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LIQUID CHROMATOGRAPHIC DETERMINATION OF ALLIIN IN GARLIC AND GARLIC PRODUCTS

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SUMMARY

A liquid chromatographic (LC) method is proposed for the determination of alliin in garlic and garlic products. The method involves heating of the sample with water in a bath of boiling water followed by homogenization and centrifugation. Interfering components are eliminated by use of a Sep-Pak C_{18} cartridge as a clean up step before injection. The LC system with ultraviolet detection at 210 nm consists of a separation on a Zorbax TMS column and isocratic elution with water as a mobile phase. Fluorometric determination by ion-pairing chromatography with tetra-*n*-butylammonium bromide on a Nucleosil 5C_{18} column is also described. The overall recoveries of alliin added to garlic products were greater than 90%. Thin-layer chromatography and enzymatic degradation of alliin were performed for the confirmation of alliin detected in garlic products.

INTRODUCTION

Garlic (*Allium sativum L.*) has been used world-wide as a seasoning, spice and folk medicine. A recent study shows that garlic extracts inhibit platelet aggregation and smooth muscle contractions[1]. In 1981, the platelet aggregation inhibitor was identified as methyl allyl trisulphide by Ariga *et al.*[2]. Cavallito and Bailey[3] found in 1944 that allicin, an enzymatic conversion product of alliin (Fig. 1), is responsible for the antibacterial and antifungal properties of garlic.

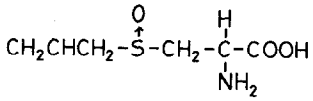

Fig. 1. Structure of alliin.

In Japan, garlic and garlic products have been popular in recent years as a drug and health food. There is now a need for a simple and rapid method of evaluating the quality of these products. Being a specific amine in garlic, alliin could be used as an indicator.

The current method for estimation of the alliin level in garlic employs allinase in an enzymatic method which rapidly converts alliin into allicin, ammonia and pyruvic acid. The ammonia is then measured by azotometry, pyruvic acid spectrophotometrically at 430 nm as the dinitrophenylhydrazone derivatives[4]. These methods are non-specific and time-consuming. Alliin is a primary amine exhibiting an absorption maximum at 210 nm. Thus a fluorophore derivative with fluorescamine was also investigated. Fluorescamine, a fluorophore labelling reagent, has been used for the determination of primary amines[5,6], and its effectiveness has been reported for the determination of various other compounds. This paper describes a liquid chromatographic (LC) method for the rapid and simple determination of alliin in garlic and garlic products, based on ultraviolet (UV) and fluorometric (FL) detection.

EXPERIMENTAL

Apparatus

A Model LC-2 liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-1 spectrophotometer operated at the wavelength of 210 nm and a Shimadzu RF-510 spectrofluorometer with excitation at 405 nm and emission at 480 nm was used.

For UV detection, the separation of alliin was performed on a Zorbax TMS (5 μm, 250 mm $\times$ 4.6 mm I.D.; DuPont, Wilmington, DE, U.S.A.) with isocratic elution using water as a mobile phase at a flow-rate of 0.5 ml/min.

For FL detection, the chromatographic column was Nucleosil $5C_{18}$ (5 μm, 250 mm $\times$ 4 mm I.D.; Macherey, Nagel & Co., Düren, F.R.G.), thermostatted at 50°C. The mobile phase used was 0.03 *M* sodium acetate (pH 7.0)–acetonitrile (66:34, v/v) with 5 m*M* tetra-*n*-butylammonium bromide, at a flow-rate of 0.5 ml/min.

Materials

Fresh garlic bulbs and garlic products were obtained commercially.

The acetonitrile and distilled water were of high-performance liquid chromatography (HPLC) grade (Wako, Osaka, Japan). Analytical grade chemicals were used unless otherwise specified. The alliin standard was donated by Wakunaga Pharmaceutical, Osaka, Japan. A 10-mg amount was weighed accurately into a 10-ml flask and diluted to volume in distilled water.

Sep-Pak C_{18} cartridges (Millipore, Bedford, MA, U.S.A.) were washed with 10 ml of methanol and then 10 ml of water before use.

Thin-layer chromatographic (TLC) plates (10 cm $\times$ 5 cm) were precoated with Silica gel 70 (Wako). The developing solvents were ethanol–25% ammonium hydroxide (63:37), *n*-propanol–25% ammonium hydroxide (67:37), chloroform–methanol–25% ammonium hydroxide (2:2:1) and *n*-butanol–acetic acid–water (3:1:1).

Allinase was prepared from fresh garlic by the method of Tsuno[7] with slight modifications as follows: 10 g of garlic were ground and homogenized with water and diluted to a volume of 100 ml. The homogenate was centrifuged at 3000 *g* for 10 min at

4°C. The supernatant was adjusted to pH 4.0 with phosphoric acid and centrifuged at 12000 *g* for 5 min at 4°C. The supernatant was discarded. The precipitate was dissolved in water, and adjusted to pH 4.0 with phosphoric acid. After centrifugation at 12000 *g* for 5 min at 4°C, the supernatant was discarded. The precipitate was dissolved in 10 ml of phosphate buffer (pH 6.4) and stored in a refrigerator.

Sample preparation

A 10-g sample was weighed in a 100-ml beaker or blender cup with addition of 40 ml hot water. After heating for 15 min in boiling water, the contents were cooled to room temperature and homogenized for 3 min. The homogenate was transferred to a volumetric flask and diluted to 100 ml in distilled water. After centrifugation at 12000 *g* for 10 min at 4°C, the supernatant was filtered through Toyo Roshi No. 5A filter-paper. A 1-ml volume of filtrate was passed through a Sep-Pak C_{18} cartridge and eluted with 8 ml distilled water and brought to a volume of 10.0 ml. The eluate was analysed directly by liquid chromatography with an ultraviolet detector at 210 nm; 10 μl of the solution were injected into the LC system. If necessary, the sample was diluted further in distilled water.

Fluorometric determination of alliin was performed as follows; 50 μl of the eluate from the Sep-Pak C_{18} cartridge were transferred to a 10-ml amber test-tube containing 2 ml of 0.2 *M* sodium borate buffer (pH 8.0), and mixed. A 200-μl volume of a solution of fluorescamine (Roche Diagnostics, Nutley, NJ, U.S.A.) in acetone (3 mg/ml) was then added and mixed well. The LC analysis was carried out with a fluorometric detector (excitation, 405 nm; emission, 480 nm).

Confirmation of alliin by TLC and enzymatic treatment

The sample solution, prepared through use of the Sep-Pak C_{18} cartridge, was evaporated to dryness below 45°C in a vacuum. The residue was dissolved in 0.5 ml of water, and applied to precoated silica gel plates. After development in different media, the plates were sprayed with 0.2% ninhydrin reagent and placed in an oven at 110°C for 10 min. Alliin was identified by comparison with the standard on the same TLC plate.

For further confirmation, enzymatic treatment was employed. A 1-ml volume of allinase solution was added to 1 ml of sample solution which had been previously analysed by LC. The pooled sample was incubated for 15 min at 37°C. The results were confirmed by the disappearance of the alliin peak on the liquid chromatogram.

RESULTS AND DISCUSSION

As alliin shows maximum UV absorption at 210 nm, UV detection was employed. The retention of alliin on different columns was examined using phosphate buffer, pH from 2.5 to 8.0, mixed with acetonitrile. A trimethylsilyl (TMS) column gave symmetrical peaks with more reasonable retention times than those on an ODS column, when water was used as a mobile phase. No significant differences between retention times and peak shapes were observed in the range pH 2.5 to 8.0. Based on preliminary studies of the chromatographic separation including interference from co-existing compounds, a Zorbax TMS column and water as a mobile phase were chosen for the UV method.

The introduction of fluorophore derivatives with fluorescamine was considered because co-existing interfering substances were found with UV detection in some liquid preparations. The optimum LC operating conditions were derived from variation of the column, column temperature and mobile phase composition. The widely used C_{18} reversed-phase column gave better resolution of the alliin–fluorescamine complex than RP-8 and TMS columns. The maximum temperature for a reasonable retention time and resolution was 50°C, when the column temperature was investigated from 25 to 55°C. The effect of the mobile phase composition on the alliin separation was investigated with different contents of acetonitrile and acetate buffer. Ion-pairing chromatography with tetra-*n*-butylammonium bromide was also studied since the alliin–fluorescamine complex possesses a carboxyl group. The effect of the sodium acetate concentration up to 50 m*M* was studied, and 30 m*M* sodium acetate buffer, pH 7.0 was employed to measure the alliin–fluorescamine complex.

As fluorescamine reacts with primary amines at pH ranging from 8.0 to 9.5, and with peptides from pH 8.0 to 8.5[8], the effect of the pH of the borate buffer was studied and a pH of 8.0 was chosen. The excitation and emission maxima for the fluorescamine–alliin reaction product were 405 and 480 nm, respectively. A fluorescamine concentration of $9.6 \cdot 10^{-4}$ *M* resulted in the maximum fluorescence intensity in the range of $1.6 \cdot 10^{-4}$–$1.3 \cdot 10^{-3}$ *M* fluorescamine against 50 μl alliin (0.1 mg/ml). The fluorophore derivatives of alliin were stable for over 9 h when kept in a dark room at room temperature.

Correlation between the two detection methods, UV and FL, for concentrations ranging from 10 to 60 μg/ml was high with a correlation coefficient, *r*, of 0.996. The detection limits with these two methods were 10 μg/ml for UV and 1 μg/ml for FL. The standard graphs were linear throughout the ranges of 10–200 μg/ml for UV and 1–1000 μg/ml for FL.

Effect of heating on alliin

Alliin is readily converted into allicin, ammonia and pyruvic acid by the enzyme in fresh plants during dropping and crushing. Thus, fresh garlic and garlic products were heated in boiling water to inactivate the enzyme. The stability of alliin to heating was studied at 100°C and it was shown to be fairly stable; the amount of alliin remaining was 94.7% after 20 min, 88.3% after 40 min and 79.4% after 60 min.

Clean up

The Sep-Pak C_{18} cartridge was examined as a rapid and easy clean-up for alliin from the extract. A 1-ml volume of alliin solution (0.5 and 2.5 mg/ml) was loaded on the prepared Sep-Pak cartridges and eluted with water. No alliin was retained on the cartridge. Even the larger amount of alliin (2.5 mg) was totally eluted from the reversed-phase cartridge; alliin was totally eluted with 6 ml water, and co-existents such as dyes were retained on the cartridge. The eluent containing tetra-*n*-butylammonium chloride did not completely separate alliin from sample coextractives. Therefore, water was chosen as the eluent for clean up.

Recovery of alliin

The recovery of alliin from selected and spiked samples is shown in Table I. The recovery from four determinations with the UV method ranged from 96.6 to 99.5%

TABLE I

RECOVERY OF ALLIIN FROM COMMERCIAL GARLIC PRODUCTS

Sample	*Added (mg/g)*	*Recovery, % (mean ± S.D.)**	
		UV	*FL*
Garlic bulbs in honey	2.5	97.8 ± 5.1	90.4 ± 3.7
Garlic bulbs in soy sauce	3.0	99.5 ± 2.4	103.3 ± 14.1
Salted bulbs	2.5	96.7 ± 4.6	
Freeze-dried powder	7.5	98.0 ± 1.4	94.9 ± 2.8
Freeze-dried tablet	6.0	99.1 ± 1.2	
Liquid formulation	7.5	96.6 ± 0.9	91.0 ± 1.3

* Average of four determinations.

with excellent precision. The recovery with the FL method ranged from 90.4 to 103.3%. The minimum detection limits for alliin were 0.5 mg/g (UV) and 0.05 mg/g (FL).

Analysis of commercial samples

The alliin contents of fresh garlic and commercial products determined by the methods proposed are shown in Table II. Alliin was found in garlic pellets, freeze-dried samples and salted products. Fresh garlic contained 4.9 mg/g alliin (coefficient of variation, C.V. = 13.8%). Other forms contained little or no alliin. Figs. 2 and 3 show typical chromatograms of commercial garlic products. Alliin was found in freeze-dried garlic powder. UV detection was simple, but susceptible to interference by compounds absorbing at 210 nm. FL detection was 10 times more sensitive and specific than UV detection.

TABLE II

CONTENTS OF ALLIIN IN GARLIC AND GARLIC PRODUCTS

Sample	*n*	*Found (mg/g)*	
		UV	*FL*
Garlic bulbs	8	4.5–6.2	4.0–6.0
Garlic bulbs in honey	5	<0.5	<0.05
Garlic bulbs in miso	1	0.6	
Garlic bulbs in mirin	1	<0.5	
Garlic bulbs in soy sauce	2	0.5–0.6	
Salted bulbs	3	0.6–0.9	
Garlic powder	2	<0.5	<0.05
Grated garlic paste	2	<0.5	<0.05
Garlic pellets	1	6.1	5.4
Freeze-dried powder	1	10.5	9.0
Freeze-dried tablet	1	3.0	2.8
Liquid formulation	2	<0.5	<0.05
Tablet formulation	1	<0.5	<0.05

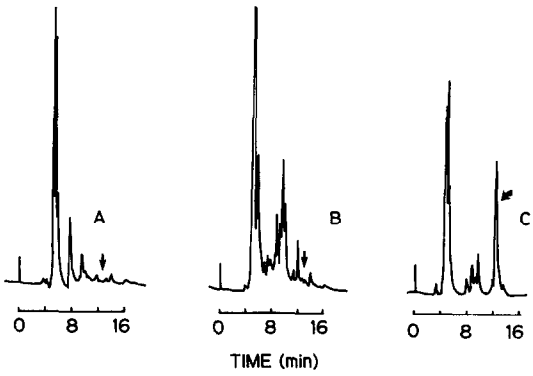

Fig. 2. Liquid chromatograms of commercial garlic products with ultraviolet (UV) detection. Arrow shows alliin peak. (A) Garlic bulbs in honey; (B) liquid formulation; (C) freeze-dried powder. Conditions: column, Zorbax TMS (250 mm × 4.6 mm I.D.); mobile phase, water; flow-rate, 0.5 ml/min.

Confirmation of alliin

TLC with several developing solvents was investigated for confirmation of alliin. Ethanol–25% ammonium hydroxide (63:37), *n*-propanol–25% ammonium hydroxide (67:33), chloroform–methanol–25% ammonium hydroxide (2:2:1) and *n*-butanol–acetic acid–water (3:3:1) gave R_F values of 0.77, 0.64, 0.20 and 0.75, respectively. By comparison of these R_F values with those of an authentic sample, TLC provided a means of confirmation of alliin.

In addition to TLC, enzymatic treatment of alliin by allinase was also studied by Tsuno's method[7]. The extract was incubated with allinase for 15 min at 37°C. This treatment resulted in the disappearance of the peak established as alliin on the LC chromatogram, as shown in Figs. 4 and 5. Alliin degradation by enzymatic treatment provided additional proof of the presence of alliin.

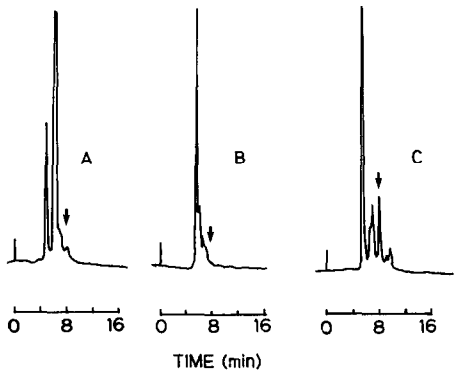

Fig. 3. Liquid chromatograms of commercial garlic products with fluorometric (FL) detection. Arrow shows alliin peak. (A) Garlic bulbs in soy sauce; (B) liquid formulation; (C) freeze-dried powder. Conditions: column, Nucleosil $5C_{18}$ (250 × 4.0 mm I.D.); mobile phase, 0.03 *M* sodium acetate (pH 7.0)–acetonitrile (66:34, v/v) with 5 m*M* tetra-*n*-butylammonium bromide; flow-rate, 0.5 ml/min; column temperature, 50°C.

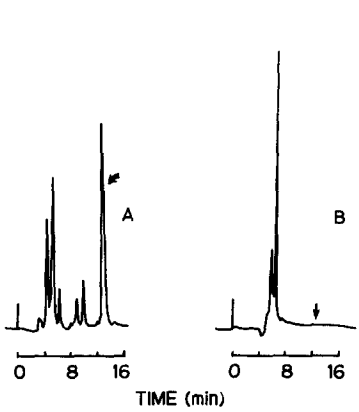

Fig. 4. Disappearance of the alliin peak in a garlic extract upon enzyme treatment (UV detection). (A) Untreated; (B) treated. Arrow shows alliin peak. Conditions: as in Fig. 2.

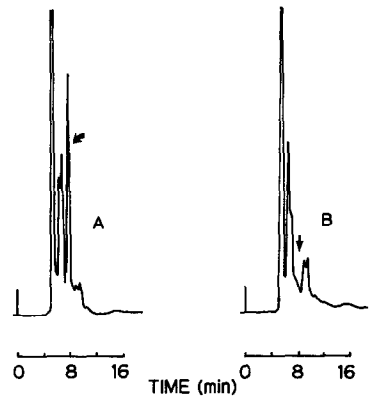

Fig. 5. Disappearance of the alliin peak in a commercial garlic product upon enzyme treatment (FL detection). (A) Untreated; (B) treated. Arrow shows alliin peak. Conditions: as in Fig. 3.

The data obtained from the present study indicated that the LC method for the determination of alliin was simple, straightforward and accurate. Ultraviolet detection enabled direct determination of alliin, and the FL method, based on a fluorometric derivatization, showed greater specificity and sensitivity. Both the UV and FL methods for the determination of alliin using LC are suitable for the quality assessment of garlic and garlic products. The present LC method can be applied to the other alliin containing plants of *Allium* species.

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