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Article

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Stability of Hydroxycinnamic Acid Derivatives, Flavonol Glycosides and Anthocyanins in Black Currant Juice

Leenamaija Mäkilä [†,*], Oskar Laaksonen [†], Aino-Liisa Alanne [‡], Maaria Kortesniemi [†], Heikki Kallio [†,§], Baoru Yang [†]

[†] Food Chemistry and Food Development, Department of Biochemistry, University of Turku, FI-20014 Turku, Finland

[‡] Instrument Centre, Department of Chemistry, University of Turku, FI-20014, Turku, Finland

[§] Department of Food Science and Engineering, Jinan University, 510632 Guangzhou, China

* Corresponding author (Tel: + 358 2 333 6871; Fax: +358 2 333 6860: E-mail: leenamaija.makila@utu.fi)

Abstract

Stability of phenolic compounds was followed in black currant juice at ambient temperature (in light and in dark) and at + 4 °C for a year. Analyses were based on HPLC-DAD-ESI-MS(-MS^2) and HPLC-DAD-ESI-Q-TOF-MS methods supported by NMR after selective HPLC isolation. Altogether 43 phenolic compounds were identified, of which 2-(*Z*)-*p*-coumaroyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile, 2-(*E*)-caffeoyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile, 1-*O*-(*Z*)-*p*-coumaroyl-β-D-glucopyranose, (*Z*)-*p*-coumaric acid 4-*O*-β-D-glucopyranoside and (*Z*)-*p*-coumaric acid were novel findings in black currant juice. Hydroxycinnamic acid derivatives degraded 20–40% at room temperature during one-year storage, releasing free hydroxycinnamic acids. *O*-Glucosides of hydroxycinnamic acid compounds were the most stable followed by *O*-acylquinic acids, acyloxymethyleneglucosyloxybutenenitriles and *O*-acylglucoses. Light induced isomerization of (*E*)-coumaric acid compounds into corresponding *Z*-isomers. Flavonol glycosides stayed fairly stable. Flavonol aglycones derived mainly from malonylglucosides. Over 90% of anthocyanins were lost at room temperature in a year, practically independent on light. Storage at low temperatures, preferably excluding light, is necessary to retain the original composition of phenolic compounds.

Keywords: anthocyanins, black currant, flavonols, hydroxycinnamic acids, storage

INTRODUCTION

Fruits and berries are rich in phenolic compounds, such as anthocyanins, flavonols and phenolic acids. The compounds have been found to influence positively on human health.[1] They are synthesized via the phenylpropanoid pathway as secondary metabolites during development of plants, especially in response to UV radiation and other stress factors.[2] Anthocyanins are water soluble pigments that deliver blue and magenta colors to various fruits, berries and vegetables.[3] Flavonols are accumulated mainly in the skin of fruits due to the biosynthesis stimulated by light.[4] The highest concentrations of phenolic acids are also found in the skin of fruits, where they are esterified with pectins and arabinoxylans constructing the plant cell wall, with decreasing trend as the fruit ripens.[3]

In nature, phenolic compounds exist as glycosides and other derivatives. The most common anthocyanins and flavonols occur as *O*-glycosides at the carbon positions 3-, 5- or 7-.[5] A less studied group of phenolics, hydroxyphenylpropenoic acids, trivially named as hydroxycinnamic acids (HCAs), exist commonly as *O*-acylglucoses, *O*-acylquinic acids or as *O*-glucosides.[2] The most common HCAs found in nature are coumaric (compound **10**, Figure 1), caffeic (**8**) and ferulic acids (**12**). The acids rarely exist in free form unless the derivatives have undergone various processing steps[6] or storage.[7] Both *E*- and *Z*-forms of HCAs occur in plants, but the *E*-form is predominant, and also found to be more stable.[8] Other types of HCA derivatives are nitrile containing glucosides. This type of compounds were identified as coumaric and ferulic acid derivatives from black currant seed.[9] The nitriles are non-cyanogenic β-glucosides of β- or γ-hydroxynitriles, which do not release toxic hydrogen cyanide upon hydrolysis as a plant defense mechanism, as opposed to α-hydroxynitrile β-glucosides.[10]

HCAs have been demonstrated to possess various biological activities *in vitro* and *in vivo*, such as antioxidative, anti-inflammatory, anti-mutagenic, anti-bacterial, and anti-cancer properties.[2,11] The antioxidant capacity of free HCAs is shown to be higher than their corresponding glucose esters.[12]

Similarly, flavonol aglycones were found with stronger antioxidant capacity than their glycosides.[13] Anthocyanins and anthocyanidins vary in their antioxidant power depending on the structures.[14] Phenolic compounds contribute to sensory quality of products rich in phenolic compounds, such as fruit and berry juices. Especially hydroxycinnamic acids and flavonols are responsible of bitter and astringent sensations of the juices.[15-17] Storage often leads to changes in phenolic compounds due to temperature, light, pH, enzymes and oxygen. These factors induce hydrolysis of derivatives, releasing free HCAs and flavonol aglycones to the food matrix. [7,18,19] Hydrolysis of anthocyanidin glycosides releases free anthocyanidins, which are unstable, especially at pH values higher than 2. Thus, anthocyanidins are usually degraded further to hydroxybenzoic acids by thermal degradation[20,21], or they may polymerize or become co-pigmented with e.g. other phenolic compounds, forming more stable stuctures.[7,22,23] Long shelf-life is often applied to the fruit and berry products, which may cause drastic changes in phenolic compounds altering the sensory qualities. It is of high importance to study the impact of storage on phenolics at molecular level. The degradation of anthocyanins is fairly widely known. There is a severe lack of knowledge on the fate of individual HCA derivatives during storage, since earlier studies have mainly concentrated on the changes in the content of free HCAs.[7,18,19] Also, the structural stability of flavonols and their derivatives has not been sufficiently investigated in natural products.

The objective of this research was to investigate the structural stability of glycosides and other derivatives of phenolic compounds in black currant juice, pressed without macerating enzymes. Thorough identification of various derivatives of HCAs, flavonol and anthocyanidin glycosides as well as their degradation products, including aglycones, was conducted. The analytical tools for identification were HPLC coupled with DAD, ESI-MS(-MS^2), Q-TOF-MS, and NMR-analysis after

preparative HPLC fractionation. The main purpose of the study was to reveal the compositional changes of the less investigated HCA derivatives during storage.

MATERIALS AND METHODS

Materials. Black currants (*Ribes nigrum* L.) of cultivar 'Mortti' were cultivated in Southern Finland [Natural Resources Institute Finland (Luke) Piikkiö, Finland] and harvested in August 2010. Berries were frozen right after harvesting and kept at – 20 °C before juice pressing. Reference compounds of anthocyanins, delphinidin and cyanidin chlorides and flavonols were obtained from Extrasynthese (Genay, France) and the phenolic acids from Sigma (St. Louis, MO).

Sample preparation. Berries were pressed with a hydraulic laboratory-scale juice extractor without enzyme supplementation.[15] The juice yield was fixed to 60% using a pressure of 14 MPa. The juice was degassed in vacuum for three minutes. Juice was pasteurized in 50 mL transparent glass test tubes in a water bath at 100 °C. The juice temperature in the central part of the test tubes was monitored with a thermo-sensor Testo 177-T4 (Testo AG, Lenzkirch, Germany). Temperature was raised to 95 °C in 20–30 min, and kept at 95–97 °C for 30 seconds. Thereafter, the juice-tubes were transferred into an ice bath to cool down to approximately 20 °C in 30 min. The samples were stored for 3, 6, 9 or 12 months in refrigerator (+ 4 °C) or at ambient room temperature (the temperature varying from 19–27 °C). The samples stored at room temperature were kept on the same laboratory shelf either in dark (RT in dark) or exposed to surrounding light (RT in light). A juice sample was taken right after pasteurization to serve as the "baseline", to which other storage samples were compared. The baseline sample and the juice from each storage time point were stored in 10 mL falcon tubes at –20 °C until further analyses.

Before juice was transferred into falcon tubes, the microbial quality was tested. Cultivations were carried out using potato dextrose agar (LAB 98) and tryptone glucose extract agar (LAB 63) by the poured plate method (Lab M Limited, Lancashire, UK). No growth was detected in any samples in any storage time points.

Anthocyanins were extracted from the juices with acidified MeOH, as described in our previous studies.[15,24,25] The rest of the compounds were extracted with ethyl acetate by modifying an earlier method.[25] A portion of 15 g of juice was weighed and extracted four times with 10 mL of ethyl acetate. The extracts were combined and the evaporated product was diluted in 2 mL of MeOH and filtered (0.2 μm) for further analysis.

HPLC-DAD-ESI-MS(-MS^2) analysis. All the phenolic compounds and peaks **17** and **18** (Figure 1, Table 1) were analyzed with HPLC-DAD-ESI-MS and hydroxycinnamic acid compounds in addition by MS^2. The apparatus was a Waters Acquity Ultra Performance LC system in combination with a Waters 2996 DAD detector and a Waters Quattro Premier mass spectrometer (Waters Corp., Milford, MA) equipped with an ion-spray interface. An analytical column used for hydroxycinnamic acid and flavonol compounds, degradation products of anthocyanins (except anthocyanidins), and peaks **17** and **18**, was a 150 mm × 4.60 mm i.d., 3.6 μm, Aeris PEPTIDE XB-C18, with a 2.00 mm × 4.60 mm i.d. guard column of the same material (Phenomenex, Torrance, CA). The elution was carried out with a binary solvent system. Solvent A was 0.1% formic acid in Milli-Q water and solvent B was 0.1% formic acid in acetonitrile. The flow rate was 1.0 mL / min and the injection volume 10 μL. The gradient program for solvent B was 0–15 min, 2–18%; 15–20 min, 18%; 20–30 min, 18–20%; 30–35 min, 20–60%; 35–40 min, 60–2%; 40–45 min, 2%. Chromatograms were recorded by scanning the absorption at 190–600 nm. Hydroxycinnamic acids, as well as peak **18** were monitored at 320 nm, flavonols at 360 nm, and the anthocyanin degradation products, protocatechuic acid (**34**, Table 1) and 4-hydroxybenzoic acid (**35**) at 250 nm, phloroglucinaldehyde (**36**) at 290 nm and peak **17** at 290 nm.

After the DAD, a flow of 0.4 mL / min was directed to the mass spectrometer. The MS-data was acquired simultaneously in positive and negative ion modes, separately for MS^2-data, by applying the methods of Liu et al.[26] with modifications. The capillary voltage was 3.5 kV and 3.6 kV, the cone voltage 15 V and 22 V and the extractor voltage 3 V and 4 V, respectively, for the positive and negative ion mode. For the MS^2 analysis the capillary voltage was 3 kV and 3.6 kV, cone voltage 22 V and 22 V and extractor voltage 5 V and 4 V, respectively, for the positive and negative ion mode. The source temperature was 120 °C, and the desolvation temperature 300 °C. The desolvation gas flow was 600 L / h, and the cone gas flow 97 L / h. The collision energy for the daughter ion scan was 30 eV. Mass spectra (MS) were obtained by scanning larger ions between *m/z* 220 and 1000 and smaller ions between *m/z* 80 and 600 (full scan). MassLynx 4.1 software was used to operate the HPLC-ESI-MS(-MS^2) system.

In MS analysis of anthocyanins, the analytical column used was a 100 mm × 4.6 mm i.d., 2.6 µm, Kinetex C18 100A, with a guard column of the same support material (Phenomenex, Torrance, CA). Solvent A was 5% formic acid in Milli-Q water and solvent B was 100% acetonitrile. The flow rate was 1.0 mL / min and the injection volume 10 µL. The gradient program for solvent B was 0–1 min, 4–6%; 1–2 min, 6–8%; 2–6 min, 8–9%; 6–7 min, 9%; 7–10 min, 9–10%; 10–14 min, 10–11%; 14–18 min, 11%; 18–20 min, 11–18%; 20–21 min, 18–24%; 21–28 min, 24–80%; 28–29 min, 80–20%; 29–31 min, 20–4%; 31–35 min, 4%. Chromatograms were recorded by scanning absorption at 190–600 nm. Anthocyanins were recorded at 520 nm. After the DAD, a flow of 0.2 mL / min was directed to the mass spectrometer operated in the positive ion mode. The capillary voltage was 3.25 kV, the cone voltage 30 V and the extractor voltage 2.5 V. The source temperature was 150 °C, and the desolvation temperature 400 °C. The desolvation gas flow was 800 L / h, and the cone gas flow 47 L / h. Mass spectra were obtained by scanning ions between *m/z* 250 and 1000 (full scan).

HPLC-DAD-ESI-Q-TOF-MS analysis. The structures of hydroxycinnamic acid and flavonol metabolites and compounds **17** and **18** were further verified with LC-DAD-ESI-Q-TOF-MS system, which consisted of an Agilent HPLC 1200 Series instrument equipped with an Agilent G1315C Starlight diode array detector (Agilent Technologies, Waldbronn, Germany) and micrOTOF$_Q$ ESI-mass spectrometer (Bruker Daltonics, Bremen, Germany). The column and the HPLC method were the same as applied for HPLC-DAD-ESI-MS(-MS^2) analysis of HCA and flavonol compounds. After the DAD, a flow of 0.3 mL / min was directed to the ion source. The HPLC system was controlled by Hystar software version 3.2 (Bruker Biospin, Rheinstetten, Germany). The mass spectrometer was controlled by Bruker Compass 1.3 for micrOTOF control software (Bruker Daltonics, Bremen, Germany) and operated in the positive and negative ion mode by applying the earlier method with modifications.[27] The capillary voltage was 4.5 kV in positive ion mode and 4 kV in negative ion mode. The end plate offset was at –500 V. The pressure of the nebulizer gas (N_2) was set at 1.6 bar. The drying gas (N_2) flow was 12 L / min, and the drying gas temperature 200 °C. The mass range was from *m/z* 50 up to 2000 (full scan). The calibration was carried out with 5 mM sodium formate injected via a six-port-valve at the end of LC-MS experiment in order to provide high-accuracy mass measurements. The internal mass spectrum calibration was performed with HPC mode. The minimal number of calibration points was seven, and the standard deviation was below 1.5 ppm. The data were handled by Bruker Compass DataAnalysis (version 4.0 SP2; Bruker Daltonics, Bremen, Germany).

Quantitative analysis. For quantitative analysis the stored samples were analyzed in duplicates and the baseline juice five times. The duplicate samples mainly from separate pasteurization batches were prepared identically. All the compounds were quantitated with a Shimadzu LC-10AVP HPLC (Shimadzu, Kyoto, Japan) with a LC-10AT pump, a SIL-10A autosampler and a SDP-M10AVP diode array detector linked to the SCL-M10AVP data handling station. The column temperature was set to 30 °C. For hydroxycinnamic acid and flavonol compounds, degradation products of anthocyanins (except

anthocyanidins), and compounds **17** and **18**, the column, the binary gradient system, the injection volume and the wavelengths for monitoring the UV absorption spectra were the same as described earlier. For anthocyanin analyses, our earlier methods were used.[15,24,25] The analysis was carried out with an external standard method based on calibration curves. The total content of anthocyanins stored at various conditions was calculated by defining the descending curve $y = A \times e^{(-x/t)} + y0$ and using Origin 8 software (Originlab Corp., Northampton, MA).[16]

Purification and NMR analysis of selected compounds. Juice (4 kg) pressed in larger scale[28] was extracted with ethyl acetate following the procedure described above. A portion of 1-4 g of the evaporated extract was diluted into Milli-Q water (40 °C) to reach a final concentration of 100 mg / mL. The diluted sample was applied in a glass column (230 mm × 26 mm i.d.) packed with 34 g of Sephadex LH-20 (average dry particle size 70 μm) swollen in Milli-Q water overnight. The extract was purified with EtOH –Milli-Q water according to the elution gradient[29] as follows: F1A (50 mL), 100% water; F1B (200 mL), 100% water; F2 (200–300 mL), 10% EtOH in water; F3 (200–300 mL), 20% EtOH in water; F4 (200–300 mL), 30% EtOH in water; F5 (200–300 mL), 40% EtOH in water. An elution volume of 200 mL was used, when the amount of sample applied into the column was 1–2 g. A volume of 300 mL was used, when the sample amount was 3–4 g. Gradient was continued by stepwise increasing ethanol content from 50% to 80%, followed by aqueous acetone of two concentrations of 30% and 70%. Selected unknown compounds **14**, **15**, **17** and **18** (Table 1, Figure 1) eluted in fractions: F1B, **17**; F2–3, **14** and **15** and F4–5, **18**. The elution speed was maintained at 21 mL / min with a peristaltic pump. The fractions were evaporated to dryness using a rotary evaporator at 39 °C. Each dried fraction was recollected in 4–5 mL of MeOH. The fractions were analyzed with HPLC-DAD and HPLC-DAD-ESI-MS, using the same methods.

Compounds **14**, **15**, **17** and **18** in the Sephadex LH-20 fractions (F1B-F5) were purified by a semi-preparative system consisting of a SIL-20A autosampler, an LC-20AB pump, a CTO-10AC column oven, an SPD-20A UV-detector and a DGU-20A5 degasser. The column was a 250 mm × 10.0 mm i.d., 5 μm, Aeris PEPTIDE XB-C18, with a 10 mm x 10 mm i.d. guard column of the same material (Phenomenex, Torrance, CA). The fractions obtained from Sephadex purification were dissolved in MeOH:H_2O (1:1) and filtered (0.2 μm) before injection. Elution was carried out with a binary solvent system. Solvent A was Milli-Q water for isolation of the peak **17**. For the rest of the compounds **14**, **15** and **18** solvent A was 0.1% formic acid in water. Solvent B was 100% acetonitrile for the isolation of all the analytes. For **17**, the gradient program for solvent B was 0–9 min, 2%; 9–20 min, 2–20%; 20–30 min, 20–35%; 30–35 min, 35–68%; 35–40 min, 68–2%; 40–45 min, 2%. For isolating **14** and **15**, the gradient program for solvent B was 0–15 min, 2–18%; 15–24 min, 18%; 24–25 min, 18–14%; 25–34 min, 14–17%; 34–38 min, 17–65%; 38–40 min, 65–2%; 40–45 min, 2%. For **18**, the gradient program for solvent B was 0–15 min, 2–18%; 15–20 min, 18%; 20–30 min, 18–20%; 30–35 min, 20–60%; 35–40 min, 60–2%; 40–45 min, 2%. The flow rate was 4.9 mL / min. The column oven was kept at 40 °C. The chromatograms were recorded at 320 nm. Injections, ranging from 500–1500 μL, were repeated 25–50 times for each peak.

For confirmation of successful collections, the fractions were re-analyzed with HPLC-DAD and HPLC-DAD-ESI-MS. Fractions of the same peaks were combined and the solvents evaporated using a rotary evaporator at 30 °C, approximately for 45 min. After evaporation, the samples were dissolved in Milli-Q water and freeze-dried.

^{1}H and ^{13}C NMR spectra were obtained with an Avance Bruker 500 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) operating at 500.13 and 125.77 MHz, respectively. Spectra were recorded at 25 °C using CD_3OD as the solvent with a non-spinning sample in a 5 mm NMR-tube. The spectra

were calibrated on the solvent residual signal of CD_3OD at 3.31 ppm for ^1H and 49.15 ppm for ^{13}C and processed by TopSpin 3.2 software.

2-(Z)-p-coumaroyloxymethylene-4-β-D-glucopyranosyloxy-2-(Z)-butenenitrile (**15**, Figure 1). ^1H NMR (CD_3OD, 500.13 MHz): δ 7.67 (2'', 6'', d, J = 8.7 Hz), 6.95 (7'', d, J = 12.7 Hz), 6.81 (3, dd, J = 6.5 Hz, J = 5.9 Hz), 6.76 (3'', 5'', d, J = 8.7 Hz), 5.81 (8'', d, J = 12.7 Hz), 4.77 (10'', s), 4.64 (4a, dd, J = 14.5, J = 5.9), 4.51 (4b, dd, J = 14.5, J = 6.5), 4.33 (1', d, J = 7.8 Hz), 3.88 (6'a, dd, J = 12.0 Hz, J = 2.1 Hz), 3.68 (6'b, dd, J = 12.0 Hz, J = 5.2 Hz), 3.39–3.26 (3', 4', 5', m), 3.21 (2', dd, J = 7.8 Hz, J = 8.9 Hz). ^{13}C NMR (CD_3OD, 125.77 MHz): 167.2 (C-9''), 160.5 (C-4''), 149.3 (C-3), 146.9 (C-7''), 134.1 (C-2'', C-6''), 127.1 (C-1''), 116.3 (C-1), 116.1 (C-3'', C-5''), 115.4 (C-8''), 112.9 (C-2), 104.4 (C-1'), 78.2, 78.1, 71.6 (C-3', C-4', C-5'), 75.1 (C-2'), 68.4 (C-4), 64.3 (C-10''), 62.7 (C-6').

UV-A and sunlight irradiation of HCAs. Each reference compound of *E*-isomers of HCAs, i.e. *p*-coumaric acid, ferulic acid, caffeic acid, chlorogenic acid (3-*O*-caffeoylquinic acid) and neochlorogenic acid (5-*O*-caffeoylquinic acid) was irradiated separately in water solutions by UV-A and by sunlight. Concentrations of the reference compound in water solutions were 0.8 mg / mL, except of neochlorogenic acid was 0.1 mg / mL due to shortage of the compound. The solutions were divided in two batches of 25 mL volume each, one batch to be irradiated with UV-A and the other with direct sunlight. The solutions were transferred into 50 mL transparent glass test tubes, same as used in the storage of juice samples. The solutions of reference compounds were irradiated with a led-light emitting UV-A radiation (360–370 nm) with intensity of 41 W/m^2. The intensity was measured with photo/radiometer HD 2102.1 using a probe LP 471 UVA (Delta OHM, Padua, Italy). The distance of the probe to led-light was the same as distance from the bottom of the test tube to led-light (1.0 cm). Height of the spout irradiated was 6.5 cm and duration of irradiation 1.0 hour. The purified peak **14** (concentration 1.8 mg / mL) was also irradiated with UV-A, but in 3 mL MeOH with a spout of 1.2 cm.

The reference compounds of (*E*)-*p*-coumaric, (*E*)-ferulic and (*E*)-caffeic acid were kept in direct sunlight for 7 days in the middle of August 2015 and chlorogenic and neochlorogenic acid for 6 days.

Statistical analyses. Influence of storage on the metabolites was determined by analyses of variance (ANOVA) together with Tukey's post hoc and LSD test. One-way ANOVA was used for comparing the storage conditions at each time-point, and for comparing the samples in different conditions at all the time points (3–12 months of storage samples) with the baseline sample. One-way ANOVA was used also to compare the changes in the content of various groups of compounds. Student's *t*-test (equal variances assumed) was applied with less than three variables. The criterion for statistical significances was $p < 0.05$. ANOVA analysis and *t*-tests were performed using SPSS 21.0 (SPSS Inc. Chicago, IL).

RESULTS AND DISCUSSION

Identification of the metabolites. Identification of the analytes extracted with acidified methanol and ethyl acetate from black currant juices were based on the HPLC retention behavior, DAD spectra, ESI-MS(-MS^2) spectra, ESI-Q-TOF-MS spectra, and ^1H and ^{13}C NMR analysis of the isolated compounds, as well as analyses of reference compounds and literature comparisons.[9,15-17,20-22,24,25,30-43] For NMR analyses the extracts were pre-fractionated before HPLC isolation. Based on the results, the compounds were classified either as identified or tentatively identified (Table 1, Figure 1). The calculations to determine the probable origin of positive ion fragments in MS^2 -data in Table 1 were based on previous reports.[30,32]

The less investigated group of phenolic compounds, hydroxycinnamic acids (HCAs), was of special interest in our study. The HCAs were identified as caffeic, coumaric and ferulic acid derivatives (Figure 2, Table 1). Previously identified 1-*O*-(*E*)-caffeoyl- (peak **3**), 1-*O*-(*E*)-*p*-

coumaroyl- (**6**) and 1-*O*-(*E*)-feruloyl-β-D-glucopyranoses (**9**)[24,25,36] (Figure 1) were confirmed based on the bathochromic shift compared to the corresponding free HCA reference compound.[32] These *O*-acylated glucoses of HCAs shared a similar MS^2 fragmentation pattern of $[M + H - Glc - H_2O]^+$ in the positive ion mode. Tentatively identified HCA *O*-glucosides, presumably 4-*O*-β-D-glucopyranosides [17,32,33] of (*E*)-caffeic and (*E*)-*p*-coumaric acids were found to co-elute (peak **2**). 4-*O*-Glucosides were identified based on similar MS and MS^2 spectra as the corresponding 1-*O*-acylglucoses, and on elution before the corresponding 1-*O*-acylglucose (Table 1).[34] UV spectra of the 4-*O*-glucosides also showed the indicative hypsochromic shift compared to the free HCA reference compound.[34] The 4-*O*-glucoside of (*E*)-*p*-coumaric acid eluted before (*E*)-caffeoylglucose.[32] Three forms of *O*-acylquinic acids were detected: neochlorogenic acid[31] [5-*O*-(*E*)-caffeoylquinic acid, peak **1**], 3-*O*-(*E*)-*p*-coumaroylquinic acid[34] (peak **4**) and chlorogenic acid[34] [3-*O*-(*E*)-caffeoylquinic acid, peak **6**]. Chlorogenic acid co-eluted with (*E*)-*p*-coumaroylglucose showing low signal intensity in MS spectra compared to (*E*)-*p*-coumaroylglucose (Table 1).

Tentatively identified derivatives and free (*Z*)-*p*-coumaric acid (peaks **5**, **7**, **11** and **15**) were more abundant in the chromatogram of Figure 2B compared to 2A. *Z*-isomers of *p*-coumaric acid compounds eluted 0.5–2 minutes after the corresponding *E*-isomers[37], and had similar MS and MS^2 spectra, but the UV spectra of the *Z*-isomers showed hypsochromic shift compared to (*E*)-isomers. [35] From previous studies,[17,35] the 4-*O*-glucoside of (*Z*)-*p*-coumaric acid was also found to elute before the corresponding *E*-isomer. (*Z*)-*p*-coumaric acid was identified with a reference compound; irradiation with UV-A light and sunlight led to partial conversion of (*E*)-*p*-coumaric acid to the *Z*-isomer (Figure 3A). To our knowledge, this was the first time (*Z*)-*p*-coumaric acid and its derivatives were reported in black currant juice. *Z*-configurations of caffeic and ferulic acids as free form or derivatives were not detected.

p-Coumaric acid derivatives dominated in black currant juice whereas ferulic acid derivatives were not common. Feruloylquinic acid and 4-*O*-glucoside of ferulic acid were either not found, or they existed in trace amounts only. HCA derivatives of special interest were the four compounds **13**–**16** with higher molecular weight (Table 1, Figure 1 and 2), of which peaks **14** and **16** have earlier been identified in black currant seeds.[9] Odd number nominal masses [M] of *m/z* 437, 421 and 451 indicated the presence of odd number of nitrogen atoms in the molecules. Q-TOF-MS-data confirmed this preliminary identification giving mass error: |error ppm| < 5.8 (Table 1). These nitrogen containing compounds shared a similar fragmentation pattern, leaving an unknown residue of *m/z* 95 in the mass spectra of every compound (caffeic / coumaric / ferulic acid + *m/z* 162 (Glc) + *m/z* 95). Also MS^2 showed a positive ion fragment with *m/z* value of 114 $[M + H - (HCA - H_2O) - Glc]^+$ for every peak **13**–**16**. Thus, determining the *m/z* 95 residue for one compound would establish the structures for the other compounds as well. The peaks **14** and **15** gave similar UV, MS and MS^2 spectra, suggesting that these compounds were geometric isomers.

Peak **14** was selected to determine the structure of the nitrogen containing *m/z* 95 residue. Peak **15** was selected to possibly reveal the *Z*-isomer of the peak **14**. Peaks **17** and **18** were isolated for identification, due to their greatly increased contents during storage (Figure 2B). The UV spectra of these compounds did not correspond to HCA. After isolation with preparative HPLC, the structures of peaks **14**, **15**, **17** and **18** were further studied by ^1H and ^{13}C NMR using 1D-TOCSY, COSY, HSQC and HMBC measurements as additional tools.

Peaks **14** and **15** (Figure 1) were identified as geometric isomers: 2-(*E*)-*p*-coumaroyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile and 2-(*Z*)-*p*-coumaroyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile respectively. The NMR data of the peak **14** was published earlier.[9] The NMR data for the later-eluting peak **15** has not been

published previously and is now reported. Peak **17** was found to contain two forms of citric acid monomethyl esters: 2-hydroxy-1,2,3-propanetricarboxylic acid-2-methyl ester (symmetric, 16%) and 2-hydroxy-1,2,3-propanetricarboxylic acid-1-methyl ester (non-symmetric, 84%).[41] The structure of peak **18** was elucidated by NMR, and the data were consistent with those of flazin: 1-(5-hydroxymethyl-2-furyl)-β-carboline-3-carboxylic acid.[42]

Compounds **13** and **16** (Figure 1) were identified as 2-(*E*)-caffeoyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile and 2-(*E*)-feruloyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile, respectively. Identification was based on similar patterns in the MS, MS^2 and Q-TOF-MS -data (Table 1) with the peak **14**. Peak **15**, 2-(*Z*)-coumaroyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile was succeeded to be purified only up to 65% (Figure 4A). The rest of the isolate, 35%, was converted into the corresponding, more stable *E*-isomer (**14**). When optimizing the chromatographic separation of the peaks **14** and **15**, the difference in the retention times of the two isomers reached even 10 minutes. Thus, the presence of *E*-isomer was not due to the lack of chromatographic resolution, but due to spontaneous conversion of the isomers. Previous research[17] has pointed out the difficulties in purifying *E*- and *Z*-isomers of coumaric acid 4-*O*-glucosides. The isolated peak **14** had also partially (5%) converted into the corresponding *Z*-form (Figure 4A). The purity of both isomers was enough for the structure determination with NMR.

The chromatograms of flavonol compounds and anthocyanins are shown in Figures 2C and 2E. Flavonol glycosides were identified as flavonol 3-*O*-glycosides based on reference compounds and earlier studies.[16,24] The tentatively identified aureusidin glucoside (peak **22**) co-eluted with myricetin 3-*O*-β-D-(6''-malonyl)-glucopyranoside. This was seen as a shoulder of UV absorption maximum of 400 nm, typical for aureusidin,[36] in the spectrum of myricetin malonylglucoside (main absorption maximum 350 nm). Aglycones myricetin (**30**), quercetin (**31**), kaempferol (**32**) and isorhamnetin

(**33**) were seen in trace amounts in the samples after twelve months of storage (Figure 2C). The four major anthocyanidin 3-*O*-glycosides[16,24] were identified as delphinidin 3-*O*-β-D-glucopyranoside (**37**), delphinidin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**38**), cyanidin 3-*O*-β-D-glucopyranoside (**39**) and cyanidin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**40**) (Figure 2E, baseline sample).

Free delphinidin was detected in trace amounts and only in the baseline sample, even though the decline of the corresponding glycosides was drastic during storage (Figure 2E, 12-month chromatogram). To some extent, the anthocyanins degraded to hydroxybenzoic acids. The pH of the juice samples was ~2.9. In higher pH than 2.0, anthocyanins are thermally degraded through formation of chalcone structure.[20,21] Protocatechuic acid (peak **34**) and 4-hydroxybenzoic acid (**35**) were two abundant peaks in the 12-month samples (Figure 2D). They are known to be derived from the B-ring of cyanidin and pelargonidin, respectively. Phloroglucinaldehyde (**36**) was detected in minor concentrations from the possible cleavage of the A-ring of anthocyanidins.[20,21] To our knowledge, phloroglucinaldehyde has not been earlier identified from black currant. Gallic acid is known to be derived via cleavage of the B-ring of delphinidin[44], but was not found. Gallic acid would have co-eluted with peak **17**.

Influence of storage. Table 2 summarizes the critical information related to stability of various compound classes and individual metabolites during storage of the juice. The total content of HCA compounds in black currant juice at baseline was 2.8 mg / 100 g of which only 6% were free acids (Table 2). (*E*)-coumaroyloxymethyleneglucosyloxybutenenitrile (peak **14**; 1.1 mg / 100 g juice) was the major compound at baseline followed by (*E*)-*p*-coumaroylglucose / chlorogenic acid (**6**; 0.4), (*E*)-caffeoyloxymethyleneglucosyloxybutenenitrile (**13**; 0.3), (*E*)-feruloyloxymethyleneglucosyloxybutenenitrile (**16**; 0.3), (*Z*)-

coumaroyloxymethyleneglucosyloxybutenenitrile (**15**; 0.2), (*E*)-*p*-coumaroylquinic acid (**4**; 0.1) and (*E*)-caffeoylglucose (**3**; 0.1). Metabolites based on (*E/Z*)-*p*-coumaric acid (2 mg / 100 g juice) formed the major group of HCAs followed by caffeic (0.5 mg / 100 g) and ferulic (0.4 mg / 100 g) acid compounds.

The total content of HCA compounds in juice stored at room temperature in light decreased in three months by ca. 26%, and remained thereafter at lower level than in the other conditions. The decrease was due to loss in the total HCA derivatives which became significant also in dark at room temperature after nine months. *Vice versa*, the sum of free HCAs increased significantly during storage at room temperature, both in light and in dark, even by a factor up to four, due to their release via hydrolysis of their derivatives. Analogous decrease in the total HCA derivatives and increase in the corresponding free acids in white wine have been reported earlier.[19] The changes were minimal and statistically insignificant when stored in refrigerator (+ 4 °C).

The most obvious change induced by storage among coumaric, caffeic and ferulic acid compounds was increase in each of the corresponding free HCAs and decrease or minor increase in the derivatives. The contents of free (*E*)-caffeic (**8**) and (*E*)-ferulic acids (**12**) increased at room temperature by a factor 3–4 during 12 months, regardless of the light conditions. Free (*E*)- and (*Z*)-coumaric acids (**10**, **11**) were released in even higher degree. The very special feature was that formation of *Z*-isomer was dominant. Especially when juice was stored in light the content increased from 11–140 µg / 100 g (Table 2).

Analogously to increase in free HCAs, the contents of (*E*)-coumaroyl- (**6**), (*E*)-caffeoyl- (**3**) and (*E*)-feruloylglucoses **(9)**, as well as (*E*)-coumaroyl- (**14**) and (*E*)-feruloyloxymethyleneglucosyloxybutenenitriles (**16**) decreased significantly at room temperature

but the caffeoyl derivative (**13**) to a lesser extent. Another special characteristic of black currant juice was the light-induced conversion of (*E*)-coumaroylglucose and (*E*)-coumaroyloxymethyleneglucosyloxybutenenitrile into the corresponding *Z*-forms. When stored at + 4 °C, the changes were minimal, except for some formation of (*Z*)-coumaric acid, which seemed to be a sensitive indicator of the shelf-life of the juice.

Stability of the sums of HCAs and their derivatives were compared to each other (Table 2). Derivatives of (*E/Z*)-*p*-coumaric acids were commonly very sensitive to light, but at 12-month time point (*E*)-ferulic acids had degraded even more. In dark, (*E*)-ferulic acid derivatives degraded the most, but (*E*)-caffeic acids stayed fairly stable. Therefore, the second OH-group in the HCA seemed to stabilize the system. As a summary, at room temperature, *O*-acylglucoses of HCAs were the most labile type of derivatives followed by acyloxymethyleneglucosyloxybutenenitriles and *O*-acylquinic acids. HCA *O*-glucosides were rather stable, showing even an increase in their contents. *O*-Acylglucoses and *O*-acylquinic acids of HCAs are degraded by the loss of sugar or quinic acid moiety, acyloxymethyleneglucosyloxybutenenitriles by the loss of the oxymethyleneglucosyloxybutenenitrile moiety, all resulting in free HCAs.

The *p*-coumaric acid derivatives deserve special attention, as sunlight and UV-light are known to induce isomerization of (*E*)-cinnamic acids into the corresponding *Z*-forms.[45] Most of the isomerization induced by UV-light in nature is due to UV-A.[46] *E*-isomers of HCA reference compounds and the purified (*E*)-coumaroyloxymethyleneglucosyloxybutenenitrile (**14**) were irradiated by UV-A (360–370 nm) and direct sunlight (Figure 3). (*E*)-*p*-coumaric acid was converted into the corresponding *Z*-isomer under UV-A (one hour) and sunlight (one week) by 25% and 34%, respectively (Figure 3A). The corresponding conversions of ferulic acid were 15% and 19%, respectively (Figure 3B). Caffeic acid, as free or *O*-acylated to quinic acid (chlorogenic and

neochlorogenic acids) (Figures 3C-E), showed only minimal conversion. The second OH-group of caffeic acid might have stabilized the ring system. The minor peaks eluting after the *E*-isomers of caffeic and chlorogenic acids might have indicated the presence of the *Z*-isomers. The purified peak of (*E*)-coumaroyloxymethyleneglucosyloxybutenenitrile (**14**) was found to contain 8% of the corresponding *Z*-isomer (Figure 3F). After UV-A irradiation, proportion of the *Z*-isomer increased up to 21%.

The total content of flavonol compounds in black currant juice was before storage 1.6 mg / 100 g of which 90% consisted of glycosides and the rest of aglycones (Table 2). Myricetin formed 60% and quercetin 30% of the phenolic components, whereas kaempferol and isorhamnetin were minor compounds. Changes of the total amount of flavonol compounds were small at + 4 °C and not statistically significant. Table 2 indicates, however, that flavonol aglycones had a common trend to be released at room temperature when compared with the samples in refrigerator. Free quercetin (**31**) increased significantly during storage and about doubled in nine months at room temperature, both in dark and in light. Myricetin was liberated more slowly reaching a significant change only after nine-month storage.

Malonylglucosides of myricetin (**22**) and quercetin (**26**) decreased gradually during storage at room temperature. Of quercetin malonylglucoside, only a fraction of the original quantity was left after six months, but the corresponding myricetin compound decreased by ca. 60% only. In refrigerator the changes did not reach the level of significance even in the more labile quercetin derivatives. Formation of flavonol aglycones seemed to be derived from malonylglucosides, not from other *O*-glycosides. Bimpilas et al.[18] found also an increase in flavonol aglycones during one year storage in wines.

Anthocyanins were clearly more affected by storage than the other phenolic compounds. In the beginning of the trial, anthocyanins at the level of 30 mg / 100 g formed the major group of phenolics and decreased by ca. 50% in one year in refrigerator (Table 2). The most drastic decline in the total anthocyanins ended up to trace amounts only (0.1 – 0.2 mg / 100 g), when stored at room temperature for one year. Reduction followed a descending curve ($y = A \times e^{(-x/t)} + y0$) and was practically independent of light, which was in contradiction to results of a previous study.[47] By using the equation above, it takes eight years for the anthocyanins to degrade in refrigerator to the same extent as they are degraded at room temperature within a year. Wilkes et al.[7] found that anthocyanins decreased in a linear manner up to five months storage at 25 °C. Stability of cyanidin and delphinidin glycosides was similar when stored at room temperature, but delphinidin glycosides were more stable when stored in refrigerator (Table 2). The sugar moieties had no effect on the stability of anthocyanins at room temperature. In refrigerator, glucosides seemed to be more stable compared to rutinosides of one and the same anthocyanidin.

Protocatechuic acid (**34**), the dominant degradation product of cyanidin glycosides, showed a four-fold increase in one year at room temperature in both storage conditions. Minor degradation products were 4-hydroxybenzoic acid (**35**) and phloroglucinaldehyde (**36**). The characteristic UV absorbance maximum of 4-hydroxybenzoic acid at 253 nm was seen only after six months of storage at room temperature. Before storage for six months the second absorbance maximum was at 217 nm.

Citric acid methyl esters (**17**, Figure 1, Figure 2B) eluted as a one broad peak. The peak **17** showed some minor increase at room temperature during storage (Table 2). Content of the peak rose approximately 650%. Before pasteurization the second UV absorption maximum of the peak **17** was at 270 nm, not at its characteristic 290 nm. Thus, the heat treatment (+ 95 °C) seemed to alter the structure of the compound.

Flazin (**18**, Figure 1), belonging to the group of β-carboline alkaloids (Figure 2B) was found in the black currant juice. Flazin is a highly fluorescent, yellow-colored compound, which has been previously isolated from fermented soy bean products.[48,49] Deshydroxymethyl flazin and flazin methyl ether have been previously revealed in fresh black currant juice, which were also formed during storage by condensation of tryptophan and ascorbic acid or its degradation products, such as furfural.[50] Shaaban et al.[49] postulated that flazin was a microbial metabolite, since a sterile nutrient broth did not contain flazin, but the concentration increased during bacterial fermentation. The content of flazin increased drastically during storage at room temperature, but no change was seen at + 4 °C. The increased concentration of flazin in stored juices without any detected microbial growth was surprising.

In summary, a comprehensive profile of phenolics was revealed in black currant juice as fresh, as well as after one-year storage in various conditions. Altogether 43 phenolic compounds were identified, eight of which were found for the first time in the juice. Novel findings related to stability and hydrolysis patterns of various derivatives and also to their *E/Z*-isomerism were obtained. Both structure of the phenolic compartment and type of the derivative had an influence on the stability. The effects of light and temperature were analyte-dependent but all in all, storage at + 4 °C retained the composition well.

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ASSOCIATED CONTENT

The Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

Table S1: List of the reference compounds used for quantitation. Figure S1: Identification of anthocyanidins. Figure S2: ^{1}H NMR (CD_3OD, 500.13 MHz) spectrum of peak **15**. Figure S3: ^{13}C NMR (CD_3OD, 125.77 MHz) spectrum of peak **15**. Figure S4: Optimizing chromatographic separation of the peaks **14** and **15.** Figure S5: Decomposition of phenolic compounds and flazin. Figure S6: Decomposition of individual HCA compounds. Figure S7: Decomposition of individual flavonol compounds and anthocyanins. Table S2: Concentrations of the metabolites. Figure S8: Increase of peak **17** during pasteurization (PDF).

AUTHOR INFORMATION

Corresponding Author

* Phone: + 358 2 333 6871. Fax: +358 2 333 6860. E-mail: leenamaija.makila@utu.fi

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Notes

The authors declare that there is no competing financial interest.

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646 FIGURE CAPTIONS

647

648 **Figure 1**. Structures of compounds 1−18. Numbering of the peaks refers to Table 1.

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650 **Figure 2.** (A–D) Chromatograms of ethyl acetate extracts and (E) acidified MeOH extract of black
651 currant juice obtained via HPLC-DAD. (A) Baseline sample for HCA compounds, citric acid methyl
652 esters and flazin and (B) for the sample stored for 12 months at room temperature in light, monitored at
653 320 nm. (C) Chromatograms of flavonols monitored at 360 nm. (D) Chromatograms of the degradation
654 products of anthocyanins monitored at 250 nm and (E) chromatograms of anthocyanins monitored at
655 520 nm. The numbering of the peaks refers to Table 1.

656

657 **Figure 3.** The UV-A and sunlight irradiation of reference compounds and purified peak **14.**
658 Chromatograms were obtained when samples were irradiated with (b) sunlight, (c) UV-A or (a) without
659 any irradiation, analyzed by HPLC-DAD.

660

661 **Figure 4.** Isolation of peaks **14**–**18**. (A) Chromatogram shows the combined fractions of F2 and F3 (**14**,
662 **15**), (B) fraction F1B (**17**) and (C) combined fractions of F4 and F5 (**18**) from Sephadex LH-20
663 purifications obtained by semi-preparative HPLC. After isolation, the purity was checked using
664 analytical HPLC-DAD.

Table 1. UV, MS, MS^2 and Q-TOF-MS -data of the Metabolites in Black Currant Juice Stored for One Year.

No			MS (*m/z*)			MS^2 (*m/z*)		Q-TOF-MS (*m/z*)					
		λ_{max} (nm)	$[M+H]^+$ / $[[M]^+$**	$[M-H]^-$	$[2M-H]^-$	Pos. ions	Neg. ions	$[M+H]^+$ meas./calc.	$[M-H]^-$ meas./calc.	$[M+H]^+$ |error (ppm)|	$[M-H]^-$ |error (ppm)|	Molecular formula	Identification of the compound
HYDROXYCINNAMIC ACID COMPOUNDS													
1	5-*O*-(*E*)-caffeoylquinic acid (neochlorogenic acid) [e]	214, 325	355	353	707	145 [M+H–quinic acid–H_2O]$^+$, **163** [M+H–quinic acid]$^+$	**135**, 191	n.d.	353.0856/ 353.0878	n.d.	6.23	$C_{16}H_{18}O_9$	Ref. [a–c], UV, MS, MS^2, TOF-MS [30,31]
2	(*E*)-caffeic acid 4-*O*-β-D-glucopyranoside [(*E*)-caffeic acid *O*-glucoside] [d,e] and (*E*)-*p*-coumaric acid 4-*O*-β-D-glucopyranoside [(*E*)-*p*-coumaric acid *O*-glucoside] [d,e]	217, 296	343 / 327	341 / 325	683 / 651	145, 163	133, 161	327.1053 / 327.1074	341.0863/ 341.0878, 325.0943/ 325.0929	6.42	4.40 / 4.31	$C_{15}H_{18}O_9$/ $C_{15}H_{18}O_8$	UV, MS, MS^2, TOF-MS, [32-34]
3	1-*O*-(*E*)-caffeoyl-β-D-glucopyranose [(*E*)-caffeoylglucose] [e]	215, 329	343	341	683	145, **163** [M+H–Glc–H_2O]$^+$	133, **161**	n.d.	341.0875/ 341.0878	n.d.	0.88	$C_{15}H_{18}O_9$	UV, MS, MS^2, TOF-MS [24,25,32,34]
4	3-*O*-(*E*)-*p*-coumaroylquinic acid [(*E*)-*p*-coumaroylquinic acid] [e]	209, 309	339	337	675	119, **147** [M+H–quinic acid]$^+$	**119**, 163	339.1035 / 339.1074	337.0920/ 337.0929	8.55	2.67	$C_{16}H_{18}O_8$	UV, MS, MS^2, TOF-MS [24,25,30,32,34]
5	(*Z*)-*p*-coumaric acid 4-*O*-β-D-glucopyranoside [(*Z*)-*p*-coumaric acid *O*-glucoside] [d]	201, 281*	327	325	651	119, **147** [M+H–Glc–H_2O]$^+$	**119**, 163	n.d.	325.0917/ 325.0929	n.d.	3.69	$C_{15}H_{18}O_8$	UV, MS, MS^2, TOF-MS [17,35]
6	1-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranose [(*E*)-*p*-coumaroylglucose] [e] and 3-*O*-(*E*)-caffeoylquinic acid (chlorogenic acid) [e]	223, 315	327	325 / 353	651	119, **147** [M+H–Glc–H_2O]$^+$	117, **145**	n.d.	325.0922/ 325.0929, 353.0862/ 353.0878	n.d.	2.15 / 4.53	$C_{15}H_{18}O_8$/ $C_{16}H_{18}O_9$	Ref.[a–c] (chlorogenic acid), UV, MS, MS^2, TOF-MS [25,32,34,36]
7	1-*O*-(*Z*)-*p*-coumaroyl-β-D-glucopyranose	218,	327	325	651	119, **147** [M+H–	117,	n.d.	325.0921/	n.d.	2.46	$C_{15}H_{18}O_8$	UV, MS,

	[(*Z*)-*p*-coumaroylglucose] [d]	309*				Glc–H_2O]$^+$	**145**		325.0929				MS^2, TOF-MS[37]
8	(*E*)-caffeic acid [e]	218, 321*	181	179	n.d.	89, 117	n.a.	n.d.	179.0344/ 179.0350	n.d.	3.35	$C_9H_8O_4$	Ref.[a–c], UV, MS, MS^2, TOF-MS[31,38]
9	1-*O*-(*E*)-feruloyl-β-D-glucopyranose [(*E*)-feruloylglucose] [e]	218, 328	357	355	n.d.	**145** [M+H–ferulic acid–H_2O]$^+$, 177 [M+H–Glc–H_2O]$^+$	**160**, 175	357.1141 / 357.1180	355.1013/ 355.1035	10.92	6.20	$C_{16}H_{20}O_9$	UV, MS, MS^2, TOF-MS[24,25,32,34]
10	(*E*)-*p*-coumaric acid [e]	223, 308*	165	163	n.d.	**91**, 119	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	Ref.[a–c], UV, MS, MS^2 , TOF-MS[31,38]
11	(*Z*)-*p*-coumaric acid	219, 297*	165	163	n.d.	**91**, 119	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	Irradiated ref.[a] (Figure 3) UV, MS, MS[39]
12	(*E*)-ferulic acid [e]	219, 321*	195	193	n.d.	**89**, 117	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	Ref.[a-c] , UV, MS, MS^2, TOF-MS[31,38]
13	2-(*E*)-caffeoyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile [(*E*)-caffeoyloxymethylenegluco-syloxybutenenitrile]	219, 328	438	436	873	**114** [M+H–(caffeic acid–H_2O)–Glc]$^+$, 163 [caffeic acid–H_2O+H]$^+$	**135**, 179	438.1373 / 438.1395	436.1243/ 436.1249	5.02	1.38	$C_{20}H_{23}NO_{10}$	Based on peak 14, UV, MS, MS^2, TOF-MS
14	2-(*E*)-*p*-coumaroyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile [(*E*)-coumaroyloxymethyleneglucosyl-oxybutenenitrile] [e]	219, 313	422	420	841	114 [M+H–(coumaric acid–H_2O)–Glc]$^+$, **147** [coumaric acid+H–H_2O]$^+$	119, 163	422.1422 / 422.1446	420.1304/ 420.1300	5.69	0.95	$C_{20}H_{23}NO_9$	NMR, UV, MS, MS^2, TOF-MS[9]
15	2-(*Z*)-*p*-coumaroyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile [(*Z*)-coumaroyloxymethyleneglucosyl-oxybutenenitrile]	219, 307*	422	420	841	**114** [M+H–(coumaric acid–H_2O)–Glc]$^+$, 147 [coumaric acid+H–H_2O]$^+$	**119**, 163	422.1407 / 422.1446	420.1314/ 420.1300	5.21	3.33	$C_{20}H_{23}NO_9$	NMR, UV, MS, MS^2, TOF-MS
16	2-(*E*)-feruloyloxymethylene-4-β-D-glucopyranosyloxy-2-(*Z*)-butenenitrile [(*E*)-feruloyloxymethyleneglucosyl-oxybutenenitrile] [e]	218, 328	452	450	n.d.	114[M+H–(ferulic acid–H_2O)–Glc]$^+$, **177** [ferulic acid+H–H_2O]$^+$	**134**, 193	452.1523 / 452.1551	450.1396/ 450.1406	5.75	2.22	$C_{21}H_{25}NO_{10}$	Based on peak 14, UV, MS, MS^2, TOF-MS[9]

FLAVONOL COMPOUNDS														
19	myricetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (myricetin rutinoside) [e]	218, 352	627	625	n.a.	n.a.	n.a.	n.a.	625.1407/ 625.1410	n.a.	0.48	$C_{27}H_{30}O_{17}$	UV, MS, TOF-MS[15,16,40]
20	myricetin 3-*O*-β-D-glucopyranoside (myricetin glucoside) [e]	210, 354	481	479	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[15,16,40]
21	myricetin 3-*O*-arabinofuranoside (myricetin arabinoside) [e]	219, 346	451	449	n.a.	n.a.	n.a.	n.a.	449.0748/ 449.0725	n.a.	5.12	$C_{20}H_{18}O_{12}$	UV, MS, TOF-MS[15,16,40]
22	myricetin 3-*O*-β-D-(6″-malonyl)-glucopyranoside (myricetin malonylglucoside) [e] and aureusidin glucopyranoside (aureusidin glucoside) [d,e]	213, 352, 400s	567 /44 9	565	n.a.	n.a.	n.a.	n.a.	565.0820/ 565.0835	n.a.	2.65	$C_{24}H_{22}O_{16}$	UV, MS, TOF-MS[15,34,40]
23	quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (quercetin rutinoside) [e]	219, 353	611	609	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[15,16,40]
24	quercetin 3-*O*-β-D-glucopyranoside (quercetin glucoside) [e]	213, 353	465	463	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[15,16,40]
25	kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (kaempferol rutinoside) [e]	221, 346	595	593	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[15,40]
26	quercetin 3-*O*-β-D-(6″-malonyl)-glucopyranoside (quercetin malonylglucoside) [e]	219, 352	551	549	n.a.	n.a.	n.a.	n.a.	549.0874/ 549.0886	n.a.	2.19	$C_{24}H_{22}O_{15}$	UV, MS, TOF-MS[15,40]
27	isorhamnetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (isorhamnetin rutinoside)[e]	220, 355	625	623	n.a.	n.a.	n.a.	n.a.	623.1591/ 623.1618	n.a.	4.33	$C_{28}H_{32}O_{16}$	UV, MS, TOF-MS[34,40]
28	kaempferol 3-*O*-β-D-glucopyranoside (kaempferol glucoside) [e]	219, 346	449	447	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[15,16,40]
29	isorhamnetin 3-*O*-β-D-glucopyranoside (isorhamnetin glucoside) [e]	219, 353	479	477	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[40]
30	myricetin [e]	220, 370*	319	317	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[15,16,40]
31	quercetin [e]	221, 370*	303	301	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b], UV, MS[15,16,40]
32	kaempferol [e]	222, 367*	287	285	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b],UV, MS[15,16]
33	isorhamnetin [e]	223, 370*	317	315	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a,b],UV, MS[43]
ANTHOCYANINS**														
37	delphinidin 3-*O*-β-D-glucopyranoside	279,	465	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	UV,

	(delphinidin glucoside) [e]	526											MS[15,16,22]
38	delphinidin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (delphinidin rutinoside) [e]	276, 526	611	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	UV, MS[15,16,22]
39	cyanidin 3-*O*-β-D-glucopyranoside (cyanidin glucoside) [e]	279, 516	449	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	UV, MS[15,16,22]
40	cyanidin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (cyanidin rutinoside) [e]	280, 518	595	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	UV, MS[15,16,22]
	Anthocyanin degradation products (hydroxybenzoic acids)												
34	3,4-dihydroxybenzoic acid (protocatechuic acid) [e]	218, 259, 293s*	155	153	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a], UV, MS[20,21,36]
35	4-hydroxybenzoic acid [e]	198, 253*	139	137	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a], UV, MS[20,21,36]
36	2,4,6-trihydroxybenzaldehyde (phloroglucinaldehyde)	218, 291*	155	153	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Ref.[a], UV, MS[20,21]
CITRIC ACID METHYL ESTERS AND FLAZIN													
17	2-hydroxy-1,2,3-propanetricarboxylic acid-2-methyl ester (symmetric) and 2-hydroxy-1,2,3-propanetricarboxylic acid-1-methyl ester (non-symmetric) (citric acid methyl esters)	195, 291*	207	205	n.d.	**143**, 171	**111**, 143	207.0499 / 207.0499	205.0348/ 205.0354	0.00	2.93	$C_7H_{10}O_7$	NMR-analysis, UV, MS, MS^2, TOF-MS[41]
18	1-(5-hydroxymethyl-2-furyl)-β-carboline-3-carboxylic acid (flazin)***	220, 284, 354s 415s*	309	307	615	**206**, 263	**205**, 233	309.0870 / 309.0870	307.0710/ 307.0724	0.00	4.56	$C_{17}H_{12}N_2O_4$	NMR-analysis, UV, MS, MS^2, TOF-MS[42]

HCA and flavonol compounds, citric acid methyl esters, flazin and anthocyanin degradation products were identified from the ethyl acetate extract, and anthocyanins from the acidified MeOH extract of black currant juices stored for one year. N.d., not detected, n.a., not analyzed, ref., reference. The bolding in the MS^2-data represented the ion with higher intensity. The calculations to determine the probable origin of positive ion fragments in MS^2 -data in were based on previous reports.[30,32] [a,b,c] UV, MS or MS^2 data were recorded form the reference compound, respectively, and compared with that of the sample. Letter [d] indicated that compound was tentatively identified, the rest of the compounds were identified. Letter [e] indicated that the compound was earlier identified from black currant juice, wine, pomace or seed. *) λ_{max} –value was the UV absorption maxima of the 12 month storage sample (in MeOH) stored at RT in light, otherwise UV absorption maxima was analyzed from the baseline sample (in MeOH, pasteurized), s, maximum of the shoulder. (-) Flavylium cation was not detectable in

negative ion mode. **[)] The MS-data for anthocyanins (excluding anthocyanin degradation products) are molecular ions $[M]^+$ (m/z). *** See UV absorption spectrum from the Figure 4. See Figure 2 for the chromatogram and the numbering of the peaks.

Table 2. Concentrations of the Metabolites in Black Currant Juices Stored for One Year.

		Concentration (μg / 100 g of juice)			
	Compounds	Baseline	12-month storage		
No			RT in light	RT in dark	+ 4 °C
HYDROXYCINNAMIC ACID COMPOUNDS					
	Caffeic acids				
1	neochlorogenic acid	13.8 ± 1.2	15.6 ± 1.3	15.5 ± 3.3	15.4 ± 4.4
2	(*E*)-caffeic acid *O*-glucoside and (*E*)-*p*-coumaric acid *O*-glucoside	31.4 ± 1.8	40.8 ± 2.2*	41.4 ± 3.4*	32.0 ± 6.2
3	(*E*)-caffeoylglucose	97.5 ± 5.6	70.8 ± 0.94*	77.4 ± 3.3*	93.0 ± 18
6	(*E*)-*p*-coumaroylglucose and chlorogenic acid	370 ± 18	133.6 ± 10.0[b,]*	288.5 ± 1.2[a,]*	320 ± 20[a]
8	(*E*)-caffeic acid	48.4 ± 3.7	145.1 ± 7.8[b,]*	169.2 ± 1.8[a,]*	53.8 ± 5.9[c]
13	(*E*)-caffeoyloxymethyleneglucosyl-oxybutenenitrile	290 ± 36	240 ± 28	240 ± 22	260 ± 37
	Tot. caffeic acid derivatives**	430 ± 36	370 ± 32	370 ± 31	400 ± 65
	Tot. caffeic acid and its derivatives**	480 ± 39	510 ± 39	540 ± 33	450 ± 71
	Coumaric acids				
2	(*E*)-caffeic acid *O*-glucoside and (*E*)-*p*-coumaric acid *O*-glucoside	31.4 ± 1.8	40.8 ± 2.2*	41.4 ± 3.4*	32.0 ± 6.2
4	(*E*)-*p*-coumaroylquinic acid	120 ± 13	62.1 ± 5.2[b,]*	102.8 ± 9.4[a]	100 ± 13[a]
5	(*Z*)-*p*-coumaric acid *O*-glucoside	26.1 ± 4.4	30.9 ± 6.2	25.7 ± 5.2	23.2 ± 6.1
6	*(E)*-*p*-coumaroylglucose and chlorogenic acid	370 ± 18	133.6 ± 10.0[b,]*	288.5 ± 1.2[a,]*	320 ± 20[a]
7	(*Z*)-*p*-coumaroylglucose	30.0 ± 7.0	48.0 ± 2.3[a,]*	12.9 ± 2.8[c,]*	27.3 ± 4.5[b]
10	(*E*)-*p*-coumaric acid	73.1 ± 7.6	180.1 ± 6.5[b,]*	276.2 ± 6.4[a,]*	85.6 ± 9.7[c,]*
11	(*Z*)-*p*-coumaric acid	11.2 ± 4.6	140 ± 12[a,]*	83 ± 15[b,]*	20.4 ± 7.8[c]
14	(*E*)-coumaroyloxymethylene-glucosyloxybutenenitrile	1100 ± 26	530 ± 26*	880 ± 200*	940 ± 190
15	(*Z*)-coumaroyloxymethylene-glucosyloxybutenenitrile	220 ± 13	260 ± 35[a,]*	110 ± 9.6[b,]*	170 ± 32[ab]
	Tot. (*E/Z*)-*p*-coumaric acid derivatives	1900 ± 63	1100 ± 81*	1500 ± 230*	1600 ± 270
	Tot. (*E*)-*p*-coumaric acid derivatives	1600 ± 56	760 ±43[b,]*	1300 ± 210[ab,]*	1400 ± 230[a]
	Tot. (*Z*)-*p*-coumaric acid derivatives	270 ± 24	340 ± 39[a]	150 ± 18*[,b]	230 ± 42[ab]
	Tot. (*E/Z*)-*p*-coumaric acids and their derivatives	2000 ± 62	1400 ± 100*	1800 ± 250	1700 ± 290
	Ferulic acids				
9	(*E*)-feruloylglucose	110 ± 20	69 ± 11*	90 ± 13	100 ± 21
12	(*E*)-ferulic acid	24.9 ± 8.1	75.7 ± 3.1[a,]*	99 ± 14[a,]*	36.4 ± 5.5[b]
16	(*E*)-feruloyloxymethylenegluco-syloxybutenenitrile	250 ± 17	120.1 ± 9.4[b,]*	190 ± 19[ab,]*	230 ± 27[a]
	Tot. ferulic acid derivatives	360 ± 25	190 ± 20*	280 ± 32*	330 ± 48
	Tot. ferulic acid and its derivatives	380 ± 27	260 ± 23*	380 ± 46	360 ± 54
	Tot. *O*-acylglucoses of HCAs	610 ± 42	320 ± 19[b,]*	470 ± 18[ab,]*	550 ± 63[a]
	Tot. *O*-glucosides of HCAs	57.4 ± 5.1	71.7 ± 8.3	67.1 ± 8.5	55 ± 12
	Tot. *O*-acylquinic acids of HCAs**	130 ± 13	77.7 ± 6.3*	120 ± 13	120 ± 17

	Tot. acyloxymethyleneglucosyl-oxybutenenitriles of HCAs	1800 ± 38	1100 ± 97*	1400 ± 240*	1600 ± 280
	Tot. free HCAs and their derivatives	2800 ± 87	2200 ± 160*	2700 ± 320	2500 ± 410
	Tot. HCA derivatives	2600 ± 79	1600 ± 130*	2100 ± 280*	2300 ± 380
	Tot. free HCAs	160 ± 20	540 ± 29[a,]*	630 ± 37[a,]*	200 ± 29[b]
FLAVONOL COMPOUNDS					
	Myricetin				
19	myricetin rutinoside	73.8 ± 2.4	85.0 ± 2.8	72 ± 18	74 ± 10
20	myricetin glucoside	650 ± 22	730 ± 66	640 ± 170	620 ± 140
21	myricetin arabinoside	26.1 ± 1.6	23.2 ± 6.4	9.7 ± 3.0	23.6 ± 4.2
22	myricetin malonylglucoside and aureusidin glucoside	120.6 ± 6.0	52.8 ± 7.4[b,]*	56 ± 10[b,]*	96 ± 14[a]
30	myricetin	102.4 ± 9.8	140 ± 18*	130 ± 17	83 ± 35
	Tot. myricetin and myricetin glycosides	970 ± 29	1000 ± 100	910 ± 220	890 ± 200
	Quercetin				
23	quercetin rutinoside	87 ± 13	120 ± 10	100 ± 19	140 ± 38
24	quercetin glucoside	360 ± 12	390 ± 42	350 ± 69	330 ± 61
26	quercetin malonylglucoside	46.4 ± 4.0	5.8 ± 1.3[b,]*	2.9 ± 0.7[b,]*	34 ± 12[a]
31	quercetin	21.0 ± 4.2	42 ± 12*	43.5 ± 1.7*	20.1 ± 3.2
	Tot. quercetin and quercetin glycosides	510 ± 24	550 ± 66	500 ± 90	530 ± 110
	Kaempferol				
25	kaempferol rutinoside	24.5 ± 4.4	32.3 ± 9.0	27.7 ± 8.1	27.4 ± 3.8
28	kaempferol glucoside	59.2 ± 2.1	77 ± 12	62 ± 14	57 ± 14
32	kaempferol	12.8 ± 7.4	12.4 ± 3.5	16.3 ± 6.2	10.441 ± 0.030
	Tot. kaempferol and kaempferol glycosides	96.4 ± 7.4	120 ± 24	110 ± 28	95 ± 18
	Isorhamnetin				
27	isorhamnetin rutinoside	20.3 ± 2.0	16.5 ± 2.4	10.3 ± 4.5*	22.6 ± 5.1
29	isorhamnetin glucoside	22.2 ± 7.1	25.6 ± 4.0	18.9 ± 6.0	16.6 ± 5.4
33	isorhamnetin	8.6 ± 3.6	11.5 ± 2.8[ab]	12.81 ± 0.96[a]	4.94 ± 0.56[b]
	Tot. isorhamnetin and isorhamnetin glycosides	51.1 ± 9.4	53.6 ± 9.2	42.03 ± 0.60	44 ± 11
	Tot. FlaGly and FlaAgl	1600 ± 57	1800 ± 200	1600 ± 340	1600 ± 350
	Tot. FlaGly	1500 ± 54	1500 ± 160	1400 ± 310	1400 ± 310
	Tot. FlaAgl	140 ± 20	210 ± 36	200 ± 24	120 ± 39
ANTHOCYANINS					
37	delphinidin glucoside	3500 ± 150	19.9 ± 1.2[b,]*	12.2 ± 2.4[b,]*	1776.9 ± 2.5[a,]*
38	delphinidin rutinoside	16000 ± 500	109.2 ± 1.0[b,]*	68.2 ± 3.4[c,]*	7400 ± 11[a,]*
39	cyanidin glucoside	1100 ± 240	16.0 ± 3.0[b,]*	10.0 ± 5.5[b,]*	670 ± 19[a,]*
40	cyanidin rutinoside	9900 ± 890	48.10 ± 0.96[b,]*	29.7 ± 5.0[b,]*	4000 ± 87[a,]*
	Tot. delphinidin glycosides	19000 ± 650	129.07 ± 0.20[b,]*	80.4 ± 5.8[c,]*	9100 ± 14[a,]*
	Tot. cyanidin glycosides	11000 ± 1100	64.1 ± 2.0[b,]*	39.67 ± 0.49[b,]*	4700 ± 110[a,]*
	Tot. anthocyanins	30000 ± 1000	193.1 ± 1.8[b,]*	120.0 ± 6.3[b,]*	14000 ± 120[a,]*
	Anthocyanin degradation products (hydroxybenzoic acids)				

34	protocatechuic acid	230 ± 12	990 ± 36[a,*]	920 ± 50[a,*]	305.6 ± 5.3[b]
35	4-hydroxybenzoic acid	n.d. ***	185.7 ± 2.5	172.6 ± 5.9	n.d. ***
36	phloroglucinaldehyde	28.9 ± 6.1	29.6 ± 1.8	28.9 ± 6.9	28.5 ± 7.8
	Tot. Ant. degradation products	260 ± 13	1200 ± 40[a,*]	1100 ± 51[a,*]	334.1 ± 2.6[b]
CITRIC ACID METHYL ESTERS AND FLAZIN					
17	citric acid methyl esters	397.2 ± 6.6	482.1 ± 2.0	470 ± 78	410 ± 38
18	flazin	290 ± 99	1300 ± 53[a,*]	1600 ± 280[a,*]	320 ± 86[b]

Means and standard deviations of two replicates, baseline sample had 5 replicates, n.d., not detected. Significant differences between storage conditions are marked with a–c. *) Significant difference between baseline and 12 month storage sample. Statistical significance was based on One-way ANOVA with Turkey's HSD and LSD test ($p < 0.05$). **) Chlorogenic acid was not taken into account in the sum of total caffeic acid and its derivatives, total caffeic acid derivatives and total quinic acid derivatives. ***) At baseline and at 12-month storage in refrigerator (+ 4 °C) 4-hydroxybenzoic acid did not show its characteristic UV absorption maximum. See Table 1 and Figure 2 for the naming and numbering of the compounds.

Figure 1.

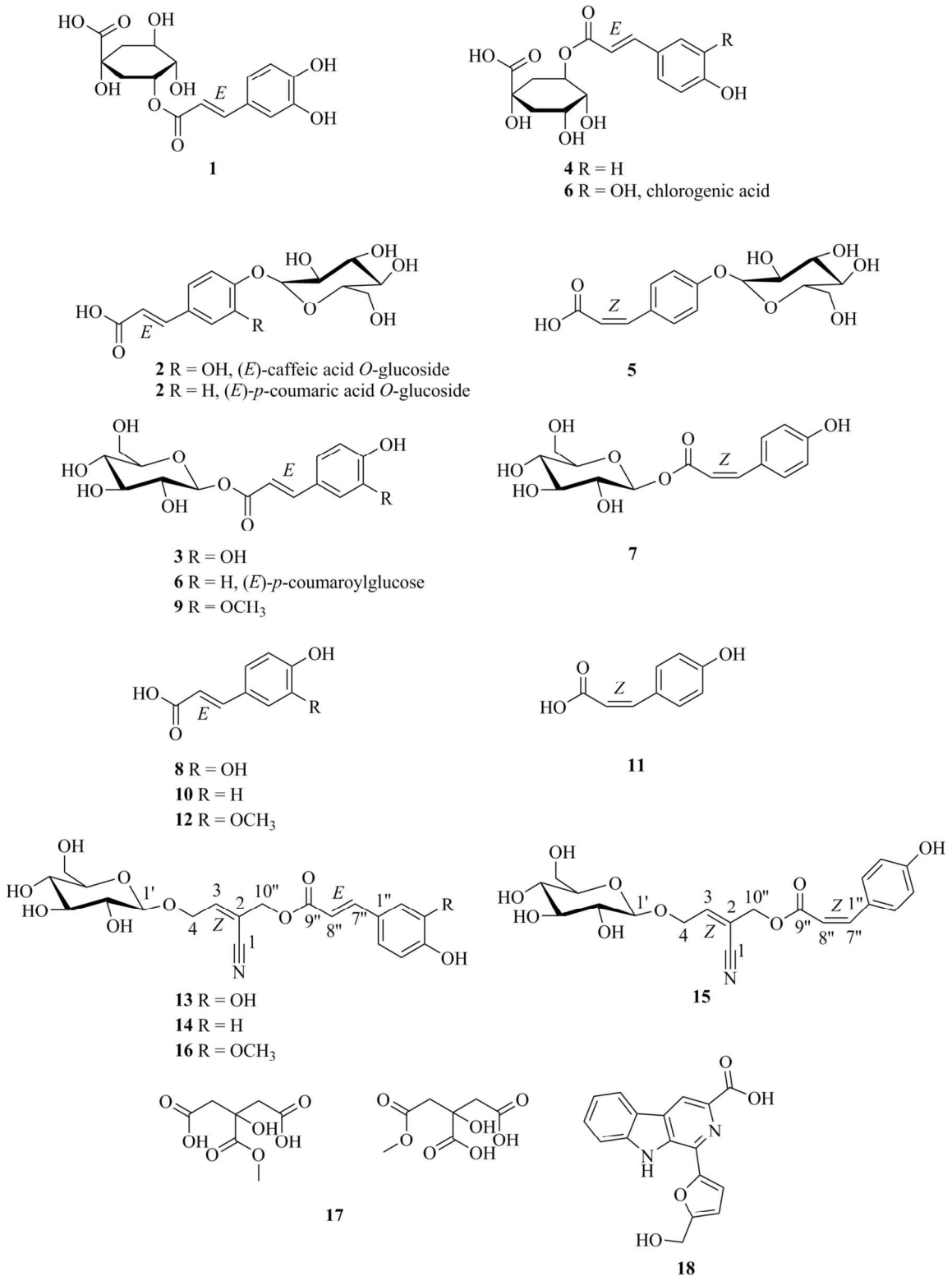

1
4 R = H
6 R = OH, chlorogenic acid
2 R = OH, (E)-caffeic acid O-glucoside
2 R = H, (E)-p-coumaric acid O-glucoside
5
3 R = OH
6 R = H, (E)-p-coumaroylglucose
9 R = OCH3
7
8 R = OH
10 R = H
12 R = OCH3
11
13 R = OH
14 R = H
16 R = OCH3
15
17
18

Figure 2.

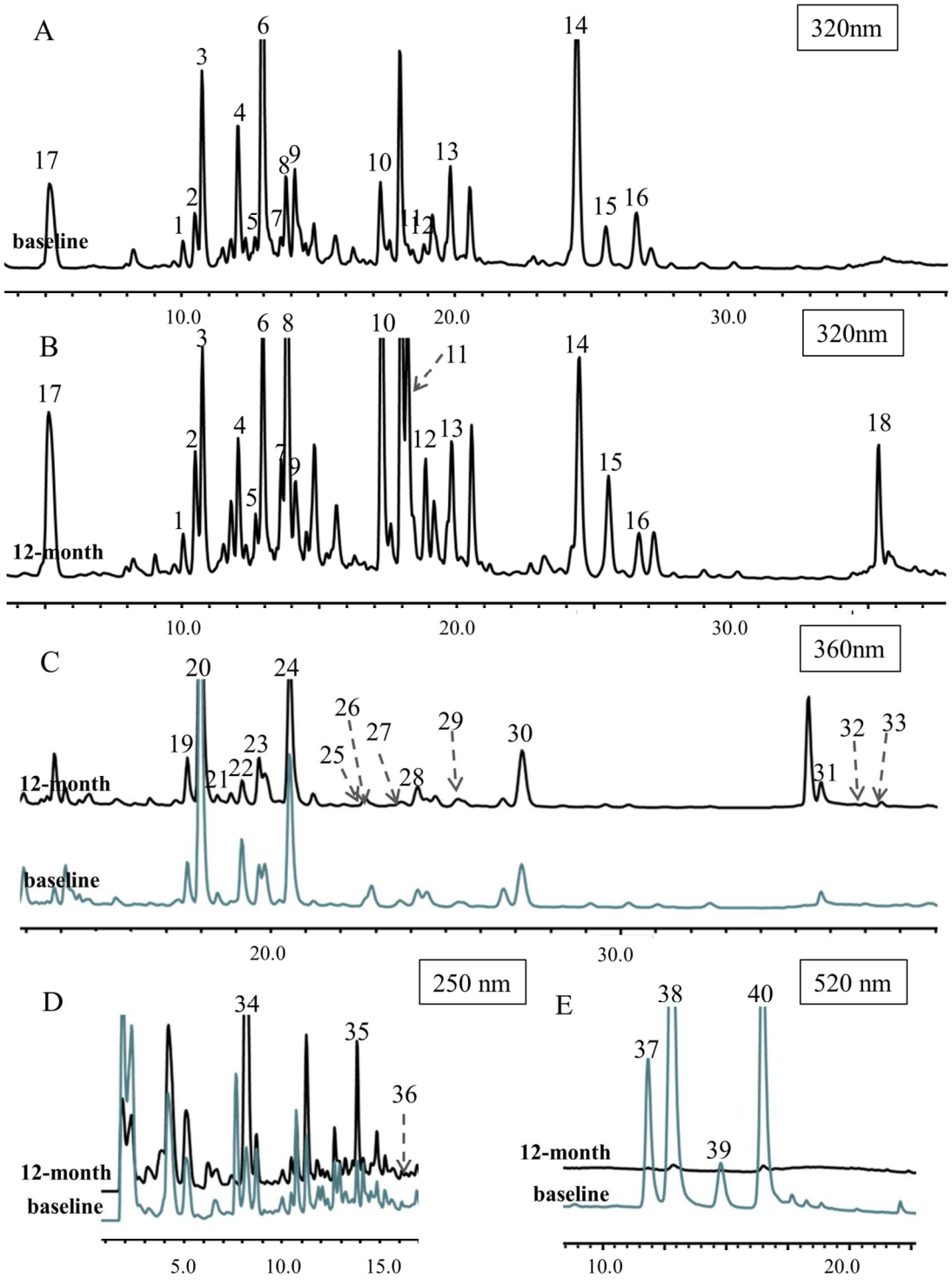

A
320nm
baseline
B
320nm
12-month
C
360nm
12-month
baseline
D
250 nm
12-month
baseline
E
520 nm
12-month
baseline

Figure 3.

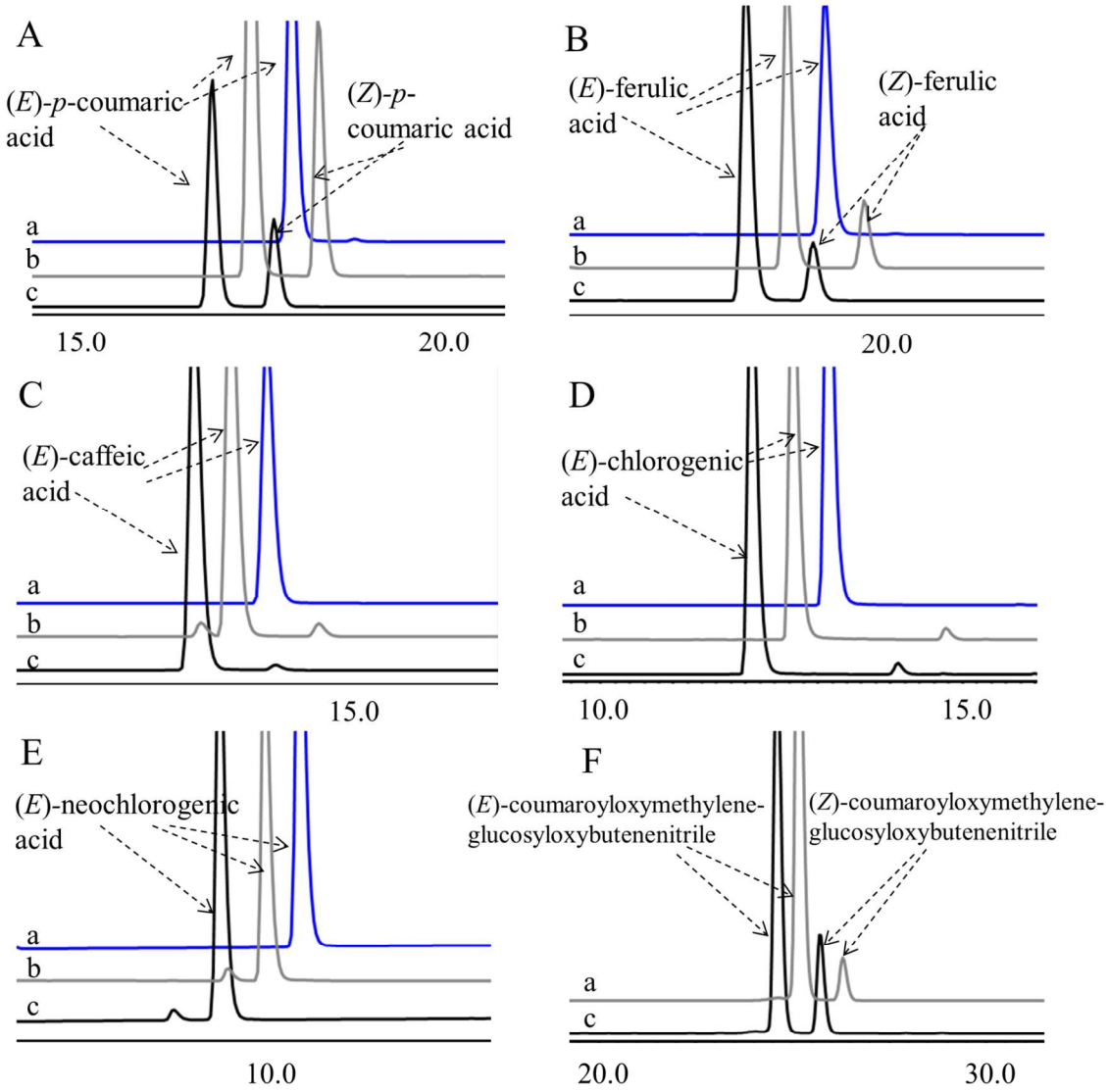
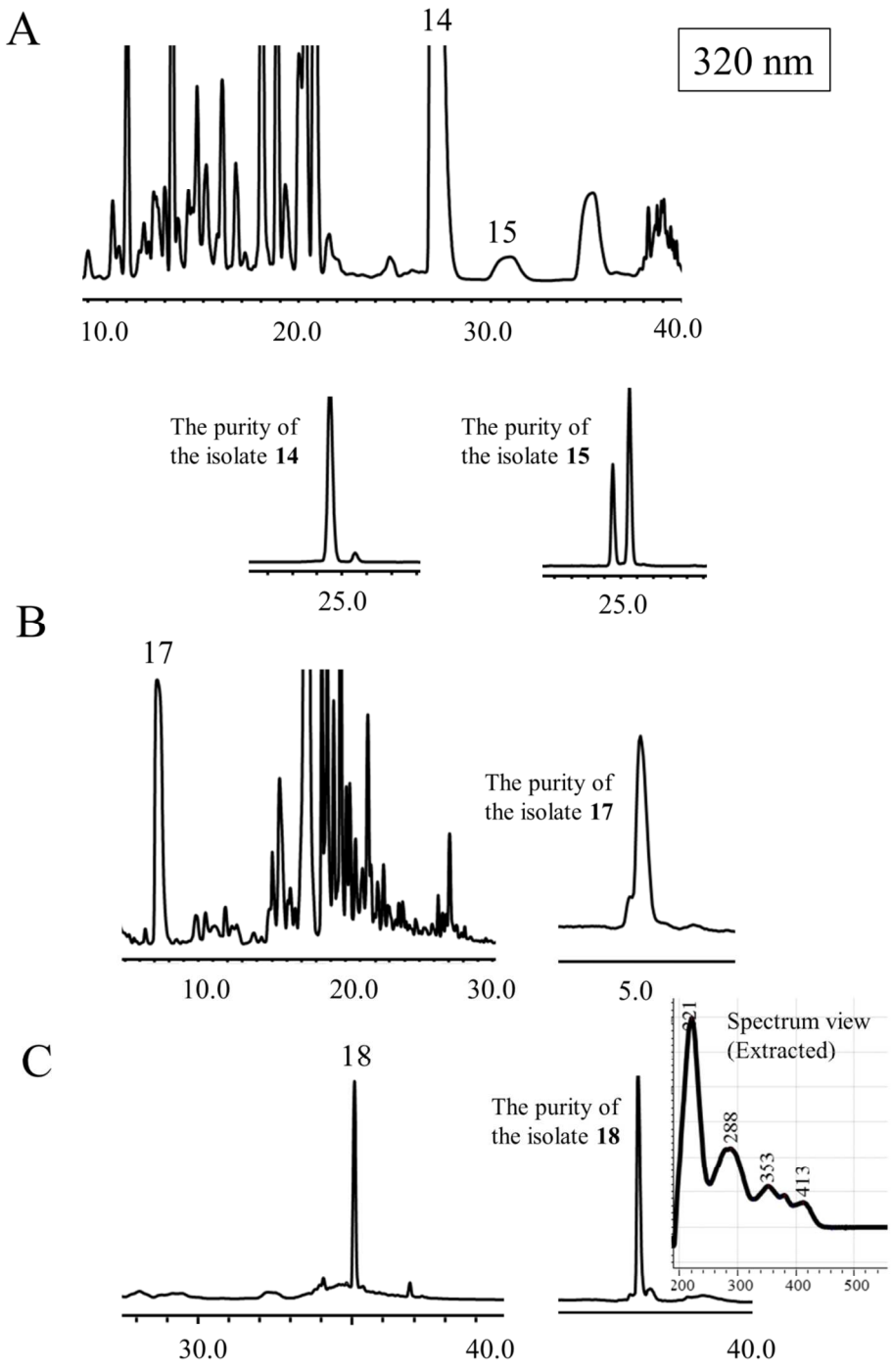

A
(E)-p-coumaric acid
(Z)-p-coumaric acid
a
b
c
15.0
20.0
B
(E)-ferulic acid
(Z)-ferulic acid
a
b
c
20.0
C
(E)-caffeic acid
a
b
c
15.0
D
(E)-chlorogenic acid
a
b
c
10.0
15.0
E
(E)-neochlorogenic acid
a
b
c
10.0
F
(E)-coumaroyloxymethylene-glucosyloxybutenenitrile
(Z)-coumaroyloxymethylene-glucosyloxybutenenitrile
a
c
20.0
30.0

Figure 4.


A
14
320 nm
15
10.0
20.0
30.0
40.0
The purity of the isolate 14
25.0
The purity of the isolate 15
25.0
B
17
10.0
20.0
30.0
The purity of the isolate 17
5.0
C
18
30.0
40.0
The purity of the isolate 18
40.0
Spectrum view (Extracted)
221
288
353
413
200
300
400
500

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