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Fractionation and identification of the phenolic compounds of Highbush blueberries (*Vaccinium corymbosum*, L.)

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A scheme for the fractionation of flavonoid and nonflavonoid compounds is presented. The phenolic compounds of Highbush blueberries (cultivar 'Coville') were analysed by high performance liquid chromatography and thin-layer chromatography. Fifteen anthocyanins were identified as the 3-monoglucoside, 3-monogalactoside and 3-monoarabinoside of delphinidin, cyanidin, malvidin, peonidin and petunidin. No acyl anthocyanin was detected. Derivatives of malvidin and delphinidin were the most abundant; the 3-monogalactoside constituted 41% of the anthocyanin. Four flavonol glycosides were also identified as the kaempferol-3-O-glucoside, 3-O-glucoside, 3-O-galactoside and 3-O-rhamnoside of quercetin. The major phenolic acid was chlorogenic acid. After hydrolysis of the phenolic neutral fraction (FA), gallic, syringic and vanillic acids were identified by TLC. These acids appeared to be present in their ester forms, probably as glucoside esters.

INTRODUCTION

Blueberries (*Vaccinium corymbosum*, L.) are extensively cultivated in the United States and more recently have become a popular commercial crop in Europe. In this paper the cultivar 'Coville', which is cultivated in the north-east of France (Vosges), was investigated. The chemical composition of these fruits has been studied by Kader *et al.* (1993, 1994).

Blueberry plantings remain productive for many years (Draper & Hancock, 1990) and can be grown on poor soils if they are properly irrigated, mulched and acidified (Hancock, 1989). Highbush blueberries are characterised by a large fruit with intensive colour (dark-blue), firmness and a pleasant acid flavour (Ballington *et al.*, 1982). Colour is an important factor in the influence of fresh-market value and the suitability of the berries for processing.

The composition and the distribution of anthocyanins in blueberries are affected by genetic factors and environmental conditions (Sapers *et al.*, 1984). Recently, other areas such as chemotaxonomy have been exploited for the classification of *Vaccinium* (Ballington *et al.*, 1987). The phenolic and flavonol glycoside profiles may be useful to taxonomists for characterising a fruit or a cultivar (McRae *et al.*, 1990). Some of these compounds also have important pharmacological properties and are used for therapeutic purposes.

Fifteen anthocyanins have been identified in blueberries cultivated in North America (Ballington *et al.*, 1982; Sapers *et al.*, 1984; Ballington *et al.*, 1987; Macheix *et al.*, 1990; Mazza & Miniati, 1993). These anthocyanins are the 3-monoarabinosides, 3-monogalactosides and 3-monoglucosides of cyanidin, delphinidin, malvidin, peonidin and petunidin. However, there has been no report on the anthocyanin content of Highbush blueberries cultivated in Europe. A knowledge of the anthocyanin profile of the French and the American blueberry would elucidate differences in their formation patterns; their genetic expression is modulated by the environment.

In addition to the detection of the flavonol aglycone (quercetin) in Highbush blueberry fruit (Bilyk & Sapers, 1986), the phenolic compounds have been studied by Schuster & Herrmann (1985). However, the flavonol glycosides remain largely unknown.

The main objective of this work is to isolate and identify phenolic compounds, including flavonoids. A fractionation scheme for these compounds and their analysis by HPLC and TLC will be presented.

It is anticipated that the flavonoid profiles could be used for the characterisation of French blueberry plantings.

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MATERIALS AND METHODS

Fruit sampling and storage

Ripe blueberries of the 'Coville' variety were harvested during August 1991 in a commercial planting in north-eastern France (Vosges) (poor soils, properly irrigated and mulched). Sample ripeness was judged on the basis of skin colour of representative berries. After picking, the berries were deep-frozen and stored at − 30°C.

Anthocyanin analysis

Extraction of anthocyanins

The skins, containing all anthocyanins, were removed from 75 g of frozen berries and twice-extracted by maceration with 100 ml of methanol for 2 h at room temperature. Fractions were pooled and then suction-filtered through a Whatman No.1 filter paper.

The extract was concentrated in a rotary evaporator under vacuum at 25°C. The concentrate (2.0 ml) was stored at 4°C under nitrogen.

HPLC of blueberry anthocyanins

20 μl of the anthocyanin extract was analysed by an HPLC (Merck Hitachi L-6200 intelligent pump equipped with a photodiode array detector Merck Hitachi L-3000) with a Lichrosorb 100 RP-18 reversed-phase column (250 x 4 mm, 5 μm) using a mobile phase consisting of water/formic acid (90:10,v/v) (solvent A) and water/acetonitrile/methanol/formic acid (40:22.5:22.5:10, v/v) (solvent B).

Elution was performed at a flow rate of 1.0 ml/min with the gradient shown in Table 1. Detection at 520 nm.

All separations were performed at 22°C and all solvents were HPLC grade.

Phenolic and flavonol glycosides compounds analysis (Fig. 1)

Juice preparation

Frozen blueberries (250 g) were blended with 200 ml of distilled water in a Braun Mx 32 blender at 4°C. The resulting mixture was centrifuged at 3000 × *g* for 20 min at 4°C. The supernatant solution was filtered through a porous glass filter (90 –150 μm) and then stored at 4°C for 24 h to allow the pectins time to gel. The solution was centrifuged twice for 15 min, at 4°C and 3000 × *g*. The supernatant was used for the flavonoids extraction.

Table 1. Elution gradient of anthocyanins

Time (min)	% A	% B
0	80	20
2.0	80	20
15.0	75	25
60.0	60	40
80.0	20	80

Extraction

The blueberry juice (200 ml) was acidified to pH 1.5 with 2 M HCl. Phenolic compounds were extracted 4 times (v/v) with ethyl acetate after the addition of sodium bisulfite (0.1%, w/v). The sodium bisulfite discolours any anthocyanin monomers occurring in the juice and keeps them in the aqueous phase. The ethyl acetate extract was dehydrated with anhydrous Na_2SO_4 and then dried in a rotary evaporator under vacuum at 30°C until all the ethyl acetate had been removed. The residue was dissolved in 10 ml of 5% (w/v) aqueous Na_2CO_3, pH 8. Neutral compounds were extracted four times from this Na_2CO_3 solution by ethyl acetate (v/v). This organic phase was removed under reduced pressure at 30°C. The dried product, FA, was dissolved in methanol (5.0 ml) and stored at 4°C under nitrogen.

The resulting aqueous phase (Na_2CO_3 solution) was acidified to pH 1.5 with 2 M HCl and the phenolic acids were extracted 4 times with ethyl acetate (v/v). This organic phase was removed under reduced pressure at 30°C. The residual product FB was dissolved in methanol (5.0 ml) and stored at 4°C under nitrogen.

TLC analysis

Fraction FA was chromatographed comparatively to standard phenolic compounds on a cellulose plate (Merck, ref. 5552) with a water–acetic acid (95:5, v/v) system. The phenolic compounds were located by spraying chromatograms with the diazotised *p*-nitroaniline and then with 15% in water (w/v) sodium bicarbonate (Van Sumere *et al.*, 1965).

Acid hydrolysis of the fraction FA

The flavonol glycosides occurring in the fraction FA were hydrolysed by heating at 100°C in 2 M HCl–methanol (v/v) for 1 h. Aglycone moieties (FA1) were extracted by ethyl acetate (v/v) and identified by TLC as described by Bilyk & Sapers (1985), on a silica gel plate (Merck, ref. 5553) in the presence of standard compounds with benzene/pyridine/formic acid (65:25:10, v/v). Aglycones were detected with Neu reagent [1% (w/v) diphenyl-boric acid–ethanolamine solution in methanol] (Neu, 1957) and under UV light. The aqueous phase (FA2) was chromatographed to identify sugar moieties on silica gel plates (Merck, ref. 5553) with a propan-2-ol/ethyl acetate/water (50:40:10, v/v) system. Sugars were located on TLC plates by spraying with 2% (w/v) naphthoresorcinol solution in acetone and 9% phosphoric acid (5:1, v/v) followed by heating for 10 min in an oven at 105°C.

Alkaline hydrolysis of the fraction FB

Alkaline hydrolysis was carried out at room temperature (22°C) in 2 M KOH under nitrogen for 2 h and stopped by acidification to pH 1.5 with 4 M HCl.

Phenolic acids (FB1) were then extracted by ethyl acetate and identified by TLC on cellulose plates with the following solvent system: water–acetic acid (95:5, v/v).

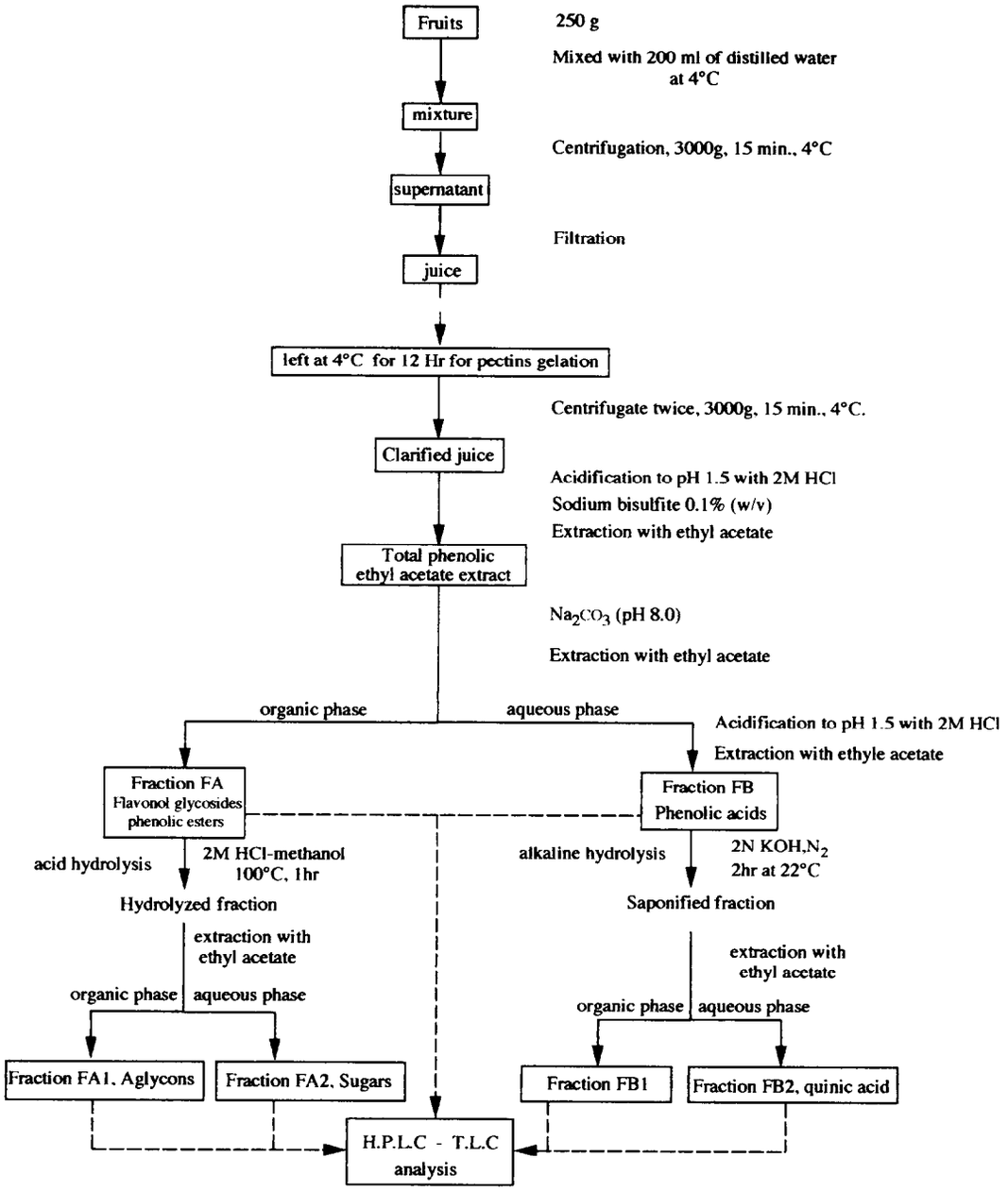

Fig. 1. Scheme for the fractionation of Highbush blueberry juice for analytical investigations.

Detection was carried out as previously described for fraction FB.

The aqueous phase (FB2) was chromatographed to identify the quinic acid released from chlorogenic acid on cellulose plates with propan-1-ol–acetic acid–water (80:2:18, v/v) system and this was located on a TLC plate with green bromocresol reagent (0.04%, w/v) in ethanol (96%); the colour of the solution was adjusted to blue with NaOH (0.1 N).

HPLC analysis

FB and FB1 were analysed by HPLC under the same conditions as the anthocyanins except that the mobile phase was water–acetic acid (98:2, v/v—solvent A) and water–acetic acid acetonitrile (88:2:10, v/v—solvent B). Elution was performed at a flow rate of 1.0 ml/min using a gradient starting with 2% solvent B and increasing to levels of 12% at 15 min, 25% at 25 min and 50% at 40 min. Detection was achieved at 280 and 330 nm.

The mobile phase for FA (flavonol glycosides) analysis consisted of water–acetic acid (97.5:2.5, v/v—solvent A) and tetrahydrofuran (solvent B) with a gradient of 100% solvent A to 50% of A in 40 min at a flow rate of 0.6 ml/min. 30 μl of the flavonol glycosides extract was injected and the elution of compounds was monitored at 365 nm.

For FA1 analysis (aglycones), the mobile phase was water–formic acid (95:5, v/v—solvent A) and methanol (solvent B), with a gradient of 80% to 30% of solvent A in 10 min and then isocratically with a flow rate of 1.2 ml/min. Detection was achieved at 280 nm.

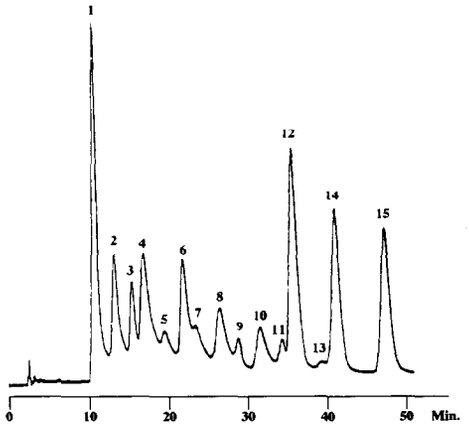

Fig. 2. Chromatogram of analytical HPLC of Highbush blueberry anthocyanins (cv. 'Coville').

RESULTS AND DISCUSSION

Anthocyanins

The anthocyanin profile which was obtained from the HPLC is presented in Fig. 2. The profile shows that the solvent program gave a convenient separation. Standards of anthocyanins available from previous work (Ballinger *et al.*, 1970; Ballinger *et al.*, 1972; Sapers *et al.*, 1984; Ballington *et al.*, 1987; Ballington *et al.*, 1988) were used to identify peaks 1–15 in Fig. 2, and 15 anthocyanins were identified (Table 2). These were the 3-monoarabinosides, 3-monogalactosides and 3-monoglucosides of cyanidin, delphinidin, petunidin and peonidin. No acyl anthocyanin was detected in the extract by HPLC analysis.

The availability of components, in order of decreasing amounts, was delphinidin-3-monogalactoside (13.5%), malvidin-3-monogalactoside (12.9%), malvidin-3-monoglucoside (11.9%), malvidin-3-monoarabinoside (11.9%) and delphinidin-3-monoarabinoside (10.3%) (Table 2). Small amounts (0.1–2.2%) of 3-monoarabinoside of peonidin and cyanidin, peonidin-3-monoglucoside and 3-monogalactoside were also present.

Table 2. Anthocyanin composition of Highbush blueberry cv. 'Coville'

Peaks[a]	Anthocyanins	R_t (min)	(%)
1	Delphinidin-3-monogalactoside	10.6	13.5
2	Delphinidin-3-monoglucoside	13.4	6.9
3	Cyanidin-3-monogalactoside	15.8	5.0
4	Delphinidin-3-monoarabinoside	17.3	10.3
5	Cyanidin-3-monoglucoside	18.4	5.0
6	Petunidin-3-monogalactoside	22.4	8.8
7	Cyanidin-3-monoarabinoside	24.6	2.2
8	Petunidin-3-monoglucoside	27.2	6.6
9	Peonidin-3-monogalactoside	29.6	0.7
10	Petunidin-3-monoarabinoside	32.3	3.1
11	Peonidin-3-monoglucoside	35.2	1.1
12	Malvidin-3-monogalactoside	36.5	12.9
13	Peonidin-3-monoarabinoside	43.3	0.1
14	Malvidin-3-monoglucoside	42.1	11.9
15	Malvidin-3-monoarabinoside	48.6	11.9
Derivatives	Glucosides		31.5
	Galactosides		40.9
	Arabinosides		27.6

[a]Refer to peak number in Fig.2.

Derivatives of malvidin and delphinidin accounted for 37 and 31% of the total anthocyanin, respectively. The 3-monogalactoside derivatives constituted about 41% of the anthocyanin content of ripe fruits of the 'Coville' blueberry.

Saper *et al.* (1984) have studied the anthocyanin profiles of some blueberry cultivars including 'Coville' cultivated in North America. In this cultivar (Coville), they identified only 10 anthocyanins. The differences could be attributed to difference in ripeness.

Indeed, for most blueberry cultivars, ripening is associated with the development of anthocyanins in the epidermal and subepidermal cells of the berry (Ballinger *et al.*, 1972). Woodruff *et al.* (1960) followed the chemical changes in blueberry fruit with ripening and found that it required 16 days for the completion of the ripening process but the anthocyanin pigmentation increased only in the first 6 days. Therefore, the anthocyanin colour of blueberries appears to be a variable criterion of ripeness which is influenced both by heredity and the environment (Ballinger *et al.*, 1972).

Nonflavonoid phenolics

The main problem, in HPLC analysis of nonflavonoid phenolics from juice, is the large amounts of sugars and pectins. They create difficulty in the extraction and preparation of samples. In this work, ethyl acetate was used for the removal of sugars and polar compounds from fruit extracts, to obtain purified phenolic fractions. This technique was reported by Azar *et al.* (1987).

The different compounds were characterised by comparing the retention times with standards from Fluka. TLC analysis was used, *a priori*, for identification of compounds by comparing Rf values and colours given with those of the standards.

Chlorogenic acid (R_t 17.60 min) was the major cinnamic derivative found in blueberries. Alkaline hydrolysis released caffeic acid (fraction FB1, R_t 18.72 min) which was identified by chromatographic comparisons (TLC, HPLC analysis) with standards. Quinic acid (fraction FB2) was identified by TLC analysis. In blueberries (cv. Bluecrop and Heerna) Schuster & Herrmann (1985) have reported that chlorogenic acid is the main phenolic compound.

The fraction FA1 obtained after acidic hydrolysis of the fraction FA, was analysed by TLC with a specific solvent for phenolic compounds. After spraying the developed chromatogram we identified three phenolic acids: gallic acid, syringic acid and vanillic acid. These compounds were better identified by TLC due to their chromogenic characteristics after spraying with PAN reagent or observation under UV light.

All these compounds have carboxylic functions, and should have been obtained in the fraction FB. Hence the presence of these compounds in the fraction FA1

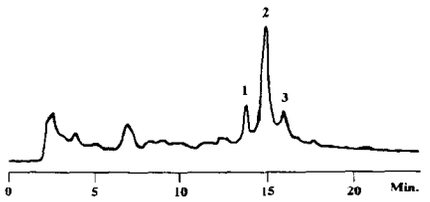

Fig. 3. HPLC analysis of the fraction FA1 (flavonol aglycons) obtained after acid hydrolysis of the fraction FA. (1) Oxidised product; (2) Quercetin 14.9 min; (3) Kaempferol 15.9 min.

signifies that they are esterified. The compounds occurred in the blueberry juice in their ester forms (glucoside esters).

Flavonoid glycosides

The flavonoid aglycones (fraction FA1) recovered from hydrolysis of the fraction FA were identified by comparing their R_f values with standards.

Quercetin and kaempferol were detected in the fraction FA1 by HPLC analysis (Fig. 3). Gradient elution was required for satisfactory resolution of the flavonol aglycones. The analysis was completed in 15 min with a column temperature of 22°C. Peaks 2 and 3 (Fig. 3) have been identified as quercetin and kaempferol, respectively. Peak 1 was collected and chromatographed on a TLC silica gel plate. After spraying (Neu reagent), no aglycone was detected even under UV light.

In their study of flavonoid aglycones from Highbush blueberries, Bilyk & Sapers (1986) detected only quercetin but not kaempferol after acid hydrolysis. The different origins of the blueberry cultivars may explain the difference between our results and those obtained by these authors, but other factors may be important such as the stage of ripeness, climacteric and agronomic factors (soil nutriments).

Chromatography of the aqueous phase, FA2, on a silica gel plate revealed glucose, galactose and rhamnose.

HPLC analysis of the flavonol glycosides (fraction FA)

The chromatographic conditions for flavonol glycosides determination were established as those proposed by MacMurrough *et al.* (1982) to separate several quercetin and kaempferol glycosides in hops.

Fig. 4 shows a chromatogram corresponding to a mixture of five flavonol monoglycosides that gave a convenient separation with the exception of quercetin 3-O-glucoside and quercetin 3-O-galactoside.

Peaks 2, 3, 4, 5 (Fig. 4) were identified by comparing their retention times with standards. Peak 1 remains unknown, although its chromatographic response suggests that it could be tentatively assigned to a derivative of quercetin.

Four flavonol glycosides were identified in Highbush blueberries as quercetin 3-O-monoglucoside, quercetin 3-O-monogalactoside, quercetin 3-O-monorhamnoside and kaempferol 3-O-monoglucoside.

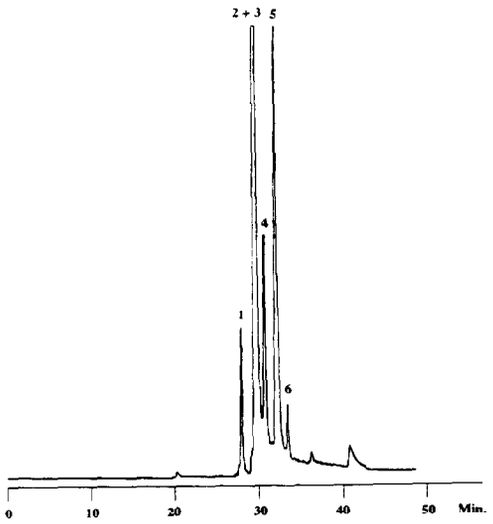

Fig. 4. HPLC analysis of the fraction FA (flavonol glycosides). (1) Unknown 27.4 min; (2) Quercetin-3-glucoside 29.3 min; (3) Quercetin-3-galactoside 29.5 min; (4) Kaempferol-3-glucoside 30.8 min; (5) Quercetin-3-rhamnoside 32.8 min; (6) unknown 33.9 min.

CONCLUSION

A scheme for the fractionation of flavonoid and non-flavonoid compounds has been developed. The application of this scheme to the study of phenolic compounds in Highbush blueberries, cultivated in the mountainous country of north-east of France (Vosges), revealed that chlorogenic acid is the major phenolic acid in this fruit. This scheme may be applied to the analysis of phenolic compounds. Methanol extraction of pigments from French blueberries revealed 15 anthocyanins in comparison to the reported 10 anthocyanins for American planting of the same cultivar. This might be due to a difference in the degree of ripeness of the samples.

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