Journal of Chromatography, 360 (1986) 452–458
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 614

Note

High-performance liquid chromatographic analysis of *p*-hydroxyacetophenone and *p*-hydroxyacetophenone-β-D-glucopyranoside, two major phenolic compounds in Norway spruce

E. HOQUE
Lehrstuhl für Forstbotanik, Ludwig-Maximilians-Universität München, Amalienstrasse 52, 8000 Munich 40 (F.R.G.)

(First received January 29th, 1986; revised manuscript received March 6th, 1986)

p-Hydroxyacetophenone (*p*-HAP) is a major phenolic compound in Norway spruce and plays a significant role in stress phenomena[1–3]. Under stress conditions, *p*-HAP accumulates in different plant organs. The ratio of *p*-HAP to *p*-hydroxyacetophenone-β-D-glucopyranoside (*p*-HAPG) was shown to increase with the increasing decline-symptoms of Norway spruce. *p*-HAP caused needle-fall, retarded apical growth and inhibited bud-sprouting in biological tests. Therefore, a suitable method for the determination of *p*-HAP and *p*-HAPG was urgently needed to handle a large number of samples in physiological investigations.

We have previously described the measurements of levels of *p*-HAP and *p*-HAPG by a combination of thin-layer chromatography (TLC), enzymic hydrolysis and gas chromatographic (GC)–flame ionization detection (FID)[1,2]. *p*-HAP and *p*-HAPG were also determined by 2,4-dinitrophenyl hydrazine reagent, TLC and spectrophotometry[4]. The early methods of quantification of *p*-HAPG were by paper chromatography, enzymatically with β-glucosidase[5,6] or colorimetrically with Million's reagent[7,8].

In the course of applying our previous methods to a study on Norway spruce decline, we became aware of the convenience of high performance liquid chromatographic (HPLC) methods for the determination of biologically active compounds[1,2]. The present report describes the determinations of *p*-HAP and *p*-HAPG by reversed-phase HPLC, in tissues, needle-segments and whole needles in the nanogram range.

EXPERIMENTAL

Plant materials

Epidermal strips and vascular bundles were detached from needles of Norway spruce trees, *Picea abies* (L.) Korst. (90–100 years old, harvest: June 28th, 1985, FA Bodenmais, Bavaria, F.R.G.) using a microdissection technique. The epidermal strips were cleaned from the mesophyll contaminants by a sharp razor blade. Additionally, needles of cloned Norway spruce (3.5 years old, clone no. 1027, supplied by Pflanzgarten Laufen, harvest: June 19th, 1985) were used.

0021-9673/86/$03.50

Chromatographic purification and derivation of authentic samples

Commercially available *p*-HAP (Sigma, Munich, F.R.G.) was purified on a silica gel partition column as described by us[1] to give pure white crystals of *p*-HAP (HPLC purity: 95.87%). Chromatographically purified *p*-HAP was acetylated with pyridine–acetic acid anhydride (1:1) for 4 h at room temperature and purified further by silica gel partition chromatography (column: 35 cm × 1.6 cm I.D., particle size: 230–240 mesh; silica gel + 20% water). Acetyl-*p*-HAP was eluted with *n*-hexane–chloroform (98:2–96:4), in 1% step increases of chloroform, to give pure white acetyl-*p*-HAP crystals (HPLC purity: 94.21%).

The trifluoroacetylation of authentic *p*-HAP (500 mg) was carried out by trifluoroacetic acid anhydride (TFAA)–pyridine (1:1) for 4 h at room temperature and purified by partitioning between chloroform and water (pH 8.0); the purity was checked by high-performance TLC (HPTLC) [($R_{F_{\text{trifluoroacetyl-}p\text{-HAP}}}$ = 0.82–0.87, $R_{F_{p\text{-HAP}}}$ = 0.59–0.61; solvent system: chloroform–ethylacetate (7:3), silica gel F_{254} and HPTLC) (HPLC purity: 95.01%)].

Trifluoroacetylation of p-HAP from plant samples

The trifluoroacetylation of plant samples was performed under the following modified conditions: modification of method 1, Pierce Catalogue (Atlanta, Heidelberg, 1983)–dried sample in 200 μl of benzene, 100 μl of 0.01 *M* triethylamine + 100 μl of TFAA for 10 min at room temperature; addition of 200 μl of phosphate buffer (pH 6.0), shaken; organic phase (3 ×) collected, evaporated and dissolved in aliquots of ethyl acetate or water–methanol (65:35) prior to injection for GC or HPLC, respectively.

High-performance liquid chromatography–ultraviolet detection

The analysis was done on a Kontron HPLC system (HPLC Pump T-414, UV detector Uvikon 722 LC).

The separation of *p*-HAP and *p*-HAPG was done on a Kontron Spherisorb ODS-2 column (250 mm × 5 mm I.D., 5 μm particle size) connected with a Vydac 201SC precolumn (50 × 5 mm I.D., 30–40 μm particle size). The data were acquired and processed by Basic-programmable Shimadzu Integrator Chromatopack C-R3A. The chromatographic conditions are given in the figure legends.

Due to unavailability of radioactive *p*-HAP and *p*-HAP-β-D-glucopyranoside and radioactivity counting equipment in our laboratory, we made some compromises in experimental design. We used acetyl-*p*-HAP as internal standard in our analysis.

Preparation of plant samples for measurement by HPLC

The plant samples were finely homogenized in water and extracted with 80% methanol [+ 100 ppm butylated hydroxytoluene (BHT) as antioxidant]. The extracts were filtered, dried *in vacuo* and diluted in the ratio 1:8000–1:24000.

The internal standard acetyl-*p*-HAP (equivalent to 2.19 μg) was added in the intermediate dilution of the sample (1:1000) in order to monitor the losses during chromatographic analysis. No influence of BHT could be detected in the analysis under the conditions described below.

Gas chromatography–electron-capture detection

The determination of *p*-HAP and *p*-HAP-β-D-glucopyranoside (after enzymic hydrolysis) following trifluoroacetylation by GC with electron-capture detection (ECD) (^{63}Ni, 10 mCi) was performed under the following conditions: column, 10% SE-30 packed on Gas Chrom Q, 100–120 mesh (1 m × 2 mm I.D.); column temperature, 130°C; injector temperature, 250°C; flow-rate of nitrogen, 25 ml/min. The measurements were done using a Packard-Becker Gas chromatograph Model 427. Under these conditions, the detection limit of trifluoroacetyl-*p*-HAP was *ca.* 5 ng.

Gas chromatography–flame ionization detection

The preparation of sample and the GC–FID measurement of *p*-HAP and *p*-HAPG were performed according to Hoque[2].

RESULTS AND DISCUSSION

p-HAP, *p*-HAPG, trifluoroacetyl-*p*-HAP and acetyl-*p*-HAP can be detected at 265 nm (near their maximum absorption wavelength). In our work on the levels of *p*-HAP and *p*-HAPG in Norway spruce needles, we observed no significant interference at this wavelength from the contaminants in the 80% aq. methanolic extracts of Norway spruce needles. Our results are summarized in Tables I, II, III and IV.

Fig. 1 shows the test of linearity of the measurements of *p*-HAP, *p*-HAP-β-

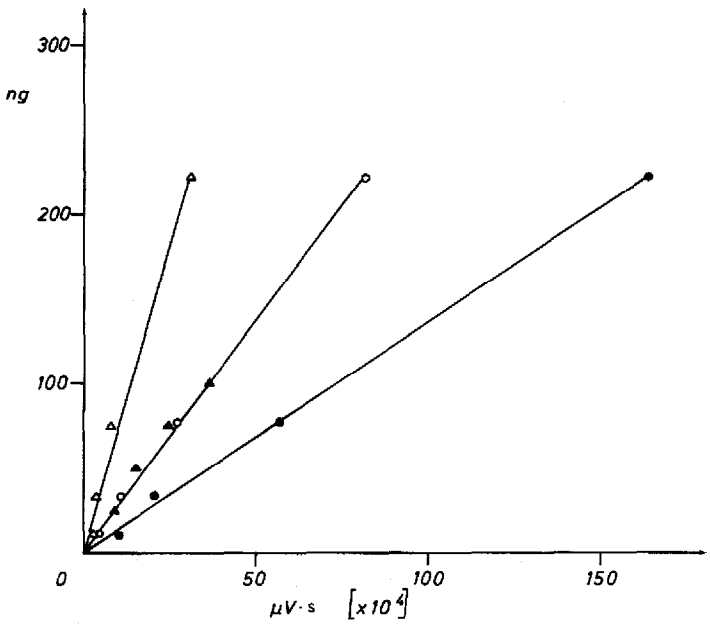

Fig. 1. Test of linearity of the measurement of *p*-HAP (●), *p*-HAP-β-D-glucopyranoside (○), trifluoroacetyl-*p*-HAP (▲) and acetyl-*p*-HAP (△) by isocratic HPLC (0.6 ml/min flow-rate; λ = 265 nm; mobile phase = 65% water + 35% methanol). The linearity of the measurements of *p*-HAP, *p*-HAP-β-D-glucopyranoside, TFA-*p*-HAP and acetyl-*p*-HAP can be described by the regression lines, $y = 0.51 + 0.000135x$ ($r^2 = 1.00$), $y = 2.39 + 0.000268x$ ($r^2 = 1.00$), $y = 2.46 + 0.000275x$ ($r^2 = 0.99$) and $y = 4.14 + 0.000715x$ ($r^2 = 0.98$), respectively.

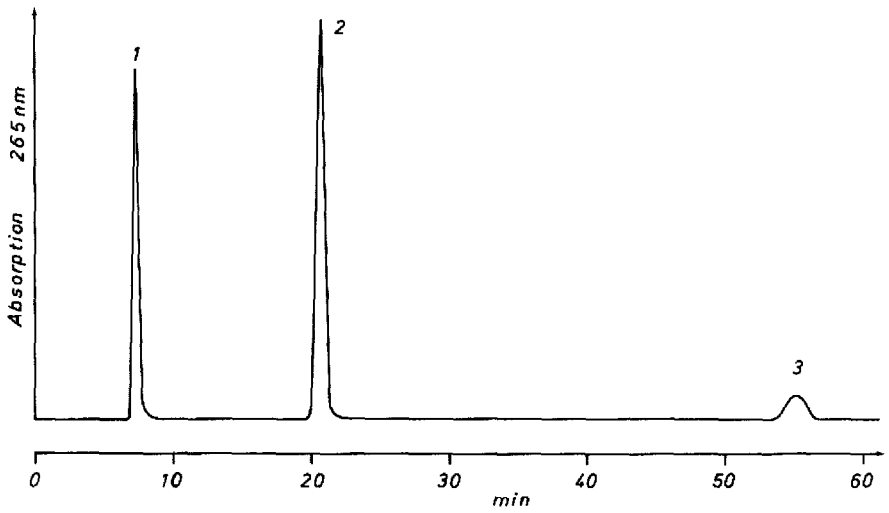

Fig. 2. Separation of authentic samples of *p*-HAPG (1), *p*-HAP (2) and acetyl-*p*-HAP (3) by isocratic reversed-phase HPLC. An aliquot equivalent to 77.77 ng of each sample was injected.

D-glucopyranoside and acetyl-*p*-HAP by HPLC. Fig. 2 shows the separation of authentic *p*-HAP, *p*-HAP-*β*-D-glucopyranoside and acetyl-*p*-HAP, and Fig. 3 shows the separation of these substances from plant material. Phenolic acids could not be separated from each other by the water–methanol (65:35) mobile phase. The elution pattern of the phenolic compounds studied here were in the following order: phenolic

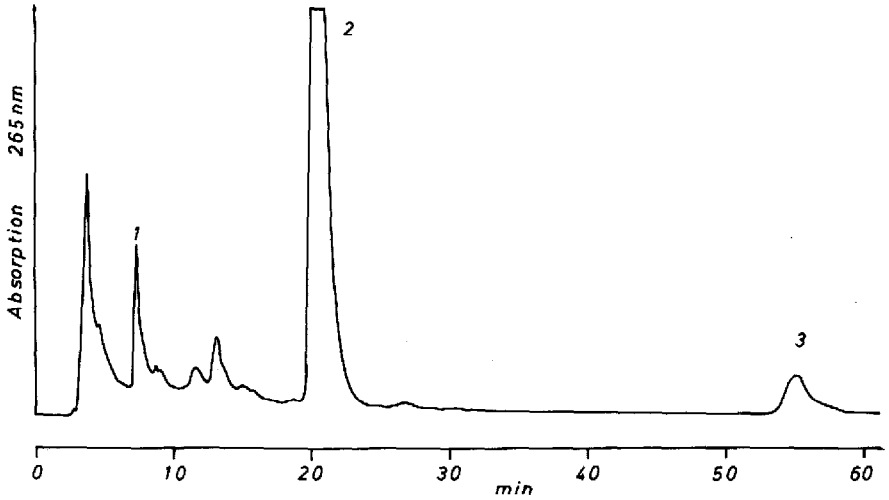

Fig. 3. Analysis of *p*-HAPG (1) and *p*-HAP (2) in plant samples by isocratic reversed-phase HPLC. Peak 3 corresponds to the internal standard acetyl-*p*-HAP. An aliquot of the diluted extract equivalent to 4.17 (affected tree)–250 μg (healthy tree) fresh weight was injected for measurement. The above chromatogram was obtained from needles of an affected tree (FA Bodenmais, harvest: 28.6.85, *ca.* 100-year-old tree). The losses of acetyl-*p*-HAP were between 15 and 20% during chromatographic analysis.

TABLE I

RETENTION TIMES OF PHENOLIC COMPOUNDS

Compounds	*Retention time (min)*
p-HAPG	8.3
p-Cumaric Acid	5.5
Ferulic Acid	5.5
Caffeic Acid	4.1
Benzoic Acid	5.3
p-HAP	22.5
Trifluoroacetyl-*p*-HAP	23.0
Acetyl-*p*-HAP	55.0

TABLE II

PRECISION AND ACCURACY OF THE MEASUREMENT OF *p*-HAP AND *p*-HAPG BY HPLC IN EPIDERMAL STRIPS AND NEEDLES OF NORWAY SPRUCE

p-HAP: μg/g fres weight, *p*-HAPG: μg/g fresh weight calculated as *p*-HAP equivalents

Object	*Tree No.*	*Substance*	*Average* ($\bar{x}$)	*Standard deviation* ($n = 10$)	*Precision****	*Accuracy*§
Epidermal strips* (3-year-old needles)	1	*p*-HAP	329	30	9.0	6.8
		p-HAPG	5781	399	7.0	5.3
Needles** (5 years old)	2	*p*-HAP	20	3.50	17.0	12.8
		p-HAPG	2531	393	16.0	12.1

* Site Bodenmais (1100 m above sea-level, harvest: June 28th, 1985).

** Site Sauerlach (670 m above sea-level, harvest: June 12th, 1984).

*** Precision = coefficient of variation (C.V.%) = (standard deviation × 100)/$\bar{x}$.

§ Accuracy ($S_{\bar{x}}$%) = (C.V.% × t)/$\sqrt{n-1}$, where t = t value of 2-tail Student's t-test at $P = 0.05$ and degrees of freedom = $n - 1$.

were measured by the TLC–GC method, obviously due to high losses of *p*-HAPG acids eluted earlier than *p*-HAP-β-D-glucopyranoside, *p*-HAP earlier than trifluoroacetyl-*p*-HAP and trifluoroacetyl-*p*-HAP earlier than acetyl-*p*-HAP (Table I).

The precision and accuracy of the measurement by HPLC as shown in Table II demonstrate the high reliability of the HPLC method in the anlysis of *p*-HAP and *p*-HAPG in needle extracts. Table III shows the comparison of the measurement of *p*-HAP and *p*-HAPG by HPLC– and TLC–GC methods. Lower values of *p*-HAPG were measured by the TLC–GC method, obviously due to high losses of *p*-HAPG in different steps of sample preparation for GC measurement (extraction, fractionation, TLC, enzymic hydrolysis, extraction)[2].

GC–ECD of trifluoroacetylated extracts before and after alkaline hydrolysis following purification by TLC confirmed the presence of *p*-HAP and *p*-HAP-β-D-

TABLE III

COMPARISON BETWEEN HPLC AND GC METHODS IN THE MEASUREMENT OF *p*-HAP AND *p*-HAPG IN NEEDLES

3-Year-old needles, 100-year old healthy trees, FA Sauerlach, harvest: June 12th, 1984, *p*-HAP: μg/g fresh weight, *p*-HAPG: μg/g fresh weight *p*-HAP equivalents.

Sample no.	*GC–FID*		*HPLC–UV*	
	p-HAP	*p-HAPG*	*p-HAP*	*p-HAPG*
1	56	405	63	405
2	192	1750	157	2763
3	137	568	131	912
4	29	1194	18	2546
5	251	409	352	566
6	41	1466	20	1840
7	134	164	105	229

glucopyranoside (trifluoroacetyl-*p*-HAP, GC–ECD, t_R = 7.2 min) in different tissues of needles as shown by HPLC.

The response of the electron-capture detector was found to be linear in the measuring range 5–62 ng and can be described by the regression line, $y = -22.07 + 0.16x$ ($r^2 = 0.82$), where y = injected amount of trifluoroacetyl-*p*-HAP (ng) and x = detected area of the peak (mm^2). However, the GC–ECD technique was found to be unsuitable for a large number of samples.

Previously, the identification of *p*-HAP was reported by us in Norway spruce needles[3]. *p*-HAPG was also inequivocally identified[9] in Norway spruce needles using UV, IR, mass spectroscopy ^1H NMR and ^{13}C NMR spectroscopy. Table IV shows the levels of *p*-HAP and *p*-HAP-β-D-glucopyranoside in different tissues and needles as measured by HPLC.

TABLE IV

CONTENTS OF *p*-HAP AND *p*-HAPG IN EPIDERMAL STRIPS (μg/g FRESH WEIGHT), VASCULAR BUNDLES (μg/g DRY WEIGHT) AND NEEDLES OF CURRENT-YEAR SHOOTS (μg/g FRESH WEIGHT) BY HPLC

Needles/parts of needles	*Tree No.*	*Plant No.**	*p-HAP*	*p-HAPG (p-HAP equivalents)*
Epidermal strips	1		353	545
	2		744	6185
	3		745	1346
Vascular bundles	1		116	250
	2		–	74
Needles of current-year shoots		1	274	8
		2	322	35
		3	372	0
		4	552	58

* Plants with planting shock.

CONCLUSIONS

The results show that the determination of *p*-HAP and *p*-HAP-β-D-glucopyranoside can be conveniently carried out by isocratic HPLC. This method is superior to the methods described previously[1–4]. The following merits of the isocratic method are important: rapid measurement (8–10 samples/day), no enzymic hydrolysation of *p*-HAP-β-D-glucopyranoside necessary prior to HPLC measurements, sensitive measurement in the nanogramme range, low cost/measurement, measurement of *p*-HAP and *p*-HAPG in the same run.

A further reduction of analysis duration could be obtained with 2,5-dihydroxyacetophenone (retention time 27.2 min) instead of acetyl-*p*-hydroxyacetophenone (retention time 55.0 min, see Table I) as internal standard. The response of the detector was found to be linear for 2,5-dihydroxyacetophenone in the range 20–840 ng.

REFERENCES

1 E. Hoque, *Eur. J. For. Pathol.*, 14 (1984) 377.
2 E. Hoque, *Eur. J. For. Pathol.*, 15 (1985) 129.
3 E. Hoque, *Phytochemistry*, 23 (1984) 923.
4 H. Esterbauer, D. Grill and G. Beck, *Anal. Chem.*, 46 (1974) 789.
5 P. Dittrich, *Thesis*, University of Munich, 1970.
6 E. Borquelot, *C.R. Acad. Sci.*, 17 (1920) 535.
7 H. Thieme, *Pharmazie*, 19 (1964) 535.
8 H. Thieme, *Pharmazie*, 19 (1964) 471.
9 E. Hoque and O. Seligmann, unpublished results.