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Research Note

A Chemotaxonomic Approach to the Fatty Acid and Tocochromanol Content of *Cannabis sativa* L. (*Cannabaceae*)

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Abstract: In this study, the fatty acid, tocopherol and tocotrienol composition in the seed oil of *Cannabis sativa* L., which is traded under the common name hemp seed oil, were determined by using GLC and HPLC techniques. While α- linolenic, linoleic, oleic and palmitic acid were the main fatty acid components, γ – linolenic (18:3 n-6) and stearidonic acid (18:4 n-3) were found as unusual minor fatty acids in the seed oil. γ – linolenic acid is an important fatty acid used both as a healthy nutrient and as a therapeutic agent. The occurrence of this fatty acid in some plant groups may have practical consequences with respect to genetic engineering or plant breeding for renewable lipid resources and may be of significant interest in plant chemotaxonomy and evolution. While the hemp seed oil was rich in tocopherols, particularly γ– tocopherol, tocotrienols were not present. The chemotaxonomic importance of the fatty acids and tocochromanols (tocopherol and tocotrienols) was discussed in the family (*Cannabaceae*) pattern.

Key Words: *Cannabaceae*, *Cannabis sativa* L., Chemotaxonomy, Fatty Acids, Tocopherols, Tocotrienols, gamma-Linolenic Acid, Stearidonic Acid

Cannabis sativa (*Cannabaceae*)'nın Yağ Asiti ve Tokokromanol İçeriği Üzerinde Kemotaksonomik Bir Yaklaşım

Özet: Bu çalışmada, kenevir adıyla ticareti yapılan *Cannabis sativa* L. tohum yağının yağ asidi, tokoferol ve tokotrienol içeriği GLC ve HPLC teknikleri kullanılarak saptanmıştır. α- linolenik, linoleik, oleik ve palmitik asit temel yağ asidi bileşeni olarak saptanırken, γ – linolenik (18:3 n-6) ve stearidonik asit (18:4 n-3) tohum yağında alışılmamış küçük yağ asidi bileşenleri olarak bulunmuştur. γ– linolenik asit hem sağlıklı besleyici hem de tedavi edici ajan olarak kullanılan önemli bir yağ asididir. Bu yağ asidinin bitki gruplarında bulunması, yenilenebilen lipid kaynaklarının bulunması ve genetik mühendisliği, bitki ıslahı konularında ayrıca bitki kemotaksonomisi ve evrimi yönünden pratik sonuçlar verebilir. Kenevir tohum yağı tokoferol ve özellikle gamma - tokoferol bakımından zengin olmakla beraber tokotrienoller tohum yağında bulunmamıştır. Yağ asidi ve tokokromanol (tokoferol ve tokotrienol)'lerin *Cannabaceae* familyası örneklerindeki kemotaksonomik önemi tartışılmıştır.

Anahtar Sözcükler: *Cannabaceae*, *Cannabis sativa* L., Kemotaksonomi, Yağ asidi, tokoferol, tokotrienol, gamma - linolenik asit, Stearidonik asit

Introduction

Cannabaceae (*Cannabinaceae*) are composed of two genera, both occurring in the northern hemisphere, in Turkey and the rest of the world (Davis, 1978; Benson, 1979). One of them is *Cannabis* L. and the other is *Humulus* L. The genus *Cannabis* (hemp) is represented by a single species, *Cannabis sativa* L. The latter is also represented by *Humulus lupulus* L.. Both genera are monotypic in Turkey Flora (Davis, 1978; 1988). *Cannabis sativa* is probably widely cultivated, but little collected and has local distribution in Turkey, occurring as a casual around ports and on rubbish tips in cooler regions. It is grown in many warmer parts of the world for fibre, oil and narcotic resin. However, for fibre and oil production in the European Union only hemp seeds with

low amounts of tetrahydrocannabinol, the narcotic agent, are allowed. Probably indigenous to C & W Asia, its exact native area has been blurred by cultivation from ancient times (Davis, 1978; Baytop, 1984). It is of economic and pharmaceutical importance all over the world. The foliage and branches with leaves have been used as a sedative and narcotic drug known as Herba cannabis (Baytop, 1984). Hempseed, which is rich in vitamins A, C and E, minerals and β-carotene, is claimed to have exceptional nutritional value (Orhan et al., 2000).

It has been demonstrated that the content and composition of fatty acids of seed lipids can serve as taxonomic markers in higher plants (Harborne & Turner, 1984; Hegnauer, 1989; Aitzetmuller, 1993). The occurrence and distribution of gamma (γ-) linolenic acid in the plant kingdom may have chemotaxonomical significance in some families. γ-Linolenic acid is highly appreciated and of considerable interest for pharmaceutical and dietary use, and medical benefits (Gunstone, 1992; Horrobin, 1992; Tsevegsüren et al., 1997). It (γ-ln, 18:3Δ6c, 9c, 12c or 18:3 n-6) is one of the important fatty acids used both as a health nutrient and as a therapeutic agent. The occurrence of γ-ln as a seed oil component has been reported by previous workers in some 12 different families, but it is of economic importance in *Onagraceae* (*Oenothera* L.), *Boraginaceae* (*Borago* L. and *Echium* L.) and *Grossulariaceae* (Ribes L.) (Gunstone, 1992; Tsevegsüren & Aitzetmuller, 1996; Tsevegsüren et al., 1997). Stearidonic acid (18:4 n-3) is another fatty acid that is relatively uncommon in the plant kingdom, but occurs in some families (Hegnauer, 1989; Aitzetmuller & Werner, 1991; Velasco & Goffman, 1999).

Tocopherols are natural antioxidants, which occur as four homologues (α-, β-, γ-, δ-tocopherols - the α–species being known as vitamin E), differing in the methylation of the tocol head group (Pongracz et al., 1995; Goffman et al., 1999). The relative content of individual tocopherols is known to be characteristic of the seed oil of different cultivated plants. The tocopherols are present in oilseeds and in the leaves and other green parts of higher plants. Kamal–Eldin & Appelqvist (1996) and Velasco & Goffman (1999) have claimed that tocotrienols are not found in the green parts of plants. The chemotaxonomic value of the tocopherols has been reported in some plant families e.g. *Brassicaceae, Boraginaceae* (Velasco & Goffman, 1999; Goffman et al., 1999, Bağcı et al., unpublished).

Seed fatty acid and the tocopherol composition of plants can be used to confirm phylogenetic and taxonomical relations in the plant kingdom. Alston and Turner (1963), regarding fatty acid patterns in the angiosperms, emphasized that little attempt had been made to use fatty acids directly to solve systematic problems. More recently, Aitzetmuller et al., (1999), Velasco & Goffman (1999), Goffman et al., and (1999a), and Bağcı et al. (unpublished) have demonstrated the taxonomic potential of the evaluation of seed fatty acids and tocochromanols in some families.

In this study, the fatty acid, tocopherol, tocotrienol and plastochromanol–8 content of *Cannabis sativa* was determined and chemotaxonomic significance was assessed in the family patterns. Although there are a few studies on the fatty acids of this drug source (Mehmedic, 1989; Ahmad, 1989; Matthaus et al., 2001) there has been no chemotaxonomic evaluation of these genera and their oil content. In addition, during the course of this study, a considerable number of new sources of the pharmaceutically interesting γ-linolenic acid and stearidonic acid have been discovered and discussed.

Experimental

Seed samples

Seed samples were obtained from the seed gene bank (Aegean Agricultural Research Institute) in İzmir. The location of the specimen is Erzurum - Şenkaya, Gülveren village, 2500 m. Altan, 90993. The seed specimens were deposited in the Aegean Agricultural Research Institute (İzmir).

Oil Extraction and preparation of fatty acid methyl esters (FAME)

Impurities were removed from the seeds, and the cleaned seeds were ground into powder using a ball mill. Lipids were extracted with heptane in a straight through extractor. The triglycerides were transesterified to methyl esters with potassium hydroxide in methanol according to ISO method 5509 (DGF, 1989).

Capillary GLC

Fatty acid methyl ester composition was determined on two different gas chromatographs, Hewlett-Packard HP5890 (A) and HP6890 (B), each equipped with a fused silica WCOT capillary and FID:

A) Silar 5 CP, 50 m x 0.25 mm ID, 0.24 mm film thickness, nitrogen as carrier gas, 1:50 split ratio, pressure 160 kPa, oven temp.: 5 min isothermal at 163 ºC, then 163 to 205 ºC at 1 ºC/min; Inj.= 230 ºC, Det. 260 ºC.

B) DB-23, 60 m x 0.32 mm (J&W), 0.25 mm film thickness, hydrogen as carrier gas, 1:50 split ratio, pressure 69 kPa, oven temp.: 1 min isothermal at 80 °C, then 80 to 150 °C at 25 ºC/min then 150 to 240 °C at 3 ºC/ min, 5 min isothermal, PTV-Inj. 80 °C, 12 °C/s to 250 °C, 5 min isothermal, Det. 250 ºC.

Data analysis was carried out with a Chromato-Integrator D 2500 (Merck-Hitachi) and Chemstation integration software, respectively. Peak identification was achieved by comparison of relative retention times with those obtained from test mixtures of known composition on two different columns.

Tocopherol analysis

Tocochromanols were determined by high-performance liquid chromatography (HPLC) according to the procedure of Balz et al. (1992). An aliquot of a solution of 50 mg oil in 1 ml heptane was injected in an HPLC system via a Rheodyne valve with a sample loop volume of 20 µl. Tocopherols were separated on a LiChrospher 100 Diol phase, 5 µm particle size (Merck, Darmstadt, Germany). HPLC column 25 cm x 4.6 mm ID with an additional guard column 4 mm long and 4 mm ID, filled with LiChrospher Si 60, 5 mm particle size. The system was operated with an eluent of heptane/tert.-butyl methyl ether (96 + 4v/v) and detection by a fluorescence detector F-1000 (Merck, Darmstadt) at 295 nm excitation wavelength and 330 nm emission wavelength.

A D-2500 Chromato-Integrator (Merck, Darmstadt) was used for data aquisition and processing. Calibration was done by external standards with α-, β-, γ- and δ-tocopherol (Calbiochem, Bad Soden, Germany). Tocotrienols were calculated with the same response factors as the corresponding tocopherols, and plastochromanol-8 was calculated with the same response factor as gamma-tocopherol (Balz et al., 1992).

Results and Discussion

In this study, the fatty acid composition and tocochromanol derivatives, α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienols and plastochromanol-8-were detected in *Cannabis sativa*. The results of the fatty acid analysis and the oil yield are shown in Table 1. The results for the tocopherol and tocotrienol contents of the studied sample are shown in Table 2. The GLC chromatogram of the *Cannabis sativa* seed oil is shown in Figure 1.

The total oil yield of the species studied reached 31.79 (wt%) of seed. The extracted seed oil of *Cannabis sativa* contained significant amounts of linoleic (50.46%), α-linolenic (20.09%) and oleic acid (16.01%), which are the major fatty acids in *Cannabis sativa*. These were the abundant fatty acid components in the *Cannabis* oil. On the other hand, palmitic (6.53) and stearic acid (2.64) and the others were found as the minor fatty acids. The sum of all saturated fatty acids (SFA) in hemp seed oil is 10.47% and the amount of unsaturated fatty acids (USFA) is 89.10% (Table 1). This means that the shelf life of hempseed oil is limited due to the high amount of unsaturated fatty acids, which are easily oxidised. For this reason care must be taken over storage and handling of the neat oil, while the oil is much more stable in the seed.

High amounts of individual main fatty acids may be useful in assessing chemotaxonomic relationships among the plant taxa, but unusual fatty acids are even more useful and important in elucidating chemotaxonomic relationships between some genera and families, because

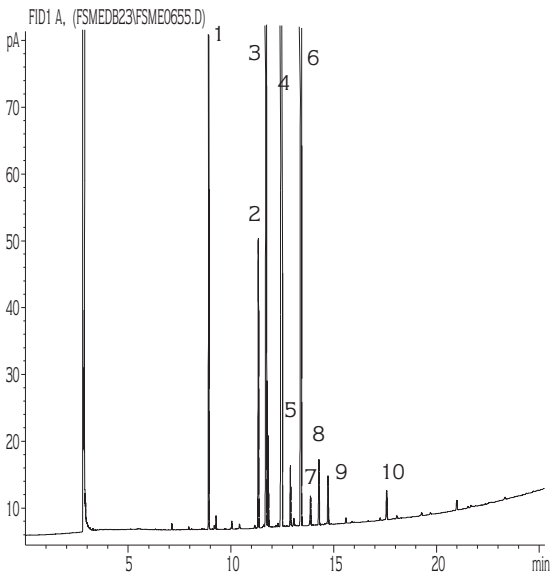

Fig. 1. Fatty acid methyl ester from hempseed oil. Peak assignment: 1 palmitic, 2 stearic, 3 oleic, 4 linoleic, 5 gamma linolenic, 6 alpha linolenic, 7 stearidonic, 8 eicosanoic, 9 gadoleic, 10 docosanoic acid.

Table 1. Fatty acid composition of *Cannabis sativa* L. Data shown are peak area - % from GLC (Fig. 1).

Fatty acid Components	GLC area %
14:0	0.035
15:0	0.000
16:0 Palmitic a.	6.532
16:1Δ7	0.034
16:1Δ9	0.104
17:0	0.068
18:0 Stearic a.	2.643
18:1D9 Oleic a.	15.21
18:1Δ11	0.801
18:2 Δ9,12 linoleic	50.46
18:3Δ 6,9,12 γ- linolenic a.	0.582
18:3 Δ 9,12,15 α- linolenic a.	20.09
18:4 Δ 6,9,12,15 Steraidonic a.	0.337
20:0 Eicosanoic a.	0.700
20:1Δ9 Gadoleic a.	0.529
20:2Δ11,14	0.596
22:0 Docosanoic a.	0.345
22:1Δ13	0.359
24:0	0.129
24:1Δ15	0.000
Tot. SFA	10.472
Tot. UFA	89.102
Oil content (wt%)	31.79

Table 2. Tocochromanols (tocopherol (T) and tocotrienol (T3) composition of *Cannabis sativa* L.

Tocochromanols	% values
α - Tocopherol	5.66
β - Tocopherol	0.33
γ - Tocopherol	89.11
δ - Tocopherol	4.90
α - Tocotrienol	--
β - Tocotrienol	--
γ - Tocotrienol	--
δ - Tocotrienol	--
Plastochromanol-8	--
Tocopherol yield (mg / 100g)	74.62

the occurrence of unusual fatty acids in seeds is often correlated to plant families (Aitzetmuller, 1993). There is a considerable potential in higher plants for the biosynthesis of unusual fatty acid structures, which are of particular interest to the chemical industry (Aitzetmueller et al., 1999).

γ–linolenic (0.582%) and stearidonic acid (0.337%), unusual fatty acids, were found in the *Cannabis* oil studied here. γ–linolenic acid, which is of great interest for dietic and pharmaceutical use, is a family characteristic in the Boraginaceae, but also occurs in sporadically clusters in other families. Stearidonic acid (18:4) is also a very important unusual fatty acid in some families, such as *Boraginaceae* (Tetenyii, 1974; Velasco & Goffman, 1999; Bağcı et al., unpublished). These two unusual fatty acids were not reported in *Cannabis* oil by Yazıcıoğlu & Karaali (1983), Mehmedic (1989) and Ahmad (1989), but γ-linolenic acid was reported in the Aitzetmuller (1996) and Orhan et al. (2000) studies. The amount of this fatty acid was reported as 2.01% in Orhan et al. (2000) studies, 1.10% in the Aitzetmuller study (unpublished) and 2.00% in the Kuhn (1997) study.

The tocochromanol (tocopherol and tocotrienol) profile of *Cannabis sativa* showed that it was very rich in tocopherol content, although tocotrienols were not determined in the seed oil. While γ-tocopherol was the most abundant component (89.11%), the others, α-(5.66%), β-(0.33%) and δ-(4.90%) tocopherol, showed only small concentrations in the seed oil (Table 2). Plastochromanol–8 was also not detected in hempseed oil. Oomah et al. (2002) reported that λ tocopherol was found as a major component in hempseed oil and that this and the fatty acids were not affected by microwave treatment, in contrast to beta - tocopherol.

The fatty acid analysis results provide very important chemotaxonomic clues among the studied and other family patterns. Investigation of the fatty acid composition of *Cannabis sativa* revealed that 18:4 n-3, stearidonic acid, is only found in *Cannabis*, and was not detected in the genus *Humulus*, the other genus in *Cannabaceae*. From the literature (see Table 3), *Humulus japonicus* Sieb. & Zucc., *H. lupulus* L. (which grows naturally in Turkey; Davis, 1978) and *H. scandens* (Lour) Merrill. do not contain stearidonic acid (Earle, 1962; Gorjaev & Evdakova, 1977; Aitzetmuller & Ivanov, unpublished). In the last study, γ- linolenic acid was detected in *Humulus lupulus* seed oil, although stearidonic

Table 3. *Cannabis sativa* and *Humulus* sp. (*Cannabaceae*) seed oil composition according to references. (nr: not reported)

Species	Fatty acid components								References
	16:0	18:0	18:1	18:2	18:3 γ	18:3 α	18:4	20:0	
Cannabis sativa	8.53	3.06	nr	54.66	2.01	31.72 + 18:1 (tr)	nr	nr	Orhan , 2000
Cannabis sativa	8.30	2.50	17.20	54.90	nr	1.16	nr	1.00	Mehmedic, 1989
Cannabis sativa	9.40	3.20	15.0	49.30	nr	23.10	nr	nr	Yazıcıoğlu, 1983
Cannabis sativa	6.70	2.60	16.40	53.10	1.10	16.10	0.40	0.80	Aitzetmuller (unpb.), 1996
Cannabis sativa	7.80	4.30	10.60	53.80	nr	18.70	nr	nr	Ahmad, 1989
Cannabis sativa	nr	nr	nr	52.00	2.00	18.00	nr	nr	Kuhn, 1997
Humulus japonicus	16.10	3.0	14.10	52.40	nr	14.40	nr	nr	Gorjaev & Evdakova, 1977
Humulus lupulus	11.20	5.90	19.70	32.80	1.50	4.60	nr	1.80	Aitzetmuller & Ivanov (unpb), 1996
Humulus scandens	nr	nr	15.00	54.00	nr	13.00	nr	nr	Earle, 1962

acid was not found. It may therefore be useful to determine this component in order to differentiate two genera from each other by these means and chemotaxonomy. Stearidonic acid has chemotaxonomic importance in *Cannabaceae* genera, particularly in the studied genus pattern. On the other hand, there are some differences between *Cannabis* and *Humulus* species with regard to usual fatty acid composition. Palmitic acid has a higher concentration in *Humulus* sp. than *Cannabis sativa* according to all researchers (see Table 3).

The chemotaxonomic importance and potential of fatty acids and tocochromanols in this family were confirmed by this study. Some indications were obtained by this study to determine the degree to which fatty acids (particularly usual as well as unusual ones) can contribute to delimiting taxonomic classes within the family. Differences in fatty acid patterns illustrate some chemotaxonomic relationships between the family members studied. However, further studies are required to confirm the results obtained from this study, particularly the family pattern all over the world.

Tocopherols and plastochromanol–8 with the addition of fatty acids possess an important chemotaxonomic value for the genus *Linum* L. (Velasco & Goffman, 2000), and the tocochromanols (Velasco & Goffman, 1999; Goffmann et al., 1999a) have chemotaxonomic importance in *Boraginaceae* and *Brassicaceae*. Among the tocopherols present in foods, the α – homologue shows the highest vitamin E activity, thus making it the most important for human health (Goffman et al., 1999). A genetic engineering approach for elevating the vitamin E content in seeds was carried out by Shintani & Dellapena (1998). The findings may suggest the fixed oil of *Cannabis sativa* oil can be new a source of unusual and usual fatty acid and tocopherol content, particularly with regard to γ-tocopherol. The results obtained from this study will give useful information to chemistry, genetic and biotechnology researchers.

More successful results have been obtained when the fatty acid analysis has been restricted to smaller plant groups, as in the investigations by Stone et al. (1969), Hohn & Meinschin (1976), Aitzetmuller et al. (1999), Bağcı et al. (2001) and Bağcı & Özçelik (2001). The occurrence of this fatty acid component in some plants may have practical consequences with respect to genetic engineering or plant breeding for renewable lipid resources, and may attract significant interest with regard to natural product chemistry and plant chemotaxonomy and evolution. Some unusual fatty acids are present in small amounts in the seed oils only. These are chemotaxonomically significant because of their constant presence in all the species of one genus or a few genera, combined with their constant absence throughout all the species of other genera (Aitzetmuller & Tsevegsüren, 1994). Unusual and technically interesting fatty acids and their occurrence in seed oils are genetically determined, and they are highly significant indicators of phylogenetic relationships (Aitzetmuller, 1995). Both further studies and more family patterns, however, are needed to determine the degree to which fatty acids can contribute to delimiting taxonomic classes within this family. The number of plant species analysed for seed

lipid composition is still limited and only a few studies have been carried out to investigate the fatty acid composition in order to assign phylogenetic relationships in this family linked to the other families.

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