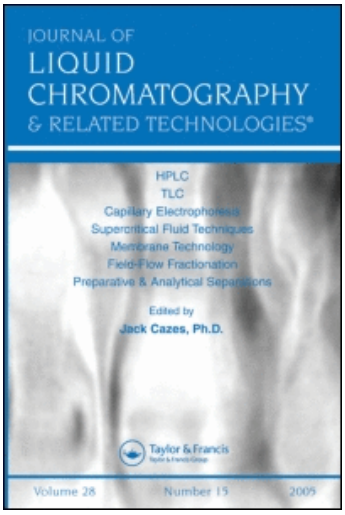

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

UV-Guided Isolation of Fungal Metabolites by HSCCC

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To cite this Article Dalsgaard, Petur W. , Nielsen, Kristian F. and Larsen, Thomas O.(2005) 'UV-Guided Isolation of Fungal Metabolites by HSCCC', Journal of Liquid Chromatography & Related Technologies, 28: 12, 2029 — 2039
To link to this Article: DOI: 10.1081/JLC-200063666
URL: http://dx.doi.org/10.1081/JLC-200063666

Journal of Liquid Chromatography & Related Technologies®, 28: 2029–2039, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1081/JLC-200063666

UV-Guided Isolation of Fungal Metabolites by HSCCC

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Abstract: Analytical standardised reversed phase liquid chromatography (RPLC) data can be helpful in finding a suitable solvent combination for isolation of fungal metabolites by high-speed counter current chromatography. Analysis of the distribution coefficient (K_D) of fungal metabolites in a series of biphasic systems consisting of *n*-heptane–ethyl acetate (EtOAc)–methanol (MeOH)–water has been used to illustrate the relationship between the RPLC retention indexes and K_D's. The relationship can be used to select a suitable solvent combination for HSCCC, as demonstrated by the isolation of two new cyclic peptides from a crude fungal extract.

Keywords: CCC, Filamentous fungi, Psychrophilin, LECA, LC-MS, Dereplication, Alkylphenones, Margraff system, *Penicillium rivulum*

INTRODUCTION

In the ongoing quest to isolate new metabolites from filamentous fungi, for drug discovery and chemotaxonomic purposes, we use high speed counter-current chromatography (HSCCC) for the isolation of presumed new metabolites. HSCCC, being a support-free liquid–liquid chromatography technique, has a 100% recovery rate since no compounds are irreversibly

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retained on the liquid stationary phase.[1–3] Furthermore, the solvent consumption is low, and it can be scaled up from analytical to preparative separation in a straightforward manner.[4] HSCCC is, thus, a powerful tool for the isolation of natural products,[5–7] and over the years a large number of solvent combinations have been developed.[1–3] A problem with HSCCC is the time consuming step of selecting a suitable biphasic solvent combination to allow the isolation of the target compound(s). The ARIZONA system, first developed by Margraff,[8] utilizes different combinations of heptane–EtOAc–MeOH–water. It is based on 23 combinations (from A to Z, with letters E, I, and O omitted), each with the same EtOAc over heptane ratio and water over MeOH ratio.[8] If the optimal distribution coefficient, K_D (between 0.5 and 2), in the ARIZONA combinations could be compared to the retention index (RI) of RPLC, it would be useful since we have screened thousands of fungal micro-extracts by RPLC using the RI system combined with diode array detection (DAD).[9–11] The RI system, based on the retention times of alkylphenones in RPLC, was used to allow retention times of mycotoxins analysed in different laboratories to be compared.[9]

In this paper, we demonstrate that data from a standardised RPLC-DAD screening method[9–11] can be applied for the selection of a suitable solvent combination from a simplified ARIZONA system consisting of 12 *n*-heptane–EtOAc–MeOH–water combinations.[12] The data from 10 metabolites from *Penicillium rivulum* define the relationship between RI and optimal K_D, and the isolation of two new cyclic peptides by HSCCC confirmed the versatility of the method.

EXPERIMENTAL

Chemicals

Solvents were gradient grade. Trifluoroacetic acid (TFA), formic acid 97% (FA), ammonium-formate, and alkylphenones for RI standards: acetophenone, propiophenone, butyrophenone, valerophenenone, hexanophenone, octanophenone, decanophenone[9] were analytical grade. The alkylphenones standards were diluted in methanol (MeOH) to 1.2, 2.4, 2.1, 2.1, 2.4, 2.8, and 2.8 mM, respectively. Water was purified to a 18.2 MΩ conductivity by a Milli-Q ion-exchanger (Millipore, USA).

Analytical LC

Analytical LC was performed on an Agilent (Waldbronn, Germany) 1100 Liquid Chromatographic system with a diode array detector (DAD), controlled by the ChemStation 6.1 software. The system was equipped with

a Phenomenex (Torrance, CA) Luna C_{18} II, 100 × 2 mm, 3 μm column and a Phenomenex SecurityGuard C_{18} pre column. A linear water–MeCN gradient system starting with 85% water and 15% MeCN, and changing to 100% MeCN in 20 min, keeping 100% MeCN for 5 min at a flow of 0.4 mL/min and 40°C was used for the separation. Both solvents contained 50 ppm TFA. An alkylphenone standard mixture was analysed (1 μL injected) as the first sample (after a blank run) in each sequence. In these chromatographic conditions, alkylphenone retention times were 5.50 min (acetophenone, RI 800), 8.85 min (propiophenone, RI 900), 11.13 min (butyrophenone, RI 1000), 12.93 min (valerophenone, RI 1100), 14.41 min (hexanophenone, RI 1200), 16.81 min (octanophenone, RI 1400) and 18.79 min (decanophenone, RI 1600).

For biphasic samples, the injector needle was set to draw 1 μL from the upper *n*-heptane–EtOAc phase (15 mm above the 2-mL vial bottom) and then 1 μL from the lower water–MeOH phase (0.5 mm above the vial bottom). Relative comparison of metabolite quantities were based on peak areas determined at their respective UV max wavelengths.

HSCCC Apparatus

HSCCC was carried out with a Model CCC-1000 high-speed counter current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three coils, connected in series (120 mL, wound with 0.86 mm I.D. PTFE tubing), on the same single bobbin. The HSCCC system was equipped with a Waters model 600 pump and a Waters model 996 photodiode array detector (Waters, Milford, USA), and a sample injection valve with a 3 mL sample loop. Data were processed using Millennium software.

LC-DAD-MS

LC-DAD-MS was performed on a similar system as described above, and was coupled to a LCT orthogonal Time Of Flight mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray source and a reference probe, and was controlled from the Masslynx 3.5 software. The MS was operated in the positive electrospray (ESI^+) mode, tuned to a resolution >6500 (at half peak height), and data were collected as centroid data from m/z 100 to 900 as described in detail by Nielsen and Smedsgaard.[10] Separation was performed as above, except that the column was 50 mm long, the flow was 0.3 mL/min, the MeCN was buffered with 20 mM FA, and the water with 10 mM ammonium formiate and 20 mM FA.

Preparation of Fungal Extracts and Dereplication of Secondary Metabolites

Penicillium rivulum Frisvad (IBT 24420) is available from the IBT Culture Collection, BioCentrum-DTU, Technical University of Denmark. Micro scale extracts for analytical LC-DAD screening were obtained from 14-day old yeast extract, sucrose (YES) and Czapek yeast autolysate (CYA) agar cultures[13] grown at 20°C, where 0.6 cm^2 was extracted with 1 mL EtOAc with 1% FA and subsequently with 1 mL 2-propanol, which was pooled, evaporated *in vacuo*, dissolved in 500 μL MeOH, and filtered through 4 mm PFTE syringe filters.[10]

Preparation of Extract

The extract for isolation of metabolites was obtained from a fungal isolates cultured on LECA nuts[14] covered by 4 × 500 mL Czapek yeast autolysate (CYA) at 20°C for 19 days in the dark. The LECA nuts inoculated with *P. rivulum* were extracted twice with EtOAc, filtered, and the combined extracts evaporated to dryness under reduced pressure.

Preparation of Biphasic Crude Extract Samples for LC-DAD Analysis

A sub sample (ca. 24 mg) of the crude preparative extract was dissolved in MeOH and filtered through a PFTE syringe filter, equal volumes distributed in twelve 2-mL vials (ca. 2 mg crude extract per vial), and evaporated under a gentle stream of nitrogen. The extracts were then dissolved in 1.6 mL of the twelve *n*-heptane–EtOAc–MeOH–water systems as listed in Table 1. After vigorous shaking, the samples were allowed to equilibrate. Equal volumes of each phase were then analysed by LC-DAD to obtain the K_D of each component. The K_D value of each component was determined from a pair of its corresponding peaks by dividing the peak height of the lower phase by that from the upper phase. The peak heights were obtained from the extracted LC chromatograms of the target compounds UV max ± 2 nm.

HSCCC Separation Procedure

The medium biphasic solvent system (1:1:1:1 v/v/v/v) was equilibrated thoroughly in a separation funnel at room temperature. The upper phase and lower phase were separated and the coils in the HSCCC were entirely filled with the lower MeOH–water rich phase as stationary phase. Then, the apparatus rotor was started and the mobile phase (upper *n*-heptane–EtOAc

Table 1. Relationship between the secondary metabolites from *Penicillium rivulum* and K_D from twelve combinations of *n*-heptane–EtOAc–MeOH–water

ARIZONA system			D	H	K	L	M	N	P	Q	R	T	W	Z
	n-heptane		1	2	3	4	5	6	6	6	6	6	6	6
	EtOAc		6	6	6	6	6	6	5	4	3	2	1	0
	MeOH		1	2	3	4	5	6	6	6	6	6	6	6
	Water		6	6	6	6	6	6	5	4	3	2	1	0
	RT	RI	K_D	K_D	K_D	K_D	K_D	K_D	K_D	K_D	K_D	K_D	K_D	K_D
Ergosterol (**10**)	24.35	2162	—	—	—	—	—	—	—	—	—	—	0.1	**0.9**
Unknown (**9**)	19.36	1658	—	—	—	—	—	—	—	—	—	—	—	0.3
Target 2 (**8**)	11.26	1007	—	—	—	0.1	0.2	**0.6**	**1.3**	3.3	8.9	—	—	—
Target 1 (**7**)	10.04	952	—	—	—	0.2	0.4	**1.1**	2.6	6.5	—	—	—	—
Griseofulvin (**6**)	9.12	912	—	—	0.2	**0.6**	**1.4**	3.5	7.2	—	—	—	—	—
Unkonwn (**5**)	8.18	880	0.2	**0.6**	**1.9**	5.9	—	—	—	—	—	—	—	—
Unknown (**4**)	7.72	866	—	—	0.1	**0.6**	**1.8**	6.2	—	—	—	—	—	—
Unknown (**3**)	5.40	797	**0.6**	**0.7**	**1**	2.4	3.8	—	—	—	—	—	—	—
Meleagrine (**2**)	5.19	791	—	—	0.1	**0.6**	**1.5**	5.0	—	—	—	—	—	—
Unknown (**1**)	1.07	668	**0.8**	2.3	3.4	—	—	—	—	—	—	—	—	—

K_D is calculated as $A_{\text{lower phase}}/A_{\text{upper phase}}$. K_D between 0.5 and 2 are most suitable for HSCCC separation (written in bold).

rich phase) was pumped into the column in tail-to-head mode, at a flow-rate of 1.0 mL/min. After the hydrodynamic equilibrium was established in the column (Sf = 75% stationary phase retention ratio), about 15 mg of the crude *P. rivulum* extract, dissolved in 3 mL of upper apolar mobile phase, was injected through the injection valve. The effluent of the column was continuously monitored with a diode array detector. Fractions of 10 mL were collected and analysed with LC-DAD as described above.

RESULTS AND DISCUSSION

To determine the relationship between RIs in RPLC and solute K_D's in the simplified ARIZONA system, the 7 alkylphenones were tested in the 12 ARIZONA combinations. The alkylphenones showed too high an affinity for the *n*-heptane–EtOAc phase, thereby precluding the determination of K_D and a usable relationship. Instead, the alkylphenones were used to determine the RI of the metabolites from a crude extract of *Penicillium rivulum.*

LC Analysis, Dereplication, and RI/K_D Relationship

Initial RPLC screening showed that CYA agar gave the highest quantities of metabolites and, thus, the fungus was grown in preparative scale on CYA loaded onto LECA.[14] Figure 1 shows the RPLC analysis of the crude extract from *P. rivulum.* In the dereplication step, RPLC-DAD and RPLC-DAD-MS analyses identified three known metabolites, meleagrine (**2**) (RI = 791, $[M + H]^+$ 434.18 amu), griseofulvin (**6**) (RI 912, $[M + H]^+$ 353.08 amu), and ergosterol (**10**) (RI = 2162, very poor ionization in positive electrospray ionisation (ESI^+)). An additional 7 unknown metabolites were selected because of their characteristic UV-spectra and the relative high peaks in the RPLC chromatogram. Two of the compounds, target 1 (**7**) and target 2 (**8**) had UV spectra identical with psychrophilin A[15] and were selected as target compounds for isolation.

Table 1 shows results from the biphasic RPLC analysis from twelve 2 mL vials with crude extract in the simplified ARIZONA solvent combinations. The result from the 24 injections (12 upper phase and 12 lower phase) shows a relationship between the RI and the optimal K_D (between 0.5 and 2) in the simplified ARIZONA system. Plotting the variation of K_D with the selected phase systems as they change in polarity (Figure 2) shows how K_D changes with polarity. It can be seen that for high RI values both RI and K_D change with polarity in a similar way, but in the mid-range it is less predictable. With the exception of Compounds **2**, **5**, and **9**, there is a rank-order correlation between RI and the polarity of the mobile upper

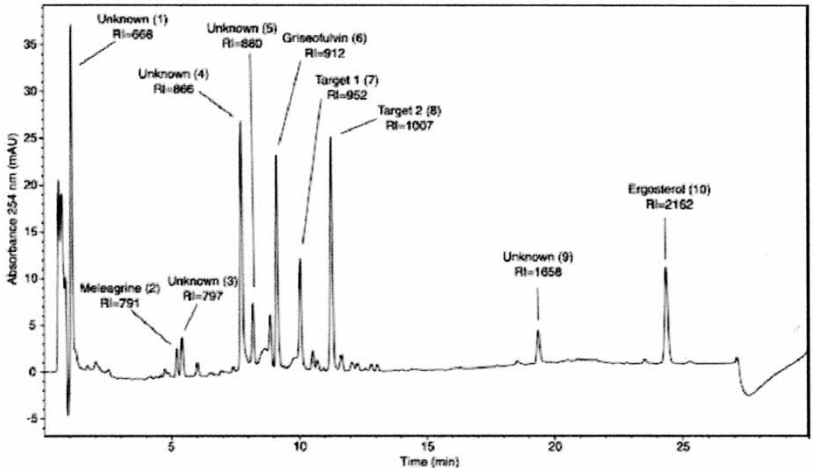

Figure 1. Chromatogram of the crude extract from *Penicillium rivulum* by RPLC analysis. Unknown (**1**), meleagrine (**2**), unknown (**3**), unknown (**4**), unknown (**5**), griseofulvin (**6**), target 1 (**7**), target 2 (**8**), unknown (**9**) and ergosterol (**10**). Conditions: column: reversed-phase Luna II C_{18} column (100 × 2 mm I.D., 3 μm); mobile phase: A: MeCN + 50 ppm TFA, B: water + 50 ppm TFA. Gradient: 30 min from 15% to 100% A; flow-rate: 0.4 mL min^{-1}; diode array detection.

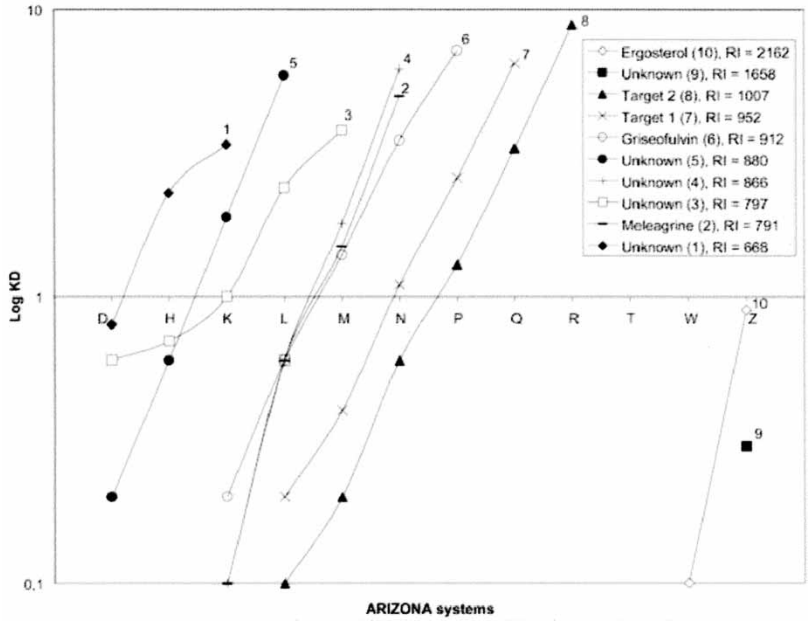

Figure 2. Variations of K_D of 10 metabolites in 12 ARIZONA solvent combinations.

phases in the Arizona solvent systems D through Z, with D being the most polar and Z being the least polar.

HSCCC Isolation

In order to isolate the target compounds **7** and **8**, the ARIZONA system designated N was selected. In this solvent combination, target 1 (**7**) has a K_D of 1.1 and target 2 (**8**) has a K_D of 0.6, which are ideal K_D's to use in HSCCC. Figure 3 shows the mirror RPLC chromatogram obtained after partitioning of the extract between the upper phase and the lower phase.

According to the HSCCC chromatogram in Figure 4, the two target compounds were separated with good resolution. Because the compounds were injected in the normal phase mode (apolar mobile phase in the tail-to-head direction and polar stationary phase), the apolar compounds elute with the solvent front. Target 2 (**8**) eluted between 80–90 min, and target 1 (**7**) between 150–170 min. From the HSCCC formula, $V_R = V_M + K_D\ V_S$ (V_R is retention volume, V_M is mobile phase volume and V_S is stationary phase volume),[16] target 2 (**8**) has an estimated K_D of 0.6 and target 1 (**7**) has a K_D of 1.4. Target 1 (**7**) has a slightly higher K_D than the one calculated from RPLC.

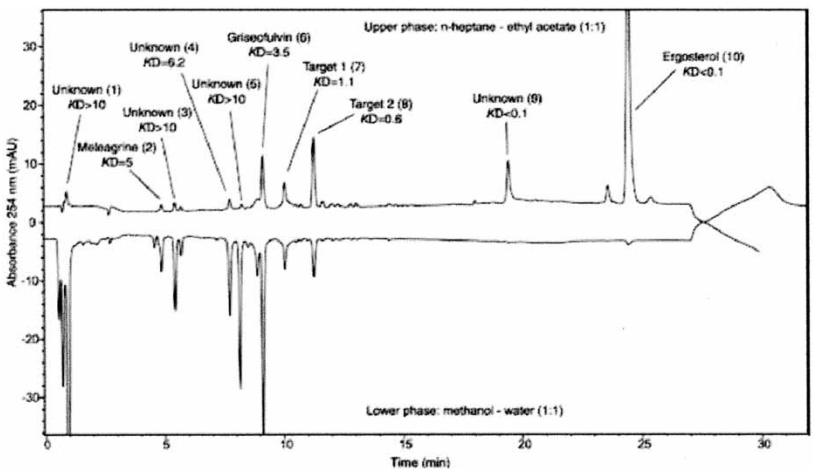

Figure 3. The mirror RPLC chromatogram of the distribution of the crude extract in the middle ARIZONA N combination of the solvents (*n*-heptane–EtOAc–MeOH–water (1:1:1:1 v/v/v/v)). The targets (**7** and **8**) are almost equally soluble in both phases. Compounds from **1** to **6** are most soluble in the lower MeOH–water-rich phase, and compounds **9** and **10** most soluble in the upper *n*-heptane–EtOAc-rich phase.

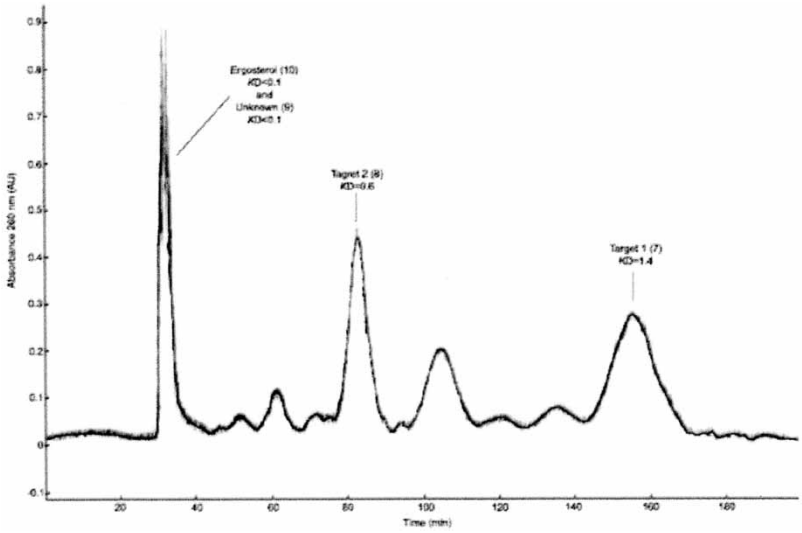

Figure 4. Chromatogram of the crude extract from *Penicillium rivulum* by HSCCC. The more polar compounds (**1, 2, 3, 4, 5, 6**) are not eluted and remain in the stationary phase. Solvent system: *n*-heptane–EtOAc–MeOH–water (1:1:1:1 v/v/v/v) (ARIZONA system N); mobile phase: apolar upper phase; flow-rate: 1.0 mL/min; sample size: 15 mg dissolved in 3 mL mobile phase; retention of the stationary phase: Sf = 75%.

The experiment shows that it is possible to isolate fungal metabolites by determining their RI, testing them in biphasic micro-scale RPLC, calculating the K_D, and directly injecting the crude extract into HSCCC. The two targets were later isolated by preparative HSCCC and found to be two new analogues of the cyclic peptide psychrophilin A.[17]

Future Application

Table 1 and Figure 2 can be used as a guide as to which ARIZONA system to use for HSCCC, after the RI has been calculated from a standardised RPLC analysis. Even though there is not a perfect rank-order correlation between the RI and the K_D, it should only be necessary to test 2–3 solvent combinations to find a suitable solvent system. An advantage with this approach is the fact that the K_D of the target compound can be determined without having the pure compound as a standard. However, the approach can be misleading since pH is not considered and different types of compounds may behave differently in RPLC and HSCCC.

When the ARIZONA system is scaled up from analytical to preparative HSCCC, the poor solubility of the sample may be a problem. This can be

compensated for by using the same ratio MeOH–EtOAc and lowering the concentration of *n*-heptane and water, or by using glyme and methyl *tert.*-butyl ether instead of EtOAc.[8]

CONCLUSION

These investigations show that the K_D of fungal metabolites can easily be determined by direct injection in RPLC and that there is a limited, but useful, rank-order relationship between the RI in RPLC and the K_D in the ARIZONA system. Utilizing this relationship eases the process of finding a suitable solvent combination for new target compounds. The K_D determined by RPLC can be used to determine the retention time of a target compound in HSCCC.

ACKNOWLEDGMENTS

This project was supported by the Danish Technical Research Council under the project "Functional biodiversity in *Penicillium* and *Aspergillus*" (grant no. 9901295). We are indebted to Jens C. Frisvad for identification of the fungal culture.

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Received September 20, 2004
Accepted December 13, 2004
Manuscript 6591G