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Occurrence and characterization of oils rich in γ-linolenic acid Part I: *Echium* seeds from Macaronesia

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Abstract

Nineteen species of the genus *Echium* (Fam. Boraginaceae) collected in Macaronesia were surveyed in a search for new sources of γ-linolenic acid (GLA, 18:3ω6). High amounts of this acid were found in all of them, ranging from 9.15% (*E. plantagineum*) to 26.31% (*E. callithyrsum*) of total seed fatty acids. The amounts of GLA related to total seed weight were also significant, ranging from 1.77% (*E. sventenii*) to 5.02% (*E. nervosum*). In addition, considerable amounts of stearidonic acid (SA, 18:4ω3) were detected, ranging from 3.03% (*E. auberianum*) to 12.94% (*E. plantagineum*) of total fatty acids. These data allow us to consider the members of the genus *Echium* from Macaronesia as one of the richest sources of γ-linolenic acid found so far in nature. The results obtained from multivariable data analysis and the taxonomic relationships among the species is discussed.

Keywords: Boraginaceae; *Echium*; γ-linolenic acid; Macaronesia; Stearidonic acid; Fatty acid; Seed oil; Multivariable data analysis

1. Introduction

GLA is a metabolite of linoleic acid (LA, 18:2ω6) and the first intermediate in the conversion of LA to arachidonic acid (AA, 20:4ω6) (Gunstone, 1992). It is of increasing biomedical, dietary and cosmetical importance (Horrobin, 1992). Commercial sources are almost limited to seed oils from three plants: evening primrose (9.6% GLA of total fatty acids) (Hudson, 1984; Pina, Graille, Grignac, Lacombe, Quenot & Garnier, 1984), borage (23%) (Uzzan, 1986; Whipkey, Simon & Janick, 1998) and black currant (15–20%) (Traitler, Winter, Richli & Ingenbleek, 1984; Lercker, Cocchi & Turchetto, 1988) and some microbiological sources, as *Mucor javanicus* (15–18%) and *Spirulina platensis* (21%) (Ratledge, 1982; Kamisaka, Yokochi, Nakahara & Suzuki, 1990; Aggelis, Komaitis, Dimitroulias, Pina & Graille, 1991a, 1991b). GLA appears in a wide variety of plants, fungi and microorganisms. In plants, only 259 out of 147,000 species belonging to ten families have been described for seed oil composition (Ucciani, 1995). Significant amounts of GLA have been found in the plant families Onagraceae, Saxifragaceae and Scrophulariaceae, but Boraginaceae is probably the best source. The latter is the only family including species with GLA contents higher than 20% of total seed fatty acids and, according to recent reviews, only five species do so. Seed oils of only 87 out of 2000 species of Boraginaceae have been analyzed to date (Ucciani, 1995). SA has only been detected together with GLA in seed oils of Boraginaceae, Primulaceae and in plants of the genus *Ribes* (Saxifragaceae) (Gunstone, 1992).

Several frutescent species of the genus *Echium* (Boraginaceae) have been described as endemics from the Macaronesia, a group of islands located in the Mid Northeast Atlantic: namely Canary, Madeira, Azores

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Table 1
Mean values for fatty acids composition in Macaronesian species of *Echium*

	Oil% (wt)	GLA (%S)[a]	GLA (%O)[b]	16:0	16:1ω7	18:0	18:1ω9	18:2ω6	18:3ω3	18:4ω3	20:0	20:1ω9	22:0	22:1ω9	24:0	24:1ω9
Sect. *Echium*																
E. plantagineum	23.65	2.16	9.15	6.42	0.12	2.83	12.98	13.76	36.65	12.94	0.10	0.81	0.00	0.17	0.00	0.16
Sect. *Gigantea*																
E. giganteum	11.99	2.60	21.68	7.64	0.27	3.52	12.33	23.34	23.07	5.70	0.27	0.78	0.25	0.17	0.22	0.16
E. aculeatum	17.07	3.81	22.29	7.73	0.44	4.04	12.72	20.19	22.42	6.64	0.00	0.83	0.00	0.46	0.00	0.98
E. triste	19.88	3.42	17.19	8.22	0.26	3.92	10.20	35.46	19.14	3.28	0.15	0.64	0.16	0.17	0.39	0.40
E. leucophaeum	17.82	4.00	22.48	7.47	0.00	3.36	12.55	23.87	24.32	5.95	0.00	0.00	0.00	0.00	0.00	0.00
Sect. *Simplicia*																
E. wildpretti	17.21	2.61	15.16	6.02	0.17	3.58	13.66	17.67	31.91	8.97	0.26	1.11	0.07	0.26	0.04	0.19
E. simplex	10.04	1.94	19.28	9.20	0.21	4.78	9.47	19.19	27.39	7.50	0.23	1.09	0.00	0.47	0.00	0.30
Sect. *Virescentia*																
E. virescens	10.89	2.35	21.60	7.77	0.15	4.01	9.79	24.00	23.15	7.04	0.00	0.84	0.00	0.45	0.00	0.23
E. fastuosum	13.68	3.25	23.77	7.49	0.24	3.02	9.75	16.17	25.52	10.90	0.00	0.78	0.00	0.14	0.00	0.24
E. sventenii	7.25	1.77	24.39	9.14	0.38	4.36	11.81	23.34	18.75	4.69	0.31	0.77	0.30	0.21	0.17	0.20
E. nervosum	20.47	5.02	24.52	7.03	0.07	3.87	9.85	21.96	24.44	7.23	0.00	0.77	0.00	0.20	0.00	0.00
E. candicans	10.51	2.48	23.66	9.13	0.27	5.01	21.05	21.39	12.33	3.37	0.30	1.11	0.20	0.39	0.07	0.33
E. acanthocarpum	15.07	3.69	24.51	5.97	0.15	3.90	11.49	19.26	24.23	7.45	0.06	0.76	0.00	0.21	0.00	0.15
E. onosmifolium	18.65	4.37	23.44	6.90	0.14	4.50	9.81	21.00	24.52	7.11	0.18	1.13	0.09	0.45	0.00	0.11
E. callithyrsum	17.83	4.69	26.31	6.38	0.00	4.13	9.64	17.62	24.92	9.40	0.18	0.94	0.00	0.29	0.00	0.00
E. hierrense	20.42	4.03	19.75	7.74	0.16	4.36	9.96	19.76	28.98	7.20	0.17	0.90	0.19	0.19	0.06	0.17
Sect. *Auberiana*																
E. auberianum	17.31	3.02	17.45	7.03	0.17	2.52	19.22	29.15	18.92	3.03	0.05	0.89	0.00	0.25	0.05	0.24
Sect. *Decaisnea*																
E. decaisnei	13.38	2.67	19.92	7.51	0.14	4.19	12.08	28.09	21.94	4.39	0.21	0.67	0.15	0.26	0.00	0.05
Sect. *Stricta*																
E. strictum	17.83	3.34	18.75	6.89	0.14	4.22	11.56	28.82	23.03	4.36	0.15	0.54	0.04	0.32	0.00	0.15

[a] Perecentage of GLA in the seed.
[b] Percentage of GLA in the oil.

and Cabo Verde Islands. Seed oils of several species of Macaronesian *Echium* were analyzed in a search for new sources of GLA. On the other hand, we have used principal component analysis as a tool to investigate phylogenetic relationships among *Echium* species on the basis of the differences in fatty acid composition.

2. Results and discussion

Seed oil content and fatty acid composition from the 19 *Echium* species analyzed are given in Table 1. Seed oil content ranges from 7.10% in *E. fastuosum* to 23.65% in *E. plantagineum* (mean = 15.83%). The percentages of GLA ranges from 9.15% of total fatty acids in *E. plantagineum* to 26.31% in *E. callithyrsum* (mean = 20.79%). The percentage of total weight ranges from 1.77% GLA in *E. sventenii* to 5.02% GLA for *E. nervosum* (mean = 3.22%).

The 19 species of *Echium* described are among the richest sources of GLA found in nature so far. These high amounts of GLA in seed oil are rarely described in literature. We have reported 11 new plant species with a GLA content over 20% of the seed oil. Only five plant species have been described to have such higher contents (Ucciani, 1995). One species (*E. plantagineum*) showed a GLA amount in good agreement to other species of the *Echium* section. Eight species showed GLA values higher than 23% and five showed GLA percentages in total seeds above 4%. For these species, the high amount of GLA in oil (>19%) is paralleled by a high oil content within the seed (>17%). *E. callythyrsum* appears as the richest source of GLA in oil, with 4.69% of GLA in total seed. Except for *E. sventenii* and *E. simplex*, all the species have more than 2% of GLA compared to total seed weight. All *Echium* species, except *E. plantagineum*, have more than 15% of GLA in the oil.

The amounts determined for other polyunsaturated fatty acids were in good agreement with previous reports (Tsevegsüren & Aitzetmüller, 1996; Wolf, Kleiman & England, 1983). SA ranged from 3.03% in *E. auberianum* to 12.90% in *E. plantagineum* (mean = 6.69). LA ranged from 16.17% in *E. fastuosum* to 35.46% in *E. triste* (mean = 22.32%). Linolenic acid (ALA, 18:3ω3) ranged from 12.33% in *E. candicans* to 36.65% in *E. plantagineum* (mean = 23.98%). With respect to monounsaturated fatty acids, we obtained similar values to those reported for other species of this family (Tsevegsüren & Aitzetmüller, 1996; Wolf et al., 1983). Oleic acid (18:1ω9) ranged from 9.47% in *E. simplex* to 21.05% in *E. candicans* (mean = 12.10%). Other monounsaturated fatty acids were found in low amounts (20:1ω9, 22:1ω9 and 24:1ω9) as it is commonly described for Boraginaceae species (Tsevegsüren & Aitzetmüller, 1996; Wolf et al., 1983). Results for saturated fatty acids also agree with the reports mentioned above. E.g. palmitic acid (16:0) ranged from 5.47 in *E. acanthocarpum* to 9.20% in *E. simplex* (mean = 7.45%).

2.1. Principal-components analysis

We have performed a multivariable data analysis based on the fatty acid composition in order to investigate relationships among these plants. Multivariable data analysis is a suitable approach to find the underlying structures in complicated biological systems. One of the most powerful and widely used method is principal component analysis (PCA), which reduces the number of variables to a limited number of principal components (PC) (Joliffe, 1986; Wold, Eseben & Geladi, 1987). Unlike measured variables, PC are orthogonal, and thereby describe independent variation structures in the data. The presence of significant PC indicates structure in the data. Graphic overviews, ideally showing a large part of the variance in the two dimensions, of the objects and variables are obtained by score and loading plots, respectively. PCA was initially applied to data corresponding to polyunsaturated fatty acids, LA, ALA, GLA and SA, from all *Echium* species considered here. The analysis was restricted to these fatty acids due to the fact that a more complex enzymatic system is required for their synthesis, and this may account for a higher amount of genetic variation. PC are linear combinations of the original variables and are determined so that the first PC explains the largest part of the total variance and the following PC successively explain smaller parts of the original variance. This means that correlated variables are explained by the same PC and less correlated variables by different PC. In the present analysis, the first two PC explained 65.8% and 27.8%, respectively, of the total variance in the four variables. A plot of the Two First Component Weights (Fig. 1) shows that the ω3 fatty acids (LNA and SA) are positively correlated ($r = 0.832$, $p < 0.01$) while no significant correlation is observed for ω6 fatty acids (LA and GLA). A possible explanation for these results is that the ω12 desaturase activity responsible for the introduction of the double bond in LA or in ALA correspond to different enzymes. Although it is generally believed that the enzymatic systems responsible for the metabolism of LA and ALA are identical (Horrobin, 1992), our results suggest that this might not be the case for the first desaturation step at least for *Echium* plants. Nevertheless, this hypothesis requires further investigation.

In this analysis, all fatty acids have a high influence on the model. Component 2 is substantially influenced by GLA. The resulting Scatterplot (Fig. 2) provides a

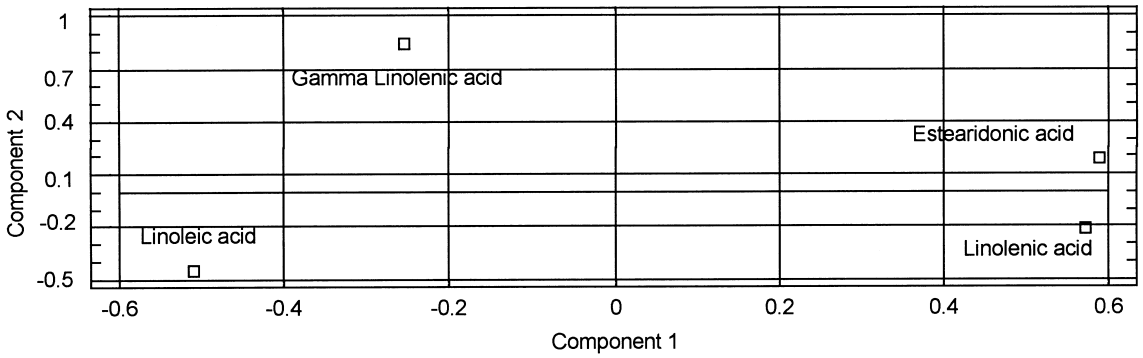

Fig. 1. Plot for first two components weights in fatty acid composition for seed oils of *Echium* from Macaronesia (93.6% of variance).

conceptual overview of the samples by showing a total of 93.6% of the variance. The relationships between those variables and the PC are defined as loadings. The pattern of covariation between the species can be seen in this graphic. Variables found in similar direction and far from the origin are positively correlated, while those found at opposite sides of the plot are negatively correlated. Therefore, the present scatterplot indicates that species can be grouped according to their contents of polyunsaturated fatty acids. The component plot and scatterplot can be interpreted together because objects with high scores for a specific PC also have high values for the variables with high loading plots and low values for those with low loadings. The scatterplot shows that similarities between species are in most cases coincident with sections of *Echium* proposed by Bramwell (1972) and compatible with the phylogenetic relationships obtained by Böhle, Hilger & Martin, (1996). Influence on group formation can be assigned to a particular fatty acid. Thus, LA has great influence in sections *Decaisnea*, *Auberiana* and *Stricta*. The scatterplot also indicates that the similarity observed between species for sections *Virescentia* and *Gigantea* is due to their high content in GLA. Therefore, section *Gigantea* seems to be highly related with section *Virescentia*. *E. triste*, a species belonging to the *Gigantea* section, is separated from the other species of this section due to its high content of LA. *Gigantea* is a complex section with a main distribution area around the western Canary Islands, especially on Tenerife. Species from this section (*E. leucophaeum*, *E. aculeatum* and *E. giganteum*), though basically similar in morphology, are of allopatric origin and can be readily distinguished (Bramwell, 1972). Species of this group are considered as vicarious taxa which have probably arisen by fragmentation of a single ancestral species. *E. triste* is a very variable species which ranges in habit from a biennial to a short-lived perennial with a very woody stock. It is adapted to extreme xeric conditions (Bramwell, 1972), and its high LA content perhaps can be due to an adaptation to this habitat or, alternatively, to a biennial biological cycle, while the other species of this section are perennial brushes, common in more humid and temperate habitats. Section *Decaisnea* is distinguished from section *Gigantea* by the even lobing of the corolla which is not laterally compressed, although both sections are considered to be close (Bramwell, 1972). In agreement to this, plants

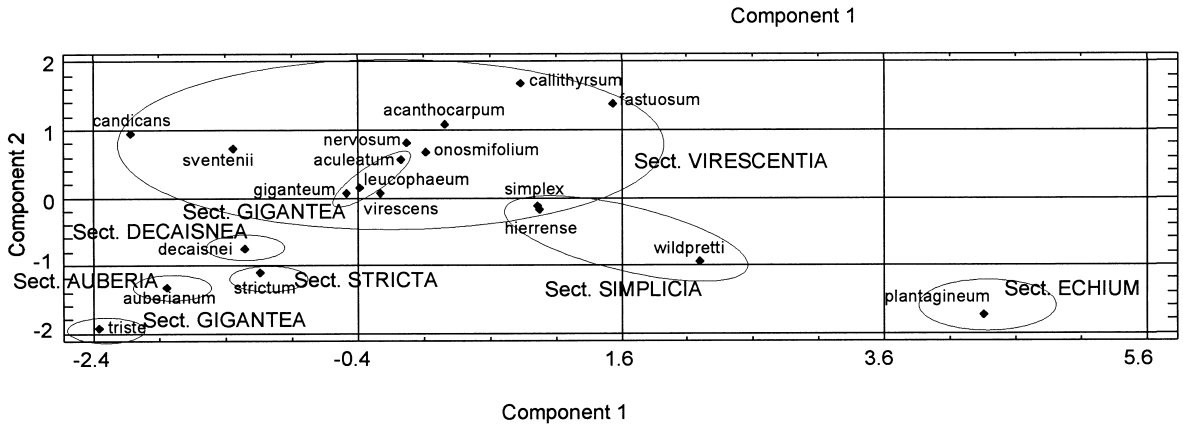

Fig. 2. Scatterplot for the first two component weights for seed oils of *Echium* from Macaronesia (93.6% of variance).

from these sections appear together in the scatterplot. Relationships between section *Auberiana*, containing a single species, *E. auberianum*, and other sections remain rather obscure. This species is considered as a transition from the woody shrubs of Macaronesia to the herbaceous plants of the Iberian–North African region (Bramwell, 1972). Nevertheless, fatty acid composition indicates that its similarity with herbaceous plants of the *Echium* section is lower than with plants from other sections. Section *Echium*, with just one species analysed (*E. plantagineum*) lies very far from the others. This species is considered as introduced into the islands, and it is therefore distantly related to the Macaronesian endemic species of *Echium* (Böhle et al., 1996). *Simplicia* species, that are related in turn with members of the *Virescentia* section grouping the majority of species analyzed, seem to be the closest to plants of the *Echium* section.

GLA content in *Echium* Macaronesian species could even be higher, as many varieties of these species remain untested. Furthermore, several *Echium* species remain to be tested in Cape Verde and Canary Islands. Herbaceous *Echium* from Europe and North of Africa show a much lower GLA and SA content, usually around 10% of the oil. The fact that a high GLA content is found in seeds of all *Echium* species tested so far in the Macaronesian islands suggests that this characteristic was present in their common ancestor. Wether a high GLA level might confer an adaptative advantage in their environments to these plants is something that deserves further investigation.

According to these data, *Echium* species from Macaronesia appear to be a potential new source of GLA which also possesses significant amounts of SA.

3. Experimental

3.1. Materials

Mature plant seeds were collected in the natural habitats of the plants. Seeds of *E. nervosum*, *E. plantagineum* and *E. candicans* were collected from Madeira in July 1998. Seeds of *E. decaisnei*, *E. onosmifolium*, *E. callithyrsum* and *E. strictum* were gathered from the island of Gran Canaria in July 1998 while seeds of *E. simplex*, *E. virescens*, *E. triste*, *E. leucophaeum*, *E. aculeatum*, *E. sventeni* and *E. giganteum*, were obtained from the island of Tenerife in May 1998. Seeds of *E. wildpretti* and *E. auberianum* were obtained from the island of Tenerife in July 1998. Seeds of *E. acanthocarpum* were collected from the island of La Gomera in July 1998. Seeds of *E. hierrense* were obtained from the island of Hierro in July 1998. Seeds of *E. fastuosum* were collected from garden plants in Almería (Spain) in May 1998. Due to the fact that these plants are endemics, a great number of precautions were accomplished in the harvesting. Only the necessary seeds to accomplish the analysis were collected (usually two seeds per plant until completing 20 mg, approximately 30 seeds for all species). For herbaceous species, only seeds from parched plants were collected. In shrubs, seeds were taken from dry branches.

3.2. Oil extraction and transesterification

Seeds were freeze-dryed and ground to powder with a mortar. Rapid simultaneous oil extraction and transesterification was made according to the method of Rodríguez-Ruiz, Belarbi, García Sánchez and López Alonso (1998). Around 10 mg of each sample were transferred to test tubes with 1 ml of the methylation mixture (methanol/acetyl chloride, 20:1 v/v) and 0.5 ml hexane and heated at 100°C for 10 min. After cooling to room temperature, 1 ml of distilled water was added and the upper hexanic layer was extracted for CG analysis. This method for fatty acid analysis provides routinely a variation of less than 5%, and all *Echium* analysis were in this range. This way, duplicates were done of every sample, and mean values are shown in the tables.

3.3. Gas–liquid chromatography (GLC)

Mixed fatty acid metyl esters (FAME) were analyzed in an Hewlett–Packard HP5890 series II gas chromatograph provided with FID and HP3394 integrator. A capillary column of fused silica of high polarity (Supelco SP2330; length: 30 m; internal diameter: 0.25 mm; thickness of the film: 0.2 μm) was used. The flow of the carrier gas (N_2) was 0.75 l/min. Split ratio in the injector was 100:1. Injector temperature was 240°C, and detector temperature was 260°C. The oven starting temperature was 205°C, and it was increased at a rate of 6°C/min until 240°C (5.83 min). Injection volume was 5 μl, and a blank was run every ten analyses. Peaks were identified by comparison with known methyl ester standards (''Rapeseed oil mix'' and ''PUFAS-1'', from Sigma) and oil contents in seeds were determined gravimetically, by using methyl heptadecanoate (17:0) as internal standard. Unidentified peaks were taken into account for further calculations.

Verification of double bonds was performed by GC–mass spectrometry (MS) in a HewlettPackard HP5890A G.C. provided with a Hewlett–Packard 5988A M.S. A capillary column of methyl silicone (HP-1; length: 25 m; internal diameter 0.2 mm; thickness of the film: 0.33 μm). The flow of the carrier gas (He) was 1 ml/min. Injector temperature was 260°C, and the pressure at the head of the column was 15 psi. The oven starting temperature was 100°C, and it was

increased at a rate of 10°C until 280°C, and then kept at 280°C for 10 min. The temperature in the interphase was 280°C, and the temperature of the source in the detector was 180°C.

Principal Components Analyses were performed with the software package Statgraphics for Windows (v. 3.0).

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