Characterization of Sterols in Refined Borage Oil by GC–MS

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ABSTRACT: Borage oil sterols were isolated by TLC and characterized using GC and GC–MS. Several diunsaturated Δ^5-sterols, some of them not previously recorded in vegetable oils, were found. Of these, 24-methylcholesta-5,23-dienol and 24-ethylcholesta-5,23-dienol could be useful markers for borage oil. Two other diunsaturated Δ^5-sterols that are rarely found in vegetable oils, 24-methylcholesta-5,24(25)-dienol and 24-ethylcholesta-5,24(25)-dienol, were identified. The diunsaturated C-24(28)-sterol, isofucosterol, was also found, as well as the monounsaturated Δ^5-sterols campesterol and sitosterol. These are normally present in vegetable oils, which makes them unsuitable as markers for borage oil.

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KEY WORDS: Borage oil, GC–MS, sterols, TLC.

Recently, the drug and health food industries have shown increased interest in the use of borage oil. This interest stems from its use as a source of γ-linolenic acid, a precursor to the prostaglandins. Borage oil, which is extracted from the plant *Borago officinalis* L. (Boraginaceae), has a high content of γ-linolenic acid as well as other n-6 FA. A characterization of the FA in borage oil, by means of GC–MS and GC, is reported elsewhere (1).

An important analytical parameter when processing vegetable oils is the sterol content. This can be used as a means of identifying the oil and, therefore, for detecting adulteration. Generally, the sterol pattern, i.e., the type of sterols and their relative proportions, is unique for each oil (2) and can therefore complement FA analysis. Furthermore, sterol analysis can be used to determine the purity of the oil. The level of purity does not significantly affect the relative proportions of the sterols but does affect the total sterol content. The individual steps in the refinement process, such as degumming, neutralization, bleaching, and deodorization, each lead to a reduction in the total sterol content (3).

Several techniques for the isolation and analysis of the 4-desmethyl-, 4-monomethyl-, and 4,4-dimethylsterol fractions have been reported. TLC, as well as solid phase extraction (SPE) or HPLC, are normally used to isolate these fractions. On-line LC in combination with GC (LC–GC) can be used for both isolation and analysis of sterols (4). However, TLC is a well-established technique for fractionation of sterols and provides compatibility with literature data. For saponification, a small-scale procedure involving ultrasonic treatment of a mixture of the oil and a potassium hydroxide solution at 100°C has been reported (5). This procedure gives comparable results and is less time consuming than the conventional reflux method. In order to obtain a recovery close to 100%, at least three *n*-hexane extractions are required (6). Normally, sterols are analyzed as silyl derivatives, as acetates, or in free form by means of GC. During the analysis of sterols in free form, adsorption onto the column might occur. In contrast, silyl derivatives are well suited to analysis by GC and provide distinct and selective mass spectra.

The objective of this study was to characterize borage oil with respect to 4-desmethylsterols (sterols), 4-monomethylsterols, and 4,4-dimethylsterols (also known as triterpenes) and to find an appropriate method of analysis.

EXPERIMENTAL PROCEDURES

Materials. Refined borage oil was obtained from AB Karlshamns Oljefabriker (Karlshamn, Sweden). The internal standards, 5-α-cholestane and lathosterol, were purchased from Sigma (Deisenhofen, Germany) and Makor Chemicals (Jerusalem, Israel), respectively; the latter was recrystallized in 99.5% ethanol before use. Campesterol and sitosterol, used as reference sterols, were bought from Aldrich (Milwaukee, WI) and Calbiochem–Novabiochem (San Diego, CA), respectively. The silylation reagent TriSil Z was obtained from Pierce (Rockford, IL).

Saponification. A lathosterol solution (6 mL), prepared in *n*-hexane/chloroform 9:1 at a concentration of 0.02 mg/mL, was added, as an internal standard, to 300 mg of borage oil in a conical tube. After evaporation of the solvent, 7.5 mL of potassium hydroxide (2 M) in ethanol was added to the mixture. The tube was carefully sealed with a screw cap and placed in an ultrasonic bath at 100°C for 10 min. When the saponification was complete, the mixture was diluted with 7.5 mL of distilled water. Subsequently, the unsaponifiable material was extracted using *n*-hexane (3 × 15 mL). The supernatants were combined and the solvent was evaporated under nitrogen. Finally, the extracts were dissolved in aliquots of chloroform to prepare them for TLC.

TLC. The main part of the unsaponifiable material, about 3 mg, was applied on a Merck 240 (Darmstadt, Germany) TLC plate as a line 1.5 cm from the base. Small portions of the material were also applied as spots on both sides of the line to act as references. The plate was developed twice in *n*-hexane/diethyl ether/acetic acid (70:30:1). When the plate had dried after the second development, it was exposed to iodine vapor in a closed glass vessel, and the sample was covered with a glass plate. On the basis of the reference spots, three zones were marked out. The R_f values of the zones were

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0.16–0.20, 0.24–0.27, and 0.29–0.33, respectively. The zones were scraped off and extracted twice with diethyl ether. The diethyl ether volumes were combined; and 2 mL of a second internal standard, 5α-cholestane in *n*-hexane at a concentration of 0.02 g/mL, was added to each sterol fraction. The solvent was then evaporated under nitrogen, and the extracts were dissolved in small volumes of *n*-hexane for subsequent analysis by GC and GC–MS.

GC. After the *n*-hexane had evaporated, the sterol fractions were silylated by incubating them with 100 μL of TriSil Z at 70°C for 45 min. Then the sample preparations were injected using split injection (1:40) onto a Hewlett-Packard 5890 gas chromatograph equipped with an FID. A nonpolar, fused-silica capillary column, CPSil 8CB (5% phenylmethylsilicone; Chrompack, Middelburg, The Netherlands), 30 m × 0.32 mm i.d., and film thickness 0.25 μm, was used for the separation. The sterols were eluted both isothermally at 260°C, for determination of the relative retention times, and by a temperature gradient of 10°C/min from 200 to 270°C for quantification. The injection and detection temperatures were 290 and 310°C, respectively.

GC–MS. The silylated sterol fractions were also analyzed by GC–MS, using a Hewlett-Packard Model 5890 gas chromatograph, equipped with a split/splitless injection system, in combination with a Hewlett-Packard 5970 mass-selective detector. In this case, the gas chromatograph was fitted with a CPSil 8CB fused-silica capillary column (25 m × 0.25 mm i.d., film thickness 0.25 μm). The samples were introduced on the column with splitless injection. The temperature in the injector was 280°C, and the column temperature was initially held at 200°C for 3 min, increased to 270°C at a rate of 5°C/min, and then held at this higher temperature for 23 min. The EI–MS conditions used were an ionization voltage of 70 eV, an ion current of 50 μA, and an ion source temperature of 200°C.

RESULTS AND DISCUSSION

Unsaponifiables. The 4-desmethylsterol fraction accounted for 0.35% of the borage oil, as determined by GC, whereas the 4-monomethylsterol and 4,4-dimethylsterol fractions accounted for only 0.08 and 0.03%, respectively. The relative proportions of the fractions are similar to those in most vegetable oils (2). The reproducibility of the GC analysis was determined by the analysis of six different sample preparations of borage oil. Obtained relative standard deviation (RSD) values were 3.0, 14, and 11%, respectively.

Chromatography. The GC separations of the fractions are shown in Figure 1. The internal standards, 5α-cholestane and lathosterol, appear near the beginning of the chromatogram of the 4-desmethylsterols. The lathosterol contained a small amount of cholesterol when purchased, but after recrystallization this was reduced to a negligible level. The separations between some peak pairs in Figure 1, i.e., campesterol/24-methylcholesta-5,23-dienol (peaks 2 and 3) and 24-ethylcholesta-5,23-dienol/a minor Δ^7 sterol (peak 5 and the adjacent unresolved peak), were not improved when other columns were tested, such as a 50-m 5%-phenylmethylsilicone and a 30-m 50%-phenylmethyl silicone column.

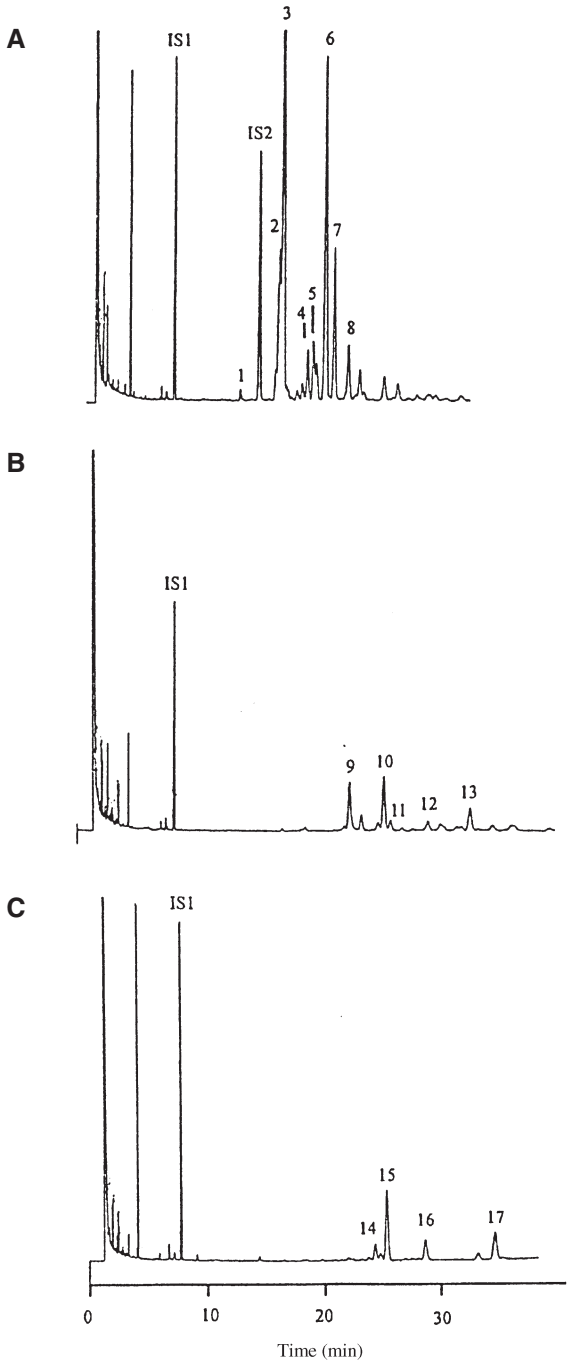


FIG. 1. GC separation of the (A) 4-desmethyl-, (B) 4-monomethyl-, and (C) 4,4-dimethylsterols from borage oil on a 30 m × 0.32 mm CPSil 8CB column (5% phenylmethylsilicone; Chrompack, Middelburg, The Netherlands). IS1, IS2, internal standards 1 and 2, respectively. See Table 2 for peak designations.

The compositions of the 4-desmethyl-, 4-monomethyl-, and 4,4-dimethylsterols are presented in Table 1, and the MS data are presented in Table 2. The sterol structures are depicted in Scheme 1.

4-Desmethylsterols. Most of the sterols identified here were Δ^5-sterols, which means that they have a double bond at

TABLE 1
The Sterol Composition of Borage Oil According to GC Analysis as Trimethylsilyl Ether Derivatives

Peak no.	Name	GC RRT[a]	Composition (%)
Desmethylsterols[b]			
1	Cholesterol	1.69	Trace[c]
2	24-Methyl-5,23-cholestadienol	2.10	14
3	Campesterol	2.15	33
4	24-Methyl-5,24(25)-cholestadienol	2.40	4
5	24-Ethyl-5,23-cholestadienol	2.46	4
6	Sitosterol	2.59	23
7	Isofucosterol	2.68	10
8	24-Ethyl-5,24(25)-cholestadienol	2.83	4
	Other unidentified sterols		6
4-Monomethylsterols[d]			
9	Obtusifoliol	2.81	26
10	Gramisterol	3.17	29
11	Cycloeucalenol	3.24	6
12	4α,24-Dimethylcholesta-7,24-dienol	3.63	5
13	Citrostadienol	4.07	13
	Other unidentified 4-monomethylsterols		21
4,4-Dimethylsterols[d]			
14	α-Amyrin	3.09	10
15	Cycloartenol	3.22	46
16	24-Methylenecycloartanol	3.65	15
17	9,19-Propanesterol, not identified	4.41	25
	Other unidentified 4,4-dimethylsterols		4

[a]The relative retention times (RRT) were determined in relation to 5α-cholestane.
[b]Lathosterol was used as an internal standard.
[c]Less than 0.5%. The common names and their equivalent systematic names are: cholesterol, cholest-5-en-3β-ol; campesterol, 24α-methylcholest-5-en-3β-ol; sitosterol, 24α-ethylcholest-5-en-3β-ol; isofucosterol, 24(*Z*)-ethylidenecholest-5-en-3β-ol; obtusifoliol, 4α,14-dimethyl-24-methylene-5α-cholest-8-en-3β-ol; gramisterol, 4α-methyl-24-methylenecholest-7-en-3β-ol; cycloeucalenol, 4α-14-dimethyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol; citrostadienol, 4α,methyl-24(*Z*)-ethylidenecholest-7-en-3β-ol; cycloartenol, 4,4,14-trimethyl-9β,19-cyclo-5α-cholest-24-3en-3β-ol; 24-methylenecycloartanol, 4,4,14-trimethyl-24-methylene-9β,19-cyclo-5α-cholest-24-3en-3β-ol.
[d]5-α-Cholestane was used as an internal standard and the figures are corrected for losses in the preparation.

position 5 in the steroid skeleton. Also, they contained 28 or 29 carbon atoms, which is typical for phytosterols. The monounsaturated Δ^5-sterols campesterol and sitosterol (peaks 3 and 6), common in vegetable oils, together constituted more than 50% of the desmethylsterol fraction. Cholesterol (peak 1) was also present but only in trace amounts. Furthermore, the fraction contained several diunsaturated Δ^5-sterols with a second double bond in the side chain. Their double bonds appeared at the positions C-23, C-24(25), and C-24(28). Two Δ^{23}-sterols were found, 24-methylcholesta-5,23-dienol and 24-ethylcholesta-5,23-dienol (peaks 2 and 5), which together constituted approximately 18% of the fraction. One sterol with its side chain double bond at C-24(28), isofucosterol (peak 7), constituted 10% of the fraction. Two Δ^5-sterols with their second double bond at position C-24(25) were also found, 24-methylcholesta-5,24(25)-dienol and 24-ethylcholesta-5,24(25)-dienol (peaks 4 and 8), which together made up 8% of the fraction. Two minor, unidentified peaks between campesterol and methylcholesta-5,24(25)-dienol, and two toward the end of the chromatogram, each one constituting less than 2% of the fraction, were recorded as "others" in Table 1. One of the peaks that eluted closely after campesterol was probably stigmasterol, but the peaks were too small to produce identifiable spectra. A small peak, believed to be a Δ^7-sterol, eluted closely after 24-ethylcholesta-5,23-dienol, and it was included in the category "others."

In the mass spectra of campesterol and sitosterol (peaks 3 and 6), ions typical of silylated Δ^5-sterols were found. The most prominent ions in the mass spectra were obtained by loss of trimethylsilyl hydroxide (TMSOH). This gave ions at M – 90, corresponding to the *m/z* values 382 and 396 for campesterol and sitosterol, respectively. Other abundant ions, at *m/z* 357 for campesterol and *m/z* 381 for sitosterol, are derived from the combined loss of TMSOH and a methyl group (M – 90 – 15). Furthermore, intense peaks at *m/z* 129 and M – 129, typical of silylated Δ^5-sterols, were found. For campesterol, M – 129 corresponds to an ion at *m/z* 343 and for sitosterol an ion at *m/z* 357. The remaining sterols identified were diunsaturated sterols with an additional double bond in the side chain. With the exception of the ions described above, which are common for both monounsaturated and diunsaturated sterols, their mass spectra contained peaks deriving from cleavages of the side chain. A peak common for most diunsaturated sterols is obtained through loss of the side chain

TABLE 2
Mass Fragmentation Patterns for Sterols in Borage Oil Analyzed as Trimethylsilyl Ether Derivatives

Peak no.		Mass fragmentations
Desmethylsterols		
1	Cholesterol[a]	
2	24-Methylcholesta-5,23-dienol	470(8), 455(26), 386(36), 380(33), 371(7), 343(47), 341(39), 296(26), 283(35), 253(24), 129(88)
3	24-Methylcholesterol (campesterol)	472(65), 457(15), 382(86), 367(35), 345(6), 343(100), 261(12), 255(16), 213(11), 129(77)
4	24-Methylcholesta-5,24(25)-dienol	470(22), 455(15), 386(100), 380(20), 371(39), 365(6), 341(15), 296(39), 281(26), 257(34), 129(75)
5	24-Ethylcholesta-5,23-dienol	484(40), 469(17), 394(31), 355(22), 343(64), 283(100), 253(22), 129(45)
6	24-Ethylcholesterol (sitosterol)	486(53), 471(4), 396(100), 381(38), 357(95), 255(33), 213(13), 129(92)
7	Isofucosterol	484(14), 469(12), 394(5), 386(100), 371(14), 355(6), 343(5), 296(62), 281(25), 257(27), 253(5), 129(48)
8	24-Ethylcholesta-5,24(25)-dienol	484(38), 469(19), 394(18), 386(100), 371(24), 355(14), 343(20), 296(57), 281(23), 257(22), 253(9), 129(56)
4-Monomethylsterols		
9	Obtusifoliol	498(88), 493(100), 469(8), 399(4), 393(78), 379(7)
10	Gramisterol	484(66), 469(28), 400(38), 394(18), 379(15), 357(100)
11	Cycloeucalenol[b]	
12	4α,24-Dimethylcholesta-7,24-dienol	498(21), 484(54), 469(29), 400(88), 379(14), 357(100)
13	Citrostadienol	498(11), 483(11), 408(4), 400(100), 393(7), 357(84)
4,4-Dimethylsterols		
14	α-Amyrin	498(100), 483(85), 393(96), 218(47)
15	Cycloartenol	498(5), 483(11), 408(100), 393(60), 365(42), 339(42), 286(20)
16	24-Methylenecycloartanol	512(7), 497(10), 422(100), 407(54), 379(43), 353(18), 300(18)
17	9,19-Propanesterol, not identified	514(3), 499(9), 424(100), 409(58), 381(43), 355(35), 302(17)

[a]See Table 1 for definitions.
[b]Spectrum missing.

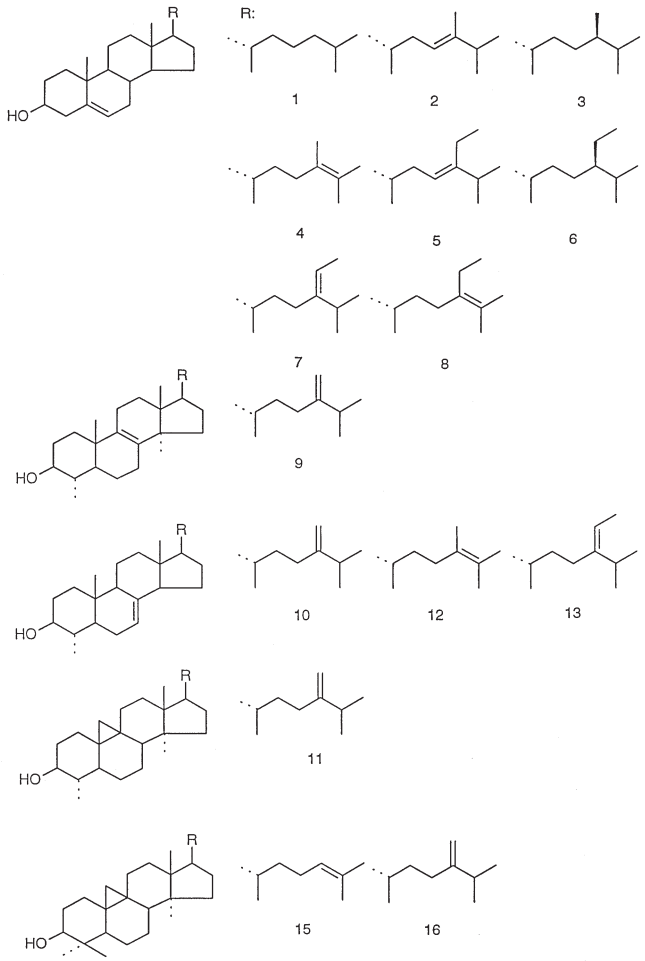
and two hydrogen atoms, which gives a fragmentation at *m/z* 343. A further loss of TMSOH gives a fragmentation at *m/z* 253 (7). These two ions were found in all the spectra of the diunsaturated sterols except for 24-methylcholesta-5,24(25)-dienol (peak 4). However, to distinguish between the diunsaturated sterol peaks, other typical fragmentations are required.

In the spectra of the Δ^{23}-sterols, 24-methyl-5,23-cholestadienol and the ethyl analog 24-ethyl-5,23-cholestadienol (peaks 2 and 5), an intense ion at *m/z* 283, characteristic of these sterols, appears (8,9). This ion is the result of an allylic cleavage from the Δ^{23} double bond combined with the loss of TMSOH. The 283 ion is stronger for the ethyl analog, indicating that in this case a more stable fragmentation is formed. A minor sterol, not fully separated from 24-ethylcholesta-5,23-dienol, could not be identified because the intensity of the ions in the spectrum was too low. The peak was, however, believed to be a Δ^7-sterol because it contained a less intense peak at *m/z* 129 and a significant peak at *m/z* 229. These features are characteristic of Δ^7-sterols (9). All the other sterols found had a large peak at *m/z* 129, which is typical of Δ^5-sterols.

For the identification of Δ^{24}-sterols, both the $\Delta^{24(25)}$-sterols and the $\Delta^{24(28)}$-sterol, an ion at *m/z* 386 is of importance. This peak is found in the spectra of 24-methyl-5,24(25)-cholestadienol, 24-ethyl-5,24(25)-cholestadienol, and the $\Delta^{24(28)}$-sterol isofucosterol (peaks 4, 8, and 7). The fragmentation at *m/z* 386 is formed by allylic cleavage from the Δ^{24} double bond. An abundant ion at *m/z* 296 derived from a combination of allylic cleavage and loss of TMSOH is also present. Another ion characteristic of Δ^{24}-sterols appears at *m/z* 257 (7). This is formed from allylic cleavage and loss of a fragmentation of 129 mass units. The loss of 129 mass units is common for most silylated Δ^5-sterols. Only slight differences between these ions typical of Δ^{24}-sterols were found when their spectra were compared. The methyl and ethyl analogs can be distinguished by their difference in M.W., but to differ between $\Delta^{5,24(28)}$- and $\Delta^{5,24(25)}$-sterols, retention times must be used. The C-24(25)-sterols have longer retention times than their corresponding C-24(28)-sterols on nonpolar columns.

The identification of campesterol and sitosterol was accomplished by comparison with a spectrum from authentic material, whereas the other sterols were identified by careful interpretation of retention data and mass spectra and comparisons with GC data and GC–MS data from the literature (2,7–11). The 24α-orientation, which is common for sterols in vegetable oils, has been assumed in the identification. To confirm this, NMR would be of great value.

4-Monomethylsterols. The predominant sterols in the 4-monomethylsterol fraction were obtusifoliol, gramisterol, and



SCHEME 1

citrostadienol (peaks 9, 10, and 13). Cycloeucalenol and 4α,24-dimethylcholesta-7,24-dienol (peaks 11 and 12) were also found in this fraction. Cycloeucalenol was identified by means of its GC retention time because no satisfactory spectrum of the sterol could be obtained. All these 4-monomethylsterols are common in vegetable oils (2).

4,4-Dimethylsterols. Two 4,4-dimethylsterols, cycloartenol and methylenecycloartanol (peaks 15 and 16), together constituted about 60% of this fraction. Both these dimethyl sterols are 9,19-cyclopropanesterols, so they have a cyclopropane ring in their steroid skeleton. Typically, 9,19-cyclopropanesterols contain a triplet of mass fragmentations at M – 133, M – 159, and M – 212 in their mass spectra. One other 9,19-propanesterol (peak 17) made up 25% of this fraction. Its mass spectrum suggested that it might be methylcycloartenol, since the spectrum was very similar to that of cycloartanol, but with peaks at *m/z*-values 14 mass units higher. However, this could not be confirmed by comparison with a spectrum of either an authentic sample or of one from the literature. α-Amyrin, a pentacyclic triterpene (peak 14), was also present in significant amounts. Except for the last 9,19-cyclopropanesterol, the 4,4-dimethyl sterols found are common in vegetable oils (2).

Sterols with a second double bond at position C-23 in the side chain have not previously been identified in vegetable oils. They have, however, been found in the pollen of broad bean (*Vicia faba* L.) (12) and in corn root lesion nematodes (10). The sterol pattern of the pollen of broad bean greatly resembles that of borage oil because it contains campesterol, sitosterol, and Δ^{23}-, $\Delta^{24(28)}$-, and $\Delta^{24(25)}$-sterols, but not stigmasterol (borage oil contains only traces of stigmasterol). In the corn root lesion nematodes, *Pratylenchus agilis*, Δ^{23}- and $\Delta^{24(25)}$-sterols were found in small amounts. Some of these sterols are involved in the biosynthesis of the major sterols of corn (10). A Δ^{23}-sterol, 24-methylcholesta-5,23-dienol (ergosta-5,23-dienol in the reference), and the 4,4-dimethylsterol, methylcycloartanol, are also believed to participate in the biosynthesis of sterol end products of sorghum (13).

Sterols in borage oil have been determined previously by Iatrides *et al.* (14). Their gas chromatograms were quite similar to ours but more compressed since they used a higher oven temperature. However, their identification results deviate from those reported here with respect to the diunsaturated sterols; they found one additional $\Delta^{24(28)}$-sterol, 24-methylenecholesterol, but no Δ^{23}- or $\Delta^{24(25)}$-sterols. The identification in the cited study was reportedly based on MS and NMR. They analyzed two different borage oils of French origin, one commercially produced and one extracted in the laboratory. Our borage oil was of Swedish origin, commercially produced and of refined quality. We analyzed several batches in our laboratory with reproducible results. It has been reported that the qualitative and quantitative composition of sterols in oil appears to be independent of the cultivar, harvesting period, and location of cultivation, with the exception of those derived from corn and wheat (3). The differing results from the analysis of French and Swedish borage oil indicate that borage oil may belong to this latter group.

Because of the presence of several diunsaturated desmethylsterols with their double bonds close to each other, such as the Δ^{23}-, $\Delta^{24(28)}$-, and $\Delta^{24(25)}$-sterols, it might be suggested that isomerization occurred during our isolation process. Ikekawa *et al.* (15) showed that the C-24(28)-sterols, fucosterol and 24-methylenecholesterol, both isomerize in the presence of iodine and heat, giving a mixture of $\Delta^{5,23}$-, $\Delta^{5,24(25)}$-, and $\Delta^{5,25}$-sterols in equal amounts. Since no $\Delta^{5,25}$-sterols were found in our desmethylsterol fraction, it is not likely that any isomerization occurred during the sample preparation. Our isolation procedure is also well known and has been frequently used by many researchers without isomerization problems.

Borage oil has a distinctive sterol pattern, which can be used to distinguish it from other oils. The 4-monomethylsterol and 4,4-dimethylsterol composition did not provide any additional information concerning the identity of the oil. Thus, the desmethylsterols can be used to distinguish borage oil from other oils. Furthermore, it has been shown that the TLC–GC method was suitable for this purpose. Alternatively, the desmethyl sterol composition can be determined without fractionation by TLC because the 4-desmethyl-, 4-monomethyl-, and 4,4-dimethylsterols elute separately with

only one overlap between the 4-desmethyl- and 4-monomethyl fractions.

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