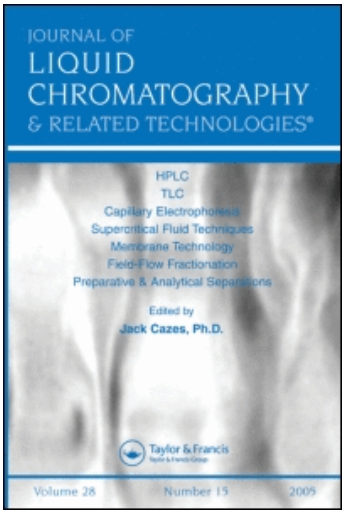

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HSCCC-MS Study of Flavonoids in the Extracts from the Seeds of *Oroxylum indicum*

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Abstract: In this study, two analytical high-speed countercurrent chromatography (HSCCC) instruments, interfaced directly with electrospray ionization and atmospheric pressure chemical ionization mass spectrometry, were explored for the separation of a mixture of three standard flavonoids. Hexane–ethyl acetate–methanol–water solvent systems with different ratios were chosen and optimized for the best separation. On-line HSCCC-MS was successfully used in the identification of flavonoids from an ethyl acetate extract of the seeds of *Oroxylum indicum.*

Keywords: HSCCC, Flavonoids, *Oroxylum indicum*

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INTRODUCTION

During the preceding decade, high-speed countercurrent chromatography (HSCCC) has been widely used for the analytical and preparative scale separation of a wide range of natural products.[1,2] There are also a lot of publications dealing with the separation of flavonoids.[3] Interfacing CCC with mass spectrometry was suggested by H. Oka in 1980.[4] HSCCC coupled to a mass spectrometer has not been widely used for the following reasons: 1) HSCCC-MS can sometimes result in the carry-over of stationary phase, which can result in high baseline noise in mass spectra; 2) the high back pressure caused by interfacing the two instruments can reduce the life of the flying leads; 3) the solvent systems usually used in HSCCC would not give good sensitivity, unless a modified solution was added to help ionization of target components; 4) most CCC instruments were of preparative scale and, therefore, unsuitable for the low flow rates required by the mass spectrometer. However, the benefits offered by coupling a mass spectrometer with many analytical instruments play a very important role in the identification and determination of components in a complex sample.

Mass spectrometry is a rapid detection method that works well with many types of chromatography because of its high sensitivity and the small amount of sample required. It is important to interface HSCCC with mass spectrometry because it combines the advantage of HSCCC with the low detection limit and identification capability of MS. Considerable effort has recently been made to develop analytical HSCCC for interfacing mass spectrometry. CCC-TSP (thermospray)-MS has been successfully applied to the separation, mainly of natural products including the analyses of alkaloids, triterponic acid, and ligans.[5–7]

The development of the CCC technique at analytical scale and the development of stainless steel, high-pressure coils allow the use of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) MS as a detection technique.[8] Hence, the CCC-ESI-MS and CCC-APCI-MS provide convenient interface methods at atmospheric pressure, suitable for analytical scale separations.

In this study, two HSCCC instruments interfacing directly with ESI and APCI mass spectrometry were explored for the separation of mixtures of four standard flavonoids, and were also successfully used for the isolation and identification of flavonoids in an ethyl acetate extract from the seeds of *Oroxylum indicum.*

EXPERIMENTAL

Solvent and Reagents

HPLC grade solvents used in this study included hexane, ethyl acetate, methanol, and water. All these solvents were purchased from Fisher

Chemicals (Loughborough, UK). AnalR grade formic acid and acetic acid were also used from the same supplier. All solvents were degassed prior to use. Standard baicalein-7-O-glucoside was isolated by our laboratory,[9] and the purity checked by UV and NMR. Baicalein, Biochanin A, and chrysin were purchased from SIGMA Company.

Instruments

Two HSCCC instruments were used in the study: a) an analytical HSCCC instrument, which was a prototype J-type coil planet centrifuge high-speed analytical instrument with a rotor radius of 110 mm. The instrument was assembled with two identical bobbins; each with 25 m of 0.76 mm bore tubing connected in series to provide a total coil volume of 25 mL with a β value of 0.88. The centrifuge is designed to rotate at speeds up to 1400 rpm. In this study, speeds up to 1100 rpm were used; b) a J-type Milli-CCC centrifuge with a rotor radius of 50 mm and gears enclosed in a lubricated gearbox to minimize noise. Its volume with one coil mounted in a cantilever style is 4.6 mL with 10 m of 0.76 mm bore tubing. Both centrifuges were provided by Brunel Institute for Bioengineering, Brunel University and the Milli-CCC centrifuge is now available from Dynamic Extractions Ltd, Brunel Enterprise Centre, Uxbridge UB8 3PH as the Mini-DE Centrifuge.

Mass spectrometry: A Finnigan AQA mass spectrometer (Thermoquest) was used and this instrument can be set up rapidly with an ESI or APCI interface.

Sample Preparation

Seeds of *Oroxylum indicum* (50 g) were refluxed for 5 hours in 90% methanol, the extracts were then filtered and evaporated. The residue was redissolved in 200 mL H_2O and extracted three times with ethyl acetate. Final evaporation yielded 6.5 g of a yellow powder.

HSCCC Method

A biphasic mixture of hexane–ethyl acetate–methanol–water was prepared in different ratios and purged for 30 minutes with nitrogen to remove any dissolved gases. First, the coil was filled with the upper phase (organic layer: hexane–ethyl acetate) of the biphasic mixture. The coils were rotated in a forward direction at a speed of 1100 rpm and the lower phase (aqueous: methanol–water) was pumped into the coil from head to tail at a flow rate of 1.0 mL/minute. When the two layers were observed, the equilibration point was determined when no more stationary phase was eluted (hydrodynamic equilibration). The retention volume of the system could then be

calculated by subtracting the volume of the stationary phase eluted at the end of the equilibration process, and dead volume from the total volume. The dead volume of the column was calculated according to the method recently described by Wood et al.[10]

HSCCC Interfacing with Mass Spectrometer

Analytical HSCCC can be interfaced directly with ESI/MS or APCI/MS without additional pumps between the mass spectrometer and the HSCCC. When ESI/MS is interfaced with HSCCC, the flow rate must be reduced splitting the flow for ESI ionization. Figure 1 shows the HSCCC interfacing with the mass spectrometer.

RESULTS AND DISCUSSION

Negative HSCCC/ESI-MS for the Separation of a Standard Flavonoid Mixture of Baicalein, Chrysin, Biochanin A, and Baicalein-7-O-Glucoside

The results of tuning with standard flavonoids showed that negative ESI ionization is better than positive ESI ionization. A 0.05% ammonium formate buffer solution of pH = 4, which is commonly used in HPLC-MS, was also added between the HSCCC and the mass spectrometer in order to improve the ionization efficiency. Figure 2 shows the separation results by negative HSCCC-ESI-MS for the separation of a standard mixture of flavonoids.

HSCCC-ESI-MS could affect the detection sensitivity of three flavonoids in the negative mode, and biochanin-A was highly retained in the stationary phase. Furthermore, a split of flow rate has to be used to provide the low flow rates required by the mass spectrometer in the ESI mode, making the sensitivity problem even worst. So, HSCCC-APCI-MS was explored for the separation of flavonoids without use of the split of the flow.

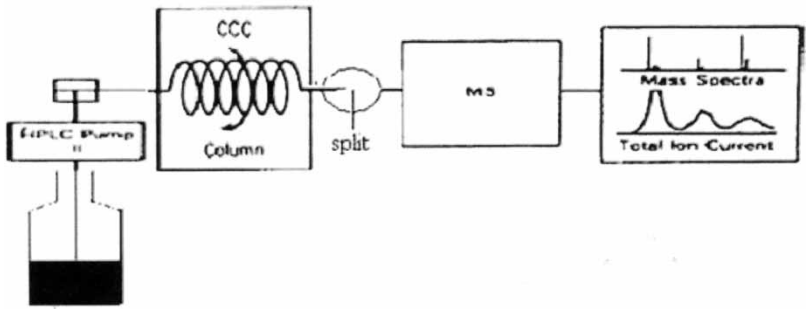

Figure 1. The HSCCC interface with mass spectrometry.

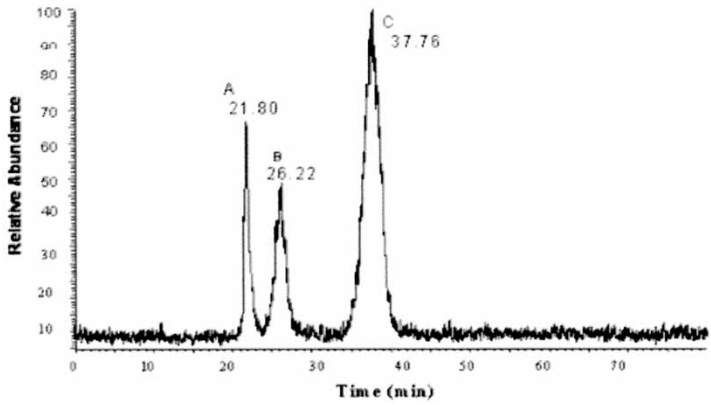

Figure 2. Total ion current (TIC) chromatogram of a standard flavonoid mixture by negative HSCCC-ESI-MS. A: Baicalein-7-O-glucoside; B: Baicalein; C: Chrysin. Sample concentration was 25 μg/mL. HSCCC conditions: Solvent system: Hexane–Ethyl acetate–Methanol–Water (1:1.2:1:1 v/v/v/v); Upper organic phase: Stationary phase; Lower polar aqueous phase: Mobile phase, 1 mL/min; Stationary retention $S_F = 57\%$; Mass Spectrometry conditions: Capillary temperature is 170°C; the AQA is 25 kV.

HSCCC-APCI-MS for the Separation of a Standard Mixture of Flavonoids

Figure 3 shows that HSCCC-APCI-MS can resolve a standard flavonoid mixture of baicalein-7-O-glucoside, baicalein, and chrysin as well. Biochanin A is the last constituent eluted but gives low signals. This result indicates that negative APCI mode could effect the ionization of flavonoids.

The study showed that HSCCC-APCI-MS in the negative mode could be used for the identification of a mixture of standard flavonoids. The next step is to show that similar results can be obtained for the separation and identification of flavonoids in the seeds of *Oroxylum indicum.*

Application of HSCCC-APCI-MS for the Separation of Flavonoids from the Ethyl Acetate Extract of the Seeds of *Oroxylum indicum*

Oroxylum indicum is a small to medium sized tree found in China and India. Its seeds are known as the crude drug 'Mu Hu Die' in China and it has been used as an analgesic, antitussive, and anti-inflammatory agent for the treatment of cough, bronchitis, and other diseases. Its importance has already been described in a previous paper.[11] HSCCC-APCI-MS can provide an important guide for the selection of solvent systems for preparative CCC. Figure 4 shows the separation of the ethyl acetate extract from the seeds

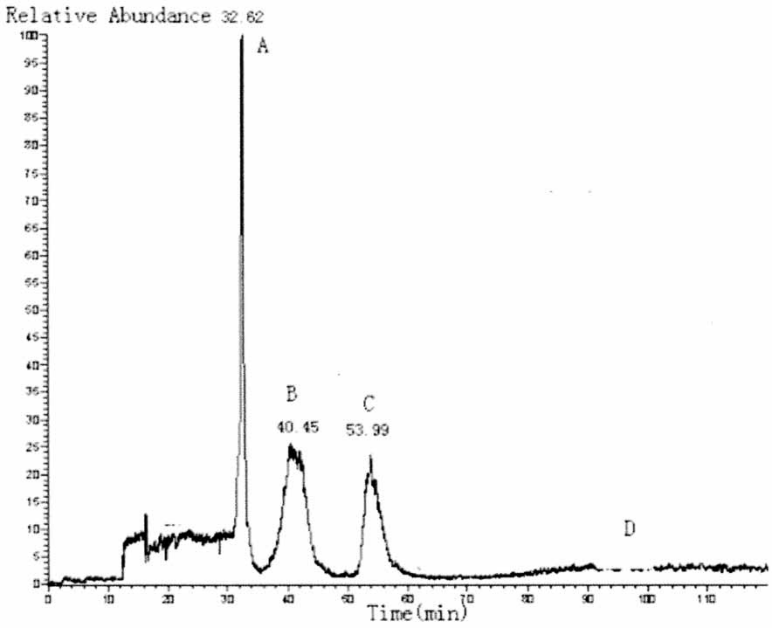

Figure 3. Reconstructed TIC chromatograms of a standard flavonoid mixture by negative HSCCC-APCI-MS. A) Baicalein-7-o-glucoside; B) Baicalein; C) Chrysin. D) Biochanin A. Sample concentration was 25 μg/mL. HSCCC condition: Solvent system: hexane–ethyl acetate–methanol–water (1:2:1:1); Upper organic phase: Stationary phase; Lower aqueous polar phase: Mobile phase; Stationary retention $S_F = 57\%$; Speed 1100 rpm; Flow rate: 1 mL/min. Mass Spectrometer conditions: Capillary temperature 250°C, AQA 20 V, Corona 3 V.

by HSCCC-APCI-MS and Figure 5 shows the mass spectra of fractions 1, 2, and 3 from Figure 4.

It was concluded that HSCCC-APCI-MS could be successfully used for the separation of flavonoid components of the ethyl acetate extract from the seeds of *Oroxylum indicum.* Three components were obtained with high purities and were identified as baicalein-7-O-glucoside, baicalein, and chrysin by comparing their mass spectra with those of standard materials.

Milli-ESI-MS for the Separation Flavonoids from the Ethyl Acetate Extract of the Seeds of *Oroxylum indicum* in Positive Mode

The Milli CCC is a recent analytical instrument. It can not only conduct separations in an analytical timescale but also has the ability to be connected to a mass spectrometry detector for on line CCC-MS separation. This device reduces the noise level since all its gearing is enclosed in a

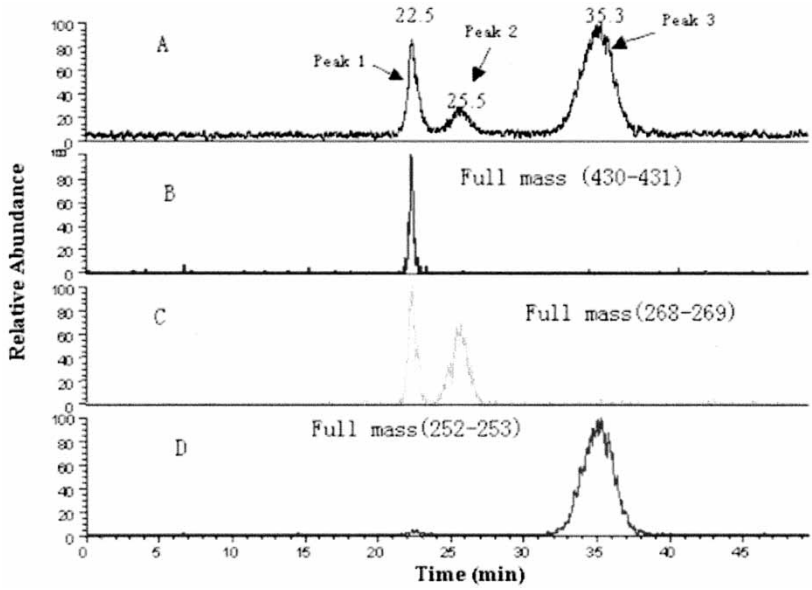

Figure 4. A) TIC (total ion current) chromatograms of the extract of the seeds of *O, indicum* by HSCCC-APCI-MS separation. Peak 1: baicalein-7-O-glucoside; Peak 2: baicalein; Peak 3: chrysin. B) SIM (Selected ion current) chromatogram of baicalein-7-O-glucoside at $m/z = 431$; C) SIM chromatogram of baicalein at $m/z = 269$; D) SIM chromatogram of chrysin at $m/z = 253$. HSCCC conditions: Solvent system: hexane–ethyl acetate–methanol–water, 1:1.2:1:1 v/v/v/v; Upper organic phase: Stationary phase; Lower aqueous polar phase: Mobile phase 1 mL/min; Stationary retention: $S_F = 50\%$; rotor rotation speed 1100 rpm.

gearbox and operates in oil. Figures 6 and 7 show the Milli-CCC-MS results for the separation of flavonoids of the ethyl acetate extract from the seeds of *Oroxylum indicum* with different flow rates. In Figure 6, the whole elution can be finished in 15 min when the flow rate is 1 mL/min, and three constituents are baseline resolved. When the flow rate is doubled (2 mL/min), the elution time is shortened to 9 minutes, but constituents 1 and 2 overlap due to the reduction of retained stationary phase volume (reduced resolution, see Figure 7 and Table 1). The Milli-CCC is an excellent instrument for interfacing with a mass spectrometer.

Comparison Between the HSCCC Centrifuge and the Milli-CCC Centrifuge

The HSCCC centrifuge has a coil, which is 4 × longer than the Milli-CCC and so, should give resolutions between peaks 2 × greater. The resolutions between the two centrifuges calculated from Figures 4 and 7 are compared

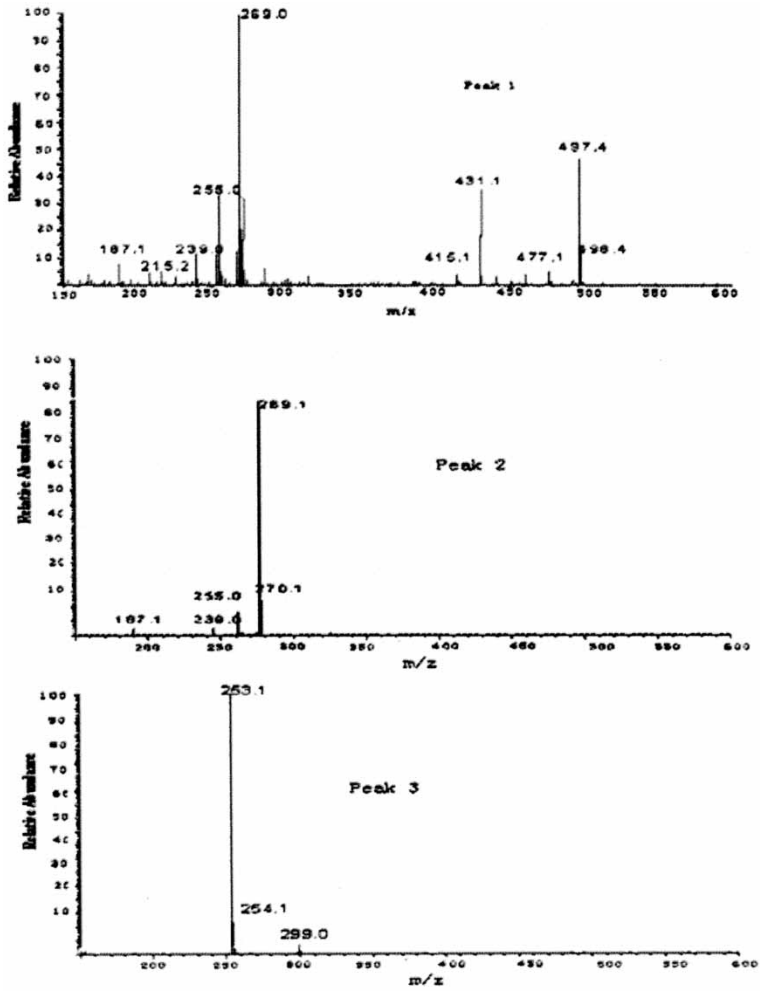

Figure 5. Full scan negative APCI mass spectra of peaks 1, 2, and 3.

in Table 1. It can be seen that the resolution between peaks 1 & 2 is actually 1.7 × better on the Milli-CCC centrifuge and between 2 & 3 is 1.6 × worse. The Milli CCC centrifuge is, therefore, more efficient than the HSCCC one for a given length of tubing. Furthermore, because the Milli-CCC has shorter coils separations are much faster, 15 min instead of 40 min for the HSCCC. This increased efficiency is mainly due to the smaller radius of rotation, which for the same g level gives more mixing and settling cycles per minute (1800 for Milli-CCC and only 1100 for HSCCC). Also, the stationary phase retention in the Milli-CCC (81%) is significantly higher than that in the HSCCC (50%). Compared to the larger analytical HSCCC, the Milli-CCC

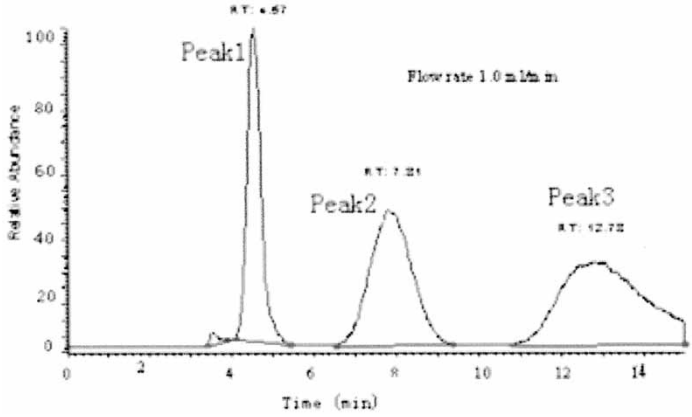

Figure 6. SIM (selected ion monitor) chromatogram of Milli-ESI-MS in positive mode in the ethyl acetate extract from the seeds of *O, indicum.* Milli CCC conditions: Coil volume: 4.9 mL; Hexane–Ethyl acetate–Methanol–Water, 1:1.2:1:1 v/v/v/v; Upper phase: stationary organic phase; Lower phase: Mobile polar aqueous phase 1 mL/min; Rotation speed: 1800 rpm; Sample loading: 50 μl. Stationary phase retention Sf = 81%; Sample concentration 50 ug/mL. A split of 1:20 was used between the HSCCC and the mass spectrometer (retention volumes and times differ). Mass Spectrometer conditions: Probe Temperature: 450°C; Ionization Mode: + ESI; Capillary: 4.5 kV; Source voltage: 40 V; RF Lens: 0.3 V.

is quieter and can maintain high resolution because it can have a better stationary phase retention factor (S_F) due to its high rotational speed. At 2 mL/min, the volume of stationary phase retained is twice lower ($S_F = 40\%$) than that at 1 mL/min. This S_F reduction produces a dramatic loss of resolution (Figure 7 and Table 1) as previously described.[12] A S_F ratio of 40% at a flow rate corresponding to one machine volume in about 2 min is still a remarkable value. This machine is portable and economical. It can complement HPLC in the modern analytical laboratory and encourage more analytical HSCCC applications for coupling with the mass spectrometer.

CONCLUSIONS

Good resolution of three flavonoids from the seeds of *Oroxylum indicum* was obtained by HSCCC-MS with two HSCCC instruments, and the small, quieter Milli-CCC was shown to be more efficient and give comparable separation in a shorter time than the large radius HSCCC running at similar operating conditions.

At present, HSCCC-MS can not compete with HPLC-MS in efficiency and resolution due to its lower separation efficiency, but its high sample loading capacity means that it can be used at an analytical scale for examining

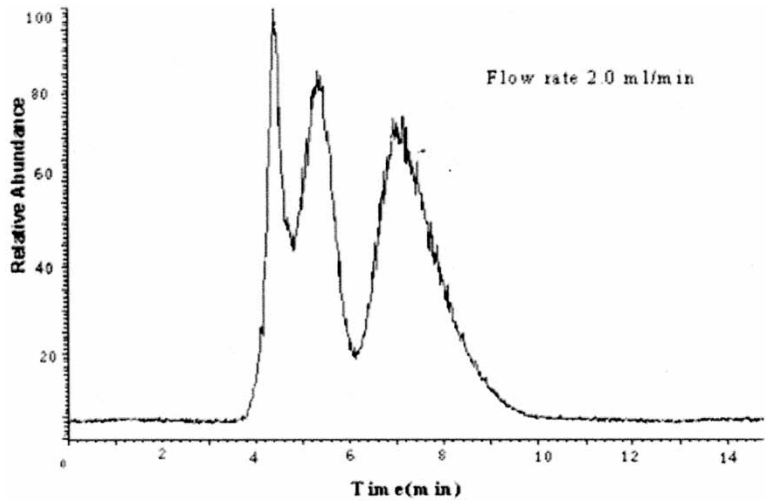

Figure 7. SIM (selected ion monitor) chromatogram of HSCCC-ESI-MS in positive mode in the ethyl acetate extract from the seeds of *O, indicum.* HSCCC conditions: Coil volume: 4.9 mL; Hexane–Ethyl acetate–Methanol–Water = 1:1.2:1:1; Upper phase: organic stationary phase; Lower phase: Mobile polar aqueous phase, 2 mL/min; Rotation speed: 1800 rpm; Sample loading: 50 μl. Stationary phase retention: $S_F = 40\%$. Sample concentration 50 ug/mL. A split of 1:20 was used between the HSCCC and the mass spectrometer. Mass Spectrometer conditions: Probe temperature: 450°C; Ionization Mode: +ESI; Capillary 4.5 kV; Source voltage 40 V; RF Lens: 0. 3 V.

Table 1. Comparison between resolution obtained with the HSCCC and the Milli-CCC for the same tubing bore (0.76 mm). The major difference between the HSCCC & Milli-CCC centrifuges was the radius of rotation and coil length which were 110 mm and 50 m, and 50 mm and 10 m respectively

Centrifuge	Flow rate (mL/min)	Retention (%)	Total time (min)	Rs12	Rs23	Rs13
HSCCC (Fig. 4)	1	50	40	1.6	2.7	4.3
Milli-CCC (Fig. 6)	1	81	16	2.5	1.7	3.5
Milli-CCC (Fig. 7)	2	40	10	0.7	0.8	1.5

the feasibility of scale-up of CCC, which appears to be both easy and predictable.[12] Its support-free, low cost, 100% recovery of target compounds makes it very attractive for the screening and identification of bioactive components and for sample clean-up procedures. The analytical HSCCC can also be used for the selection of solvent systems due to low consumption of solvent and short separation times. Furthermore, HSCCC-MS

can be a complementary analytical instrument to solve some separation and identification problem, which is difficult to be solved by HPLC-MS.

REFERENCES

1. Zhang, T.Y.; Pannell, L.K.; Pu, Q.L.; Cai, D.G.; Ito, Y. J. Chromatogr. **1988**, *442*, 445.
2. Lu, H.T.; Jiang, Y.; Chen, F. J. Chromatogr A. **2004**, *1025*, 185.
3. Cao, X.L.; Yu, T.; Zhang, T.Y.; Li, X.; Ito, Y. J. Chromatogr A. **1999**, *855*, 709.
4. Lee, Y.-W.; Voyksner, R.D.; Fang, Q.-C.; Cook, C.E.; Ito, Y. J. Liq. Chromatogr. **1988**, *11*, 153.
5. Lee, Y.-W.; Voyksner, R.D.; Pack, V.; Cook, C.E.; Fang, Q.-C.; Ito, Y. Anal. Chem. **1999**, *62*, 244.
6. Lee, Y.-W.; Pack, T.W.; Voyksner, R.D.; Fang, Q.-C.; Ito, Y. J. Liq. Chromatogr. **1990**, *13*, 2389.
7. Janaway, L.; Hawes, D.; Ignatova, S. J. Liq. Chromatogr. **2003**, *26* (9&10), 1345.
8. Chen, L.J.; Games, D.E.; Jones, J. J. Liq. Chromatogr. **2003**, *26* (9&10), 1623.
9. Wood, P.L.; Hawes, D.; Janaway, L.; Sutherland, I.A. J. Liq. Chromatog. & Rel. Technol. **2003**, *26*, 1417.
10. Chen, L.J.; Games, D.E.; Jones, J. J. Chromatogr A. **2003**, *988*, 95.
11. Fisher, D.; Garrard, I.J.; van den Heuvel, R.; Chou, F.E.; Fahey, J.W.; Sutherland, I.A. J. Liq. Chromatog. & Rel. Technol *ibid* **2005**.
12. Berthod, A.; Brown, L.; Leitao, G.; Leitao, S. Comprehensive analytical chemistry. In *CCC, The Support-Free Liquid Stationary Phase*; Berthod, A., Ed.; 2002; Vol. XXXVII, Ch. 2, pp. 28–29.

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