JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantitative Determination of Allicin in Garlic: Supercritical Fluid Extraction and Standard Addition of Alliin

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A quantitative method is described for the determination of allicin (2-propene-1-sulfinothioic acid *S*-2-propenyl ester) in garlic, using standard additions of alliin (L-(+)-*S*-allylcysteine sulfoxide) in conjunction with supercritical fluid extraction (SFE) and high performance liquid chromatography analysis with UV−vis absorbance detection. Optimum CO_2-SFE conditions provided 96% recovery for allicin with precision of 3% (RSD) for repeat samples. The incorporation of an internal standard (allyl phenyl sulfone) in the SFE step resulted in a modest improvement in recovery (99%) and precision (2% RSD). Standard additions of alliin were converted to allicin in situ by endogenous alliinase (L-(+)-*S*-alk(en)ylcysteine sulfoxide lyase, EC 4.4.1.4). Complete conversion of the spiked alliin to allicin was achieved by making additions after homogenization-induced conversion of the naturally occurring cysteine sulfoxides to thiosulfinates had taken place, thus eliminating the likelihood of competing reactions. Concentration values for allicin determined in samples of fresh garlic (*Allium sativum* L. and *Allium ampeloprasum*) and commercially available garlic powders (*Allium sativum* L.) by standard addition of alliin were found in all cases to be in statistical agreement (95% confidence interval) with values determined using a secondary allicin standard (concentration determined using published extinction coefficients). This method provides a convenient alternative for assessing the amount of allicin present in fresh and powdered garlic, as alliin is a far more stable and commercially prevalent compound than allicin and is thus more amenable for use as a standard for routine analysis.

KEYWORDS: Alliin; allicin; alliinase; cysteine sulfoxides; thiosulfinates; standard addition; supercritical fluid extraction; high performance liquid chromatography

INTRODUCTION

The use of garlic as a means of preventing diseases and treating common ailments is one of the earliest documented examples of a plant being used as a medicinal agent (*1*), and its consumption has become a widely accepted general dietary course for promoting overall human health. Many of the health benefits associated with garlic consumption have been attributed to the thiosulfinates, the single most abundant class of organosulfur compounds found in freshly chopped or crushed garlic (*2*). Allicin (2-propene-1-sulfinothioic acid *S*-2-propenyl ester) is the most abundant thiosulfinate, typically accounting for 70% (w/w) of the total thiosulfinates (approximately 0.4% by fresh mass) found in fresh garlic (*3*). Studies have shown allicin to be an antimicrobial agent (*4*, *5*), and substantial evidence suggests that allicin may be the active component responsible for observed anticancer (*6*) and antioxidant (*7*) activity.

The routine determination of allicin is complicated by its instability and its reactive nature. The half-life of allicin can vary depending on the concentration, temperature, and solvent in which it was stored. At room temperature, the half-life of allicin as a crystalline solid is 16 h, but in crushed garlic it is approximately 2.5 days (*3*). Allicin is most stable when stored at low temperatures in solvents that exert a high degree of hydrogen bonding. In water, at concentrations ranging from 100 to 1000 ppm, the half-life of allicin is 30−40 days at 23 °C. At −70 °C the half-life increases to the point where no detectable loss is observed over a two-year period (*3*). Allicin decomposition can proceed by one of several possible pathways to produce a variety of products such as allylsulfides, vinyldithiins, and ajoenes, with the mode, rate, and products being determined by the conditions under which it is stored (*8*).

The instability of allicin has precluded its widespread use as an analytical standard. It has been documented that some commercially available allicin standards do not contain the purported concentration due to decomposition (*9*). As a result, the majority of the published methods for determining allicin call for it to be synthesized and purified "in-house" as a standard (*10*−*13*), with its exact concentration being determined using published extinction coefficients (*12*). This is a laborious task

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10.1021/jf034853x
Published on Web 01/24/2004

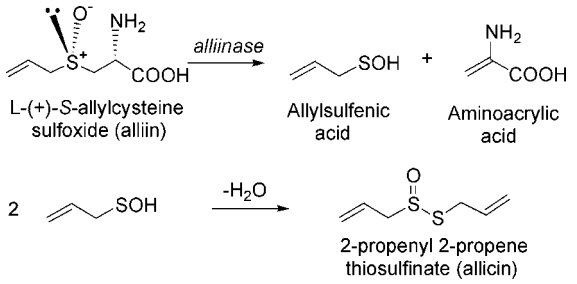

Figure 1. Enzymatic formation of allicin from alliin.

that is impractical in many laboratory settings. Alternative indirect methods for determining allicin have also been developed (*14*, *15*), such as that suggested by Han et al. based on the stoichiometric reaction of allicin with cysteine (*15*). Excess cysteine is determined by the spectrophotometric measurement of the 2-nitro-5-thiobenzoate formed after the reaction of cysteine with 5,5′-dithio-bis-(2-nitrobenzoic acid).

Whereas allicin is a challenging compound to work with, its enzymatic precursor alliin (L-(+)-*S*-allylcysteine sulfoxide) (**Figure 1**), a cysteine sulfoxide, is more amenable for use as a standard. Allicin is formed when fresh, raw garlic is chopped or crushed, rupturing the intracellular compartments that keep alliin and alliinase (L-(+)-*S*-alk(en)ylcysteine sulfoxide lyase, EC 4.4.1.4, an α,β-eliminating endogenous lyase from *Allium* spp.) physically separated from each other, allowing them to mix and react (*16*). The transformation of alliin to allicin is rapid and complete, taking less than 10 s at 23 °C in fresh garlic (*17*). Alliin is a water-soluble compound that is relatively stable; a 10 mg mL^{-1} solution in water stored at 4 °C will show no signs of decomposition after more than a year (*18*). Alliin has been used to generate allicin for use as a standard in allicin analyses (*18*−*20*). To eliminate the possible formation of thiosulfinates other than allicin, the enzymatic process has traditionally been conducted in vitro with alliinase (*18*, *19*). Although this ensures the purity of allicin, it requires the laborious extraction (*20*) and purification (*18*, *19*) of the alliinase. To date, there has been no attempt to use alliinase in situ to convert an exogenous alliin addition completely to allicin. In principle, addition of a series of systematically increasing masses of alliin to a series of identically prepared samples should allow the determination of allicin by the method of standard additions.

Extraction of allicin and other thiosulfinates, ajoenes, cepaenes, and disulfides by supercritical fluid extraction (SFE) with CO_2 and separation by liquid chromatography provide an efficient, environmentally friendly, and qualitatively accurate means of isolating the small organosulfur compounds found in plants of the genus *Allium* (*21*−*24*). This methodology effectively extracts the thiosulfinates from the bulk of the carbohydrates and proteins present in the bulb, resulting in a relatively clean analytical matrix. Because organosulfur compounds are highly reactive in organic solvents and artifact formation has been a problem, SFE has been used as a means of extracting organosulfur compounds without using organic solvents. This methodology, however, has not been rigorously evaluated as a means of quantitative analysis.

The objective of this study was 2-fold: to develop and evaluate an SFE method suitable for the quantitative extraction and determination of allicin in garlic and to develop and evaluate a method for determining allicin in garlic by standard addition of alliin using the endogenous alliinase enzyme to form allicin in situ. Results are reported for the determination of allicin in fresh and powdered garlic samples using the method of additions, and are compared to external standards of allicin whose concentration were determined using published extinction coefficients (*12*). The accuracy, precision, and comparability of both methods are discussed.

MATERIALS AND METHODS

Standards and Samples. A series of aqueous standards of alliin for standard additions was prepared by dissolving commercially available, optically pure L-(+)-alliin (LKT Laboratories, St. Paul, MN) in 0.2-μm filtered distilled deionized water (DDW) and serial dilution to concentrations of 0.01, 0.1, 1.0, and 10 mg mL^{-1}. This allowed spiked volumes to remain approximately constant (within a factor of 2), and in all cases, small with respect to the sample volume.

External calibration standards were prepared for allicin with concentration based on the published extinction coefficient. Allicin calibration standards were prepared by serial dilution of a commercially available allicin standard (5 mg dissolved in approximately 0.5 mL of 60:40 mixture of water and methanol) (LKT Laboratories). Because the stated concentration of the allicin solution was approximate, the exact concentration was verified by measuring the absorbance of a 1:4 dilution of the standard with DDW at both 240 and 254 nm, using published extinction coefficients (*12*). The concentration of the allicin standard was determined to be 14.6 mg mL^{-1}. The stated chemical purity of the allicin standard (98.5%) was verified by HPLC.

Fresh and powdered garlic samples for analysis were purchased from various grocery stores in the metropolitan Washington, DC area.

Sample Preparation. *Fresh Garlic.* Whole garlic cloves were first finely chopped by hand and then ground to a paste using an 8-mL Potter-Elvehjem tissue grinding apparatus (Kontes Glass Co., Vineland, NJ) mechanically driven at 1050 rpm by a drill press. Grinding the garlic in this manner ensured that both the maximum enzymatic yield of thiosulfinates was achieved and that the remaining pieces of vegetable tissue were as fine as possible. For samples of white garlic, 2 g of sample was mixed with 2 mL of DDW directly in the tissue grinder; for elephant garlic, samples were ground without dilution. To minimize frictional heating of the sample during the grinding process, the tissue grinder was chilled prior to and during the grinding process with an ice bath.

Fresh garlic samples to be analyzed by direct comparison to the external allicin standards were prepared by mixing 50 mg of the ground garlic paste (prepared as described above) with 2.5 mL of DDW in 15-mL polypropylene conical tubes (Falcon Blue Max, Becton Dickinson, Franklin Lakes, NJ). Samples to be analyzed by standard additions were prepared by transferring 50 mg of the ground garlic paste to the bottom of a 15-mL conical tube and mixed with 30−100 μL of an alliin standard or a blank (DDW). Relatively small spiking volumes were used to ensure that the alliinase activity of the sample was affected minimally by dilution. The samples were then vortex mixed for 30 s and allowed to stand for 10 min to allow complete allicin formation. Aqueous homogenates were then prepared by diluting the samples with 2.5 mL of DDW. Allyl phenyl sulfone (TCI America, Portland, OR) was then added to all homogenates as an internal standard (10 mg of allyl phenyl sulfone per g^{-1} of fresh garlic starting material). Homogenates were either analyzed immediately or refrigerated at 4 °C and analyzed within 24 h.

Powdered Garlic. The first step was to induce complete conversion of the endogenous cysteine sulfoxides to thiosulfinates. This was done by mixing 20 mg of garlic powder with 100 μL of DDW in the bottom of a 15-mL conical tube, vortex mixing for 30 s and incubating for 10 min at room temperature. The dilution used in this incubation step was a conservative estimate based on previously published work with powdered garlic (*2*, *25*). Lawson and Wang (*2*) reported that the maximum allicin formation from powdered garlic occurred at 200 mg mL^{-1}, and Lawson and Hughes (*25*) reported that, for powdered garlic diluted in water at room temperature to a concentration of 50 mg mL^{-1}, 50% of the maximum allicin yield was present after 6 s and >97% after 30 s.

Aqueous homogenates for powdered garlic samples being determined by comparison to external allicin standards were prepared by diluting the incubated sample with 2.5 mL of DDW. For standard additions,

incubated samples were spiked with 30−100 μL of an alliin standard or a blank (DDW). The samples were then vortex mixed for 30 s and allowed to stand for 10 min to allow complete allicin formation. Aqueous homogenates were then prepared by diluting the samples with 2.5 mL of DDW. Allyl phenyl sulfone was then added to all homogenates as an internal standard (25 mg of allyl phenyl sulfone per g^{-1} of fresh garlic starting material). Homogenates were either analyzed immediately or refrigerated at 0−4 °C and analyzed within 24 h.

Allicin Extraction and Chromatographic Analysis. Sample extraction was achieved using SFE (Prepmaster SFE system and Accutrap sample collector, Suprex (now ISCO, Lincoln, NE)) with supercritical fluid grade CO_2 (Airgas Inc., Radnor, PA) as the extraction solvent. Approximately 0.5 mL of aqueous homogenate was mixed with 5 mL (~1 g) of diatomaceous earth (Hydromatrix, Varian, Walnut Creek, CA) and transferred to a 5-mL stainless steel extraction cell. The cell was first equilibrated (the static extraction stage) at a temperature of 35 °C and a CO_2 pressure of 240 atm for 5 min. During the dynamic extraction stage, the cell was purged with 50 g (approximately 10 extraction cell void volumes) of CO_2 at a flow rate of 2.0 mL min^{-1} (controlled by a flow restrictor maintained at a temperature of 50 °C). Solutes extracted during the dynamic stage were desolvated by depressurization of the supercritical CO_2 flow and collected in a 7.0- × 4.5-mm i.d. trap packed with 80/100 mesh borosilicate glass beads cryogenically cooled to a temperature of 1 °C. Upon completion of the dynamic extraction stage, the collected solutes were desorbed from the trap at 25 °C with 4.0 mL of a 32% (v/v) solution of acetonitrile (HPLC grade, Fisher, Fair Lawn, NJ) in DDW and filtered through a 0.22-μm polyvinyldifluoride syringe filter (Acrodisc LC 13 mm, Pall Life Sciences, Ann Arbor, MI). Homogenates that were analyzed by HPLC with no SFE treatment were simply filtered (0.22 μm) prior to injection. All samples were immediately refrigerated and stored at 4 °C prior to analysis to prevent artifact formation.

Supercritical fluid extracts were analyzed on an HPLC system equipped with diode array UV-vis absorbance detection and a Peltier-cooled autosampler (System Gold model 126 solvent delivery module, model 168 detector, and model 508 autosampler, Beckman Coulter, Fullerton, CA). Chromatographic separation was achieved with a 250- × 2.0-mm i.d., 4-μm particle size, 80-Å pore size C_{18} reversed phase column (YMC J'sphere ODS-M80, Waters Corporation, Milford, MA) and a linear gradient of DDW and acetonitrile (HPLC grade, Fisher) pumped at a flow rate of 200 μL min^{-1} (*21*). The elution program was held at a mobile phase composition of 36.5% CH_3CN for 10 min, followed by a 15 min linear gradient to a final mobile phase composition of 59% CH_3CN that was held for 15 min. In all cases, 20 μL of sample was injected, and the temperature of the autosampler tray was maintained at 4 °C to prevent thiosulfinate decomposition. The identity of the peaks in the chromatogram were confirmed either by injecting pure standards (where available), or by comparing the UV-vis absorbance spectra or mass spectra obtained by LC/MS with atmospheric pressure chemical ionization (LCQ Classic, Finnigan MAT (now Thermo Finnigan, San Jose, CA)) with those that appear in the literature (*21*, *22*).

RESULTS AND DISCUSSION

Quantitative SFE of Allicin. Qualitative and semiquantitative SFE studies of allicin and other thiosulfinates from garlic (*21*−*24*) and related *Alliums* (*26*) have been reported previously. In the present study, the SFE conditions (extraction at conditions of 35 °C and 240 atm with collection on glass beads cooled to 1 °C) proposed by Calvey et al. (*21*, *22*) were initially used. Under these conditions, it was found that at least 30 g of CO_2 was needed to achieve maximum extraction of allicin from an aqueous homogenate of white garlic deposited on diatomaceous earth in a 5-mL extraction vessel (**Figure 2**). A minimum of 3 mL of 35% (v/v) acetonitrile in water was needed to accomplish maximum allicin desorption from the cryogenic trap (**Figure 3**).

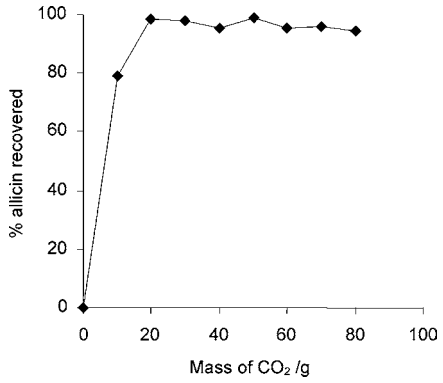

Figure 2. SFE recovery of allicin as a function of extraction solvent mass (g CO_2). Extraction conditions: 35 °C, 240 atm. CO_2 flow rate: 2.0 mL min^{-1}. Cryotrap desorption solvent: 4 mL of 35% (v/v) acetonitrile in water.

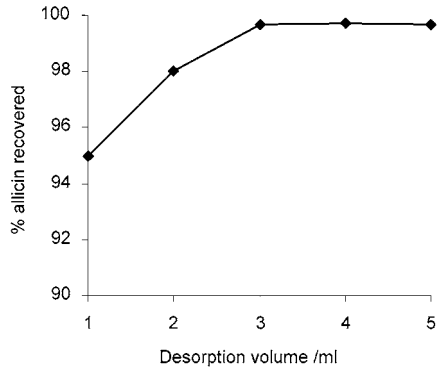

Figure 3. SFE recovery of allicin as a function of cryotrap desorption solvent volume. SFE conditions: 35 °C, 240 atm. Mass of extraction solvent (CO_2): 50 g. CO_2 flow rate: 2.0 mL min^{-1}. Cryotrap desorption solvent: 35% (v/v) acetonitrile in water.

A systematic study of the effect of extraction temperature (35, 40, 45, 50 °C) and pressure (240, 275, 310, 345 atm) was conducted on a fresh garlic homogenate to determine the ideal conditions for maximum allicin recovery (as compared to direct aqueous extraction). Maximum recovery was observed to occur at temperature/pressure conditions that provided the theoretical maximum density for CO_2 of 0.90 g mL^{-1}. Maximum density occurred at conditions of 40 °C/275 atm and 45 °C/310 atm, as well as 35 °C/240 atm proposed by Calvey et al. (*21*, *22*). Higher temperature and pressure conditions do not increase the density of CO_2 and do not provide any improvement in recovery. On the contrary, at 50 °C/345 atm, the allicin recovery was 40% lower. Allicin thermal breakdown products have been observed in SFE experiments performed on *allium* homogenates at 50 °C (*22*), and it is likely that the low recovery of allicin observed here is due to thermal decomposition.

Using 50 g of CO_2, 4 mL of desorption solvent, and SFE conditions of 35 °C and 240 atm, the recovery of allicin from a fresh white garlic homogenate was found to be 96% relative to direct injection of the exact same homogenate (0.22 μm filtered) when determined by HPLC (allicin peak area). Repetitive extractions ($n = 6$) performed on the same homogenate were reproducible, with an RSD of 3%. By comparison, the reproducibility of the allicin signal for repeated injections ($n = 5$) of the same SFE sample was 0.4% (RSD). The SFE reproducibility remained unchanged at 3% (RSD) for extractions performed on different aqueous homogenates ($n = 6$) prepared from the same ground white garlic sample paste.

Sulfones, such as diallyl sulfone (*27*) have been used as internal standards for the determination of allicin. It was

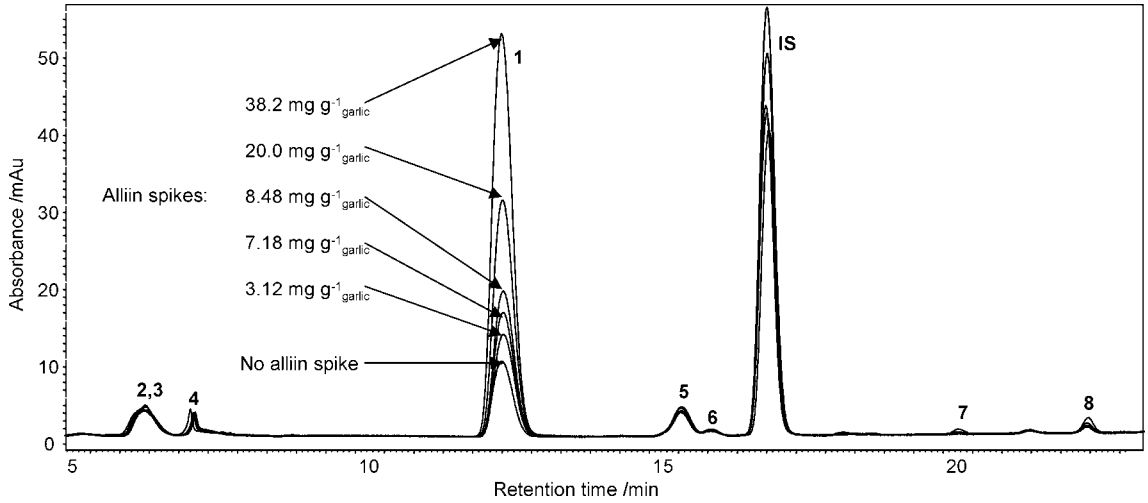

Figure 4. Overlaid chromatograms for aliquots of a garlic (*Allium sativum* L., fresh) sample spiked with increasing amounts of alliin. Peak identities: **1**, 2-propene-1-sulfinothioic acid *S*-2-propenyl ester (allicin); **2**, 2-propene-1-sulfinothioic acid *S*-methyl ester; **3**, methyl-1-sulfinothioic acid *S*-2-propenyl ester; **4**, *E*-1-propene-1-sulfinothioic acid *S*-2-methyl ester; **5**, 2-propene-1-sulfinothioic acid *S*-*E*-1-propenyl ester; **6**, *E*-1-propene-1-sulfinothioic acid *S*-2-propenyl ester; **7**, 2-vinyl-4*H*-1,3-dithiin (1,3-vinyldithiin); **8**, *E*,*Z*-4,5,9-trithiadodeca-1,6,11-triene 9-oxide (ajoene); **IS**, allylphenyl sulfone (internal standard).

hypothesized that sulfones would exhibit SFE characteristics similar to that observed with the thiosulfinates and would be unlikely to occur in most plants. Due to its relatively obscure commercial availability, the more prevalent compound allyl phenyl sulfone was chosen as an internal standard. In practice, allyl phenyl sulfone proved to be unreactive in the ground garlic homogenates and had extraction characteristics that were virtually identical to those observed for allicin. When allyl phenyl sulfone was added to the homogenates as an internal standard, the recovery for allicin improved to 99% ± an RSD of 2%.

Method of Standard Additions. The validity of using alliin in a standard additions scheme to determine allicin in garlic rests on the premise that the alliinase enzyme endogenous to the sample is capable of converting the exogenous alliin completely to allicin in situ. **Figure 4** illustrates that this is possible, provided the addition is made after complete conversion of the endogenous cysteine sulfoxides to thiosulfinates has taken place. The overlaid chromatograms in **Figure 4** show that an increase in the allicin signal was observed for each aliquot of the homogenized fresh garlic sample to which alliin was added, and that the increase in the allicin signal was proportional to the alliin spike size. Furthermore, the signals for other thiosulfinates were not affected by the addition of alliin. These observations confirmed that the conversion of exogenous alliin to allicin was occurring in these samples in the absence of any detectable competing reactions from other endogenous cysteine sulfoxides. The only signals that appeared to show any increase with the amount of alliin added to the aliquot were those for 1,3-vinyldithiin (2-vinyl-4*H*-1,3-dithiin) and ajoene ((*E*,*Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide) (**Figure 4**). It is important to note that vinyldithiins and ajoenes are not directly produced by alliinase-catalyzed reactions but rather are decomposition products of allicin in solvents less polar than water (*8*). As expected, the small amounts of 1,3-vinyldithiin and ajoene found in the samples generally increased with the amount of allicin in the sample, and hence, the amount of alliin added to the sample.

A plot of the allicin signal as a function of added allicin is shown in **Figure 5**, along with a calibration curve obtained from serial dilution of a commercial allicin standard. In both cases, the signal is expressed as the ratio of the allicin absorbance

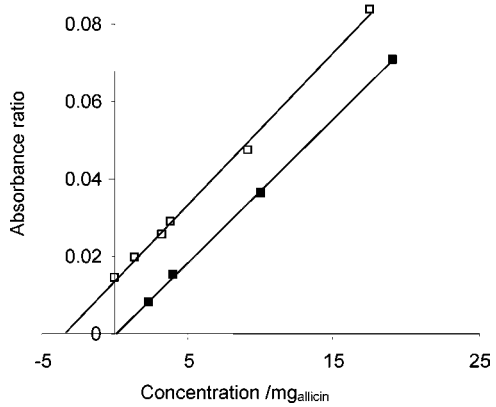

Figure 5. Calibration curves for allicin. ■, external allicin standards; □, standard addition of alliin (from data in **Figure 4**).

and the internal standard absorbance. The standard addition data is the same as that shown in **Figure 4**. Complete conversion of alliin to allicin in the standard additions samples was verified by comparing the slopes of the two calibration curves. The identical slopes confirm the lack of a multiplicative interference (i.e., complete conversion took place). Standard additions of alliin were performed on a total of four fresh garlic samples (3 white garlics and 1 elephant garlic) and two commercial garlic powders (white garlic). The average slope of these six standard additions curves was 3.8 (± 0.2) × 10^{-3} [A_{allicin} (A_{IS} g^{-1}_{IS})$^{-1}$] g^{-1}_{allicin}, as compared to the slope of 3.72 (± 0.03) × 10^{-3} [A_{allicin} (A_{IS} g^{-1}_{IS})$^{-1}$] g^{-1}_{allicin} obtained for the external allicin calibration curve. Spike recovery calculations (assuming conversion of 2 alliin molecules to 1 allicin molecule, **Figure 1**) gave an average value of 96 ± 8% for the same six samples whose slopes were compared above.

The effect of dilution of the homogenates (prior to SFE extraction) on the conversion of exogenous alliin to allicin was evaluated. Dilution of fresh white garlic homogenates with water to concentrations as low as 50 mg mL^{-1} prior to addition of alliin was found to have no noticeable effect on the ability of the sample to generate allicin when incubated for 10 min at room temperature. Though there exist no comparable kinetic studies involving the addition of exogenous alliin to garlic,

Table 1. Determination of Allicin in Garlic Samples, Comparison of Methods

sample	sample no.	concentration of allicin found/ mg g^{-1} of sample[a] std addition of L-(+)-alliin	external stds (allicin)
white garlic (*Allium sativum* L.)	1	3.98 ± 0.12 ($n = 7$, $r^2 = 0.996$)	4.13 ± 0.05 ($n = 5$)
	2	3.37 ± 0.37 ($n = 6$, $r^2 = 0.992$)	3.20 ± 0.08 ($n = 6$)
	3	4.60 ± 0.20 ($n = 6$, $r^2 = 0.9995$)	4.81 ± 0.10 ($n = 4$)
elephant garlic (*Allium ampeloprasum*)	1	0.73 ± 0.05 ($n = 7$, $r^2 = 0.997$)	0.73 ± 0.05 ($n = 6$)
garlic powder (*Allium sativum* L.)	1	2.62 ± 0.16 ($n = 5$, $r^2 = 0.9994$)	2.25 ± 0.06 ($n = 5$)
	2	3.05 ± 0.23 ($n = 7$, $r^2 = 0.992$)	2.93 ± 0.05 ($n = 6$)

[a] Concentration is expressed as mg g^{-1} fresh mass for garlic samples and mg g^{-1} dry mass for garlic powders.

allicin formation from garlic powders under similar conditions is known to be complete in 5 min (*25*). The effect of using standard additions of L-alliin (racemic mixture of ± forms) in place of L-(+)-alliin was also evaluated. The racemic mixture was found to be equally effective at generating allicin in samples of fresh white garlic. This was surprising, as alliinase is known to be specific to naturally occurring (i.e., L-(+)-) alliin, with the reaction rate being considerably slower for L-(−)-alliin and nonexistent for D-(±)-alliin (*16*). It is important to note, though, that the 10 min incubation period used in the present study was not controlled (i.e., the alliinase enzyme was not deactivated following incubation). As a result, the possibility of further reaction of the abundant alliinase enzyme with the slower-reacting L-(−) isomer exists in each sample until SFE is performed. Because the time between sample incubation and SFE may be as long as 24 h, ample time may have elapsed for the conversion of L-(−) alliin to allicin to have taken place, even with sample homogenates being stored at 4 °C until extraction. Alliinase is known to be highly active, even at temperatures as low as 2 °C (*25*).

Initial experiments with fresh white garlic produced standard additions curves that had reduced slopes (compared to the external allicin calibration curve) and were nonlinear (bent toward the *x*-axis). It was determined that the grinding process generated an excessive amount of frictional heating that resulted in either partial or complete denaturation of the alliinase enzyme present in these samples and that this was causing incomplete conversion of the added alliin to allicin. Diluting the garlic sample with water and refrigerating the apparatus during the grinding process (see Methods and Materials) corrected this problem. By analyzing multiple samples at a time (with one preferably being fresh garlic) and making multiple standard additions to each sample, cases of incomplete allicin formation could be detected simply by comparing the slopes of the standard addition curves.

Accuracy and Precision. Using SFE extraction and HPLC separation and detection as described above, the allicin concentrations of four fresh garlic and two commercially available garlic powders were determined by both multiple standard additions of alliin and external standardization with allicin (**Table 1**). In each case, the standard additions value was based on at least five additions (one point from an unspiked aliquot and at least four points from alliin-spiked aliquots) to ensure that an adequate representation of the regression statistics and the 95% confidence interval determined by extrapolation (*28*) was achieved. The concentration range of the additions used in each determination was limited so that the allicin concentration achieved with the most concentrated addition was approximately twice that of the endogenous allicin. Using this approach, linear standard additions curves with r^2 values ranging from 0.992 to 0.9996 were obtained for every sample, and the relative uncertainty of the calculated allicin concentrations ranged from 3 to 11%. For each of the six samples analyzed, the allicin concentration determined by standard addition of alliin was found to be in statistical agreement (within the 95% confidence interval) with the value determined using secondary external standards (i.e., using the published extinction coefficient for allicin) (*12*). The relative error in determining allicin by standard additions of alliin ranged from −4.3 to +16% (compared to values obtained from external allicin calibration standards) and showed an insignificant bias toward higher values with an average of 3 ± 8%. Accuracy was generally better for the fresh garlic samples (relative error range of −4.3 to +5.3%, average of −1 ± 4%) as compared to the garlic powders.

With the exception of the elephant garlic sample, the uncertainty in the allicin concentrations determined by standard additions was generally 2−4.5 times higher than that obtained using external calibration. Analysis of the variances using an *f*-test ($p = 0.05$) revealed that this difference was significant in each case. The higher uncertainties are attributable to the different ways that measurement errors manifest themselves in the two methods. First, the deviation of the points about the standard additions calibration curves tended to be greater than that observed for the external allicin calibration curve. This is because the points of the standard additions curves have essentially three additional sources of error: the garlic homogenization and sampling process, the allicin generation process for both the endogenous and exogenous alliin, and the SFE process. Second, the allicin concentration and its associated uncertainty are both determined by extrapolation from the more imprecise standard additions curve, rather than repetitive interpolation of samples bracketed by external allicin standards of greater precision.

Supercritical fluid extraction coupled with HPLC separation and UV−vis absorbance detection provides an accurate and precise method for the extraction, separation, and detection of allicin in garlic samples. The use of standard additions of alliin as a means for determining allicin concentration eliminates the need for purification and calibration of commercial allicin standards. This method has been demonstrated to be practical for samples in which the alliinase enzyme is intact. Samples for which this method would be applicable are fresh vegetables and suitably dehydrated powders akin to those found in seasonings or in tablet supplement form. This method cannot be used for samples where the alliinase activity has been deactivated due to the manufacturing or preparation process used. It is highly recommended that, when using this method of standard additions, standard additions of alliin are made to multiple aliquots from each sample, and that it be performed on more than one sample at a given time (with one of the samples preferably being fresh garlic). By doing so, the appearance and slope of each standard addition curve will be known to a greater degree of certainty and can be compared with those obtained from other samples, facilitating the detection of cases where incomplete allicin conversion may have taken place. Although this work has looked strictly at *A. sativum* L. (white garlic) and *A. ampeloprasum* (elephant garlic) samples, this approach should be valid for species throughout the genus

Allium. It should be noted, however, that only a few other species would be of any interest (e.g., *A. ursinum* L. (wild garlic), *A. vineale* (field garlic), *A. victoralis* (alpine leek), and *A. tricoccum* (ramp)), as many *Alliums* do not contain significant amounts of alliin (e.g., *A. cepa* (onion) varieties) and as a result produce little to no allicin.

ACKNOWLEDGMENT

We thank Clifford Rice of the Environmental Quality Laboratory, USDA, for the generous loan of the SFE system used in this study, Vincent P. Flanagan, for his assistance with the LC/MS work, and Gary R. Beecher, Robert J. Pawlosky and Wayne Wolf, for helpful collegial discussions.

LITERATURE CITED

(1) Rivlin, R. S. Historical perspective on the use of garlic. *J. Nutr.* **2001**, *131* (3S), 951S−954S.
(2) Lawson, L. D.; Wang, Z. J. Low allicin release from garlic supplements: a major problem due to the sensitivities of alliinase activity. *J. Agric. Food Chem.* **2001**, *49*, 2592−2599.
(3) Lawson, L. D. Garlic: a review of its medicinal effects and indicated active compounds. In *Phytomedicines of Europe: Chemistry and Biological Activity*; Lawson, L. D., Bauer, R., Eds.; ACS Symposium Series; American Chemical Society: Washington, DC, 1998; Vol. 691, pp 176−209.
(4) Harris, J. C.; Cottrell, S. L.; Plummer, S.; Lloyd, D. Antimicrobial properties of *Allium sativum* (garlic). *Appl. Microbiol. Biotechnol.* **2001**, *57*, 282−286.
(5) Reuter, H. D.; Koch, H. P.; Lawson, L. D. Therapeutic effects and applications of garlic and its preparations. In *Garlic: the Science and Therapeutic Application of Allium sativum L. and Related Species*, 2nd ed.; Koch, H. P., Lawson, L. D., Eds.; Williams & Wilkins: Baltimore, MD, 1996; pp 135−212.
(6) Hirsch, K.; Danilenko, M.; Giat, J.; Miron, T.; Rabinkov, A.; Wilchek, M.; Mirelman, D.; Levy, J.; Sharoni, Y. Effect of purified allicin, the major ingredient of freshly crushed garlic, on cancer cell proliferation. *Nutr. Cancer* **2000**, *38* (2), 245−254.
(7) Parsad, K.; Laxdal, V. A.; Yu, M.; Raney, B. L. Antioxidant activity of allicin, an active principle in garlic. *Mol. Cell. Biochem.* **1995**, *148*, 183−189.
(8) Iberl, B.; Winkler, G.; Knobloch, K. Products of allicin transformation: ajoenes and dithiins, characterization and their determination by HPLC. *Planta Med.* **1990**, *56* (2), 202−211.
(9) Jaeger, W.; Koch, H. P.; Pfaff, K. Standard allicin adsorbates on silicates. *Phytother. Res.* **1992**, *6* (3), 149−151.
(10) Bocchini, P.; Andalo, C.; Pozzi, R.; Galletti, G. C.; Antonelli, A. Determination of diallyl thiosulfinate (allicin) in garlic (Allium sativum L.) by high-performance liquid chromatography with a postcolumn photochemical reactor. *Anal. Chim. Acta* **2001**, *441* (1), 37−43.
(11) Mondy, N.; Naudin, A.; Christides, J. P.; Mandon, N.; Auger, J. Comparison of GC-MS and HPLC for the analysis of Allilum volatiles. *Chromatographia* **2001**, *53*, S356−S360.
(12) Lawson, L. D.; Wood, S. G.; Hughes, B. G. HPLC analysis of allicin and other thiosulfinates in garlic clove homogenates. *Planta Med.* **1991**, *57* (3), 263−270.
(13) Iberl, B.; Winkler, G.; Müller, B.; Knobloch, K. Quantitative determination of allicin and alliin from garlic by HPLC. *Planta Med.* **1990**, *56* (3), 320−326.
(14) Miron, T.; Shin, I.; Feigenbalt, G.; Weiner, L.; Mirelman, D.; Wilchek, M.; Rabinkov, A. A spectrophotometric assay for allicin, alliin, and alliinase (alliin lyase) with a chromogenic thiol; reaction of 4-mercaptopyridine with thiosulfinates. *Anal. Biochem.* **2002**, *307*, 76−83.
(15) Han, J.; Lawson, L.; Han, G.; Han, P. Spectrophotometric Method for Quantitative Determination of Allicin and Total Thiosulfinates in Garlic. *Anal. Biochem.* **1995**, *225* (1), 157−160.
(16) Stoll, A.; Seebeck, E. Chemical investigations on alliin, the specific principle of garlic. *Adv. Enzymol.* **1951**, *11*, 377−400.
(17) Lawson, L. D. The composition and chemistry of garlic cloves and processed garlic. In *Garlic: the Science and Therapeutic Application of Allium sativum L. and Related Species*, 2nd ed.; Koch, H. P., Lawson, L. D., Eds.; Williams & Wilkins: Baltimore, MD, 1996; pp 37−107.
(18) Mochizuki, E.; Yamamoto, T.; Horie, M.; Ikai, Y.; Nakazawa, H. Simultaneous determination of alliin and allicin in *Allium* plants and their products by liquid chromatography. *J. AOAC Int.* **1997**, *80* (5), 1052−1056.
(19) Miron, T.; Rabinkov, A.; Mirelman, D.; Weiner, L.; Wilchek, M. Spectrophotometric assay for allicin and alliinase (*alliin lyase*) activity: reaction of 2-nitro-5-thiobenzoate with thiosulfinates. *Anal. Biochem.* **1998**, *265* (2), 317−325.
(20) The United States Pharmacopeial Convention. Garlic Delayed-Release Tablets. *United States Pharmacopeia/National Formulary* **2003**, *26/21*, 2752−2754.
(21) Calvey, E. M.; Matusik, J. E.; White, K. D.; DeOrazio, R.; Sha, D.; Block, E. *Allium* chemistry: supercritical fluid extraction and LC-APCI-MS of thiosulfinates and related compounds from homogenates of garlic, onion, and ramp. Identification in garlic and ramp and synthesis of 1-propanesulfinothioic acid *S*-allyl ester. *J. Agric. Food Chem.* **1997**, *45* (11), 4406−4413.
(22) Calvey, E. M.; Block, E. Supercritical fluid extraction of *Allium* species. In *Spices*: *Flavor Chemistry and Antioxidant Properties*; Risch, S. J., Ho, C.-T., Eds.; ACS Symposium Series; American Chemical Society: Washington, DC, 1997; Vol. 660, pp 113−124.
(23) Calvey, E. M.; Matusik, J. E.; White, K. D.; Betz, J. M.; Block, E.; Littlejohn, M. H.; Naganathan, S.; Putman, D. Off-line supercritical-fluid extraction of thiosulfinates from garlic and onion. *J. Agr. Food Chem.* **1994**, *42* (6), 1335−1341.
(24) Calvey, E. M.; Roach, J. A. G.; Block, E. Supercritical-fluid chromatography of garlic (*Allium sativum*) extracts with mass-spectrometric identification of allicin. *J. Chromatogr. Sci.* **1994**, *32* (3), 93−96.
(25) Lawson, L. D.; Hughes, B. G. Characterization of the formation of allicin and other thiosulfinates from garlic. *Planta Med.* **1992**, *58* (4), 345−350.
(26) Calvey, E. M.; White, K. D.; Matusik, J. E.; Sha, D. Y.; Block, E. Allium chemistry: identification of organosulfur compounds in ramp (*Allium tricoccum*) homogenates. *Phytochemistry* **1998**, *49* (2), 359−364.
(27) Rosen, R. T.; Hiserodt, R. D.; Fukuda, E. K.; Ruiz, R. J.; Zhou, Z. Y.; Lech, J.; Rosen, S. L.; Hartman, T. G. *J. Nutr.* **2001**, *131* (Suppl. 3), 968S−971S.
(28) Bruce, G. R.; Gill, P. S. Estimates of precision in a standard additions analysis. *J. Chem. Educ.* **1999**, *76* (6), 805−807.

Received for review July 29, 2003. Revised manuscript received December 4, 2003. Accepted December 14, 2003.

JF034853X