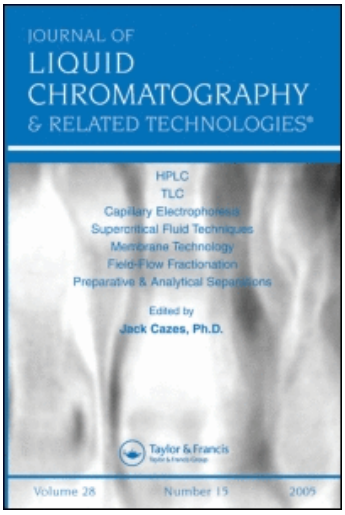

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Lucas R. Chadwick[a]; Harry H. S. Fong[a]; Norman R. Farnsworth[a]; Guido F. Pauli[a]
[a] UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, USA

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CCC Sample Cutting for Isolation of Prenylated Phenolics from Hops

Lucas R. Chadwick, Harry H. S. Fong, Norman R. Farnsworth, and Guido F. Pauli

UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, USA

Abstract: One of the most potent estrogenic substances from the plant kingdom is the prenylated flavanone, 8-prenylnaringenin (8PN), present in hops (*Humulus lupulus* L., Cannabaceae) in low (<10 ppm) concentrations. The prenylated chalcone desmethylxanthohumol (DMX), which is also present in this plant material, serves as a precursor to 8PN. A method for the preparative isolation of these and related bioactive phytochemicals has been developed that involves the use of multiple chromatographic techniques including complementary countercurrent chromatography (CCC) solvent systems. This paper describes the isolation of 8PN and DMX employing "sample-cutting CCC". The Hexanes-EtOAc-MeOH-H_2O (HEMWat) solvent systems found to be effective for isolation of prenylated phenolics from hops range from HEMWat 8-2-8-2 for diprenylxanthohumol and 6,8-diprenylnaringenin, to HEMWat 6-4-6-4 for xanthohumol (XH), 6PN and 8PN, to HEMWat 5-5-5-5 for DMX, hydroxylated derivatives of XH, and humulinones.

Keywords: Sample cutting, Prenylated phenolics, Isolation, CCC, Hops

INTRODUCTION

Hops, the resinous inflorescences of the twining vine *Humulus lupulus* L. (Cannabaceae), are used today primarily for their bitter and aromatic properties in the manufacture of beer. In this respect, aqueous ethanolic tinctures of

Address correspondence to Guido F. Pauli, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA. E-mail: gfp@uic.edu

hops are commonly consumed worldwide. They contain estrogenic prenylated flavanones[1] as well as chemopreventive prenylated chalcones,[2] and are often reported to have sedative-like activity.[3–5] We wish to evaluate the hormonal activity of a standardized hop extract in a clinical setting. In order to produce a properly standardized extract, it is necessary to (i) identify the relevant bioactive/marker compounds present in the extract, and (ii) obtain bioactive/marker compounds in a purified state for use as reference standards.

One of the greatest advantages of countercurrent chromatography (CCC) is the fact that no irreversible adsorption occurs and the sample is therefore entirely recovered at the end of each separation. We have introduced a new term for CCC that highlights this fact, namely, "sample cutting". Methods that are worked out using this technique can be readily developed into multi-dimensional CCC procedures, as described by Yang et al. and Tian et al.,[6,7] who have demonstrated the effectiveness of combining multiple CCC dimensions using multiple instruments coupled with switching valves. Sample cutting CCC takes full advantage of (i) 100% sample recovery offered by CCC technology and (ii) the fact that results in one dimension are predictive of behavior in the next dimension.

In order to purify phytochemicals present in the low ppm or ppb levels, it is often necessary to process large amounts of plant material (1–10 kg) yielding crude extracts on the order of 100–1000 g, and to employ a combination of chromatographic separations. When compared with traditional preparative separation techniques such as adsorption chromatography, the only significant and general limitation of the average countercurrent chromatographic instruments available in natural products laboratories is their limited loading capacity. Thus, in the present work, two consecutive vacuum-liquid chromatographic procedures using silica gel as a solid adsorbent were employed, in order to produce fractions of sufficiently small mass for separation by standard HSCCC instrumentation.

EXPERIMENTAL

Instrument and General Experimental Procedures

The HSCCC apparatus consisted of a J-type instrument (Model CCC-1000; Pharma-Tech Research Corporation, Baltimore, MD) containing a self-balancing three-coil centrifuge rotor (radius 7.5 cm) equipped with three 40 mL columns ($\beta = 0.53–0.60$) with Zeus #20 PTFE tubing (0.85 mm ID/1.3 mm OD) connected in series, solvent pump (Shimadzu LC-10), UV detector (Shimadzu SPD-10A UV dual wavelength with preparative flow cell or SPD-6AV single wavelength with analytical flow cell) and a Foxy Jr. fraction collector (Isco, Inc.). The total volume of the flying leads was 1.5 mL. Samples were injected through a 2 mL sample loop using a mixture of upper and lower phases of the solvent system. In addition to the UV detector,

fractions were analyzed offline by thin layer chromatography (TLC). Silica gel Si F_{254} (Merck, Darmstadt, Germany) plates were used for TLC, and $FeCl_3$ (3% in dry EtOH) was sprayed on the plates to detect phenolics, and duplicate plates were dipped into anisaldehyde-H_2SO_4-HOAc (1-1-48), followed by heating, as a general detection reagent. TLC solvent systems: A: $CHCl_3$-MeOH (95 : 5); B: Hexanes-EtOAc (3 : 1). In most cases, the TLC plates were allowed to dry and then eluted a second time before applying detection reagents. NMR spectra of isolated compounds were recorded on Bruker Avance 300, 360, or 500 MHz instruments. Mass spectra were recorded on a Micromass Q-ToF-2 mass spectrometer. UV spectra were recorded on a Beckman DU-7 spectrometer.

Solvents

Petroleum ether (petr. ether) and ethyl acetate (EtOAc) were ACS grade from Fisher Scientific Co., Pittsburgh, PA, USA. ACS-grade EtOAc was distilled prior to use for CCC. Hexanes and MeOH were HPLC grade from Fisher and were used without further purification. Deionized water was passed through a Nanopure ultrapure water system (Barnstead-Thermoline; Dubuque, IA, USA) prior to use.

Plant Extraction and Sample Preparation

Spent Nugget hops: Plant material remaining after CO_2 extraction of pelletized strobiles of *Humulus lupulus* cv. Nugget (642 g) provided by Yakima Chief, Inc. (Sunnyside, WA) was macerated in methanol (1.5 l) twice overnight, and the combined filtrates were concentrated under reduced pressure to a total volume of 800 mL. The concentrated methanol extract (**E1**) was partitioned by adding H_2O (200 mL) and extracting with petr. ether (**P2**; 600 mL × 1; 400 mL × 2). The lower phase remaining after petr. ether extraction was subsequently extracted with $CHCl_3$ (**P3**; 500 mL × 1; 400 mL × 2). The $CHCl_3$ partition **P3** was first fractionated by vacuum liquid chromatography (VLC) using a petr. ether → EtOAc → MeOH gradient (50 × 500 mL fractions, using recycled solvent from rotavapor) and all subfractions were combined into three pooled fractions, namely, **F1** (least polar, 0–40% EtOAc), **F2** (moderately polar, 40–50% EtOAc), and **F3** (most polar, 50% EtOAc → MeOH wash). VLC was performed using a modified version of the procedure described by Coll and Bowden,[10] in that higher packing heights were used. Each of the fractions **F1**–**F3** were separately fractionated again by VLC, but with a different solvent gradient ($CHCl_3$ → MeOH) to yield a total of 31 combined fractions. The chromatographic behavior of the VLC fractions that were fractionated by countercurrent chromatography using HEMWat systems is shown in Table 1.

Table 1. Preparative chromatographic behavior of subfractions of the $CHCl_3$ partition of spent hops[a]

VLC1	VLC2	%MeOH ($CHCl_3$)	CCC1	Partitioning?[b]	CCC2	Partitioning?[b]	Isolates
F1	F1-1	0-1.0	8-2-8-2	y			**8, 14**
	F1-1	**0-1.0**	**7-3-7-3**	y			**8, 14**
	F1-2	**1.0-1.0**	**6-4-6-4**	y			**18**
	F1-3	**1.0-1.4**	**6-4-6-4**	y			**1,2**
	F1-5	**2.0-2.0**	**6-4-6-4**	y			**1,3,10,15,16**
	F1-6	2.0-2.8	6-4-6-4	n (cutting)	**5-5-5-5**	y	**7**
	F1-7	2.8-10.0	6-4-6-4	n (cutting)	**5-5-5-5**	y	
F2	**F2-3**	**1.0-1.3**	**6-4-6-4**	y			**1**
	F2-4	**1.3-2.0**	**6-4-6-4**	y			**1**
	F2-5	2.0-2.3	6-4-6-4	y			
	F2-6	2.3-3.0	6-4-6-4	n (cutting)	**5-5-5-5**	y	**4-7,9-13**
	F2-7	3.0-3.7	6-4-6-4	n (cutting)	5-5-5-5	y	
F3	**F3-2**	**0-5.0**	**6-4-6-4**	y			**17,20,21**
	F3-3	5.0-5.0	6-4-6-4	n (cutting)	5-5-5-5	y	
	F3-4	5.0-5.0	6-4-6-4	n (cutting)	5-5-5-5	y	
	F3-5	5.0-5.0	6-4-6-4	n (cutting)	5-5-5-5	y	**19** (ppt from F3-5)
	F3-6	5.0-5.0	6-4-6-4	n (cutting)	5-5-5-5	y	
	F3-7	6.0-6.0	6-4-6-4	n (cutting)	5-5-5-5	n	

[a]Solvent systems that were suitable for the given fraction are shown in bold font.
[b]y = yes; n = no

HSCCC Separation

Prior to injection, the two-phase solvent mixture was separated and the instrument was loaded with stationary phase. The instrument was consistently operated in head-to-tail (descending) mode, with the aqueous phase being mobile. The sample was injected in a mixture of the two phases, either after hydrodynamic equilibration of the two phases (Injection After Equilibration, IAE) or just before introduction of the mobile phase (Injection Before Equilibration, IBE), as indicated. In the IBE method, the system was loaded with stationary phase and the sample loop was filled with sample and, at time $t_R = 0$, mobile phase was introduced, the coils were set in motion at 900 rpm, and the sample was injected. Partition ratios presented in Figure 4 were calculated using the marker method (K_M) where unretained peaks were defined as the marker with $K \equiv 0$. For sample cutting, the IBE method was used and the column contents were collected after each run. The most hydrophobic VLC fraction (F1-1) from the $CHCl_3$ partition of spent hops (**P3**) was effectively separated using the Hexane : Ethylacetate : Methanol : Water 7.5-2.5-7.5-2.5 (Table 1). The system HEMWat 6-4-6-4 was effective for a broad range of xanthohumol-containing fractions. Those fractions that were too hydrophilic to be effectively separated using HEMWat 6-4-6-4, but were nonetheless "cleanly cut" using this system. The "cleanly cut" samples, when partitioned with a more polar system, HEMWat 5-5-5-5, as in Figure 1, could be separated sequentially as shown in Figure 2.

Referring to Figure 1, components with $0 \leq K \leq 0.2$ in the bottom chromatogram were combined and the solvent was removed by rotary evaporation. This "cleanly cut" sample (78 mg) was then dissolved in a mixture of 1 mL each phase of HEMWat-0 for injection (Figure 1, top). For both separations, the 125 mL coil and flow rate = 0.5 mL/min was used; relative absorbance units are shown along with partition ratios calculated using both the marker method (K_M) where the peak front was defined as the point $K \equiv 0$, and the carryover method (K_{CO}) as described by Ito and Conway.[11]

Figure 2 shows the sequential separation of samples that were "cut" with HEMWat-3 into HEMWat-0. This approach led to the isolation of isoxanthohumol (A), desmethylxanthohumol (B), and two mixtures of hydroxyprenylchalcones (C, D) that were further purified by HPLC. In terms of solvent consumption, this represents a very economical preparative chromatographic method. In general, stationary phase retention (S_F) was much greater for the cleanly cut samples, and analytes with $0 \leq K \leq 0.2$ in HEMWat-3 have $0 \leq K \leq 0.6$ in HEMWat-0.

Initial Solvent System Selection

In order to identify a potential solvent system (SS) for preparative isolation of prenylated flavonoids from hops using CCC, the partition ratio of

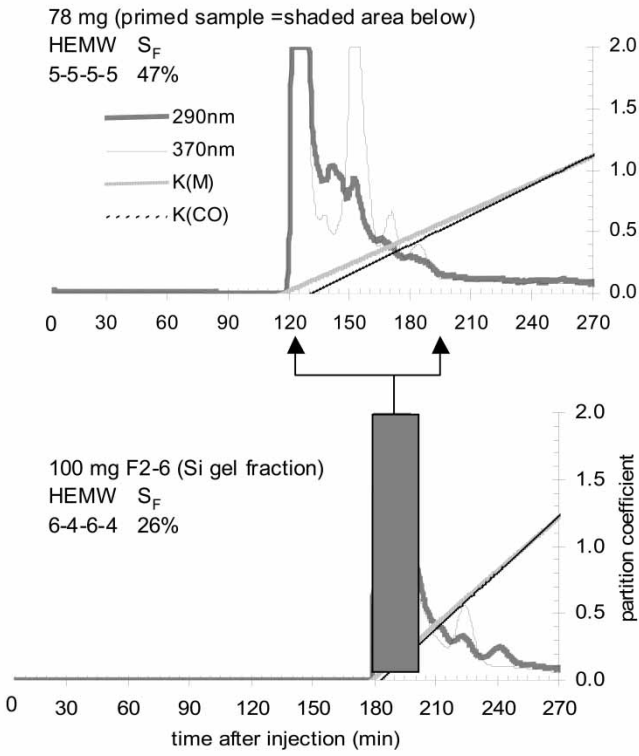

Figure 1. "Sample-cutting" with CCC using HEMWat 6-4-6-4 (HEMWat-3) for cutting (lower figure; IBE method), and HEMWat 5-5-5-5 (HEMWat-0) for separation (upper figure; IAE method).

6-prenylnaringenin (**16**; 6PN; 0.8 mg sample isolated previously via a combination of adsorption-, size exclusion, and high-pressure liquid chromatography) was determined in petr. ether–EtOAc–MeOH–H_2O (PEMWat) solvent systems until a system showing $K_p \sim 1$ was identified (PEMWat = 5-5-6-4). Partition ratios were approximated with the micro-shake-flask method,[12] and quantification by UV (absorbance at 290 nm). Results using petr. ether indicated that this heterogeneous mixture yielded somewhat inconsistent behavior as an eluent. HPLC grade hexanes yielded more reproducible results, and were used exclusively thereafter and for all of the experiments presented herein.

RESULTS AND DISCUSSION

In order to explore the potential of CCC for high-throughput preparative fractionation of multiple components of varying polarity, a series of silica gel

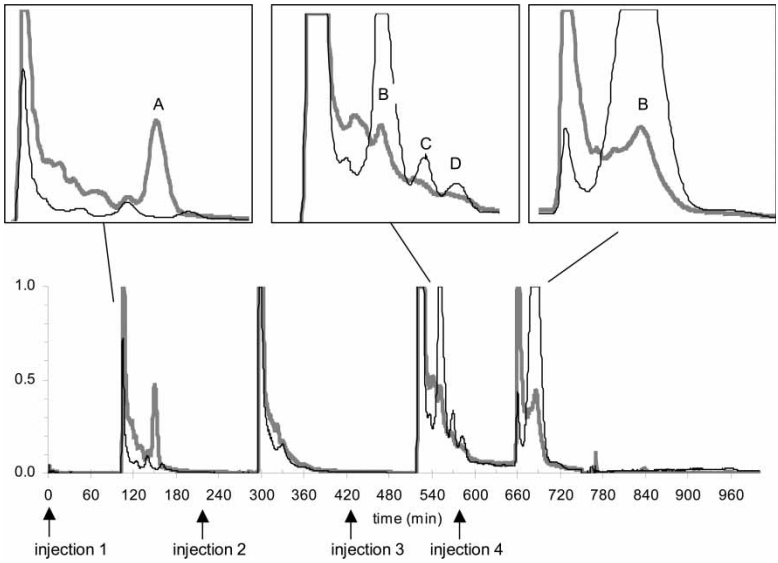

Figure 2. Sequential separations of multiple cleanly cut samples. Enlargements of bottom chromatogram are shown in the boxes above. Separations of cut samples: Injection 1 (23.3 mg), Injection 2 (43.9 mg), Injection 3 (78.5 mg), Injection 4 (32.0 mg). The traces represent dual wavelength detection as shown in Figure 1.

fractions were "cleanly cut" by HSCCC using the same (HEMWat 6-4-6-4) SS. Using the 120 mL coil, 100 mg aliquots of silica gel fractions were "cut" using the IBE method (see Experimental). In every case, there was on one hand a group of unretained analytes eluting with the solvent front, and on the other hand a group of highly retained components remaining in the column at the end of each experiment. The relative proportions between the unretained front and the highly retained column contents varied with the polarity of the silica gel fractions, but in every injection of a VLC fraction, there were unretained components ($K = 0$) and components that do not elute in any reasonable amount of time ($K \rightarrow \infty$). If such VLC fractions were to be injected sequentially, there would be significant overlap (cross-contamination). Poorly retained components were therefore regarded as being "cleanly cut" for further HSCCC fractionation using a slightly more polar SS.

Figure 3 illustrates a potential caveat to sequential injection of cut samples (e.g., Figure 2), which is the dynamic nature of phytochemicals and the potential for artifact formation. A silica gel fraction (F1-7) containing desmethylxanthohumol (**7**; DMX) was first cut using HEMWat-3. Components with $0.05 \leq K \leq 0.3$ in the bottom chromatogram were re-injected (after removal of solvent and ca. 1 week) using the same solvent system. Both 6- and 8-prenylnaringenin (**16, 15**; 6PN, 8PN) with K values of 0.82 and 0.62, respectively, had formed spontaneously in the course of 1 week,

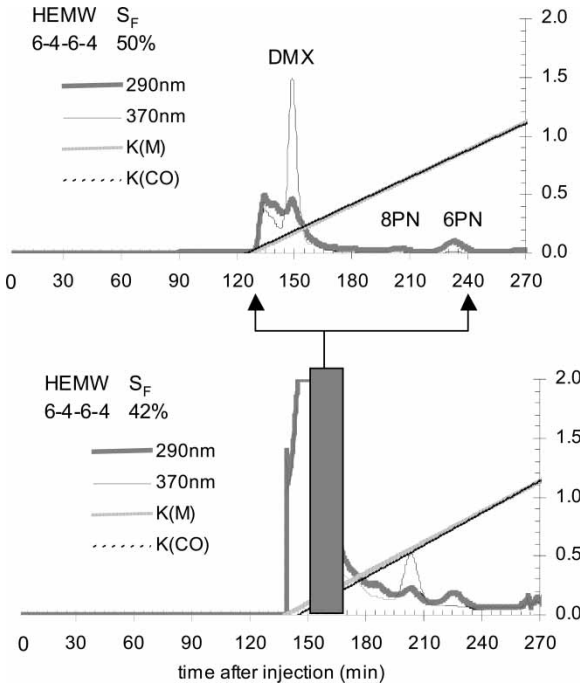

Figure 3. Sample-cutting CCC using the same solvent system (HEMWat-3) for both dimensions.

including 2 days in solution. If multiple "cleanly cut" samples are injected sequentially, such artifacts might be buried under a subsequently injected sample. In the context of bioassay-guided fractionation, such a scenario could potentially confound bioassay results. It must be noted that such a scenario is possible regardless of the chromatographic method employed, and that the adsorbent-free nature of CCC provides the greatest possible chance to avoid artifact formation.

A benefit of sample cutting is that several "cleanly cut" samples can be injected in succession with a very much reduced risk of cross-contamination. In the absence of artifact formation, a caveat that is illustrated in Figure 3, there is no possibility of cross-contamination between samples, as the cleanly cut samples each represent a small window of the polarity spectrum. In general terms, those samples that eluted between 1.0 and 2.0 % MeOH from silica gel using a $CHCl_3 \rightarrow$ MeOH gradient, were adequately partitioned in the HEMWat 6-4-6-4 SS (Table 1). By applying this "sample cutting" method to silica gel fractions of hops, the compounds shown were obtained in sufficiently concentrated form after the second CCC dimension for rapid purification by HPLC. Details of the structure elucidation have been described elsewhere.[13]

CONCLUSIONS

Sample-cutting "CCC" is an effective method to explore multidimensional CCC using a single instrument. This approach led to the isolation of more than 20 prenylated phenolics from spent hops (Figure 4). The IBE method was found to be most appropriate for cutting crude samples. The IAE method can also be used with crude samples, but it is still necessary to elute the column after each run. Equilibration of the solvents prior to injection had no noticeable effect on separation, and, therefore resulted only in increased solvent use. However, the IAE method was found to be most appropriate for the 2nd through the last sample in a set of sequential separations of "cleanly cut" samples, where the first sample in each set was injected using the IBE method. Because cleanly cut samples only represent a narrow polarity window (they are "primed" for purification), it is not necessary to elute the column between injections. An advantage of this method is that unretained constituents of cut samples can be used as markers to define t_0 and therefore K_0 for accurate determination of partition ratios. The only caveat with this approach is due to the dynamic nature of some phytochemicals such as prenylchalcones, which even in plain solution isomerize to their corresponding flavanones. If such chemistry occurs between separations, it is possible to observe the formation of artifacts that elute outside the theoretically possible range for the cleanly cut samples (Figure 3). Future applications of CCC sample cutting will be greatly enhanced with the advent of high-capacity CCC instrumentation. It is noteworthy that only very recently laboratory-size CPC instrumentation has become available in 5.0 l sizes, offering a much-increased loading capacity

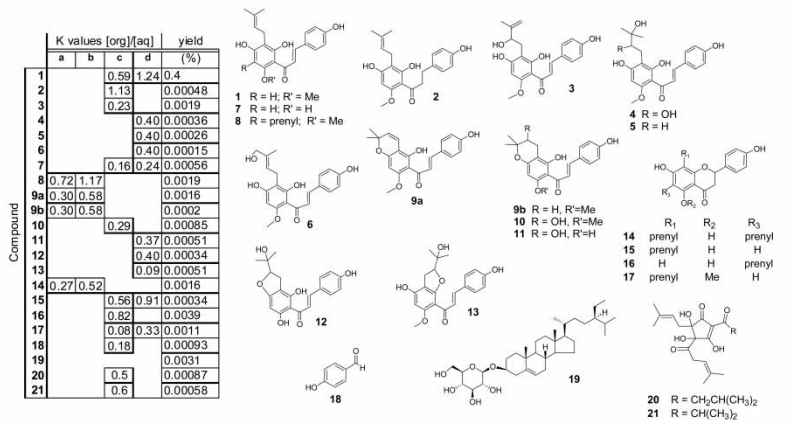

Figure 4. Structures, partition ratios, and % yields of isolates from spent Nugget hops.[a-d] Partition ratios determined for CCC solvent systems using the marker method [a]Hexanes-EtOAc-MeOH-H_2O (HEMWat) 4-1-4-1 [b]HEMWat 3-1-3-1 [c]HEMWat 6-4-6-4 [d]HEMWat 5-5-5-5.

combined with very high flow rates (>30 mL min^{-1}). Such equipment is promising for sample cutting of typical low kg quantities of natural crude extracts. In the present work VLC was used for "prefractionation", and the HEMWat 6-4-6-4 system was, in general, ideal for VLC fractions eluting from silica gel between 1–2% MeOH (in $CHCl_3$). An infinite number of complementary solvent systems may be devised for sample-cutting CCC in the future, for samples of any polarity.

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