Identification and Quantification of Organosulfur Compliance Markers in a Garlic Extract

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Marker compounds are needed to determine dietary compliance in free-living human study populations participating in dietary intervention trials for cancer research. Nine organosulfur marker compounds were detected and identified in an aged garlic extract. A convenient method that involves solvent extraction and gas chromatographic/mass spectrometric analysis was developed to quantify the organosulfur compounds in the garlic extract. Although the garlic extract proved to be unstable and the concentration of the organosulfur compounds varied with time, one analysis of the extract gave the following results: methyl disulfide (0.607 μg/g), methyl trisulfide (0.181 μg/g), allyl sulfide (2.02 μg/g), allyl disulfide (0.784 μg/g), allyl trisulfide (0.795 μg/g), allyl methyl sulfide (1.64 μg/g), allyl methyl disulfide (0.411 μg/g), allyl methyl trisulfide (0.695 μg/g), and ethyl 2-propenesulfinate (11.4 μg/g).

INTRODUCTION

Many natural components of food have been designated anticarcinogens because they inhibit various initiating and promoting mechanisms of chemical carcinogenesis (Ames, 1983; Wattenberg, 1985). Plants of the genus *Allium*, including garlic (*A. sativum* L.), onion (*A. cepa* L.), and caucas (*A. victorialis* L.), are sources of various organic sulfides with anticarcinogenic activity (Belman, 1983; Block, 1985). For example, garlic oil contains allyl sulfide, which inhibits color cancer in mice exposed to 1,2-dimethylhydrazine (Wargovitch, 1987). Also, allyl methyl trisulfide, allyl methyl disulfide, allyl trisulfide, and allyl sulfide all inhibited benzo[*a*]pyrene-induced neoplasma of forestomach and lung of female A/J mice when administered 96 and 48 h prior to carcinogen challenge (Sparnins et al., 1988).

Compliance markers are needed to determine dietary compliance in free-living human study populations participating in dietary intervention trials for cancer research. Detection of the markers at appropriate concentrations in the serum of participants in the trials would provide evidence that the participants are in fact following the prescribed diet. Anticarcinogenic foods such as garlic extract that contain naturally occurring phytochemical compliance markers would have the advantage of not being restricted by new drug requirements and would possess adequate safety as defined by the Food and Drug Administration. Southern Research Institute (SRI) undertook the identification and quantification of major organosulfur compounds present in an aged garlic extract on a contract with the National Cancer Institute.

Volatile, nonpolar sulfur-containing compounds previously reported in *Allium* species such as garlic or caucas include allyl mercaptan (Nishimura et al., 1988), allyl methyl sulfide (Nishimura et al., 1971, 1988), allyl methyl disulfide (Brodnitz et al., 1971; Nishimura et al., 1971, 1988; Yu et al., 1989), allyl methyl trisulfide (Brodnitz et al., 1971; Nishimura et al., 1971; 1988; Yu et al., 1989), allyl *n*-propyl sulfide (Nishimura et al., 1988), allyl *n*-propyl disulfide (Nishimura et al., 1971, 1988; Yu et al., 1989), allyl sulfide (Nishimura et al., 1971, 1988; Yu et al., 1989), allyl disulfide (Brodnitz et al., 1971; Block et al., 1986); Nishimura et al., 1971, 1988; Yu et al., 1989), allyl trisulfide (Brodnitz et al., 1971; Nishimura et al., 1988; Yu et al., 1989), and allyl tetrasulfide (Block et al., 1986).

EXPERIMENTAL PROCEDURES

Materials. *Reagents.* High-purity acetone, methanol, and methylene chloride and GC[2] grade pentane were obtained from Burdick and Jackson (McGaw Park, IL). Acetone-d_6 (minimum isotopic purity 96.96 atom %), peracetic acid (32 wt %), and hydrogen peroxide (30 wt %) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sodium chloride (AR grade) was obtained from Mallinckrodt Inc. (St. Louis, MO). Phenylthiourea (BDA microanalytical reagent) was obtained from British Drug Houses Ltd. (Poole, England). Deionized water was prepared at SRI using a Millipore Milli-Q water purification system.

Sample. Twelve 60-mL bottles of aged garlic extract were provided to SRI by the National Cancer Institute, which had obtained the garlic extract from Wakunaga of America (Mission Viejo, CA) on a materials-transfer agreement. The extract was stored at 2–6 °C in a refrigerator.

Components. Methyl disulfide, allyl sulfide, and *allyl methyl sulfide* were obtained from Aldrich. The purity of each compound was listed as 97–99%. *Methyl trisulfide* was obtained from Pfaltz & Bauer, Inc. (Waterbury, CT), and the purity was listed as 97%. *Allyl disulfide* and *allyl methyl disulfide* were obtained from Columbia Organic Chemicals (Cassatt, SC), and the purity was listed as 95 or 97%. Gas chromatographic/mass spectrometric (GC/MS) analysis of each compound produced one major gas chromatographic peak and a mass spectrum that exhibited the expected parent and fragment ions.

Allyl trisulfide was obtained from Penta Manufacturing Co. (Fairfield, NJ). The purity was listed as 35%. Pure allyl trisulfide was isolated from the mixture by high-pressure liquid chromatography (HPLC) using a Keystone Scientific Co. (Bellefonte, PA) 250 × 10 mm, 12 μm, 120 Å, Hyperprep ODS semipreparative column. Isocratic elution (77% aqueous methanol) was used, and the flow rate was 3 mL/min. Because late eluting compounds were present, 1 mL of a solution prepared by saturating 10 mL of methanol with the allyl trisulfide solution was injected. All of the fraction that eluted between 5 and 30 min was collected. The fraction was diluted with water and extracted with pentane. The pentane was evaporated, and the residue was taken up in 1 mL of methanol. Injections (100–200 μL) were then made, and allyl trisulfide was collected over a period of 3 min. Each collected fraction was diluted with water and extracted with pentane. The pentane extracts were concentrated and reserved. The entire purification process was repeated four times. GC/MS of each product showed one major peak and a mass spectrum that was consistent with that of allyl trisulfide.

The four products were quantitatively transferred using pentane to a 10-mL pear-shaped flask. The solution was rotary evaporated to dryness using gentle heating and a water aspirator

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vacuum. The residue was dissolved in 1.00 mL of acetone-d_6 containing 0.0209 mmol of ethyl acetate. The molar ratio of allyl trisulfide and ethyl acetate was determined by proton NMR spectroscopy. The concentration of allyl trisulfide was then calculated to be 9.48 mg/mL of acetone-d_6 solution. The concentration of ethyl acetate in acetone-d_6 was originally determined by NMR spectroscopy using phenylthiourea as a primary standard.

Allyl methyl trisulfide was obtained from Columbia. The purity was listed as 93%, but GC/MS analysis revealed that the purity was much lower than this. Pure allyl methyl trisulfide was isolated from the mixture using semipreparative HPLC. The same column and flow rate previously described were used. Initially, isocratic elution (70% aqueous methanol) was used. Because late eluting compounds were present, 1 mL of a solution that was prepared by adding 1.2 g of crude methyl trisulfide to 3 mL of methanol was injected. All of the fraction that eluted between 13 and 22 min was collected. The fraction was diluted with water and extracted with pentane. The pentane was evaporated, and the residue was taken up in 1 mL of methanol. Injections (300 μL) were then made, and eluting allyl methyl trisulfide and an unknown component were collected over a period of 3 min.

Each collected eluent was diluted with water and extracted with pentane. The pentane extracts were combined, evaporated to dryness, and taken up in 0.5 mL of methanol. Allyl methyl trisulfide was isolated using gradient elution (85% aqueous methanol for 1 min, ramp to 56% aqueous methanol over 1.25 min, hold for 24 min, and then return to 85% aqueous methanol). Each collected fraction was diluted with water and extracted with pentane. The pentane extracts were concentrated and reserved. GC/MS showed one major peak and produced a mass spectrum expected for allyl methyl trisulfide.

The extract was quantitatively transferred using pentane to a 10-mL pear-shaped flask. The solution was rotary evaporated to dryness using gentle heating and a water aspirator vacuum. The residue was dissolved in 1.00 mL of acetone-d_6 containing 0.0209 mmol of ethyl acetate. The molar ratio of allyl methyl trisulfide and ethyl acetate was determined by proton NMR spectroscopy. The concentration of allyl methyl trisulfide was then calculated to be 23.6 mg/mL of acetone-d_6 solution.

Ethyl 2-propenesulfinate was synthesized in a two-step procedure. The method of Block et al. (1986) was modified to synthesize allyl 2-propenethiosulfinate (allicin) from allyl disulfide. The disulfide (0.0113 mol) was treated in cold methylene chloride with a small excess of 32% peracetic acid. After 30 min, the cold solution was neutralized with excess potassium carbonate and the suspension was filtered. The cold filtrate was concentrated in vacuo and gave what appeared to be a nearly quantitative yield of the crude thiosulfinate. However, the crude thiosulfinate was not identified or purified, but was used in the next step.

The method of Takata et al. (1982) was modified to synthesize ethyl 2-propenesulfinate from crude allicin. The thiosulfinate was dissolved in 70 mL of cold absolute ethanol. Hydrogen peroxide (30%, 0.0106 mol) and iodine (0.00014 mol) were quickly added, and the suspension was stirred until it was nearly homogeneous. The solution was allowed to stand at ambient temperature for about 18 h and was then diluted with an equal volume of water. The aqueous alcohol solution was further diluted with water and then extracted with methylene chloride. Evaporation of the extract produced the target compound.

Pure ethyl 2-propenesulfinate was isolated from a mixture using semipreparative HPLC. The same column and flow rate described above for allyl trisulfide was employed. Isocratic elution (40% aqueous methanol) was used. Because late eluting compounds were present, 200 μL of a solution prepared by adding 100 μL of the isolated product to 100 μL of methanol was injected, and all of the fraction that eluted between 5 and 12 min was collected. The eluent was diluted with water and extracted with methylene chloride. The solvent was evaporated, and the residue was taken up in 1 mL of methanol. Injections (100 μL) were then made, and ethyl 2-propenesulfinate was collected over a period of 3 min. Each collected fraction was diluted with water and extracted with methylene chloride. The methylene chloride extracts were concentrated and reserved. The entire purification process was repeated twice. GC/MS showed one major peak, and the mass spectrum of the component producing the major peak is shown in Figure 10b in the supplementary material. The molecular ion (M) is present at m/z 134, and major fragment ions include m/z 93 (M − allyl) and 64 (sulfur dioxide). (The ion at m/z 122 disappears when the temperature of the injection port, column, transfer line, and ion source is reduced.)

The extract was quantitatively transferred to a 10-mL pear-shaped flask. The solution was rotary evaporated to dryness using gentle heating and a water aspirator vacuum. The residue was dissolved in 1.00 mL of acetone-d_6 containing 0.0209 mmol of ethyl acetate. The molar ratio of ethyl 2-propenesulfinate and ethyl acetate was determined by proton NMR spectroscopy. The concentration of ethyl 2-propenesulfinate was then calculated to be 11.9 mg/mL of acetone-d_6 solution. The NMR spectrum is consistent with the assigned structure: ^{1}H NMR (acetone-d_6) δ 5.81 (m, 1, =CH−, J = 17.2 Hz, J = 10.1 Hz, J = 7.5 Hz), 5.37 and 5.36 (two overlapping multiplets, 2, CH_2=), 4.06 (q, 2, CH_3CH_2−), 3.48 (m, 2, −CH_2−), 1.29 (t, 3, CH_3CH_2−). (In addition, the sample contained 1.7 mmol of ethyl acetate/mol of ethyl 2-propenesulfinate. The δ values for ethyl acetate are 4.05, 1.97, and 1.20 ppm).

Surrogates and Internal Standards. 3-Chlorothiophene and thianaphthene were obtained from Aldrich. The purity was listed as 98 or 99%. 1,4-Dichlorobenzene-d_4 (99 atom % D) was obtained from Cambridge Isotope Laboratories (Woburn, MA). GC/MS of each component showed one major peak, and the mass spectrum of the component producing the peak exhibited the expected parent and fragment ions.

Instrumentation. A Hewlett-Packard Co. (Atlanta, GA) Model 5890 gas chromatograph was coupled to a VG 70S high-resolution mass spectrometer by means of a direct inlet for capillary column gas chromatography. A Waters (Division of Millipore, Milford, MA) Model 600 E multisolvent delivery system was connected to a Waters Model 484 variable-wavelength detector. Data from the variable-wavelength detector was sent to a Hewlett-Packard Model 3396 recording integrator, which in turn is interfaced to a CompuAdd (Austin, TX) Model 286 personal computer. The communication between the recording integrator and personal computer was established using Chrom Perfect software distributed by Justice Innovations, Inc. (Palo Alto, CA). The LC/UV data were collected as hardcopy on the recording integrator and stored on IBM-compatible disks in the personal computer. A Nicolet Co. (now General Electric NMR Instruments, Fremont, CA) NT 300B nuclear magnetic resonance spectrometer operated at 300.635 MHZ for observing protons. Data were stored on a magnetic disk, and hardcopy is produced using a Nicolet Zeta 8 digital printer.

Methods. *Large-Scale Extraction of Garlic Extract.* Garlic extract (66.9 g) was shaken with 60 mL of pentane and centrifuged, and 50 mL of the pentane phase removed. The pentane extract was concentrated to 0.5 mL in a Kuderna-Danish apparatus.

Gas Chromatographic/Mass Spectrometric Analyses. The gas chromatographic capillary column was a 25-m, 0.32-mm-i.d., HP-5 column coated with a 0.52-μm bonded-phase film of methyl silicone. The column was maintained at 35 °C for 3 min and then heated to 220 °C at 8 °C/min. The final temperature was held for 30 min. The solvent delay was 3.25 min. Splitless injections were made. The injection port was maintained at 220 °C, and the flow of helium carrier gas was maintained by a head pressure of 4.2 psig. The mass spectrometer transfer line was operated at 300 °C and the ion source at 260 °C. The ionization voltage was 70 eV. The magnet was scanned from mass 225 to 45 at 1 s/decade and with a rest time of 0.5 s. The nominal resolution was 1000.

Preparation of Standards. The surrogate solution contained the following components in pentane: thianaphthene (15.25 ng/μL) and 3-chlorothiophene (16.45 ng/μL). The internal standard solution contained 1,4-dichlorobenzene-d_4 (15.80 ng/μL) in pentane. An instrument calibration solution was prepared that contained in pentane methyl disulfide (1056 ng/mL), methyl trisulfide (761 ng/mL), allyl sulfide (9715 ng/mL), allyl disulfide (2278 ng/mL), allyl trisulfide (3240 ng/mL), allyl methyl sulfide (2666 ng/mL), allyl methyl disulfide (1237 ng/mL), allyl methyl trisulfide (4720 ng/mL), and ethyl 2-propenesulfinate (5250 ng/mL). By serial dilution, solutions that contained $^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$, and $^1/_{32}$ of these concentrations were then prepared. An additional

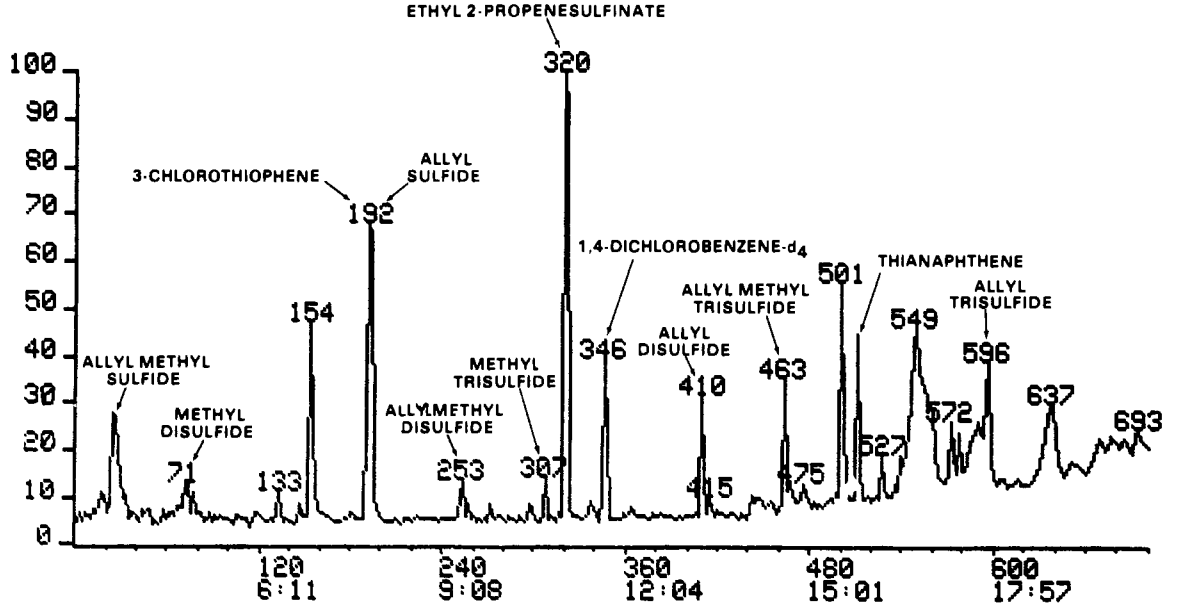

Figure 1. Total-ion chromatogram of garlic extract containing surrogates and internal standard.

Table I. Difference in Gas Chromatographic/Mass Spectrometric Retention Times of Components and 1,4-Dichlorobenzene-d_4 in Extracts from Garlic Extract and in Instrument Calibration Standards

component	relative GC/MS retention time difference (Δ scans)			
	sample run	calibration runs		
	1	1	2	3
1,4-dichlorobenzene-d_4[a]	0	0	0	0
methyl disulfide	274	274	274	275
methyl trisulfide	39	39	39	40
allyl sulfide	154	153	153	155
allyl disulfide	−64	−64	−64	−64
allyl trisulfide	−250	−250	−251	−250
allyl methyl sulfide	321	320	321	320
allyl methyl disulfide	93	93	93	94
allyl methyl trisulfide	−118	−117	−118	−117
ethyl 2-propenesulfinate	25	25	25	25

[a] Internal standard.

solution that contained ethyl 2-propenesulfinate at a concentration of 31 500 ng/μL was also prepared. Instrument calibration solutions were prepared by adding to 1 mL of each solution 25 μL of the surrogate solution and 25 μL of the internal standard solutions described above. Matrix spiking solutions that contained all of the components at appropriate concentrations were prepared. The solutions were stored in a refrigerator at 2–6 °C when not in use.

Extraction and Quantitative Determination of Target Compounds in Garlic Extract. A surrogate solution (50 μL), excess sodium chloride, and 2 mL of pentane were added to 1 g of garlic extract present in a disposable culture vial. The surrogate solution contained 3-chlorothiophene (822 ng) and thianaphthene (762 ng). The suspension was vigorously shaken for 3 min, 1 mL of the top pentane phase was removed, and 25 μL of an internal standard solution was added. The internal standard solution contained 1,4-dichlorobenzene-d_4 (395 ng). A small quantity of the pentane solution was then analyzed by GC/MS. A typical total-ion chromatogram is shown in Figure 1.

RESULTS AND DISCUSSION

Identification of Organosulfur Compounds in a Garlic Extract. A large quantity of garlic extract was extracted with pentane, and the pentane extract was concentrated to a small volume. The extract was then analyzed using capillary column GC/MS. On the basis of the mass spectral data, methyl disulfide, methyl trisulfide, allyl sulfide, allyl disulfide, allyl trisulfide, allyl methyl sulfide, allyl methyl disulfide, allyl methyl trisulfide, and ethyl 2-propenesulfinate were tentatively identified.

For the definitive identification of a compound, the mass spectrum of a tentatively identified compound must be essentially identical to that of the mass spectrum of the authentic compound. Although some extraneous peaks from the matrix may be present in the mass spectrum of the tentatively identified component, especially when the component is present at low concentration, peaks such as the parent peak, base peak, and key fragment peaks must have similar m/z values and relative intensities. In addition, the GC retention time of a tentatively identified component relative to that of an internal standard should be within 2 scans (i.e., about 2 s) of the GC retention time of the authentic compound relative to that of the same internal standard when the samples are analyzed under the same conditions.

Pure samples of methyl disulfide, methyl trisulfide, allyl sulfide, allyl disulfide, allyl methyl sulfide, and allyl methyl disulfide were purchased. Comparison of the mass spectrum of each tentatively identified compound (Figures 2a, 3a, 4a, 5a, 6a, and 7a in the supplementary material) with the mass spectrum of the corresponding authentic compound (respectively, Figures 2b, 3b, 4b, 5b, 6b, and 7b) shows that a good match is obtained in all cases. Also, the retention times of the components present in garlic extract listed in Table I meet the requirement specified above.

Impure allyl trisulfide and allyl methyl trisulfide were purchased and purified using semipreparative HPLC.

Table II. Gas Chromatographic/Mass Spectrometric Relative Response Factors (RRF) for Identified Components in Garlic Extract

component	quantifn ion, m/z	linear regression curve RRF[a]	corr coeff
methyl disulfide	94	1.01	0.988
methyl trisulfide	126	1.18	0.984
allyl sulfide	114	0.241	0.984
allyl disulfide	146	0.196	0.992
allyl trisulfide	113	0.414	0.984
allyl methyl sulfide	88	0.487	0.985
allyl methyl disulfide	120	0.778	0.982
allyl methyl trisulfide	87	0.670	0.983
ethyl 2-propenesulfinate	93	0.182	0.999
3-chlorothiopene[b]	192	0.791	NA[c]
thianaphthene[b]	134	2.10	NA

[a] Response factor relative to 1,4-dichlorobenzene-d_4 (m/z 150). [b] Surrogates. [c] A constant concentration of each surrogate was employed.

Table III. Measured Concentration of Identified Components in Garlic Extract and Recovery of Surrogates

component	concn, ng/g aliquot 1	2	3	4	av	% RSD[a]
methyl disulfide	548	560	622	697	607	11.3
methyl trisulfide	187	176	204	158	181	10.7
allyl sulfide	2122	1979	2065	1899	2016	4.7
allyl disulfide	848	740	723	825	784	7.9
allyl trisulfide	799	813	864	704	795	18.4
allyl methyl sulfide	1797	1528	1690	1555	1643	7.6
allyl methyl disulfide	408	388	454	393	411	7.3
allyl methyl trisulfide	742	788	745	506	695	8.4
ethyl 2-propenesulfinate	10857	11053	11379	12244	11383	5.4
3-chlorothiopene[b]	89	85	89	83	86	3.6
thianaphthene[b]	109	104	102	101	104	3.3

[a] % RSD, percent relative standard deviation. [b] Surrogates (values represent % recovery).

Comparison of the mass spectrum of each tentatively identified compound (Figures 8a and 9a in the supplementary material) with the mass spectrum of the corresponding authentic compound (respectively, Figures 8b and 9b) shows that good matches are obtained. Also, the retention times of the components present in garlic extract listed in Table I meet the requirement specified above.

No reference to ethyl 2-propenesulfinate was found in the literature. Apparently ethyl alcohol, which is used by the supplier to extract garlic to produce the original garlic extract employed in this study, reacts with allicin in the garlic to form ethyl 2-propenesulfinate during the extraction process. Comparison of the mass spectrum of the tentatively identified compound (Figure 10a) with the mass spectrum of the synthesized compound (Figure 10b) shows that a good match is obtained. The retention times of the tentatively identified component present in garlic extract listed in Table I also meet the requirement specified above.

Quantification of Components in a Garlic Extract. *Calibration of the Gas Chromatograph/Mass Spectrometer.* GC/MS was selected for the analysis procedure because it is a very sensitive technique. In addition, a low concentration of a target compound can be quantified in the presence of a high concentration of an interfering compound if the target compound generates a mass spectral quantification ion that is not produced by the interfering compound. Standard solutions that contained the nine identified organosulfur compounds, two surrogates, and an internal standard were used to calibrate the GC/MS. After an initial analysis of the garlic extract, new standards were prepared that contained components whose concentrations bracketed the estimated concentrations of the components in the garlic extract. A six-point calibration curve for each component was then generated. By linear regression analysis with a force of the curve through zero the typical relative response factors and correlation coefficients shown in Table II were obtained.

3-Chlorothiophene and thianaphthene, neither of which was detected in garlic extract, were selected as surrogates for the organosulfur compounds. The extraction behavior of the surrogates should resemble that of the target compounds because of their structural similarity. Consequently, the recovery of the surrogates should be indicative of the recovery of the target compounds during the analytical procedure. 1,4-Dichlorobenzene-d_4, which is very stable and produces a mass spectrum with only a few intense peaks, was used as the internal standard. The use of an internal standard generally increases the accuracy of an analysis because the internal standard responds to some extent to instrument fluctuations in the same manner as the target compounds and surrogates. In addition, with an internal standard present, it is not necessary to inject the identical volume of a sample during each GC/MS determination.

Extraction and Analysis of the Garlic Extract. The goal was to generate a convenient method for rapidly but accurately determining the nine organosulfur compounds in garlic extract. Pentane was selected as the extracting solvent because it readily dissolves the target organosulfur compounds but will not easily dissolve potentially interfering polar compounds. Sodium chloride was added to reduce the solubility of the organosulfur compounds in the garlic extract, and the suspension was vigorously agitated for several minutes to equilibrate the phases. One milliliter of the top pentane phase was removed, and a solution containing the internal standard was added. The chances of losing the more volatile components and the time required for an analysis are both decreased by analyzing the extract without concentrating it. Analysis of four aliquots of the garlic extract gave the results shown in Table III. The reproducibility of the analyses is

Table IV. Recovery of Components Spiked into Garlic Extract

component	initial quantity in 1.00 g of garlic extract, ng	quantity spiked into 1.00 g of garlic extract, ng	% recovery sample 1	sample 2	sample 3	sample 4	sample 5	av % recovery	% RSD[a]
methyl disulfide	607	264	76	52	83	45	69	65	16
methyl trisulfide	181	190	88	24	77	62	63	67	24
allyl sulfide	2016	2428	57	62	91	51	66	65	15
allyl disulfide	784	560	78	70	117	48	114	85	30
allyl trisulfide	695	810	78	62	82	54	80	71	12
allyl methyl sulfide	1643	666	89	107	149	66	70	96	34
allyl methyl disulfide	411	310	80	67	75	39	87	70	19
allyl methyl trisulfide	795	1180	82	65	83	54	59	69	13
ethyl 2-propenesulfinate	11363	22312	35	74	158	38	86	78	50

[a] % RSD, percent relative standard deviation.

excellent, and no interference was detected in a blank analyzed at the same time. The recovery of the surrogates is also quite good. The results of the analysis of five matrix spikes are shown in Table IV. The recoveries of the organosulfur compounds are lower than expected.

Stability of Garlic Extract. It was evident at an early stage that odorous sulfur-containing compounds are not necessarily present in a plant as such but are formed enzymatically when the cellular tissue is disrupted (Stoll et al., 1947, 1951; Virtanen et al., 1959, 1965; Yu et al., 1989). It was discovered in this study that the concentration of most of the components in the garlic extract increased in concentration with time, presumably as a result of the decomposition of precursors to the organosulfur compounds. For example, after 12 weeks at 25 °C, the sulfides and disulfides had nearly tripled in concentration and the trisulfides had increased in concentration in garlic extract by an order of magnitude. These observations were confirmed in an independent study of the same extract by another organization (private communication from the National Cancer Institute).

When *both* the garlic extract and the spiked garlic extract were analyzed at the same time, better recoveries of the spiked components were found. However, further study is required to more fully validate the analytical protocol. It must be determined if the exposure of the garlic extract to pentane induces a change in the total quantity of the volatile organosulfur compounds present in the two phases. It must be determined what effect the extraction conditions have on extraction efficiency. It must also be determined if the precursors to the organosulfur compounds are extracted into pentane and, if so, if thermal decomposition of the precursors in the injection port of the gas chromatograph adversely affects the determination of the organosulfur compounds.

Conclusion. Methodology was developed to detect, identify, and quantify methyl disulfide, methyl trisulfide, allyl sulfide, allyl disulfide, allyl trisulfide, allyl methyl sulfide, allyl methyl disulfide, allyl methyl trisulfide, and 2-propenesulfinate compliance markers in garlic extract. However, in view of the instability of the experimentally formulated garlic extract, more study of methods for analyzing the extract should be undertaken.

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Supplementary Material Available: Figures 2–10 showing the mass spectra of the compounds (9 pages). Ordering information is given on any current masthead page.

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