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SEPARATION OF SULFUR-CONTAINING FATTY ACIDS FROM GARLIC, *ALLIUM SATIVUM*, USING SERIALLY COUPLED CAPILLARY COLUMNS WITH CONSECUTIVE NONPOLAR, SEMIPOLAR, AND POLAR STATIONARY PHASES ◈

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SUMMARY

A GC–MS method with serially coupled capillary columns containing consecutive nonpolar, semipolar, and polar stationary phases has been used for determination of fatty acids in garlic (*Allium sativum*). Saturated (14:0, 15:0, 16:0, and 18:0), unsaturated (7-16:1, 7-18:1, 9-18:1, 9,12-18:2, 9,12,15-18:3), and unusual cyclic sulfur-containing fatty acids in garlic were identified by GC–MS.

INTRODUCTION

Sulfolipids were originally discovered in diatom species, in 1966, by Morris Kates [1–3]. High levels of these sulfolipids have been found in the alga *Ochromonas danica* (Chrysophyceae, Chrysophyta) where they constitute 15% of total lipids and 3% of the dry weight of heterotrophically grown, stationary-phase cells [4,5]. More recently, sulfolipids have also been identified in more than 30 species of green (Chlorophyceae), brown (Phaeophyceae), and red (Rhodophyceae) freshwater and marine macrophytic algae [6–8] and other microalgal species [9–14]. The presence of sulfolipids in algae, diatoms, and marine invertebrates has been reported in several review articles [15–20].

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Sulfur-containing fatty (carboxylic) acids (SFA) are rare in nature. Several sulfur-containing fatty acids have been isolated from garlic; they are also produced by some microorganisms. 1,2-Dithiacyclopentane-3-carboxylic acid (also known as tetra-norlipoic or tetranorthioctic acid) was recently isolated from two varieties of garlic (*Allium sativum* var. *sativum* and *Allium ampeloprasum* var. *holmens*) cultivated in the North of Iran [21,22]. 3-(Methylsulfinyl)alanine has been detected in different onion cultivars (*Allium cepa*) [23] and is present among the free amino acids in two species of *Allium sativum* var. *opioscorodon* (hardneck) and *Allium sativum* var. *sativum* (softneck) [24]. (*S*)-2-Propenyl-D-cysteine has been isolated from garlic (Aomori Prefecture, Japan) after heating from 30 to 65°C [25]. 3-Allylthiopropionic acid has been detected among the volatile organic compounds obtained from fresh and frozen dried garlic (*Allium sativum*, collected in South Korea) [26] and in this study. Three-hundred and forty *Streptomyces* species grown in the presence of DL-methionine synthesized 3-methylthiopropionic and *trans*-3-methylthioacrylic acids [27]. 3-Methylthioacrylic acid, a potential herbicide, also produced by *Streptomyces kasugaensis* SK 619, inhibited growth of *Photobacterium phosphoreum* at concentrations of 50–200 μg per disk [28]. Derivatives of 3-methylthiopropionic and *trans*-3-methylthioacrylic acids, named entadamide A and entadamide B, have been isolated from the dry seeds of *Entada phaseoloides* [29,30]. Their glycosides were obtained from the same plant [31]. Entadamide C and entadamide A has been isolated from the leaves of *Entada phaseoloides* [32].

The structure of 4-thiapentanoic (methylthiopropionic) acid formed from methionine has also been reported [33,34]. Three isomeric epithio stearic acids have been isolated as minor components of canola oil [35]. The biological activity of these fatty acids and some their medicinal effects have been reported recently [36].

In this study we continued our investigation of food products [37] and attempted to characterize the sulfolipids of garlic which grows in the Middle East.

EXPERIMENTAL

Samples

Garlic cloves (*Allium sativum*) were purchased in local stores in Jerusalem. The garlic was obtained directly from the growers – Vegetable

Beth-Shemesh Farm, near Jerusalem. For irrigation Vegetable Beth-Shemesh Farm uses underground water containing a high concentration of H_2S [37].

Extraction and Preparation of Methyl Esters

Fresh garlic cloves (1.8 kg) were peeled and the kernels were homogenized at high speed. The resulting suspension was filtered, at +2°C, through gauze then a large porous membrane. Fatty acids were extracted three times with a cold (−5°C) 1:1 (*v*/*v*) mixture of dichloromethane and methanol. The combined dichloromethane–methanol extracts were evaporated under reduced pressure at 10°C. The garlic oil obtained was divided into two parts, one of which was agitated with benzene and sodium methoxide in methanol for 2 h at room temperature and left to stand overnight. Methyl esters of the fatty acids were obtained by transesterification, as described elsewhere [33,34], and crystallized overnight at 0°C from a solution of methanol and urea (50 g urea dissolved in 300 mL methanol). The precipitate was filtered, washed with cold methanol saturated with urea, and, after dilution with water, the methyl esters were extracted with diethyl ether. Methyl esters of both fractions were separated on TLC plates (20 cm × 20 cm, 0.25 mm, with fluorescent indicator; Merck) using hexane–diethyl ether–acetic acid 80:20:10 (*v*/*v*) as mobile phase. The two main zones (visible under UV illumination) on TLC plates were scraped from the plate, extracted with diethyl ether, and stored at −20°C until GC–MS analysis.

GC–MS

GC–MS was performed with a Hewlett-Packard (HP) 5890 (series II) chromatograph modified for use with glass capillary columns and coupled with an HP 5971B MSD mass-selective detector. Sulfur compounds and fatty acid methyl esters were analyzed by gas chromatography on three coupled capillary columns, as described elsewhere [16] – first, a 10 m × 0.32 mm i.d. column coated with a 0.25 μm film of HP-5, second a 30 m × 0.32 mm i.d. column coated with a 0.25 μm film of RTX-1701 (Restek, PA, USA), and third a 30 m × 0.32 mm column coated with a 0.25 μm film of HP-FFAP. The GC oven was maintained at 40°C for 2 min then programmed at 2° min^{-1} to 300°C which was maintained for 20 min. The injector was used in splitless mode at 180°C. Helium was used as carrier gas at a flow rate of 25 cm s^{-1}. The MS detector was operated at 194°C,

with an ionization energy of 70 eV. Scanning between *m*/*z* 30 and 650 was performed at 0.9 scans s^{-1}. The solvent delay was 10 min. The fatty acid methyl esters were identified from their mass spectral fragmentation and by use of the Wiley (7th edition) mass spectral library search. Mass spectra obtained for SFA were compared with those obtained from synthesized analogues.

RESULTS AND DISCUSSION

Sulfur compounds and methyl esters of fatty acids from garlic were analyzed by GC–MS on three coupled capillary columns [38]. By using both a typical injection port and also 'cryogenic GC–MS' analysis [37] we identified 2-vinyl-4*H*-1,3-dithiin and 3-vinyl-4*H*-1,2-dithiin as the two major compounds, in addition to di-2-propenyl trisulfide, in garlic extract. Separation of the sulfur compounds and the fatty acid methyl esters is shown in Fig. 1. Among the fatty acids identified are palmitic and stearic

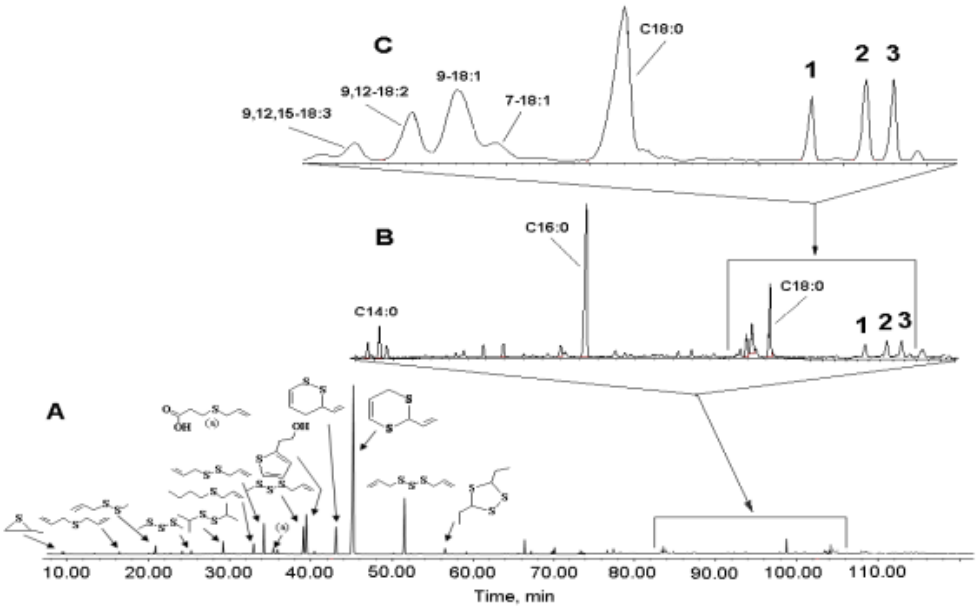

Fig. 1

GC–MS chromatogram of organosulfur compounds and methyl esters of fatty acids from garlic (*Allium sativum*). Separation was achieved by use of serially coupled capillary columns with stationary phases of different polarity. A. Full chromatogram (up to 120 min). B. Part of the chromatogram showing separation of the fatty acid methyl esters. C. Part of the chromatogram showing separation of the C_{18} family of acids, including the novel SFA (**1**, **2**, and **3**)

acids as major compounds, with C14:0, C15:0, 7-16:1, 7-18:1, 9-18:1, 9,12-18:2, 9,12,15-18:3, and three novel cyclic SFA – 8-(5-hexylthiophen-2-yl)octanoic acid, **1**, 8-(5-hexyl-tetrahydrothiophen-2-yl)octanoic acid, **2**, and 8-(6-pentyl-tetrahydro-2*H*-thiopyran-2-yl)octanoic acid), **3**. The structures of SFA isolated from garlic and from other plants are shown in Fig. 2. Mass spectra of these SFA were obtained and are shown in Fig. 3.

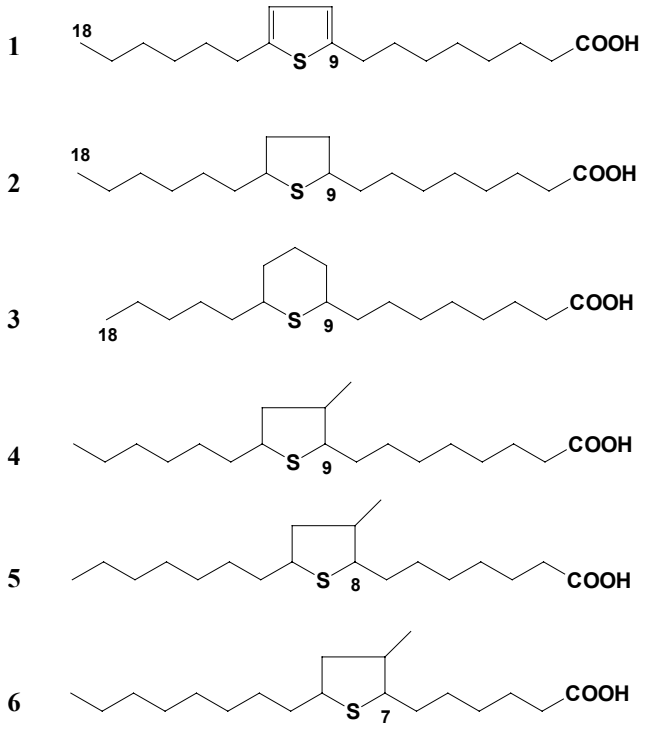

Fig. 2

Thiopyran and thiophene derivatives and SFA isolated from garlic (**1**, **2**, **3**) and from canola oil (**4**, **5**, **6**)

8-(5-Hexylthiophen-2-yl)octanoic acid methyl ester (**1**, Fig. 3A) was characterized by the presence of a major fragment formed by β-cleavage of the thiophene ring of the alkylcarbomethyl moiety (*m*/*z* 181), from the M − 31 fragment (loss of a methoxy group, *m*/*z* 293), and from the molecular ion (*m*/*z* 324). β-Cleavage of the alkyl moiety (*m*/*z* 253) is less intense and the relative abundance of this ion is also reduced. Loss of both side-chains and transfer of one hydrogen atom to the thiophene ring gives rise

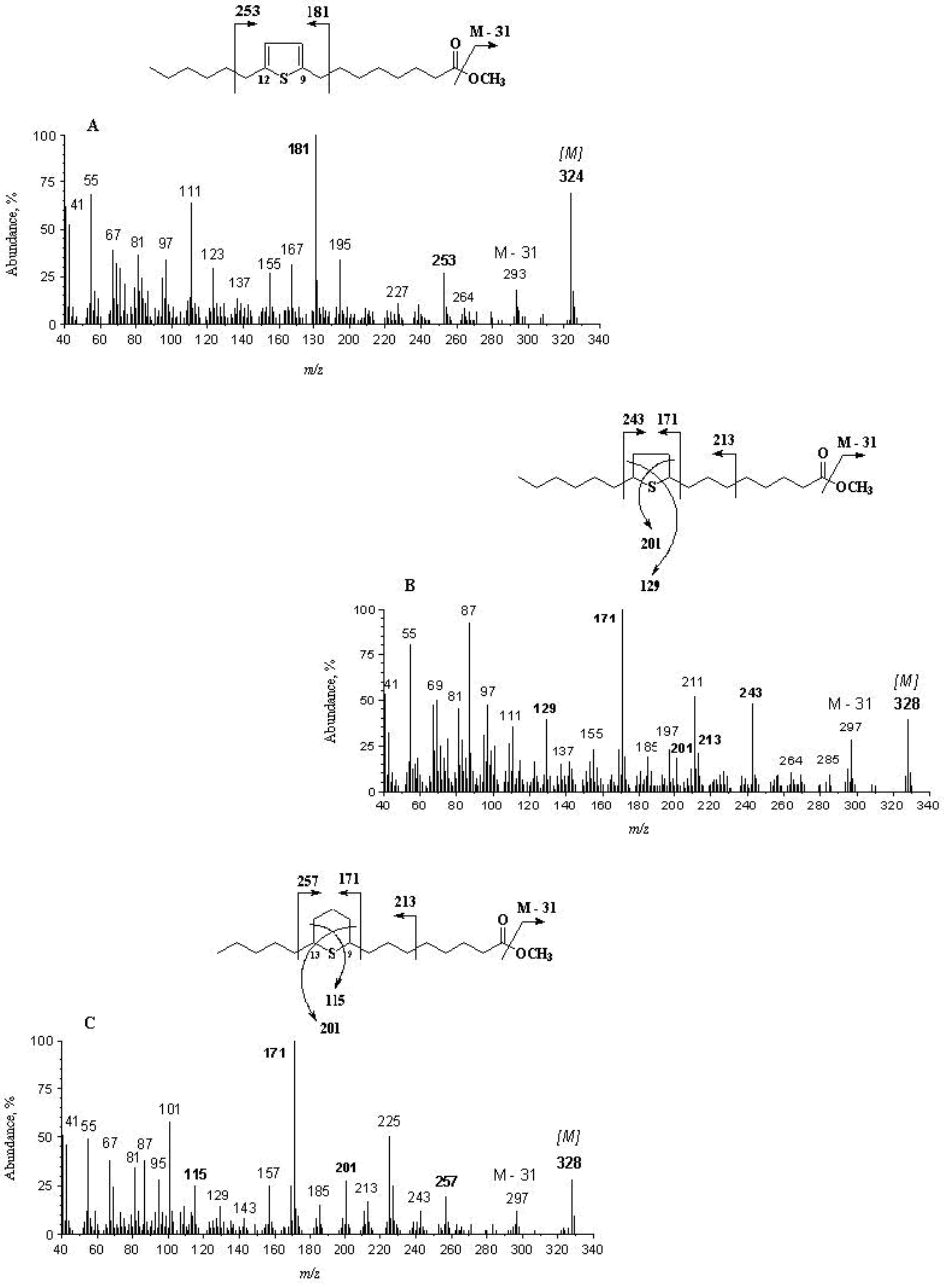

Fig. 3

Mass spectral fragmentation pathways proposed for the three unusual C_{18} sulfur-containing fatty acids isolated from garlic. Further details of these fragmentations are given in the text.

to secondary fragments (*m*/*z* 97, 111, and 123). The McLafferty rearrangement ion (*m*/*z* 74) for the carboxymethyl group is not significant and fragmentation of the whole molecule is determined by the thiophene ring structure.

Fragmentation of the methyl ester of 8-(5-hexyltetrahydrothiophen-2-yl)octanoic acid (**2**, Fig. 3B) furnished two main fragments, *m*/*z* 243 and 171, produced by α-cleavage of the ring of the alkyl and alkylcarboxymethyl moieties, respectively. Cleavage of both moieties and subsequent hydrogen transfer to the thiolane ring gives ions of *m*/*z* 87 and 101. Fragmentation through the ring gives ions of *m*/*z* 129 and 201.

The major fragmentation of 8-(6-pentyl-tetrahydro-2*H*-thiopyran-2-yl)octanoic acid methyl ester (**3**, Fig 13C) is similar to that of the methyl ester of the alkylthiolane acid (**2**, Fig. 1B). α-Cleavage of the side chains of the ring gives fragments of *m*/*z* 171 and 257. Loss of both chains and hydrogen transfer to the ring gives an ion of *m*/*z* 101. Fragmentation through the ring gives ions of *m*/*z* 115 and 201. The mass spectra obtained for all the SFA were compared with spectra of sulfur-containing compounds isolated from sedimentary organic matter and crude oil [39–42].

Epithio acid structures may be related to furanoid fatty acids with oxygen in the ring in lieu of sulfur, which are known, and found in oils from algae, plants, fish, and invertebrates [43,44]. A mechanism for their formation in nature has been published [45,46].

The two cyclic SFA **1** and **2** contain thiophene and tetrahydrothiophene rings, respectively. Derivatives of thiophene are widespread in plant species and also identified in the genus *Allium*: *A. chinense* [47], *A. ampeloprasum* var. *bulga* [48], *A. albidum, A. altaicum, A. carolinianum, A. hymenorrhizum, A. lineare, A. nutans, A. ramosum*, and *A. tuberosum* [49], *A. cepa* and *A. porum* [50], *A. sativum* var. *sativum* and *A. sativum* var. *holmense* [21,22], among others.

The cyclic SFA **3** contains a thiopyran ring. Thiopyran derivatives are rare in nature, but have been detected in garlic and some other plants. 3,6-Dihydro-2*H*-thiopyran and 2*H*-thiopyran-3(6*H*)-one have been detected in dried Jimbu (*Allium wallichii*) [51]. Derivatives of thiophene have been isolated from fresh Greek garlic (*Allium sativum*) cloves [52] and from Chinese garlic [53]. Tetrahydro-2*H*-thiopyran-2-carboxylic acid has been isolated from the rod-shaped bacterium *Tubercle bacillus* [54].

Three isomeric epithio stearic acids, 9,12 (**4**), 8,11 (**5**), and 7,10 (**6**), each with a methyl substituent on the ring (Fig. 2), have been isolated

as minor sulfur-bearing fatty acids from unprocessed canola oil (*Celtic mythology*) [35].

Analysis of garlic extracts has shown that samples collected from different locations have different fatty acid profiles. The flavor compounds of *Allium mairei* collected in Kunming Province, China were prepared by use of simultaneous distillation and extraction equipment. Six fatty acids were detected: C7:0, C9:0, C14:0, C15:0, C16:0, and C18:0 [55,56]. Analysis of the fatty acids of the seed oil of *Allium tuberosum* collected in Shanghai Province revealed the presence of many important fatty acids, including linoleic (9,12-18:2; the amount varying in different samples from 57.0 to 71.6%), palmitic (C16:0, 6.6–9.7%), 9-18:1, 11-20:1, C20:0, C22:0, C23:0, and C24:0 [57]. The flowers of *Allium tenuissimum* from Shanxi Province contain C16:0, C19:0, and 9,12-18:2 as major fatty acids [58].

The unique fatty acid (*Z*,*Z*)-2,5-octadecadienoic acid has been identified with C16:0, 11,14-eicosadienoic, and 2,3-dihydroxybutanedioic acids in the aerial part of *Allium tuberosum* [59].

CONCLUSIONS

We have demonstrated that garlic contains low levels of cyclic SFA. It is possible that the SFA in garlic oil are derived from stable polar lipids and/or triacylglycerides. We have also found that the polar lipid fraction contains other sulfur compounds. Identification of these sulfolipid compounds is in progress.

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