0014-4754/94/010080-06$1.50 + 0.20

Elemental sulphur (S_8) in higher plants – biogenic or anthropogenic origin?

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Received 23 June 1993; accepted 8 September 1993

Abstract. In the course of investigating lipophilic air pollutants in the epicuticular wax of *Pinus sylvestris* L. needles, elemental sulphur, S_8, was found in all samples. An investigation was conducted to determine the origin of this substance. No correlation between the level of S_8 in the needles and human activities in the sampling area could be found, contrary to what would have been expected of an anthropogenic compound. The internal lipids of *P. sylvestris* as well as the epicuticular wax of historical herbarium material and seedlings grown in clean, filtered air, and the epicuticular wax of several other species, both gymnosperms and angiosperms, also contained S_8. Quantitation of S_8 in *P. sylvestris* gave levels of 7.2 ± 2.9 μg/g wax, 3.8 ± 1.9 μg/g internal lipid and 0.43 ± 0.17 μg/g total needle dry weight. Almost 0.1% of the total sulphur in pine needles is S_8, and approximately half of the total S_8 is found in the wax. The results suggest that S_8 is endogeneous in many higher plants. A function for S_8 as part of an antifungal defence system is possible.
Key words. Elemental sulphur; S_8; *Pinus sylvestris*; epicuticular wax; higher plants; antifungal activity.

Lipophilic air pollutants transported in the vapour state are partitioned between the air and lipophilic surfaces such as the epicuticular wax of higher plants[1,2]. Particulate material in the surrounding air will also be deposited on the surface of the plants. Plants have, therefore, been used to monitor the distribution of lipophilic pollutants in the atmosphere on both subcontinental[3,4] and local scales[5]. It is also possible to include previously unknown air pollutants in such studies. Evergreen species, such as pines, are particularly suitable for this purpose since the needles accumulate persistent lipophilic substances over several years[6,7], and the rate of accumulation reflects the average concentration of the pollutant in the surrounding air. It is therefore possible to distinguish between pollutants and endogeneous substances, since the concentration of a pollutant in the needles as well as its accumulation rate would be expected to vary in parallel with human activities.

We report the discovery of elemental sulphur in the form of S_8 in *Pinus sylvestris* L. (Scots pine) needles, and other higher plants, during a project aimed at monitoring the distribution of lipophilic air pollutants. The origin of S_8 in the pine needles was investigated to ascertain if it is an air pollutant or of natural, endogenous origin.

Materials and methods

Except for the chemical and mass spectrometric characterization of S_8, the methods used were primarily developed for the determination of organochlorine pollutants. Therefore only an outline of the sampling and clean-up procedures are given here. Complete descriptions can be found in a previous report from this project[4].

Chemicals. Dichloromethane and hexane were pesticide grade (Fisons, Loughborough, England). Benzene and sulphuric acid were both pro analysi, and were purchased from Merck, Darmstadt, Germany, as was the silica gel (Kieselgel 60, 0.063-0.200 mm). Potassium hydroxide was pro analysi (EKA Nobel, Bohus, Sweden), deionized water was produced in an Elgastat (Elga, High Wycombe, England) and absolute ethanol was from Kemetyl (Stockholm, Sweden). For identification and quantitation of S_8, an authentic standard of sulphur flower was used (Kebo, Stockholm, Sweden). Metallic mercury and copper were also from Kebo. 2,3,3′,4,4′,5,5′-heptachlorobiphenyl, synthesised according to Sundström[8], was used as internal standard for quantitation.

Samples. Recent field samples of *Pinus sylvestris* were collected in 'background' areas (> 10 km from major cities or roads) throughout Western Europe and Poland during the years 1985–1989. Historical field samples of *P. sylvestris* were obtained from the herbarium of the Swedish Museum of Natural History in Stockholm. These samples were collected during the years 1834–1943 (3 samples 1834–1887, 7 samples 1900–1917, 1 sample 1943) in Central and Northern Scandinavia, where influence of anthropogenic sulphur emissions is expected to have been minimal. Seedlings of *P. sylvestris* grown in clean, filtered air, were obtained from the phytotron of the Swedish University of Agriculture and Forestry, Stockholm.

The epicuticular wax of other species was also investigated, including *Pinus mugo* Turra and *P. nigra* Arnold (field samples from Europe), *P. radiata* D. Don (field samples from New Zealand), *P. contorta* Loudon (field

samples from Canada), *Picea abies* (L.) Karst. (field samples from Europe), *Yucca sp.* and *Nerium oleander* L. (indoor pot plants), *Phragmites communis* Trin., *Sorbus aucuparia* L., *Tilia cordata* Mill. and *Geranium sylvaticum* L. (field samples from Stockholm, Sweden).

Extraction and clean-up. Needles were divided into year-classes, put into large test tubes and internal standard added, and a fraction operationally defined as the epicuticular wax was obtained by extraction with dichloromethane. The bulk of the wax material was removed by silica gel chromatography with dichloromethane as eluent. The sample was further purified by elution with hexane:benzene 1:1 through silica gel coated with concentrated sulphuric acid (SiO_2:H_2SO_4 2:1, w:w). The solvent was evaporated and the sample redissolved in hexane.

To extract the internal lipids, the material remaining after the wax extraction was cut into small pieces, and homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in dichloromethane in an extraction funnel[9]. The solvent was removed and the process repeated twice. The extracts were then cleaned up as described above for the wax. The final solutions were analyzed by gas chromatography with an electron capture detector.

Chemical characterization of S_8. Chemical evidence for the identity of elemental sulphur includes the disappearance of the peak corresponding to S_8 when the samples were treated with any of the following: 1) potassium hydroxide[9] which converts S_8 to sulphate[10], 2) metallic copper[11] or mercury[12] converting S_8 to insoluble sulphides, 3) tetrabutylammonium sulphite reagent[13] converting S_8 to thiosulphate. To check that the presence of S_8 was not an artifact formed during the clean-up of the samples, the methods for removing S_8 were applied after each step in the clean-up procedure to aliquots of a large sample of wax from Stockholm. Blank samples were also subjected to the entire clean-up procedure and checked for the presence of S_8 after each step.

Instruments. Gas chromatography (GC) was performed on a Varian 3700 or 3400 instrument equipped with an electron capture detector (ECD) (Varian, USA) operated according to Jensen et al.[4]. For identification purposes a flame photometric detector (FPD) in the sulphur mode was used. Gas chromatography-mass spectrometry (GC-MS) was performed using a Finnigan 4500 GC-MS system (Finnigan, USA) using helium as carrier gas, but otherwise similar chromatographic conditions as for the GC-ECD. Methane was used as reagent gas for negative ion chemical ionization (NICI). The ion source was held at 125 °C, 450 mTorr and the ionization energy was either 25 or 125 eV. Operation was done either as selected ion monitoring for m/z 256 (25 eV) or scanning m/z 60 to 270 (125 eV, 1 scan/second). Mass spectrometry-mass spectrometry (MS-MS) was performed on a Finnigan TSQ 700 triple quadrupole instrument with a direct inlet probe. The probe was temperature-programmed from 34 °C held for 5 min, 10 °C/min to 350 °C. NICI was performed, selectively monitoring the daughter ions m/z 64, 96 and 128 of the parent ion at m/z 192, using 70 eV electron energy with methane at 4300 mTorr as reagent gas, ion source temperature 125 °C, argon at 1.44 mTorr as collision gas and 20 eV collision energy. Electron ionization (EI) was performed, selectively monitoring the daughter ions m/z 128, 160 and 192 of the parent ion at m/z 252, with 70 eV electron energy, argon at 1.43 mTorr as collision gas and 20 eV collision energy.

Results

The gas chromatographic peak, identified as a sulphur containing analyte by GC-FPD, disappeared when the samples were treated with any of the S_8-removing reagents mentioned above. GC-MS was indicative of S_8, but the detection limits were not sufficiently low to give a positive identification of S_8 except in a pooled sample. Operation at 125 eV ionization energy in the NICI mode did not give any molecular ion; instead, the base peak was m/z 192. Operation at 25 eV gave a molecular ion ($M^{\bullet -} = 256$) but the ionization efficiency was so low that selected ion monitoring had to be used. Electron ionization at 70 eV was tested, but the detection limits were so high that the GC-column had to be overloaded to give a mass spectrum. MS-MS in both EI and NICI mode proved useful for a positive identification of S_8 in individual samples. Figure 1 shows a direct inlet mass spectrum of an S_8 standard obtained in the NICI mode, figure 2A is the daughter ion spectrum of m/z 192 of the S_8 standard, and figure 2B is the daughter ion spectrum of a sample of pine needle wax. The probe temperature at which daughter ion spectra of this type was registered was 35–38 °C both for the standard and the samples. Checking the blank samples and the individual clean-up steps after treatment with reagents to remove S_8, showed that the S_8 observed was not an artifact introduced by the clean-up procedure.

Quantitation of S_8 in recent field samples of epicuticular wax from *P.sylvestris* taken throughout Europe showed no observable geographical (table 1) or temporal (table 2) trends. As no significant differences in S_8 levels were observed, historical and clean air samples were obtained and tested. All these contained a peak corresponding to S_8 (table 1). The levels of S_8 in the historical and clean air samples were well within the variation of the recent field samples.

To investigate whether S_8 was also present in the interior tissues, recent field samples, historical samples and samples from clean air of *P. sylvestris* were homogenized after the extraction of the epicuticular wax and the amount of S_8 determined. Average concentrations of S_8 are given in table 3. As the wax comprises 1–4%

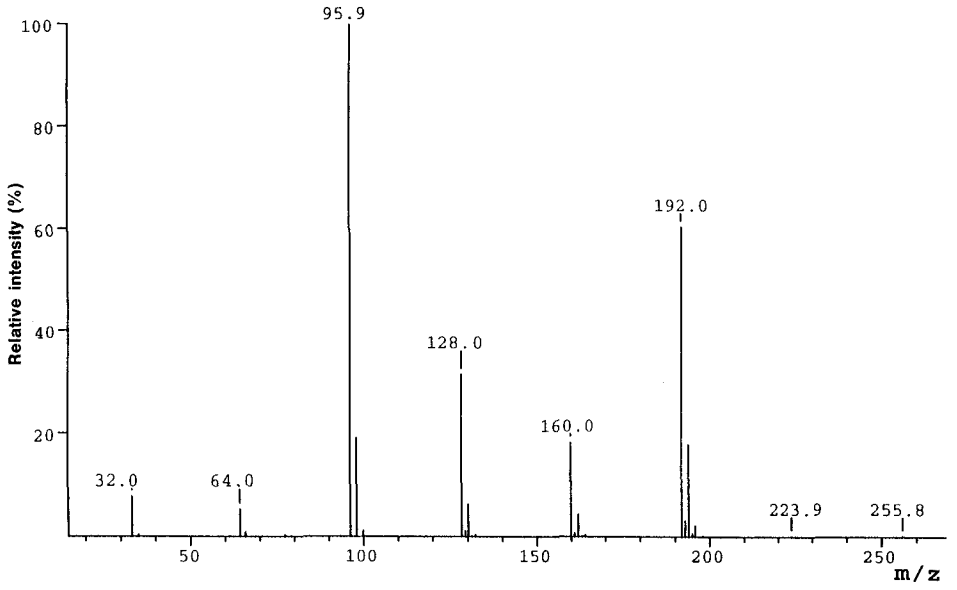

Figure 1. Full scan mass spectrum of sulphur flower obtained in the negative ion chemical ionization mode. Parameters as in text.

and the internal lipids 2–8% of the total dry weight, approximately half of the total S_8 is present in the wax. Comparing the levels of S_8 with published levels of total sulphur in Southern Central Sweden[14], approximately 0.08% of the total sulphur in pine needles is S_8 (table 3). All tested wax samples of other species, both gymnosperm and angiosperm, also contained a GC-ECD peak corresponding to S_8. However, quantitation of S_8 was done only on *P. sylvestris*.

Discussion

The identification of S_8 in the pine needle samples was facilitated by our experience with other types of environmental samples. Elemental sulphur is often a problem when determining organochlorines in environmental samples, as 1) it has a good response on an ECD, 2) it is lipophilic and hence will be extracted together with the target analytes, and 3) it is resistant to one of the more common clean-up steps, namely treat-

Table 1. Amount (μg/g ± S.D.) of S_8 in the epicuticular wax of *Pinus sylvestris* from different regions of Europe, from historical samples and samples from clean air.

Central Europe	Southern Scandinavia	Northern and central Scandinavia	Historical samples, northern and central Scandinavia	Clean air samples
7.4 ± 3.2 range 1.5–15.0 n = 53	7.1 ± 2.8 range 2.0–12.0 n = 108	7.2 ± 2.7 range 2.0–14.0 n = 92	7.2 ± 2.5 range 4.5–10.8 n = 11	7.3 ± 2.3 range 4.1–9.8 n = 10

Means of all samples of different year-classes.

Table 2. Amount (μg/ ± S.D.) of S_8 in the epicuticular wax of *Pinus sylvestris* from the first 3 year-classes of needles in recent samples from Central Europe and Scandinavia.

Recent samples from:	10 months old	22 months old	34 months old
Central Europe	7.3 ± 3.0 range 1.5–13.0 n = 16	7.6 ± 3.3 range 2.1–15.0 n = 16	7.5 ± 2.9 range 2.3–13.8 n = 14
Scandinavia	7.2 ± 3.0 range 2.0–14.0 n = 54	7.2 ± 2.8 range 2.1–13.4 n = 76	7.3 ± 3.2 range 2.0–13.5 n = 53

The needles were sampled in April–May, approximately 10 months after reaching full length. At this time of year most trees in Central Europe carry 3 year-classes of needles, but a variation of between 2 and 5 year-classes per tree occurs. In Northern Scandinavia trees with 5 or more year-classes are frequent. Only the first 3 year-classes are included in the table.

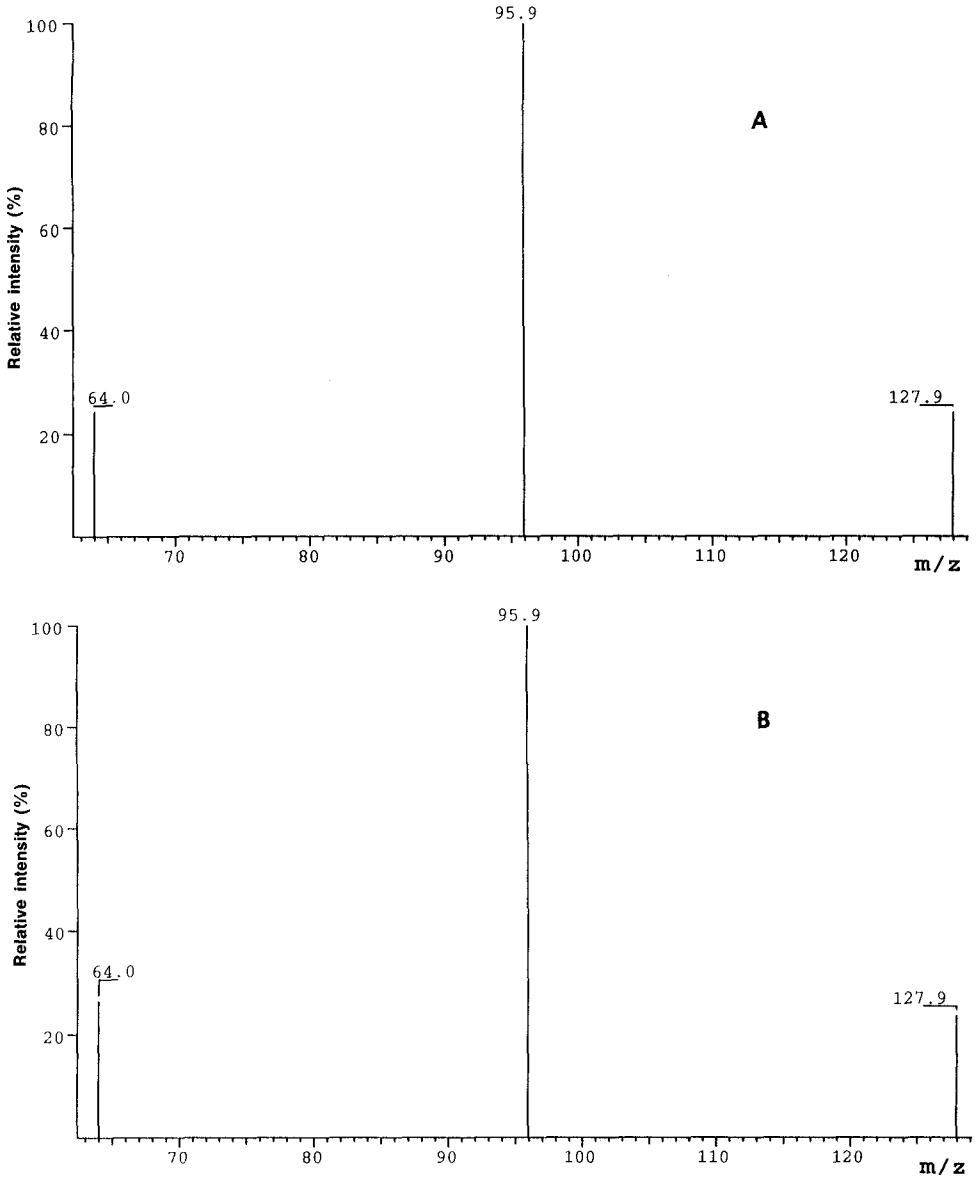

Figure 2. Daughter ion spectra of the parent ion at m/z 192, negative ion chemical ionization mode. *A* Sulphur flower standard. *B* Sample of *P. sylvestris* epicuticular wax.

Table 3. Amount of elemental sulphur (μg/g ± S.D.) in different compartments of *Pinus sylvestris* needles.

S_8 μg/g wax	S_8 μg/g internal lipids	S_8 μg/g total needle dry weight	% of total needle S_8 in wax	% of total sulphur[14] in needles as S_8
7.2 ± 2.9 range 1.5–15 n = 274	3.8 ± 1.9 range 1.1–13 n = 56	0.43 ± 0.17 range 0.09–1.2 n = 56	55 ± 18 range 39–65 n = 56	0.08 ± 0.032 range 0.02–0.2

The wax comprises approximately 1–4% and the internal lipids 2–8% of the total dry weight. The concentration of S_8 in the wax was obtained from 253 recent field samples, 11 historical herbarium specimens and 10 specimens grown in clean air conditions. The concentration in the interior lipids was obtained from 35 recent field samples, 11 historical specimens and 10 clean air specimens. The total sulphur content in *P. sylvestris* was obtained from literature[14], which accounts for the levels in an experimental forest in southern central Sweden (Jädraås).

ment with concentrated sulphuric acid. It is a particular problem when analyzing sediment and sludge samples where sulphur is produced by anaerobic microbiological processes[13]. However, the discovery of S_8 in pine needle samples was not expected, since, as far as we know, there had been no reports of endogenous S_8 in higher

plants, and only a few reports where S_8 had been identified as an air pollutant in areas remote from direct input of S_8 into the atmosphere[15].

There are several anthropogenic, biogenic or geothermal sources of S_8[16–18]. Anthropogenic sources of S_8 include agricultural uses as fertilizer[19] and fungicide[20] as well as vehicular[21] and industrial emissions[16,22]. Several prokaryotes, such as photosynthetic and chemoautotrophic bacteria and cyanobacteria, possess the ability to produce elemental sulphur[23–30]. In eukaryotes, however, the production of S_8 has only been described in a few cases including fungi[31] and a few algae[32–34]. Recently, production of S_8 was also described for suspensions of spinach chloroplasts[35]. To our knowledge, the present report is so far the only one concerning naturally occurring S_8 in higher plants in vivo.

The presence of S_8 in the historical and clean air samples, as well as the lack of geographical trends in concentration or accumulation rate between year-classes, shows that the S_8 found in *P. sylvestris* needles is not of anthropogenic origin. Its presence in the clean air material also precludes other exogenous biogenic or geothermal sources, and leads to the conclusion that S_8 is endogenous in *P. sylvestris*. The possibility that S_8 is produced by microorganisms on the needle surface is unlikely. The most likely substrate for production would be sulphate and most common sulphate reducing microbiological processes leading to S_8 are anaerobic[18,22]. The fact that S_8 can be produced by spinach chloroplasts[35] makes it conceivable that formation of S_8 is possible in vivo in spinach and other higher plants. In the light of its presence in all of the species investigated by us, it seems that elemental sulphur is, indeed, endogenous in many higher plants. We cannot exclude, however, that a minor fraction of the S_8 found in the pine needles is of anthropogenic or other exogenous origin, but so much endogenous S_8 is evidently present that no exogenous influence can be detected in the field material. A possible method to determine minor amounts of anthropogenic S_8 would be to determine the ratio of the ^{34}S to ^{32}S isotopes in the needle material, and compare this with the ratio from possible anthropogenic sources[22].

Although the wax comprises only 1–4% of the total dry weight of *P. sylvestris*, it contains half of the elemental sulphur (table 3). The epicuticular wax, therefore, seems to enrich and function as a main store for S_8. Physiologically, it is possible that the presence of S_8 in the plant tissues is due to sequesterir.g of a small part of the sulphur at this oxidation state during the reduction of sulphate to sulphide. Wheat leaves[36] and spinach chloroplasts[35] have been shown to metabolize S_8. The reason for enrichment of S_8 in the wax, therefore, might be that once it has diffused into the wax it is no longer available for metabolism. For a persistent substance passively accumulating in the wax, higher concentrations in the wax would be expected in older needles. There should, however, be ample opportunity for the oxidation of S_8 on the needle surface, which might deplete the temporal accumulation expected, and explain why no temporal trends can be seen in an individual tree.

Finally, it is possible to envisage an ecological role for S_8, particularly in the epicuticular wax, as part of an antifungal defence system. Elemental sulphur is a fungicide in its own right[20], but has also been shown to act synergistically with other fungicides[37]. Endogenous fungistatic substances are known from the epicuticular wax of pines[38]. Thus it is conceivable that S_8 could enhance the efficacy of such substances. An antibiotic effect of S_8 has been suggested in a red alga[32]. The role of S_8 in higher plants and its contribution to the terrestrial sulphur cycle needs further investigation.

Acknowledgments. Dr. I. Dormling and Dr. M. Kähr are thanked for the clean air samples and Dr. F. Björkbäck for the historical samples. V. Scorcec skilfully operated the MS-MS and Prof. Å. Bergman provided the helptachloro-biphenyl internal standard and valuable discussion of the manuscript. Financial support by the Swedish Environmental Protection Board and the Swedish National Bank Tercenary Foundation is gratefully acknowledged.

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