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Forced-flow multi-phase liquid extraction, a separation method based on relative and absolute counter-current distribution

I. Description of the method and basic possibilities

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ABSTRACT

A novel variant of continuous relative and absolute counter-current distribution (CCD), called forced-flow multi-phase liquid extraction (FFMLE), is introduced as a new separation method. This method is a special case of liquid–liquid extraction requiring three immiscible liquid phases and movement of inert gas bubbles through the three liquid phases as the fourth constituent of the multi-phase system.

On using three immiscible liquid phases, two basic situations prevail: two phases may be stationary and one mobile, or one stationary and two mobile. Among the five basic possibilities, four belong to relative CCD in the ascending and descending modes. The extraction columns are filled with the immiscible stationary phase or phases and mobile phase or phases are forced through the stationary phase or phases by means of pumps. For the fifth possibility, the two mobile phases move in opposite directions as very small droplets. This method constitutes absolute CCD, and results in exhaustive and rapid extraction. The efficiency of FFMLE is achieved not just because the mobile phases move in opposite directions, but is also a result of three other effects that are discussed. The different types of relative and absolute CCD methods may also be varied by connecting two or more columns together.

A typical three-phase system can be obtained on mixing *n*-hexane, acetonitrile and water as the basic ternary system, with one of diethyl ether, dichloromethane, toluene or chloroform as the fourth solvent (auxiliary solvent). A virtual solvent strength (S_v) value is introduced for characterization of the three-phase liquid system. The solvent and phase ratio of some three-phase systems are given for different S_v values and selectivity.

The method can be used for the rapid purification of various substance classes occurring as complex matrices. Experiments carried out with a laboratory extraction column (3.5 l) show that the separation of *ca.* 10 g of raw extract into two or more fractions requires less than 1 h.

INTRODUCTION

The separation of molecules as a result of their differential solubility between two immiscible phases has provided the basis for various separation techniques. Martin and Synge [1] first reported liquid–liquid chromatography: one liquid was used as a sorbent and another liquid was allowed to percolate through the former, thus

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making the method a chromatographic process [Martin–Synge distribution (MSD)]. Craig invented countercurrent distribution (CCD) [2] and performed such separations with CCD instruments [3,4]. The selectivity of CCD arises from the fact that the partition coefficient basically reflects the intermolecular forces, which can be strongly influenced by phase composition and by selection of a suitable temperature.

The development of CCD methods leads to liquid–liquid chromatography, called countercurrent chromatography (CCC). The theory and advantages of CCC have recently been summarized in detail by Mandava and Ito [5] and by Conway [6]. The application of CCC to natural product isolation was summarized by Hostettmann *et al.* [7]. All of the CCC methods are concerned with two immiscible phases; therefore, relative CCD was generally carried out. Recently, Lee *et al.* [8] reported the application of dual CCC. In this method, the separation system used a combination of centrifugal and planetary motion to produce an unique hydrodynamic effect which allows two immiscible phases to flow countercurrently through the coiled column. This method constitutes absolute CCD, working continously with two phases.

The other direction of development of CCD leads to liquid–liquid extraction, where the aim of the process is not to separate substances with closely related structures, but to divide a given matrix into different fractions or to isolate a pure compound from a complex mixture. Generally, modern liquid–liquid extraction methods are carried out in the relative CCD mode using two immiscible phases [9–11].

Discontinuous CCD using three immiscible liquid phases has been known for many years [3]. The efficiency of discontinuous three-phase CCD was clearly demonstrated as long ago as 1958 for the isolation of brain lipids by Meltzer [12] and Meltzer *et al.* [13]. However, this relative three-phase CCD has not found widespread use either for extraction or for chromatography, because of experimental difficulties encountered both in the selection of suitable solvents for the preparation of immiscible multi-phase systems and in the development of convenient phase separation devices. Advances in multiple liquid–liquid partition systems have not gone beyond three-phase systems.

Combining the advantage of relative and absolute CCD, the forced-flow technique with a continuous operating mode, and the three-phase solvent systems which ensure the fractionation and purification of a given biological matrix or the isolation of pure compounds from a complex mixture, we recently developed a new separation method, called forced-flow multi-phase liquid extraction (FFMLE) [14,15]. In this paper we report the principle of FFMLE, the design of the prototype apparatus and the preparation of basic three-phase liquid systems, and also describe the fundamental operating possibilities.

EXPERIMENTAL

Glass extraction columns (1500 × 60 mm I.D. and 1000 × 40 mm I.D.) were obtained from Möller (Zürich, Switzerland). The mobile phases were delivered with two Lewa lab® (Leonberg, F.R.G.) M 5 pumps.

Analytical-reagent grade solvents were used for the preparation of the three-phase solvent systems. Saturation of the multi-phase systems was always carried out for over 3 h in a shaker.

The furocoumarin-containing raw extract of *Heracleum sphondylium* was ob-

tained using chloroform as extraction solvent. The flavonoid glycoside-containing raw extract from Betulae folium was extracted with ethyl acetate. For monitoring of the extraction process, 250-ml fractions were obtained. Samples of the effluents were applied with a Linomat IV TLC spotter from Camag, (Muttenz, Switzerland). Process monitoring was by thin-layer chromatography (TLC) on silica gel 60 F_{254} TLC alufoils from Merck (Darmstadt, F.R.G.) in unsaturated chromatographic chambers. The mobile phase for TLC checks on the extraction of furocoumarins consisted of *n*-hexane–dichloromethane–tetrahydrofuran–chloroform (72.8:10.8:8.3:8.1) [16] and for the flavonoids ethyl acetate–tetrahydrofuran–*n*-hexane–ethanol–water–formic acid (70:20:15:10:5:2) [17]. The densitograms in the absorption mode were recorded at 313 nm for furocoumarins and 254 nm for the flavonoid glycosides with a Camag TLC scanner II coupled with an HP 9000-216 computer.

RESULTS AND DISCUSSION

Description of the method

The separation of a certain sample into fractions of different polarity can be achieved with the new multi-phase extraction technique. Successful separation requires that the equilibrium between the phases be maintained constantly, which means that sufficient time must be allowed for mass transfer between the phases.

On using three immiscible liquid phases, two basic situations prevail: two phases may be stationary and one mobile, or one stationary and two mobile. The five basic possibilities are shown in Fig. 1. The first four cases (Fig. 1a–d) belong to relative CCD; cases (a) and (c) represent the descending mode and cases (b) and (d) the ascending mode. The extraction columns are filled with the immiscible stationary phase or phases and mobile phase or phases are forced through the stationary phase or phases by means of one or two pumps. The directions of movement of the mobile

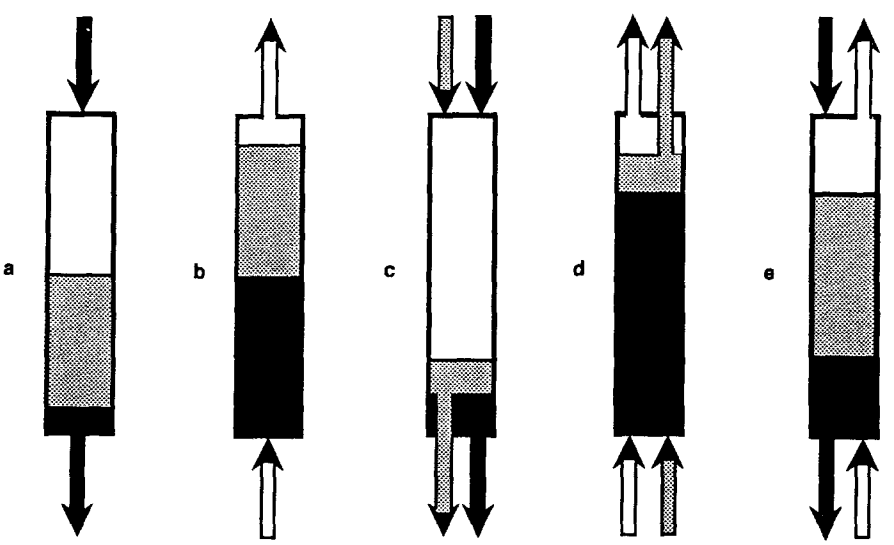

Fig. 1. Basic possibilities for extraction with three liquid phases.

phase or phases are marked with arrows. For the fifth possibility (Fig. 1e), the two mobile phases move in opposite directions as very small droplets. This method constitutes absolute CCD.

The operating principle and design of the FFMLE set-up in the absolute CCD operating mode are shown schematically in Fig. 2. The extraction column is filled with an appropriate volume of the stationary phase (shown in grey, Fig. 2). The lowest of the three immiscible liquid phases, shown in black, is pumped through the stationary (middle) phase. It moves as droplets from the top of the column to the bottom, where it is collected and removed from the system. The uppermost of the three immiscible phases (white) is pumped from the bottom to the top of the extractor, where it is collected and removed from the extraction column. The driving force for movement of the droplets is, in addition to the forced flow, the difference between the specific gravities of the phases travelling in opposite directions.

The extraction column can be thermostated; the whole system is made of inert materials. Both ends of the glass column are closed with a PTFE stopper unit. The lower unit incorporates an injector system for the upper phase, a lower phase outlet and an inlet for inert gas. The top unit consists of the lower phase injector, the upper

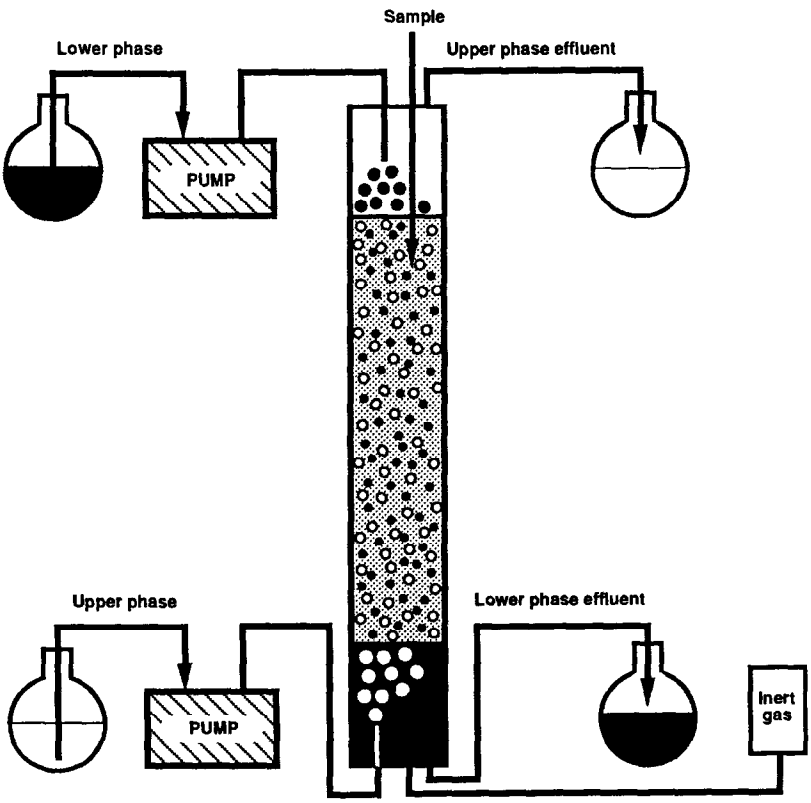

Fig. 2. Operating principle and design of the FFMLE set-up in the absolute CCD operating mode. Upper (mobile) phase is white, middle (stationary phase) is grey and the lower (mobile) phase is black.

phase outlet, a sensor for recording the level of the middle and upper phases and the sample inlet. The two mobile phases are forced through the injector systems with a programmed pump system. This is connected to an electronic sensor, which ensures that the same volume of mobile phases leave the extraction column at each end.

The high velocity (10–50 ml/min) of the mobile phases and the small diameter (0.3 mm) of the injectors ensure that both mobile phases enter the system in the form of an extremely fine spray. In the absolute CCD operating mode, the adjustable injector systems are located such that the upper phase is injected into the lower phase and the lower phase into the upper phase. At the phase boundary the droplets can be disintegrated into numerous much smaller droplets, thus significantly increasing the surface area of the droplets. In the middle phase millions of tiny droplets are present; these droplets can only be shown schematically in Fig. 2. The distance travelled by such a droplet is much longer than the length of the column, because both mobile phases move in opposite directions and therefore the droplets do not move in a straight line but, owing to the many collisions, in a zig-zag manner. The movement of inert gas (e.g., nitrogen) bubbles through the three liquid phases further enhances this effect.

The length and the inside diameter of the extraction column determine its capacity; therefore, both dimensions should be selected according to the separation problem. In the absolute CCD operating mode, the sample is always injected into the middle phase; however, the ratio of the three phases can be chosen freely.

Among the main features of FFMLE, the variety of possible operating modes, which include relative and absolute CCD and also the descending and ascending modes, warrants particular mention. Other operating factors are the geometry of the extraction column, the physico-chemical properties of the three-phase liquid system, the ratio of the phases, the flow-rate of the mobile phase(s), extraction time, pressure and temperature and amount of sample.

Needless to say, the different types of relative and absolute distribution methods may also be varied by connecting different columns together. For example, for the isolation of alkaloids after a three-phase absolute CCD the upper phase may contain alkaloids in addition to other compounds. These may then be passed through a second extraction column operating in the relative CCD mode, in which the middle stationary phase has been modified with 2% sulphuric acid; the purified alkaloid fraction is concentrated in the modified middle phase of the second column. Further, various other variations of the extraction columns are also possible. With suitable selection of three-phase liquid systems, one of the mobile phases can be passed through a second or third three-phase liquid system, with a similar solvent composition to the first.

Preparation of multi-phase solvent systems

FFMLE is a special case of liquid–liquid extraction requiring three immiscible phases which are generally obtained from three or four solvents. A three-phase liquid system can be achieved on mixing three suitable solvents, *e.g.*, a 1:1:1 mixture of *n*-hexane or *n*-octanol, water and nitromethane or nitrobenzene. However, from the practical point of view, the uses of these mixtures are limited.

Based on our experience, the solvent strength values of the individual solvents (S_i) and Snyder's solvent classification [18] seem to be helpful for selection of suitable

three-phase liquid systems. Snyder classified the commonly used solvents into eight groups (I–VIII) according to their properties with regard to proton-acceptor, proton-donor and dipole interactions. Typical three-phase systems can be obtained on mixing hexane, acetonitrile (VI) and water (VIII) as the basic ternary system, with one of the following as the fourth (auxiliary) solvent: the basic ternary system, with one of the following as the fourth (auxiliary) solvent: diethyl ether (II), dichloromethane (V), toluene (VII) or chloroform (VIII). Some of the possible three-phase liquid systems are given in Table I, together with the ratio of the upper, middle and lower phases.

TABLE I

TYPICAL TERNARY AND QUATERNARY SOLVENT SYSTEMS

	Solvent mixture	Solvent strength	Selectivity group	Solvent ratio	Phase ratio[a]
(A)	*n*-Hexane	0.1	–	1	1:1:1
	Water	10.2	VIII	1	
	Nitromethane	6.0	VII	1	
	$S_v = 5.43$				
(B)	*n*-Octanol	3.4	II	1	1:1:1
	Water	10.2	VIII	1	
	Nitromethane	6.0	VII	1	
	$S_v = 6.53$				
(C)	*n*-Butanol	3.9	II	1	23:36:41
	n-Hexane	0.1	–	1	
	Water	10.2	VI	1	
	Nitromethane	6.0	VIII	1	
	$S_v = 4.17$				
(D)	Diethyl ether	2.8	I	1	19:37:44
	n-Hexane	0.1	–	2	
	Acetonitrile	5.8	VIII	2	
	Water	10.2	VII	1	
	$S_v = 4.13$				
(E)	Dichloromethane	3.1	V	1	23:32:45
	n-Hexane	0.1	–	2	
	Acetonitrile	5.8	VI	2	
	Water	10.2	VIII	1	
	$S_v = 4.18$				
(F)	Toluene	2.4	VII	1	21:51:58
	n-Hexane	0.1	–	2	
	Acetonitrile	5.8	VI	2	
	Water	10.2	VIII	1	
	$S_v = 4.07$				
(G)	Chloroform	4.1	VIII	1	21:51:58
	n-Hexane	0.1	–	2	
	Acetonitrile	5.8	VI	2	
	Water	10.2	VIII	1	
	$S_v = 4.35$				

[a] Lower phase:middle phase:upper phase.

To the best of our knowledge, no value exists for the characterization of three-phase liquid systems. It appeared to us that the solvent strength values serve this purpose with a certain modification indicating that the total solvent strength (S_T) of the system is divided among three phases. Theoretically, all three phases may be characterized by an S_T value and the solvent strength of the system would be the average of these three S_T values. However, as the calculation of these values would be complicated, we propose the introduction of the virtual solvent strength (S_v) value for the characterization of three-phase liquid systems.

The S_v of a three-phase liquid system is the arithmetic average of the solvent strengths of the pure solvents (S_i) in the system, weighted according to the volume fraction (φ) of each solvent. For a quaternary three-phase liquid system composed of solvents A, B, C and D, the S_v can be defined as

$$S_v = \varphi_A S_A + \varphi_B S_B + \varphi_C S_C + \varphi_D S_D$$

As shown in Table I, the three-phase liquid systems always have various S_v values, which indicate the different polarities. All the mixtures presented also have different selectivities. Three-phase liquid systems can be obtained from three solvents (see Table I, mixtures A and B) or more solvents (see Table I, mixtures C–G). In four of these cases (D, E, F and G) the ratio of all the solvents in each system is the same, but there is a difference in the quality of the fourth. Diethyl ether, as a representative of group I (see in Table I), is a proton acceptor, whereas in mixture G, the fourth solvent, chloroform (group VIII), is a typical proton-donor solvent. In the other two examples the fourth solvent is from group V (mixture E), whereas toluene is from group VII (mixture F). Despite these general combinations, it has been possible to formulate a large number of three-phase solvent systems that appear to be useful for the separation of complex mixtures.

It should be noted that the selectivity can be further increased by adding a fifth solvent. A three-phase system containing eight solvents, *e.g.*, of *n*-hexane–diethyl ether–methanol–tetrahydrofuran–dichloromethane–acetonitrile–chloroform–water (68.2:3.6:4.4:7.7:25.8:50:2.4:37.8), is also known.

In Fig. 3 the ratios of the three basic solvents (water, acetonitrile and *n*-hexane) are represented in a triangular diagram, where the volume fractions of the three solvents are given as three-digit numbers, called combination points (P_c). The fourth solvent (chloroform) is added (5–25%) to achieve the three immiscible phases. The combination points, where three phases could be obtained, are shown by black circles. It is clearly seen that three-phase liquid systems can be achieved only at given P_c. These difficulties could be one of the reasons why liquid–liquid partition systems have not been exploited beyond three-phase systems.

The ratio of the solvents determines not only the selectivity, but also the volume ratio of the three immiscible phases, as is shown in Fig. 4, where the three-digit numbers represent the same three-phase solvent combinations as in Fig. 3b. The linear relationship found is always valid in the horizontal direction if the amounts of two of the four solvents (forming the three-phase system) are constant, *e.g.*, along the lines between P_c = 127 and 172 the volumes of water and chloroform were constant and only the ratio of acetonitrile and *n*-hexane was varied. The same is valid, *e.g.*, between P_c = 613 and 253, where the amount of *n*-hexane was constant (30%) and the added volume was always 10%.

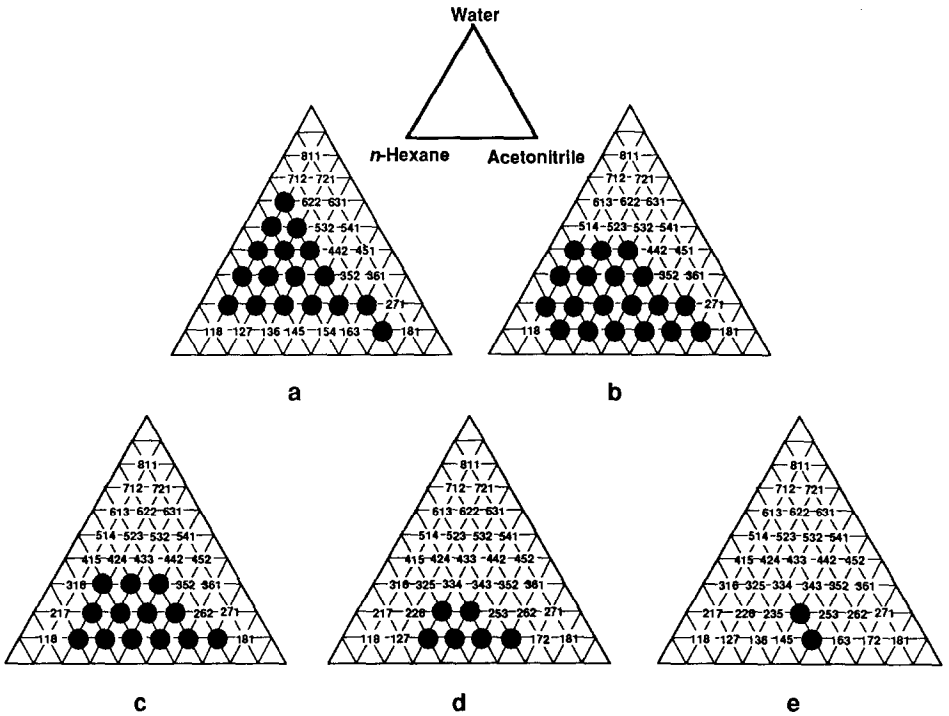

Fig. 3. Preparation of three immiscible phases for FFMLE from the three basic solvents (water, acetonitrile and *n*-hexane) and from a fourth, auxiliary solvent (chloroform). The volume fractions of the three basic solvents are given as three-digit numbers. The solvent compositions where three phases could be obtained are shown by black circles.

At a given P_c there also exists a vertical function between the fourth (auxiliary) solvent and the proportion of phases. Fig. 4b shows the connections of the amount of chloroform (5–25%) and the ratio of the three immiscible phases at P_c = 244. It should be noted that the change of lower phase is small and almost linear, whereas the middle and upper phases show quadratic relationships. Increasing the content of chloroform causes the volume of upper phase to decrease. At the critical volume of chloroform, where the curves of two phases meet, the third phase is eliminated.

Separation examples

Pimpinella saxifraga was selected as an example of purification for non-polar compounds containing eight furocoumarin isomers in various ratios [19]. A 860-g sample of plant material was subjected to exhaustive forced-flow solid–liquid extraction [20] with chloroform for a combined solution and diffusion time [21] of 6 h. The residue of the extract was dissolved in the middle phase of the saturated three-phase system, which consisted of chloroform–*n*-hexane–acetonitrile–water (5:20:60:20). The extraction column was filled with 10% of lower phase, 80% of middle phase and 10% of upper phase. The sample was injected into the middle layer of the three-phase system. FFMLE was carried out in the absolute CCD operating mode at a velocity of

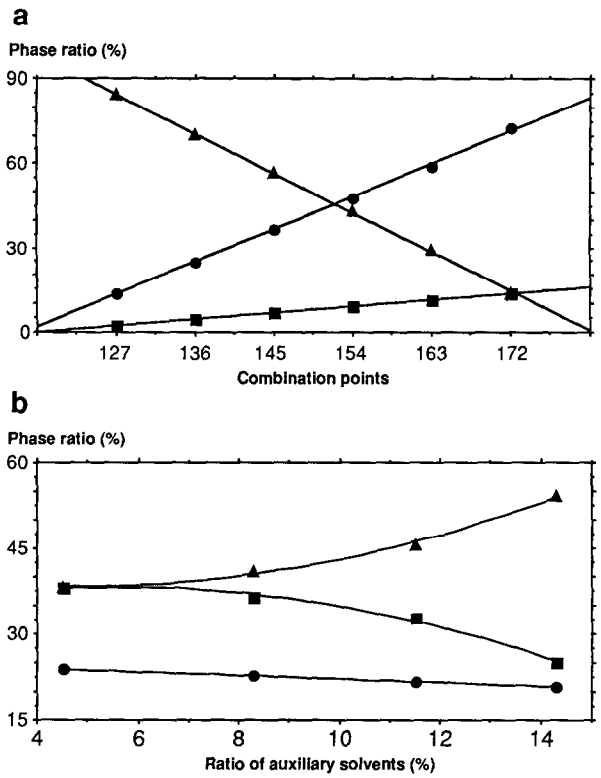

Fig. 4. Ratio of three immiscible phases using the solvent composition from Fig. 3b. ● = Upper phase; ▲ = middle phase; ■ = lower phase. (a) The horizontal linear relationship for a quaternary solvent system. The amounts of any two solvents (water and chloroform shown) are constant; only the ratio of the others (acetonitrile and *n*-hexane shown) are varied. (b) The vertical relationship for a quaternary solvent system. Only the amount of the auxiliary solvent (chloroform shown) is varied.

23 ml/min for both mobile phases. Upper phase fractions of 250 ml were monitored by TLC; the total area of the densitograms between 0.1 and 0.8 was determined at 313 nm. The extraction was complete in 40 min. After the process was finished, the two other (middle and lower) phases were also checked by analytical methods [22]. It could be stated that the extraction was exhaustive.

As an example of the extraction of polar compounds, flavonoid glycosides were separated from Betulae folium. The forced-flow solid–liquid extraction was started with chloroform to purify the plant material (1650 g) from non-polar compounds. Extraction of flavonoid glycosides was subsequently carried out with ethyl acetate for a combined solution and diffusion time [21] of 8 h. The residue of the extract was dissolved in the middle phase of the three-phase liquid system [chloroform–*n*-hexane–acetonitrile–water (13:26:4:13)]. FFMLE was carried out in the absolute CCD operating mode at a velocity of 10 ml/min for both mobile phases. The lower phase fractions containing the flavonoid glycosides were evaporated and the amount of flavonoid glycosides was determined in each fraction by analytical methods [23]. The two other immiscible phases were also checked. The exhaustive extraction of flavonoid glycosides was completely finished in 65 min.

CONCLUSIONS

This novel CCD method permits the rapid and complete extraction of various substance classes from complex matrices. It utilizes the forced-flow technique and works with three immiscible liquid phases within an extraction column. The whole process is continuous, including the sample application and the outlet of effluents.

The most important advantage of this separation technique, as with other liquid–liquid partition methods, lies in the absence of solid supports. Additionally, it can be applied as a thorough yet mild purification and/or isolation method owing to the permanent present of inert gas in the extraction column.

The technique can be used on both the micropreparative and preparative scales by variation of the column size, is more readily applied to separations of polar compounds, is applicable to both small and macromolecules and operates at low pressure (< 10 p.s.i.). The equipment is simple to operate, comparatively inexpensive, requiring minimum bench space, and is easily cleaned between runs. Also macromolecules that have been purified by the slower CCD procedure may be efficiently separated by FFMLE. It is perhaps best regarded as being complementary to, rather than competitive with, other purification and/or extraction methods.

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