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Structure elucidation of saccharides in anthocyanins and flavonols by means of methylation analysis and gas chromatography

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ABSTRACT

The monosaccharide composition of anthocyanins and flavonols was elucidated by capillary gas chromatography after derivatization to the corresponding alditol acetates. Methylation analysis using gas chromatography revealed the types of linkages in these saccharides. Three anthocyanins isolated from carrot cell suspension cultures were shown to contain galactose, xylose and glucose in equimolar amounts, forming a branched triglycoside. Therefore, the structures of these carrot anthocyanins could be identified as cyanidin 3-(6^G-glucosyllathyroside) and the same triglycoside acylated with sinapic or ferulic acid.

INTRODUCTION

The carbohydrate part of flavonoids is usually investigated by using paper (PC) or thin-layer chromatography (TLC) after acid or enzymic hydrolysis or after peroxide degradation of the aglycone [1,2]. Sugar analyses using gas chromatography (GC) require the conversion of the sugars into volatile derivatives. Two techniques for derivatization have been reported: trimethylsilylation of the sugars [3] and reduction of the sugars to their alditols followed by peracetylation to form the alditol acetates [4,5]. In order to reveal the monosaccharide composition of anthocyanidin and flavonol glycosides, we applied a slightly different procedure for alditol acetate formation [6] and separated the resulting derivatives using capillary GC.

Additional information about the linkages between the sugars was obtained by methylation analysis, a technique commonly used in the structure elucidation of proteoglycans, polysaccharides and oligosaccharides [6–9]. The carbon atoms involved in glycosidic bonds could be identified by methylation of the sugars prior to hydrolysis and subsequent reduction, acetylation and GC.

These GC techniques were used to examine the glycoside component of three anthocyanins isolated from carrot cell suspension cultures. The structure of the major pigment of these cells has been reported to be cyanidin 3-(sinapoylxylosylglucosylgalactoside) [10,11] with a linear triglycoside chain [12]. Recently, the question arose whether the trisaccharide of the pigment could be branched through the galactose moiety with the acyl group located on the glucose residue [13,14]. Methylation analysis provided a suitable means of deciding between these two structure proposals by determining the positions of the linkages.

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EXPERIMENTAL

Chemicals

Cyanidin 3-glucoside was a gift from Professor J. B. Harborne, University of Reading, UK; cyanin, hyperoside, quercitrin and rutin were purchased from Roth (Karlsruhe, Germany). Hyperoside and quercitrin were purified before use by TLC on cellulose (20 × 20 cm plates, 0.1-mm layer; Merck, Darmstadt, Germany) with chloroform–acetic acid–water (50:45:5) as solvent. The compounds were detected under UV light (354 nm, R_F = 0.35 for hyperoside and 0.32 for quercitrin) and eluted with methanol. Amberlite IR-120 was obtained from Fluka (Buchs, Switzerland).

Dimethylsulphinyl carbanion (potassium salt) was synthesized from potassium hydride and dimethyl sulphoxide (DMSO) using the method of Harris *et al.* [6] with minor modifications [15]. Partially methylated alditol acetate (PMAA) standards were prepared according to York *et al.* [8] and identified by gas chromatography–mass spectrometry (GC–MS) [15].

Isolation of anthocyanins

Anthocyanins were isolated from suspension-cultured cells of an Afghan cultivar of *Daucus carota* L. [16] maintained as described previously [17]. Cells were extracted with methanol–acetic acid–water (50:8:42) using a mortar and pestle. After washing with ethyl acetate, anthocyanins were purified by repeated PC in the descending mode on Whatman 3MM paper with *n*-butanol–acetic acid–water (4:1:5, upper phase, overrun) and with 15% acetic acid as solvents, followed by column chromatography on Sephadex LH-20 (17 × 2 cm I.D., gravity flow) using 8% acetic acid as the eluent. The purity of the samples was monitored at 530 nm by reversed-phase high-performance liquid chromatography (HPLC) on a Hypersil ODS (5 μm) column (250 × 5 mm I.D.) (Grom, Herrenberg, Germany) using a gradient with 10% formic acid in water (solvent A) and in methanol (solvent B), with solvent B increasing from 10% to 13% within 10 min, to 20% during 10–20 min, to 40% during 20–25 min and to 98% during 25–30 min at a flow-rate of 1.0 ml/min. The major compound (1) proved to be identical with the substance characterized by Harborne and co-workers [10,13] by co-chromatography with HPLC and TLC on cellulose with three solvents (Table I).

Deacylation of anthocyanins and identification of acyl residues

Alkaline hydrolysis was carried out according to Albach *et al.* [18] with 10% potassium hydroxide solution for 4 h in the dark at room temperature. The hydrolysate was acidified with prewashed Amberlite IR-120 (H^+ form). The liberated hydroxycinnamic acids were extracted with diethyl ether and identified (Table I) by HPLC on a Hypersil ODS (5 μm) column (250 × 5 mm I.D.) (Grom) using a linear gradient with 5% acetic acid and methanol as solvents (from 10 to 40% methanol in 20 min at a flow-rate of 1.5 ml/min, detected at 280 nm). Caffeic acid (elution time 7.77 min), *p*-coumaric acid (11.79 min), ferulic acid (13.66 min), sinapic acid (14.36 min) and *p*-hydroxybenzoic acid (6.03 min) were used as standards. The aqueous phase containing the deacylated pigments was also subjected to sugar analysis.

TABLE I

CHROMATOGRAPHIC DATA FOR THREE CARROT ANTHOCYANINS FROM HPLC AND TLC

For HPLC, see Experimental. TLC on cellulose (0.1 mm, 20 × 20 cm) (Merck). Solvent 1 = *n*-butanol–acetic acid–water (4:1:5, upper phase); solvent 2 = 1% hydrochloric acid; solvent 3 = acetic acid–hydrochloric acid–water (15:3:82).

Anthocyanin	Elution time in HPLC (min)	R_F in TLC			Acyl group
		Solvent 1	Solvent 2	Solvent 3	
1	22.38	17.5	15	28.5	Sinapic acid
2	24.36	21	15	31	Ferulic acid
3	17.15	14	32	58	–

Peroxide degradation

Degradation of the aglycone of the purified anthocyanins was performed by treatment with 2 *M* ammonia solution and 30% hydrogen peroxide as described by Markham [3]. The remaining carbohydrate parts of the anthocyanins were derivatized for GC analysis.

Monosaccharide analysis

Samples containing 50–500 μg of flavonoid standard or purified anthocyanin were dried in a stream of nitrogen and dissolved in 490 μl of water. After the addition of 50 μg of *myo*-inositol as internal standard, the samples were brought to 2 *M* trifluoroacetic acid (TFA) and hydrolysed at 120°C for 60 min. The liberated aglycones could be extracted from the hydrolysate with ethyl acetate and identified by TLC or HPLC. The TFA was then removed by evaporation with nitrogen and the monosaccharides were reduced and acetylated according to Harris *et al.* [6] to obtain volatile alditol acetates. Aromatic acyl groups are probably detached from the saccharides during this procedure. The alditol acetates were extracted with 1.5 ml of dichloromethane and the organic phase was washed four times with water. Then the dichloromethane was removed in a stream of filtered air and the dry samples were stored at −18°C. For GC analysis, the samples were dissolved in 50 μl of dichloromethane.

GC was performed on a Shimadzu GC-9A instrument equipped with a flame ionization detector (275°C). Separation of the alditol acetates was carried out on an SP-2340 capillary column (30 m × 0.25 mm I.D.) isothermally at 235°C. The carrier gas was nitrogen at an inlet pressure of 4.5 bar. Volumes of 1.0–1.5 μl of the samples were injected into a glass inlet at 275°C with a splitting ratio of 10:1. The alditol acetates were identified by comparing their relative retention times with respect to *myo*-inositol hexaacetate with those of known standards. Quantification was based on relative response factors obtained by the analysis of standard mixtures of monosaccharides.

Methylation analysis

A modification of the procedure described by Harris *et al.* [6] was used [15]. Rutin in different amounts ranging from 0.2 to 0.8 mg or 0.2–2 mg of purified anthocyanins were dried carefully in a Speed Vac and taken up in 0.2 ml of dry DMSO. A 0.2-ml volume of dimethylsulphinyl carbanion (potassium salt) was added under argon and the samples were stirred at ambient temperature for at least 60 min. During this deprotonation [7], aromatic acyl groups are likely to be removed from the anthocyanins by alkaline hydrolysis. The samples were cooled on ice and 150 μl of methyl iodide were added to the frozen samples. After thawing and mixing, the methylation reaction was allowed to proceed for at least 1 h or overnight. Subsequently, 3 ml of chloroform–methanol (2:1, v/v) were added and the organic phase was washed four times with 2 ml of water. The methylated samples were then dried under a stream of filtered air at 40°C. A 50-μg amount of *myo*-inositol was added as an internal standard and the permethylated saccharides were hydrolysed in 2 *M* TFA. This procedure also removes the aglycones from the saccharide part. The partially methylated monosaccharides were then reduced and acetylated as described above for the monosaccharide analysis.

GC was performed on a Shimadzu GC-9A instrument equipped with a DB-1701 capillary column (30 m × 0.25 mm I.D., 0.25-μm film thickness) with temperature programming from 180 to 240°C at 2.5°C/min. The carrier gas, injector and detector were as described above. The peaks were identified by comparing their relative retention times with those of PMAA standards analysed by GC–MS. Cellobiose was used as the external standard. Quantitative results were obtained by using the relative response factors provided by Sweet *et al.* [19].

RESULTS AND DISCUSSION

Monosaccharide composition

The derivatization of the sugar residues to their alditol acetates produces volatile derivatives suitable for GC analysis [4]. A mixture of the alditol acetates derived from eight standard sugars and the internal standard *myo*-inositol was well separated by capillary GC (Fig. 1a). The alditol acetates could also be chromatographed with the column and GC programme used for methylation analysis.

The investigation of the flavonoid standards quercitrin (= quercetin 3-rhamnoside), hyperoside (= quercetin 3-galactoside), rutin (= quercetin 3-rhamnosylglucoside), cyanin (= cyanidin 3,5-di-

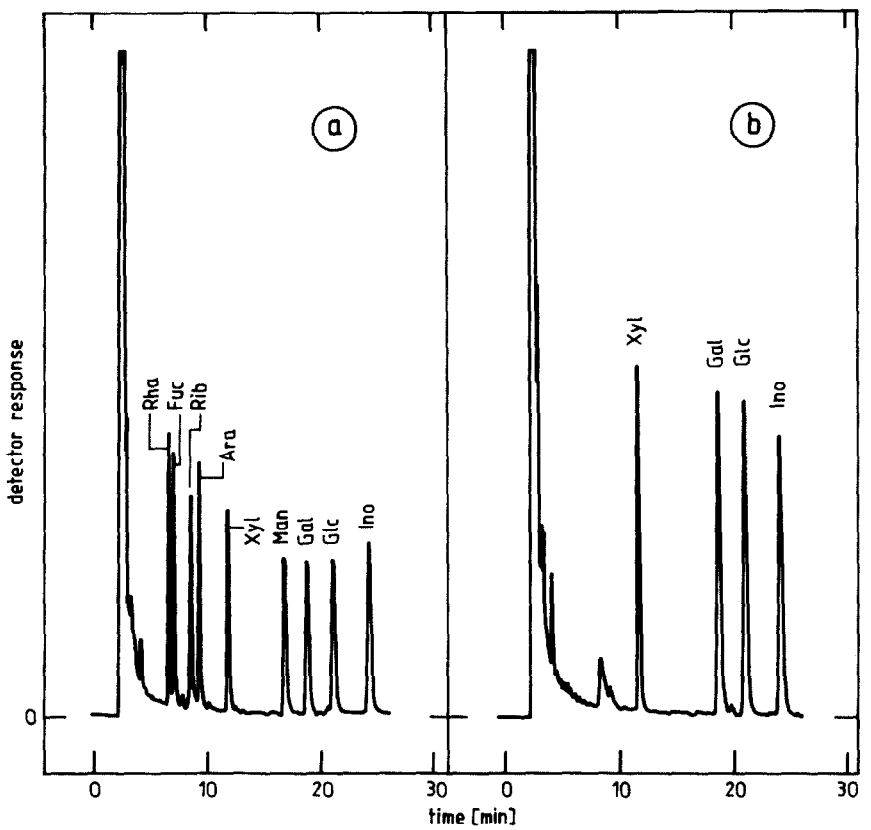

Fig. 1. GC separation of alditol acetates on a Shimadzu GC-9A instrument equipped with an SP-2340 capillary column (30 m × 0.25 mm I.D.) run isothermally at 235°C with flame ionization detection. Derivatization of the samples was carried out as described under Experimental. (a) Standard mixture containing alditol acetates derived from Rha = rhamnose, Fuc = fucose, Rib = ribose, Ara = arabinose, Xyl = xylose, Man = mannose, Gal = galactose, Glc = glucose and Ino = *myo*-inositol. (b) Alditol acetates derived from the purified major anthocyanin from carrot cell suspension cultures with *myo*-inositol as internal standard.

glucoside) and cyanidin 3-glucoside yielded the expected monosaccharide derivatives (data not shown). In the chromatograms for quercitrin and hyperoside, some impurities were present: traces of glucose could be identified, probably originating from the cellulose TLC plates. The flavonoid aglycones were split off during acid hydrolysis and could be extracted with ethyl acetate and identified by TLC or HPLC.

Capillary GC of the alditol acetates derived from the major anthocyanin of carrot cell cultures after TFA hydrolysis and derivatization yielded three peaks (Fig. 1b) which could be clearly identified as xylitol pentaacetate, galactitol hexaacetate and glucitol hexaacetate by comparison with the standard mixture (Fig. 1a). Hence the carbohydrate component of the pigment is composed of xylose, glucose and galactose in equimolar amounts. These results confirm earlier findings by Harborne *et al.* [10] and Hopp and Seitz [11] obtained with PC and TLC. Two further carrot anthocyanins proved to have the same monosaccharide composition. Hemingson and Collins [20] tried to determine the monosaccharides in carrot anthocyanins using a similar method, but had problems with extraneous sugars probably originating from the chromatography paper. Their identification of two mono- and two diglycosides is obviously connected with degradation products [10].

The monosaccharide composition of samples containing as little as 50 μg of purified pigment could be unambiguously determined with the outlined proce-

dure. In contrast, GC analysis of sugars after trimethylsilylation requires about 250–500 μg [3]. Despite some limitations (difficulties with ketoses and some sugar pairs have been discussed [5]), this derivatization technique has proved to be a useful method for identifying most of the monosaccharides naturally occurring in anthocyanins and other flavonoids.

Methylation analysis

The PMAA obtained by methylation of the free hydroxyl groups, hydrolysis of the glycosidic bonds and subsequent acetylation were separated by capillary GC and identified by comparison with PMAA standards and the external standard cellobiose [15]. Cellobiose yielded two peaks originating from a terminal glucose residue bound through C-1 and a glucose bound through C-4. Rutin exhibited one peak clearly representing a 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol and a peak that could not be distinguished from a terminal rhamnose (2,3,4-tri-O-methylrhamnositol). These two peaks corresponded to the expected derivatives which were present in equimolar amounts (data not shown). A series with different quantities of rutin from 200 to 800 μg was analysed, revealing that 200 μg were sufficient for detection. Thus, methylation analysis requires much smaller amounts of analyte than ^{13}C NMR studies necessary for structure elucidation with spectroscopic methods.

Methylation analysis of the glycoside part of the major carrot anthocyanin yielded three PMAA peaks separated with capillary GC (Fig. 2). Their relative retention times (RRT) compared with the internal standard were 0.330 (peak 1), 0.455 (peak 2) and 0.835 (peak 3). Peak 3 corresponded well to 1,2,5,6-tetra-O-acetyl-3,4-di-O-methylgalactitol (RRT 0.838) and definitely not to a tri-O-acetyltri-O-methylgalactitol (RRT 0.586–0.687) [15]. This unequivocally proved that the galactose residue is branched at the 2- and 6-positions, as had been postulated from fast atom bombardment (FAB) MS data [21]. Peak 1 represented 1,5-di-O-acetyl-2,3,4-tri-O-methylxylitol and peak 2 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol originating from terminal xylopyranose and glucopyranose, respectively. Chromatograms of the external standard cellobiose confirmed the peak identification. The three peaks represented equimolar amounts as calculated

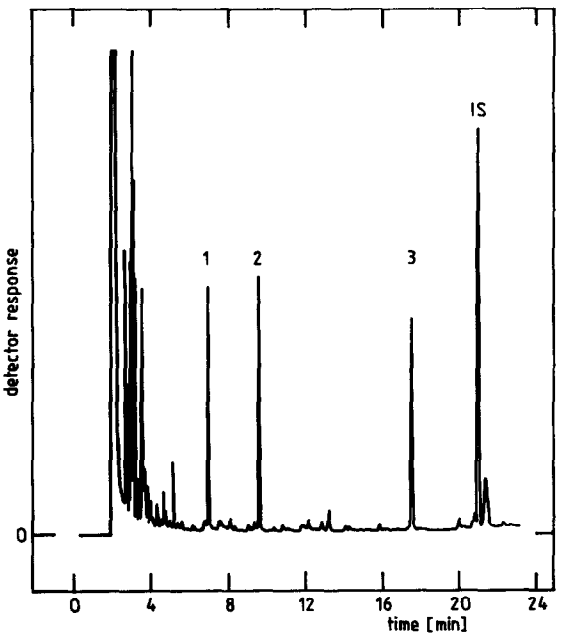

Fig. 2. GC analysis of partially methylated alditol acetates prepared from the purified major anthocyanin component of carrot cell cultures. GC was performed on a Shimadzu GC-9A instrument equipped with a DB-1701 capillary column (30 m × 0.25 mm I.D., 0.25-μm film thickness) with a temperature gradient from 180 to 240°C at 2.5°C/min and flame ionization detection. IS = Internal standard (*myo*-inositol).

from the relative response factors [19]. Hence the results on the monosaccharide composition are corroborated by methylation analysis.

The occurrence of nine methylated hydroxyl groups on derivatization of the trisaccharide indicated that the acyl group must have been removed prior to methylation by the alkali treatment with dimethylsulphinyl carbanion. The attachment site of the acyl group therefore cannot be determined with this technique. Attempts to evaluate the position of this linkage by GC analysis of methylated samples of anthocyanins [18,22,23] were hampered in each instance by the removal of the acyl group during methylation. Considering the detection of sinapoylglucose fragments with FAB-MS [21], the acyl group must be attached to the glucose moiety. On the basis of indirect evidence, the glucose–galactose link has been concluded to be β (1–6) [12]. As the acyl group was clearly identified to be sinapic acid by HPLC

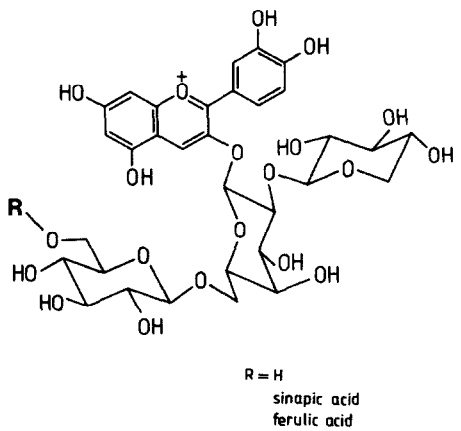

Fig. 3. Structures of three anthocyanins from carrot cell suspension cultures.

(data not shown), the structure of the major anthocyanin from carrot cell suspension cultures is cyanidin 3-(6^G-glucosyllathyroside), *i.e.*, cyanidin 3-O-(2″-xylopyranosyl-6″-glucopyranosylgalactopyranoside), acylated with sinapic acid. This conclusion is consistent with NMR data obtained recently [24]. Another two anthocyanins derived from the same cell culture were analysed. They exhibited the same carbohydrate component but contained ferulic acid or had no acyl residue (Fig. 3).

A comparable methylation procedure with methyl iodide in dimethylformamide in the presence of silver oxide has been described for the examination of acylated betacyanins [23]. The identification of partially methylated sugars by PC and TLC made possible the discovery of a branched trisaccharide in the betacyanins of *Bougainvillea glabra* [25].

Deacylation of the anthocyanins with potassium hydroxide prior to derivatization had no substantial effect on either the methylation data or on the monosaccharide analysis. Since peroxide degradation of the cyanidin aglycone did not improve the GC profiles either, we suggest that both derivatization procedures are carried out without additional pretreatment. The advantage of the outlined GC analyses is their sensitivity and the option of combining them with detection by mass spectrometry. The monosaccharide composition can be elucidated with only 25 μg of carbohydrate. Complete methylation allows reliable determinations of the interglycosidic linkages (except the α or β configuration at the anomeric carbon atoms) and terminal sugars with samples containing as little as 200 μg of purified pigment. Methylation analysis is especially suitable for investigations of complex glycosides, *e.g.*, for branched saccharides in anthocyanins and other flavonoids.

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