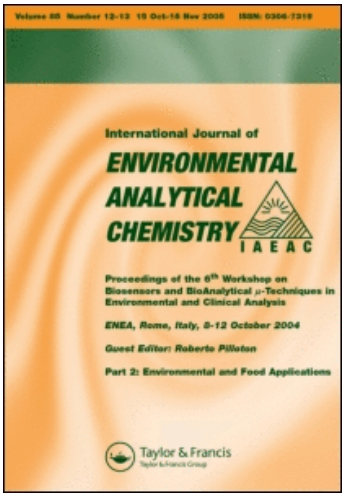

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Single and Combined Effects of Continuous and Discontinuous O_3 and SO_2 Immission on Norway Spruce Needles: II. Metabolic Changes

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Single and Combined Effects of Continuous and Discontinuous O_3 and SO_2 Immission on Norway Spruce Needles

II. Metabolic Changes

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(*Received 14 August 1987*)

The analysis of the cytological effects of SO_2, O_3 or SO_2/O_3 exposure on the structure of spruce needles (*Picea abies* Karst.) revealed an extremely high amount of phenolic compounds in the vacuoles of the mesophyll cells of primary needles (see part 1 of this study). This observation prompted us to identify and quantify different spruce needle phenolics, their changing amount during SO_2, O_3 or SO_2/O_3 exposure as well as their effect on the adenylate energy charge (AEC) as an integrate stress index of the plant cell.

Discontinuous fumigation with O_3 combined with low constant-concentration exposure to SO_2, overlapped by occasional peak concentration of SO_2, seems to affect

the phenolic metabolism and the adenylate energy charge the most. The high phytotoxicity of this fumigation pattern is indicated by a drastically reduced adenylate energy charge and extremely high levels of distinct intermediates of the phenolic pathway.

KEY WORDS: Forest die-back, ozone, sulfur dioxide, needle phenolics, adenine nucleotides.

INTRODUCTION

Monomeric and dimeric phenols as well as related polyphenols are widely distributed in plants. Practically all higher plant phenolics are formed from shikimate, via the shikimic acid pathway and their production through the intermediacy of phenylalanine, the enzyme phenylalanine ammonia lyase, and cinnamic acid as illustrated in Figures 1 and 2. Several flavonoids are also formed by the acetate-malonate pathway (see Figure 1) and many simple plant phenols are derived directly by the polyketide pathway from acetyl and malonyl coenzyme A.

Chemically, phenols are reactive substances and this must be noticed when considering their function in plants. They are usually acidic and can often be separated from other plant constituents by their solubility in aqueous sodium carbonate. Unless sterically hindered, all phenols are capable of taking part in hydrogen bonding. This may be intramolecular, as between the 5-hydroxyl and 4-carbonyl in many flavonoids. More importantly, it may be intermolecular and bring about interactions between plant phenols and the peptide links of proteins and enzymes. Another biological important property of those phenols bearing diol (catechol) groups is their ability to chelate metals. Finally, phenols are very susceptible to photo-oxidation and there are special enzymes present in plants—the phenolases—which catalyse the oxidation of monophenols to diphenols and of diphenols to quinones and hence often to polymeric materials.

There is still considerable uncertainty about the physiological role of phenolic compounds in plant growth and metabolism. Many phenols are clearly able to exert significant effects on growth processes when applied to plant tissues at physiological concentrations, but this does not necessarily imply that they have an

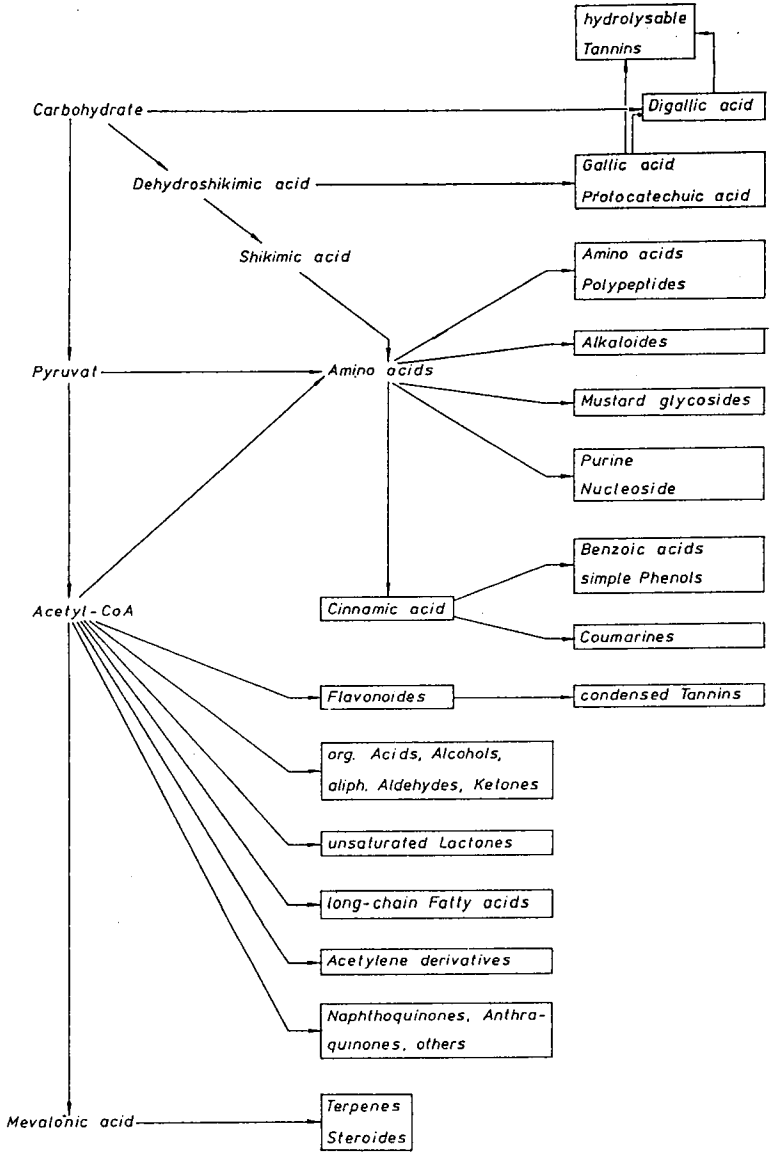

Figure 1 Pathway of biosynthesis of related plant phenolics.

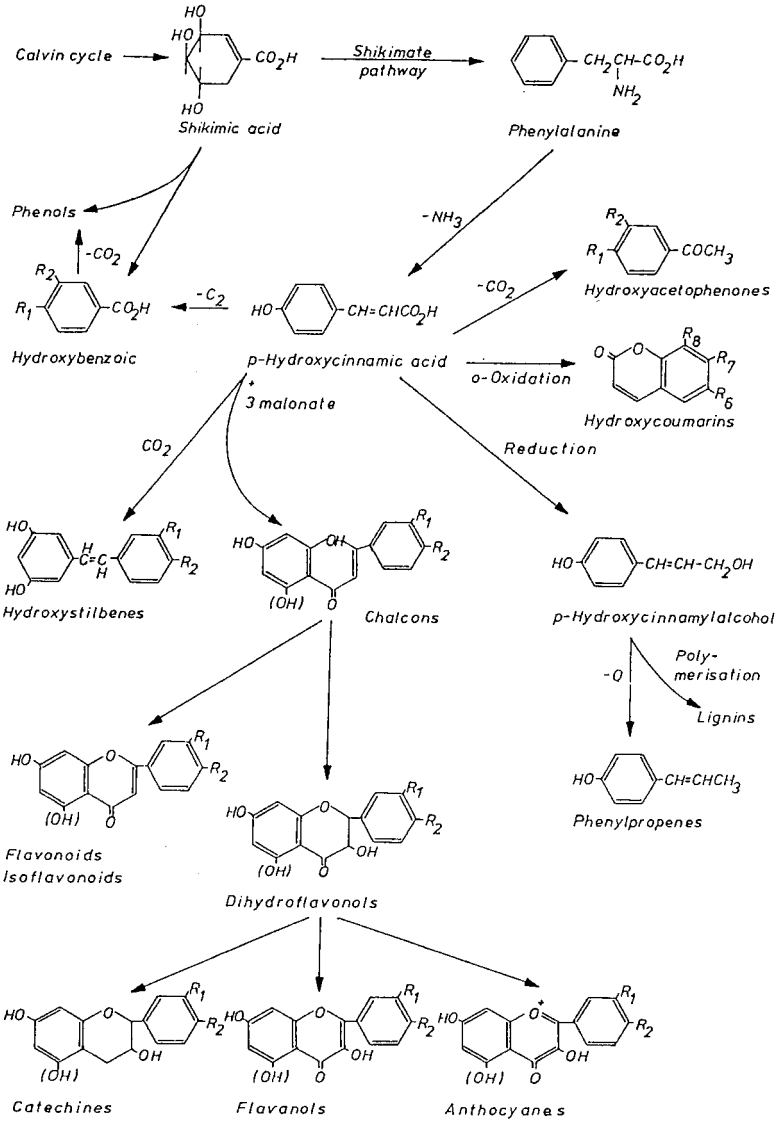

Figure 2 Biosynthetic origin of plant phenolics from shikimate and phenylalanine.

endogenous role. The putative physiological role of phenols in plants has been discussed by a number of authors, including Galston,[1] Gross,[2] Kefeli and Kutacek,[3] McClure[4] and Stenlid.[5]

Even if most of the phenolics are not hormones themselves, they may affect plant growth by interaction with one or other of the major classes of plant hormones such as the auxins.[6] Another point of hormone control that phenolics might affect is the biosynthesis of ethylene.[7]

Phenolics may react with other hormones by synergism or inhibition and both situations have been recorded in the case of plant growth stimulated by gibberellic acid.[8] On the other hand phenolics may have indirect effects on intermediary metabolism. For example, many phenolics are capable of inhibiting oxidative ATP synthesis in mitochondria, of uncoupling respiration and of inhibiting ion absorption in spruce roots.[5] There are also a variety of enzymatic activities which may be inhibited in the presence of compounds such as quercetin.[9] The significance of these effects in the normal growth pattern of the plants, especially spruce needles, has yet to be determined. In this paper we discuss the identification and quantification of different spruce needle phenolics, their changing amount during SO_2, O_3 or SO_2/O_3 exposure as well as their possible effects on the adenylate energy charge (AEC) of the plant cell.

The adenine nucleotides (ATP, ADP, AMP) play a central role as key metabolites and allosteric modulators in the phosphate and energy transfer reactions of the plant cell. ATP-utilizing and ATP-regenerating processes are known to be controlled by the adenylate energy charge[10] (AEC = [ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP]), which ranges from 0 to 1.

Temporary-metabolic induced-deviations from the steady state value (0.8 ± 0.1) of a balanced energy metabolism are compensated in the intact plant cell by an increased (reduced) ATP production or a reduced (increased) ATP consumption. Thus, lower AEC values found in damaged plant cells should reflect an elevated ATP consumption, a reduced photosynthetic and/or mitochondrial ATP production or a reduced adenylate kinase activity.

The photosynthetic generation of ATP is a membrane-bound process and thus sensitive to air-pollutant-induced ultrastructural changes of the thylakoid membrane system. These primary morphological damages can cause a decoupling or inhibition of photo-

phosphorylation, thereby leading to an irreversible change of the AEC. Furthermore, a similar reduction of the AEC value can occur by the action of decoupling or inhibitory active secondary plant metabolites as, for example, the aforementioned phenolic compounds. Finally, a metal ion deficiency also can influence the regulatory effect of the AEC as, e.g., magnesium is an essential cofactor for many nucleotide-dependent enzymes (e.g., adenylate kinase; CF_1-ATPase).

Thus, the AEC ratio integrates endogenous and exogenous factors affecting the plant metabolism and should reflect the net effect of environmental stresses, such as the exposure to gaseous pollutants.

EXPERIMENTAL

Plant material

Primary and secondary Norway spruce needles were collected from 3–5 year old spruce trees (*picea abies* Karst.) after treatment with SO_2, O_3 and SO_2/O_3 as well as of the reference series (classification of the plant material and fumigation conditions, see experimental procedure; part 1 of this study, this journal). The needle samples were frozen immediately with liquid nitrogen in order to avoid any biological change and transferred to Dewar vessels containing dry ice during transport. Liquid-nitrogen-frozen samples that were subjected to grinding in a ball triturator the following day were placed in a freezer overnight. All samples were ground within one day.

Sample preparation (phenolics)

A scheme of the method is shown in Figure 3. It is designed to fit into our general secondary plant products screening methodology. Crude needle extracts were obtained by extraction of 0.5 g nitrogen-frozen ground needles with methanol (60 °C) in an ultrasonic bath. The suspension was centrifugated and 1 μg of gallic acid (internal standard) in 100 μl of methanol was added to the crude extracts. For the clean-up procedure these extracts were rapidly forced through a 0.45 μm Spartan-3-filter (Schleicher & Schüll, Einbeck, FRG) and

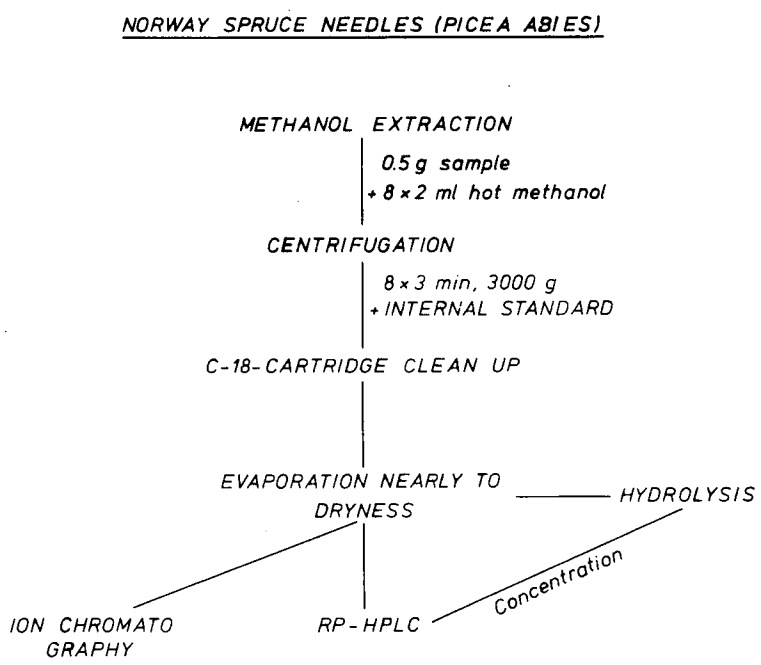

Figure 3 Schematic for sample extraction and clean-up for phenolic compounds in Norway spruce needles.

applied to a home-made C-18 cartridge (Bond–Elut column, refilled with 10 ml LiChrosorb C-18, 5 μm) to remove lipophilic compounds and photosynthesis pigments. The volumes of the cartridge effluents containing the phenolic compounds were reduced to approx. 500 μl by blowing with nitrogen, refilled up to 2 ml with methanol and forced through a 0.45 μm Spartan-3-filter. Samples of 25 μl or 50 μl were finally injected for RP-HPLC and ion chromatographic analysis respectively. To minimize experimental error three parallel needle extractions and five chromatographic measurements were performed for each fumigation series.

The enzymatic hydrolysis of the glycosylated phenolic compounds as well as the acidic hydrolysis of the catechin dimers and trimers (procyanidin) was carried out as described by Galensa *et al.*[11] and Endres.[12]

Analytical methodology of needle phenolics

The HPLC system comprised an HP 1090 liquid chromatograph (Hewlett Packard, Waldbronn, FRG) equipped with a 1040 HP detection system combined with a HP85-PC, a 9121 D flexible disc drive and a 2225 inkjet printer for storing and recording chromatograms and UV-VIS spectra, respectively. Separations were performed on a LiChrosorb RP-18 column, 10 μm (250 mm $\times$ 4.6 mm I.D., Merck, Darmstadt, FRG), equipped with a LiChrosorb RP-8 precolumn, 5 μm (4 mm $\times$ 4 mm I.D., Merck). Methanol-water gradients were used for the elution.[13,14] To avoid the tailing of phenolic compounds the solvents were adjusted to a pH of about 3.2 with acetic acid (5 ml of glacial acetic acid per litre of solvent). The temperature of the oven thermostat was set at 40°C. The flow rate was 1.5 ml/min and the column pressure 110–150 bar. The diode array detector was set at 254 nm, 280 nm, 300 nm and 365 nm, optical bandwidth 4 nm (cf. Figure 4). The recovery for the HPLC phenol assay was found to be (85–94) $\pm$ (3–5)%.

The quantitative determination of quinic acid and shikimic acid was obtained by ion chromatographic separation and conductivity detection using a Dionex 2020i IC-system (Weiterstadt, FRG).

Separations were performed on a Dionex-AS 1 column with 0.001 M hydrochloric acid as eluent and 0.005 M potassium hydroxide as suppressor. The recovery of quinic acid was found to be 83 $\pm$ 12% and that of shikimic acid to be 91 $\pm$ 8%.

Sample preparation (adenine nucleotides)

Crude needle extracts were obtained by extraction of 500 mg triturated and lyophilized needle material with 5 ml of 0.1 M NaH_2PO_4, pH 2.5 for 30 min at 4 °C in an ultrasonic bath. The water-insoluble matrix constituents were separated by centrifugation for 3 min at 5000 g. The supernatant was membrane filtered (0.2 μm, Schleicher & Schüll) and an aliquot subjected to HPLC analysis.

'On-line' HPLC analysis of adenine nucleotides

The adenine nucleotides adenosine-5'-mono-, di- and triphosphate (AMP, ADP, ATP) were separated from the residual matrix con-

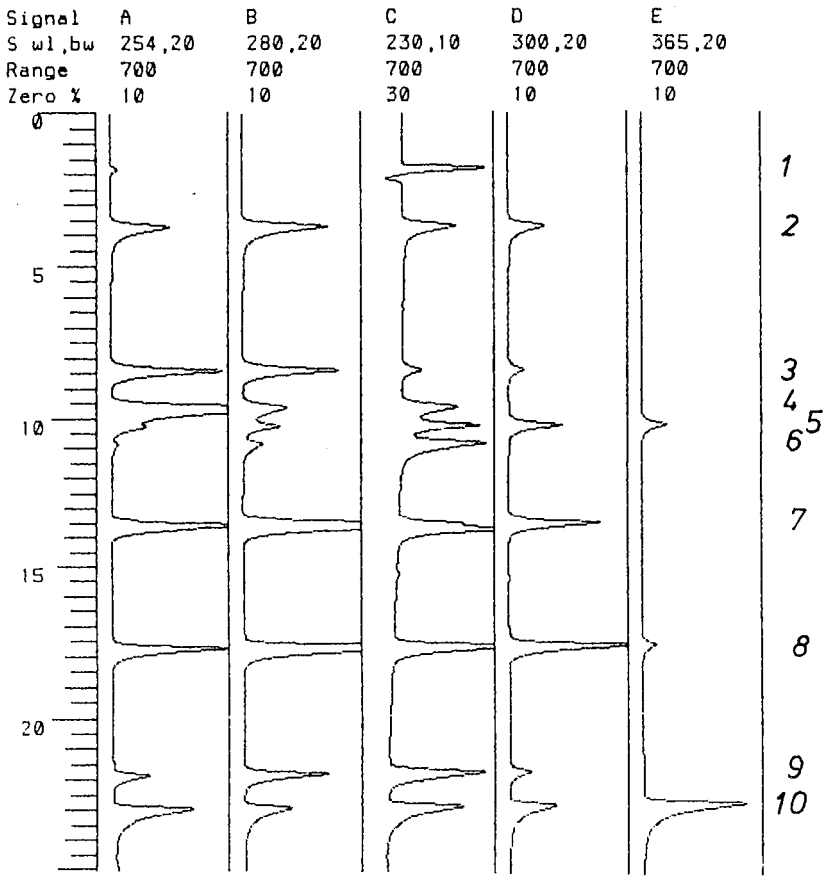

Figure 4 Chromatogram of a 'phenolic' standard solution on a C-18 column. Chromatographic conditions, see experimental part; detection wavelength: 230 nm, 254 nm, 280 nm, 300 nm, 365 nm; sample: 1. shikimic acid, 2. gallic acid, 3. picein, 4. *p*-hydroxy benzoic acid, 5. esculin, 6. *d*-catechin, 7. (—)-epicatechin, 8. *o*-coumaric acid, 9. 1-naphthylacetic acid, 10. kaempferol.

stituents of the aqueous extract and analytically resolved by the use of a dual HPLC column switching technique.

The HPLC apparatus (cf. Figure 5) was built up from two different modular units: a sample processing unit, which consists of

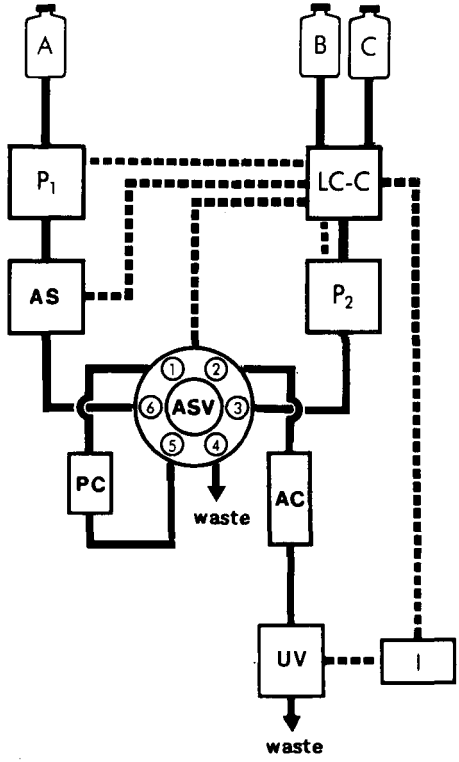

Figure 5 "On-line" system set-up, A, B, C: eluent reservoirs; LC-C: liquid chromatography controller; P1, P2: pumps; AS: autosampler; ASV: automatic switching valve; DC: pre-column; AC: analytical column; UV: UV-monitor; I: integrator.

pump 1 (Model 655A-12, E. Merck) an autosampler AS (Model 655A-40) and a pre-column PC (LiChrosorb RP-18, 7 μm, 30 × 40 mm I.D.) for bonded-phase extraction and on-column fractionation, respectively. An analytical unit, which consists of an LC controller LC-C (Model L 5000), an analytical column (LiChrosorb RP-18, 7 μm, 250 × 4 mm I.D.), an UV-monitor (Model 655 A) and an integrator (3390 A, Hewlett–Packard).

In order to perform a dual column switching between an off-line and an on-line mode, the pre-column and the analytical column were

connected via an automatic six-port valve ASV (Model ELV 7000), whose configurations are shown in Figure 6.

Prior to analysis the pre-column and the analytical column are equilibrated in the valve configuration "off-line" for 10 min at a flow-rate of 1.5 ml/min with eluent A (H_2O/acetone, 99/1, v/v) and eluent B (0.25 M NaH_2PO_4, 0.01 M tetrabutylammoniumsulfate, pH 4.0), respectively.

Simultaneous with the sample injection (100 μl standard mixture or aqueous needle extract) the pre-column and the analytical column are series-connected by switching into the "on-line" mode. Under these conditions the adenine nucleotides are fractionated and quantitatively transferred at a flow rate of 1.5 ml/min within 1.5 min to the top of the analytical column.

Switching back into valve position "off-line" allows the regeneration of the pre-column simply by backflushing the residual matrix constituents to waste. At the same time the analytical resolution and detection of the adenine nucleotides is performed by starting pump 2, which delivers an acetonitrile step gradient (0–6 min, 100% eluent B; 6–15 min, 0–15%, v/v, acetonitrile, eluent C; 15–17 min, 85% eluent B, 15% eluent C; flow-rate: 1.5 ml/min; cf. Figure 7).

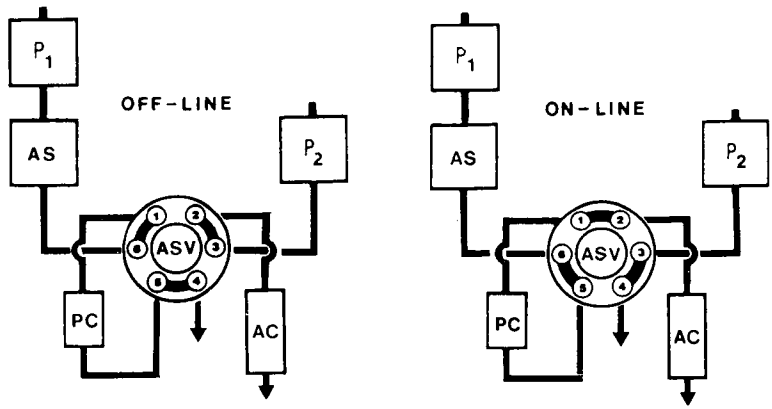

Figure 6 Valve switching positions. Left: off-line mode; pre-column (PC) and analytical column(AC) can be run separately. Right: on-line mode; pre-column and analytical column are series-connected through valve positions 6-5-1-2.

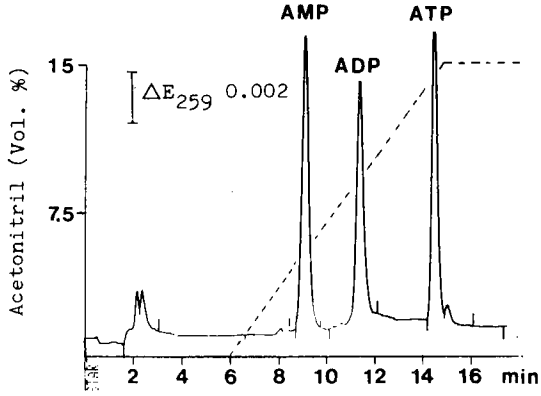

Figure 7 On-line chromatogram of a standard mixture of adenine nucleotides.

RESULTS AND DISCUSSION

Spruce needle analysis by RP-HPLC and UV-VIS diode array detection

Reversed-phase chromatography on octadecylsilyl bonded columns with methanol-water has been successfully employed in many separations of polar flavonoid aglycones and flavonoid glycosides.[13,16–18] As needle extracts of *Picea abies* usually contain phenolic compounds of different polarity and oxidation states an elution with acidified water–methanol gradients turned out to be an ideal solution system.

Chromatograms representing the separation of a purified spruce needle extract from the first year's growth period of a Norway spruce tree of the reference series are shown in Figures 8 and 9. The availability of a photodiode array detector for HPLC enables a remarkable improvement in peak identification. Simultaneous detection at different wavelengths and measurement of the UV spectrum of each separated compound during the elution allows an easy and rapid identification of Norway spruce needle phenolics. Among the classes present are shikimic acid, the most important intermediate of spruce needle phenolic biosynthesis, some derivatives of benzoic acid, *o*-coumaric acid, *p*-coumaric acid and *p*-hydroxyacetophenone, the 3-glycosides of the flavonoids kaempferol and quercetin, the stilbenes piceatannolglycoside and isorhapontin as well as several catechins.

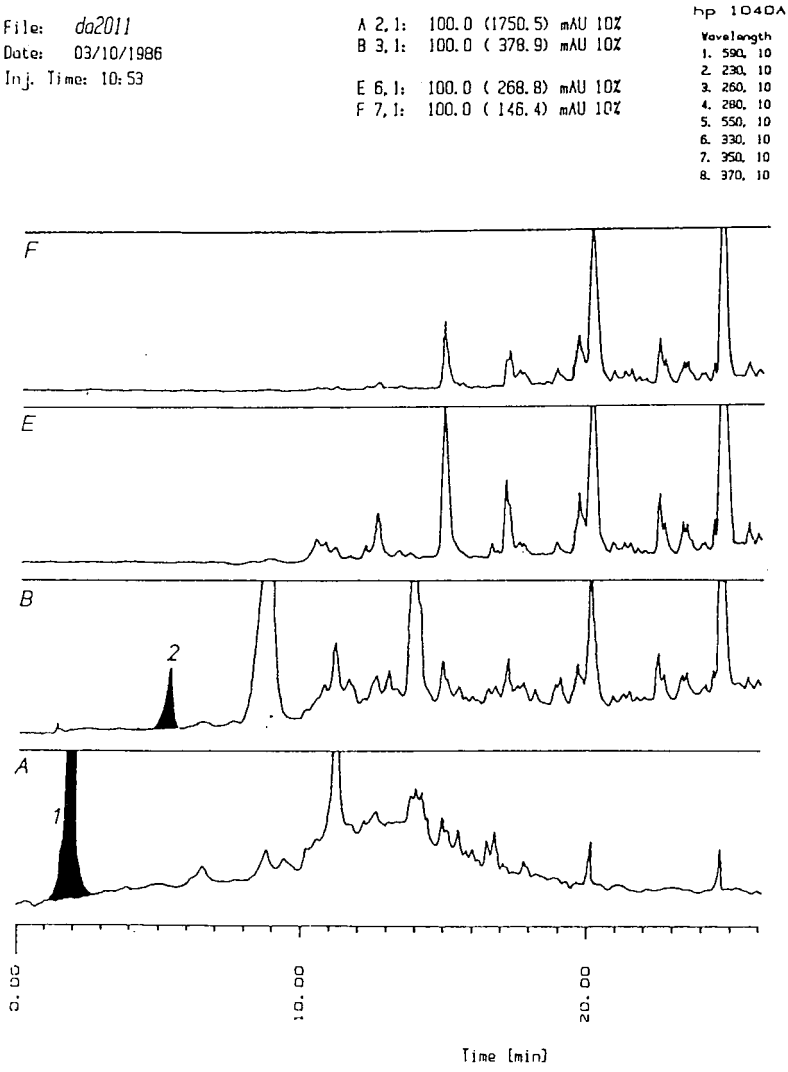

Figure 8 Multi-signal plot of a purified spruce needle extract (reference series). 1. shikimic acid, 2. *p*-hydroxybenzoic acid glycoside.

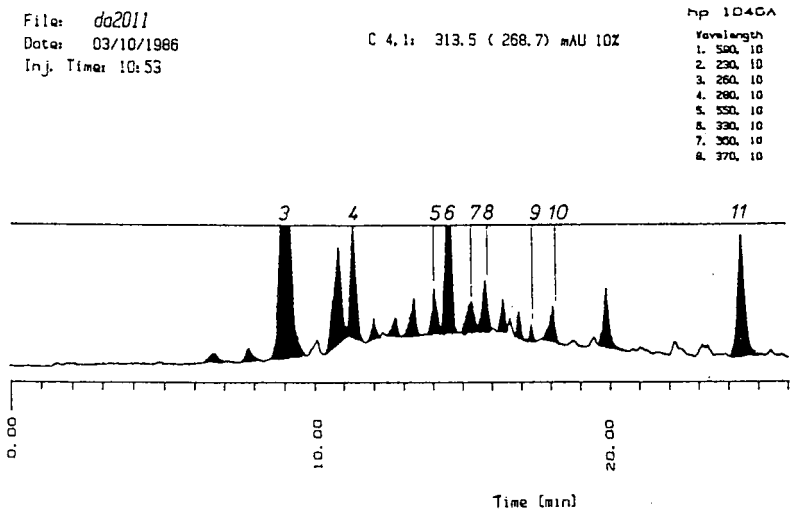

Figure 9 Separation of phenolic compounds from a purified spruce needle extract. Detection: 280 nm, 3. picein, 4. *d*-catechin, 5. (–)-epicatechin, 6. *p*-hydroxyacetophenone, 7. piceatannol, 8. *p*-coumaric acid, 9. benzoic acid, 10. *o*-coumaric acid, 11. isorhapontin.

Peaks 1 and 3–11 (Figure 9) could be identified by comparison of their retention times and UV spectra with those of authentic compounds from the standard mixture (cf. Table 1). Analysed standard solutions indicated that except for *p*-hydroxyacetophenone (6), *p*-coumaric acid (8), *o*-coumaric acid (10), catechin (4) and epicatechin (5) all extracted phenolics are in their natural glycoside linkage or procyanidin B/C form. To ascertain the compounds 2 and 12–20 (Figure 10) separation was repeated after enzymatic and hydrochloric acid hydrolysis. Chromatograms of the hydrolysates are presented in Figures 10b and 10c. The retention, peak area and UV data of the identified "new" monomeric compounds may be directly correlated with the unhydrolysed glycosides, although the sugar compound was not analysed. This technique proves useful for a number of studies on needle phenolics. The following needle phenolics have been identified in this manner: *p*-hydroxybenzoic acid glycoside (2), *p*-coumaric acid glycoside (14), *o*-coumaric acid glycoside (17), kaempferol-3-glycoside (20), benzoyl-glycose (18), quercetin-3-

Table 1 Retention times, capacity factors and measuring wavelength of plant phenolics on LiChrosorb RP-W column; chromatographic conditions: see experimental part; $t_0 = 1.35$ min

Compound	t_R (*min*)	k'	λ (*nm*)
Gallic acid	3.90	1.9	280
Picein	8.80	5.5	280
p-Hydroxybenzoic acid	9.70	6.2	250
d-Catechin	11.20	7.8	280
(–)-Epicatechin	13.70	9.2	280
p-Hydroxyacetophenone	14.00	9.4	280
Piceatannolglycoside	14.70	9.9	280
p-Coumaric acid	15.40	10.4	350
Benzoic acid	17.00	11.6	280
o-Coumaric acid	18.00	12.3	330
Quercetin	21.45	14.9	350
Kaempferol	23.00	16.0	350
Isorhapontin	24.50	17.2	280

glycoside (19) as glycoside compounds and the procyanidins B-3 (13), B-4 (15), B-6 (16) and C-2 (12).

The analysed Norway spruce needles of the seven expose chamber experiments provides information about:

— quantitative data on the amount of phenolic compounds in the needles of *Picea abies*
— variation of these amounts with the age of spruce needles
— comparison of these amounts between exposed and non-exposed Norway spruce trees.

The chromatographic measurements of primary and secondary needles showed that during the exposure to SO_2, O_3 and SO_2/O_3 containing atmosphere the amount of shikimic acid, quinic acid, kaempferol-3-glycoside, *p*-coumaroyl glycoside, *p*-hydroxyacetophenone, picein, catechin and *p*-hydroxybenzoic acid glycoside changed most extremely, whereas the amount of the other identified phenolic compounds decreased only slightly. The most distinct alterations were observed for *p*-hydroxyacetophenone and its glycoside picein after SO_2 exposure (admitting to both simulation models: clean-air area and industrial urban area) as can be seen in

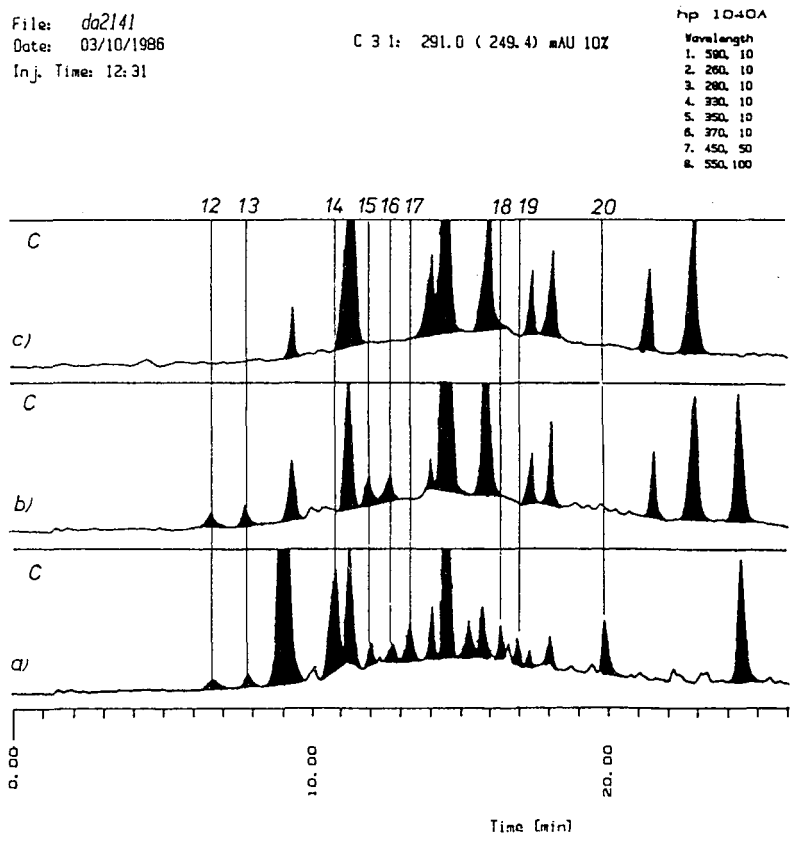

Figure 10 Separation of needle phenolics: (a) from a purified extract, (b) after enzymatic hydrolysation, (c) after acid hydrolysation, 12. procyanidin C-3, 13. procyanidin C-2, 14. *p*-coumaric acid glycoside, 15. procyanidin B-4, 16. procyanidin B-6, 17. *o*-coumaric acid glycoside, 18. benzoyl-glycose, 19. quercetin-3-glycoside, 20. kaempferol-3-glycoside.

Figure 11. Table 2 shows that the levels of *p*-hydroxyacetophenone are always very high in the affected needles and that the ratio *p*-hydroxyacetophenone/picein is larger than 1 whereas in the undamaged spruce needles of the reference series this ratio is smaller than 1. The measurements also showed that the level of *p*-hydroxyacetophenone is always lower (factor 90–120) in undamaged needles than in SO_2 damaged needle material. This effect is irrespec-

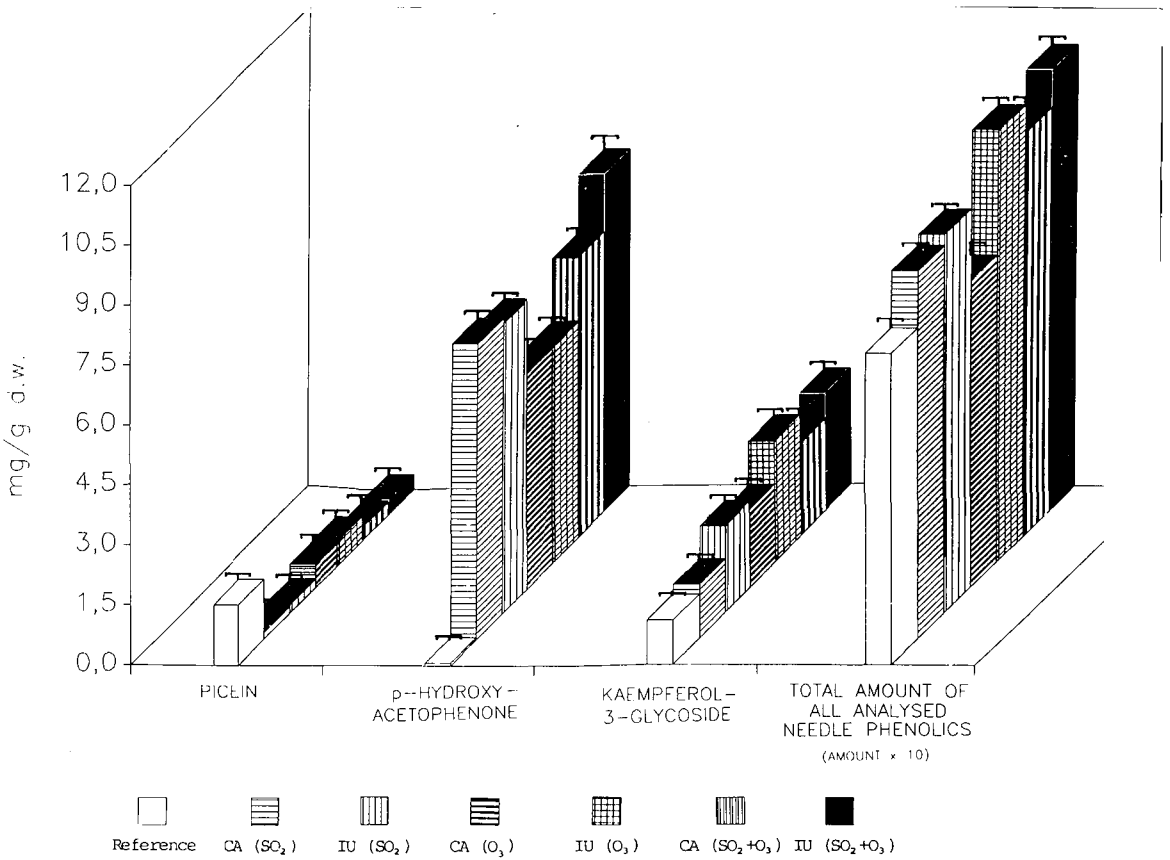

Figure 11 in after O_3 and SO_2/O_3

Table 2 *p*-Hydroxyacetophenone/picein amount in primary spruce needles after SO_2, O_3 and SO_2/O_3 exposure[a]

Sample	*Fumigation conditions*	*Amount (mg/g d.w.)*		*Ratio H/P*
		H	*P*	
Reference	Charcoal-filtered air	0.06	1.5	0.04
CA (SO_2)	60± 7 $\mu g/m^3$ SO_2 cont. +500 $\mu g/m^3$ SO_2 2h/14d	7.40	0.2	37.0
IU (SO_2)	99± 9 $\mu g/m^3$ SO_2 cont.	7.30	0.2	36.5
CA (O_3)	130±25 $\mu g/m^3$ O_3 cont.	5.30	0.6	8.8
IU (O_3)	132±24 $\mu g/m^3$ O_3 5/h/d	5.50	0.6	9.2
CA (SO_2+O_3)	130±28 $\mu g/m^3$ O_3 cont. + 60± 8 $\mu g/m^3$ SO_2 cont. +500 $\mu g/m^3$ SO_2 2h/14d	7.00	0.3	23.3
IU (SO_2+O_3)	125±30 $\mu g/m^3$ O_3 5h/d + 98± 7 $\mu g/m^3$ SO_2 cont.	8.50	0.4	21.3

[a]H, *p*-Hydroxyacetophenone; P, picein; CA, clean-air area; IU, industrial urban area.

tive of seasonal rhythm and is most distinct in primary needles as confirmed by our measuring on reference material for studying the annual rhythm and also by literature.[19] The exposure of ozone (130 ±25 μg continuously or 130±24 μg for 5 h day^{-1}) showed similar effects in the alteration of the amounts of these phenolic compounds (see block 1, column 4 and 5 in Figure 11).

However, the decrease of picein and the increase of *p*-hydroxyacetophenone were not as extreme as during SO_2 exposure. The ratio *p*-hydroxyacetophenone/picein of the O_3 series yields only to a fourth of that of the SO_2 series. *p*-Hydroxyacetophenone was unequivocally identified as the major growth inhibition compound of Norway spruce.[20] The large accumulation in needles of affected trees seems to be a reaction of spruce trees on stress.[21] The function and the mode of accumulation of this compound in the diseased needles of *Picea abies* remain open. *p*-Hydroxyacetophenone may accelerate the production of auxinoxidizing enzymes (IAA-oxidase), thereby reducing the level of IAA in different plant parts.[22] The reduction of the IAA level may result in accelerated needle senescence leading to loss of photosynthetic activity.[19]

Block 3 in Figure 11 illustrates the increasing amount of kaempferol-3-glycoside during the exposure to SO_2 and O_3. Concomitant to the noxious pollutants the amount increases approximately two-fold after SO_2 exposure (column 3), and three-fold after O_3 treatment (column 5) in comparison to the reference series. Schneider and coworkers[23] found that the flavonol kaempferol completely inhibits photophosphorylation of broken chloroplasts at a concentration of about 5×10^{-5} M. These results are in good agreement with our findings on the adenine nucleotide (AMP, ADP, ATP) concentrations and adenylate energy charge.

The amount of quinic acid and shikimic acid, which are the most important intermediates of phenolic biosynthesis, were determined by ion chromatographic separation and conductivity detection, as the high absorption rate and noisy baseline of the purified extracts below 230 nm did not allow quantitative UV measurements of these phenolics in RP-HPLC..

In the youngest needles the amount of the quinic acid is very high, indeed, but it decreases rapidly within the next growth period as can be seen in Figure 12. The change of the amount of shikimic acid in relation to the needle age is contrary to what is observed with quinic acid. The primer phenolic starts with lower values in youngest needles and increases approximately two-fold within one year. In the damaged needle species we always observed higher amounts of quinic acid and shikimic acid, where the increase of quinic acid was most distinct.

The possible function of *p*-coumaroyl glycoside (large decrease in the youngest needles) as well as of catechin (slight increase in secondary needles) and *p*-hydroxybenzoic acid glycoside (not detectable in SO_2-damaged needles admitting to both simulation models) is not known yet. The decreased amounts of the other phenolic compounds listed before points to increased needle senescence. In the case of the transformation of quinic acid to shikimic acid as well as the transformation of *p*-hydroxyacetophenone to picein the metabolism seems to be disturbed by enzymatic inhibition.

The total amount of all needle phenolics of primary and secondary needles in dependence of the different SO_2 and O_3 exposure models (single or combined, continuous or discontinuous) is shown in Figure 11, block 4. During simulation of clean-air area conditions by a continuous exposure to O_3 ($130\,\mu g\,m^{-3}$), no alteration of the total phenolic amount was observed (cf. block 4 column 4 in Figure 11).

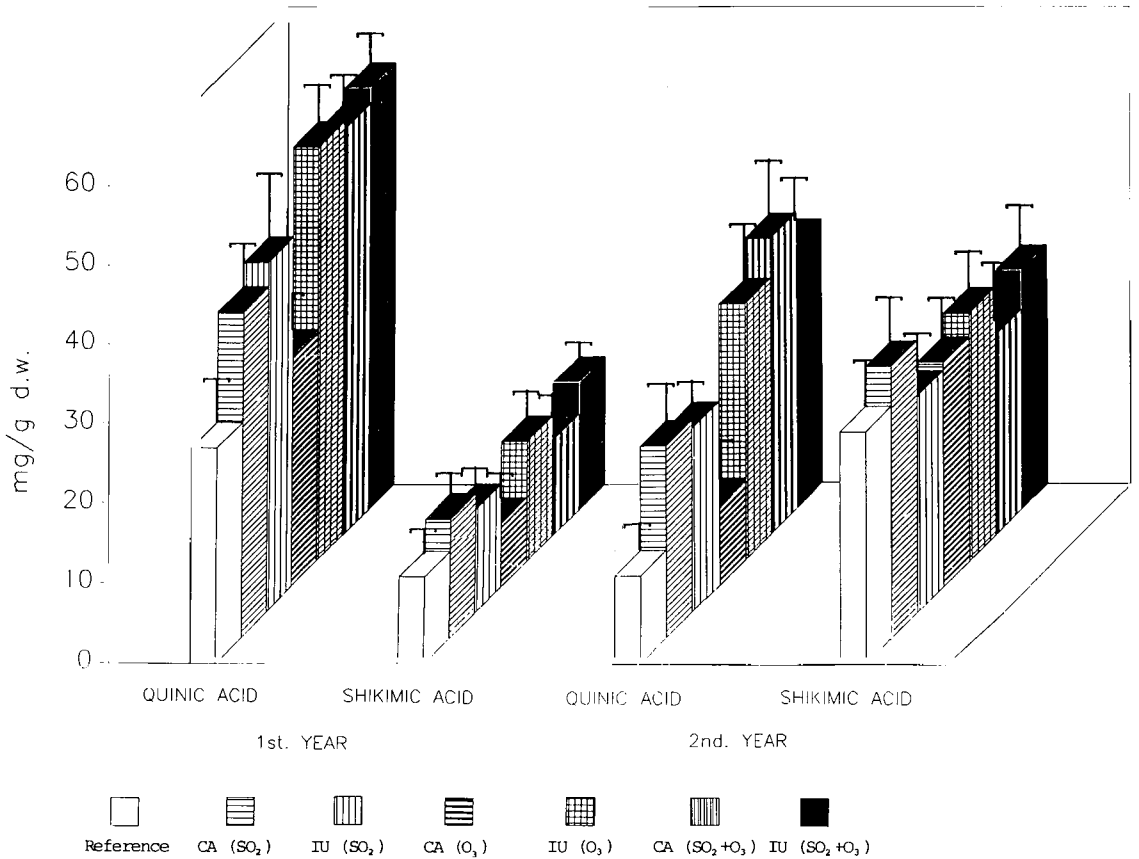

Figure 12 Amount of quinic acid and shikimate in primary and secondary spruce needles after SO_2, O_3 and SO_2/O_3 exposure.

However, with regard specific to compounds this experiment demonstrates an increase of kaempferol-3-glycoside (70%) as well as a marked increase of *p*-hydroxyacetophenone (800%). In summary, the increasing amount of both needle phenolics is compensated by the drastically reduced amount of picein and *p*-coumaric acid glycoside. The intermittent O_3 exposure ($130\,\mu g\,m^{-3}$) for 5 h day^{-1} over the whole fumigation time of 1.5 year mimics the O_3 situation for spruce needle trees growing in industrial urban areas. This type of O_3 treatment results in a 40% increase of the total phenolic amount, with primarily the amounts of quinic acid, *p*-hydroxyacetophenone and kaempferol-3-glycoside increasing. The intermittent application of pollutants seems to induce or to force phenolic synthesis, especially of those compounds which are discussed as inhibitors of photophosphorylation and growth. SO_2 fumigation, continuously or intermittently with a maximum concentration of $500\,\mu g\,m^{-3}$ for 2 h every 14 days, indicates a more distinct variation of the *p*-hydroxyacetophenone/picein ratio, contrary to a discontinuous O_3 exposure (see Table 2).

Mixtures of O_3 and SO_2 caused the greatest increase of the total phenolic amount (30–40%). This rise is not only based on the high levels of quinic acid, *p*-hydroxyacetophenone and kaempferol-3-glycoside but on the elevated amount of shikimate too. The drastic increase in the production of these secondary plant products could be explained as follows: combinations of SO_2 and O_3 in the simulation model clean-air area consist of a continuous O_3 and SO_2 application ($130\,\mu g\ O_3\,m^{-3}$ and $60\,\mu g\ SO_2\ m^{-3}$) with intermittent extreme values of $500\,\mu g\ SO_2\ m^{-3}$ each 14th day for 2 h. This combination permanently generates acidic oxidation products (sulfite, sulfate) with the intermittent SO_2 peak being compensated by O_3. Thus, the increase of phenolic compounds can be attributed to the action of acidic oxidation products. This is documented by the very good correlation between column 2 of block 4 of Figure 11 and column 6 of the same block.

Combinations of SO_2 and O_3 in the simulation model industrial urban area consist of a continuous treatment of SO_2 ($100\,\mu g\,m^{-3}$) as well as a discontinuous O_3 application ($150\ \mu g\,m^{-3}$) for 5 h per day. During this time O_3 reacts with SO_2 yielding the aforementioned acidic oxidation products. During the remaining 19 h of each day, however, an excess of SO_2 persists. This interactive air pollutant

pattern subsisting to O_3, SO_2, SO_3^{2-} and SO_4^{2-} obviously affects the phenolic metabolism the most. In this case not only the inhibitory-active phenolic compounds reach extremely high levels, but the most important intermediates of phenolic biosynthesis too. Beyond the enhanced biosynthesis of quinic acid and shikimate we expect a reduction of the carbohydrate metabolism by product inhibition.

Analysis of adenine nucleotides

Our approach to use a coupled dual column system for the online sample clean-up and trace analysis of the adenine nucleotides AMP, ADP and ATP led to the development of a very reliable and accurate method for the determination of the adenylate energy charge of plant cells.[24, 25] Due to its high analytical precision, its automation and its practicability this on-line HPLC method is superior to the commonly used enzymatic assays for the quantitation of adenine nucleotides in complex biological matrices[26, 27].

Figure 13 shows a typical on-line chromatogram of an aqueous needle extract from the first year's growth period of a Norway spruce tree of the reference series. Peak identification was performed by spiked, authentic samples and enzymatic peak shift, i.e., partial hydrolysis of the crude extract by acidic phosphatase (EC 3.1.3.2).

The quantitation of the individual adenine nucleotides and their total amount relative to the dry weight of the exposed and untreated needle material and the calculation of the ATP/ADP ration as well as the adenylate energy charge (AEC) are documented in Table 3.

A comparison with the reference series shows that in both simulation models (clean-air and industrial urban area) the AEC values and the ATP/ADP ratio are significantly reduced. The lower energy charge and ATP/ADP ratio could be due to an increased phosphatase activity and/or a reduced adenylate kinase activity. Both enzyme activities could result in the 5- to 6-fold higher AMP amount relative to the reference series. However, an elevated phosphatase activity can be almost excluded as the total amount of adenine nucleotides remains nearly constant.

Another explanation would be that an increased ATP consumption and/or a reduced photophosphorylation rate is induced by these fumigation patterns. Both effects could result in the 2- to 4-fold reduced ATP content relative to the reference value. However, the analysis of the phenolic compounds points to a reduced photo-

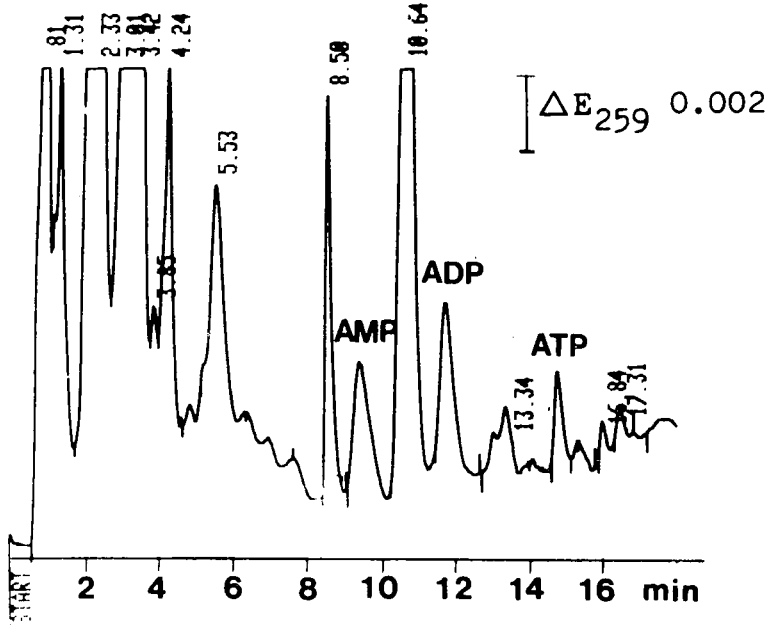

Figure 13 On-line chromatogram of an aqueous spruce needle extract. Sample volume: 100 μl; AMP: 0.42 nmol; ADP: 0.62 nmol; ATP: 0.25 nmol; recovery: $93 \pm 5\%$ ($n = 2$).

phosphorylation rate as the principal reason. Kaempferol is known as a potent inhibitor of light-dependent ATP-synthesis.[23] *p*-Hydroxyacetophenon indirectly accelerates needle senescence and, consequently, reduces the photosynthetic activity.[19] The amount and, thus, the inhibitory power of both phenolic derivatives are drastically (20-fold) increased in both simulation models.

A comparison of the clean-air (continuous O_3 exposure) and industrial urban (discontinuous O_3 exposure) area shows that the ATP/ADP ratio as well as the cellular energy charge is more decreased by a discontinuous treatment with ozone.

A comparison of the intermittent and continuous SO_2 fumigation in both simulation models reveals that the phytotoxicity of the intermittent (14-day rhythm) SO_2 peak in the clean-air model is more pronounced. This effect is documented by a smaller ATP/ADP ratio and AEC value.

A comparison of the combined fumigation in both simulation

Table 3 Adenine nucleotide (AdN) amount and adenylate energy charge (AEC) of primary spruce needles (*picea abies*)[a]

Sample	*AMP* (*nmol/g d.w.*)	*ADP* (*nmol/g d.w.*)	*ATP* (*nmol/g d.w.*)	*AdN* (*nmol/g d.w.*)	*ATP/ADP*	*AEC*
Reference	5.8 ± 1.2[b]	61.0 ± 0.9	54.4 ± 0.7	122.0 ± 1.7	0.88 ± 0.01	0.70[c]
CA (SO_2)	38.8 ± 0.8	51.0 ± 2.0	25.5 ± 2.1	115.3 ± 3.0	0.50 ± 0.04	0.44
IU (SO_2)	32.8 ± 0.8	61.2 ± 0.6	38.2 ± 0.8	131.2 ± 1.3	0.62 ± 0.01	0.52
CA (O_3)	27.6 ± 4.0	44.4 ± 0.8	36.5 ± 0.8	108.5 ± 4.2	0.82 ± 0.02	0.54
IU (O_3)	30.6 ± 1.7	61.7 ± 0.6	21.4 ± 0.9	113.6 ± 2.0	0.35 ± 0.01	0.46
CA ($SO_2 + O_3$)	34.3 ± 1.0	44.8 ± 0.2	30.7 ± 0.3	109.8 ± 1.1	0.68 ± 0.01	0.48
IU ($SO_2 + O_3$)	33.6 ± 0.1	47.5 ± 0.6	14.3 ± 0.9	95.4 ± 1.1	0.30 ± 0.02	0.40

[a]AdN = AMP + ADP + ATP; CA, clean-air area; IU, industrial urban area.
[b]Standard deviation (s); $n = 2$.
[c]$s \leqq 0.005$.

models shows that the energy charge as well as the ATP/ADP ratio are reduced to the lowest value in the industrial urban area model. As discussed for the drastic increase of phenolic compounds, the generation of acidic oxidation products and the additional persistence of SO_2 in the industrial urban area model, i.e., a distinct interactive pollutant pattern, exhibit the highest phytotoxicity with respect to the energy metabolism of the spruce needles investigated.

From other studies it is also known that SO_2 and its reaction products with water, i.e. bisulfite, sulfite and sulfate, inhibit photophosphorylation and oxidative phosphorylation[28] and that ozonation decreases the ATP content in plant leaves.[29] In contrast to our findings Benz and Hampp[30] determined by enzymatic analysis a higher ATP amount and a significantly higher ATP/ADP ratio in damaged spruce needles than in healthy ones. On the other hand, this group found a decreased AEC value in damaged needles too.

CONCLUSIONS

As shown in part 1 of this study for the histological and cytological changes, the spruce needles suffer the most distinct damage in the simulation model of an industrial urban area with a discontinuous (5 h/day) ozone and a continuous sulfur dioxide fumigation. These findings correlate with the pollutant-induced subcellular changes of the amount of marker molecules investigated in this study. Furthermore, our investigations point to a disturbed H_2O-balance, i.e. a water deficiency, as the physiological consequence of the exposure to SO_2 and O_3.

The leaf and needle permeability, which determines the uptake of pollutants, is known to be enhanced by ozone- and acid-induced disintegration of the cuticula.[31–33] The resulting increased diffusion and evaporation rate generates a water deficiency, which subsequently causes a premature senescence. The increased catabolic activity during senescence results in a starch congestion,[34] an increased synthesis of polyphenols,[35] a degeneration of chloroplasts[36] and chlorophylls[37] and a reduced photosynthesis rate.[38] Water-stress also inhibits the phytohormone-controlled cell growth and division rate.[39–42]

In order to prove our working hypothesis of water-stress as the physiological reaction of spruce needles on the exposure to air pollutants, we will also investigate the single and combined effects of O_3 and SO_2 fumigation on the phytohormone content.

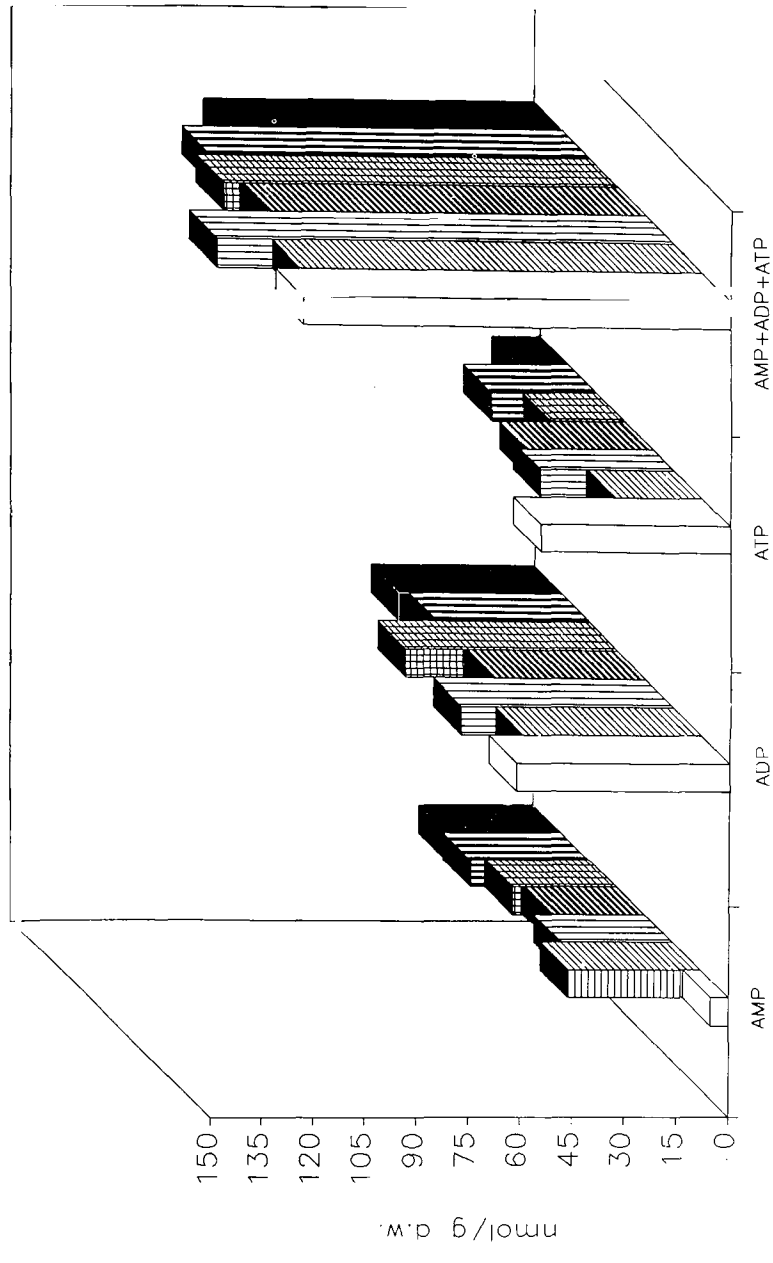

Figure 14 Amount of adenine nucleotides in primary spruce needles after SO_2, O_3 and SO_2/O_3 exposure.

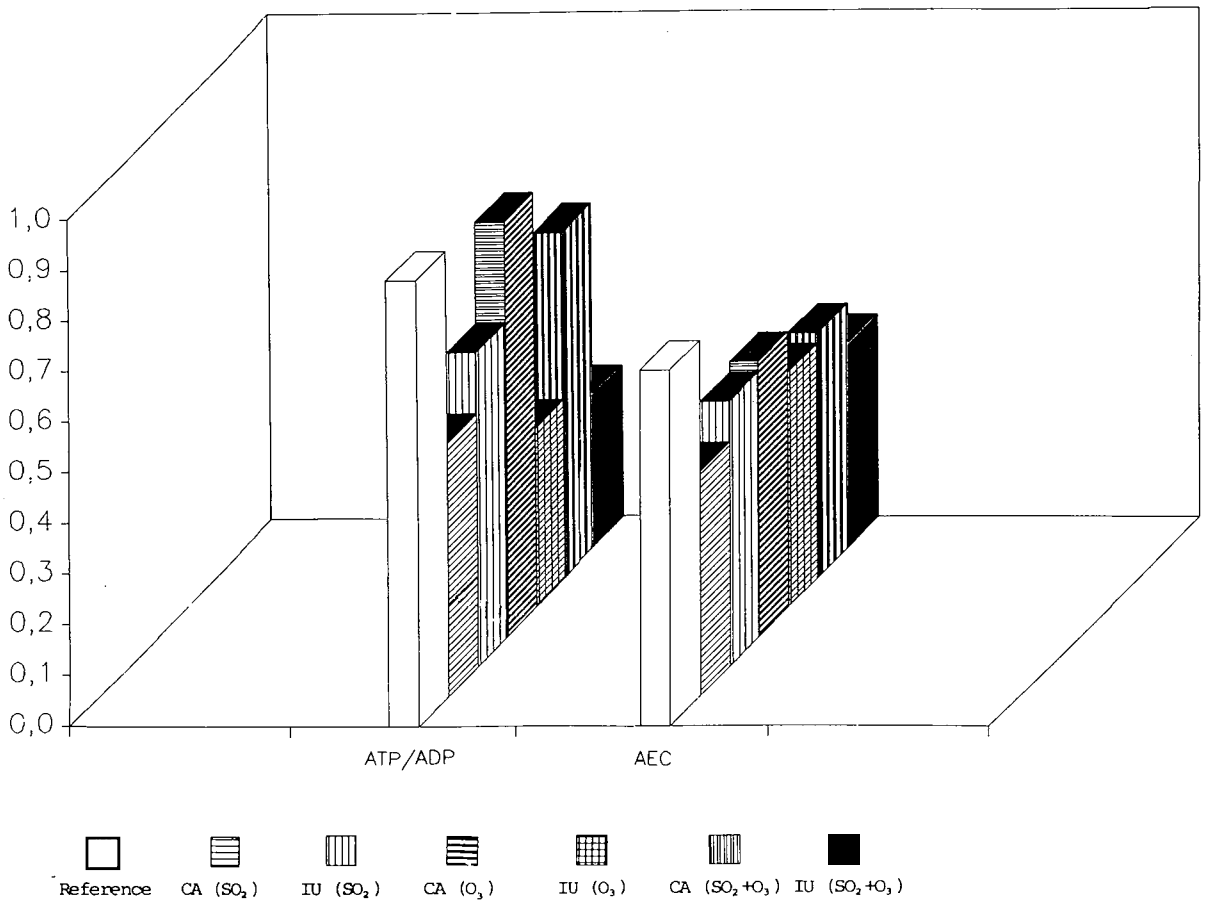

Figure 15 ATP/ADP ratio and adenylate energy charge (AEC) in primary spruce needles after SO_2, O_3 and SO_2/O_3 exposure.

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