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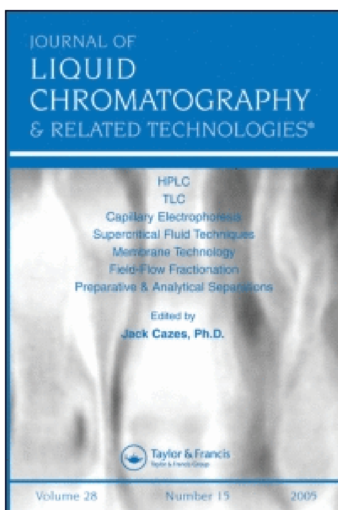

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Purification of a Flavonoid Sample by Countercurrent Chromatography

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PURIFICATION OF A FLAVONOID SAMPLE BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

A quercitrin rich vegetal flavonoid sample was fractionated using a new hydrodynamic CCC apparatus based on the Ito scheme IV design. The Kromaton CCC apparatus has two spools with a tube coiling allowing work with two different apparatus volumes. The small “analytical” volume was 94 mL and the large “preparative” volume was 1.07 L. The fractionation of the flavonoid sample was done using the two machine volumes. The bi phasic liquid system was a non chlorinated solvent system, butanone/water. UV detection was possible at 330 nm. In one run, 330 mg of the flavonoid sample could be fractionated by the CCC machine in six peaks that were collected in nine fractions.

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The HPLC and MS analysis of the fractions allowed identification of the main constituents of the sample. Quercitrin, a rhamno-flavonoid, was the major compound making up more than 88% w/w of the studied sample. The three minor identified flavonoids were quercetin, kaempferol, and kaempferol-3-rhamnoglucoside. Six very minor impurities were detected by the HPLC analysis of the fractions, but they were not unambiguously identified.

INTRODUCTION

Flavonoids are extracted from various plants. They all derived from flavone, a tricyclic aromatic molecule. Many flavonoids have a biological activity. They were used as capillary protectants, vasodilatators, anti anginal agents, and free radical quenchers, as non-exhaustive therapeutic categories. These polyphenols have a high polarity that renders them difficult to purify by classical liquid chromatography (LC).

Countercurrent chromatography (CCC) is a liquid chromatography technique that uses a liquid biphasic system: the stationary phase is one of the two phases and the mobile phase is the other liquid phase.[1] Since its inception by Yoishiro Ito in 1966,[2] the development of the CCC technique is the subject of numerous works and books.[3-6] CCC is a preparative technique.

The CCC machines have internal volumes ranging from 30 mL to liters. The chromatographic efficiency being limited, it is the loading capability and the versatility of the technique that make it attractive. The denser liquid phase or the lighter one can be used as the stationary phase. It is even possible to exchange the phase role during a run.[4-5]

CCC is able to separate highly polar solutes using a polar biphasic liquid system such as the water/butanol system. Tannins were separated with such a liquid system[7] or with the butanol/propanol/water system.[8] Flavonoids such as genkwanin were separated by CCC with a chloroform/methanol/water system.[9] Apigenin and leuteolin were separated using a similar liquid system.[10]

Chloroform and most chlorinated solvents should not be used due to environmental concern. Soon these solvents will be completely banned in any industrial process. It is important to find alternative solvent systems to perform the separation and purification of polar solutes. In this work, the fractionation of a flavonoid sample is performed using a new CCC machine and a non-chlorinated solvent system.

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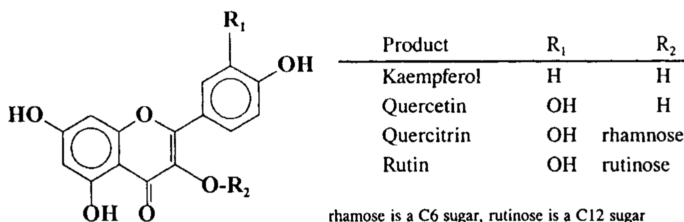

Product	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Quercitrin	OH	rhamnose
Rutin	OH	rutinose

rhamose is a C6 sugar, rutinose is a C12 sugar

Figure 1. Molecular structure of the flavonoids studied in this work.

EXPERIMENTAL

The Flavonoid Sample

A commercial sample denominated "quercitrin" was obtained from L. Light & Co Ltd., Colinbrode, England, and was used for the separation. 4 grams of a bright yellow powder were received. The flavonoid quercitrin is stated to be the main component of the yellow powdered sample. The other components that could be found with quercitrin are kaempferol and quercetin.

Quercitrin, $C_{21}H_{20}O_{11}$, m.w. 448, m.p. 178°C, is soluble in alcohols and alkaline solutions (intense yellow) where it oxidizes rapidly turning brown. It is not soluble in cold water nor in ether. It has a strong UV absorption at 258 and 350 nm. It is a rhamnoglycoside with the structural formula shown in Figure 1, in which the spatial structure (chiral structure) of the 3 sugar alcohols and the one of the methyl group are not given.[11] Quercetin, $C_{15}H_{10}O_7$, m.w. 302, which decomposes at 314°C, is soluble in alcohols, alkaline solutions, and acetic acid, and is not soluble in cold water. It has also a strong UV absorption at 258 and 375 nm due to the flavone group. It is the aglucon part of the glycoside quercitrin.[11] Kaempferol, $C_{15}H_{10}O_6$, m.w. 286, m.p. *277*°C, is soluble in warm alcohols, alkaline solutions, and ethers; it is also slightly soluble in cold water. Kaempferol absorbs UV at 265 and 365 nm (Figure 1).

The CCC Machine

A Kromaton II from S.E.A.B. (Villejuif, France) was used. This CCC machine is adapted from the Ito Scheme IV multilayer-coil planet centrifuge design.[12] Two spools are symmetrically held in a rotor that rotates horizontally. A gear arrangement produces a planetary motion of the spools at twice the rotor speed. The rotor and spool radiuses are respectively, 15 and 12.5 cm, producing

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Table 1

Physico-Chemical Solvent Parameters*

Parameter and its Unit	Butanone or Methylethylketone	Water
Mol. Weight (g/mol)	72	18
Refractive Index	1.3788	1.333
Density (g/cm^3)	0.805 (0.821)	0.998 (0.945)
Viscosity (cP)	0.43	1
Boil. Point (°C)	79.6	100
UV Cutoff (nm)	329	180
Dipol. Mom. (D)	2.76	1.87
Diel. const.	15.2	80.1
Reichardt Polarity	32.7 (53)	100 (95)
Solvent in Water (%w/w)	24%	100
Water in Solvent (%w/w)	10%	100
$P_{oct/water}$	2	0.033

* Values in parentheses correspond to the water-saturated organic phase and the solvent-saturated aqueous phase.

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a β ratio of 0.83. Each spool contained 30 turns of 5/8 in. (1.6 mm) i.d. PTFE tubing (length 23.5 m, volume 46.5 mL) and 320 turns of the same tubing (length 242 m, volume 488 mL) coiled in two parallel layers. A four-valve arrangement allowed use of the full volume of the apparatus (1070 mL) with the two spools and 700 turns and 530 m of PTFE tubing, or only the small volume of the spools (94 mL, 60 turns, 47 m of tubing). A #1 valve was used to select the "preparative" volume (1.07 L) or the "analytical" volume (94 mL). A #2 valve was used for the sample injection. A #3 valve allowed bypassing of the CCC column and flushing the phase in the pump, detector and fraction collector, and Valve #4 was used to select the CCC mode: head-to-tail or tail-to-head.[1,4,5] The machine has a cooling unit that permitted maintaining the rotor temperature at 20°C. An electric engine with electronic regulation was able to rotate the 30-kg rotor, spools and liquids at a maximum speed of 600 rpm.

Solvent Choice and CCC Procedure

After different tries, the very simple biphasic system obtained with two solvents only, 2-butanone and water, was selected. 2-butanone, or methylethylketone (MEK), and water physico-chemical parameters are recalled in Table 1.

It should be noted that the MEK phase contains 10% w/w of water which is 30.6% in molar fraction. The aqueous phase contains 24% w/w of MEK at 20°C which is only 7.3% in molar fraction. This is a high mutual solubility that changes, significantly, the physico-chemical properties of the mutually equilibrated liquid phase. The liquid phase density and Reichardt polarity are indicated in parentheses in Table 1.[13] The mutual solubility is sensitive to temperature changes. The CCC machine was thermostated at 20°C±1°C in all studies.

In a typical run, the CCC machine is first filled with the stationary phase (aqueous denser phase) without rotation at the maximum pump flow rate (10 mL/min). Then, the rotor is started at 400 rpm and the mobile phase, the lighter organic phase, is pumped in the tail-to-head direction at 2 mL/min. The organic mobile phase displaces part of the aqueous stationary phase and the stationary phase leaves the machine. When the organic phase is seen leaving the CCC apparatus, the equilibrium is reached. The displaced stationary phase volume corresponds to V_M, the mobile phase volume inside the machine. The phase retention ratio, Sf, is defined as

$$Sf = V_S/V_T = (V_T - V_M)/V_T = 1 - V_M/V_T$$

in which V_T is the CCC machine volume used (94 mL or 1070 mL). Once the CCC machine is equilibrated, the sample can be injected. The retention volume of a peak, V_R, allows one to calculate the liquid-liquid partition coefficient of the corresponding molecule, P, using

$$V_R = V_M + PV_S$$

and

$$P = (V_R - V_M)/V_S.$$

The solutes have a high UV absorption band around 350 nm. The MEK UV cutoff value is 329 nm. Continuous UV detection at 330 nm will be done using a Shimadzu LC10A UV detector.

Other Experimental Set-ups

The chromatographic conditions for the HPLC analysis of the CCC fractions are as follows:

Column: 15 cm, 4.6 mm i.d., 5μm bonded phase Nucleosil 100 ODS (C 18) (Macherey-Nagel, Interchim, Montlucon, France).

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Mobile phase: methanol/water 50/50 v/v + drops of acetic acid for pH 4, flow rate 1 mL/min.

Injection volume: 20 μL of the fractions (Rheodyne 7125 valve)

Detection: UV 330 nm.

Hardware: pump LC-6A Shimadzu, UV detector PU 4025 Pye Unicam Philips, recorder integrator CR-3A chromatopack Shimadzu (Touzart et Matignon, Courtabeuf, France).

The fractions were also diluted to 1% by a methanol/water 80/20 v/v solution at pH 4 (acetic acid) and directly introduced using a syringe pump in the electrospray ionization needle of an LCQ mass spectrometer (Finnigan Mat, Thermo Quest, Les Ulis, France). The mass spectrometers were electronically recorded on a Gateway Pentium P5-133 that fully drives the LCQ mass spectrometer.

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ANALYSIS WITH THE SMALL KROMATON COLUMN

The analytical (94 mL) column of the Kromaton machine was first used to have a rapid idea of the capability of the biphasic liquid system selected. The whole volume of the machine was filled with the aqueous phase (10 mL/min, two hours) because the rotor is better balanced when the whole tubing contains a liquid. The analytical column was selected by the rotation of Valve #1. The centrifuge was started at 400 RPM and the MEK phase was pumped at 2 mL/min in the FLUSH position of valve #3 until the MEK phase is seen at the outlet of the UV detector. Then, Valve #3 was turned to the COLUMN position and, at the same time, the effluent was collected. The MEK phase is the lighter upper phase; that is why it is pumped in the tail-to-head way.

13 mL of water was displaced before the MEK phase appeared. Then, the Sf ratio is 86% (Sf = Vs/Vt, Vs = stationary phase volume = aqueous phase volume, Vt = column volume = 94 mL, Vm = MEK mobile phase volume = 13 mL, Vs = Vt - Vm = 94 - 13 = 81 mL, Sf = 81/94 = 86%). This value, 86%, for the retention ratio Sf, is fair.

It indicates that the machine is able to retain an acceptable volume of the aqueous phase. Then, 500 μL of a 6g/L quercitrin solution in MEK water saturated were injected (mass = 3 mg) using the injection valve (Valve #2). Figure 2 shows the chromatogram obtained with a continuous 330 nm UV detection. Two peaks are seen with the Table 2 chromatographic parameters.

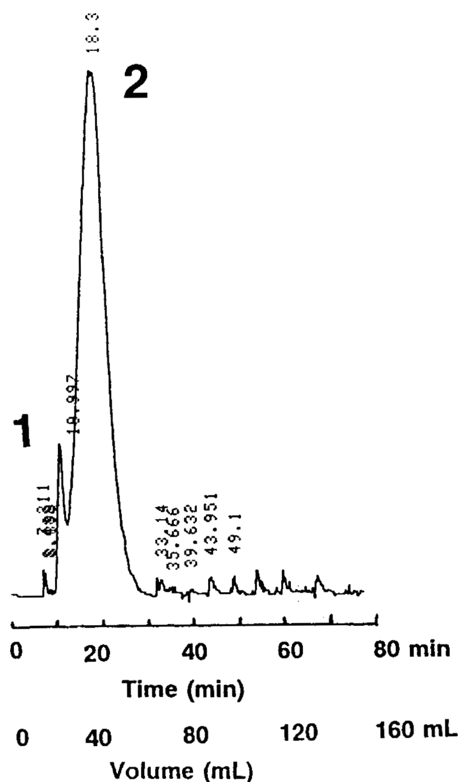

Figure 2. CCC chromatogram of the flavonoid sample obtained with the analytical column. Mobile phase: MEK, 2 mL/min, tail-to-head, stationary phase: aqueous phase, 400 RPM, injected volume: 500 μL, injected mass: 3 mg. The peak numbers refer to Table 2.

The efficiencies of the Figure 2 peaks are 250 and 50 plates for the first and second peak, respectively. The second peak seems to correspond to several products. This efficiency corresponds to 4 and 0.8 plates per tubing turn, respectively.

Such efficiency is acceptable when referred to the number of tube turns in the spool. It is low when referred to the column volume.[14] Several 100 mL CCC machines were able to produce a 500-plate chromatographic efficiency.[3,14]

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Table 2

Chromatographic Parameters of the Figure 2 Chromatogram*

Peak	tr Min	Vr mL	N Plates	P MEK/Water	P Water/MEK
1	11	22	250	10.2	0.10
2	18.3	36.6	50	3.6	0.28

* P values are partition coefficient calculated as $P_{water/MEK}$ = (Vr-Vm)/Vs with the indicated Vr values and Vm = 12 mL and Vs = 81 mL. $P_{MEK/water}$ is the opposite of $P_{water/MEK}$.

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SEPARATION ON THE PREPARATIVE COLUMN

The next set of fractionation of the flavonoid sample was done using the preparative column (1.07 L of internal volume). It was previously filled with the aqueous phase (water saturated by MEK). The lines were rinsed with the organic phase (MEK saturated by water). The centrifuge was cranked at 400 RPM following the same procedure that was used for the analytical column, except the lighter organic phase was pushed at 3 mL/min in the tail-to-head way. The equilibration procedure allowed collection of 102 mL of aqueous phase. The retention factor is Sf= 90.4 % (V_M = 102 mL, V_S = 1070-102 = 968 mL, Sf = V_S/V_T = 968/1070 = 0.904). The large volume column, with its large number of turns (700) of tubing in the two spools retains a significantly higher aqueous phase volume than the small volume (only 60 turns) column. It should be noted that the phase retention factor, Sf, always decreases when the flow rate increases.[1,3,4]

A first separation was tried injecting 9 mL of a 115 g/L quercitrin solution, which was 1 g injected. To make a homogeneous solution at such high concentration (11.5% w/v), a mixture of methanol-water-MEK 33.3-33.3-33.3 v/v/v was used. The problem was that precipitation occurred inside the CCC column. The precipitated solid pushed the whole volume of the mobile and stationary phase out of the Kromaton machine. No separation was obtained. This problem shows that the way the solutes are introduced in a CCC machine may be critical.[3]

Another solution of the flavonoid sample was prepared at a lower concentration (37 g/L) in water saturated by MEK, *i.e.,* the stationary phase. The CCC Kromaton was equilibrated as previously described. The same phase retention factor (Sf = 90.4%) was obtained. 9 mL of the 37 g/L solution were injected using Valve #2. It corresponds to 330 mg of solid flavonoid yellow

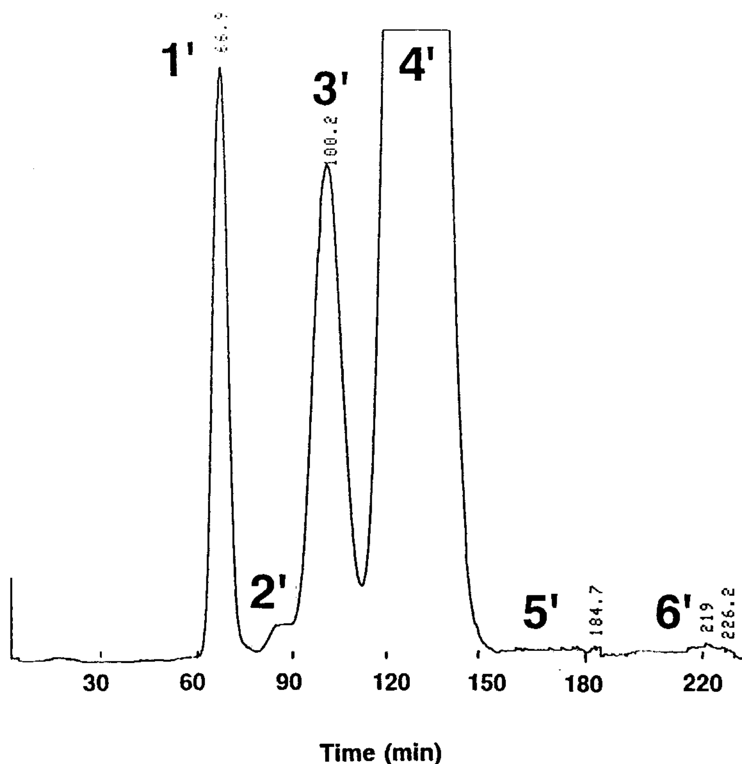

Figure 3. CCC chromatogram of the flavonoid sample obtained with the preparative column. Mobile phase: MEK, 3 mL/min, tail-to-head, stationary phase: aqueous phase, 400 RPM, injected volume: 9 mL, injected mass: 330 mg. The peak numbers refer to Table 3.

powder. The separation shown by Figure 3 was obtained in four hours at 3 mL/min. 720 mL of the water saturated organic phase was pumped at 20°C in the tail to head way. 21 mL of the aqueous stationary phase were displaced in the same time that corresponds to a 90 µL/min bleed flow rate which is usual with a liquid system with such mutual solubility. Also, it should be taken in account that the 9 mL injection volume in the aqueous phase added more aqueous phase and contributed to 21 mL displaced. A continuous 330 nm UV detection was possible although the microscopic droplets of the aqueous phase contained in the organic mobile phase generated noise in the detector. A preparative cell with a short path length was used to minimize this noise. Figure 3 shows the chromatogram and Table 3 lists the relevant chromatographic parameters.

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Table 3

Figure 3 Chromatographic Parameters*

Peak Fraction Code	Vr mL	tr Min	N Plates	P MEK/Water	P Water/MEK
1' (A, B)	201	67	800	10	0.10
2' (C)	258	86	700	6.2	0.16
3' (D,E)	300	100	640	4.9	0.20
4' (G, H)	387	129	off scale	3.4	0.29
5' (1)	471	157	---	2.6	0.38
6' (J)	615	205	---	1.9	0.53

* Partition coefficient P calculated using $P = (V_R - V_M)/V_S$ with $V_M = 102$ mL and $V_S = 968$ mL.

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Comparing Table 2 and Table 3, it is clear that the two peaks of Figure 2 correspond to Peak #1' and Peak #4' of Figure 3. The 10x higher efficiency of the preparative column allows a much better resolution in the separation of the sample. Quercitrin is likely Peak #4', the major peak corresponding to a $P_{water/MEK} = 0.29$. As the $P_{water/MEK}$ coefficient increases, the solute has more affinity for the aqueous phase: it is more polar, its molecule may contain more hydroxyl groups, and vice-versa. Peak #6', the last eluted peak, corresponds to a compound with a $P_{water/MEK}$ value of 0.53 only. The corresponding retention volume, 615 mL, is just above half the CCC column volume. This separation illustrates the difference between classical LC and CCC. With only about 600 plates, a CCC machine is able to resolve solutes with low partition coefficients because the stationary phase over mobile phase volume ratio is high.[1]

The mobile MEK phase was collected using a fraction collector set to collect 18 mL fractions (6 min). Figure 4 locates the studied fractions lettered from A to J. Figure 4 was obtained using exactly the same experimental conditions as the ones described for Figure 3. A high amplification factor was selected to show the minor peaks (A, I, and J). The 10 collected fractions were analyzed by HPLC and mass spectrometry.

ANALYSIS OF THE CCC FRACTIONS BY HPLC AND MS

A classical HPLC system was set up to analyze the A-J fractions as they were obtained (no concentration). For MS analysis, the fractions were diluted

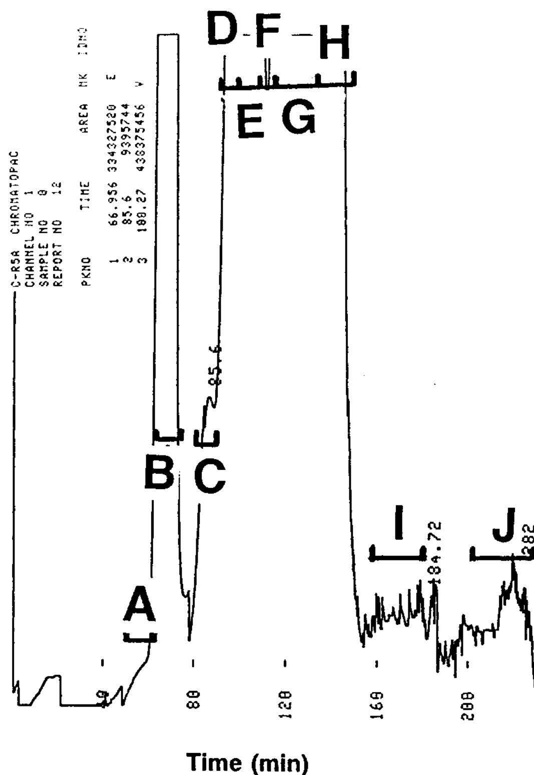

Figure 4. Location of the A-J collected fractions. Same CCC conditions as in Figure 3 except, the signal is 10 time amplified.

at 1% with a methanol/water 80/20 v/v solution and introduced in the LCQ mass spectrometer with electrospray ionization. Figure 5 shows the HPLC chromatogram of the flavonoid sample. 10 peaks could be noted. In Table 4 were gathered the retention factors, peak areas and corresponding masses found in the MS spectra for the HPLC and MS analyses of the 10 CCC fractions. Figure 6 shows the mass spectra of the E and G fractions obtained with the negative ionization mode.

Peak #1 was found in the CCC fraction H, it was not possible to determine certainly its molecular mass. Peak #2 was found in Fractions F-H. As shown by the mass spectrum of the G fraction (Figure 6), this major peak corresponds to a molecular mass of 448.7 which is Quercitrin (Figure 1). Peak #3 was found in Fractions C to F, with a large concentration in Fractions D and E.

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Table 4

HPLC and MS Analyses of the CCC Fractions

HPLC Peak CCC Fraction Figure 5	1 **3.4** 130	2 **4.0** 130	3 **5.3** 5	4 **5.9** 25	5 **9.3** 2.7	6 **16.3** f.p.	7 **18.2** f.p.	8 **20.5** --	9 **29** f.p.	10 **31.3** f.p.
A					**9.4** 8.4 285.5	**16.4** 9.7 301.4				
B					**9.4** 9.1 285.5	**16.5** 1.0 301.4				
C		**4.4** 1.0 418	**5.2** 0.9 431.7							
D			**5.3** 27 431.7	**5.9** 5 321.8					**29** 0.1 ?	
E			**5.3** 35 431.7	**5.9** 5 321.8				**21** 0.4 499?	**29** 0.5 ?	
F		**4.0** 1.6 448.7	**5.3** 6 431.7	**5.9** 3.4 321.8				**21** 0.1 499?	**29**-- ?	
G		**4.0** satur. 448.7								**31.3** 0.2 484?

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H	**3.4** 8	**4.0** 49	
	?	448.7	
I	*		*
	?		
J	*		*
	?		

The numbers in **bold** are the retention factor, k', of the peaks observed on the corresponding chromatograms. The numbers following the retention factors correspond to the area values in 10^6 μV/s obtained at 330 nm UV light for a 20 μL injection of the CCC fraction. It is a very rough indication of the possible component concentration. The number under the retention factor indicates the possible mass of the solute corresponding to the peak. satur. = the integrator was saturated. f.p. = fused peak without integration possibility. * = no peak up to k' = 45.

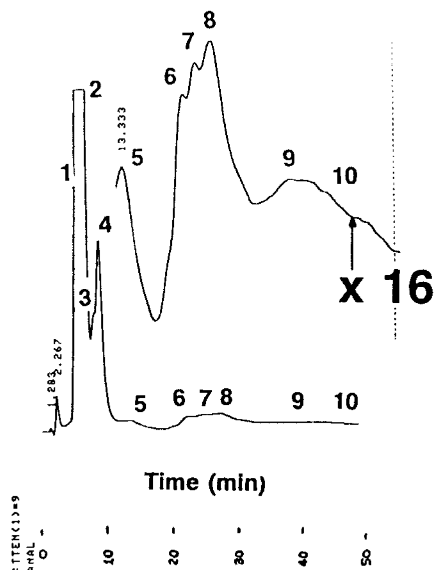

Figure 5. HPLC chromatogram of the flavonoid sample. Mobile phase: methanol-water 50-50 v-v, 1 mL/min; sationary phase: column 15 cm, 4.6 mm i.d., Nucleosil 100 ODS. After 10 min, the 16x amplified chromatogram is shown. The peak numbering refers to Table 4.

The corresponding molecule has a mass of 431.7 (Figure 6). It corresponds to the flavonoid Kaempferol-3-rhamnoglucoside, i.e. R1=H and R2=rhamnose in Figure 1. Peak #4, found associated with Peak #3, corresponds to a molecule that may have a molecular mass of 321.8 (Figure 6). The flavonoid with such a molecular mass was not identified. It may not be a flavonoid. Peak #5, found in Fractions A and B, was associated to a molecule of 285.5 molecular mass. It is the tetrahydroxyflavone or Kaempferol (R1=R2=H in Figure 1). Similarly, Peak #6 was identified as Quercetin (R1=OH, R2=H and m.w.=301.4, Figure 1). Peak #7, a minor peak in the original flavonoid mixture chromatogram (Figure 5), was not found in the A-J CCC fraction nor identified. The minor peaks, Peaks #8-10, were found as traces in the CCC fractions indicated on Table 4. Peak #8 could correspond to a product with a 499 molecular mass. Peak #10 could correspond to a 484 molecular mass.

Considering the identified molecules, their CCC retention order: Kaempferol, Quercetin, Kaempferol-3-rhamnoglucoside and Quercitrin, is also the increasing polarity order: Kaempferol has only four hydroxyl groups on the flavone structure, Quercetin has five hydroxyl groups, and the sugars add more

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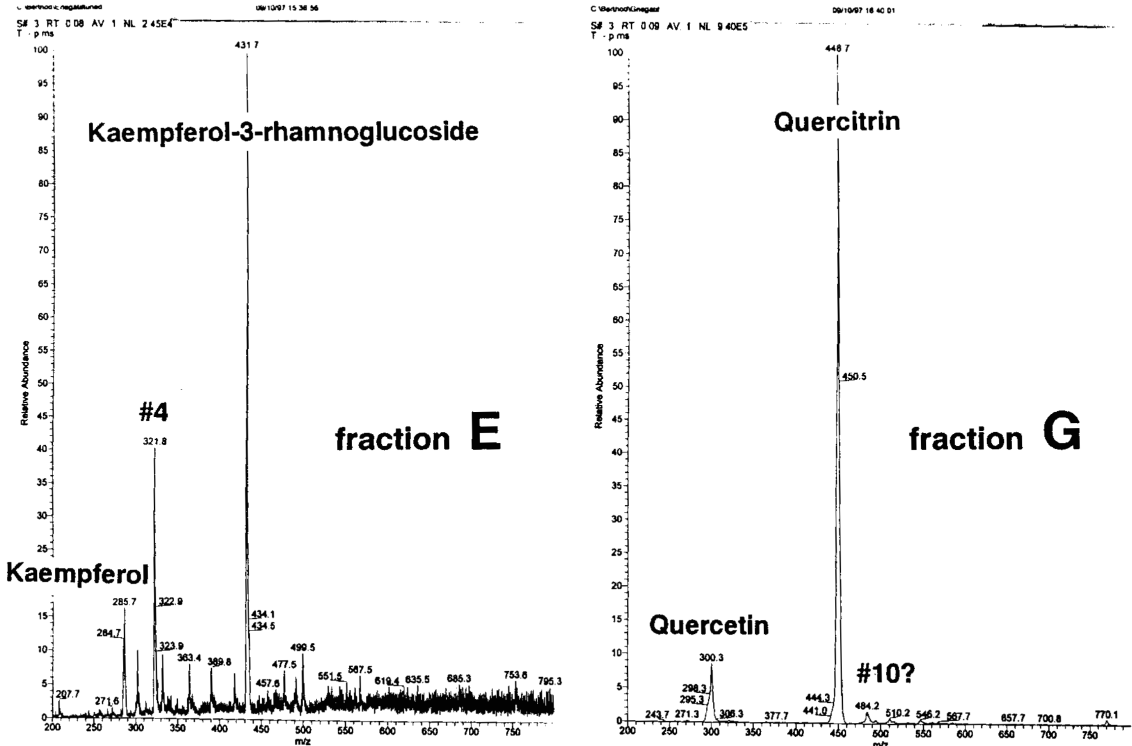

Figure 6. Mass spectra of the E (left) and G (right) CCC fractions. In the E spectrum, kaempferol (mass 285.7) is obtained as a fragment of the rhamnoglucoside. Mass 321.8 could correspond to Peak #4 in Figure 5. In the G spectrum, quercetin (mass 300) is a fragment of quercitrin. Mass 484.2 could correspond to Peak #10.

hydroxyl groups on the larger flavonoid molecules. This CCC retention order with a polar aqueous stationary phase is opposite to the one obtained in HPLC with an apolar C_{18} stationary phase (Table 4).

Once the fraction's analysis was completed, the Fractions G and H of two successive CCC separations of 330 mg were joined together and evaporated at room temperature under a nitrogen stream. The quercitrin mass obtained was 585 mg which is 88.6% of the 660 mg initially injected mass. The mass recovery ratio cannot be estimated because the other fractions were not dried and weighted.

CONCLUSION

The studied sample, labeled Quercitrin, contains more than 88% of the quercitrin flavonoid. The remaining 12% are essentially made of three different flavonoids, kaempferol-3-rhamnoside, quercetin, and kaempherol. There are also at least six very minor impurities. Countercurrent chromatography (CCC) is a powerful tool able to separate significant mass of a sample in more simple fractions. 330 mg of the flavonoid sample could be fractionated in one run (3 hours, 900 mL of organic phase). With the biphasic liquid system made of butanone (methylethylketone) and water, an easy liquid phase retention was possible in both the small and the large columns of the KROMATON apparatus. Continuous UV detection @ 330 nm was possible because the aqueous stationary phase was well retained by the CCC machine.

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