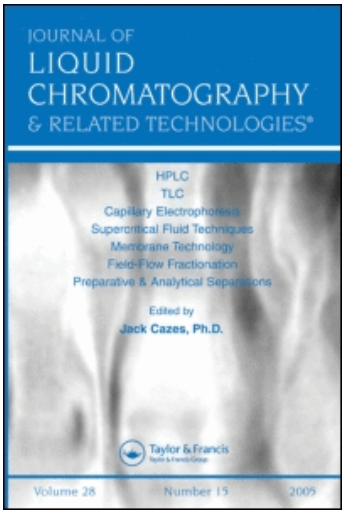

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Purification of Xanthohumol from *Humulus lupulus* by Centrifugal Partition Chromatography Using an Original Acetone Based Solvent Scale

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Abstract: Centrifugal partition chromatography, using an original acetone-based solvent scale was successfully used to purify xanthohumol, isoxanthohumol, 6,8-diprenylnaringenin, and (*E*)-2″-(2‴-hydroxy-isopropyl)-dihydrofurano[2″,3″:4′,3′]-2′,4-dihydroxy-6′-methoxychalcone from a xanthohumol-rich fraction obtained from an ethanol extract of the cones of *Humulus lupulus* L. The composition of the selected quaternary biphasic system was heptane/toluene/acetone/water 24.8:2.8:50:22.4 v/v.

Keywords: Centrifugal partition chromatography, Prenylflavonoid, Purification, Xanthohumol, *Humulus lupulus*

INTRODUCTION

Humulus lupulus L., Cannabinaceae, commonly named the hops plant, is widely cultivated throughout the temperate zones. Hops (i.e. the female inflorescences, or cones) are used in the brewing industry as a preservative and for

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giving beer its characteristic flavor and aroma. Even today, hops are used as a mild sedative in folk medicine in Europe.[1] The essential components involved in the organoleptic properties of hops are bitter acids, essential oils, and flavonoids. The latter compounds are prenylated chalcones, the two major representatives being xanthohumol (XN) and its flavanone isomer, isoxanthohumol (Figure 1). Studies have shown several interesting biological activities for XN and its derivatives as an antifungal or estrogenic agent, or more recently, as an antiproliferative agent against human breast, colon, or ovarian cancer cells.[2–4]

Isolation of flavonoids using traditional purification techniques on solid support is often very tedious, requiring many chromatographic steps and generally resulting in low yields.[5] The principal problem is the phenomena of irreversible adsorption and/or degradation on silica-based solid supports. In order to avoid these problems, centrifugal partition chromatography (CPC)[6] was applied to a xanthohumol-rich fraction obtained from an ethanol extract of the hop cones, using an original acetone-based solvent scale.[7]

EXPERIMENTAL

Reagents

Acetonitrile, acetone, toluene, methanol, heptane, and formic acid were purchased from Carlo Erba (Rodano, Italy). Water was purified by

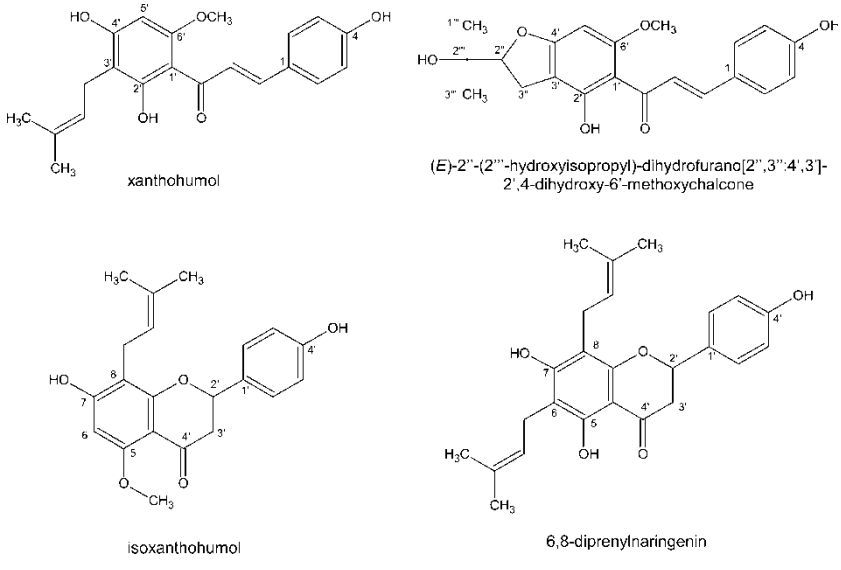


Figure 1. Structure of the isolated prenylflavonoids.

de-ionization and inverse osmosis. Amberlite XAD 1600 resin was purchased from Sigma-Aldrich (Lyon, France).

Xanthohumol Prepurification

An 11 g sample of an ethanol extract of the hop cones was fractionated on Amberlite XAD1600 using a water-methanol gradient to increase the XN content.

Rapid Estimation of the Partition Coefficients

An XN enriched fraction (2 mg) was dissolved in 2 mL of the biphasic solvent system (1 mL of each phase). After agitation and decantation, 15 μL of both the organic and the aqueous phase were separately spotted on silica gel TLC. Estimation of the partition coefficient was achieved by comparing the respective intensities of the XN spot on the developed TLC (eluent: $CHCl_3$/MeOH/H_2O 85:15:1 v/v).

CPC Apparatus

Separations were performed on a FCPC Kromaton Technologies apparatus (Figure 2) (Angers, France), using a rotor of 20 circular partition disks (1320 partition cells, column capacity: 200 mL). Rotation speed could be adjusted from 200 to 2000 rpm, producing a centrifugal force field in the partition cell of about 120 g at 1000 rpm and 470 g at 2000 rpm.

The solvents were pumped using a Dionex P580HPG 4-way binary high pressure gradient pump (Sunnyvale, CA, USA). The samples were introduced into the CPC column via a low pressure injection valve (Upchurch, CIL Cluzeau, Sainte-Foy-La-Grande, France) equipped with a 21 mL sample loop. The effluent was monitored with a Dionex UVD 170S detector equipped with a preparative flow cell (6 μL internal volume, path length of 2 mm). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). The experiments were conducted at room temperature ($22 \pm 1^{\circ}C$).

Solvent Systems Preparation

Biphasic systems were prepared by mixing heptane, toluene, acetone, and water in suitable proportions (24.8:2.8:50:22.4 v/v) in a separatory funnel, shaking them vigorously, and allowing them to settle until the phases became limpid.

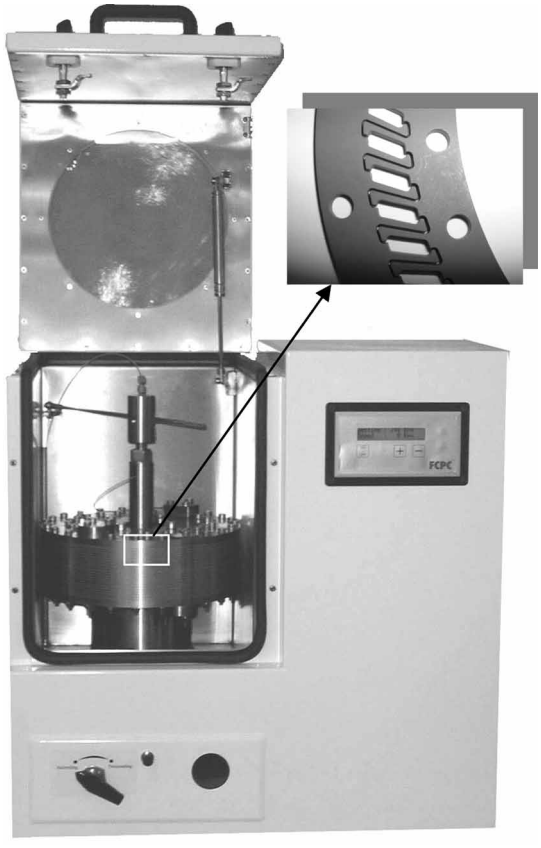


Figure 2. Kromaton Technologies FCPC® 200 mL rotor and partition disk. Note the connecting ducts centered on the bottom and the top of each cell. The upper and lower cell walls are made of inter-disk Teflon® gaskets.

Injection and CPC Operating Procedure

The rotor was first entirely filled with the stationary phase while rotating at 300 rpm. The sample was then injected (1 g XN-rich fraction dissolved in 15 mL of organic phase and 5 mL of aqueous phase). The rotation speed was increased to 1400 rpm, and the organic mobile phase was pumped into the column in the ascending mode at a flow rate of 8 mL/min, for 74 min. The mobile phase displaced 35% of the stationary phase and, therefore, the retention of the stationary phase was 65%. The back pressure was 36 bars. A dual mode was then performed by pumping the aqueous phase in the descending mode at 8 mL/min, for 43 min. Detection was performed at 364 nm and the fraction size was 1 min.

Fraction Analysis

Analytical HPLC

Analyses of the collected fractions were performed on a Dionex Summit HPLC system, equipped with a P580 pump, an ASI-100 autosampler, a STH column oven, and a UVD 340S diode array detector and a C_{18} Kromasil (250 × 4.6 mm i.d., 5 μm particle size) column (A.I.T., Saint-Nom La Bretèche, France). The mobile phases were acetonitrile and HCOOH (1% in water). The gradient profile was linear from 40% to 100% of acetonitrile in 20 min, at a flow rate of 1 mL/min. The temperature of the column oven was set at 22°C. The effluent was monitored at 205, 285, 320, and 363 nm by DAD. All of the chromatographic data management was ensured by the Chromeleon software 6.0.1 version (Dionex, USA).

NMR Analysis

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 in CD_3OD (^{1}H at 500 MHz and ^{13}C at 125 MHz); 2D experiments were performed using standard Bruker microprograms, and XWIN-NMR software (version 2.6) package for data acquisition and processing.

Semipreparative HPLC

Final purification of impure fractions from CPC was performed using the above described HPLC system and a C_{18} VYDAC (Ref. 201SP510, 250 × 10 mm i.d., 5 μm particle size) column (Dionex, USA). The mobile phases were acetonitrile and HCOOH (1% in water). The gradient profile was linear from 40% to 100% of acetonitrile in 20 min, at a flow rate of 5 mL/min.

RESULTS AND DISCUSSION

The Acetone Based Solvent Scale

Selection of the appropriate biphasic system is essential in order to optimize selectivity and solubility using solid free liquid–liquid chromatography (counter current chromatography or centrifugal partition chromatography). Although, numerous combinations are available to achieve this task, the large number of possible combinations becomes a drawback of this technique. Certain rules rationalizing the biphasic system selection have been described in order to produce rapid results in routine laboratory experiments.[8] The "best solvent" approach, where the biphasic system is built around a good solvent for the sample, is certainly the most intuitive way

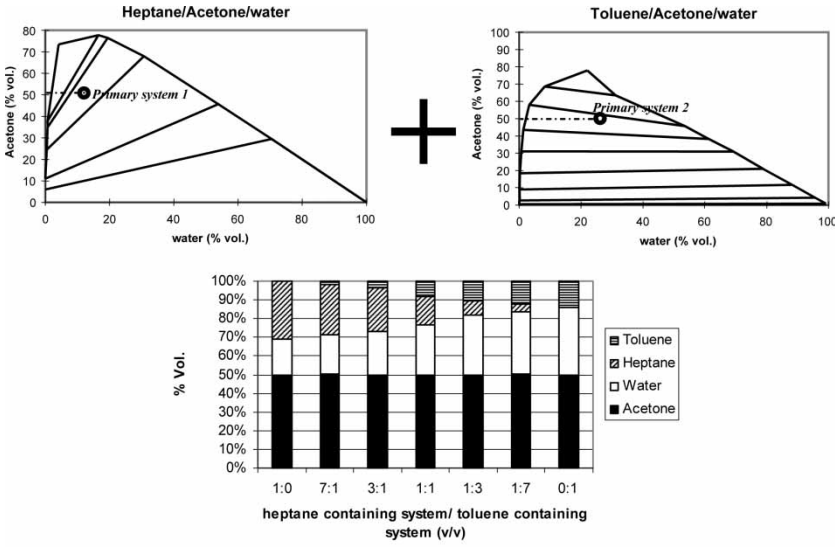
to find a system adapted to a given sample. Another approach consists of ingeniously combining solvents to form a nearly continuous series, which is made by gradually varying the solvent proportions. The resulting mixtures have a stepwise increasing polarity. Many scales have been reported in the literature, such as the ARIZONA scale (combinations of the ethyl acetate-water and *n*-heptane-methanol systems) or the OKA scale (sixteen mixtures of *n*-hexane, ethyl acetate, *n*-butanol, methanol and water). Two major problems are encountered with the existing scales: they are built with certain solvents unusable in industry and, especially for the ARIZONA scale, poor solubility of the sample is often observed. We have developed the acetone based solvent scale in order to remedy these problems.

Acetone is a good solvent for many crude mixtures of secondary plant metabolites. It is, thus, highly desirable to use it as the "best solvent" in ternary systems. If "A" is the molecule of interest, its partitioning in a heptane-acetone-water system is generally in favor of the water rich phase because acetone has more affinity for water than for heptane, as shown by the slope of the tie lines (Figure 3). In addition, when dissolved in a toluene-acetone-water system, "A" is mainly present in the toluene rich phase, owing to the higher affinity of acetone with toluene. A quaternary system containing toluene, heptane, acetone, and water is thus able to distribute "A" in nearly equivalent amounts between the two phases. This strategy permits a high acetone content in both the aqueous and the organic phases, thus allowing high concentrations of "A" in the system. For example, starting with two ternary systems–one based on heptane, the other based on toluene, and both containing 50% of acetone–one can build a solvent scale with the same high and constant acetone content. All of these solvents can be employed in industry under the usual safety conditions.

Purification of Xanthohumol

Selection of a Suitable Biphasic System

In the elution mode, the distribution constant of the compound to be purified in the chromatographic system (i.e. the two liquid phases) should be ≈ 1.[9] A suitable combination of the two initial ternary biphasic systems (heptane-acetone-water 31:50:19 v/v and toluene-acetone-water 14:50:36 v/v) was selected by evaluating the partition coefficient of XN contained in the fraction of interest in the different quaternary biphasic systems mentioned above (see Figure 3). The best solvent composition corresponded to 4 volumes of the heptane system and 1 volume of the toluene system. The resulting composition of this quaternary biphasic system was heptane-toluene-acetone-water 24.8:2.8:50:22.4 v/v.



Composition in % vol. for the 7 systems

Systems	Acetone	Water	Heptane	Toluene
1:0	50,0	19,0	31,0	0,0
7:1	50,0	21,1	27,1	1,8
3:1	50,0	23,3	23,3	3,5
1:1	50,0	27,5	15,5	8,0
1:3	50,0	31,8	7,8	10,5
1:7	50,0	33,9	3,9	12,3
0:1	50,0	36,0	0,0	14,0

Figure 3. Design of the solvent scale with high acetone content (50% in this example).

Selecting the Operating Conditions

Since this biphasic system was used for the first time in CPC, preliminary work was necessary to define the operating conditions (rotation and flow rate). The influence of the flow rate and the column rotation speed on the stationary phase retention and the back pressure was studied without sample injection. Figure 4 shows that this system is stable (retention $>75\%$ without sample injection) under classical conditions. Previous work has shown that a high rotation speed enhances column efficiency, the limiting factor being the back pressure (60 bars for this CPC column).[8] As a result, a flow rate of 8 mL/min and a rotation of 1400 rpm were selected.

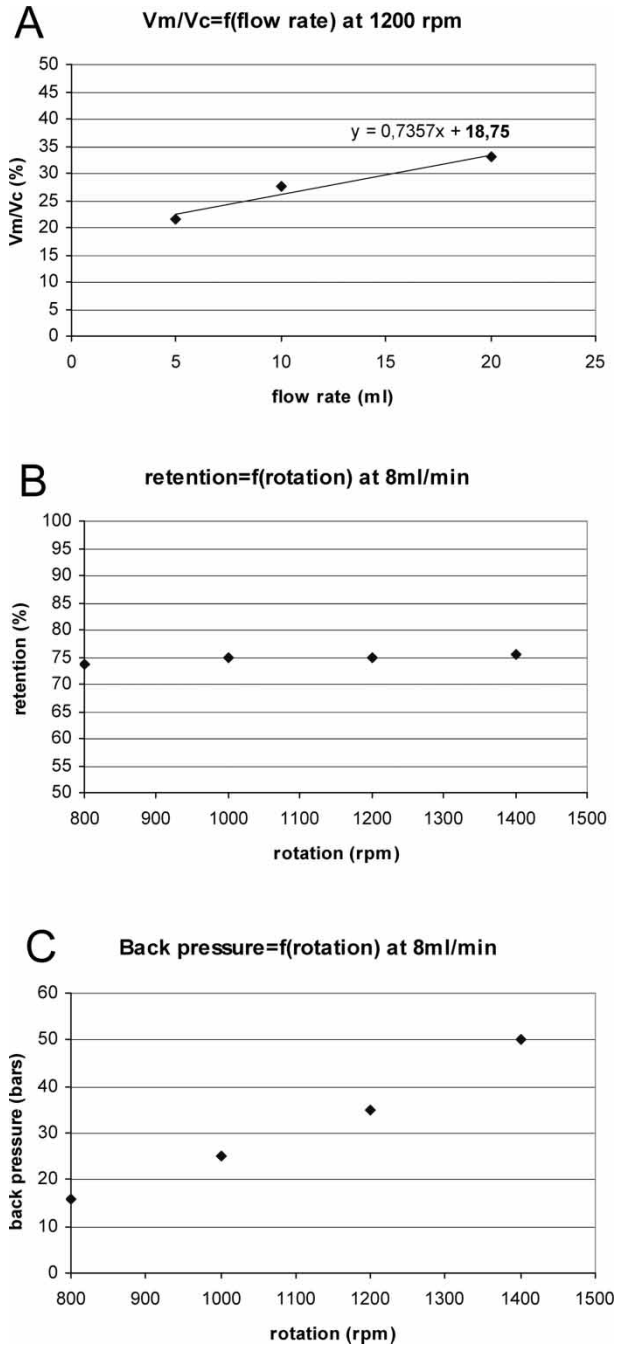


Figure 4. Influence of the flow rate (A) on the stationary phase retention and the column rotation speed (B) on the stationary phase retention and the back pressure (C).

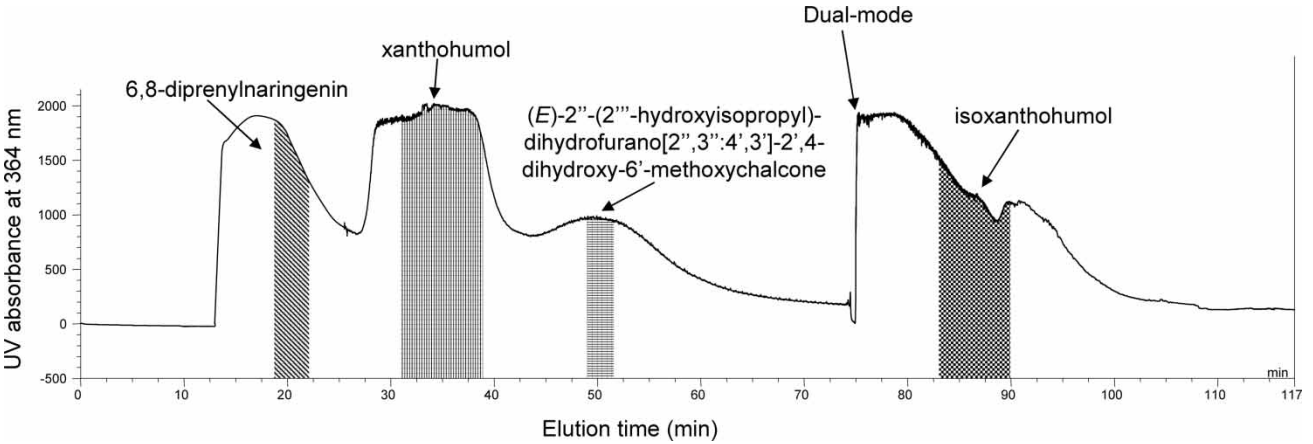


Figure 5. Chromatogram of *Humulus lupulus* extract purification on a FCPC® Kromaton 200 mL column. Sample size: 1 g; Biphasic solvent system: heptane-toluene-acetone-water 24.8:2.8:50:22.4 v/v; Ascending mode for 74 min, rotation speed: 1400 rpm, flow rate: 8 mL/min, back pressure: 36 bars, retention: 65%; then descending mode for 43 min, rotation speed: 1400 rpm, flow rate: 8 mL/min; Fraction duration: 1 min; UV detection: 364 nm.

Application to the Fractionation of an XN Enriched Fraction from Humulus Lupulus

Figure 5 shows the CPC chromatogram, recorded at 364 nm, obtained after injection of 1 g of the XN enriched fraction (dissolved in 15 mL of aqueous stationary phase and 5 mL of the organic mobile phase). Identical fractions 31 to 39 were pooled, evaporated to dryness, and then analyzed by RP-HPLC and NMR. They contained 40 mg of pure xanthohumol.[4] Similarly, fractions 83 to 90 yielded 50 mg of pure isoxanthohumol.[9] Further purification by semipreparative RP-HPLC of fractions 19 to 22 and 49 to 52 gave 1 mg of pure 6,8-diprenylnaringenin[10] and 2 mg of pure (*E*)-2″-(2‴-hydroxyisopropyl)-dihydrofurano [2″,3″:4′,3′]-2′,4-dihydroxy-6′-methoxychalcone,[4] respectively. Structural confirmation of the isolated compounds was achieved using NMR data.[4,9,10] A second CPC run was carried out using a larger sample size (2 g dissolved in 15 mL of aqueous stationary phase and 5 mL of the organic mobile phase). The major consequence was a loss of resolution between the elution peaks of the different compounds, probably due to a π-stacking phenomena in the aqueous phase, often encountered with polyphenolic compounds in liquid–liquid support free chromatography.[11]

CONCLUSION

Centrifugal partition chromatography has been successfully applied to the purification of four prenylflavonoids from *Humulus lupulus* using an original acetone based solvent scale. The results highlight the performance of this scale that combines the rapidity and the versatility of the solvent scale approach and the capacity aspect of the "good solvent" strategy, the latter being provided by the high acetone content in both the aqueous and the organic phases.

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