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Green pigment in crushed garlic (*Allium sativum* L.) cloves: Purification and partial characterization

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Abstract

Unknown green pigment, responsible for greening in crushed garlic cloves, was purified and characterized by using a series of column chromatography, liquid chromatography–electrospray ionization (LC–ESI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. The purified green pigment was highly polar and slightly viscous, with a garlic flavor, and easily turned to a yellow or brown color with exposure to room temperature. The absorption spectrum in methanol showed a crude methanolic green pigment-like profile with two absorbance maxima at 440 and 590 nm.

A compound based on MS spectra showing the ion peak at m/z 412 is responsible for greening. Although complete isolation of the molecular weight (MW) 411 compound for proper structure elucidation was not achieved in our experiments, the MS and NMR spectra of the MW 411 green pigment suggested the ambiguous structural assignment of one sulfur atom and odd numbers of nitrogen atoms, with 25–30 carbons including aromatic ring. Therefore, we suggest the possibility that the green pigment MW 411 observed in crushed garlic cloves is a new sulfur-containing nitrogenous water-soluble compound differing significantly from all previously reported green pigments in plants.

Keywords: *Allium*; Garlic; Greening; Discoloration; Green pigment; Spectrometry; Spectroscopy

1. Introduction

Garlic (*Allium sativum* L.) is a common food crop used in all parts of the world. Modern garlic likely originated from a wild ancestor in Central Asia. As one of the oldest of cultivated plants, garlic has been cultivated for more than 5000 years and is valued as a food, a medicine and as a flavoring. The genus *Allium* contains abundant amounts of sulfur compounds, which are primarily responsible for its therapeutic and biological activities (Abdullah, Kandil, Elkadi, & Carter, 1988). In addition to its well recognized medicinal properties, garlic as a health food has a therapeutic value with its antivirus, antifungal, and antibiotic characteristics, as well as an antioxidative action. Therefore, many medicinal uses of garlic have been developed (Ankri & Mirelman, 1999; Cavallito & Bailey, 1944; Mayeux et al., 1988).

Garlic is processed in various forms including purees, juice, powder, and oleoresin. Recently, the steady growth in retail sales of fresh ready-to-use vegetables, occurring in direct response to marketplace demands, has lead to minimally processed pre-peeled and chopped garlic products (Hong & Kim, 2001). During processing, intensely colored pigments are often formed. The formation of green pigmentation, called greening, represents the phenomenon of discoloration from a cream to green color in crushed

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garlic cloves. This phenomenon occurs only in mechanically bruised or finely cut tissue, but not in carefully sliced garlic cloves. Recently, development of green pigmentation, similar to the greening during garlic processing, has been reported in the traditional homemade Chinese ''Laba'' garlic product, which is pickled in 5% acetic acid solution (v/v, pH 2.33). After garlic cloves are soaked in acetic acid (such as vinegar) for 2 days, a green color develops. After 4 days, the pickling solution itself begins to turn green (Bai et al., 2005).

It has been previously suggested that the greening is a multi-step process similar to the pink discoloration of onion (Kubec, Hrbacova, Musah, & Velisek, 2004; Lukes, 1986; Shannon, Yamaguchi, & Howard, 1967a, Shannon, Yamaguchi, & Howard, 1967b). There was an essential enzyme step in the first phase of the pigment formation and at least three steps were involved, suggesting the possible role of a wide range of amino acids (Lukes, 1959). Unknown colorless ether-soluble compounds (also called ''color developers'') reacted with certain amino acids in onions to form a second colorless compound insoluble in ether. The latter compound then reacted with formaldehyde or naturally occurring carbonyl to form the final pigment (Shannon et al., 1967a, 1967b). Soon after, this essential enzyme was considered an alliinase. The activity of alk(en)yl-L-cysteine sulfoxides as flavor precursors, especially S-1-propenyl cysteine sulfoxide (1-PeCSO, isoalliin), an abundant lachrymatory compound in onion, became the focus of study. The role of 1-PeCSO in pinking (Bandyopadhyay & Tewari, 1973) and greening (Lukes, 1986) was suggested. This proposal appears to be supported by the results of recent study (Kubec et al., 2004), which show that at pH 5.5, a dark blue pigment was formed as the result of a system containing isoalliin, alliin, glycine, and alliinase. Also, the formation of pink pigment in onion and green pigment in garlic were confirmed to be of similar in nature, with 1-PeCSO serving as the primary precursor. Several factors, such as pH, temperature, time of heating, salt, and sulfite content, which influence the formation of green pigment in garlic tissue macerates, have also been investigated by researchers (Ahmed, Pawanpreet, & Shivhare, 2001; Cho, Ku, & Kim, 1999; Hong & Kim, 2001; Lukes, 1986).

Although the reactions, which remain theoretical, have been extensively studied, little is known about the green pigment responsible for greening, as there have been no attempts to isolate, identify, or document it. Furthermore, the structures of the pigment remain unknown (Kubec et al., 2004). It has previously been reported that the pigment observed in macerated onion bulbs tissues may be a new nitrogenous water-soluble red pigment (Joslyn & Peterson, 1958). Green pigment extracted from crushed garlic cloves were found to be readily soluble in water and in polar solvents but only sparingly soluble in hexane, ether, or other non-polar solvents. Also, the pigments did not behave like chlorophyll or a related porphyrin pigment (Joslyn & Sano, 1956). The absorption spectrum of a methanol extract of the crude green pigment, with maximum absorption at 440 and 590 nm (Bai et al., 2005), was also quite different from that of chlorophyll broadly observed in plants.

Knowledge of the molecular structure would be useful in explaining the mechanism of greening in crushed garlic cloves. This, in turn, could lead to better selection of garlic bulbs, and control of factors influencing greening. Thus, the primary aim of this study is to characterize the unknown green pigment observed in crushed garlic cloves. As a first step, the molecules involved were identified according to the standard procedure for characterization of unknown compounds, including: extraction, ultra-violet (UV) spectrometry, flash column chromatography (CC), high performance liquid chromatography (HPLC), mass spectrometry (MS) using electrospray ionization (ESI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), and nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Chemicals

All solvents and chemicals used were of analytical grade or purer. Amberlite XAD-16 and Sephadex LH-20 resins were purchased from Sigma Chemicals (MO, USA) and Amersham Pharmacia Biotech. (Uppsala, Sweden), respectively. Solvent grade methanol, ethanol, trifluoroacetic acid (TFA), petroleum ether, chloroform, sodium hydroxide (NaOH), glycerol, tetrahydrofuran (THF), and cyano-4-hydrocynamic acid were purchased from Sigma Chemicals and methanol-d_4 was purchased from Merck (NJ, USA). Distilled and deionized water was used throughout this study.

2.2. Plant materials

Garlic (*Allium sativum* L.) was purchased from local retail shops in Korea. Garlic bulbs were stored at 0 °C for at least 5 months, and showed greening upon complete crushing.

2.3. Green pigment extraction

For pigment analysis, 4 kg peeled garlic cloves were ground in a blender and held at room temperature for maximum green color development. Green color garlic homogenates were extracted with 3 l of 100% methanol at 5 °C for 1 day. The methanol was then removed and extraction was repeated once in fresh methanol (1 l) under the same conditions as above. The green solution was separated from the plant tissues using a Büchner funnel with filter paper (Whatman No. 4). To achieve complete pigment extraction from the plant material, the filtered residue was rinsed twice with the extraction solution and finally with 80% methanol (500 ml). The combined extracts were filtered

and concentrated in vacuo (35 °C) to a small volume (about 300 ml). The condensate was treated with petroleum ether to remove fats and other ether-soluble components (Saito, 2000). Condensate free of ether was re-suspended in 100 ml of purified water, and flushed with nitrogen before freezing at −70 °C until purification.

2.4. Precipitation of hydrocolloids and proteins

Hydrocolloids and proteins were removed by adding 2 ml of 96% cold ethanol to 1 ml of green pigment solution. After 5 min, the mucilages were separated from the aqueous phase with filter paper on a Büchner funnel and washed with an ethanol:water mixture (2:1, v/v) until the residue was colorless. Ethanol was removed under reduced pressure at 35 °C and the residue dissolved in 50 ml of acidified water (pH 3) before being used for CC. Acidified water was prepared by adding TFA to purified water until the pH reached 3.0 (Stintzing, Schiber, & Carle, 2002a, 2002b).

2.5. Desalting and fraction by Amberlite XAD-16 polymeric resin

Green pigment were purified using a series of the following CC described in Fig. 1. All procedures (Kim, Choi, & Chung, 2000; Lee & Chou, 2000; Park et al., 2000; Stintzing et al., 2002a, Stintzing et al., 2002a, 2002b; Yang, Oh, Lee, Kim, & Song, 2002) were slightly modified and optimized throughout the study.

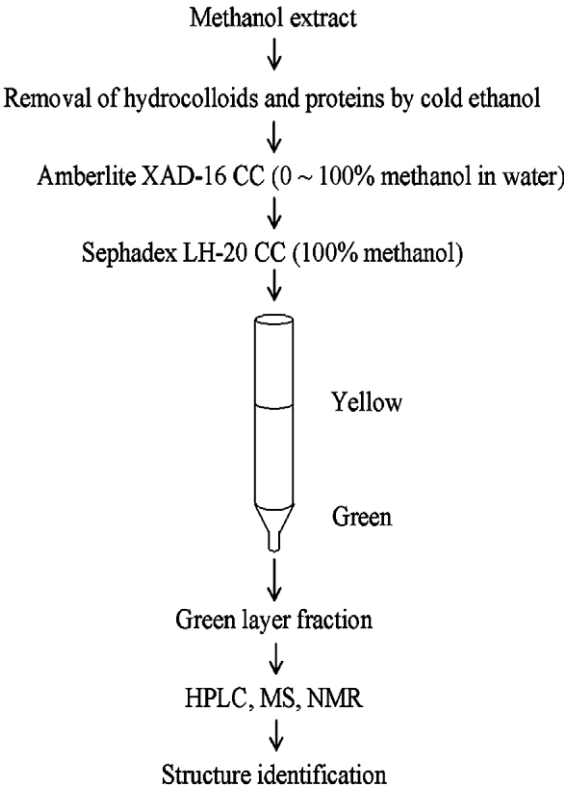

Fig. 1. Purification and identification procedures for green pigment extracted from crushed garlic cloves.

Amberlite XAD-16 resin was slurred in purified water and held overnight at 4 °C. Before use, the materials were rinsed with purified water until the supernatant became clear. A glass column (500 × 20 mm i.d.) was filled with resin. The sorbent material was rinsed with 1 l of purified water and activated with 500 ml of 2% aqueous NaOH. After neutralization by rinsing with purified water (500 ml), the material was conditioned to pH 3 by washing with 1 l of acidified water. The sample (20 ml) was applied to the resin and subsequently desalted by rinsing with 1 l acidified water at a flow rate of 13 ml/min. The green pigment were then eluted with a gradient of 500 ml methanol in water from 0% to 100%, and the absorbance of each fraction measured at 590 nm with a spectrometer (UV 1601, Shimadzu Co., Kyoto, Japan). The methanol extract fractions (from 80% to 100%) showing the highest absorbance at 590 nm were combined and evaporated for the next Sephadex LH-20 CC procedure.

2.6. Fractionation by Sephadex LH-20 CC

On a Sephadex LH-20 column (700 × 15 mm i.d.) equilibrated with 100% methanol, concentrated green color fraction obtained from XAD-16 CC was loaded and eluted with 100% methanol at a flow rate of 4.5 ml/min. Fractions were pooled into two major partitions; upper-layer yellow color and lower-layer green color. The green layer fraction was further subjected to HPLC, MS, and NMR spectroscopy.

2.7. Reversed-phase HPLC

HPLC conditions were as follows: an analytical scale (250 × 4.6 mm i.d.) *Alltima* C_{18} column (Alltech Associates, Inc., Deerfield, IL, USA) with particle size of 5 μm; injection volume, 10 μl; column temperature, 20 °C; mobile phase, an isocratic mode of 0.1% TFA in water and methanol (50:50, v/v); flow rate, 1.0 ml/min; detector, UV–vis (210, 240, 280, 445, and 595 nm). The peaks of interest were automatically collected using a Gilson FC 205 fraction collector with a total of approximately 30 injections. The pooled fractions (10 ml) were concentrated to a small volume in a rotary evaporator and analyzed by MS and NMR spectroscopy.

2.8. MS

To obtain mass spectra, all samples were dissolved in 100% methanol and 1–2 μl per sample was applied to the probes. FAB MS spectrum was recorded on a JMS-AX505WA (Jeol, Tokyo, Japan) using glycerol as a matrix. LC-ESI MS spectrum was obtained by a Quattro LC triple–quadruple mass spectrometer equipped with HPLC (Hewlett-Packard, HP1100, CA, USA) and nanoflow-ZTM electrospray source using both the positive and neg-

ative ion modes. MALDI-TOF MS spectrum was obtained on a Voyager-DE STR Biospectrometry Workstation spectrometer (Applied Biosystems, Inc., CA, USA) in the positive ion modes. The instrument was operated at an accelerating voltage of 20 kV and an extractor voltage of 9 kV. The pressure was $\sim 5.0 \times 10^{-6}$ Torr. Cyano-4-hydrocynamic acid was used as a matrix for MALDI-TOF MS, and a calibration mixture I (Applied Biosystems Inc., CA, USA) was used as the standard.

2.9. NMR spectroscopy

Three milligrams of green pigment were dissolved in 500 μl of methanol-d_4. ^{13}C and DEPT135 NMR spectra were recorded on a Bruker Avance 600 spectrometer (600 MHz), using the methanol solvent peak as a reference.

2.10. UV spectrometry

Crude or purified green pigment dissolved in 100% methanol were filtered and placed into a quartz cuvette for spectral measurement.

3. Results and discussion

Successful extraction or separation of green pigment from crushed garlic cloves was only available with polar solvents such as water, acetic acid, methanol, and ethanol, rather than with ethyl acetate, diethyl ether, chloroform, and dichloromethane. Green pigment of interest was highly polar, and therefore the choice of solvent was an important consideration to perform CC and thin-layer chromatography (TLC). By starting with a non-polar solvent, most of the green compound remained at the top of the column or bottom of the TLC plate. Although TLC has been used for the purification of mixed green pigment extracts, no separation was attained even with the various solvent systems used. The efficiency of this technique was not significant for separation of the green pigment from the impurities (data not shown).

Green pigment purified by various chromatography techniques was slightly viscous, had garlic flavor, and easily developed yellow or brown color at room temperature. Its absorption spectrum in methanol showed a crude methanolic green pigment-like profile with two absorbance maxima at 440 and 590 nm (Fig. 2). The discolored extracts (yellow and brown) from the original green color had only a maximum absorption at 440 nm. High absorbance at 590 nm was the most important feature of green pigment extracted from crushed garlic cloves (Joslyn & Sano, 1956). With increasing time at room temperature, the purified green pigment turned from yellow to brown, and finally disappeared. This decline was reflected in the absorbance spectra: the 590 nm absorbance declined, while the 440 nm absorbance of the yellow color intensely increased with time at room temperature. It was previously assumed that at least two species of pigment existed in ''Laba'' garlic pickling solution; one species with a yellow color, and a second with a blue color. The combination of both species would therefore result in the observed green color of the pickling solution (Bai et al., 2005). However, our present results indicated that two maximal absorbances at 440 and 590 nm in the visible region were characteristics of the purified green pigment molecule itself. Green pigment may be reaction product of ether thiosulfinates (R–S(O)–S–R′, R = allyl, R′ = (*E*) or (*Z*)-propenyl; or R = (*E*)-propenyl, R′ = allyl) with glycine at pH 5.5 (Kubec et al., 2004).

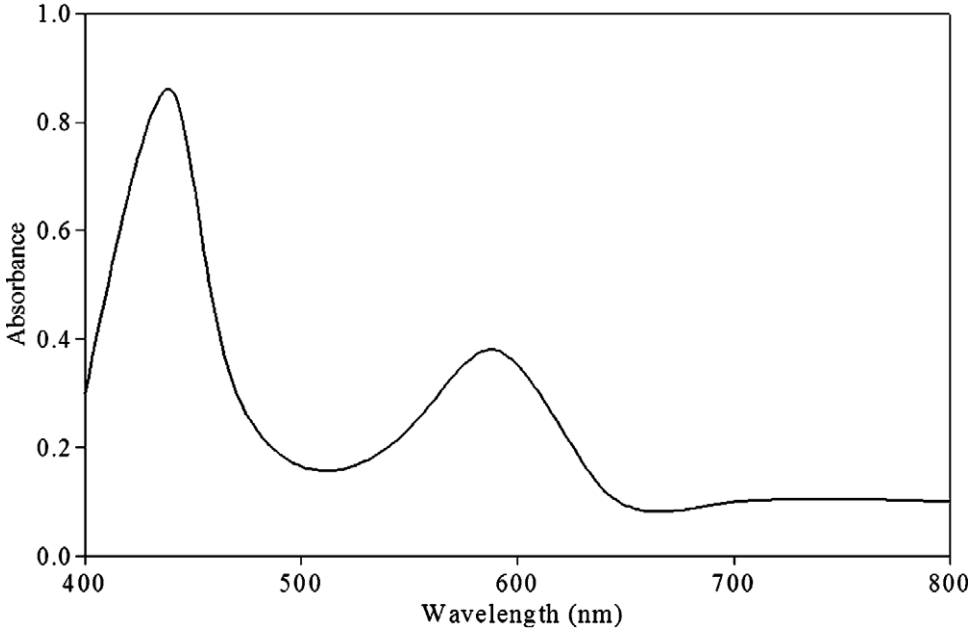

Fig. 2. Absorption spectrum of green pigment (dissolved in 100% methanol) purified from crushed garlic cloves.

The isolation and purification procedure for green pigment from garlic extracts were detailed in Fig. 1. The recovery efficiency was not considered in this procedure as the primary objective of the work was to characterize the unknown green pigment.

Organic constituents from examined samples were found to be complex mixtures of hydrocarbons, alcohols, aldehydes, acids, phenols, and pigments. Thus, the extracts were simplified into sub-fractions prior to instrumental analysis (Barth, Tjessem, & Aaberg, 1981). Although polymeric XAD-16 (Lepane, 1999) and Sephadex LH-20 resins (Archambault & Bègue, 1984; Johnels, Edlund, & Wold, 1982; Ramos & Prohaska, 1981; Van der Watt & Pretorius, 2001; Vercruysse, Delcour, & Dondeyne, 1985) have been widely applied in various organic compound analyses, they have not previously been used for the separation of the unknown green pigment extracted from crushed garlic cloves. Separation of this crude green pigment could be achieved on both sorbents by using the green pigment solvent polarity characteristics. After desalting by acidified water (pH 3), green color fractionation on XAD-16 was possible with stepwise gradients of methanol in water from 0% to 100%. Green pigment exhibited retention characteristics and could not be completely separated from the resins before eluting with 80–100% methanol.

The green color fraction eluted with 80–100% methanol was further purified by gel chromatography on Sephadex LH-20 with chloroform and stepwise gradients of chloroform, methanol, and THF. Barth et al. (1981) reported that the elution of gradient polar constituents was performed using chloroform to elute hydrocarbon, alcohols, hydroperoxides and all other non-hydroxy-containing polar

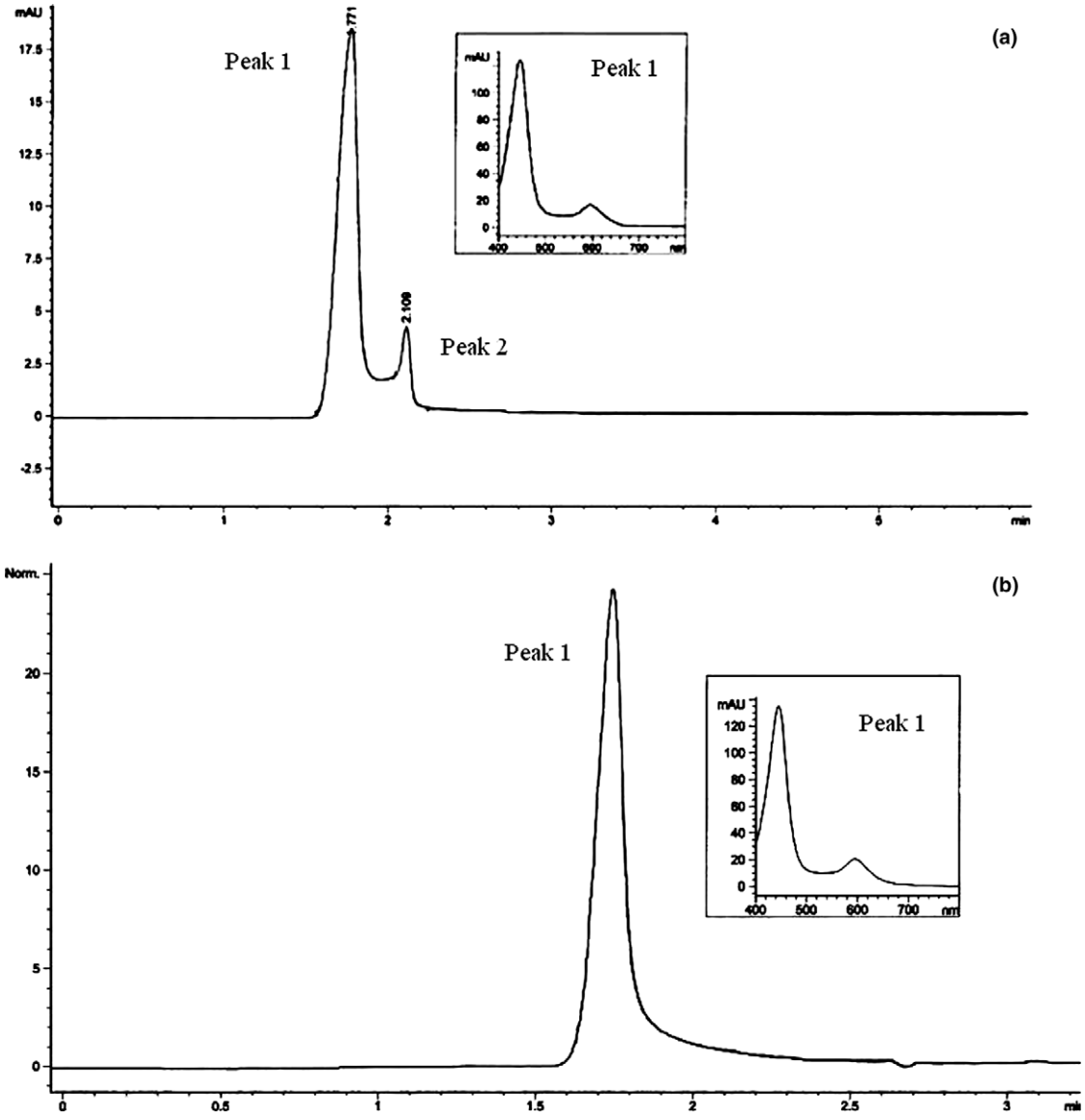

Fig. 3. HPLC chromatogram of lower-layer green fraction on Sephadex LH-20 CC. Peak 1 was determined to be the green pigment of interest, according to the absorption spectrum inserted in chromatogram (a). Only Peak 1 was collected using a fraction collector repeatedly, and re-injected into HPLC to confirm separation (b).

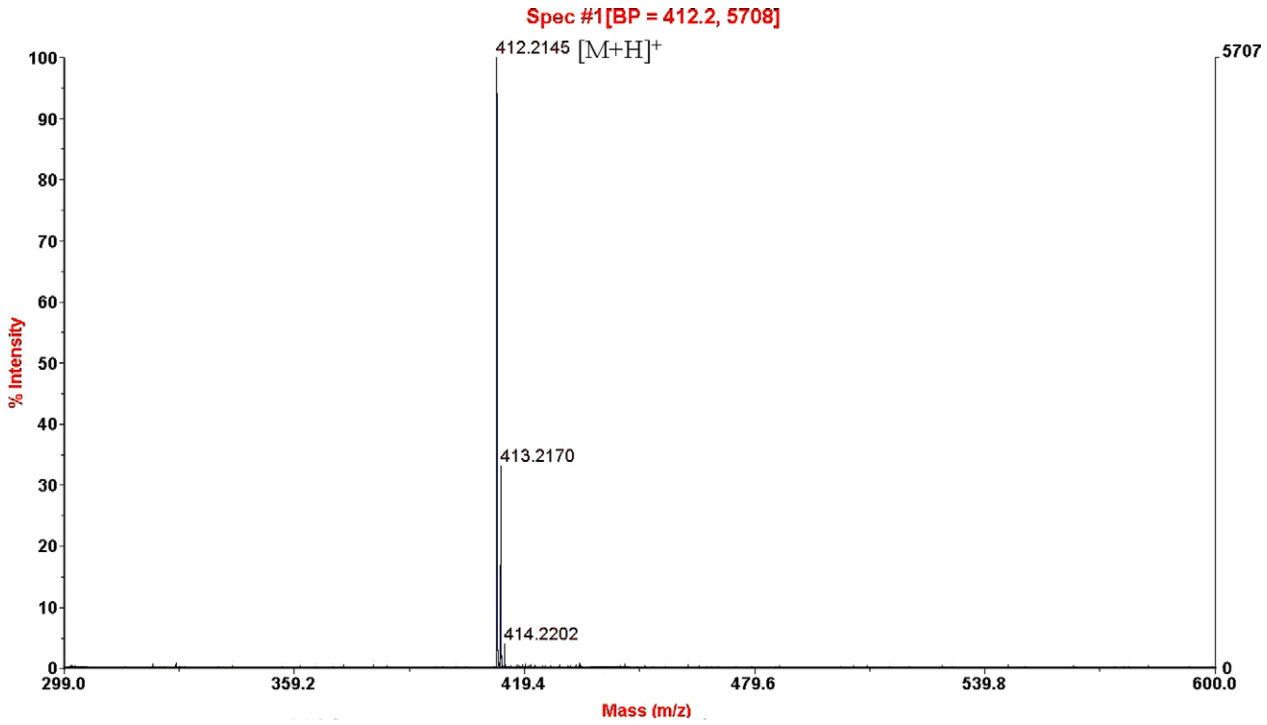

Fig. 4. MALDI-TOF MS spectrum of the green pigment isolated from crushed garlic cloves. Cyano-4-hydrocynamic acid and calibration mixture I were used as the matrix and the standard, respectively.

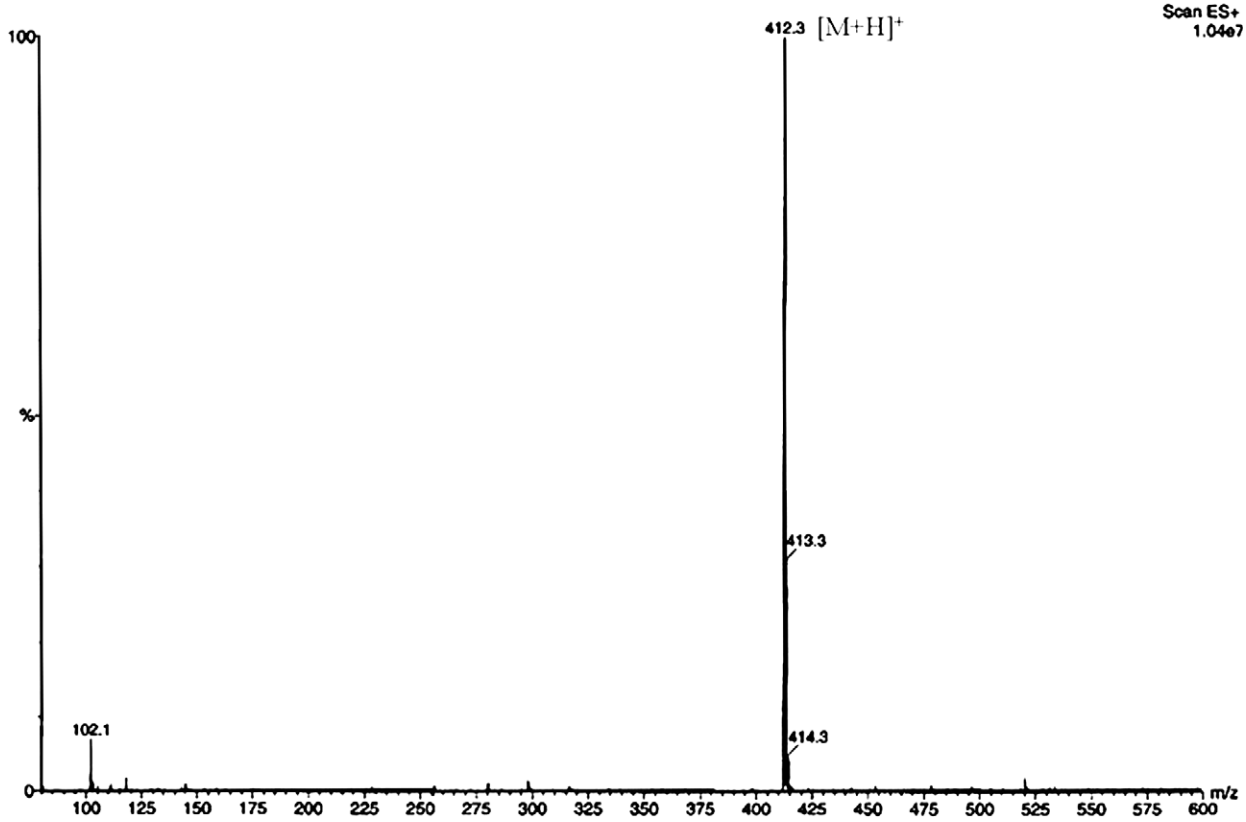

Fig. 5. Positive LC–ESI MS spectrum of the green pigment isolated from crushed garlic cloves.

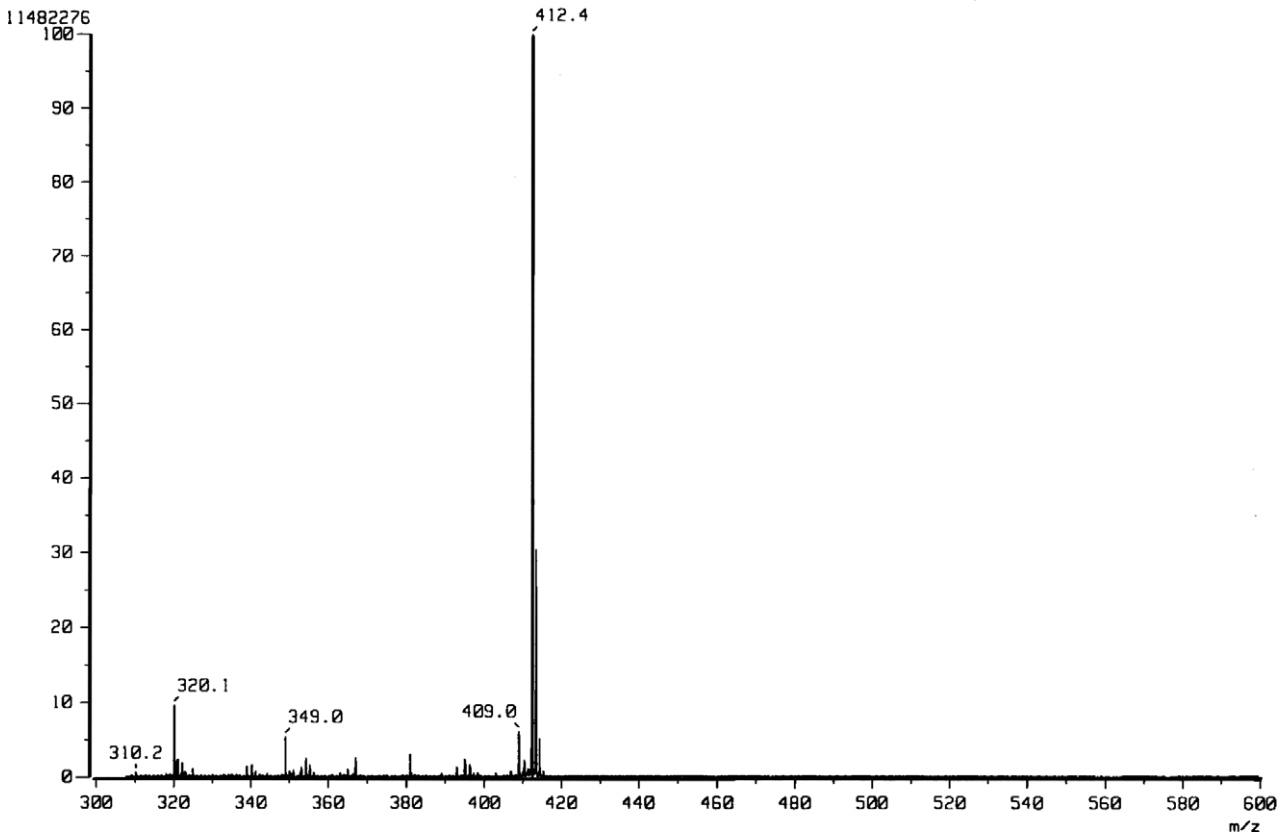

Fig. 6. Positive FAB MS spectrum of the green pigment isolated from crushed garlic cloves. Glycerol was used as a matrix.

components, 5% methanol–chloroform to elute phenol, 10% methanol–chloroform to elute diols and dihydroxy compounds, 20% THF–chloroform to elute fatty acids and other monoacids, and 30% THF–methanol to remove the remainder of the polar material sticking to the gel bed. However, these solvent systems did not effectively elute green pigment. The green pigment adsorbed to the Sephadex LH-20 beads was removed only by washing gently with 100% methanol, which then resulted in the division of the solution into two fractions: an upper-layer yellow color and a lower-layer green color.

To achieve efficient resolution of the target compound, HPLC condition was examined by varying mutual solvent volume ratios and flow rates. After several attempts, an isocratic HPLC separation method on an analytical *Alltima* C_{18} column was developed. The best results were obtained with a mobile phase consisting of 0.1% TFA in water and methanol (50:50, v/v). As a result, green fractions on Sephadex LH-20 could be separated within 5 min. Green fractions consisted of two major peaks, Peak 1 and Peak 2, although the two peaks were not completely separated (Fig. 3a). The first peak, at a retention time of 1.7 min, was the green pigment of interest, according to the absorption spectrum inserted in Fig. 3a. The second peak (Peak 2) had an absorbance only at 440 nm, and it is yellow. Therefore, only Peak 1 was collected using a fraction collector, and then was re-injected into HPLC to confirm separation (Fig. 3b). Finally, the pigment was prepared for MS and NMR spectroscopy to characterize the molecular structure.

ESI and MALDI-TOF MS under soft ionization conditions are useful in determining relative purity and molecular mass of a compound. A wide mass range scan, with a lower voltage, is typically used to ensure that all components were detected as their protonated adducts (Pasch, Pizzi, & Rode, 2001). The acquired mass spectra of this green pigment showed a single prominent protonated molecular ion peak at m/z 412 $[M+H]^+$ by MALDI-TOF MS (Fig. 4), consistent with results obtained from LC-ESI (Fig. 5) and FAB MS spectra, although some impurity peaks at m/z 310, 320, 349, and 409 were observed in FAB MS spectrum (Fig. 6). Possible molecular formulas of the green pigment were presented in Table 1, according to

Table 1
Possible molecular formula of the green pigment isolated from crushed garlic cloves

C25H17N1O3S1
C25H21N3O1S1
C25H33N3O1S1
C25H49N1O1S1
C26H21N1O2S1
C26H37N1O1S1
C27H25N1O1S1
C28H13N1O1S1

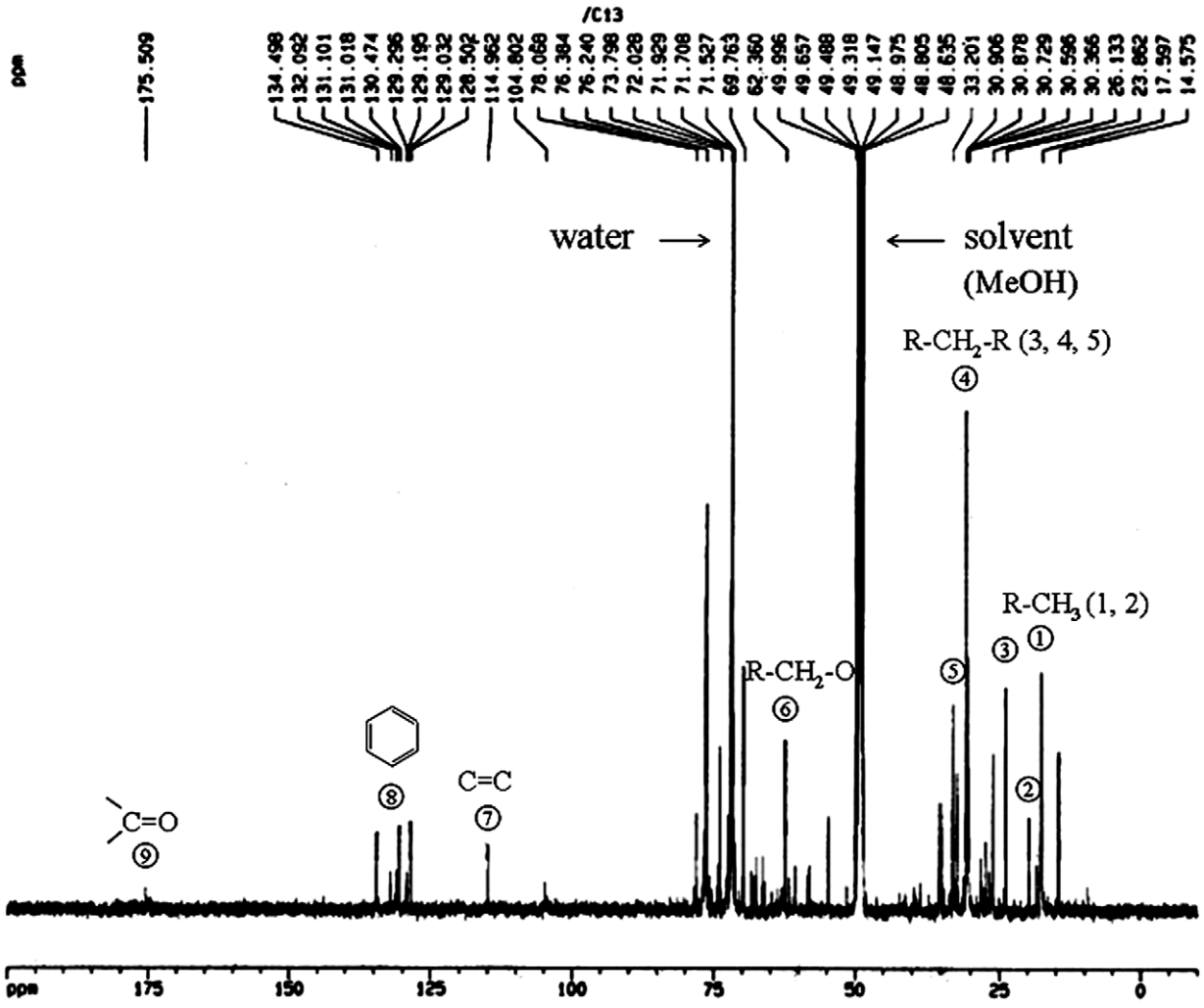

Fig. 7. ^{13}C NMR spectrum of the green pigment isolated from crushed garlic coves.

the results of these MS spectra, which showed one sulfur atom (4–5% of [M+1] ion peak), odd nitrogen numbers (odd number of molecular weight, 411), and between 25 and 30 carbons (~32% of [M+1] ion peak).

The NMR spectra of the green pigment were not clear enough to elucidate its exact structure due to the instability of the pigment during purification and NMR scanning procedures. However, the presence of some carbon groups could be assigned through ^{13}C and DEPT135 spectrum (Figs. 7 and 8). Saturated carbon atoms appeared at high field (8–60 ppm); R–CH_3 (① and ②), R–CH_2–R (③, ④, and ⑤). The next section (40–70 ppm) demonstrated the effect of electronegative atoms; R–CH_2–O (⑥). The chemical shifts of unsaturated carbon (⑦, alkene) and aromatic ring carbon atoms (⑧) appeared at 100–150 ppm, respectively. Carbonyl group (⑨) finally appeared at low field values (155–220 ppm). The observed values for aromatic ring were 137.6, 130.0, 126.2, and 128.2 ppm, respectively, and the substituents were related to benzene ring in *meta* position. In DEPT 135 spectrum, methine and methyl carbons gave rise to positive peaks, while methylene carbons appear as inverse peaks. The methylene carbons produced the inverted peaks: carbon ③, ④, and ⑤ appeared at 25.1, 30.1, and 33.4 ppm, respectively. Carbon ⑥ was deshielded since it is near the electronegative oxygen atom. The carbonyl carbon ⑨ did not appear in the DEPT135 spectrum since it has no attached hydrogen atoms (Fig. 8).

4. Conclusion

Confirmation studies by mass spectral data implied that the MW of green pigment observed crushed garlic cloves was 411. Although sufficient data from spectroscopy was not obtained, purification procedures and NMR data described here provided useful information regarding the unknown green pigment extracted from crushed garlic cloves. This pigment was made up of one sulfur atom, odd numbers of nitrogen, and 25–30 carbon atoms with aromatic ring. The green pigment, with a MW of 411, was finally suggested to be a new sulfur containing a nitrogenous water-soluble compound differing significantly from previously reported green pigment in plants.

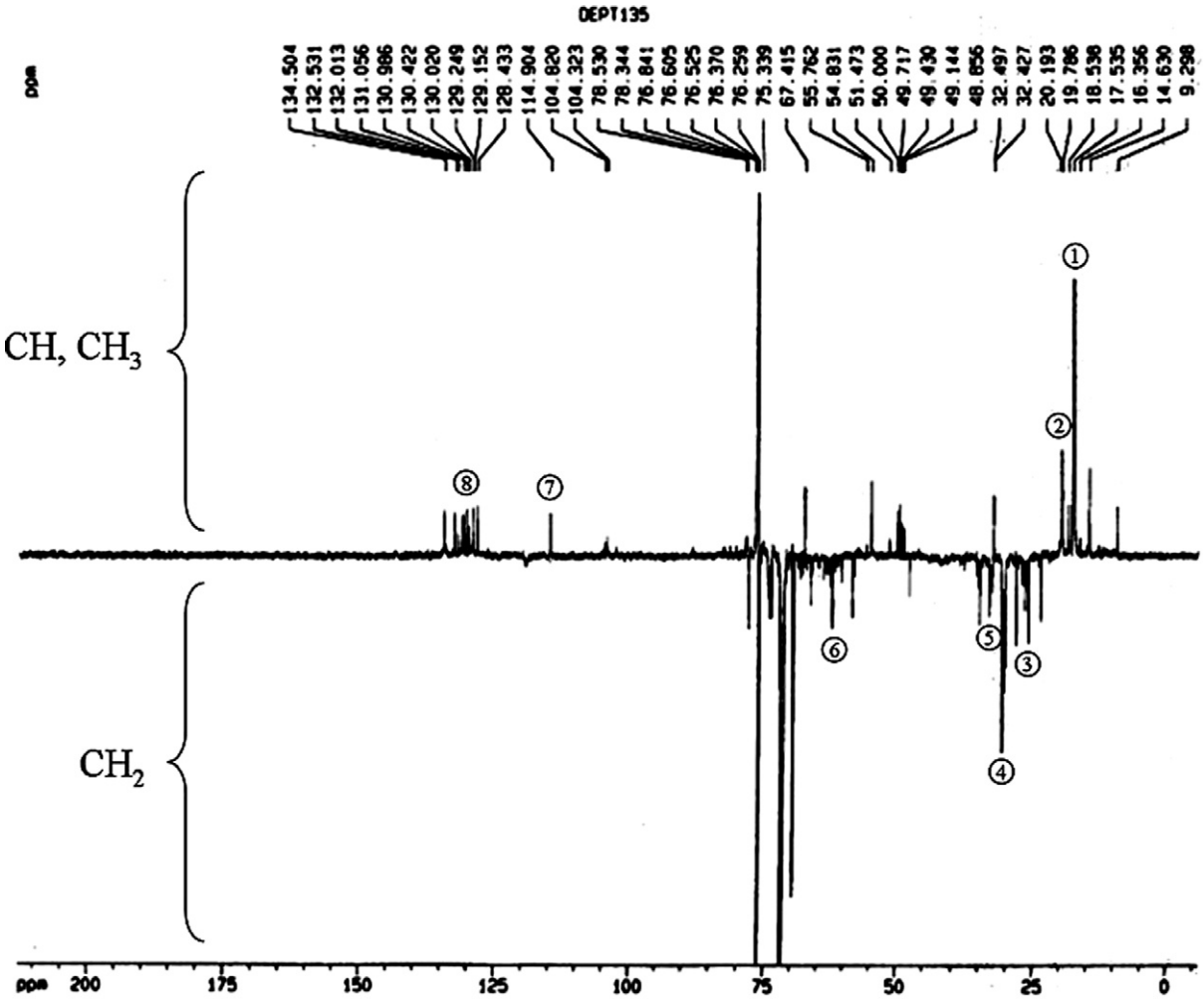

Fig. 8. DEPT135 NMR spectrum of the green pigment isolated from crushed garlic coves.

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