Commonwealth of Australia
COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION
Reprinted from: "Journal of Chromatography"
Vol. 43 No. 2 Page Nos. 250–252 August (1969)

CHROM. 4156

A microanalytical method for the determination of dihydroquercetin in wood

Dihydroquercetin (3,5,7,3′,4′-pentahydroxyflavanone) is a major constituent of the alcohol soluble materials in the wood of Larch species and the wood and bark of Douglas-fir. A sensitive analytical method is needed to enable rapid assessment of amounts of dihydroquercetin (DHQ) when processing commercial materials and for studies of biochemical aspects of DHQ formation. Ability to analyse very small wood samples is a most important criterion from a biochemical standpoint.

Most quantitative estimates of the amount of DHQ in wood have employed the method of BARTON AND GARDNER[1,2]. This method suffers from limitations in that it is specific for 3-hydroxyflavanones not only DHQ, has a relatively low sensitivity, and is extremely dependent upon reagent impurities.

SQUIRE *et al.*[3], using a quantitative paper chromatographic (PC) technique found

that Douglas-fir wood contained from 0.5 to 1.0 % DHQ, 0.1 to 0.3 % dihydrokaempferol (3,5,7,4'-tetrahydroxyflavanone) and about 0.2 % pinobanksin (3,5,7-trihydroxyflavanone). Their sensitive PC method is, however, slow and tedious. We have been unable to improve the reproducibility of the method which has given large coefficients of variation (approx. 15%).

SATO *et al.*[4] and FURUYA[5] have shown that the trimethylsilyl (TMS) ethers of flavanoids can be separated by gas–liquid chromatography (GLC). KLEBE *et al.*[6] have reported that N,O-bis(trimethylsilyl)acetamide (BSA) is a very reactive silylating reagent and does not produce interfering side products. Using this reagent we have developed a rapid sensitive and accurate method for the determination of DHQ in Douglas-fir wood.

Experimental and results

Douglas-fir wood was ground (—40 mesh), freeze dried and 1–5 mg of it and 150 μl of 40–60° petroleum ether were added to a 1 ml tube. After the mixture had stood overnight, the petroleum ether was drawn off with a syringe and the wood quickly vacuum dried. The wood was then extracted with 50 μl of a pyridine solution containing 0.2 mg/ml of phloretin (2',4, 4',6'-tetrahydroxy dihydrochalcone) to serve as an internal standard. The pyridine extraction was allowed to continue for 8–12 h in a desiccator over P_2O_5. (Precautions were taken to insure that the pyridine was dry and that the sample was not exposed to moisture during the extraction procedure.) The stirred extract (10 μl) was transferred to a 1 ml tube with a dry syringe and BSA (20 μl) added. The tube was quickly capped and stored over P_2O_5 for 1–2 h at room temperature and 3 μl were then injected into each of two columns of a Varian 2100 Gas Chromatograph. From each wood sample two 10 μl extract samples were treated as described above giving a total of four chromatograms.

The chromatograph oven was set to 195° and programmed so that the temperature rose 1°/min to 215° and then remained constant. The injection port and detector temperatures were 250°. Columns were 6 ft. long glass U tubes 3 mm I.D. packed with 2 % SE-30 on acid washed, DMCS treated Chromosorb W (80–100 mesh). The nitrogen carrier gas flow rate was 40 ml/min while hydrogen and air flows were 25 and 250 ml per min respectively.

TMS-DHQ was well separated from TMS-dihydrokaempferol under the above conditions. Retention times relative to TMS-DHQ for TMS-pinobanksin and TMS-dihydrokaempferol were 0.55 and 0.88 respectively.

The calibration curve was prepared by plotting DHQ concentration in pyridine against the ratio peak areas (TMS-DHQ/TMS-phloretin). Peak areas were measured by weighing cut out peaks from copies of the chromatograms.

The extraction procedure was checked for completeness in several ways. Extraction time periods from 12 h to 4 days did not result in an increased yield of DHQ. Neither did intermittent (3 min on, 12 min off) ultrasonic vibration over a period of 12 h.

As a further check, ground wood (5 g) was extracted for 8 h with 40–60° petroleum ether followed by methanol for 8 h. The methanol was evaporated *in vacuo*, and the extract dissolved in an appropriate amount of pyridine solvent. Samples (10 μl) of this extract, together with BSA (20 μl), were added to a 1 ml tube. The materials were reacted for 1–2 h at room temperature over P_2O_5 and then examined by GLC. Matched samples of ground wood (1–5 mg) were examined by the micromethod as described

above. The micro extraction gave an average of 0.99 % DHQ while the conventional alcohol extraction gave an average of 0.95 % DHQ.

The silylation reaction was tested in two ways. Silylations with hexamethyldisilazane and trimethylchlorosilane in pyridine were compared with the BSA silylation and gave identical results. The amount of DHQ in a methanol extract of 5 g of wood was determined by the PC method of SQUIRE *et al.*[3]. The results were compared with the amount of DHQ obtained by the micro GLC method from matched 2 mg ground wood samples. The two methods gave a similar content of DHQ in the wood, *i.e.*, GLC gave 0.95 % while PC gave 0.87%.

The GLC method showed a coefficient of variation of 4–6 % while PC resulted in a much higher variation of about 12–15%. The microdetermination by PC of SQUIRE *et al.* allows only one estimation from each wood sample while 4 determinations are easily obtained with the GLC method. The distribution of sample means at the 95 % confidence level for the GLC method is then about 6 % while that of the PC method is about 30%. Since the reacted extract sample is diluted to 30 μl and only 3 μl are used for each chromatogram additional GLC chromatograms are easily obtained.

An advantage of the GLC method is the large range in which the calibration curve is linear (from at least 0.1 mg/ml to 2.0 mg/ml). The lower limit and linear range could undoubtedly be extended. However, present use of this method has not demanded greater sensitivity. The PC method results in a curvilinear calibration curve and when applying 10 μl spots the sensitive portion of the curve is between 1 and 3 mg/ml.

In terms of a readable signal for absolute quantities of DHQ the PC method requires about 5×10^{-6} g while the GLC method as presently used requires about 3×10^{-7} g. Reducing the amount of BSA added from 20 μl to 5 μl would halve the lower limit and still provide a large excess of the BSA reagent. It would appear that acceptable analyses could be made at more dilute DHQ conditions. The GLC method is thus at least 30 times more sensitive than the PC method. Assuming a DHQ concentration of 1 % in Douglas-fir heartwood, the minimum wood sample size would appear to be about 3×10^{-5} g under present use of the method.

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Receive May 9 1969

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