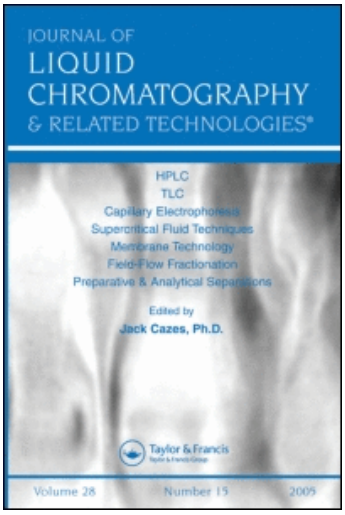

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

COLUMN-SWITCHING TECHNIQUE FOR SELECTIVE DETERMINATION OF FLAVONOIDS IN FINNISH BERRY WINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE ARRAY DETECTION

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Online publication date: 18 May 2000

J. LIQ. CHROM. & REL. TECHNOL., 23(9), 1339–1351 (2000)

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ABSTRACT

A high performance liquid chromatographic (HPLC) method with column-switching and diode array detection was developed for the determination of the flavonoids isoquercitrin, kaempferol, myricetin, quercetin, quercitrin, and rutin in three Finnish berry wines. Flavone was chosen as internal standard. Filtering was the only sample preparation before injection. The method was applied to the determination of the flavonoid profiles of wines made from black currants, blueberries, and crowberries. Myricetin, quercetin, and kaempferol were found in all three wines. In addition, the black currant wine contained considerable amounts of rutin and isoquercitrin. The repeatability of the relative retention times was good (RSD ≤ 0.5%). Detection limits were ≤ 60 ng/mL.

INTRODUCTION

Flavonoids are a class of naturally occurring and structurally related compounds, many of them present in plant tissues in relatively high concentrations as sugar conjugates.[1] Flavonoids contribute to the colour and flavour of fruits, flowers, and leaves, and protect the plant against animals, microbes and stress factors.[2] Interest in the flavonoids, and plant phenolic compounds in general, has recently been aroused because of their antioxidative,[3-5] anti-inflammatory,[6] anti-allergic,[7] and anticarcinogenic[8-10] activities. Dietary flavonoids have also been associated with reduced risk of coronary heart disease.[11,12]

Berries are a traditional part of the Finnish diet, the annual consumption standing at about 11 kilograms per person.[13] Among berries that are particularly rich in flavonoids are cranberries, lingonberries, black currants, red currants, blueberries, and crowberries.[14] In wines, flavonoids and other phenolic compounds contribute to several organoleptic characteristics, such as colour,[15] flavour, astringency,[16] and hardness. They also are an essential element in the evolution of wines.

Recently, a column-switching technique in liquid chromatography was applied to the analysis of quercetin, apigenin, and acacetin in human urine.[17,18] Otherwise, the column-switching technique has not been applied to the analysis of flavonoids in complex biological matrices. Column switching makes possible highly selective separations through the choice of different transfer technique and switching function. As well, the chromatographic modes of the separation can be changed during the overall process. The goals in applying column switching are to increase chromatographic resolution, selectivity and sensitivity, to enrich trace amounts of the sample, to protect sensitive detectors, and to speed up the column stabilisation.[19]

We describe an HPLC method with column switching for the separation of the flavonoids isoquercitrin, kaempferol, myricetin, quercetin, quercitrin, and rutin. The method was applied to the screening of the flavonoid profiles of three Finnish berry wines.

EXPERIMENTAL

Chemicals

The flavonoids were donated by the Division of Pharmacognosy (University of Helsinki, Department of Pharmacy, Helsinki, Finland). Acetonitrile and methanol were HPLC grade from Merck (Darmstadt, Germany) and J. T. Baker (Deventer, Holland). Distilled water was further purified with a Water-I instrument using 0.2 μm filters (Gelman Sciences, Ann Abor, MI, USA). All other chemicals were analytical grade.

Standards and Samples

Standard stock solutions of flavonoids were prepared by dissolving 1000 μg of flavonoid in 5 mL of methanol (200 μg/mL). The working standard solutions were prepared by diluting from the stock solutions with 25 mM Na_2HPO_4 buffer (pH=7.0). The samples of Finnish berry wines were donated by the Alcohol Testing Laboratory (Alko, Helsinki, Finland) and Raunin Tupa, Saariselän Marjat Oy. The chemical structures of the flavonoids and internal standard are presented in Figure 1.

Instrumentation

The HPLC system consisted of a Hewlett-Packard (Waldbronn, Germany) 1090 system with two pumps and a diode array detector (DAD). In addition,

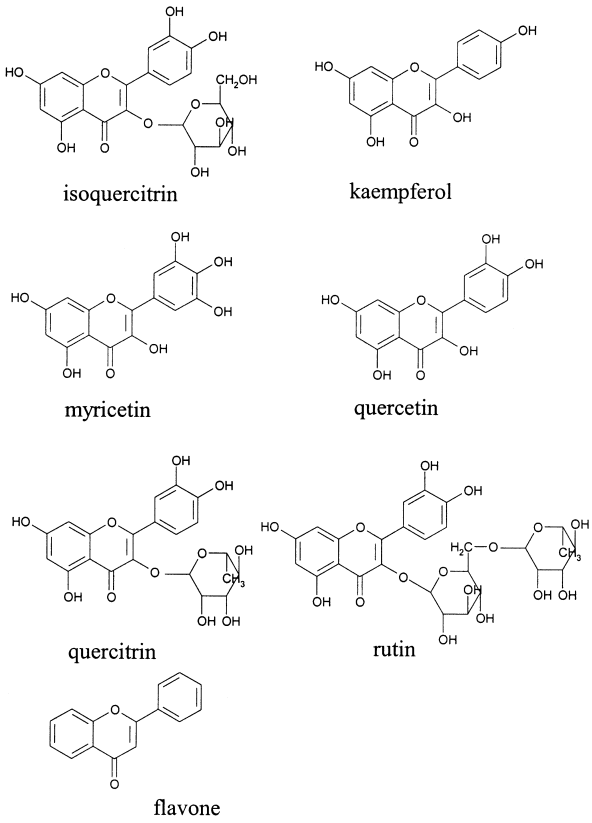

Figure 1. Chemical structures of the flavonoids studied and the internal standard flavone.

the system had an extra Jasco PU-980 pump (Tokyo, Japan) and a manual six-port column-switching valve (Valco Instruments Co. Inc., Houston, USA). The volume of the injection loop was 50 µL. The column switching system is described in detail in section column-switching procedure. The data were collected and analysed with a Hewlett-Packard computing system (HP ChemStation for LC, Rev. A.04.02).

Chromatographic Conditions

The sample clean-up column (50 x 2.1 mm) was packed with Capcell Pak C-18 material (SG, 5 µm, 120 Å, Shiseido, Japan). The analytical column (250 x 2.1 mm) was Capcell Pak C-18 (UG, 5 µm, 120 Å, Shiseido, Japan). The buffers were prepared daily by dissolving 0.8873 g of Na_2HPO_4 in 250 mL of water-I. The pH was adjusted to 2.5 and 7.0 with 25% HCl. The pH of the solutions was determined with a PHM 220 pH meter using combined pH electrode type pHC2401 (Radiometer, Copenhagen, Denmark). The analytical separation was achieved with the gradient presented in Table 1. The flow rate of the eluent was 0.2 mL/min and the column temperature was ambient. The diode array detector was set to monitor the signals of the analytes at wavelength 365 nm and the signal of the internal standard, flavone, at wavelength 330 nm.

Column-Switching Procedure

The mode of operation of the column-switching system can be seen from Figure 2.

Step 1 (0-2 min): Valve is in position A (injection). With pump 1, the clean-up column is rinsed with acetonitrile for 5 minutes (flow 1.0 mL/min) and with eluent consisting of 90% of 25 mM phosphate buffer (pH=7) and

Table 1

The Gradient Program for the HPLC Analyses

Time (Min)	ACN Concentration (%)
0	12
2	12
16	30
26	60
34	60

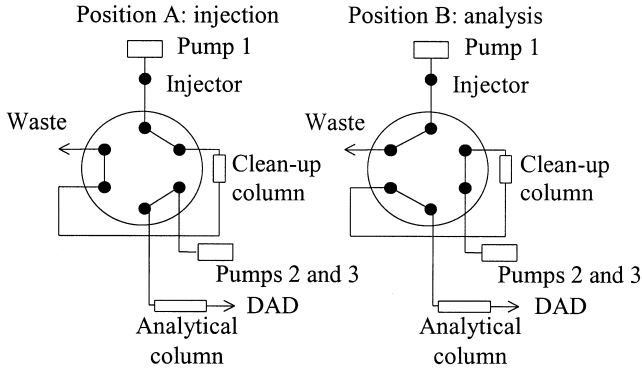

Figure 2. Schematic diagram of the column-switching system.

10% acetonitrile (v/v) for 5 minutes (flow 0.5 mL/min). The standard or sample solution of 50 µL is injected into the column with the latter solvent and for two minutes the disturbing matrix compounds are allowed to elute to waste. At the same time, eluent of 12% acetonitrile and 88% of 25 mM phosphate buffer (pH 2.5) is pushed through the analytical column with pumps 2 and 3.

Step 2 (2-26 min): Valve is switched to position B (analysis). A gradient of the mobile phase is passed through the clean-up column with pumps 2 and 3 and the retained analytes are eluted to the analytical column, where they were separated.

Step 3 (26-34 min): Valve is switched back to position A and rinsing of the clean-up column begins. The analytical column is allowed to stabilise before the next injection.

RESULTS

Optimisation of the Column-Switching System

Packing Material

Three different packing materials (C-18, C-8 and cyclohexyl) were tested for the retention of the analytes in clean-up column. The highest peak areas and heights under the selected chromatographic conditions were obtained with the C-18 material.

Eluent Composition

The eluent composition for the clean-up column (packed by Capcell Pak C-18) was optimised by testing methanol and acetonitrile alone and with different proportions of buffer. Berry wines also contain phenolic acids and they elute faster when being in ionised form. This was accomplished by setting the pH of the buffer to 7.0. The compounds were retained too strongly when the eluent contained only the buffer solution (25 mM, pH=7), so 10% of acetonitrile (v/v) was added into it. Acetonitrile, with its lower viscosity, was preferred to methanol because it gave narrower peaks, and the quantitation was easier because of the better separation.

Column-Switching Time

The two minutes for the valve switching was a compromise between time required for analysis and amounts recovered. Recoveries were not improved significantly with longer analysis times. At two minutes most of the disturbing polar compounds had eluted to waste. The total analysis time was 34 minutes.

Validation

Recovery

Recoveries were calculated by comparing the peak heights or areas of spiked samples passed through both columns with the peak heights or areas of a standard solution of the same concentration injected directly onto the analytical column. A blank berry wine matrix was not found; even white wines contained one or more of the flavonoids investigated. However, the white wine Aino gave flavonoid peaks that were easy to quantitate exactly. The heights and areas of the flavonoids in Aino were subtracted from the heights and areas of the spiked sample. The concentration of each flavonoid used in the recovery test was 2 μg/mL. The results with the relative standard deviations (RSD) are presented in Table 2.

Linearity and Detection Limits

The flavonoid standards were diluted with 25 mM Na_2HPO_4 buffer (pH=7) when the linearity was tested without the wine matrix. Over the concentration range of 0.25-8.0 μg/mL, the correlation coefficients were the same or better than 0.999, except for myricetin 0.666. It has been reported in the literature that myricetin is difficult to quantitate due to problems with instability.[20,21] However, when Aino matrix was spiked with the flavonoids, myricetin was stabilised and exhibited linearity with correlation coefficient higher than 0.997. Linearity of the compounds was good at the concentrations found in the wine samples. The detection wavelengths and limits (S/N=3) are given in Table 3.

Table 2

Recoveries of the Flavonoids According to Peak Height and Area*

Flavonoid Standard	Recovery % (Height ± RSD)	Recovery % (Area ± RSD)
Rutin	70 ± 3	97 ± 1
Isoquercitrin	65 ± 1	85 ± 1
Quercitrin	66 ± 2	98 ± 2
Myricetin	61 ± 4	86 ± 7
Quercetin	60 ± 5	85 ± 6
Kaempferol	52 ± 6	76 ± 7
Flavone	46 ± 3	94 ± 5

* When n = 6 and the concentration of each flavonoid was 2μg/mL.

Repeatability

Figure 3 shows the chromatogram for the standard solution of 2 μg/mL containing six flavonoids in Aino. The compounds are well resolved; even rutin and isoquercitrin were baseline separated. The retention times for the Aino matrix shown in Table 4 are given relative to the internal standard, flavone, which elutes at 30.4 minutes and is detected at wavelength 330 nm. The relative retention times of the six replicate analyses of the spiked wine sample were between 0.7 and 1.7%.

Table 3

Detection Limits for the Flavonoid Standards*

Flavonoid Standard	Wavelength (nm)	Detection Limit (ng/mL)
Rutin	365	60
Isoquercitrin	365	40
Quercitrin	365	60
Myricetin	365	30
Quercetin	365	20
Kaempferol	365	25
Flavone	330	20

* When S/N = 3

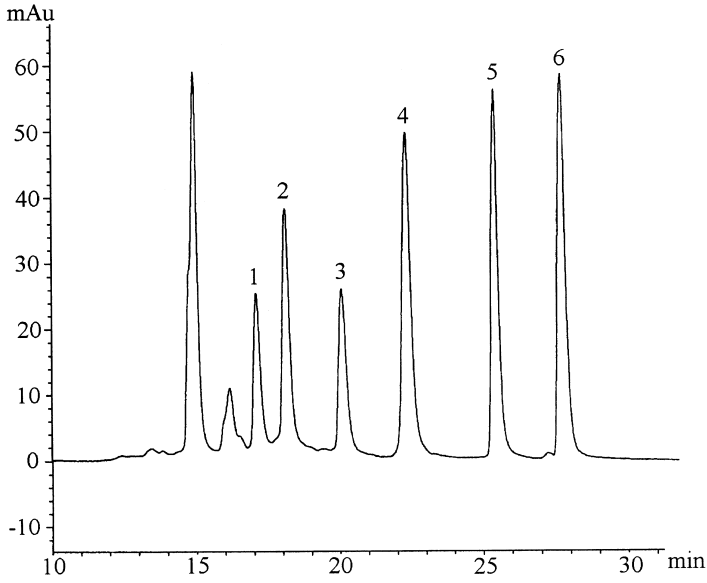

Figure 3. Chromatogram of the standard solution prepared in white wine Aino. Peaks: (1) rutin, (2) isoquercitrin, (3) quercitrin, (4) myricetin, (5) quercetin and (6) kaempferol. Concentration of the analytes was 2 μg/mL.

The day-to-day repeatability of the relative retention times was good (0.2-0.6%, n=8). The effect of the berry wine matrix on the retention times was assessed with raspberry wine, and the RSD was 0.3-0.5% (n=6). The repeatabilities of the peak heights and areas were 0.7-1.6% and 0.7-1.3% (n=6), respectively.

Table 4

Retention Times Relative to Flavone

Flavonoid Standard	Retention Time (Min)*
Rutin	14.8
Isoquercitrin	13.9
Quercitrin	12.0
Myricetin	9.9
Quercetin	6.8
Kaempferol	4.5

* Relative to flavone.

Quantitation

The LC chromatograms of the different berry wines are shown in Figure 4. Peak height was preferred for the quantitation because of nearby eluting matrix compounds. The results of the quantitation with standard deviations are listed in Table 5. The quantitation was done by internal standard method because the intensity of the peaks also depends on the matrix. The only wine matrix that contained quercitrin was the blueberry wine. Because of slightly overlapping peaks the compound could be identified but not quantitated.

DISCUSSION

Oszmianski et al.[22] have developed an off-line method for the fractionation of phenolic compounds in red wine. By passing the sample through the C-18 Sep-pak cartridge, they divided the red wine into four fractions containing phenolic acids (fraction I), catechins, procyanidins, anthocyanin monomers (fraction II), flavonols (fraction III), and anthocyanin monomers (fraction IV). The extraction efficiency for the quercetin was as high as 93%, compared with our recovery of only 85% (area). This can partly be explained by the different chromatographic conditions in our recovery test, which was done by the column-switching system. While the gradient was the same, the pressure in our system was lower and the distance that the sample had to elute was shorter when the sample was injected solely through the analytical column. Thus, the compounds eluted faster, with narrower peaks, and the efficiencies were higher. When the sample was injected with and without the column-switching valve, the column plate numbers for quercetin were 78260 and 48894, respectively, confirming the effect of the column-switching system on the peak efficiency and, thereby, also on the recovery results.

The flavonol concentrations of black currant, blueberry, and crowberry wines are reported here for the first time. However, berries have been investigated by other methods and the results have been expressed in various ways (e.g. fresh weight vs. dry weight). In addition, berries have elsewhere always been hydrolysed prior to analysis, leading to increased concentrations of aglycons such as quercetin. In our study the berry wine samples were analysed without hydrolysis, making it possible to detect the glycoside flavonols such as isoquercitrin, quercitrin, and rutin as well. Justesen et al.[21] found black currants to contain 37 mg/kg (fresh weight) and blueberries 73 mg/kg of quercetin after hydrolysis. The black currants also contained 1 mg/kg of kaempferol, but blueberries none. Myricetin was not found in either berry. Häkkinen et al.[23] have reported the amounts of quercetin, myricetin, and kaempferol in black currants after hydrolysis in seedless dry weight as 124.8, 66.56, and 24.96 mg/kg, respectively. We also determined the amounts of those three flavonols in the three berry wines we investigated.

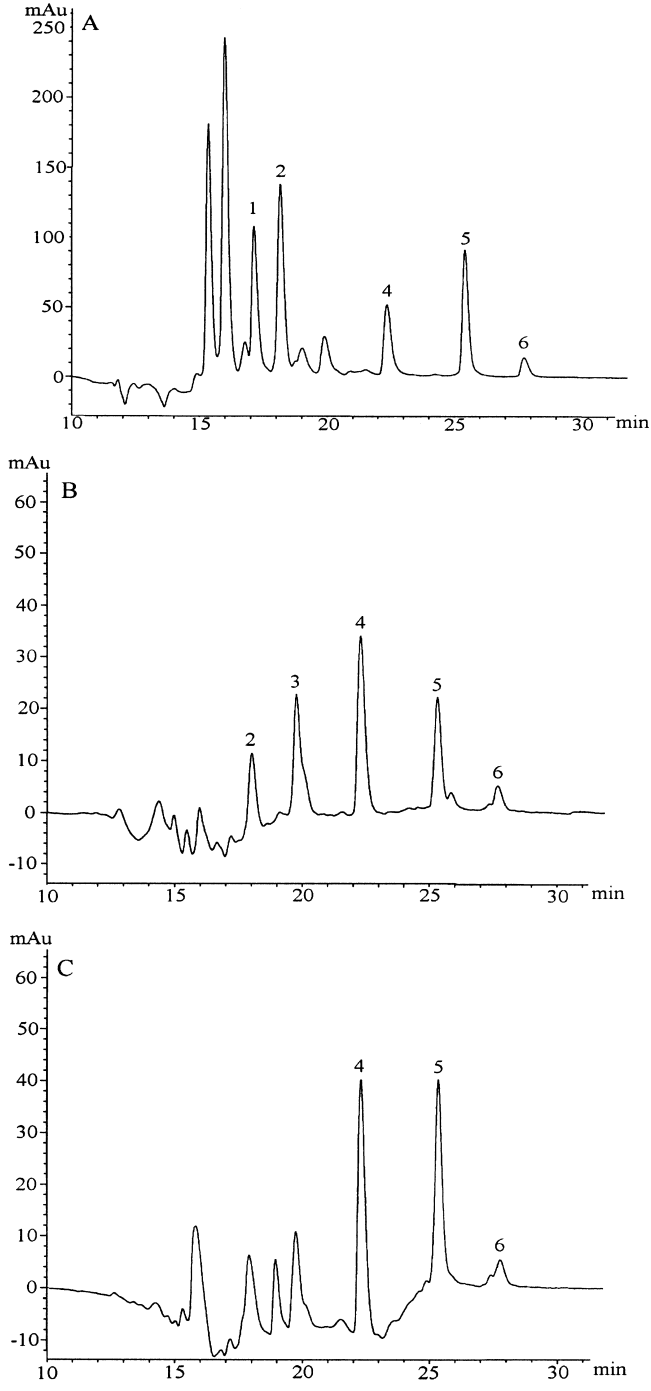

Figure 4. Chromatograms of the (A) black currant (B) blueberry and (C) crowberry wines. See Figure 3 for identification of peaks.

Table 5

Flavonoid Contents of Black Currant, Blueberry, and Crowberry Wines*

Wine	Rutin	Isoquercitrin	Myricetin	Quercetin	Kaempferol
Black currant	4.5 ± 0.5	4.8 ± 0.4	1.5 ± 0.1	2.4 ± 0.1	0.5 ± 0.05
Blueberry		0.9 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	0.2 ± 0.05
Crowberry			1.9 ± 0.2	1.4 ± 0.1	0.3 ± 0.1

* Presented as average value ± standard deviation (μg/mL), n = 4.

The only report of the antioxidant activity of berry and fruit wines is that of Heinonen et al.[24] In a study of the antioxidant activity and total phenolic content of the berry wines, they concluded that the antioxidant activities of wines made of mixtures of black currants and crowberries or billberries are superior to those of other berry wines, as well as to red wine. It is known that berry wines in general contain smaller amounts of phenolic compounds than red grape wines. Because it was also shown that the total phenolic content does not accurately correspond to the true antioxidant nature of their phenolic constituents, it is premature to draw any conclusions about the true antioxidant nature of the berry wines studied here.

CONCLUSIONS

We have described a selective method for the determination of flavonoids in berry wines. To our knowledge, ours is the first method that employs column-switching in the selective analysis of berry wines. The method was successfully applied to three berry wine matrices. All contained myricetin, quercetin, and kaempferol. In addition, considerable amounts of rutin and isoquercitrin were found in the black currant wine. The manual column-switching that was used can be easily automated with computer-programmable equipment, making it suitable for routine analysis.

ACKNOWLEDGMENT

The donation of the analytical column and packing material by the Shiseido Company (Japan) is gratefully acknowledged.

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Received August 22, 1999 Author's Revisions January 12, 2000
Accepted December 5, 1999 Manuscript 5160