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APPLICATION OF CPC AND RELATED METHODS FOR THE ISOLATION OF NATURAL SUBSTANCES – A REVIEW

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Abstract: A review of research on the isolation of various alkaloids from plant material by centrifugal partition chromatography (CPC) and related preparative techniques was made, in order to provide various conditions for separation of these important plant derived secondary metabolites. First of all, the construction of the CPC apparatus was presented as well as the principle of isolation of natural products with its help, and then the influence of operating apparatus parameters on the separation efficiency. Finally, a review of the alkaloids separation conditions was made, specifying used parameters and best solvent system.

Keywords: alkaloid separation, CPC separation, centrifugal partition chromatography, counter current chromatography

Centrifugal partition chromatography (CPC) is a type of counter-current chromatography (CCC), and the advanced form of liquid chromatography (LC) (1).

CPC was developed by Yoichiro Ito in 1964 and since then it is being used for the separation and purification of compounds (mainly of herbal origin). It remains an analytical preparative liquid – liquid technique, that does not require solid sorbent, but only two solvents that are immiscible two phases – stationary and mobile (2).

Typical CPC device is a group of cable channels combined in a cascade, located in the cartridges forming circles around the rotor. When the rotor is in motion, system is subjected to continuous centrifugal force and (by the action of pump) the mobile phase flows through the stationary phase. Due to the state of two liquid phases, their roles can be reversed – ascending mode (when the mobile phase is the lighter phase) or descending mode (when the mobile phase is heavier phase) may be selected. During the flow of one phase against the other, a process of elution (percolation) of the substance from one phase to another, and the process of retention of the substance in the stationary phase (3) are taking place. Substances are separated between the mobile and the stationary phase based on differences in the partition coefficients (4).

CPC compared to other modern chromatographic techniques, is a technique well suited to large scale separation (3), and capable of being used for the separation of substances with a wide range of polarities (4).

Selection of isolation parameters. The choice of solvent system, flow rate and rotation speed

The choice of solvents remains an important stage in the preparation of the analysis. Distribution of the substances dissolved in the sample between the two liquid phases, on the basis of the partition coefficient values, is essential for the CPC separation (2). The compounds contained in the test material should be soluble in both mobile and stationary phases. The most efficient solvent is a combination of such, at which the distribution ratio of a chosen compound will range around 1 (preferably) or within the values of 0.2–5. After mixing the two phases should be separated quickly – preferably within 30 s (5).

Retention of active compounds depends largely on the partition coefficient and the volume of each phase. Following equation shows this relationship:

$$Vr = Vm + P\,Vs \quad (1)$$

where Vr – retention volume, Vm – volume of the mobile phase, P – partition coefficient, Vs – volume of the stationary phase.

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Retention of the substance should not be too high – not to let the substance remain too long on the column, nor too low for it not to be washed out immediately. For this reason, it is important to select a solvent suitable to maintain the partition coefficient and hence the corresponding retention (6). Solvents are chosen also on the basis of other parameters, that is the difference in density between the two liquid phases, on the viscosity of the liquid, or on the interfacial tension. To determine the appropriate solvents, thin layer chromatography (TLC) method is often used. It may be done by developing the sample on a silica plate with solvents. The solvents that give retention factor in the range R_f = 0.4–0.6 are best suited for CPC analysis (7).

The column is a place where substances are separated. Centrifugal force is necessary to keep the stationary phase on place, while the mobile phase flows through. Thus, the stationary phase is stable only when the device rotor sets the column in motion, and is creates centrifugal forces (6).

In addition to the appropriate solvents selection, it is also important to set the right flow rate and speed of rotation of the rotor in the CPC apparatus. The impact of these factors on the distribution of the substance was conducted by Matsuda et al. (8). It appeared that low rotation of the rotor and high flow rate can result in insufficient retention. The results also showed that the flow rate reduction improves peak resolution and increased the number of theoretical plates. It was concluded that the higher flow rate increased the minimum value of the rotor speed, which allowed for adequate resolution (8).

CPC apparatus

There are two types of liquid-liquid chromatography columns: hydrostatic and hydrodynamic ones. On the primary column of the first type (hydrostatic), the mobile phase passed through the stationary phase only by gravity (DCCC – Droplet Counter Current Chromatography), however, it requires long elution time (1). Modern hydrostatic counter-current known as CPC (centrifugal partition chromatography) enables to speed up the process. CPC unlike DCCC produces a centrifugal force of the rotor, which allows faster movement of the mobile phase through the stationary phase. This gives a wider range of solvents that can be used in this method (7). CPC displaced DCCC method because it is much more efficient (9).

The most widely used techniques are two slightly different types of CPC apparatus. One is produced *inter alia* by Pharma-Tech Research Corp., and the second by Sanki Engineering Ltd. (2). The second in its original form had a column separator in the form of rolls arranged around the rotor connected by narrow tubes. Column is filled with the stationary phase through which the mobile phase is passed as a stream thanks to the rotation of the rotor (5). The other components of the device were: solvent pump, valve regulating the flow, the dispenser of the sample, a detector and a recording device (2). Diagram of such a device is presented in Figure 1.

Advantages of CPC

CPC as the analytical technique is much more advantageous than other types of chromatography

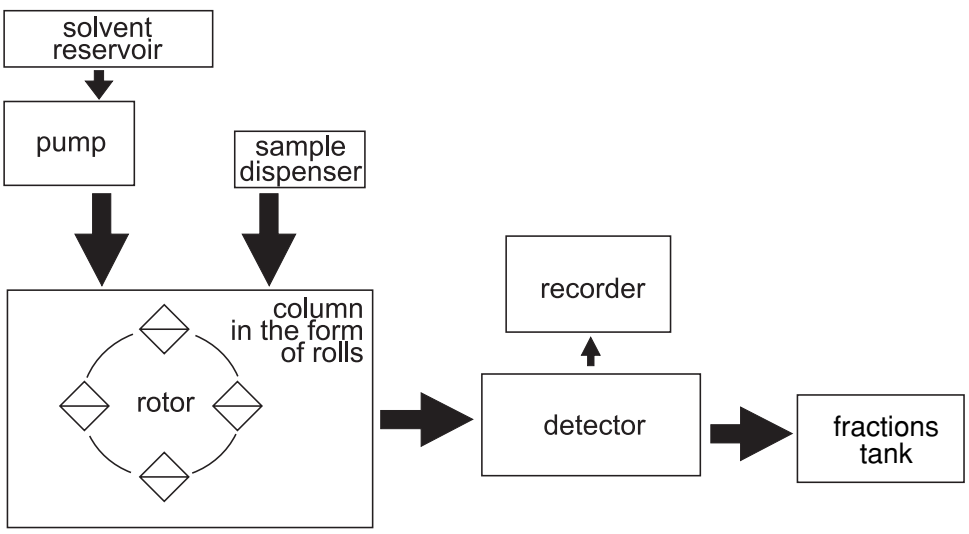

Figure 1. Diagram of CPC device

Table 1. Parameters of alkaloids isolation.

Type of alkaloids	Used phases stationary / mobile	Parameters: Speed of rotation / flow rate
Ergoline alkaloids lysergol, chanoclawine (10)	methyl *tert*-butyl ether, acetonitrile, water (4 : 1 : 5 v/v/v)	1250 rpm 3 mL/min
Indole alkaloids vindoline, vindolinine, catharanthine and vincaleukoblastine (11)	methyl *tert*-butyl ether, acetonitrile, water (4 : 1 : 5 v/v/v)	800 rpm 3 mL/min
Isoquinoline alkaloids palmatine, jatrorrhizine, columbamine, pseudocolumbamine (12)	dichloromethane, methanol, water (12 : 4 : 9 v/v/v)	700 rpm 9 mL/min (first stage) 3 mL/min (second stage)
Isoquinoline alkaloids palmatine, berberine, worenine, epiberbine, coptisine, jatrorrhizine (13)	n-hexane, ethyl acetate, methanol, water (2 : 5 : 2 : 5 v/v/v/v)	850 rpm 2 mL / min and then 650 rpm 5 mL / min
protopine, tetrahydropalmitine, bicuculline (14)	methyl *tert*-butyl ether, acetonitrile, water (2 : 2 : 3 v/v/v) + triethylamine and hydrochloric acid	800 rpm 2 mL / min
huperzine A , huperzine B (15)	N-heptane, ethyl acetate, n-propanol, water (5 : 15 : 35 : 45 v/v/v/v)	1400 rpm 6 mL / min
michellemine B (16)	chloroform, methanol, 0.5% hydrobromic acid	300 rpm 15 mL / min
Tropane alkaloids darlirgine (17)	(5 : 5 : 3 v/v/v) chloroform, methanol, water (13 : 7 : 8 v/v/v)	No data

such as high performance liquid chromatography (HPLC). In contrast to the HPLC, CPC gives complete recovery of the sample introduced. There is no denaturation of the molecules, no irreversible absorption on the solid absorbent and the solvent consumption is very low (7). The main advantage of this technique is due to significant reduction of analysis costs, allowing the use of this method on a larger scale. CPC has a high capacity. Relatively large volume of stationary phase makes the separation of substances effective (4).

Survey on using isolated alkaloids CPC

CPC is an analytical method used increasingly for the separation and purification of individual substances from raw plant material. Below an overview of research aimed at different groups of alkaloids separated using centrifugal partition chromatography is presented. Due to the similarity of CCC to the CPC method, several examples of the isolated alkaloids will be also presented by this method. Summary of methods used for the isolation of alkaloids is presented in Table 1.

The first example of the preparation of the alkaloid purification with CPC is a separation of clavine alkaloids from methanolic extract of *Ipomoea muricata* L. seeds. The separation of these substances was not easy because of their sensitivity to temperature and light, however, Mauri and Srivastava coped with this problem by using a modified CPC method, which separated the substances with an electric charge depending on the pH value. They managed to separate the two alkaloids: lysergol and chanoclavine from each other. CPC apparatus used for the separation contained 1320 cells (total column capacity of 200 mL) with a modification of rotation speed from 200 to 2000 rpm. As the stationary and the mobile phases used methyl *tert*-butyl ether, acetonitrile and water in a ratio (4 : 1 : 5 v/v/v) were used. Separation process was performed in the descending mode under the rotor speed of 1250 rpm and at the flow rate of 3 mL/min. From 4 grams of dry extract, 210 mg lysergol (97% purity) and 182 mg chanoclavine (79% purity) were obtained (10).

Renault and co-workers (11) implemented pH-zone refining mode in their separation for the

purification of indole alkaloids from the crude alkaloid extract from plant *Catharanthus roseus* (L.) Titer to obtain four alkaloids: vindoline, vindolinine, catharanthine and vincaleukoblastine. The study used a apparatus HPCPC with 2136 channels, and the total capacity of the column 250 mL. As the stationary and mobile phases the following solvents were used: methyl *tert*-butyl ether, acetonitrile and water (4 : 1 : 5 v/v/v) (the lower – the water phase was acidified). During the run, the apparatus was adjusted to 800 rpm and the flow of 3 mL/min. Attempts were made on four different amounts of the injected substance, starting from 0.8 g through 1.6, 2.4 to 7 g. After injections, the smallest mass of the sample received only two purified substances: catharanthine and vindoline. All four alkaloids were isolated, when mass of the sample was increased. Analyses were performed in the ascending mode. The following purity was obtained for the individual substances: vindoline (about 43% purity), catharanthine (about 55% purity), vincaleukoblastine (about 70% purity) (11).

Another group of alkaloids, which has been successfully separated using the technique of high-performance CPC was a group of isoquinoline alkaloids. From methanolic extract of *Enantia chlorantha* stem bark, four alkaloids were obtained: palmatine, jatrorrhizine, columbamine and pseudocolumbamine, using the following solvent system: dichloromethane, methanol and water (48 : 16 : 36 v/v/v). The aqueous phase was used as the stationary phase and organic one as the mobile phase, to isolate each of the alkaloids. Six hundert milligrams of palmatine was isolated (purity above 95%) and a mixture of the other three alkaloids was received and submitted to further separation. In the second phase, the mixture of jatrorrhizine, columbamine and pseudocolumbamine was separated, and two modifications of the above system were used. In the first one, addition of potassium perchlorate was performed and in the second system, the alkaline sodium hydroxide was added. Separation was performed as follows: in the first phase, with the addition of $KClO_4$, the rotation speed was set at 700 rpm and the flow rate at 9 mL/min in the descending mode, then, using the solvent system alkalized with sodium hydroxide, at 700 rpm and the flow rate of 3 mL/min. Jatrorrhizine and columbamine were washed out by 1100 mL of mobile phase in descending mode, and pseudocolumbamine was washed only after introduction of the dual mode by 200 mL of the mobile phase. Sixteen milligrams of jatrorrhizine, 13 mg of columbamine and 16 mg of pseudocolumbamine were received, all with a purity exceeding 95% (12).

To obtain alkaloids from the same group also the CCC technique was used. As the stationary and mobile phases, a mixture of n-hexane/ethyl acetate/methanol/water (2 : 5 : 2 : 5 v/v/v/v) at a flow rate of 2 mL/min and a rotation speed of 850 rpm were used. After 2.5 h of separation, the flow was accelerated to a speed of 5 mL/min and rotation speed was reduced to 650 rpm. The process yielded 4.7 mg of palmatine (purity 98.5%), 7.1 mg of berberine (purity 94.1%), 0.8 mg of worenine (purity 90.4%), 1.5 mg of epiberbine (purity 95.5%), 1.9 mg of coptisine (purity 88.4%) and 0.6 mg of jatrorrhizine (purity 91.1%) from 20 mg of dry extract obtained from ethanolic extract of *Coptis chinensis* Franch. rhizome (13).

The next group of alkaloids was separated using a CPC from 3.1 g sample of evaporated to dryness ethanolic extract of rhizome *Corydalis decumbens* (Thunb.) Pers. For separation, a mixture of methyl *tert*-butyl ether, acetonitrile and water was used in proportions of 2 : 2 : 3 by volume. To the top organic phase triethylamine (5–10 mM) was added, and to the lower aqueous phase hydrochloric acid (5–10 mM) was added. The mobile phase was pumped into the column at 2 mL/min while the column rotated at 800 rpm. Protopine (495 mg), tetrahydropalmityne (626 mg) and bicuculline (423 mg) were obtained. Each of the compounds was over 93% pure (14).

The pH-zone refining centrifugal partition chromatography technique was used to separate the alkaloids of the club moss *Huperzia serrata* (Thunb. ex Murray) Trevis. pH zone refining mode was used as a better modification of displacement method. n-Heptane/ethyl acetate/n-propanol/water (5 : 15 : 35 : 45 v/v/v/v) were used as solvent system. Triethylamine (8 mM) and methanesulfonic acid (6 mM) were also added to the solvent system. The pH of the upper and the lower phase was adjusted to pH 10 by addition of ammonia solution. Alkaloids were injected in the form of salts by lowering the pH to 2. The flow rate during the injection of the sample was increased gradually from 2 to 6 mL/min. Rotation speed of the column was set to 1600 rpm. The amount of 0.4 g of dry plant extract gave 9 mg huperzine A and 7 mg of pure huperzine B (15).

CPC technique was used as one of the three stages of receiving alkaloid michelemine from the dichloromethane and methanol extract from leaves and twigs of the liana *Ancistrocladus korupensis* D.W. Thomas & Gereau. Optimization of the alkaloids isolation from the above raw material was performed, and as the best solvent, mixture of chloroform – methanol – 0.5% hydrobromic acid (5 : 5 : 3

v/v/v) was selected. Separation was carried out initially at a flow rate of 16 mL/min and a rotation speed of 400 rpm, however, a reduction in these values, respectively, to 15 mL/min and 300 rpm significantly improved resolution. During the entire three-step process, about 0.5 g of pure michelemine B was obtained from about 8–9 g of a crude mixture of alkaloids (16).

The next example illustrates the use of the method for the CCC separation of tropane alkaloids from dichloromethane and methanol extract from the bark of *Darlingia darlingiana* (F. Muell.) L.A.S. Johnson. With 100 g of the extract from the bark, 74 mg of darlirgine were received. Solvent system was used as a mixture of chloroform – methanol – water (13 : 7 : 8 v/v/v) (17).

SUMMARY

CPC technique is cheap and efficient separation method, and often superior to other chromatographic techniques. However, in order to obtain the expected results, the appropriate analysis parameters must be chosen: from the choice of biphasic mixtures, the flow rate of the mobile phase up to the rotation speed of the rotor. In the present work, a few studies on the separation of alkaloids from plant material were shown. The most commonly used solvent system for alkaloids was a system containing: methyl *tert*-butyl ether, acetonitrile, water. Flow rates ranged from 2 mL/min, usually 6 mL/min up to 15 mL/min. The rotor speed values ranged from 300 to 1400 rpm.

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