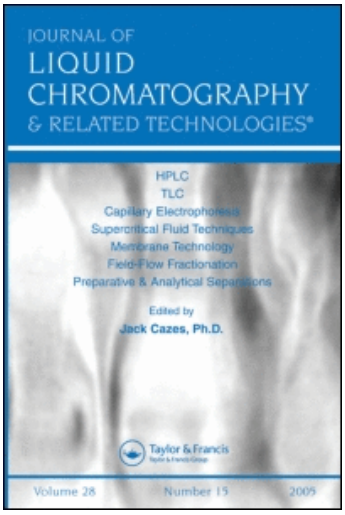


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Speed Countercurrent Chromatography : A Promising Method for the Separation of the Annonaceous Acetogenins[1]

P. Duret[a]; A. -I. Waechter[a]; R. Margraff[b]; A. Foucault[c]; R. Hocquemiller[a]; A. Cavé[a]
[a] Laboratoire de Pharmacognosie URA CNRS 1843-BIOCIS Faculté de Pharmacie, Université Paris XI rue Jean-Baptiste Clément, France [b] Natural Products Department, RhÔne-Poulenc Rorer SA Vitry Research Center, Vitry-sur-Seine, Cedex, France [c] Unité de Recherche Marine 2, Nantes, cedex, France

To cite this Article Duret, P. , Waechter, A. -I. , Margraff, R. , Foucault, A. , Hocquemiller, R. and Cavé, A.(1997) 'High-Speed Countercurrent Chromatography : A Promising Method for the Separation of the Annonaceous Acetogenins[1]', Journal of Liquid Chromatography & Related Technologies, 20: 4, 627 — 635
To link to this Article: DOI: 10.1080/10826079708010949
URL: http://dx.doi.org/10.1080/10826079708010949

J. LIQ. CHROM. & REL. TECHNOL., 20(4), 627–635 (1997)

HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY : A PROMISING METHOD FOR THE SEPARATION OF THE ANNONACEOUS ACETOGENINS[1]

P. Duret,[a] A.-I. Waechter,[a] R. Margraff,[b]
A. Foucault,[c] R. Hocquemiller,[a] A. Cavé[a]

[a]Laboratoire de Pharmacognosie
URA CNRS 1843-BIOCIS
Faculté de Pharmacie
Université Paris XI
rue Jean-Baptiste Clément
92296 Châtenay-Malabry, France

[b]Rhône-Poulenc Rorer SA
Vitry Research Center
Natural Products Department
13, quai Jules Guesde
94403 Vitry-sur-Seine Cedex, France

[c]Unité de Recherche Marine 2, IFREMER
rue de l'Ile d'Yeu, BP 1049
44037 Nantes cedex 01, France

ABSTRACT

High-speed countercurrent chromatography (HSCCC) was applied, for the first time, to the separation of Annonaceous acetogenins. The two major acetogenins of the seeds of *Annona atemoya*, squamocin and rolliniastatin-2, were isolated in one

step using a biphasic mixture of heptane/ethyl acetate/methanol/water. In addition, this method yielded four pairs of highly purified acetogenins: squamocin/molvizarin, rolliniastatin-2/asimicin, neoannonin/atemoyin and isodesacetyl-uvaricin/desacetyluvaricin. These pairs were further resolved by HPLC, leading to a total of eight pure acetogenins in two steps. Isolated acetogenins, which are homologous, position and configurational isomers, have been identified by comparison with standards and by spectroscopic techniques.

INTRODUCTION

Annonaceous acetogenins are recently discovered polyketides which were isolated, until now, only from the tropical and subtropical plants of the Annonaceae family.[2-4] They are characterized by common features: a long chain of 35-37 carbon atoms, including one or two tetrahydrofuran rings, several oxygenated functions (hydroxyl, ketone), and a γ-methyl-γ-lactone terminal group. In addition to these natural biologically active products, some natural precursors have been isolated, which bear only a γ-lactone and either hydroxyl groups and/or 1,2-epoxides with double bonds on the alkyl chain. Most of these compounds exhibit interesting cytotoxic activity with promising antitumor potential, and some have parasiticide, insecticide and immunomodulating properties.[2-4] They exist in the plant as mixtures of numerous isomers or homologs of close polarities making their isolation difficult. Various successive and repeated chromatographic steps on silica gel, Sephadex LH 20, preparative TLC, and normal and reversed phase HPLC, were needed to purify acetogenins, with generally poor overall yields.

We were interested in applying, for the first time, high-speed countercurrent chromatography (HSCCC)[5,6] for purification of acetogenins and we report, in this paper, the isolation of two major acetogenins, squamocin[2] and rolliniastatin-2,[3] from the seeds of *Annona atemoya*.[7,8]

MATERIALS AND METHODS

Extraction

Seeds of *Annona atemoya* were collected in September 1993 in Australia and authenticated by Dr. D. J. Batten (Tropical Fruit Research Station, Alstonville, New South Wales, Australia). The dried and pulverized seeds

(930g) were macerated with MeOH. The MeOH extract (79.7 g) was partitioned between H_2O and hexane to yield 6 g of hexane extract. The aqueous alcohol fraction was partially evaporated and extracted with dichloromethane to afford 29.6 g of CH_2Cl_2-soluble extract.

Apparatus

CCC runs were performed using a HSCCC[5] (P.C. Inc., Potomac, MD, USA); the large coil of the triple-coil set was used (i.d. = 1.68 mm, total volume V_c = 240 mL). The rotational speed was 800 rpm. The pump was a model LKB P-500 (Pharmacia, Uppsala, Sweden), and the injector a medium pressure Rheodyne model 50110 injector with a 5 mL sample loop. Fractions (5 mL) were collected with a fraction collector model 202 (Gilson, Villiers-le-Bel, France).

Fractionation by HSCCC

The HSCCC experiment was performed with the quaternary system heptane/ethylacetate/methanol/water (HEP/EtOAc/MeOH/H_2O) [3:10:10:7] v/v/v/v. The coil was first entirely filled with the upper phase and rotation was set up at the desired speed (800 rpm). The lower phase was then pumped into the column at a flow-rate of 4 mL/min, in the head to tail mode. After the mobile phase front emerged and the equilibrium between the two phases was established (mobile phase volume = 86 mL), the sample solution (500 mg of the dichloromethane extract in 5 mL of mobile phase) was injected through the injection valve at a flow-rate of 1 mL/min. The flow-rate was then gradually increased to 4 mL/min and the effluent was collected from the outlet of the column. After the fraction 190 had been collected, the rotation was stopped and the content of the column was pushed out by the mobile phase and collected.

Analysis of the Collected Fractions

The composition of each fraction was determined by TLC on silicagel $60F_{254}$ plates (Merck, Darmstadt, Germany) using toluene/EtOAc/EtOH [30:70:5] v/v/v as mobile phase. Solutes were visualized by vanillin-H_2SO_4 spray and heat (100°C for 10 min.) while acetogenins were characterized by the more specific Kedde reagent (typical of an unsaturated γ-lactone). Fractions

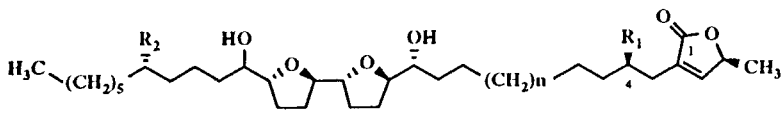
containing only one spot were analyzed by HPLC, MS and NMR and identified by comparison with authentic samples of molvizarin **[1]**, squamocin **[2]**, rolliniastatin-2 **[3]** (=bullatacin), asimicin **[4]**, neoannonin **[5]**, atemoyin **[6]**, isodesacetyluvaricin **[7]** and desacetyluvaricin **[8]**.[2-4] Analyses were performed with an HPLC system equipped with a Beckman pump 112 (Beckman Instruments, Berkeley, CA, USA), a Varian 9050 spectrophotometer (Varian, San Fernando, CA, USA) and a U6K Waters injector (Millipore, Waters Chromatography Div., Milford, MA, USA). A Spherisorb S5-ODS2 C_{18} column, 25 x 0.46 cm (Prolabo, Paris, France) and mobile phases MeOH/H_2O [85:15] v/v or [90:10] v/v were used. The flow-rate was 0.8 mL/min. and the effluent was monitored at 214 nm. Similar fractions were pooled, evaporated to dryness, and pairs of acetogenins were separated with a preparative HPLC Millipore-Waters system equipped with a model 590 pump, an SSV injector, and a 484 UV detector (214 nm). A μBondapak C_{18} prepacked cartridge (10 mm, 25 x 100 mm), eluted with MeOH/H_2O [82:18] v/v for molvizarin **[1]** / squamocin **[2]** and rolliniastatin-2 **[3]** / asimicin **[4]** or [90:10] v/v for neoannonin **[5]** / atemoyin **[6]** and isodesacetyluvaricin **[7]** / desacetyluvaricin **[8]** was used. Pure isolated products were finally analyzed by NMR and MS. NMR spectra ($CDCl_3$) were obtained with a Bruker AC-200 instrument (Bruker, Wissembourg, France) while EIMS and CIMS (methane) were performed on a Nermag R10-10C spectrometer (Nermag, Rueil-Malmaison, France).

Solvents

All organic solvents were of analytical grade and came from Prolabo (Paris, France). Authentic samples of acetogenins have been previously isolated from several Annonaceae species and were supplied by Prof. A. Cavé and collaborators.

RESULTS AND DISCUSSION

The large number of acetogenins present in the same annonaceous species, and their very close chemical structures (homologous, positional and configurational isomers), account for the difficulty in separating these kinds of compounds.[2-4] Many successive and repeated conventional column chromatographic operations are needed to isolate pure acetogenins. They are often obtained as mixtures and must be purified by HPLC. In recent years, HSCCC has gained increasing popularity for isolation of bioactive natural products. HSCCC, based on the fundamental principle of liquid-liquid



X/trans/threo/trans/threo

	R_1	R_2	n	X
Molvizarin [1] :	OH	H	4	*erythro*
Squamocin [2] :	H	OH	6	*erythro*
Rolliniastatin-2 [3] :	OH	H	6	*erythro*
Asimicin [4] :	OH	H	6	*threo*
Ncoannonin [5] :	H	H	4	*erythro*
Atemoyin [6] :	H	H	4	*threo*
Desacetyluvaricin [7] :	H	H	6	*erythro*
Isodesacetyluvaricin [8] :	H	H	6	*threo*

Figure 1. Acetogenins isolated from *Annona atemoya* seeds.

partition, utilizes a particular combination of coil orientation and planetary motion to produce a hydrodynamic phenomenon in the distribution of two immiscible solvents in a coiled column.[5,6] We found it very attractive to use this method, for the first time, to purify acetogenins.

The dichloromethane extract of *Annona atemoya* seeds is a very complex mixture, having its components spread over a wide range of polarity. So a compromise between resolution and run time was necessary. Considering the solubility of the compounds present in our extract, classical biphasic solvent systems like CH_2Cl_2/MeOH/H_2O and EtOAc/MeOH/H_2O were first examined. The adjustment of the partition coefficients to desired levels by changing the ratio of MeOH was impossible, acetogenins partitioning always entirely into the organic phases. Addition of heptane as a modifying solvent was required to decrease the solubility in the organic phase by increasing its hydrophobicity and expelling more polar components into the aqueous phase. Finally, the quaternary system HEP/EtOAc/MeOH/H_2O [3:10:10:7] v/v/v/v, combining a large capacity for dissolving extract with different partition coefficients for the acetogenins (checked by TLC[9]) was selected. Two pure compounds have been obtained in one step, using this solvent system after injection of 500 mg of the dichloromethane extract; the major acetogenins of the *Annona atemoya* seeds, squamocin **[1]** (fractions 58 to 59, 12 mg, 2.4% of the dichloromethane extract, 94% pure by HPLC) and rolliniastatin-2 **[3]** (fractions 67 to 69, 5 mg, 1% of the extract, 98% pure by HPLC). These two acetogenins differ only in the position of one hydroxyl group, *i.e.*, C-28 for squamocin and C-4 for rolliniastatin-2 (Figure 1). Four pairs of acetogenins, molvizarin **[1]** /

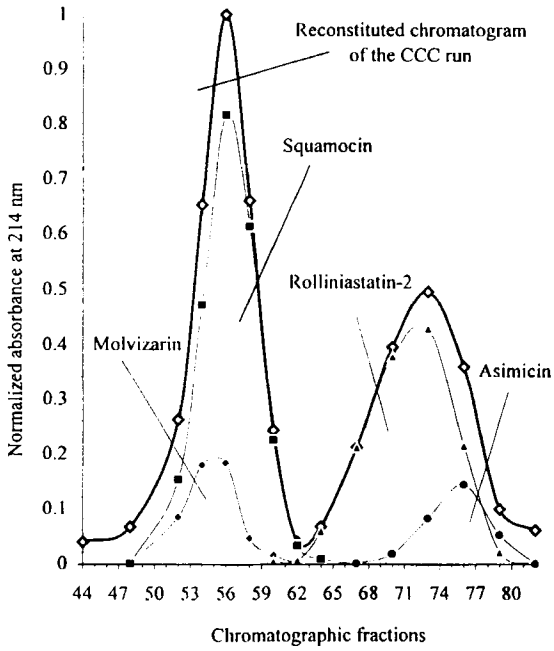


Figure 2. Chromatogram drawn from HPLC analyses of HSCCC fractions 44 to 82, showing the separation of molvizarin **[1]**, squamocin **[2]**, rolliniastatin-2 **[3]** and asimicin **[4]**.

squamocin **[2]** (fractions 52 to 57, 87 mg, 17.4% of the extract), rolliniastatin-2 **[3]** / asimicin **[4]** (fractions 70 to 78, 34 mg, 6.8% of the extract), neoannonin **[5]** /atemoyin **[6]** (fractions 130 to 155, 4 mg, 0.8% of the extract) and isodesacetyluvaricin **[7]** / desacetyluvaricin **[8]** (fractions 170 to 181, 6 mg, 1.2% of the extract) were also obtained with these chromatographic conditions. These mixtures were further purified by HPLC and pure compounds were identified by comparison (HPLC, MS and NMR) with standards previously isolated from the same crude extract by combination of numerous successive chromatographies on silica gel and tedious repeated reversed phase HPLC.[7]

The HSCCC chromatogram drawn from the HPLC analysis of fractions 44 to 82 (Figure 2) showed an elution in conformity with the reversed phase mode of chromatography which was used. Differences in polarities of acetogenins are mainly due to their oxygenated groups, carbon atom number, and relative configurations of their THF rings. Although squamocin **[2]** and rolliniastatin-2

[3] are both bis-THF acetogenins with 37 carbon atoms, three hydroxyl groups and the same relative configuration (*threo/trans/threo/trans/erythro*), squamocin is eluted first. This is probably due to the 4-OH in **3** which takes part in a hydrogen bond with the carbonyl group of the lactone. The 28-OH of **2** should be more easily submitted to interactions of polar solvents, explaining the differences in partition of the squamocin and rolliniastatin-2.

Elution of acetogenins in HSCCC follows an order similar to that found in C_{18} reversed phase HPLC: molvizarin **[1]** first with squamocin **[2]**, then rolliniastatin-2 **[3]**, then its configuration isomer asimicin **[4]** (HSCCC chromatogram shows a small but significant difference in their elution times) then neoannonin **[5]** / atemoyin **[6]** which are configuration isomers with 35 carbon atoms and two hydroxyl groups, and finally a mixture of two configuration isomers, isodesacetyluvaricin **[7]** / desacetyluvaricin **[8]**, which contain 37 carbon atoms and two hydroxyl groups (Figure 1).

Efficient separations of acetogenins are possible by optimization of the chromatographic conditions. For instance, HEP/EtOAc/MeOH/H_2O [3:10:10:7] v/v/v/v is an appropriate solvent system to separate **[1]** or **[2]** and **[3-8]**, **[3]** or **[4]** and **[5-8]**, **[5]** or **[6]** and **[7-8]** but inappropriate for **[1]** and **[2]**, **[5]** and **[6]**, **[7]** and **[8]**. Although a combination of HSCCC in initial fractionation work, with HPLC in the final step of purification seems to be a good approach (large amounts of mixtures can be treated and crude extracts pose no problem), pure compounds can be also obtained with this method in one step. Therefore, a judicious choice of the biphasic solvent system, determining the relative proportions of solutes passing into each of the two phases, is crucial to the success of the purification.

CONCLUSION

The two main acetogenins of the seeds of *Annona atemoya*, squamocin and rolliniastatin-2 were obtained in one step and with a high purity by using high-speed countercurrent chromatography. In addition, this method yielded four pairs of acetogenins, the separation of which, by HPLC, led to six additional compounds in a second step (molvizarin, asimicin, neoannonin, atemoyin, isodesacetyluvaricin and desacetyluvaricin). HSCCC allowed us to separate acetogenins with very closely related chemical structures: neoannonin/atemoyin and isodesacetyluvaricin/desacetyluvaricin, which differ by two methylene, position isomers (squamocin and rolliniastatin-2), as well as relative configurational isomers (rolliniastatin-2 and asimicin). HSCCC seems to be a very effective and promising tool for the purification of biologically

active annonaceous acetogenins, and would be a good alternative to column chromatography by using appropriate biphasic solvent systems and well adjusted partition coefficients.

ACKNOWLEDGMENTS

This research was sponsored by the Direction de la Recherche et des Etudes Doctorales (DRED), through a biennial contract with the Réseau de Recherche "Pharmacochimie". We wish to thank J. C. Jullian for NMR measurements, and S. De Barros and T. Becue for MS spectra (SAMM). We are specially grateful to Dr. D. J. Batten for supplying and for the identification of the plant material.

REFERENCES

1. Part 49 in the series "Acetogenins from Annonaceae". For part 48, see C. Gleye, A. Laurens, R. Hocquemiller, and A. Cavé, J. Org. Chem., (1996), submitted.

2. A. Cavé, D. Cortes, B. Figadère, R. Hocquemiller, O. Laprévote, A. Laurens, M. Lebœuf, "Recent Advances in Acetogenins of Annonaceae," in **Recent Advances in Phytochemistry: Phytochemical Potential of Tropical Plants, Vol. 27**, K. R. Downum, J. T. Romeo, H. A. Stafford, eds., Plenum Press, New York, 1993, pp. 167-202.

3. X.-P. Fang, M. J. Rieser, Z.-M. Gu, G.-X. Zhao, J. L. McLaughlin, Phytochem. Anal., **4**, 27-67 (1992).

4. Zhe-Ning Gu, Geng-Xian Zhao, N. Oberlies, Lu Zeng, J. L. McLaughlin, "Annonaceous Acetogenins : Potent Mitochondrial Inhibitors with Diverse Applications," in **Recent Advances in Phytochemistry: Phytochemistry of Medicinal Plants, Vol. 29**, J. T. Arnoson, R. Mata, J. T. Romeo, eds., Plenum Press, New York, 1995, pp. 249-310.

5. Y. Ito, W. D. Conway, **High-Speed Countercurrent Chromatography**, Chemical Analysis Series, Vol. 132, John Wiley & Sons Inc., New York, 1996.

6. A. Foucault, Anal. Chem., **63**, 569A (1991).

7. P. Duret, R. Hocquemiller, A. Laurens, A. Cavé, Nat. Prod. Lett., **5**, 295-302 (1995).

8. P. Duret, A. I. Waechter, R. Hocquemiller, A. Cavé, D. J. Batten, Nat. Prod. Lett., **8**, 89-95 (1996).

9. A. Foucault, **Centrifugal Partition Chromatography**, Chromatographic Science Series, Vol. 68, J. Cazes, ed., Marcel Dekker Inc., New York, 1995, chapter 4, pp.71-97.

Received June 23, 1996
Accepted July 4, 1996
Manuscript 4220