Bull. Environ. Contam. Toxicol. (2002) 68:155–160
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DOI: 10.1007/s00128-001-0232-6

Bulletin of Environmental Contamination and Toxicology

Seasonal Variation of the Terpene Content, an Overlooked Factor in the Determination of Environmental Pollutants in Pine Needles

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Received: 22 June 2001/Accepted: 29 September 2001

The epicuticular wax that covers the cuticles of the green parts of higher plants can be used to monitor the occurrence of airborne hydrophobic environmental pollutants. Needles of conifers have been used to monitor persistent organic pollutants (POPs) on global, regional and local scales, and have also been used to monitor polycyclic aromatic hydrocarbons (PAHs) (Calamari et al. 1991, Eriksson et al. 1989, Jensen et al. 1992, Kylin 1994). In the summer of 1994 we used needles of Scots pine (*Pinus sylvestris*) to perform a survey of the PAH contamination from the road traffic to and from Arlanda International Airport north of Stockholm, Sweden (Söderkvist and Undeman 1994). During the analytical work several unexpected problems were encountered that could be related to a high content of terpenoids in the needles. Realising that variations in terpenoid content might also affect the partitioning of hydrophobic pollutants between the air and the plant, we were prompted to investigate the seasonal variation of the amount of terpenoids in the needles. The results from this study are presented here together with a discussion on how the terpenoid content of the needles has to be taken into account when interpreting data on the concentrations of airborne hydrophobic contaminants in the needles.

MATERIALS AND METHODS

Sampling started the second week of August 1994 at the Stockholm University campus in Frescati on the northern outskirts of Stockholm. Samples were taken from an approximately 50 year old tree at the eastern shore of Lake Brunnsviken on the south-western side of the tree facing the open lake. Subsequent samples were taken in the second week of the month for 16 months. Samples consisted of 20 g of fresh needles. Both the current-year growth and one-year old needles were sampled. Five replicates were analysed at every sampling occasion.

The original analytical method had previously been used for the determination of PAHs in pine needles (Kylin 1994). In short the method included extraction of the wax off the fresh, whole needles by submersion in dichloromethane for 24 hours, followed by filtration and evaporation of the dichloromethane. The residue was dissolved in hot benzene, which was then cooled to form a precipitate of estolides, high molecular weight wax esters. The benzene was pipetted off and evaporated

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and the residue dissolved in hot acetonitrile, which was then cooled to form a precipitate of wax alkanes. The two combined precipitates constitute the major part of the epicuticular wax. The acetonitrile was pippeted off and evaporated after which the residue was dissolved in hexane and fractionated on silica gel to obtain a fraction containing the PAHs. Full descriptions of the method have been published previously (Franich et al. 1994, Kylin 1994, Kylin et al. 1996)

As the fractions from the silica gel chromatography contained unexpectedly high amounts of hydrophobic material, we added a further sample separation step by partitioning between equal volumes hexane (Hx1) and acetonitrile:water 9:1 (volume:volume) followed by dilution of the acetonitrile with water to the final proportions acetonitrile:water of 1:1, which was again extracted with hexane (Hx2). This Hx2 phase was expected to contain the PAHs. However, there was still a serious interference problem in the gas chromatography mass spectrometry (GCMS) determination of the PAHs. Therefore, the composition of the Hx1 and Hx2 phases was investigated by GCMS. Details on instruments and conditions are given in Franich *et al.* (1994).

To enable the determination of hydrophobic material in the time response experiments, the method was simplified. Extraction, filtration, precipitation and chromatography were made as above, but the amount of easily extractable material was determined gravimetrically using an analytical balance. The combined weight of the precipitates is called "wax" and combined weight of this "wax" together with the residue after silica gel chromatography is called the Total Extractable Surface Hydrophobic Material (TESHM). As used in this simplified method, the silica gel chromatography mainly removed the small fraction of pigments that is extracted from the needles using this extraction methodology. No instrumental quantification of the constituents was done in the time series, but every month one sample was subjected to the original method and Hx1 and Hx2 were checked by GC-MS to confirm that the major constituents were qualitatively the same throughout the year.

RESULTS AND DISCUSSION

Evaporating the two extracts Hx1 and Hx2 gave oily residues with a pleasant rosy odour. The less polar Hx1 extract was colourless, while the more polar Hx2 extract was yellowish in colour. The qualitative investigation using GC-MS showed that the main constituents of Hx1 were mono- and sesquiterpenes, while Hx2 also contained some oxygenated compounds suggesting terpene alcohols, ketones or aldehydes.

Most samples we had analysed prior to this project had been collected during autumn, winter, and spring throughout Europe (Eriksson et al. 1989, Jensen et al 1992, Kylin 1994). In these samples the TESHM usually comprised 1-4 % of the sample weight. However, in the needle samples collected in August 1994, the wax extracts comprised more than 10 % of the sample weight.

In a previous study, the TESHM extraction method was tested by analysing the extracts for chlorophyll and unsaturated fatty acids. Usually our procedure gave an extract with a minute proportion of the total amount of these compounds compared with the extract from whole needles which had three to five orders of magnitude more chlorophyll. As long as the extraction was done by submersing fresh needles in an organic solvent, the water in the apoplast (cell walls and other extracellular compartments of the needles) acted as an extraction barrier, effectively protecting most of the internal tissues from the solvent. Therefore, the extracts were considered to consist mainly of the epicuticular wax and compounds dissolved in or sorbed on the wax (Kylin *et al.* 1996). Apart from the wax compounds (and pollutants) during those times of the year when we have normally sampled, a minor fraction of the wax extracts are terpenoids (Franich et al. 1994). Much of the terpenoids presumably originates from resin ducts in the needles. However, most of the "excess" weight of the wax extract in the samples from August 1994 was accounted for as terpenoids.

As can be seen in Table 1, the TESHM varied between 1-3 % during most of the year to a maximum of more than 10 % during the hottest period. This means that the total amount of hydrophobic material in the needles was higher during the warm periods of the year than during colder times. This in turn may influence the partitioning of hydrophobic air-borne pollutants between the needles and the surrounding air. Most physico-chemical models of hydrophobic pollutant behaviour in the air/plant system indicate sorption of contaminants from the gas phase to the plants during cold weather and volatilisation during warm weather, a process driven by the vapour pressure of the compound (McLachlan and Horstman 1998). However, if the amount of endogenous hydrophobic material in the needles increases during summer when temperatures are elevated, this may counteract the volatilisation. The total amount of extractable lipid has frequently been used to normalise the concentration of hydrophobic environmental pollutants in biological samples of animal origin (Kylin 1994). In the light of the findings presented here using the extractable amount of hydrophobic material to normalise concentrations in plant material has to be done with caution. If the total amount of hydrophobic compounds in the plant material varies substantially during the year this variation has to be known to permit correct assessment of the partitioning behaviour of environmental pollutants during different seasons.

In addition, it is our experience that it is difficult to use the extractable amount of lipid in an adequate way when dealing with plants. One reason is that not all of the hydrophobic constituents of the plants can be extracted. The bulk of the cuticle is constituted of cutin, which is made up of cross-linked hydroxy fatty acids (Kolattukudy 1976, Franich et al, 1982). The cutin will take part in the sorption of hydrophobic contaminants, but will not be accounted for in the determination of extractable "lipid" since it is not soluble in the solvents used. Another complicating factor is that many of the terpenoids have fairly low boiling point and high vapour pressure and will be lost on evaporation.

Table 1. Total extractable surface hydrophobic material (TESHM) in needles of Scots pine (*Pinus sylvestris*), percent of dry weight and relative standard deviation (RSD) based on five individual determinations.

Sampling month	1995 year growth % of dry weight (RSD)		1994 year growth % of dry weight (RSD)		1993 year growth % of dry weight (RSD)	
	TESHM	Wax	TESHM	Wax	TESHM	Wax
August-94			10.5 (8.3)	1.5 (10.1)	11.5 (7.8)	0.7 (9.8)
September-94			5.8 (10.2)	2.3 (7.3)	4.2 (18.5)	0.5 (12.5)
October-94			2.3 (8.0)	2.1 (11.8)	1.4 (8.1)	1.2 (13.1)
November-94			1.2 (22.6)	1.0 (20.3)	2.2 (11.1)	0.9 (8.1)
December-94			2.4 (8.4)	1.7 (10.5)	3.2 (7.6)	1.3 (15.6)
January-95			1.5 (6.7)	0.7 (8.4)	0.8 (24.0)	0.4 (25.6)
February-95			2.0 (10.7)	0.9 (18.7)		
March-95			1.9 (13.5)	1.1 (10.8)		
April-95			2.6 (9.9)	1.3 (11.1)		
May-95			4.6 (10.0)	2.6 (15.4)		
June-95			8.2 (5.6)	3.1 (13.8)		
July-95	15.8 (4.6)	2.2 (5.9)	12.0 (8.6)	2.1 (8.9)		
August-95	12.0 (14.9)	3.1 (8.2)	11.1 (10.8)	2.8 (13.7)		
September-95	6.0 (20.4)	1.7 (14.6)	4.7 (15.1)	2.0 (10.0)		
October-95	1.8 (7.3)	0.9 (12.3)	1.2 (8.6)	0.8 (10.0)		
November-95	2.5 (15.2)	1.6 (16.2)	2.3 (11.0)	1.3) (9.9)		
December-95	1.8 (8.7)	1.3 (3.4)	1.8 (8.3)	0.7 (14.2)		

NOTE: In the Stockholm area bud-burst and needle emergence occurs in Scots pine around June 1. Elongation of the needles is completed in July and during their second winter the needles start senescing. Therefore no TESHM determination was made on the current year shoots until July or on needles older than 18 months.

Volatilisation during sample preparation is even more problematic with toluene, which is produced by many plants, including Scots pine, especially when the plant is under stress (Heiden et al. 1999). One such stress may be drought stress during summer. In Radiata pine (*P. radiata*) as much as 23 % of the volatiles may be toluene (Zabkiewicz and Allen, 1975). If toluene is a substantial part of the hydrophobic endogenous compounds the total amount of hydrophobic material in the plant will be underestimated, as the toluene will almost certainly be lost while evaporating the solvent.

It is interesting to speculate on why the content of terpenoids is higher during the warm season than during the cold. There are examples of where terpenoids increase the heat resistance of the photosynthetic process by stabilising the thylacoid membranes (Delfine et al. 2000, Singsas 2000). There are also suggestions that terpenoids may have a function to in the drought resistance of plants in hot, semiarid environments (Dell and McComb 1978) and that terpenes may function as protection against photochemical oxidants that also have higher concentrations in the air during the summer (Strömvall and Petersson 1991). One may also speculate that terpenoids have a function as plasticizer of the epicuticular wax. In our experience, extracted and purified epicuticular wax is very brittle and it is possible that a plasticizer is necessary to avoid cracks in the wax on the leaf or needle surface. Cracks in the wax would decrease the drought resistance drastically. As the evaporation of the terpenoids would be higher in the summer it might be necessary for the plant to produce more terpenoids to obtain the correct plasticity of the epicuticular wax.

The levels of TESHM during the summer found in this study compares well to other studies that have found a higher content of essential oils in various plants during the summer than during the winter (Yatagai et al. 1995). The absolute amount of extractable material is higher in our study than when the measurements are done on the essential oils only. This is probably due to different extraction methods where we have used solvents that extract compounds with a higher molecular weight and not only the low molecular weight compounds of the essential oils produced with steam distillation. It may also in part be due to the very hot summers during which this study was performed. If terpenoids play a role in the heat and drought adaptation of the plants, the sampling tree may have been adjusted to produce a higher amount of terpenoids than summers with more normal temperatures.

Acknowledgments. This study was started as part of the research school "Urban Environment" initiated by Dr. Henrik Mickos. The continuation of the project was done within a project financed by the Swedish Environmental Protection Agency.

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