*Short Communication*

# IDENTIFICATION OF SWEET CHERRY ANTHOCYANINS AND HYDROXYCINNAMIC ACIDS USING HPLC COUPLED WITH DAD AND MS DETECTOR

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## Abstract

In absence of standards, HPLC coupled with DAD offers identification of polyphenols by scanning UV-Vis spectra of individual components, which spectral characteristics are unique, but not selective. At the same time HPLC-DAD determination methods of polyphenols differ in mobile phase solutions resulting in DAD scanned spectra deviation between different studies, aggravating the precise identification based on agreement to UV-Vis data from literature. Mass spectrometry (MS) detection with molar weight determination of the individual components in the sample enables more precise identification of compounds eluted from the column. Sweet cherry Petrovka polyphenols were separated on C18 Hypersil PEP 300 column (250 x 4.6 mm, 5μm) using gradient solvent mixture consisting of methanol, water and formic acid. MS and UV-Vis spectra of eluted anthocyanins and hydroxycinnamic acids were recorded. HPLC-MS analyses were performed using a LCQ$^{TM}$ ion trap, Finnigan, MAT mass spectrometer by atmospheric pressure chemical ionisation (ACPI). Molecular and fragmented ion masses of sweet cherry hydroxycinnamic acids and anthocyanins were determined and with UV-Vis spectra, in the range of 190-600 nm, used for identification of compounds. Electro spray mass spectrum of two hydroxycinnamic acids produced ions with *m/z* ratios of 353.0 and 337.0, which corresponded to molecular weights of neochlorogenic acid and 3'-*p*-coumarylquinic acid. The molecular weights of 5 anthocyanins corresponded to cyanidin-3-glucoside (449.0), cyanidin-3-rutinoside (595.1), peonidin-3-glucoside (463.1), pelargonidin-3-rutinoside (579.1) and peonidin-3-rutinoside (609.2).

**Key words:** sweet cherries, anthocyanins, hydroxycinnamic acids, HPLC-DAD/MS

## Introduction

Phenolic compounds are important components of many fruits, vegetables, and beverages and contribute to their colour and sensory properties such as bitterness and astringency.[1] Phenolic compounds occur in all fruits as a diverse group of secondary metabolites. Their role is shown in oxidation processes as either antioxidants or substrates in browning reactions.[2]

Epidemiological studies have shown that consumption of food rich in phenoliccontents is in correlation with reduced incidence of heart diseases.[3] They retard the progression of arteriosclerosis by acting as antioxidants toward low density lipoproteins (LDL)[4] and inhibit the *in vitro* oxidation.[5,6]

As other fruits, sweet cherries also contain polyphenols.[7,8] It was confirmed that phenolic components from sweet cherries show antioxidant activity toward liposomes and LDL.[9]

Traditional methods for the determination of phenolic components in fruits relied on measurements of total phenol because of their association with browning reaction. These reactions, based on colorimetry, and because of the need for profiling and identifying individual phenolic compounds were replaced by high performance chromatographic analyses.[2] Reverse phase-high performance liquid chromatography (RP-HPLC) currently represents the most popular and reliable technique for phenolic analysis. Compounds elution is typical of RP-HPLC; that is polar compounds elute first, followed by those of decreased polarity.[10] Detection is usually based on absorption of UV or less commonly visible radiation at various wavelengths, characteristic of the class of phenolic compounds.[11] Other methods of detection, like mass spectrometry, have been developed and used in a number of analyses.[12]

That sweet cherries contain anthocyanins has been known since the beginning of the 20$^{th}$ century. Willstätter and Zolingen (1916), and later Robinson and Robinson (1931) identified 3-rutinoside and 3-glucoside of cyanidin in ripe sweet cherries. Lynn and Luh (1964) also reported the presence of peonidin and two of its glycosidic derivatives in cv. Bing cherries. Okobi (1979) identified peonidin-3-rutinoside as the main pigment of cv. Napoleon cherries.[13] The mentioned authors characterised anthocyanins by paper and/or thin layer chromatography.

As far as we are aware, before the study of Gao and Mazza,[13] HPLC of anthocyanins in cherries has not been performed. They characterised and quantified anthocyanins and colourless phenolics in different cultivars by HPLC-diode array detector (DAD) and gas chromatography (GC), using acid hydrolysis and other fruit as a source of anthocyanins to confirm the identity of detected hydroxycinnamic acids and anthocyanins peaks. All the dark coloured cherry genotypes were found to contain cyanidin-3-rutinoside and cyanidin-3-glucoside as major anthocyanins and the same

glycosides of peonidin as minor anthocyanins. Another minor anthocyanin, pelargonidin-3-rutinoside, was characterised in sweet cherries for the first time, with a help of a strawberry, a well-known source of pelargonidin anthocyanin derivatives.[14] Later only a few studies were reported which dealt with anthocyanins in cherries, using the HPLC coupled with DAD or UV-Vis detector.[9,15]

Sweet cherries are also rich in phenolic acids. The most abundant among them are derivatives of the hydroxycinnamic acid (HCA) such as caffeic acid and *p*-coumaric acid. The most common colourless phenolics in sweet cherries are neochlorogenic acid (3'-caffeilquinic acid) and 3'-*p*-coumaroylquinic acid (Figure 1).[8,13,16]

**Table 1.** Spectral properties of major sweet cherry phenolic compounds ($\lambda_{max}$).[8]

| Class of compounds | UV B | UV A | Visible |
|---|---|---|---|
| Hydroxycinnamic acids | (290-300)[a] | 305-330 | |
| Anthocyanins | 270-280 | (315-325)[b] | 500-550 |

Note: solvent methanol, except for anthocyanins where the solvent was methanol containing 0.01% HCl (v/v). [a] shoulder. [b] in the case of acylation by hydroxycinnamic acids.

A lack of polyphenol standards is a huge problem in polyphenol identification. In the absence of standards, HPLC-diode array detector (DAD) offers the identification of polyphenols by UV-Vis spectra scan of individual components, isolated also from other fruits and compared to the already published spectra of individual polyphenols.[13,17] Spectral characteristics of fruit polyphenols are unique, but not selective.[8] Typical absorption spectra for hydroxycinnamic acids and anthocyanins are found in Table 1. The identification referring to the data in literature is often aggravated by mobile phase influence on spectral characteristics of polyphenols.[18]

HPLC coupled with mass spectrometry detector (HPLC-MS) is a powerful toll in polyphenol determination, and the molar weight of eluted peaks is a strong proof for precise compound identification. In our study we describe the identification of anthocyanins and hydroxycinnamic acids present in the local Slovenian dark colored sweet cherry cultivar Petrovka based on DAD scans in the 190-600 nm range. The HPLC investigation of sweet cherry polyphenols was for the first time completed with the identification of the compounds based on molar weight determination using mass spectrometry.

## Results and Discussion

The obtained HPLC-DAD profile of hydroxycinnamic acids (Figure 1, left) has shown two major peaks in the 0–17 minute region. The recorded spectra of peaks 1 and 2 showed distinctive features in the 320 nm region and no absorbance at 520 nm (Figure 2). In accordance with literature data[13] we concluded that peak 1 ($\lambda_{max}$= 242 nm and 324 nm, shoulder at 305 nm) corresponds to neochlorogenic acid and peak 2 ($\lambda_{max}$= 312 nm) to 3'-*p*-coumaroylquinic acid. The UV-Vis spectra of peaks 1 and 2 are shown in Figure 2.

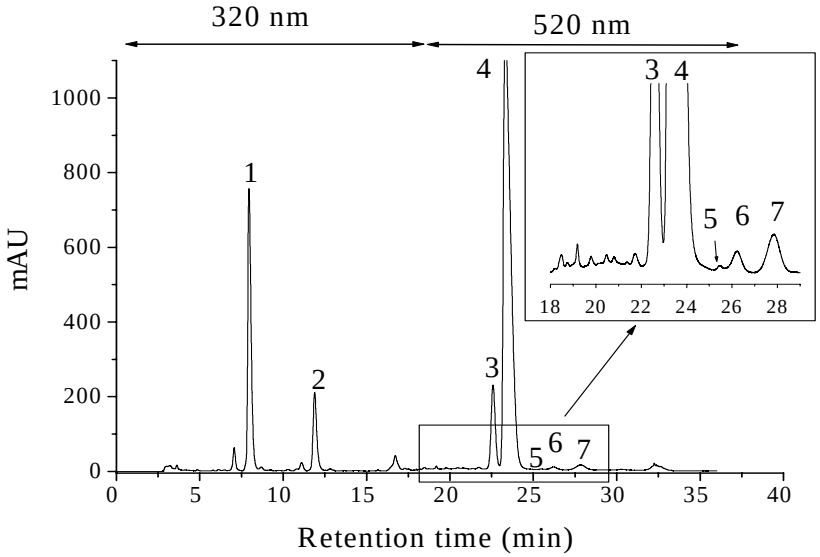


**Figure 1.** HPLC-DAD separation of methanol extract of Petrovka cherries at full ripeness, monitored at 320 nm (0-17 min of chromatographic run) and 520 nm (17-35 min of chromatographic run). Numbers denote the following components: **1**: neochlorogenic acid; **2**: 3'-*p*-coumarylquinic acid; **3**: cyanidin-3-glucoside; **4**: cyanidin-3-rutinoside; **5**: peonidin-3-glucoside; **6**: pelargonidin-3-rutinoside; **7**: peonidin-3-rutinoside.

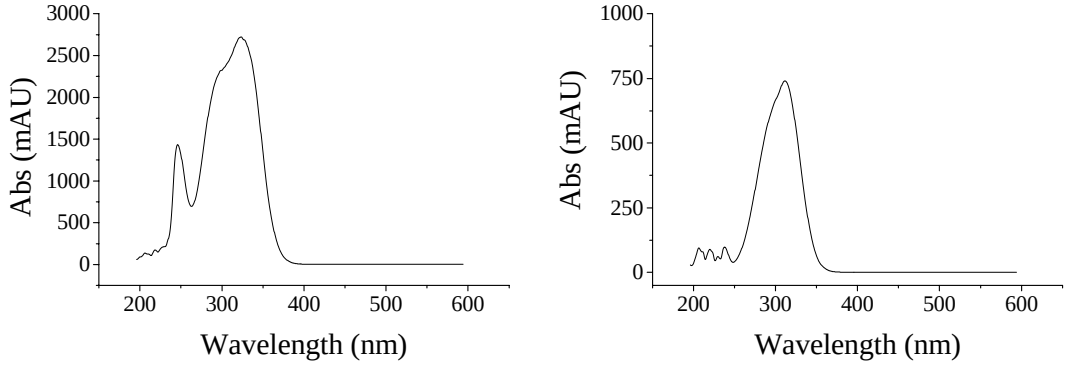


**Figure 2.** UV-Vis spectra of neochlorogenic acid (left) and 3'-*p*-coumarylquinic acid (right).

The electrospray mass spectrum of sweet cherry hydroxycinnamic acids produced ions with *m/z* ratios of 353.0 and 337.0, which corresponded to molecular weights of neochlorogenic acid (353) and 3'-*p*-coumarylquinic acid (337) (Table 2, left).

**Table 2.** HPLC- MS spectra of detected peaks in the Figure 1: (*m/z)* values of molecular and fragment ions.

| Hydroxycinnamic acids | HPLC-MS spectra $M^+$-H *m/z* values | Anthocyanins | HPLC-MS spectra [M-H]+ *m/z* values |
|---|---|---|---|
| peak 1 | 353, 179, 191.2 | peak 3 | 449, 287.3 |
| peak 2 | 337, 163, 191.4 | peak 4 | 595.1, 287 |
| | | peak 5 | 463.1, 301 |
| | | peak 6 | 579.2, 271.3 |
| | | peak 7 | 609.2, 301 |

Five of the peaks detected at 520 nm (Figure 1, 17-35 min time scale) corresponded to anthocyanins, since only that group of polyphenols absorbed in the 500-530 nm region (Figure 3).[8]

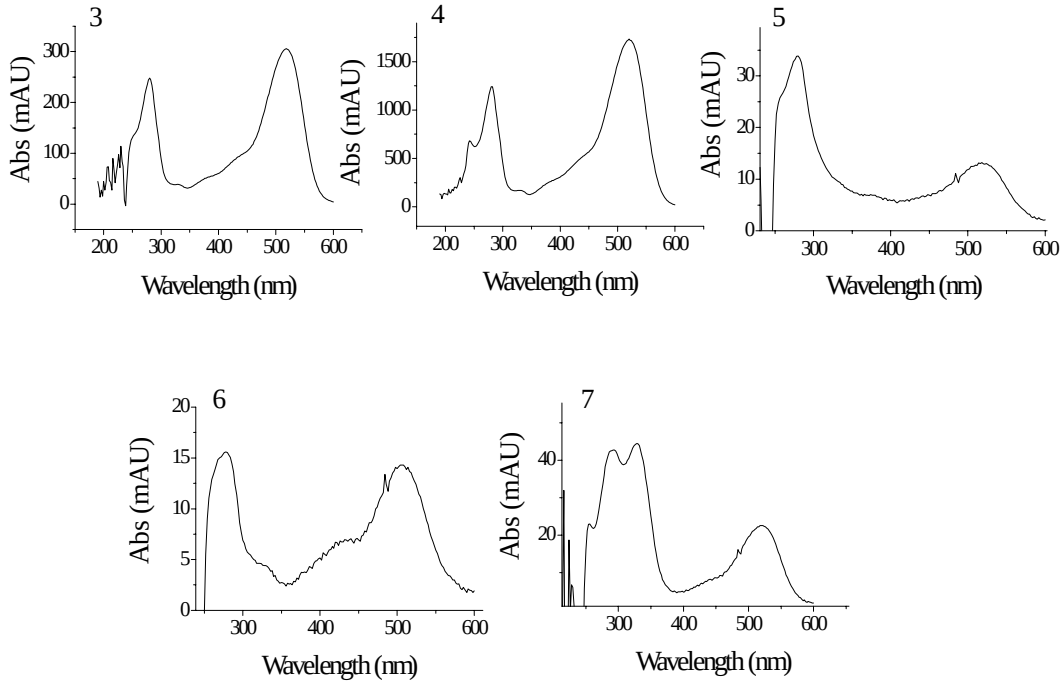


**Figure 3.** UV-Vis spectra of eluted peaks, numbers denote the peaks on the Figure 1.

Peak 4 matched the retention time and spectral characteristics of cyanidin-3-glucoside ($\lambda_{vis\ max}$ = 518 nm) while peak 5 matched the chromatographic and spectral

characteristics of cyanidin-3-rutinoside ($\lambda_{vis\ max}$ = 518 nm), which is in accordance with literature data. [13, 15] UV-VIS spectra and elution order of peaks 5, 6 and 7 indicated that they are most probably peonidin-3-glucoside ($\lambda_{vis\ max}$ = 518 nm), pelargonidin-3-rutinoside ($\lambda_{vis\ max}$ = 506 nm) and peonidin-3-rutionoside ($\lambda_{vis\ max}$ = 518 nm), respectively, which were also previously reported in sweet cherries.[13,15]

The HPLC-MS confirmed their identification consistently as reported in literature, and standards as described previously. The MS spectra of 5 anthocyanins (peak 3 - peak 7; Figure 1 - right, Table 2) showed molar and fragmented ion masses (anthocyanidins-agliconic parts of anthocyanins). Based on the agreement between scanned molecular masses using the MS detector and known anthocyanin molecular weights we confirmed peak 3 to be cyanidin-3-glucoside (449.0), peak 4 corresponded to cyanidin-3-rutinoside (595), peak 5 to peonidin-3-glucoside (463.1) peak 6 to pelargonidin-3-rutinoside (579.2) and peak 7 was recognized as peonidin-3-rutinoside (609). In addition, we confirmed anthocyanin identification with the masses of fragmented ions, which gave information about the agliconic part of the anthocyanin. The data of the fragments are shown in the Table 2.

## Experimental

Methanolic extracts of Petrovka cherries were first analysed using HPLC-DAD method, as previously described.[19] According to this method sweet cherry polyphenols were separated on the C18 Hypersil PEP 300 column (250 × 4.6, 5 μm) and than scanned in the UV-Vis region (190-600 nm) with DAD detector.

The HPLC-MS analyses were performed on the same methanolic extracts of Petrovka polyphenols in the Laboratory of Food Chemistry of the National Institute of Chemistry in Ljubljana, Slovenia. For the separation, Thermo Separation Products HPLC system was employed, involving gradient pump (Constametric 4100), autosampler (AS 3000) with 50 μL injection volume. In spite of the same column as with HPLC-DAD analysis, the different HPLC system needed a slightly modified gradient of the HPLC-DAD method[19] to provide optimal separation of all previously detected cherry peaks of anthocyanins and HCAs in the HPLC-DAD analysis.

Eluted components from the column were subjected to the electrospray mass spectrometric assay. Spectra were registred in the negative and positive mode using a

LCQ$^{TM}$ ion trap, Finnigan, MAT mass spectrometer by atmospheric pressure chemical ionisation (ACPI). Nitrogen gas was used as nebulizing gas under pressure of 0.05 MPa in a temperature of 350 °C. The capilary temperature was 250 °C, the source voltage 4.5 kV, source current 80 μA, the capilary voltage 10 V (positive polarity) and –10 V (negative polarity), tube lens offset –50.00 V for the negative mode and 0.00 for the positive mode.

## Conclusion

Molar mass determination confirmed our previous assumptions about the identity of sweet cherry hydroxycinnamic acids and anthocyanins. Once again, it proved to be an excellent tool in plant polyphenol analysis, since common HPLC-DAD identification is limited in market availability of polyphenol standards. We confirmed all the previous identifications of cherry phenolics based on literature data[15,19] and for the first time presented the HPLC-MS analysis of sweet cherry anthocyanins and hydroxycinnamic acids.

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## Povzetek

Brez standardnih komponent lahko polifenole po ločbi s tekočinsko kromatografijo visoke ločljivosti prepoznamo na podlagi posnetih spektrov v območju UV in vidne svetlobe s pomočjo DAD detekcije. Njihovi spektri so karakteristični, vendar ne specifični. HPLC-DAD metode za analizo polifenolov se razlikujejo v sestavi mobilne faze, kar povzroči odklone v posnetih spektrih in s tem ovirajo identifikacijo na podlagi podatkov iz literature. Z masno spektrometrijo lahko določimo molekulske mase komponent, ki jih ločimo s tekočinsko kromatografijo visoke ločljivosti. Na koloni C18 (Hypersil PEP 300, 250 x 4.6 mm, 5 μm) smo ločili polifenole češenj kultivarja Petrovka. Uporabili smo gradientni sistem metanola, vode in metanojske kisline. Eluiranim antocianom in hidroksicimetnim kislinam smo izmerili spektre v UV in vidnem območju, ter jih podprli z masnimi spektri. HPLC-MS analize smo opravili z masnim spektrometrom LCQ$^{TM}$ ion trap, Finnigan, MAT z ACPI ionizatorjem. Masa dveh hidroksicimetnih kislin je ustrezala neoklorogenski kislini (353) in 3'-*p*-kumarilkininski kislini (337). Molske mase 5 antocianov so potrdile identiteto cianidin-3-glukozida (449.0), cianidin-3-rutinozida (595.1), peonidin-3-glukozida (463.1), pelargonidin-3-rutinozida (579.1) and peonidin-3-rutinozida (609.2).