Application of Phase-Trafficking Methods to Natural Products Research

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A novel simultaneous phase-trafficking approach using spatially separated solid-supported reagents for rapid separation of neutral, basic, and acidic compounds from organic plant extracts with minimum labor is reported. Acidic and basic ion-exchange resins were physically separated into individual sacks (“tea bags”) for trapping basic and acidic compounds, respectively, leaving behind in solution neutral components of the natural mixtures. Trapped compounds were then recovered from solid phase by appropriate suspension in acidic or basic solutions. The feasibility of the proposed separation protocol was demonstrated and optimized with an “artificial mixture” of model compounds. In addition, the utility of this methodology was illustrated with the successful separation of the alkaloid skyanthine from Skytanthus acutus Meyen and the main catechins and caffeine from Camellia sinensis L. (Kuntze). This novel approach offers multiple advantages over traditional extraction methods, as it is not labor intensive, makes use of only small quantities of solvents, produces fractions in adequate quantities for biological assays, and can be easily adapted to field conditions for bioprospecting activities.

In 1963, R. B. Merrifield revolutionized peptide synthesis by introducing solid-phase reagents. This brilliantly simple idea allowed the use of reagents in excess and simplified purification, leading to higher yields and fast isolation.1 Subsequent elaboration using combinatorial techniques have led to peptide compound libraries of thousands of compounds. Since then, an impressive number of inventive modifications have been introduced in a wide range of fields in academia and industrial laboratories.2,3 Particularly, organic chemists have taken advantage of specific interactions between small organic molecules and solid-supported reagents (SSR) to achieve quick purification of desired nonpeptide products applying creative phase-switching strategies.4 Furthermore, the isolation process using solid-phase protocols involves only simple operations of filtration and solvent removal that are suitable for automation and high-throughput applications and has found particular value in combinatorial chemistry laboratories.5 Despite the multiple advantages of SSR for isolation of small synthetic organic molecules, this method has yet to find application in resolving natural product extracts. Ion-exchange resins have long been used for purification of particular natural products (i.e., quinine6,7) at a scale only occasionally used in fractionation schemes. The few examples of applications to natural products research include recovery and concentration of thiamine from rice bran extract,8 isolation of alkaloids from Lindelolfoia achusoides9 and Aconitum septentrionale,10 simultaneous determination of phenolics and alkaloids in methanolic extracts of Gentisina species,11 and selective adsorption of tea polyphenols.12 Generally, the use of exchange resins as column chromatography material in labor-intensive schemes is a common feature in these reports. Wider applications of SSR in natural products research have yet to appear.

The importance of natural products as a source of new therapeutics and as starting materials in medicinal chemistry is undeniable and has been recently reviewed.13 However, natural products-based drug discovery has become unpopular recently in many industrial laboratories. Particularly, the initial biological activity evaluation of crude extracts has multiple disadvantages, namely, the frequent occurrence of nonselective and nonspecific inhibitors (e.g., polyphenols), the occurrence of a number of chemically diverse compounds with potentially opposite biological activities, and the low concentration of active metabolites.14 Therefore, false-positive and false-negative outcomes are possible in both biochemical and cellular screenings, reducing the rate of success and increasing cost. In order to address these difficulties, improved fractionation methods have been developed, including pretreatments to reduce tannins,15 automated fractionation,16 single or multiple solid-phase extraction (SPE),17,18 counter current chromatography,19 preparative high-pressure liquid chromatography (HPLC), and elaborated applications of complex and costly devices.20 These methods require either a substantial investment or lengthy and tedious protocols, preventing their implementation, especially in the remote regions of current bioprospecting interest. Consequently, the need for applications that can generate samples conveniently with suitable quality for initial bioassay is of great current interest. Such a method should not only increase the relative concentration of potentially active compounds but also reduce interference from other components in the initial mixture. Also, some additional desirable features would include being fast, inexpensive, environmentally benign, non-labor-intensive, and adaptable to field conditions. To address these needs, we designed and optimized a phase-switching application that takes advantage of weak ion-exchange resins for a simultaneous rapid recovery of neutral, basic, and acidic components from plant crude organic extracts.

Results and Discussion

Normally, the acid-base character of natural products has allowed selective isolation of compounds based upon their functional groups by using pH manipulation in liquid–liquid partition protocols. However, these more tedious and solvent-demanding conventional solution-phase chemistries can be replaced in principle with simultaneous catch-and-release methodologies using immobilized reagents for natural products extract resolution, as is now commonly done in combinatorial chemistry laboratories.21 The necessary resins are kept separate from one another by use of porous bags dipped simultaneously in the stirred plant extracts. As illustrated in Scheme 1, groups of acidic and basic compounds can be selectively trapped using an appropriate ion-exchange resin, leaving behind the neutral compounds in solution so that they can each be isolated by simple evaporation. Those operations can, in principle, be adapted to field conditions.

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Since spatially separated resins do not interfere with each other’s functions, weakly basic and weakly acidic resins can be confined into separate “packets” followed by their joint immersion into a solution of an organic plant extract, allowing for partitioning of its components based upon their acid–base characteristics. In order to work out the conditions necessary to accomplish this, an “artificial extract” was prepared by mixing known amounts of basic, acidic, and neutral model compounds (quinine, 3,4,5-trimethoxybenzoic acid, and methyl 3,4,5-trimethoxybenzoate, respectively), and this was subjected to the separation scheme shown in Scheme 2 using the polyacrylic-divinylbenzene resins Dowex MAC-3 (carboxylic acid functional group) and Dowex Marathon WBA (dimethylamino functional group). These resins were chosen because of their large exchange capacities, stability over a wide pH range, and relative ease of regeneration for repeated use.

The resins were packed into the tea-filter bags and cleaned according to the manufacturer’s guidelines before use. Adsorption of model compounds was followed by HPLC during a 24 h period (Figure 1). Complete sequestration (ca. 98%) of acidic and basic compounds from the extract solution (1–2 g in 500 mL of MeOH–H$_2$O, 1:1, v/v) was achieved in 8–12 h, while, nonspecific adsorption to the resins of neutral compounds was 14% in the same period using a resin-to-sample ratio of 200:1. A simple saturation experiment showed that a resin-to-sample ratio smaller than 50:1 failed to achieve complete adsorption in a 12 h period (Figure 2). Finally, a change to a water and methanol solvent mixture (data not shown) revealed 1:1 (v/v) as the optimum solvent ratio to use during the trapping step, showing the best balance of solubilization and polarity while reducing the nonselective adsorption of neutral compounds into resins, but still promoting the rapid “switch” of acidic and basic organic compounds from solution onto the respective solid phases.

* In the first phase the acidic and basic resins are kept spatially separated by employing porous bags. In the second phase the resin bags are withdrawn and separately eluted.

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* The neutral components remain in the original methanol–water solution and are recovered by evaporation.
After removal of the loaded resins from the processed solution, the neutral fraction was simply recovered by removal of solvents under reduced pressure. Acidic and basic fractions were released from the corresponding resins by dipping the bags separately in basic and acid solutions, respectively, under optimized conditions (see Experimental Section). Model compounds were recovered in 75%, 91%, and 98% yields for neutral, basic, and acidic fractions, respectively. In addition, the recovered compounds were highly pure on the basis of HPLC traces (Figure 2). These results demonstrated that the desired selective separation could be achieved with the proposed methodology. Small quantities of the neutral compounds adhered to the resins, presumably due to their lipophilic polymeric backbone (Figure 3). If desired, these compounds could be recovered more completely by washing the resins with pure solvent before release of the ionic contents. The method thus clearly worked efficiently.

Plant alkaloids exhibit a wide range of potent pharmacological activities and are considered very important for drug discovery purposes. Therefore, generation of alkaloid-enriched fractions from plant extracts is very valuable for initial biological screening.

![Figure 1. Saturation curves for a 12 h adsorption period of quinine (black diamonds) and 3,4,5-trimethoxybenzoic acid (gray squares).](image)

![Figure 2. HPLC profile of artificial mixture (A) and recovered fractions: acidic (B), basic (C), and neutral (D).](image)

In addition, removal of alkaloids from the plant extract can allow for the evaluation of different types of bioactivities due to other
components possibly masked by the activity of the alkaloids in the original extract.

With the optimized protocol in hand, the plant extracts of *Skytanthus acutus* Meyen (Apocynaceae) and *Camellia sinensis* L. (Kuntze) (Theaceae) were next subjected to the same separation steps in order to examine the utility of the methodology for practical applications to natural product research. The plant *S. acutus* was used as an alkaloid-containing model plant to test and validate our new method in much more complex mixtures. First, the main monoterpene alkaloid present in the methanolic extract of *S. acutus*, skytanthine (Figure 4), was isolated and purified using a traditional isolation scheme as described in the literature. Finally, for comparison purposes, the green tea extract was then submitted to the general catch-and-release procedure, and the resulting fractions were analyzed by LCMS. In addition, a portion of *S. acutus* extract (10 g) was suspended in water, and 10% HCl was added dropwise to pH < 4 and then extracted three times with CH₂Cl₂. The aqueous layer was then neutralized with NH₃ concentrated to pH > 9 and extracted again with CH₂Cl₂. The resulting alkaloid extract (540 mg, 5.4%) was separated using silica gel SPE (Phenomenex, 20 mm), washing with hexanes, 1:1, mp 134.6 °C. The structure was confirmed by 1H NMR, 13C NMR, two-dimensional NMR experiments, IR, UV, and HRMS. The data were in agreement with those previously reported in the literature. Finally, for comparison purposes, the green tea extract (2.5 g) was suspended in water and extracted successively with CH₂Cl₂ and EtOAc to generate caffeine- and catechin-rich fractions, respectively.

**Plant Extraction and Isolation.** *S. acutus* and *C. sinensis* biomass was extracted exhaustively with mixtures of MeOH and CH₂Cl₂ (1:1, v/v); then the organic solvents were removed under vacuum to afford the crude organic extract. Each crude extract (2.5 g) was submitted to the general catch-and-release procedure, and the resulting fractions were analyzed by LCMS. In addition, a portion of *S. acutus* extract (10 g) was suspended in water, and 10% HCl was added dropwise to pH < 4 and then extracted three times with CH₂Cl₂. The aqueous layer was then neutralized with NH₃ concentrated to pH > 9 and extracted again with CH₂Cl₂. The resulting alkaloid extract (540 mg, 5.4%) was separated using silica gel SPE (Phenomenex, 20 mm), washing with hexanes, 1:1, mp 134.6 °C. The structure was confirmed by 1H NMR, 13C NMR, two-dimensional NMR experiments, IR, UV, and HRMS. The data were in agreement with those previously reported in the literature. Finally, for comparison purposes, the green tea extract (2.5 g) was suspended in water and extracted successively with CH₂Cl₂ and EtOAc to generate caffeine- and catechin-rich fractions, respectively.

**General Catch-and-Release Procedure.** Plant organic extract (2.5 g) was suspended in 500 mL of MeOH–H₂O (1:1, v/v). Prewashed tea bags containing 20 g of Dowex Marathon WBA anion-exchange resin and 20 g of Dowex MAC-3 cation-exchange resin were dipped into the solution and left shaking at 25 rpm overnight (8–10 h). Tea bags were then removed and washed with MeOH twice, then submitted to recovery conditions. Anion-exchange resin was immersed into 500 mL of 2% HCl (v/v in MeOH–H₂O, 1:1) and left overnight under agitation (25 rpm); then the HCl was neutralized with NH₂OH to pH 6–7, the MeOH removed under reduced pressure, and the aqueous phase extracted three times with EtOAc or CH₂Cl₂. Removal of the separated organic extract afforded the phenolic/acidic fraction upon evaporation. Cation-exchange resin was immersed into 500 mL of NH₄OH 2% (v/v in MeOH) and left overnight under agitation, and the resulting solution was concentrated under reduced pressure to afford the alkaloidal fraction. The original working solution was also concentrated under reduced pressure to yield the neutral fraction.

**Artificial Extract Preparation and Separation.** Approximately 100 mg of each of the model compounds (quinine, 3,4,5-trimethoxybenzoic acid, and methyl 3,4,5-trimethoxybenzoate) was weighed with an analytical balance and dissolved in 500 mL of a mixture of MeOH and H₂O (1:1) and was submitted to the general catch-and-release procedure. During the first 6 h, every hour a 1.0 mL sample was taken and analyzed using HPLC; then additional aliquots were taken after...
10 and 24 h. Concentrations were determined by interpolation from a calibration curve prepared for each compound by appropriate dilution of a mother solution of 20 mg/mL to final concentrations of 0.1 to 1.0 mg/mL.

HPLC/MS Analyses. The online HPLC/MS analyses of extracts and fractions were performed using an Agilent 1200 Series liquid chromatography system coupled to the Agilent Ion Trap LCMS 6310 mass spectrometer. The positive ion ESIMS experimental conditions were as follows: HV capillary voltage, 3.5 kV; drying temperature, 350 °C; drying gas, 12.0 L/min; nebulizer, 15 psi; and capillary exit voltage, 124.8 V. The Frag Amp was set to 1.0 V, and the smart fragmentation function was used (Smart Frag Amp was 30–200%). HPLC separations were done using an Agilent Eclipse XDB-C18 column (5 µm, 4.6 × 150 mm), and the flow rate was 1.0 mL/min (approximately 80 bar). The mobile phase for S. acutus samples was a linear gradient of acetonitrile and water from 10:90 (v/v) (t = 25 min), then 100:0 until (t = 30 min), and finally 10:90 during 10 min (t = 40 min) for recovery. On the other hand, the mobile-phase gradient program for C. sinensis samples was acetonitrile and 5 mM formic acid [5:95 (v/v) (t = 0 min), 15:85 (t = 15 min), 100:0 (t = 35 min)], wash for 5 min, and finally recovery to 5:95 (t = 50 min). All samples were dissolved in the mobile phase to a concentration of 1.0 mg/mL and filtered using 13 mm filters with 0.45 µm PTFE membranes (VWR). The injection volume was 25 µL.

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Supporting Information Available: 1H and 13C NMR of skytanthine. HPLC and LCMS traces of S. acutus and C. sinensis extracts and fractions. Adsorption curve of green tea catechins in solid-phase reagent. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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