:4}$ (n-3).

The critical stages limiting the endosynthesis of LC PUFA indispensable for normal functioning of the human body are the first transformations LA → GLA and ALA→ SDA, controlled by both the amount and the activity of the enzyme delta-6 desaturase (Δ^6 des) (Horrobin, 1993). When the amount or the activity of Δ^6 des decreases due to aging, alcoholism, or diabetes, the entire chain of transformations of LA and ALA, leading to the formation of LC PUFA, is suppressed. In such a case, the administration of increased doses of LA and ALA with the diet is pointless, since it cannot lead to a mitigation of the deficiency of LC PUFA. However, mitigation of LC PUFA deficiency is possible if the diet is supplemented with "ready" metabolites, i.e., GLA and SDA, present in some plant oils.

Within the plant world, large amounts of GLA are found mainly in the seed oil of various species of the following families: *Onagraceae*, *Boraginaceae*, *Saxifragaceae, Scrophulariaceae* and *Primulaceae* (Wolf *et al.*, 1987; Gunstone, 1992; Tsevegsuren & Aitzetzmuller, 1996; Velasco & Goffman, 1999; Sayanova *et al.*, 1999). Particularly important are plant seed oils in which more FA from the n-3 family are found, i.e. ALA and SDA, as compared to the n-6 family FA, i.e. LA and GLA. The number of reports concerning SDA is much smaller. To date, the main commercial source of SDA, described already in 1982–1984, has been the oil from the seeds of *Ribes nigrum* (Traitler *et al.*, 1984), which contains 3–4% SDA, 15–19% GLA and 12–14% ALA in its FA composition. Significant amounts of SDA are present in seed oil of the *Primulaceae* family, e.g. from *Dodecatheon meadia* L., in which SDA comprises 11.9% of FA composition together with 4.5% GLA and 19.9% ALA, or in the *Aleuritia* genus, e.g. *A. scotica* W.J. Hooker, the oil of which contains 22.5% SDA, 29.0% ALA, 2.2% GLA and 26.9% LA (Sayanova *et al.*, 1999).

The best recognized sources of SDA, where it is found next to GLA and ALA, are the oils from the seeds of the *Boraginaceae* family (Tsevegsuren & Aitzetzmuller, 1996; Velasco & Goffman, 1999), e.g.: of *Lithospermum arvense* L. (Velasco & Goffman, 1999) which contains up to 17.1% SDA, 5.1% GLA and 41.1% ALA within its FA. A particularly rich source of SDA, as well as of GLA and ALA, can be found in the seed oil of the *Echium* L. genus (Guil-Guerrero *et al.*, 2000; 2001) e.g., *E. plantagineum*, which contains about 12.9% SDA next to 9.1% GLA and 36.6% ALA. A similar FA composition: 12–14% SDA, 10.5% GLA and 39–40% ALA occurs in the seed oil of *E. vulgare* L., commonly found in Poland (Rostański, 1963; Zając & Zając, 2001).

The high contents of SDA, GLA and ALA in seed oil of the *Echium* genus prompts the question of how the biosynthesis of these unique FA takes place. The literature available on the subject is relatively scanty. In leaves of *Borago officinalis* the greatest accumulation of SDA was found in the mono- (MGDG) and digalactosylo-diacyloglycerols (DGDG) of the chloroplasts (Griffiths *et al.*, 1996). The authors suggest that, in *B. officinalis* leaves, SDA is synthesized in the chloroplasts through Δ^{15} desaturation of LA and, subsequently, by Δ^6 desaturation of ALA. In the available literature, however, there is a lack of information concerning the biosynthesis of SDA and GLA during the development of seeds of the *Echium* genus. Understanding these processes should be helpful in selection of proper harvesting period of *Echium* seeds from commercial plantations in order to ensure the highest yield and proportions of SDA/GLA/ALA in the seed oil.

The objective of this study was to monitor the process of biosynthesis of LA, GLA, ALA and SDA in the developing *E. vulgare* L. seeds, beginning with the 1st day until their maturation on the 20th day after flowering.

EXPERIMENTAL

Plant material. *Echium vulgare* is characterized by a paniculate inflorescence, composed of cymes, in which flowers open day by day from the rhachis of the inflorescence towards the upper parts of the cymes. Opening flowers were tagged daily, thus furnishing information on the age of the developing ovaries with ovules and then seeds. On the 20th day of tagging, the infructescence was cut and the seeds collected for analyses, from the most ripe, 20 day old ones, to the one day old ovaries with ovules within.

Isolation of lipids and preparation of fatty acid methyl esters for analysis. Each of the samples collected were freeze dried separately. After freeze drying the material was carefully crushed with a glass rod in a cone-shaped thick-walled glass vial. Double extraction of lipids was done using 2 × 1 ml of a mixture of chloroform and methanol 2 : 1 (v/v). The extracts were decanted to another cone-shaped glass vial and 0.2 volume of water, with respect to the volume of extract, was added. After vigorous shaking and separation of layers the bottom layer containing extracted lipids was separated and dried over anhydrous sodium sulphate. The solvents were evaporated under hood to dryness, in a stream of nitrogen. Then, 0.8 ml of 14% solution of BF_3 in methanol was added to the dry residue. The vial was closed tightly with a screw cap equipped with a Teflon gasket. Trans-methylation was performed in a boiling water bath for 25 min. After cooling, 3 ml of a saturated NaCl solution was added to the vial. FA methyl esters (FAME) were extracted twice using 2 × 2 ml of hexane, Lichrosolv grade (Merck Darmstadt).

The joint extracts were concentrated by evaporation of hexane in a nitrogen stream in a cone-shaped vial to approx. 0.2 ml in volume. The FA composition was determined using HP 6890 GC, split injection 50 : 1, Rtx 2330 column (Restek, Bellefonte, USA) of 105 m; 0.25 mm ID, d_f = 0.2 μm. The temperature program: 175°C/55 min, next 1.5°C/min, final temp. 210°C. The identification of the FA composition was done and the weight % determined using a FAME standard mixture with a certified % (w/w) composition: Mix 81 (Larodan, Sweden).

RESULTS AND DISCUSSION

Changes in the FA composition during the development and ripening of seeds

At particular stages of the development of ovaries with ovules and later of seeds the FA composition of extracted lipids undergoes significant changes both in quantity and quality. As it can be noticed from the comparison of chromatograms (Fig. 1A), showing FA composition in early stages of development of ovaries with ovules (3rd day), the short-chain and saturated FA are much more abundant than in ripe nutlets on 20th day after flowering (Fig. 1B).

The most notable changes in FA composition are: (*ripe nutlets in parentheses)* $C_{14:0}$ from 4.8% to (0.5%); $C_{16:0}$ from 21.4% to (6.0%); $C_{16:1}$ 9c from 2.6% to (0.3%); $C_{18:0}$ from 8.4% to (3.1%). Particularly characteristic, as noticed from Fig. 1B, is the presence of 1.1% of petroselinic acid $C_{18:1}$ 6c, in the ovary with ovules on the 3rd day after flowering. The presence of $C_{18:1}$ 6c may potentially indicate that in the developing nutlets the already active Δ^6 desaturase, also found in leaves, can cause partial Δ^6 desaturation (–2H) of stearic acid. In mature nutlets, such a process occurs at a very low rate, if at all.

As can be noticed in Fig. 2, the fraction of saturated FA, mainly $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$, decreases systematically from over 30% to about 9% of total FA as the nutlets mature, while the fraction of monoenic acids, among which oleic acid $C_{18:1}$ 9c is dominant, stabilizes at the level of 12% on the 10th day after flowering.

The fraction of the n-6 and n-3 FA families combined grows in a steady manner during the development and maturation of seeds. However, sig-

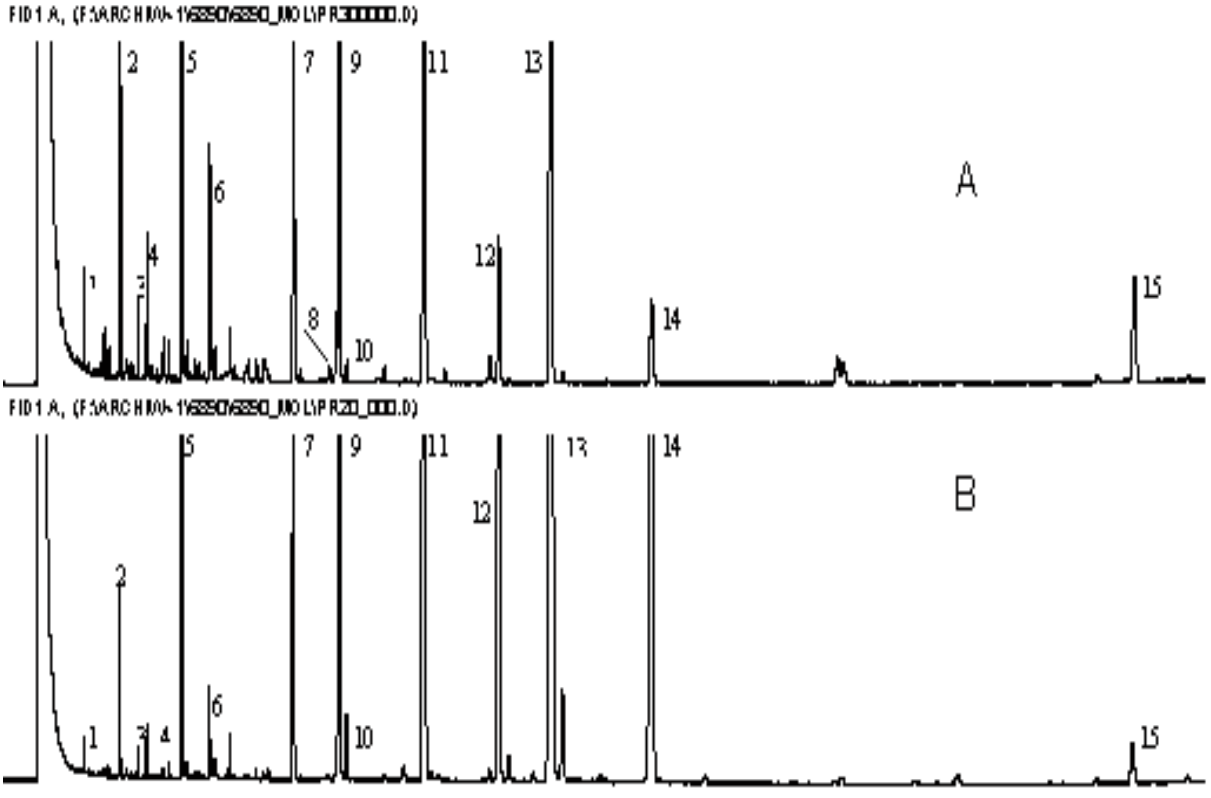

Figure 1. FA composition of lipids isolated from ovaries with ovules on the 3rd day (A) and from the seeds on the 20th day after flowering (B).
Identification: 1. $C_{12:0}$; 2. $C_{14:0}$; 3. $C_{14:1}$; 4. $C_{15:0}$; 5. $C_{16:0}$; 6. $C_{16:1}$ c 7. $C_{18:0}$; 8. $C_{18:1}$ 6c (petroselinic acid); 9. $C_{18:1}$ 9c; 10. $C_{18:1}$ 11c; 11. $C_{18:2}$ 9,12cc (LA); 12. $C_{18:3}$ 6,9,12 all *cis* (GLA); 13. $C_{18:3}$ 9,12,15 all *cis* (ALA); 14. $C_{18:4}$ 6,9,12,15 all *cis* (SDA); 15. erucic acid $C_{22:1}$.

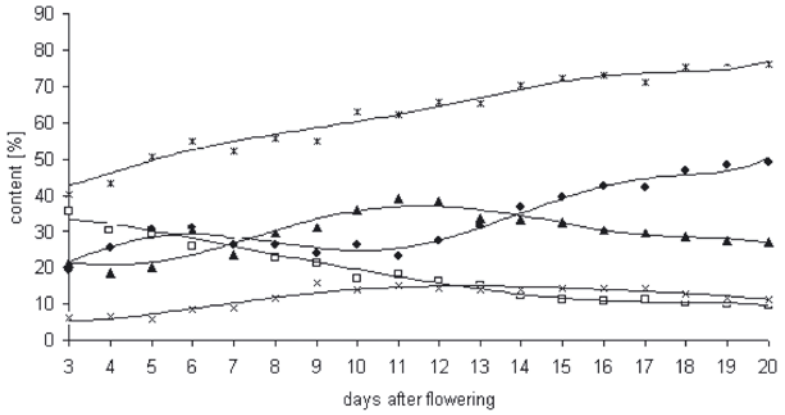

Figure 2. Changes in the content of saturated, monounstaurated, n-6 and n-3 FA in lipids isolated from developing seeds of *Echium vulgare* L.
Symbols: ♦, saturated FA; x, monounsaturated FA; ▲, total n-6 FA; ●, total n-3 FA; ∗, total (n-3) + (n-6) FA.

nificant differences were observed in the mutual proportions of FA from the n-6 and n-3 families. Starting with the 8th day after flowering, the total content of FA of the n-6 family increased steadily reaching a maximum of 38% on the 14th day, while the changes in the FA of the n-3 family were an exact mirror image of the former, with a minimum value (25%) on the 11th day after flowering. This was probably the result of the prevalence of Δ^6 desaturation, caused by a greater activity of the Δ^6 des enzyme, as compared to the lesser amount and/or activity of Δ^{15} des.

Starting with the 14th day after flowering, the rate of Δ^{15} desaturation prevails over Δ^6 desaturation, since a significant increase was observed in the rate of biosynthesis of FA from the n-3 family (up to 49.4%) — mainly of ALA (up to 39.3%) on the 20th day after flowering. At the same time, the content of FA from the n-6 family decreases significantly from 39.2% on the 11th day to 26.8% on the 20th day, after flowering.

The hypothetical route of SDA synthesis

On the basis of the literature concerning FA composition of lipids isolated from various plant sources, it can be concluded that SDA biosynthesis requires the simultaneous presence of both desaturases, Δ^{15} as well as Δ^6. This is because SDA is found in nature only when ALA is also present. For example: in *Ribes nigrum* (4% SDA, 12–14% ALA) (Traitler *et al.*, 1984), in *Myosotis latifolia* (7.9% SDA, 15.8% ALA) (Guil-Guerrero *et al.*, 2000) in the *Primulae* genus: *Dodecatheon meadia* L. (11.9% SDA, 19.9% ALA) (Sayanova *et al.*, 1999) in the *Aleuritia* genus: *A. scotica* W.J. Hooker (22.5% SDA, 29.0% ALA next to 2.2% GLA and 26.9% ALA), in *D. tetrandum* L. (12.2% SDA, 27.9%ALA) (Sayanova *et al.*, 1999). On the other hand, in the case of oils containing GLA $C_{18:3}$ (6, 9, 12, all *cis*) but not ALA $C_{18:3}$ (9, 12, 15, all *cis*), SDA does not occur. A characteristic example is the oil from the seeds of *Oenothera* L. species and hybrids (*Onagraceae*) (Mol *et al.*, 2001), where GLA is found in the FA composition at the 7–13% level, while the participation of ALA in the FA composition of this oil is marginal — below 0.3% and, what is very indicative, SDA is not found.

It is very symptomatic that at the same time, ALA is present in significant quantities, about 44.8%, in cuticular lipids isolated from leaves of *Oe. paradoxa* Hudziok. This would indicate that Δ^{15} des is found not in the seeds but in the leaves of this

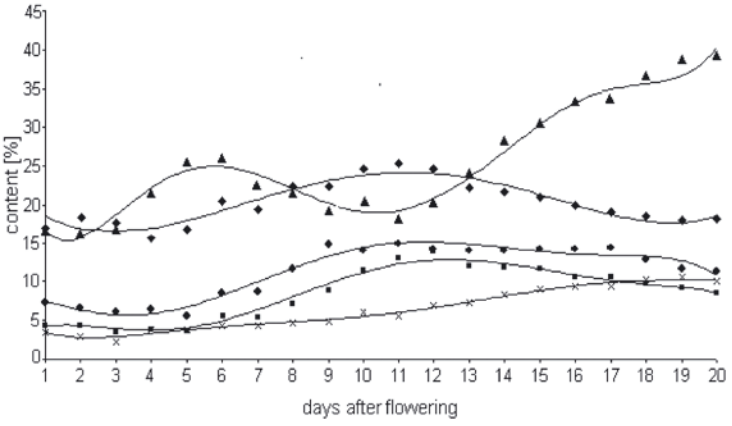

Figure 3. Changes in the content of: GLA, SDA, GLA + SDA, ALA and sum of monoenoic FA in developing seeds of *Echium vulgare* L.
Symbols: ♦, LA; ■, GLA; ✖, SDA; ▲, ALA; ●, sum of monoenoic FA.

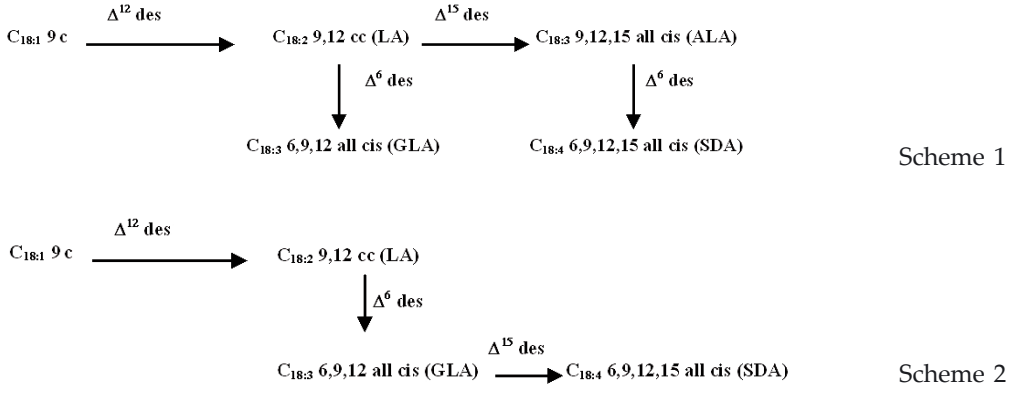

Figure 4. Likely (Scheme 1) and rather unlikely (Scheme 2) course of synthesis of SDA in developing seeds of *Echium vulgare* L.

plant only (Jankowski & Stolyhwo, 1995). The case of the differences in the composition of FA of lipids isolated from seeds and leaves of *Borago oficinalis* is also very distinctive. In the seed oil of *B. officinalis*, containing up to 23.1% GLA and 40.1% LA, the participation of ALA does not exceed 0.2%, and SDA is practically absent. However, in the FA composition of lipids isolated from leaves of *B. oficinalis*, SDA is found together with ALA at 23% and 43%, respectively (Griffiths *et al.*, 1996).

The above observations concerning the critical dependence of the presence of SDA on the presence of ALA are consistent with the observed changes in LA and GLA, and ALA and SDA in lipids isolated from the developing seeds of *Echium vulgare* L. (Fig. 3). The changes in the fraction of GLA are parallel to those of its precursor, LA, which may indicate that during the development of nutlets, the LA → GLA transformation, controlled by Δ^6 des, is dependent on the concentration of the precursor LA. On the other hand, the increase of SDA content, as shown in Fig. 3, does not depend on the increase of GLA concentration, since starting on the 11th day after flowering, the GLA concentration decreases as a result of the decreasing LA content, while the content of SDA grows systematically in this period.

The systematic increase of the SDA concentration in lipids from developing seeds corresponds with the increase in ALA content, which suggests that the probable path of SDA biosynthesis in seeds of *E. vulgare* can be as represented by Scheme 1 (Fig. 4).

The alternative course of biosynthesis, as shown in Scheme 2 (Fig. 4), is rather unlikely.

The probable course of SDA biosynthesis in the developing seeds of *E. vulgare*, as shown in Scheme 1, is consistent with earlier results (Griffiths *et al.*, 1996) according to which the biosynthesis of SDA in leaves of the *Boraginaceae* family occurs in chloroplasts through subsequent Δ^{15} and Δ^6 desaturation of LA esterified to galactolipids.

Activity of Δ^6 des is suppressed by the increase of activity of Δ^{15} des in the course of ripening of seeds

The plateau reached by Δ^6 unsaturated FA around the 12th day after flowering, as shown in Fig. 2 should also be commented upon. Suppression of the increase in the concentration of the sum of GLA + SDA can possibly be explained by depletion of the amount and/or activity of Δ^6 des in competition with the growing amount and/or activity of Δ^{15} des. This may be supported by the fact that, starting on the 13th day after flowering, the rate of Δ^{12} desaturation, connected with the formation of the intermediate LA is lower than that of Δ^{15} desaturation and the formation of ALA.

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