

An Optimized Method for Analysis of Phenolic Compounds in Buds, Leaves, and Fruits of Black Currant (*Ribes nigrum* L.)

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ABSTRACT: Although the fruits are the economic driver for the black currant industry, the buds and leaves are excellent sources of beneficial phenolic compounds that may contribute to the future value of the crop. In this study, extraction of phenolic compounds for different parts of the black currant plant was optimized, and an efficient method for their separation by HPLC was developed. This allowed the simultaneous quantification of a range of hydroxycinnamic acids, flavan-3-ols, flavonols, and anthocyanins by DAD following their identification by HPLC-ESI-MSⁿ. A total of 23 compounds were detected in the buds, 22 of which were found in fruit and leaves. To the best of our knowledge, this is the first report of flavonol glycosides of quercetin, myricetin, isorhamnetin, and kaempferol along with hydroxycinnamic acids such as neo-chlorogenic acid and chlorogenic acid in the buds. Additionally, we provide the first evidence of kaempferol-3-O-rutinoside in black currant leaves. This approach offers avenues for superior combined compositional identification and cultivar selection targeted at the generation of polyphenol-rich products derived from the whole crop and not just the fruit.

KEYWORDS: anthocyanins, ascorbic acid, bioactive compounds, extraction, flavonol, functional food, HPLC, polyphenols

INTRODUCTION

The benefit of fruits and berries in the human diet has received considerable attention in the recent years due to their rich amount of phenolic compounds with different biological activities.^{1–3} Especially small fruits have high contents of several polyphenols. Using different *in vitro* and animal models, phenolic extracts of black currant fruit have been reported to exhibit, for example, antioxidant, anti-inflammatory, vasomodulatory, antihemostatic, and muscle relaxing effects, improvement of visual function, as well as neuroprotective and anticancer activities.^{4–10} In humans, it has been shown that intake of anthocyanins improved shoulder stiffness due to typing work by increasing peripheral blood flow and reducing muscle fatigue.¹¹ In addition, oral intake of black currant juice in healthy women has induced peripheral vasodilatation and led to increase in blood flow and decrease in blood pressure.¹²

The phenolic profile of black currant fruit has therefore been extensively studied during recent years, and several papers have been published on the use of HPLC-DAD-ESI-MSⁿ for identification and quantification of phenolic compounds.^{13–21} The profile includes a range of compounds, particularly phenolic acids (both hydroxybenzoic and hydroxycinnamic acids), flavonoids, and tannins. Among flavonoids, flavanols (catechins, procyanidins), flavonols (glycosides of myricetin, quercetin, kaempferol, and isorhamnetin), and anthocyanins are commonly present. Proper characterization of the composition of phenolic compounds is important, because a specific compound may contribute with specific health benefits.

The leaves and buds of black currant might be of even higher interest than the fruits as a source of bioactive compounds. They constitute a potential raw material for the health industry as food additives and supplements that could be of relevance as

nutraceuticals.^{22,23} Although black currant leaves are popular as tea products, phenolic profiles of black currant buds and leaves have not received much attention yet. Significant anti-inflammatory activity has, however, been reported using both *in vitro* and *in vivo* models.^{24,25} Studies by Tabart et al.²² demonstrated that black currant leaves contained more total phenolic content than what is found in ripe fruits, and that the total phenolic content correlated with antioxidant capacity. Recent studies have shown that the leaves contain significant amounts of kaempferol, quercetin, and phenolic acids.^{26,27}

The presence of certain phenolic acids, flavan-3-ols, flavonols, and anthocyanins has recently been reported in the buds.²⁸ The buds are also rich sources of volatile compounds, the majority of which are hydrocarbons and oxygenated fractions of terpenes.²⁹ Tabart et al.²² have reported that buds possess higher antioxidant ability than do other black currant parts. The essential oils of buds have been reported to possess significant antimicrobial activity against pathogenic bacteria.³⁰ Hence, the buds could be a natural alternative for the treatment of infectious diseases.

Phenolic compounds are usually analyzed by different HPLC methods using UV–vis, diode array detector (DAD), and tandem mass spectrometric (MSⁿ) detection. Mass spectra detection coupled with electrospray ionization (ESI) provides information about the molecular mass and fragmentation pattern of the analyte tested.²⁰ Ionization in positive ion mode is used for the identification of anthocyanins in their

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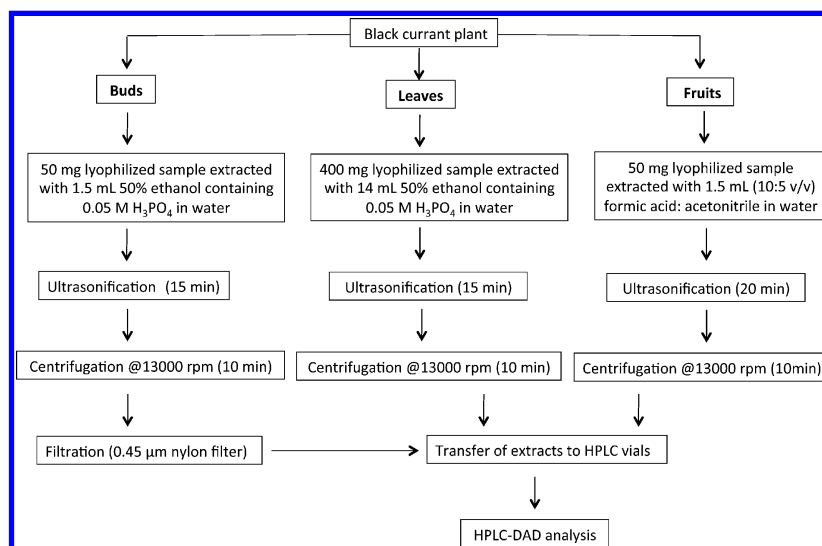


Figure 1. Flowchart depicting the extraction procedures for phenolic compounds in black currant buds, leaves, and fruits.

native form (positive flavylum cations³¹), and hydroxybenzoic and hydroxycinnamic acids, flavonol glycosides, and condensed tannins are mainly identified in negative ion mode.^{20,32} For extraction of phenolic compounds from foods, the commonly used organic solvents are aqueous mixtures of ethanol, methanol, water, and acetone, sometimes in combination with weak or strong acids. The yield and composition of phenolic compounds in extracts depend on the extraction conditions and are, in addition to the polarity of the solvent, influenced by, for example, temperature, duration of extraction, number of steps, solvent-to-sample ratio, and use of enzymes.³³ The stability of anthocyanins in fruits is influenced by pH, exposure to light, temperature, storage, and processing.³⁴ Hence, the stability is an important aspect to be considered notably during analysis but also for use of anthocyanins as ingredients in innovative products with health promoting properties.

The objectives of the current study were to (1) optimize the conditions for extraction of phenolic compounds in different black currant parts, (2) separate and identify the main phenolic compounds using HPLC-ESI-MSⁿ, and (3) develop an efficient HPLC-DAD method for quantification of these compounds in black currant buds, leaves, and fruits.

MATERIALS AND METHODS

Reagent and Chemicals. Acetonitrile (isocratic grade, >99.8%), methanol (HPLC grade, >99.8%), Folin-Ciocalteu phenol reagent, 85% ortho-phosphoric acid (H₃PO₄), sodium dihydrogen phosphate monohydrate (NaH₂PO₄), sodium acetate (CH₃COONa), and potassium chloride (KCl) were obtained from Merck KGaA (Darmstadt, Germany). Formic acid (pro analysi, 98–100%), sodium carbonate (Na₂CO₃), ascorbic acid (AsA), gallic acid, chlorogenic acid, and quercetin were purchased from Sigma (St. Louis, MO). Neochlorogenic acid, epigallocatechin, (+)-catechin, (–)-epicatechin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside were purchased from Extrasynthese (Genay, France). Ultra pure water was obtained by use of Elgastat prima UHQPS (Buckinghamshire, England). Ethanol (99.9%) was purchased from VWR (Fontenay-Sous-Bois, France).

Plant Material. The plant materials were harvested from black currant bushes grown at Balsgård (56°06' N, 14°10' E), The Swedish University of Agricultural Sciences, Sweden. Fruits were sampled at full

ripeness in July 2010; approximately 500 mL of berries were picked and divided into two subsamples of 50 g each. Leaf and bud samples were collected in September and October 2010, respectively, as during that period of the year these plant parts are fully developed. Healthy full-grown leaves without petioles were detached from the shoots all over the bush. Dormant buds were plucked with scissors from around 7 twigs from all over the bush, to attain representative samples. The apical buds on each twig were avoided during sampling. All plant material was collected in the afternoon, lyophilized for 1 week, and then kept at –20 °C before sample preparation and analyses.

Extraction of Phenolic Compounds. In initial screening experiments, three concentrations of ethanol (30%, 50%, and 70%) containing 0.05 M H₃PO₄ in different combinations with water and different extraction times (10, 15, 20, and 30 min) were tested for buds and leaves to optimize the extraction efficiency measured as total phenolic (TP) content.

For the fruits, three different extraction solutions, (1) acetonitrile/formic acid (5:10 v/v) in water, (2) acidified methanol 95:5 v/v (70% methanol containing 1% HCL:100% methanol), and (3) ethanol solution (0.05 M H₃PO₄ in ethanol 50% (v/v)), were tested for the stability of anthocyanins. The stability of anthocyanins was measured over a storage period of 6 days at room temperature as total anthocyanins (TA) content.

On the basis of the results obtained from the initial extraction screening experiments, the following extraction solutions and procedures were applied to the experimental design. The lyophilized leaves were crushed and ground to a fine powder using an analytical mill (Yellow line, A10, IKA-Werke, Staufen, Germany). Approximately 50 mg of the samples was weighed in triplicates in 2 mL tubes and extracted by 1.5 mL of 50% ethanol containing 0.05 M H₃PO₄. The extracts were vortexed (Janke & Kankel, Staufen, Germany) and sonicated in an ultrasonic bath (Bandelin Sonorex, Berlin, Germany) for 15 min at room temperature and centrifuged for 10 min at 13 000 rpm. The supernatant was transferred to HPLC vials for analysis.

From the lyophilized buds, approximately 400 mg of samples was weighed in 15 mL tubes and homogenized with 14 mL of 50% ethanol containing 0.05 M H₃PO₄ using a blender (Janke & Kunkel GmbH & Co. KG, Staufen, Germany). The extracts were vortexed and kept in an ultrasonic bath for 15 min at room temperature. The extracts were then centrifuged for 10 min at 13 000 rpm. As small residues of the buds still remained after centrifugation, the supernatant was filtered through a 0.45 µm filter (Sarstedt, Nümbrecht, Germany) (unlike for leaves and fruits) into HPLC vials for analyses. Each sample of buds and leaves was extracted in triplicate.

For extraction of the fruits, approximately 50 mg of the lyophilized samples was weighed from the two subsamples in 2 mL tubes extracted and stabilized by 1.5 mL of 10% formic acid and 5% acetonitrile in

water as previously used by Nielsen et al.³⁵ with slight modifications. The samples were vortexed, and extraction took place in an ultrasonic bath for 20 min at ambient temperature. The extracts were centrifuged at 13 000 rpm for 10 min, and the supernatant was transferred to HPLC vials. Extraction was performed in duplicate. A flowchart representing extraction procedure is presented in Figure 1.

Total Phenolic Compounds. The total phenolic (TP) content was determined using the Folin–Ciocalteu method.³⁶ Briefly, 0.5 μL of the extracted sample, 100 μL of ethanol (5% v/v), 200 μL of Folin–Ciocalteu reagent, and 2 mL of 15% Na_2CO_3 solution were added followed by addition of 1 mL of ultra pure water. The solutions in the cuvette were mixed by use of a pipet and allowed to equilibrate at room temperature for 2 h. The absorbance was recorded at 765 nm using a UV-2101PC spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid was used for the construction of a standard curve, and the results were expressed as milligrams of gallic acid (GA) per gram of dry weight (DW) of plant material. The analyses were performed in triplicate.

Although not a precise method for the quantification of phenolic compounds due to interferences from, for example, ascorbic acid (AsA), the Folin–Ciocalteu method however provides an estimate of the total phenol content.³⁷ Because AsA biased the results of the Folin–Ciocalteu method, a correction factor was calculated to correct the overestimate of the content of the total phenols. This was done by analyzing known amounts of AsA over a range of typical concentrations for the samples tested and measuring the absorbance using the Folin–Ciocalteu method. A plot of absorbance against concentration resulted in a linear equation for the relative response of AsA. The AsA equation was then calculated against a gallic acid standard curve to obtain a correction factor. A correction factor of 0.4442 was then used to correct for AsA in the samples measured by HPLC-analysis, by subtracting the absorbance contribution from the spectrophotometrically determined TP values calculated using the gallic acid standard curve and thus estimating the TP content in the samples.

AsA Analysis. The AsA content of the samples was determined on a Shimadzu (Kyoto, Japan) HPLC system (SIL-10A autosampler, SCL-10AVP control unit, LC-10AD pump, SPD-10AV VP UV–vis detector unit) controlled by Class-VP software (6.13 SP2). The isocratic mobile phase consisted of 0.05 M NaH_2PO_4 and H_3PO_4 (8.5%), and pH of the eluent was adjusted to 2.8. The separation was performed using a Restek (Bellefonte, PA) column (150 \times 4.6 mm, 5 μm particle size), operated at 30 $^\circ\text{C}$ (Column Chiller, Sorbent AB), and a guard column at a flow rate for 1 mL min^{-1} . Detection was carried out at 254 nm.

Total Anthocyanins. The total anthocyanins (TA) content in fruits of black currant was measured by the pH differential method.³⁸ Briefly, 25 μL of the extracted sample was dissolved in 2 mL of KCl buffer at pH 1.0 and 2 mL of CH_3COONa buffer at pH 4.5. After 15 min, the absorbance was measured at 516 and 700 nm, respectively, using a UV-2101PC (Shimadzu, Kyoto, Japan) spectrophotometer.

The absorbance (A) of the diluted sample was then calculated as follows:

$$A = (A_{516} - A_{700})_{\text{pH}1.0} - (A_{516} - A_{700})_{\text{pH}4.5}$$

The TA content in the sample was expressed as cyanidin-3-glucoside equivalents according to the formula:

$$\text{TA content, mg/L} = (A \times \text{MW} \times \text{DF} \times 1000) / (\epsilon \times 1)$$

A molecular weight (MW) of 449.2 for cyanidin-3-*O*-glucoside was used, DF is the dilution factor, and ϵ (26900) is the molar absorption of cyanidin-3-*O*-glucoside. The results were expressed as mg/g dry weight (DW) of the sample. Analyses were performed in duplicate on each sample.

Chromatographic Conditions. During the course of method development for HPLC analysis, different concentrations of formic acid (1%, 5%, and 7%; mobile phase A) and mixtures of acetonitrile–water–methanol (90:5:5, v/v/v; mobile phase B) were tested for the chromatographic separation of phenolic compounds using the gradient

program described by Li et al.³⁹ However, the separation was not satisfactory. Therefore, several gradient programs were tried until the peaks were satisfactorily separated.

The method development in the present study resulted in the following chromatographic conditions further applied during the HPLC analysis. The separation was executed with a mobile phase consisting of formic acid/water (7:93 v/v; mobile phase A) and acetonitrile/methanol/water (90:5:5 v/v; mobile phase B) with gradient elution performed as follows: The linear gradient started with 0–2 min, 8% B; 2–21.5 min, 8–16% B; 21.5–51.5 min, 16–23% B; 51.5–56.5 min, 23–40% B; and 56.5–61.5 min, back to 8% B followed by re-equilibration of the system for 2 min with initial conditions. The linear binary gradient was set to a flow rate of 1.2 mL min^{-1} , and total run time was 63.5 min. Injection volume of samples in HPLC-DAD was 10 μL for buds and leaves and 20 μL for fruits as initial studies with 10 μL showed low peak intensities. In LC-ESI-MS and LTQ Orbitrap (MS/MS), the injection volume was 8 μL , and the concentration of formic acid was compromised to 0.4% (mobile phase A). Separation was achieved on a Synergie hydro RP- 80A column (250 \times 4.60 mm i.d., 4 μm particle size), protected with a C-18 guard cartridge (4 \times 3.0 mm) both from Phenomenex (Værløse, Denmark). The column was maintained at 24 $^\circ\text{C}$ using a Shimadzu CTO-10AS thermostatically controlled column compartment.

HPLC-DAD and ESI-MS. The HPLC-DAD apparatus consisted of a Shimadzu instrument (Kyoto, Japan) with a LC-20AB model dual pump, and a SPD-M10A model diode array detector equipped with Waters 717 plus autosampler (Milford, MA) linked to a Shimadzu SCL-10A model system controller. The acquisition wavelength was set in the range of 260–550 nm, and chromatograms were recorded at 320 nm for conjugated forms of hydroxycinnamic acids, at 280 nm for flavan-3-ols, at 360 nm for flavonols, and at 520 nm for anthocyanins. The chromatographic data were collected using Class VP software (Shimadzu 5.0).

HPLC-ESI-MS detection was performed using a Sciex (Toronto, Canada) API 150EX Single Quadrupole mass spectrometer equipped with a Turbo Ion Spray interface. The HPLC consisted of a Perkin-Elmer system (Norwalk, CT) equipped with two pumps connected to an autosampler (Serie 200) controlled by Analyst software (Sciex 1.3). The eluent was split into 0.3 mL min^{-1} before being introduced to the ESI-MS system. Nitrogen was used as nebulizing gas. The electrospray chamber was operated at 4.0 kV. Mass spectra of phenolic compounds were obtained by acquiring data at 90–1500 amu after electro spray ionization (ESI) in negative mode. The general conditions were: nebulizer gas, 9.0 L min^{-1} ; curtain gas, 12 L min^{-1} ; and dry gas temperature, 300 $^\circ\text{C}$.

HPLC-ESI-MSⁿ. For identification of the novel compounds, an Accela system (Thermo Scientific, Hemel Hempstead, UK) with ESI-MSⁿ detection was used. The system consisted of a PDA detector (at 200–600 nm), a quaternary 600 pump connected to an autosampler cooled to 6 $^\circ\text{C}$, a column oven, and a LTQ Orbitrap XL linear iontrap mass spectrometer with an ESI source controlled by Xcalibur (Thermo Scientific 2.0.7). Ionization was performed in negative mode with orbitrap set at 30 000 resolving power. The full scan covered a mass range from m/z 100 to 2000. Nitrogen and helium were used as nebulizing gas. Operational parameters were as follows: capillary temperature and voltage, 300 $^\circ\text{C}$ and -25 V, respectively; sheath gas flow rate, 40 units; spray voltage, 3.40 kV; and spray current, 100.00 μA .

Statistical Analysis. Statistical analysis was performed using Minitab software, version 16.1 (State college, PA). Data were subjected to one-way analysis of variance (ANOVA), and Tukey's test was applied to reveal any significant differences between treatments at $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of Sample Extraction and HPLC Analysis. In the present study, optimization of the extraction procedures for different phenolic compounds in an efficient and time-saving way was evaluated. Various methods were tested to

select the best extraction procedure measured as total phenolics (TP) in buds and leaves, and as total anthocyanins (TA) in fruits of black currant. High yields of phenolic compounds were obtained from both buds and leaves, with a solvent containing 50% ethanol as compared to 30% and 70% ethanol and an extraction time of 15 min together with an ultrasonic bath. It was also observed that the TP content (Figure 2) was higher in

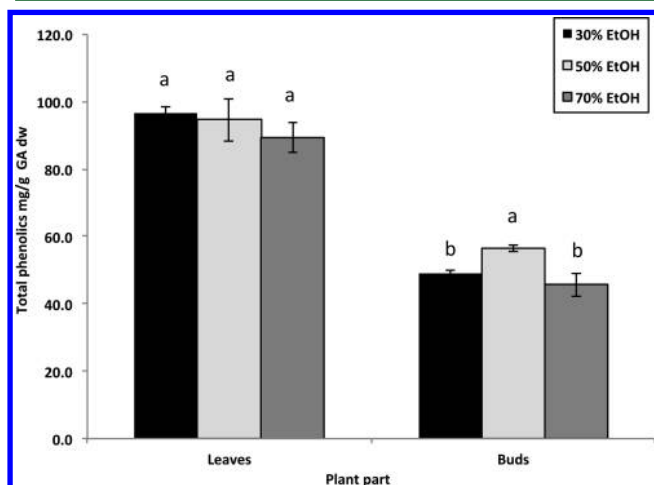


Figure 2. Total phenolics (mg/g GA DW) of leaves and buds using EtOH/water (30, 50, 70% v/v) with 0.05 M H_3PO_4 as solvent and ultrasonic bath for 15 min for extraction. 50% EtOH resulted in a high yield of phenols for both leaves and buds. Letters a and b indicate significant differences ($p < 0.05$), for each plant part, respectively. Error bars indicate \pm SD of three independent extractions.

the leaves (89–97 mg/g GA DW), with no significant difference ($p = 0.208$) between the treatments, than in the buds (45–56 mg/g GA DW), with significant difference ($p = 0.002$) between the treatments. Tabart et al.²⁸ using the black currant variety “Noir de Bourgogne” obtained 46.0 mg/g and 45.1 mg/g chlorogenic acid equivalent (CAE) fresh weight (FW) TP content in leaves and buds, respectively, with acetone/acetic acid/water (70:28:2) as solvent. Previously, acetone/acetic acid/water mixtures have been shown to extract higher levels of total phenols in black currant buds and leaves.²³ The choice of 50% ethanol in water (v/v) as extraction solvent for this study was based on the high yield of phenolic compounds. In addition, it is a moderately polar solvent and thus useful for extraction of both polar (water-soluble) and less polar (ethanol-soluble) bioactive compounds.

For analysis of a large number of samples, the stability of anthocyanins in fruits over a period of storing in lab conditions must be considered along with high extraction efficiency. Degradation of TA over time was observed in all of the extracts tested (Figure 3). Maximum stability was achieved in extracts with acetonitrile/formic acid (ACN/formic acid) (5:10 v/v in water), with no significant difference due to the number of storage days (the content, however, decreased insignificantly by 8.1% and 15.7% on days 3 and 6, respectively). By contrast, 50% ethanol (EtOH) containing 0.05 M H_3PO_4 was the most unstable extraction solution (with a significant decrease of 15.7% and 33.7% for days 3 and 6, respectively). Using acidified methanol (MeOH) 95:5 v/v (70% methanol containing 1% HCL:100% methanol), slightly higher TA levels could be extracted as compared to the other solvents (but with a significant decrease of 13.0% on day 3 and 22.3% on day 6).

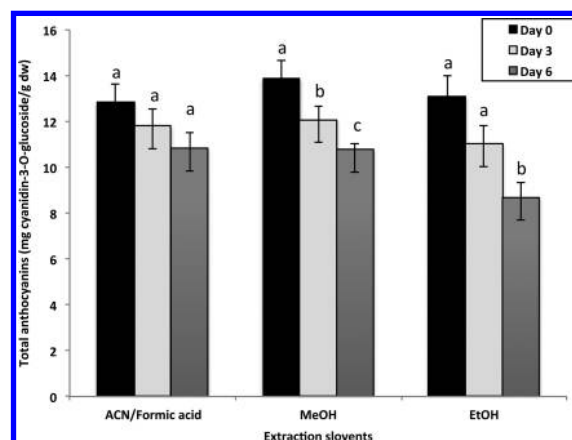


Figure 3. Content of total anthocyanins (mg cyanidin-3-O-glucoside/g DW) in different fruit extracts (ACN/formic acid, MeOH, EtOH) stored at room temperature and analyzed after 0, 3, and 6 days. The highest stability was obtained using ACN/formic acid for extraction. Letters a–c indicate significant differences ($p < 0.05$) for the specific extraction solvents within 0, 3, and 6 days of storage. Error bars indicate \pm SD of two independent extractions.

Therefore, for extraction of fruits, a solution of formic acid and acetonitrile (10:5 v/v) in water was used in the remainder of this study.

For HPLC analysis of phenolic compounds, various concentrations of formic acid (1%, 5%, and 7% v/v) in water as mobile phase A, and a mixture of acetonitrile–methanol–water (90:5:5) as mobile phase B, were tested using the gradient protocol from Li et al.³⁹ We found that 1% or 5% formic acid in water (v/v) did not result in a satisfactory separation with slight trailing peaks being observed. The use of a high concentration of acid in the mobile phase was found to be essential to achieve complete separation and improve peak trailing of compounds, change pH of the mobile phase, and improve the resolution of the compounds.⁴⁰ Therefore, the concentration of formic acid was increased to 7%, and several gradient programs were tested until better separation between the compounds eluted was achieved. Methanol was chosen for mobile phase B because it is considered to be the best solvent for catechin extraction and separation. Finally, a modified gradient program with 7% formic acid in water (v/v) as mobile phase A and a mixture of acetonitrile–methanol–water (90:5:5 v/v) as mobile phase B considerably improved the separation of phenolic compounds, resulting in sharper peaks and good resolution (Figure 4).

Identification of Phenolic Compounds. Identification of phenolic compounds was carried out by comparing their UV–vis spectra (260–550 nm) with available literature and retention times relative to available external standards as well as through peak spiking whenever possible to support the identification. A total of 23 kinds of phenolic compounds were identified in different plant parts in this study. The identified phenolic compounds are classified into conjugated forms of phenolic acids (hydroxycinnamic acids), flavan-3-ols, flavonols, and anthocyanins. A summary of all of the phenolic compounds within each class identified in buds, leaves, and fruits is given in Table 1. The HPLC-DAD chromatograms obtained for buds, leaves, and fruits at four wavelengths (280 nm for flavan-3-ols, 320 nm for hydroxycinnamic acids, 360 nm for flavonols, and 520 nm for anthocyanins) are represented in Figures 5–7.

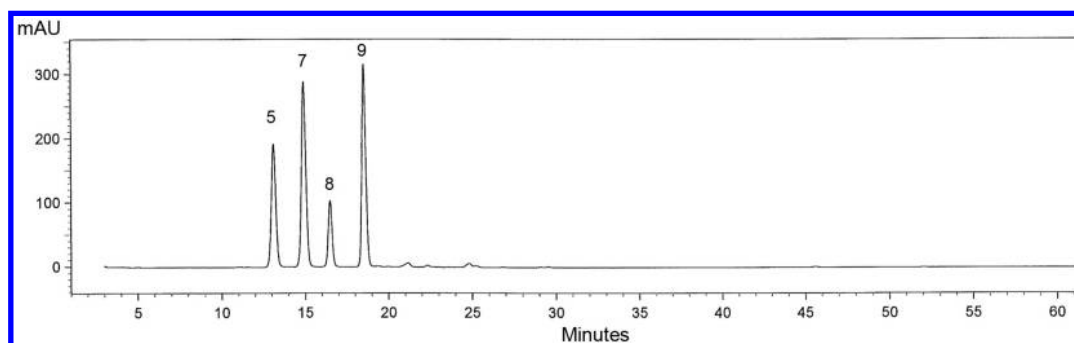


Figure 4. HPLC-DAD traces of anthocyanins in fruits detected at 520 nm using eluent A, 7% formic acid in water (v/v), and eluent B, acetonitrile–methanol–water (90:5:5), which considerably improved the separation between the peaks (peak numbers refer to Table 1).

Table 1. Retention Times (t_R), Wavelength for Detection (UV), and Mass Spectral Data for Analyses of Phenolic Compounds in Buds, Leaves, and Fruits Using HPLC-DAD-ESI-MSⁿ Detection

peak	t_R (min)	UV (nm)	mass spectra		compound identity
			$M - H^- (m/z)$	$MS^2 (m/z)^-$	
1	4.6	320	353	191, 179, 111	neo-chlorogenic acid
2	6.1	280	305	125, 179	epigallocatechin
3	7.0	280	289	245, 205	catechin
4	8.8	320	353	191	chlorogenic acid
5	13.3	520	463.1	301	delphinidin-3- <i>O</i> -glucoside
6	12.7	280	289	245, 205, 179	epicatechin
7	15.1	520	609	463, 301	delphinidin-3- <i>O</i> -rutinose
8	16.6	520	447	285	cyanidin-3- <i>O</i> -glucoside
9	18.5	520	593	447, 285	cyanidin-3- <i>O</i> -rutinose
10	21.2	360	436.1	179, 135	unidentified
11	23.4	360	565	521, 316	myricetin malonylglucoside
12	24.6	360	565	521, 316	myricetin malonylglucoside (isomer)
13	27.1	360	609.2	301, 179	quercetin-3- <i>O</i> -rutinose
14	27.6	360	463.1	301	quercetin-3- <i>O</i> -galactoside
15	28.4	360	463.1	301	quercetin-3- <i>O</i> -glucoside
16	31.8	360	549	505.1, 301	quercetin-3-6-malonyl-glucoside
17	32.8	360	593	285	kaempferol-3- <i>O</i> rutinose
18	35.9	360	447.1	285	kaempferol-3- <i>O</i> -glucoside
19	36.8	360	623	315	isorhamnetin-3- <i>O</i> -rutinose
20	38.3	360	477	315	isorhamnetin-3- <i>O</i> -glucoside
21	41.7	360	533	489, 285	kaempferol-malonylglucoside
22	45.5	360	533	489, 285	kaempferol-malonylglucoside (isomer)
23	53.0	360	301	151, 179	quercetin

Phenolic Acids. In black currants, hydroxycinnamic acids constitute the major group of phenolic acids.¹⁴ The profile of buds, leaves, and fruits revealed two major phenolic acids: neo-chlorogenic acid (5-*O*-caffeoylquinic acid) (peak 1) and chlorogenic acid (3-*O*-caffeoylquinic acid) (peak 4). Comparing the retention time with authentic standards with absorption at 320 nm and ($M - H^-$) at m/z 353 and characteristic MS/MS ions at m/z 191, 179, and 111 for neo-chlorogenic acid and m/z 191 for chlorogenic acid confirmed the identification. Both of these compounds have previously been reported in black currant fruits and leaves.^{16,26,27} However, to the best of our knowledge, we are the first to report the presence of neo-chlorogenic acid and chlorogenic acid (Figure 5 at 320 nm) in the buds of black currant.

Flavan-3-ols. Generally flavanols occur as catechin (flavan-3-ols) or in polymerized form. The commonly present flavan-3-ols in black currant are catechin, epicatechin, epigallocatechin, and their galloyl derivatives.¹⁵ In the present study, peak 2 had a ($M - H^-$) at m/z 305 and fragment ion at m/z 125 and 179,

indicating that it was epigallocatechin. Raudsepp et al.²⁶ have earlier reported the presence of this compound in black currant leaves. We identified peaks 3 and 6 as (+)-catechin and (–)-epicatechin with ($M - H^-$) at m/z 289 and fragmentation at m/z at 245, 205, and 179, respectively. The presence of epigallocatechin, (+)-catechin, and (–)-epicatechin has previously been reported in the fruits and buds of black currant.^{15,21,28} Flavan-3-ols were all identified at 280 nm in buds (Figure 5), leaves (Figure 6), and for fruits (Figure 7) according to retention times with comparison to standards, and further confirmed by LC-ESI-MSⁿ characteristics.

Flavonols. In this study, major flavonols were present as conjugates of myricetin, quercetin, kaempferol, and isorhamnetin in the different plant parts. Peaks 11 and 12 with ($M - H^-$) at m/z 565, both producing fragmentation ions at m/z 521 and 316, were identified as myricetin malonyl-glucoside and an isomer of myricetin malonyl-glucoside, respectively. Four compounds (peak 13, 14, 15, and 16) were identified as glycosides of quercetin because all compounds yielded a 301

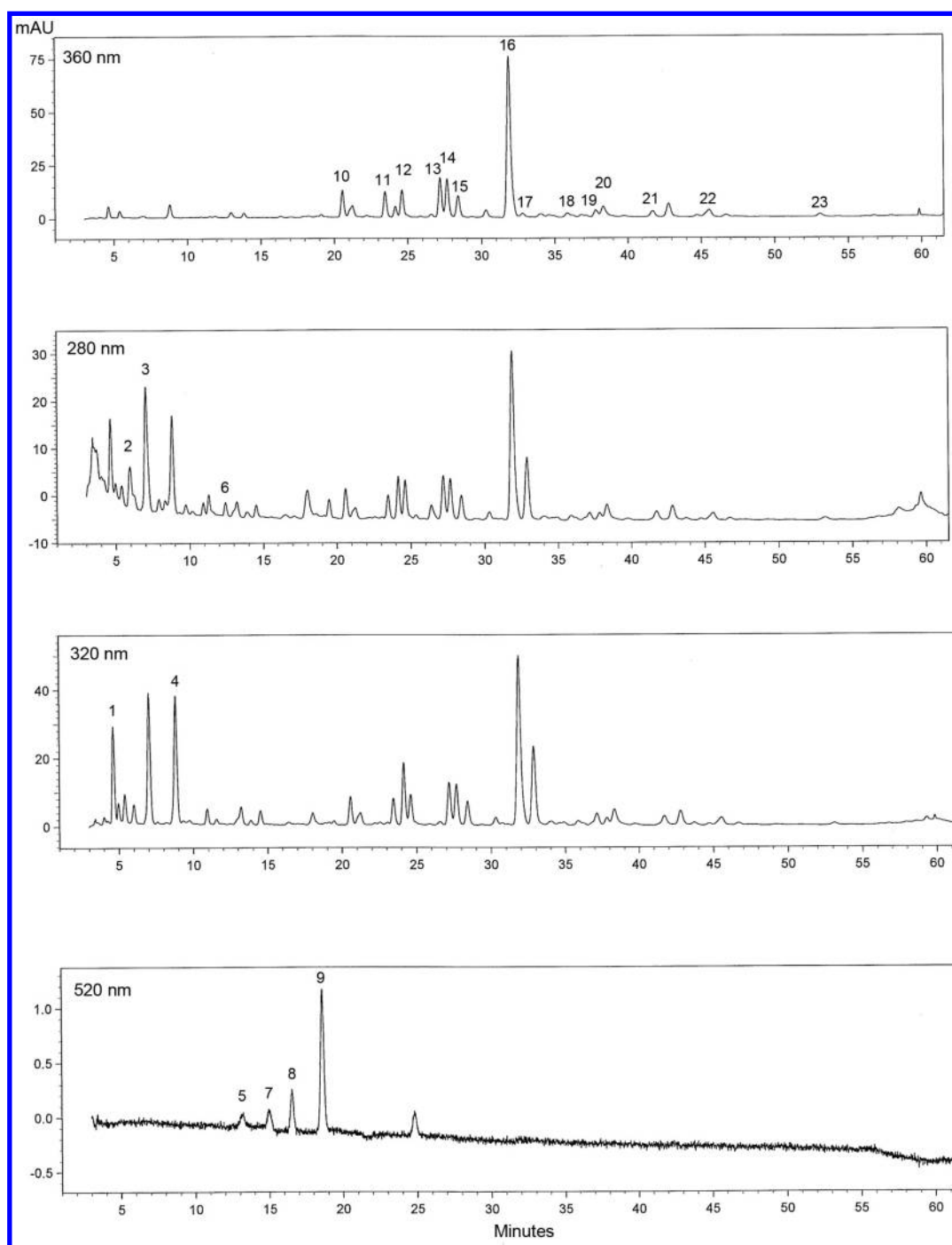


Figure 5. HPLC-DAD chromatogram of phenolic profiles for black currant buds monitored at 360 nm for flavonols, 280 nm for flavan-3-ols, 320 nm for phenolic acids, and 520 nm for anthocyanins. (Peak numbers refer to Table 1.)

(m/z) ion fragment in the MS/MS spectrum. The detected glycosides were identified as quercetin-3-*O*-rutinoside with ($M - H^-$) at m/z 609.2, quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside both with ($M - H^-$) m/z at 463.1, and quercetin-3-6-malonyl-glucoside with ($M - H^-$) at m/z 549 producing fragment ions at m/z 505.1 and 301. Four kaempferol glycosides were identified: kaempferol-3-*O*-rutinoside (peak 17) with ($M - H^-$) at m/z 593 producing a fragment ion at m/z 285, kaempferol-3-*O*-glucoside (peak 18) with ($M - H^-$) at m/z 447.1 and fragment ion with m/z 285, and kaempferol malonyl-glucoside (peak 21) and kaempferol malonyl-glucoside isomer (peak 22) both sharing the same

molecular ion ($M - H^-$) at m/z 533 resulting in fragment ions at m/z 489 and 285. Peaks 19 and 20 produced a fragment ion m/z at 315, suggesting glycosides of isorhamnetin: isorhamnetin-3-*O*-rutinoside with ($M - H^-$) at m/z 623 and isorhamnetin-3-*O*-glucoside with ($M - H^-$) at m/z 477, respectively. Peak 10 could not be identified, although we obtained information that the molecular ion at m/z 436.1 ($M - H^-$) produced two-fragment ions at m/z 179 and 135.

In a recent study, quercetin, myricetin, and kaempferol (aglycones) were reported by Tabart et al.²⁸ in hydrolyzed extracts of both buds and leaves. However, this study first reports the presence of glycosides of quercetin, myricetin,

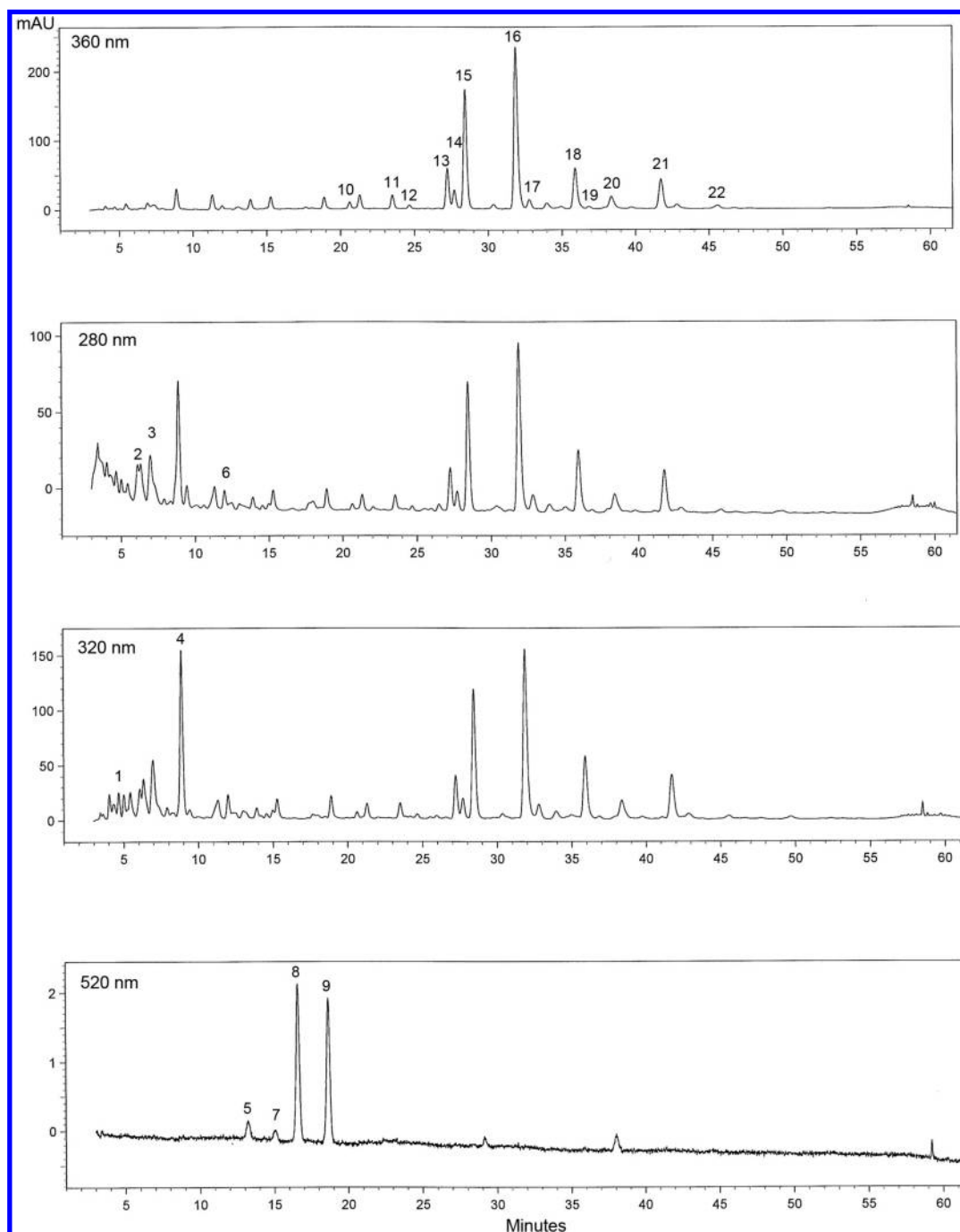


Figure 6. HPLC-DAD chromatogram of phenolic profiles for black currant leaves monitored at 360 nm for flavonols, 280 nm for flavan-3-ols, 320 nm for phenolic acids, and 520 nm for anthocyanins. (Peak numbers refer to Table 1.)

isorhamnetin, and kaempferol in the buds of black currant (Figure 5 at 360 nm). In addition, peak 23 with ($M - H^-$) at m/z 301 was identified as quercetin aglycone with fragment ions at m/z 151 and 179 present only in the buds. Kaempferol-3-*O*-rutinoside (peak 17) with ($M - H^-$) at m/z 593 producing a fragment ion at m/z 285 was for the first time identified in black currant leaves (Figure 6). This compound has previously been identified in the leaves of, for example, strawberry and raspberry.^{27,41}

In this study, the flavonols identified at 360 nm in buds (Figure 5), leaves (Figure 6), and fruits (Figure 7) are in agreement with published literature examining the phenolic profile in black currant.^{15,16,26,27}

Monomeric Anthocyanins. In this study, four major monomeric anthocyanins, delphinidin-3-*O*-glucoside with ($M - H^-$) at m/z 463.1, delphinidin-3-*O*-rutinoside with ($M - H^-$) at m/z 609, cyanidin-3-*O*-glucoside with ($M - H^-$) at m/z 447, and cyanidin-3-*O*-rutinoside with ($M - H^-$) at m/z 593, were identified at 520 nm in buds (Figure 5), leaves (Figure 6), and fruits (Figure 7), respectively. The dominant anthocyanins in the fruits were glucosides and rutinosides of delphinidin and cyanidin. In both buds and leaves, cyanidin-3-*O*-glucoside (peak 8) and cyanidin-3-*O*-rutinoside (peak 9) were the most abundant anthocyanins. Previous studies have shown that anthocyanins are the most abundant phenolic compounds in black currant fruits and extracts, contributing up to about 97%

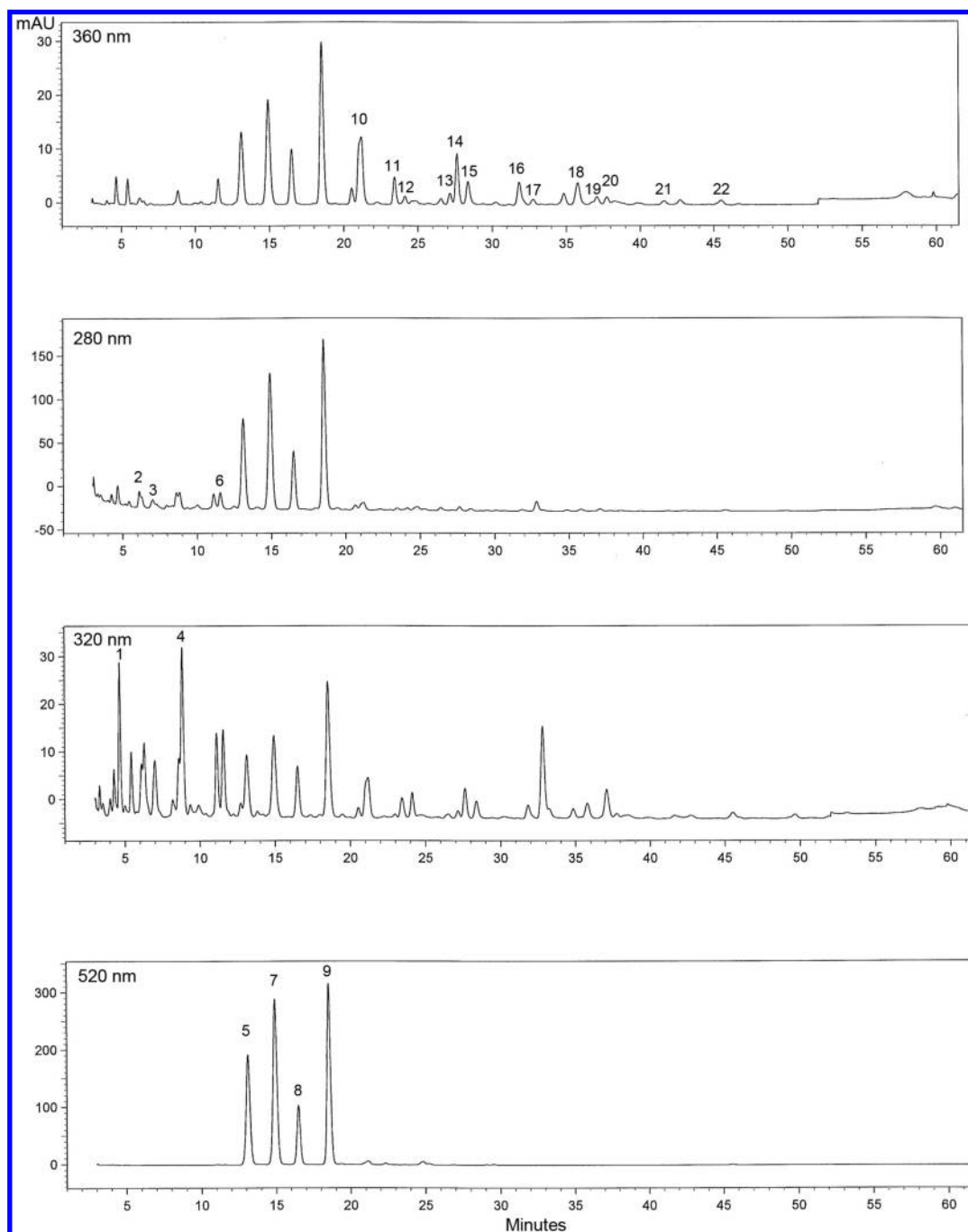


Figure 7. HPLC-DAD chromatogram of phenolic profiles for black currant fruits monitored at 360 nm for flavonols, 280 nm for flavan-3-ols, 320 nm for phenolic acids, and 520 nm for anthocyanins. (Peak numbers refer to Table 1.)

of the total anthocyanin content.^{15,20} The identified monomeric anthocyanins and the content of total anthocyanins were in accordance with previous studies.^{14,16,20,35}

In conclusion, on the basis of different extraction solvents, extraction time, choice of mobile phase, and gradient, the conditions for identification and characterization of phenolic compounds were successfully optimized. The extraction procedures developed for specific plant parts are less laborious and more efficient as compared to existing protocols,²⁶ thereby making the procedure suitable for analysis of large number of samples. A total of 23 compounds were identified in the buds, and 22 were detected in fruits and leaves. This is by far the most comprehensive study on characterization of phenolic

compounds in the buds of black currant. Furthermore, an HPLC-DAD method was developed for simultaneous quantification of several derivatives of hydroxycinnamic acids, flavan-3-ols, flavonols, and anthocyanins with good resolution and separation between the compounds in different plant parts of black currant. The HPLC-DAD method developed can assist in the quantification of different bioactive compounds, and thus be useful in breeding of plant material with enhanced nutritional content. The compounds identified in the less emphasized buds and leaves can be a great source of extracts for specific compounds and fractions for the functional food and health industry. This could offer possible avenues for breeding of black currants targeted for production of phenolic extracts

for pharmaceutical and medical use. Further investigations will focus on the effects of developmental stage, genotype, and environmental effects on these compounds, especially in the underutilized bud and leaves.

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Notes

The authors declare no competing financial interest.

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