Gas Chromatography/Mass Spectrometry Method for the Determination of Sulforaphane and Sulforaphane Nitrile in Broccoli

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Phytochemicals, especially the secondary metabolites synthesized by plants, play key roles in human nutrition, health, wellness, and disease prevention. Some phytochemicals may be harmful to human health. For example, two closely related 4-methylsulfinylbutyl glucosinolate [glucoraphanin] hydrolysis products from broccoli (*Brassica oleracea* L. var. Botrytis), 1-isothiocyanato-4-(methylsulfinyl)butane (sulforaphane) and 5-(methylsulfinyl)pentanenitrile (sulforaphane nitrile), may have beneficial or deleterious effects on human health, respectively. Preliminary studies using a gas chromatography/mass spectrometry (GC/MS) system operated under split/splitless conditions revealed that ≈80% of sulforaphane was degraded to 3-butenyl isothiocyanate. A GC/MS method was developed wherein thermal degradation of sulforaphane was reduced to 5% through the use of an appropriate injector liner and precise control of the carrier gas flow rates. The method provides a simple, rapid technique for the analysis of both sulforaphane and sulforaphane nitrile that is suitable for routine screening of plant materials.

Keywords: *Sulforaphane; sulforaphane nitrile; GC/MS; isothiocyanate; Brassica oleracea; broccoli*

INTRODUCTION

Consumers are increasingly aware of the health benefits associated with the phytochemicals or secondary metabolites found in fruits and vegetables. The effects of these phytochemicals on human health and nutrition depend upon numerous factors including the chemical structure, matrix, and quantity consumed. Glucoraphanin, a glucosinolate found in broccoli (*Brassica oleracea* L. var. Botrytis), produces sulforaphane and sulforaphane nitrile when it is hydrolyzed by the enzyme thioglucosidase (Cole, 1976). Sulforaphane is a potent inducer of phase II enzymes, including quinone reductase and glutathione *S*-transferases, which protect against carcinogens and other toxic electrophiles (Zhang et al., 1992, 1994). In contrast, nitriles have been shown to be toxic to both rats and chicks (Ringenberg and Wallig, 1997; Srivastava et al., 1975; VanEtten et al., 1969) and thus could have implications in human health.

Investigators have used various methods, including high-performance liquid chromatography (HPLC) (Zhang et al., 1992; Daxenbichler et al., 1977), gas chromatography (GC), and GC/MS (VanEtten et al., 1976; Cole, 1976) to analyze sulforaphane, sulforaphane nitrile, and other glucosinolate hydrolysis products from the Brassica family (Itoh et al., 1985; Kirk, 1984). These methods do not lend themselves to the routine, rapid quantitation that is necessary for repetitive assays for various reasons. Among them are the relatively low sensitivity of HPLC methods compared with GC methods, the lack of a characteristic UV absorbance which limits the choice of solvent for HPLC separations, and the elaborate isolation or sample preparation procedures employed for GC methods. A new analytical method is reported here that is capable of screening a large number of samples without elaborate sample preparation and cleanup procedures. The identification of 3-butenyl isothiocyanate as a primary thermal degradation product of sulforaphane is also reported. Furthermore, this technique allows for the separation and quantitative analysis of the predominant glucoraphanin hydrolysis products using small quantities of fresh or dried plant material. The relative simplicity of sample preparation and the higher sensitivity afforded by GC/MS combine to make this a rapid, efficient, and reliable method for the analysis of sulforaphane and sulforaphane nitrile.

MATERIALS AND METHODS

Materials. Field-grown broccoli was harvested from the Carl F. Rehnborg Agricultural Research Center, Lakeview, CA, or was purchased from local supermarkets. Extracts were prepared either from fresh material or from material that had been frozen at −60 °C. Sulforaphane standard was provided by Johns Hopkins University School of Medicine (Baltimore, MD). Sulforaphane nitrile was a generous gift from Dr. A. Wallig at the University of Illinois. Thioglucosidase (EC 3.2.2.1) was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium sulfate was of reagent grade, methylene chloride was of GC grade, and water was of HPLC grade.

Phytochemical Extraction. One gram of freshly harvested broccoli was homogenized in 10 mL of warm (50 °C) distilled water at medium speed for 5 min using an Omni 5000 mixer (Omni International, Waterbury, CT). For frozen broccoli, 25 units of thioglucosidase was added before homogenization to ensure complete hydrolysis. The homogenate was centrifuged at 8000*g* for 5 min, and the supernatant was filtered through a 0.45 μm Teflon filter (Whatman, Inc., Clifton, NJ). Twenty-five milliliters of methylene chloride was added to the filtered supernatant, and the combination was vortexed for 1 min. The mixture was centrifuged at 4000*g* for 5 min to form two phases. The lower methylene chloride layer

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was collected by Pasteur pipet. The aqueous fraction was extracted a second time with 25 mL of methylene chloride as described above. The methylene chloride layers were pooled, dried over sodium sulfate, and filtered through the 0.45 μm Teflon filter. The filtrate was concentrated under reduced pressure at 35 °C to <1 mL using a rotary evaporator. The concentrated extract was diluted to 1.0 mL with methylene chloride prior to injection into the GC/MS.

Gas Chromatography/Mass Spectrometry. Analyses were performed on a Hewlett-Packard (HP) 5890 Series II Plus gas chromatograph with electronic pressure control (EPC) connected to an HP 7673 autosampler and an HP 5972 mass selective detector. The split/splitless injector was operated in splitless mode using a 4 mm inside diameter (i.d.) injection liner (Hewlett-Packard, Palo Alto, CA). An HP-5MS fused silica capillary column (Hewlett-Packard, 30 m, 0.25 mm i.d., 0.25 μm film thickness, cross-linked to 5% phenyl methyl siloxane stationary phase) was used. The entire system was controlled by MS ChemStation software (Hewlett-Packard, version B.02.04). Injector and detector temperatures were 250 and 300 °C, respectively. Column oven temperature was initially set at 40 °C for 2 min, then increased to 270 °C (ramp, 10 °C/min), and held for 5 min. The EPC was used to provide the desired carrier gas flow rates for the various experimental conditions. For the constant flow conditions, the electronic pressure controller was programmed to maintain a flow rate of 1.0 mL/min throughout the chromatographic separation. For fast initial injection flow conditions, the carrier gas flow rate was programmed for an initial pressure of 25.0 psi (3.0 mL/min). After 1 min, the pressure was reduced at a rate of 20.0 psi/min to a pressure of 7.1 psi (1.0 mL/min). To maintain a constant 1.0 mL/min flow rate throughout the chromatographic separation, the pressure was increased at a rate of 0.47 psi/min to a final pressure of 20.3 psi, which was then held for an additional 5 min. Mass spectra were obtained by electron ionization (EI) over a range of 50−550 atomic mass units. Ion source temperature was 177 °C, and the electron multiplier voltage was 1753 eV.

RESULTS AND DISCUSSION

GC/MS Confirmations. MS(EI) of sulforaphane, *m/z* (%): 72 (100), 39 (12), 45 (12), 55 (33), 60 (5), 64 (17), 85 (57), 114 (8), 119 (3), 160 (68), 177 (M^+, 1). MS(EI) of sulforaphane nitrile, *m/z* (%): 55 (100), 39 (19), 41 (29), 45 (11), 63 (21), 64 (57), 78 (10), 82 (33), 145 (M^+, 10). MS(EI) of 3-butenyl isothiocyanate, *m/z* (%): 72 (100), 53 (8), 55 (27), 85 (8), 113 (M^+, 44). These data, as shown in Figure 1,were in agreement with previously published findings (Kore et al., 1993; Ohashi, 1963).

Method Improvements. The split/splitless mode of operation of the GC/MS, employing a 4.0 mm i.d. splitless inlet liner with a constant carrier gas flow rate controlled by the EPC, is typically satisfactory for the analysis of small, volatile, and thermostable molecules. Preliminary experiments demonstrated that these conditions were not acceptable for the analysis of the relatively thermolabile sulforaphane molecule. Degradation of sulforaphane nitrile was not observed. Mass spectral detection of a sulforaphane standard solution (150 μg/mL) indicated that ≈80% of the sulforaphane was degraded to 3-butenyl isothiocyanate (Figure 2A). The identification of 3-butenyl isothiocyanate as a primary thermal degradation product of sulforaphane is reported here for the first time. Confirmation that the formation of 3-butenyl isothiocyanate was the result of the thermal degradation of sulforaphane was obtained by comparison with the technique of on-column injection with programmed temperature vaporization (PTV). By minimizing exposure of the sample to heat, essentially 100% of the standard was detected as sulforaphane (Figure 2B).

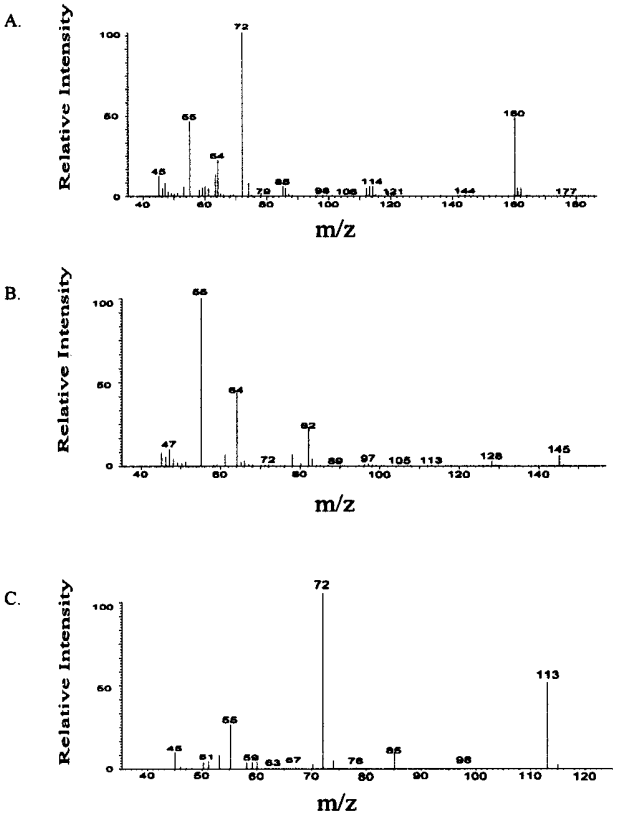

Figure 1. Mass spectra of (A) sulforaphane, (B) sulforaphane nitrile, and (C) 3-butenyl isothiocyanate.

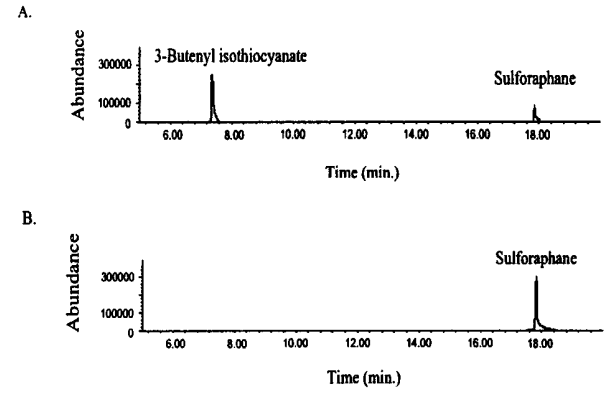

Figure 2. GC/MS chromatograms of sulforaphane standard using (A) split/splitless injector with 4.0 mm i.d. splitless inlet liner and constant flow and (B) on-column injection with PTV.

Having confirmed the thermal degradation of sulforaphane, different approaches to minimize exposure to heat were considered. Decreasing the injection port temperature is not acceptable since this could result in the deposition of material on the column and have a negative effect on performance characteristics. Similarly, the technique of on-column injection and PTV has only limited value for screening numerous samples of plant materials. This is due to the likelihood of accelerated column deterioration from the deposition of large or nonvolatile components or compounds with very high boiling temperatures (Jennings, 1987). Two modifications to the GC/MS system and its operating parameters were selected. First, using the EPC, the carrier gas flow rate was adjusted from a constant flow throughout the analysis to conditions giving rapid flow of the sample through the injector (fast initial injection flow). A

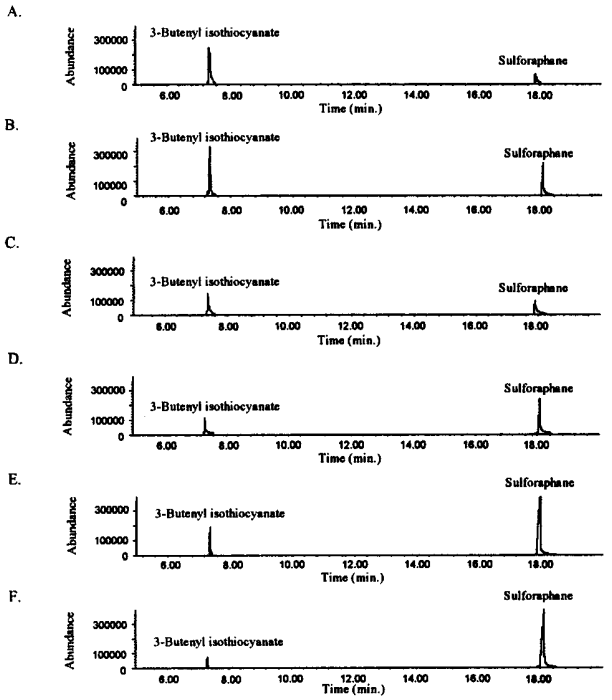

Figure 3. GC/MS chromatograms of sulforaphane standard under six different combinations of inlet liner geometry and carrier gas flow: (A) 4.0 mm i.d. splitless inlet liner with constant flow; (B) 4.0 mm i.d. splitless inlet liner with fast initial injection flow; (C) 4.0 mm i.d. neck-down inlet liner with constant flow; (D) 4.0 mm i.d. neck-down inlet liner with fast initial injection flow; (E) 1.5 mm i.d. direct inlet liner with constant flow; (F) 1.5 mm i.d. direct inlet liner with fast initial injection flow.

second modification, injector liner geometry, was also varied using either 4.0 mm i.d. neck-down inlet liner or 1.5 mm i.d. direct inlet liner configurations to further reduce residence time in the injector and exposure to high temperatures. Various combinations of these two modifications were then evaluated.

Experiments using fast initial injection flow with a 4.0 mm i.d. splitless liner reduced the sulforaphane degradation from ≈80% to 50%. Using the 4.0 mm i.d. neck-down inlet liner with fast initial injection flow also decreased degradation of the sulforaphane to ≈50%. A 1.5 mm i.d. direct inlet liner with constant flow further reduced the degradation to ≈15%. A combination of the 1.5 mm i.d. direct inlet liner and fast initial injection flow resulted in the degradation of 5% of the sulforaphane to 3-butenyl isothiocyanate. Method improvements and corresponding decreases in the degradation of sulforaphane are summarized in Figure 3. The optimal method, using a 1.5 mm i.d. direct liner in conjunction with fast initial injection flow, offers the benefits of on-column injection, that is, minimal thermal degradation, without the associated problem of column deterioration.

Linearity and Calibration. The response factors were based on single characteristic ion fragments of *m*/*z* 160 and 145 for sulforaphane and sulforaphane nitrile, respectively. Five-point calibration curves were linear over the concentration range of 13−266 μg/mL for both sulforaphane and sulforaphane nitrile. Linear regression of peak area vs concentration gave correlation coefficients of 0.996 for sulforaphane and 0.992 for sulforaphane nitrile. The limits of detection were 2 μg/g for both sulforaphane and sulforaphane nitrile.

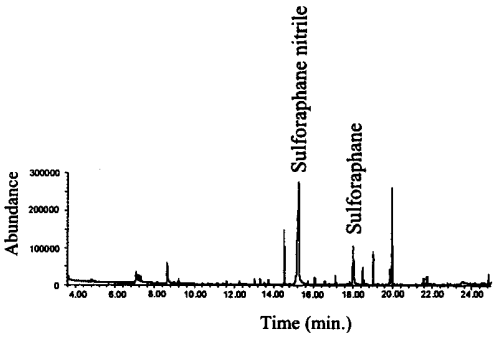

Figure 4. GC/MS chromatogram of fresh broccoli.

Table 1. Sulforaphane and Sulforaphane Nitrile Content of Selected Varieties of Broccoli

variety	sulforaphane (μg/g)	sulforaphane nitrile (μg/g)
Super Dome	3703	208
Mariner	2179	328
Green Belt	2596	192
Sultan	1885	167

Reproducibility and Recoveries. Recovery rates and reproducibility of the method were tested. For the determination of the reproducibility, a randomly selected sample of lyophilized broccoli was extracted and analyzed seven times. The relative standard deviations (RSD) for sulforaphane and sulforaphane nitrile were 4.11% and 4.00%, respectively. Furthermore, triplicate analyses of a broccoli sample following the addition of known amounts of sulforaphane standard and sulforaphane nitrile standard provided recoveries (mean ± RSD) for sulforaphane and sulforaphane nitrile of 87.5% ± 2.4% and 81.4 ± 5.8%, respectively.

Method Applicability. The method was used to quantitate sulforaphane and sulforaphane nitrile in a sample of fresh broccoli (Figure 4). Table 1 shows the sulforaphane and sulforaphane nitrile content (triplicate analyses) of four different varieties of broccoli. The method has been used to determine the content of glucosinolate hydrolysis products in other members of the Brassica family and will be the subject of a future publication.

Conclusion. Broccoli is of great interest in the field of human nutrition and disease prevention. Sulforaphane and sulforaphane nitrile, two compounds produced by the enzymatic hydrolysis of the primary glucosinolate found in broccoli, are of particular interest. A new GC/MS method for the analysis of sulforaphane and sulforaphane nitrile is sensitive, rapid, reproducible, and suitable for routine analysis. This method is now being used to screen >70 different varieties of broccoli and other *Brassica* vegetables to identify those that are high in glucoraphanin and which yield high levels of sulforaphane and low levels of sulforaphane nitrile upon hydrolysis.

NOMENCLATURE AND ABBREVIATIONS USED

Sulforaphane, 1-isothiocyanato-4-(methylsulfinyl)-butane; sulforaphane nitrile, 5-(methylsulfinyl)pentanenitrile; glucoraphanin, 4-methylsulfinylbutyl glucosinolate; HPLC, high-performance liquid chromatography; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; EPC, electronic pressure control; i.d., inside diameter; PTV, programmed temperature vaporization; EI, electron ionization; RSD, relative standard deviation.

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