

Analysis of a Reported Organosulfur, Carcinogenesis Inhibitor: 1,2-Dithiole-3-thione in Cabbage

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The putative occurrence of a reported naturally occurring chemical carcinogenesis inhibitor, 1,2-dithiole-3-thione, in cabbage (*Brassica oleracea* L.) was investigated by utilizing both reversed-phase high-performance liquid chromatography and capillary gas chromatography-mass spectrometry. Liquid-solid preparatory chromatography, charcoal adsorption, and mercuric chloride precipitation were all used to isolate the compound. Although a number of previously identified organosulfur compounds were isolated in various cabbage extracts, this study did not reveal 1,2-dithiole-3-thione naturally occurring in cabbage. In addition, two novel organosulfur compounds have been preliminarily identified in cabbage, but these compounds, as well as previously reported dithiolethiones, may have originated by thermal sulfurization reactions during the isolation procedures.

INTRODUCTION

It is established that many Brassicaceae vegetables, e.g., cabbage, Brussels sprouts, broccoli, and cauliflower, possess anticarcinogenic properties in laboratory animals (Boyd et al., 1982; Wattenberg, 1983) and in humans (Michonovicz and Bradlow, 1990). Numerous investigations have focused on various organosulfur compounds found in these vegetables that possess these effects. For example, the isothiocyanates and their metabolites have been characterized as to their biological activities (Wattenberg, 1977; Miller and Stoewsand, 1983), and the allylic and alkylic (poly)sulfides have been shown to have some carcinogenesis inhibition activity (Wargovich and Goldberg, 1985; Sparnins et al., 1988). In addition, there have been studies regarding reportedly very potent carcinogenesis inhibitors in cabbage, 1,2-dithiole-3-thiones (Figure 1) (Ansher et al., 1986; Kensler et al., 1987). This natural sulfur compound had been shown to be a component in cabbage over 30 years ago (Jirousek and Starka, 1958).

This study investigated the putative occurrence of dithiolethiones in cabbage by utilizing both reversed-phase high-performance liquid chromatography (HPLC) and capillary gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Materials and Reagents. Cabbage (*Brassica oleracea* L.) was purchased from a local supermarket and rinsed, and the outer leaves were removed. 1,2-Dithiole-3-thione was kindly provided by Dr. T. W. Kensler of The Johns Hopkins University and was reconstituted with methanol or 2-propanol. Octadecyl (C_{18}) bonded silica gel chromatographic packing (40 μ m) was purchased from Baker, Inc. (Phillipsburg, NJ). Charcoal was activated lignite based granular Darco (20-40 mesh) purchased from American Norit Co., Inc. (Atlanta, GA). All chemical reagents and redistilled solvents were of analytical grade.

Isolation of Dithiolethione-Containing Extracts. Five 1.5-kg aliquots of cabbage were initially chopped and macerated with 3.0 L of 80% boiling ethanol in a Waring blender for 30 s as suggested by Harborne (1973). After 10 min, extracted liquid (pH 6) was cooled on ice to room temperature, filtered first by using cheesecloth with a pneumatic hand press at 1000 psi, and then vacuum filtered by using Whatman No. 4 and No. 1 filter papers to remove visible waxes. After addition of 400 mL of water, the extract was separated twice with 400 mL of methylene chloride. The organic layer was washed with aqueous sodium

chloride, concentrated in vacuo by using a Rotovap (<60 °C), and transferred into 25 mL of methanol. This extract was then loaded onto an open preparatory chromatographic column (21 cm \times 2.5 cm i.d.) containing C_{18} bonded silica gel and eluted by using 100% methanol at a flow rate of 2.0 mL/min. After the void volume (approximately 70 mL) was discarded, 100 mL of eluate was collected. All aliquots were combined, concentrated, and re-eluted at a flow rate of 1.0 mL/min through another preparatory column (30 cm \times 1.5 cm i.d.) providing better separation. One and a half column volumes of eluate was again collected, concentrated, and filtered with a 0.5- μ m Millipore PTFE filter, yielding a total volume of 25 mL.

The extract was first analyzed by reversed-phase HPLC using a methanol gradient and then transferred into 30 mL of hexane, dried over sodium sulfate, and reanalyzed via GC-MS.

Isolation of Mercuric Chloride Precipitated Fractions. To assess the validity of the original dithiolethione isolation protocol (Jirousek and Starka, 1958), two 2-kg aliquots of fresh cabbage were initially chopped, macerated with 80% ethanol in a Waring blender, filtered as previously described, and passed through a Büchner funnel containing 500 g of charcoal (Darco 20-40 mesh) previously rinsed with chloroform and benzene. The adsorbed material was eluted from charcoal initially with chloroform, followed by benzene, concentrated, and transferred into methanol. Lipids were separated by chilling the methanol solution at 2 °C.

Some sulfur-containing compounds were then precipitated overnight at 2 °C by using methanolic mercuric chloride ($HgCl_2$). The precipitate was collected with a 1.0- μ m Gelman A/E glass fiber filter and resuspended by the following two methods. One portion of the precipitate was redissolved in aqueous potassium iodide and separated by using methylene chloride. The other precipitate portion was redissolved in pyridine, divided with hydrogen sulfide, and transferred into benzene by salting out with sodium chloride. Both fractions were dried over sodium sulfate, concentrated in vacuo as mentioned previously, and analyzed by GC-MS.

HPLC Analysis of Dithiolethiones. Quantitative analysis of 1,2-dithiole-3-thione was performed by utilizing reversed-phase HPLC as determined by Masoud and Bueding (1983) except that a methanol gradient elution (30-100%) was employed for samples containing crude extracts. System hardware consisted of two Rainin Rabbit HP pumps (with mixer), a 20- μ L sample loop, a 5- μ m (22 cm \times 4.6 mm i.d.) C_{18} column (Brownlee Labs) enclosed in a column heater (kept at 35 °C), and a Gilson Holo-chrome UV-vis variable-wavelength detector. Detection of 1,2-dithiole-3-thione was monitored at 415 nm (log $E = 4$). Limit of detection ($S/N = 5$) was 2 ng injected. Data analysis was generated by using the Rainin Dynamax HPLC Method Manager

which controlled both the operational aspects of the HPLC system and the acquisition, analysis, and editing of generated data.

GC-MS Analysis. A Hewlett-Packard Model 5890A capillary gas chromatograph equipped with mass selective detector (MSD) Model 5970B was used for the detection and mass spectral display of the 1,2-dithiole-3-thione standard and suspected dithiolethione-containing extracts. A Restek Rtx-5 (5% phenylmethylsilicone, Restek Corp., Bellefonte, PA) bonded phase fused silica capillary column (30 m \times 0.25 mm i.d., d_f = 0.2 μ m) was coupled directly to the MSD capillary interface.

Operating conditions of the GC were as follows: injection volume, 1.0 μ L; injector temperature, 275 $^{\circ}$ C; helium flow rate, 1 mL/min; oven temperature, 60 $^{\circ}$ C (held for 1 min) programmed to 260 $^{\circ}$ C at 5 $^{\circ}$ C/min. Linear retention indices were calculated against C₆-C₂₃ *n*-paraffins as references. Mass spectra were obtained by electron impact ionization (EI) over the range 35–500 amu at 0.86 scans/s. The ion source temperature was 240 $^{\circ}$ C, and electron impact energy was 70 eV.

To increase sensitivity, some analyses utilizing selective ion monitoring (SIM) were performed. The selected amu ions used in quantitation of 1,2-dithiole-3-thione had the following *m/z* values: 134 (the molecular ion), 136, 101, and 69. Limit of detection (*S/N* = 20) using SIM (*m/z* of 134) was 0.4 ng injected.

GC-FPD Analysis. In addition to the MSD, a Tracor Model 222 flame photometric detector (FPD) with sulfur filter (394 nm) was also used to identify sulfur-containing compounds. A Restek Rtx-5 (5% phenylmethylsilicone, Restek) bonded phase fused megabore column (30 m \times 0.53 mm i.d., d_f = 1.5 μ m) was used to separate compounds in the sample extract.

Operating conditions of the GC were as follows: injection volume, 1.0 μ L; injector temperature, 210 $^{\circ}$ C; helium flow rate, 10 mL/min; nitrogen flow rate, 80 mL/min; oven temperature, 80–260 $^{\circ}$ C at 4.5 $^{\circ}$ C/min. The detector was kept at 175 $^{\circ}$ C with hydrogen (flow rate, 80 mL/min) and air (flow rate, 150 mL/min). As GC and column conditions were slightly different between the MSD and FPD, internal standards (pesticides: eptam, methyl parathion, captan, and trithion) were used to correlate relative retention times between the two GC's and detectors.

RESULTS AND DISCUSSION

In their original paper, Jirousek and Starka (1958) used an unknown amount of plant material from either *Brassica oleracea* var. *Capitata* (cabbage) or var. *Acephala* (kale) to isolate reported dithiolethiones. Since conditions about cultivar, season of harvest, or stage of plant development were not mentioned in their original paper, isolation of these compounds in the present study was attempted by using commercial cabbage purchased from a local supermarket.

Efficiency of the isolation method used in the present study was monitored by spiking cabbage with a standard containing pure 1,2-dithiole-3-thione. The standard was dissolved in methanol and introduced into the boiling ethanol extractant. Recovery of the 1,2-dithiole-3-thione spike was greater than 75%.

In preliminary experiments involving Brussels sprout extracts, a compound corresponding to the retention time of 1,2-dithiole-3-thione was detected and the fraction was collected (post detector) from the HPLC eluate by bracketing the retention time around the pure standard. This fraction as well as other fractions of the HPLC eluate that showed strong absorbance at 415 nm were collected, concentrated, transferred into 1 mL of hexane, and analyzed by GC-MS. These fractions showed no significant mass spectral fragmentation patterns characteristic of dithiolethiones, indicating that neither the parent compound, 1,2-dithiole-3-thione, nor any other dithiolethione was present in the Brussels sprout extracts at limits of detection for the various instruments. The mass spectrum of unsubstituted dithiolethione is shown in Figure 2. Pedersen and Moller (1972) reported on the

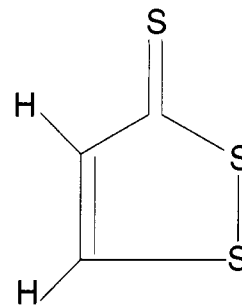


Figure 1. Structure of unsubstituted 1,2-dithiole-3-thione.

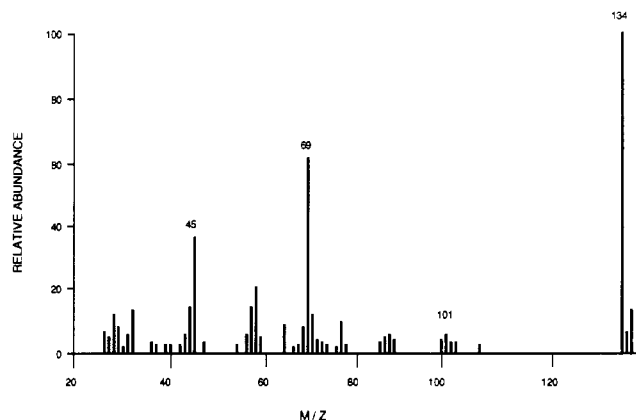


Figure 2. Mass spectrum of 1,2-dithiole-3-thione.

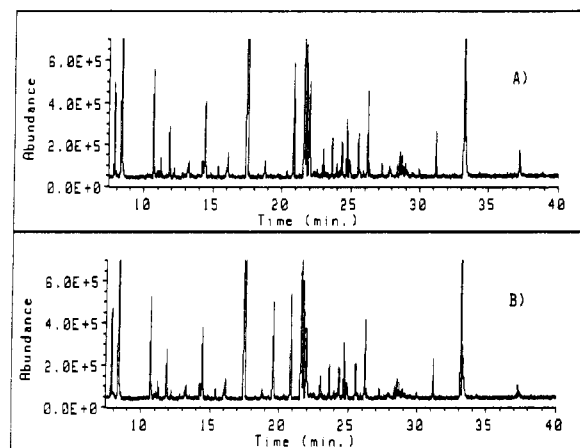


Figure 3. GC-MS total ion chromatograms of (A) cabbage extract and (B) cabbage extract spiked with 50 ng of 1,2-dithiole-3-thione (retention time of 19.58 min).

mass spectra of methyl- and phenyl-substituted dithiolethiones. In all instances, the molecular ion was the base peak. Strong losses of 33 ($M - HS$) and 65 amu ($M - HS_2$) accounted for other abundant ions dependent on position of substitution.

In the present study, even though the HPLC analysis of the crude cabbage extract showed no peaks corresponding to the retention time of 1,2-dithiole-3-thione, GC-MS analysis utilizing SIM was employed to improve detection sensitivity. Results of this analysis also indicated that 1,2-dithiole-3-thione was not present in cabbage at a concentration greater than 1 ppb. GC traces of the cabbage extract with and without a dithiolethione spike are shown in Figure 3.

To assess the possibility that this study perhaps had somehow failed to isolate dithiolethione in cabbage as reported by Jirousek and Starka (1958), additional procedures that followed their original isolation protocol were performed.

Preliminary work indicated that only 4.4% of a spiked 1,2-dithiole-3-thione standard was eluted off charcoal with chloroform. Elution of the charcoal with benzene increased recovery of 1,2-dithiole-3-thione by 21.2%. However, since chloroform was used by Jirousek and Starka (1958), elution with both chloroform and benzene was performed in the remaining experiments. In their original work, the mercuric chloride precipitate was resuspended with pyridine, transferred into benzene, and divided with hydrogen sulfide. There was some question as to how the released compounds were originally transferred into benzene, i.e., by salting out or acidification. Since aqueous potassium iodide is frequently used in the liberation of thiocarbonyl-containing precipitates including dithiolethiones (Challenger et al., 1953), this method of resuspension was also attempted.

Quantitative differences were mainly found between the liberated compounds by using the two methods (i.e., via hydrogen sulfide or potassium iodide). Although some qualitative differences in isolated sulfur compounds were found between both methods of resuspension, analysis by GC-MS indicated 1,2-dithiole-3-thione was not present in either extract at minimum levels of detection. Indeed, GC-MS data of the mercuric chloride precipitate did indicate the presence of some of the higher boiling, previously characterized, sulfur-containing volatile compounds, e.g., isothiocyanates, sulfur-containing nitriles, and (poly)sulfides (Buttery et al., 1976). However, some of the lower boiling sulfur volatiles were not detected, probably due to volatilization during the concentration procedures. In addition, two novel organosulfur compounds were detected both by GC-FPD and by GC-MSD, which have not been mentioned previously in the literature. Ongoing research is currently in progress to identify these compounds, although mass fragmentation tentatively indicates polysulfide thiophene derivatives.

During the investigation, concern was raised whether these novel polysulfides were indeed naturally occurring or simply present as an artifact of isolation procedures. Since charcoal is known to contain low percentages of elemental sulfur by weight (American Norit Co., personal communications), investigations into the contents of charcoal were initiated. By use of chloroform or benzene solvent extraction of charcoal, classic GC-MS spectra of elemental sulfur were indeed observed. Since thermal reactions of sulfur with hydrocarbons (thermal sulfurization) are known to occur, it could be possible that the sulfur from charcoal reacts with various naturally occurring compounds present in cabbage. Mayer (1977) reported that many organic compounds react or cyclize to form thiophenes or dithiolethiones when heated with sulfur. Even at temperatures as low as 130 °C, olefins can react with sulfur to form dithiolethiones.

In their original protocol, Jirousek and Starka (1958) steam distilled the resuspended mercuric precipitated residue. Although conditions of the steam were not given, thermal sulfurization most likely could occur. In the present study, the resuspended mercuric precipitate was separated into methylene chloride and injected into the GC. Thermal sulfurization reactions probably occurred within both injection port and column of the GC system. In fact, retention times of both novel compounds identified and elemental sulfur were concomitant. Even though elemental sulfur was not detected in this extract, some sulfur may have initially been present and thus thermally reacted, forming artifact compounds.

Many of the organosulfur compounds found in cabbage in this investigation have been reported to be inhibitors

of chemical carcinogenesis. However, using comparatively modern analytical techniques, this study does not indicate the presence of 1,2-dithiole-3-thione occurring naturally in cabbage at concentrations greater than 1 ppb. Keeping in mind the purpose of this study was to determine if a measurable concentration of 1,2-dithiole-3-thione naturally existed in cabbage, our results indicate that this is not the case. The presence of 1,2-dithiole-3-thione naturally occurring in cabbage at levels of biological significance should be reconsidered.

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