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Borohydride-Coupled HPLC−FPD Instrumentation and Its Use in the Determination of Dimethylsulfonium Compounds

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Novel HPLC instrumentation has been developed which employs an in-line sodium tetrahydroborate (borohydride) reaction step to generate volatile sulfur species from a variety of sulfonium compounds. Transfer of the resulting volatile sulfur-containing products into the gas phase permits them to be monitored using sulfur-specific flame photometric detection. The system has been evaluated for the determination of a collection of dimethylsulfonium compounds, comprising (dimethylsulfonio)propionate, *S*-methylmethionine (SMM), (dimethylsulfonio)-2-methylpropionate, dimethylsulfocholine, (dimethylsulfonio)-acetate, (dimethylsulfonio)butanoate, and (dimethylsulfonio)pentanoate. Following their separation by either cation- or anion-exchange HPLC, these compounds react in-line with the tetrahydroborate, generating dimethyl sulfide, which is then swept into a flame photometric detector. The development of chromatographic conditions for the resolution of the seven sulfonium compounds is described. In an example application of the instrumentation, the levels of SMM in parsley and cabbage were found to be 16 and 74 mmol kg^{-1}, respectively, on a fresh weight basis.

The release of dimethyl sulfide (DMS) into the atmosphere from aquatic and terrestrial sources is now recognized as being an important natural component of the global sulfur cycle[1] which leads to both increased acidification of precipitation[2] and the possible control of climate through the generation of cloud condensation nuclei.[3] It has been estimated that the oceanic release of DMS may account for 90% of the sulfur flux from the oceans.[4] Highly specific HPLC instrumentation having been previously developed for the measurement of the compound which is believed to be the most commonly occurring natural precursor of DMS, β-(dimethylsulfonio)propionate (DMSP),[5−7] it became necessary to extend the procedures to include a wider range of potential DMS sources.

In recent years, a number of naturally occurring dimethylsulfonium compounds have been identified which might also be expected to break down to yield DMS. Such compounds include *S*-methylmethionine (found in a wide range of flora including barley,[8] asparagus,[9] cabbage,[10] onion seedlings, tomatoes,[11] and green tea[12]), (*S*)-4-(dimethylsulfonio)-2-methoxybutanoate and (*R*)-3-(dimethylsulfonio)-2-methoxypropionate,[13−15] (both found in red macroalgae), gonyauline and gonyol[16−18] (found in the dinoflagelate *Gonyaulax polyhedra*), and the sulfonium analogue of phosphatidylcholine.[19] Dimethylsulfocholine (DMSChol) has been shown to be produced by the hydrolysis of the sulfonium analogue of phosphatidylcholine from the diatom *Nitzschia alba* with cabbage phospholipase D[19] (Figure 1).

While HPLC is a highly versatile analytical tool which is well suited to the separation of ionic sulfonium DMS precursors, the commonly employed detection systems generally exhibit poor selectivity and sensitivity. In contrast, GC detectors have been developed which are capable of high degrees of selectivity and sensitivity, and much attention has therefore focused on attempts to adapt gas chromatographic detectors for use in liquid chromatography. The obvious problem encountered when interfacing a liquid chromatograph to a GC detector is the liquid mobile phase in which the analyte is dissolved. Ideally, the detector should be able to accept the total column effluent so as to give the best sensitivity. To date, there have been two principal means by which an analyte in solution can be introduced to the GC detector, either by the use of a spraying device (nebulizer) or by evaporation of the solvent from the column effluent. While these processes have had some success with NPD and ECD detectors,[20] there is a problem with the FPD, namely, the quenching potential of the cointroduced solvent.[21] Julin et al.[22] nebulized up to 5 mL/min of column effluent and directed it into a cool hydrogen flame and

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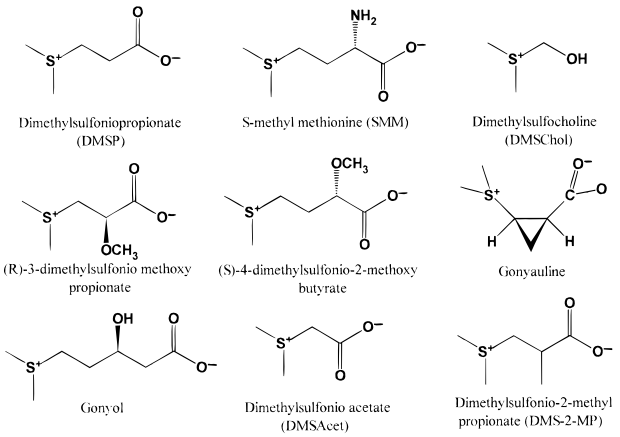

Figure 1. Chemical structures of potential dimethyl sulfide precursors employed in this study.

then monitored the emission at 383 nm, but only a relatively poor detection limit of 6.25 mmol of sulfur could be achieved. The quenching effect arising from cointroduced solvent can be reduced by the use of a dual-flame photometric detector (DFPD) system. Bernard et al.[23] achieved reduced quenching with their elaborate HPLC/FPD interface which involved vaporizing a methanolic column effluent at 750 °C to fuel a methanol−oxygen flame. Subsequently hydrogen was added, generating hydrogen sulfide in a reducing combustion step. Finally, this mixture was burned in the presence of oxygen, and it was the emission from this second flame that was recorded.

The development of packed capillary HPLC columns has permitted the sulfur chemiluminescence detector to be directly coupled to an HPLC column eluted at flow rates up to 10 μL/min.[23] Detection limits of ∼3 pg of S/s were quoted using methanol/water systems.

In the above examples, the aim was to produce a general purpose detector that would detect a range of sulfur compounds without preference, with sensitivity being of secondary importance. For this work, however, both low detection limits and good selectivity toward potential DMS precursors were required. The HPLC/FPD apparatus reported here satisfies these criteria by employing a chemical derivatization step to transfer the sulfur from the liquid phase to a gas phase, thereby circumventing the problems encountered with the direct introduction of liquids into a flame photometric detector.

EXPERIMENTAL SECTION

(i) Preparation of Compounds. Dimethylsulfoniopropionate (DMSP) was prepared by the addition of DMS to acrylic acid using the method described by Larher et al.[24]

***S*-Methylmethionine (SMM)** was obtained by the methylation of methionine with iodomethane in a formic acid/acetic acid mixture according to the method described by Toennies and Kolb.[25]

(Dimethylsulfonio)-2-methylpropionate (DMS-2-MP) was prepared using a modification of the method employed for the preparation of DMSP. Methacrylic acid (Lancaster, 30 mL; 30.45 g, 353.7 mmol) was stirred for 3 days with 30 mL of DMS (Janssen; 25.89 g, 416.7 mmol). Subsequently, 100 mL of calcium sulfate-dried toluene was added, and HCl gas was bubbled through the mix. After 2 h, a brown oil was produced which, upon cooling overnight, became a yellow-white solid. The crude product was recrystallized from a 50/50 ethanol/diethyl ether mixture to give brilliant white crystals. Yield: 5.58 g, 30.21 mmol, 8.54%. ^{1}H NMR (D_2O): δ 1.4 (m, 3H), 3.0 (s, 6H), 3.15 (m, 1H), 3.55 (m, 2H). IR (cm^{-1}, Nujol): ν 1720 (C═O stretch), 1180 (C−O stretch). CHN microanalysis. calcd: C, 39.02; H, 7.10. Found: C, 38.29; H, 6.34.

Dimethylsulfocholine (DMSChol). Bromoethanol (Aldrich, 15 mL; 26.445 g, 211.6 mmol) was mixed with a small excess of DMS (Janssen, 16 mL; 13.536 g, 217.9 mmol) and refluxed at 55 °C for 2 days. Subsequently, the unreacted starting material was evaporated off under reduced pressure, leaving a viscous liquid that was frozen at −20 °C. Yield: 30.593 g, 163.51 mmol, 77.3%. ^{1}H NMR (D_2O): δ 3.1 (s, 6H), 3.65 (t, 2H), 4.15 (t, 2H). ^{13}C NMR (D_2O): δ 28.25, 49.15, 58.78. IR (cm^{-1}): ν 3300 (O−H stretch), 1420 (O−H bend), 1050 (C−O stretch).

(Dimethylsulfonio)acetate (DMSAcet). Bromoacetic acid (Aldrich; 19.80 g, 142.5 mmol) was mixed with DMS (Janssen, 13 mL; 10.998 g, 177.0 mmol) and heated to 55 °C. The reaction was essentially complete within 2 min, giving a white solid. The product was recrystallized twice from ethanol, the last crystals being assisted out of solution by the addition of a little diethyl ether. Yield: 17.48 g, 86.9 mmol, 61.0%. ^{1}H NMR (D_2O): δ 3.0 (s). IR (cm^{-1}, Nujol): ν 1720 (C═O stretch), 1180 (C−O stretch). CHN microanalysis. Calcd: C, 23.89; H, 4.51. Found: C, 23.70; H, 4.16.

(Dimethylsulfonio)butanoate (DMSBut). Bromobutyric acid (Lancaster; 5.04 g, 30.2 mmol) was refluxed at 70 °C for 7 h with DMS (Janssen, 3 mL; 2.538 g, 40.8 mmol). No solid formed, but evaporation under reduced pressure gave an oily brown liquid which was shown to be DMSBut. Yield: 5.231 g, 22.8 mmol, 75.6%. ^{1}H NMR (D_2O): δ 2.15 (2H, t), 2.6 (2H, m), 2.9 (6H, s), 3.4 (2H, t). IR (cm^{-1}): ν 1730 (C═O stretch), 1200 (C−O stretch). CHN microanalysis. Calcd: C, 31.45; H, 5.72). Found: C, 30.27; H, 5.19.

(Dimethylsulfonio)pentanoate (DMSPent). Bromovaleric acid (Aldrich; 5.01 g, 27.7 mmol) was refluxed for 5 h at 55 °C with DMS (Janssen, 3 mL; 2.538 g, 40.8 mmol). Upon cooling, a white solid formed which was recrystallized from ethanol. Yield: 1.682 g, 6.916 mmol, 24.9%. ^{1}H NMR (D_2O): δ 1.82 (m, 4H), 2.5 (t, 2H); 2.9 (s, 6H), 3.4 (t, 2H). IR (cm^{-1}, Nujol): ν 1720 (C═O stretch), 1200 (C−O stretch). CHN microanalysis. Calcd: C, 35.19; H, 6.50. Found: C, 34.81; H, 5.64.

Gonyol and Gonyauline. Samples of synthetic gonyauline and natural gonyol were kindly provided by Prof. Nakamura of the University of Hokkaido, Sapporo, Japan.

Chromatographic Eluents. Eluents covering the pH ranges 3.13−3.66 and 5.31−7.04 were obtained by adjusting the pH of KH_2PO_4 (0.05 mol dm^{-3}) with hydrochloric acid or sodium hydroxide solution, respectively. pH 4.51 and 4.77 were obtained by adjusting the pH of $KH(C_6H_4(COO)_2)$ (0.05 mol dm^{-3}) with sodium hydroxide solution.

Samples. Cabbage and parsley were purchased from a local grocers. Pine needles (*Abies grandis*) were collected from Ashurst, West Sussex, U.K.

(ii) Instrumentation. The instrumentation employed in this work consisted of three distinct modules (Figure 2).

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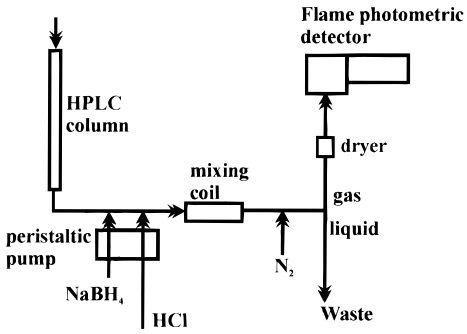

Figure 2. Schematic diagram of the HPLC-borohydride-FPD instrument.

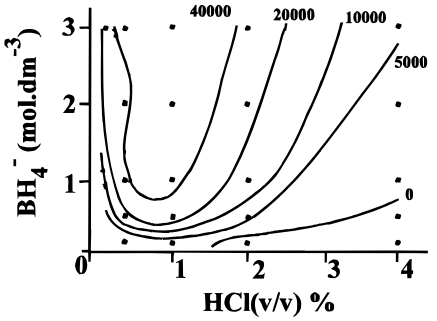

Figure 3. Isoresponse profile showing the variation of instrument sensitivity to β-(dimethylsulfonio)propionate with borohydride concentration and acid strength (% hydrochloric acid (v/v)). [Points denote experimental conditions.]

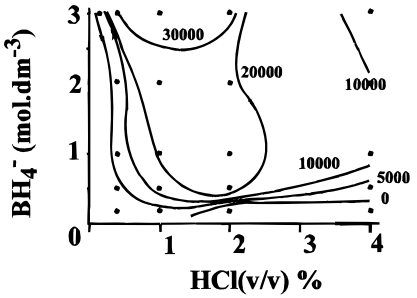

Figure 4. Isoresponse profile showing the variation of instrument sensitivity to *S*-methylmethionine with borohydride concentration and acid strength (% hydrochloric acid (v/v)). [Points denote experimental conditions.]

The first of these, the HPLC system, consisted of a duPont high-pressure pump delivering eluent through a Rheodyne 7125 loop injection valve to an ion-exchange column at a typical flow rate of 1 mL min^{-1}; 200 μL injection volumes were employed. Chromatographic separations were carried out using 25 cm × 4.6 mm id columns containing anion- (5 μm particle size Techsphere SAX) and cation-exchange (5 μm particle size Techsphere SCX) packings. The columns were supplied by HPLC Technology Co. Ltd., Macclesfield, U.K.

In the derivatization step, the column eluent was mixed with sodium borohydride solution (0.75 mL/min, typically ~2 M) and hydrochloric acid (0.75 mL/min, typically ~0.1 M). The reaction mixture was passed through a PTFE coil (2.10 m, 0.74 mm i.d.) held in a duPont column oven at 85 °C. A gas flow of nitrogen (typically 40 mL/min) then carried the mixture on to a simple gas−liquid separator consisting of a glass T tube leading the liquid products to waste.

The gas stream was dried by passing through two, 2 mL drying chambers. The first was empty and was employed to trap residual spray; the second was filled with anhydrous magnesium perchlorate (granular ACS reagent grade, Aldrich). In view of the inevitable band-broadening resulting from the introduction of these chambers into the gas stream, attempts were made to replace this drying system with a Nafion dryer held in molecular sieve. This approach, however, failed due to the inability of the Nafion system to remove the volume of water from the gas stream, resulting in excessive signal noise and occasional extinction of the flame due to water droplets. In this apparatus, a small amount of apparent chromatographic efficiency had to be sacrificed to minimize the problems associated with water transport to the detector.

The dried gas stream then mixed with hydrogen and combusted in the flame photometric detector. This detector, which was constructed in-house, was based on an EMI 6256B photomultiplier tube viewing an air/hydrogen flame through a BG12 (Oriel) glass filter having a transmission maximum at 400 nm. Hydrogen and air flow rates were typically 140 and 150 mL/min, respectively, in the final configuration, but due to sensitivity changes resulting from hydrogen generated by the borohydride reaction, these flow rates were optimized to obtain maximum sensitivity for each set of new acid/borohydride conditions. The photomultiplier current signal was converted to a voltage and then amplified. The resulting output was monitored using both a chart recorder (Bryans 28000) and a chromatographic computing integrator (Axxiom 717).

(iii) Sample Extraction Procedure. Four subsamples were taken from each sample, and each subsample was extracted using the following procedure:

(i) ~10 g of material was blotted dry and weighed;

(ii) the sample was homogenized to a fine powder by grinding in liquid nitrogen;

(iii) without allowing the sample to thaw, the sample was extracted into 10 mL of 2% (v/v) HCl;

(iv) the mixture was centrifuged for 10 min (2800*g*);

(v) the supernatant liquid was filtered (Whatman GF/C); and

(vi) immediately prior to the chromatographic analysis, the aqueous extract was buffered to the column eluent pH and diluted to give a response in the calibration range.

RESULTS AND DISCUSSION

(i) The Borohydride Link Step. Reaction Conditions. Reactant concentrations were optimized using DMSP and SMM, as these are currently believed to be of greatest environmental significance and because they differ significantly in their susceptibility to base hydrolysis. The responses of the system to DMSP and SMM solutions (1.2×10^{-5} and 2.0×10^{-5} mol dm^{-3}, respectively) were recorded under a range of BH_4^- and HCl concentrations (Figures 3 and 4).

A distinct difference was observed in the behavior of the two compounds. At a borohydride concentration of 1%, for example, more highly acidic conditions were required to obtain a maximum signal from SMM than were necessary for DMSP. This is believed to reflect the relative ease by which DMSP undergoes simple base hydrolysis to dimethyl sulfide and acrylic acid under the alkaline conditions generated by the sodium borohydride. Both plots point to optimum responses being obtained at the highest borohydride concentrations. Working with such high concentrations (~3.0 mol dm^{-3}), however, was impracticable due to the excessive signal noise which resulted from the large amount of hydrogen that was

Table 1. Relative Response Factors for Borohydride and Acid Flow Injection System

compd	3.0 mol dm^{-3} BH_4^-, 1.0% HCl	2.0 mol dm^{-3} BH_4^-, 1.0% HCl	1.0 mol dm^{-3} BH_4^-, 2.0% HCl	0.5 mol dm^{-3} BH_4^-, 1.0% HCl
DMSP	1.0	1.0	1.0	1.0
DMS-2-MP	0.91 ± 0.10	0.84 ± 0.06	0.90 ± 0.06	0.67 ± 0.08
DMSBut	0.37 ± 0.01	0.35 ± 0.02	0.34 ± 0.03	0.21 ± 0.03
SMM	0.24 ± 0.03	0.13 ± 0.01	0.56 ± 0.06	0.25 ± 0.03
DMSAcet	1.19 ± 0.10	1.18 ± 0.07	4.41 ± 0.87	1.90 ± 0.18
DMSPent	0.68 ± 0.05	0.64 ± 0.06	0.29 ± 0.05	0.31 ± 0.05
DMSChol	0.45 ± 0.03	0.44 ± 0.04	0.78 ± 0.08	0.57 ± 0.07

produced. The conditions selected to give reasonable response from both DMSP and SMM were a borohydride concentration of 2.0 mol dm^{-3} and a hydrochloric acid concentration of 1.0% (v/v) (0.115 mol dm^{-3}).

Nature of the Sulfur-Volatile. The nature of the sulfur volatile generated in the system was investigated by gas chromatographic analysis using flame photometric detection. A PTFE GC column (350 cm × $^1/_8$ in.) containing Chromosorb 101 was employed at 150 °C with a carrier gas flow rate of 30 mL/min. All seven dimethylsulfonium compounds generated a single recorded sulfur compound, having a retention time identical to that of dimethyl sulfide.

Performance. Calibration. The characteristic nonlinear response of the FPD dictates that a log/log plot must be used to give a linear calibration curve; the gradient of this plot is usually below the theoretical value of 2 (typical values ∼1.8). During routine operation, the detection limit (∼3 σ) of the system is ∼2.0 × 10^{-6} mol of DMSP dm^{-3}. With a 200 mL injection loop, this corresponds to an absolute mass of 12.8 ng of sulfur.

Relative Response Factors. The sensitivity of the detection system to the various sulfonium compounds was determined by feeding solutions of each compound into the detection system at a point corresponding to the end of the HPLC column. In this way, a steady state response could be achieved without being subject to the effects of peak width which would have occurred if sensitivity had been assessed by injection onto the chromatography column.

In assessing the sensitivity of the system to the various compounds, it was assumed that the detector response (R) to a compound was proportional to the square of the gas phase sulfur concentration, [S_{gas}], and that the gas phase sulfur concentration was linearly related to the concentration of sulfonium compound in solution, [S_{liq}], prior to the borohydride reaction step. Sensitivities were then normalized to that of DMSP.

For a compound under test, therefore,

$$R \propto [S_{gas}]^2$$

$$\text{or} \qquad R = k_1[S_{gas}]^2$$

If the concentration of sulfur in the gas phase, [S_{gas}], is linearly related to the concentration of the sulfonium compound in solution, [S_{liq}], i.e., [S_{gas}] = k_2[S_{liq}], then

$$R = k_1k_2[S_{liq}]^2$$

giving

$$k_1k_2 = R/[S_{liq}]^2 = k_S$$

The constant k_S reflects the sensitivity of the apparatus to the dimethylsulfonium compound under test; a high value implies a high yield of DMS and a high response. If DMSP is taken as the reference compound, then the sensitivity of a test compound ($k_{S(test)}$) can be related to the sensitivity to DMSP, $k_{S(DMSP)}$:

$$K_{test} = k_{S(test)}/k_{S(DMSP)}$$

where K_{test} is the relative response factor for the test compound.

All studied sulfonium compounds gave relative response factors that were within an order of magnitude of that of DMSP (Table 1).

Some relative response factors varied as the borohydride and acid concentrations were altered. It was apparent that, under conditions where the ratio of acid to borohydride was higher, the response from SMM was favored, as were the responses from DMSChol and DMSAcet.

Gonyol (1.12 × 10^{-5} mol dm^{-3}) and gonyauline (2.57 × 10^{-5} mol dm^{-3}) produced measurable detector responses, but, unfortunately, insufficient material was available for the investigation of response factors.

(iii) Chromatography of Dimethylsulfonium Compounds. Cation-Exchange HPLC. Cation-exchange chromatography was carried out using a Technosphere SCX 5 mm cation-exchange column (250 mm × 4.6 mm i.d.) eluted with aqueous eluents at 1 mL/min. The retention times of the six compounds were recorded over a range of eluent pH values (Figure 5).

Six of the seven available compounds were zwitterionic. DMSChol cannot be recovered from the column under the elution conditions investigated here. Interpretation of the observed chromatographic behavior would be assisted by a knowledge of the pK_a values of the sulfonium compounds. The authors are, however, not aware of these values being currently available in the literature.

A pH of 5.66 was chosen to provide a satisfactory separation of the compounds (Figure 6). A secondary effect of altering the pH of the column eluent was that, with low pH values, the sensitivity of the detection system toward DMSP and DMS-2-MP was reduced. This is believed to be due the acidity of the eluent reducing the initial solution conditions at the point of entry of the sodium borohydride solution, reducing the base hydrolysis of these two compounds. If it had been necessary to do so, the problem could have been readily overcome by adjustment of the conditions employed in the postcolumn reaction step.

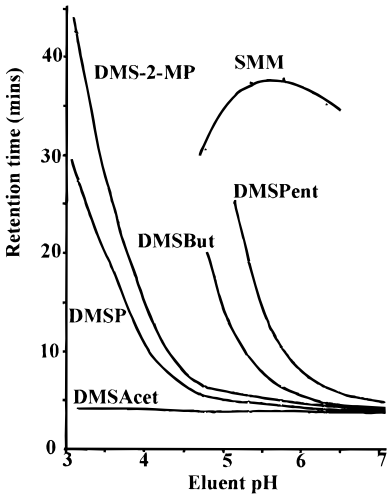

Figure 5. Effect of phosphate eluent pH on the apparent retention times of sulfonium compounds separated by cation-exchange chromatography. [Apparent retention times due to postcolumn holdup in the detection system.]

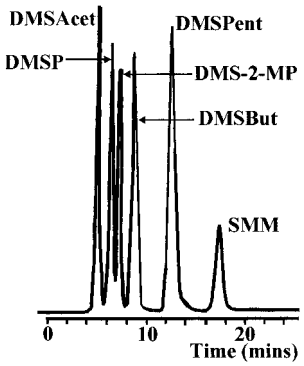

Figure 6. HPLC/FPD chromatogram of sulfonium compounds separated by cation-exchange chromatography. Eluent pH = 5.66.

Anion-Exchange HPLC. The pH dependency of the retention times of all seven prepared DMS precursors was assessed using a Technosphere SAX column (5 mm particle size, 250 mm × 4.6 mm i.d.) eluted with a range of aqueous pH-adjusted, phosphate-based eluents (Figure 7). Two distinct trends were observed in the retention times of the separated compounds, with the retention times of SMM and DMSChol being more severely influenced by pH changes above pH 6.5 than the other compounds.

In general, the anion-exchange separation of the compounds was inferior to the cation-exchange separations, with many of the compounds being grouped together and unable to be separated by altering the eluent pH. At the selected pH of 7.22 (Figure 8), as at all pH values, SMM and DMSChol were unresolved. The concentration of the eluent (pH 7.22) was reduced in order to investigate whether the resolution could be improved (Figure 8).

DMSChol and SMM were only resolved over a narrow range of eluent concentrations, the best separation being achieved with an eluent concentration of 0.0025 mol dm^{-3}. At these lower concentrations, however, the resolution of DMSP and DMS-2-MP worsens. As was found when investigating the pH of the eluent, the behavior of SMM and DMSChol seems to differ from those of the other five compounds, in that their retention times were much more susceptible to changes in the buffer strength.

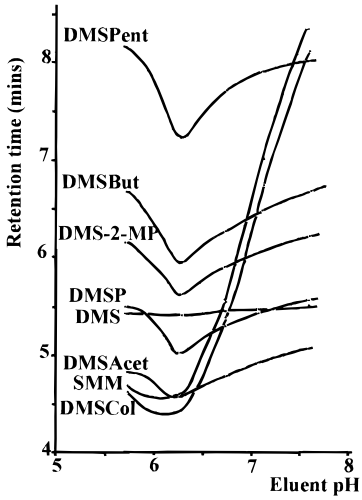

Figure 7. Effect of phosphate eluent pH on the apparent retention times of sulfonium compounds separated by anion-exchange chromatography. [Apparent retention times due to postcolumn holdup in the detection system.]

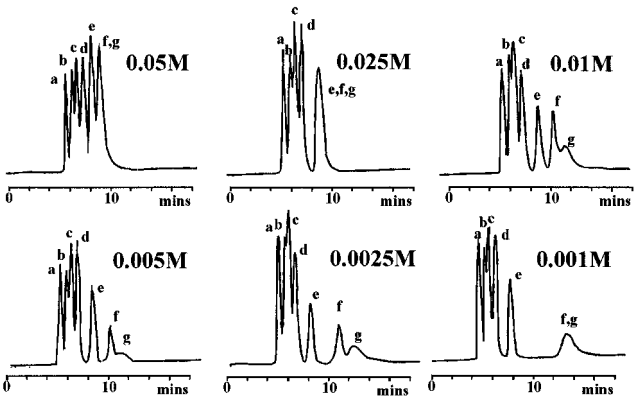

Figure 8. HPLC/FPD chromatograms of sulfonium compounds separated by anion-exchange chromatography, showing the effect of changing eluent concentration (pH = 7.22). (a) = DMSAcet, (b) DMSP, (c) DMS-2-MP, (d) DMSBut, (e) DMSPent, (f) DMSChol, (g) SMM.

(iv) SMM in Terrestrial Plants. While the presence of DMSP in the oceanic environment is well established, the origins of DMS which has been detected in a number of terrestrial ecosystems, including forests, grasslands, and freshwater wetlands,[1,25−31] has yet to be identified. One possible source of this terrestrial release of DMS is the breakdown of SMM, a compound which has been shown to be present in a number of plants, including asparagus,[8,9,32] tomatoes,[33] and tea.[11] The HPLC/FPD apparatus not only provides the means of surveying a range of samples for potential DMS precursors such as SMM but also can be employed in the quantitative evaluation of these sources.

Cabbage, fresh parsley, and pine needles (*Abies grandis*) were extracted into dilute hydrochloric acid, filtered, centrifuged,

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Table 2. SMM Content (mmol kg^{-1}) of Terrestrial Flora[a]

	SMM content (mmol kg^{-1} FW)	SMM content (mmol kg^{-1} DW)
cabbage	74.32 ± 7.00	701.16 ± 66.05
parsley	16.11 ± 0.78	130.27 ± 6.32

[a] Data are represented as mean ± standard deviation, $n = 4$. FW, fresh weight; DW, dry weight.

buffered to the column eluent pH, and then analyzed using the HPLC-borohydride-FPD instrumentation. No measurable DMS precursor could be found in the pine needle extracts, but SMM was found to be present in both the cabbage and parsley (Table 2).

CONCLUSIONS

The measurement of sulfonium compounds in the environment provides important information on the contribution made by these compounds to the global sulfur cycle. The analysis of these compounds has, in the past, been particularly difficult, which has resulted in little information being available on both the chemical nature and the presence of these compounds and, as importantly, the quantities involved. The instrument described in this paper goes some way to providing a reliable, selective, and sensitive instrumental means of both identifying and quantifying such compounds. The selectivity inherent in the postcolumn reaction allows naturally occurring sulfonium compounds to be measured in relatively impure sample extracts, obviating the need for lengthy sample "cleanup" protocols.

The chemical derivatization concept developed in this paper could be extended to a wide range of additional compound groups and reagent systems. Further enhancement of the instrument sensitivity could potentially be obtained by replacing the conventional flame photometric detector by one of the now commercially available sulfur chemiluminescence or pulsed flame photometric detectors.

The HPLC/FPD instrumentation described in this paper is not restricted to applications in the environmental field. SMM, for example, is important in a wide range of biochemical processes. DMS is an important flavor component of beer,[34] the source of which has been identified as the SMM[35−37] which is present in green malt.[38]

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