Hyphenating Centrifugal Partition Chromatography with Nuclear Magnetic Resonance through automated Solid Phase Extraction.

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ABSTRACT: Centrifugal Partition Chromatography (CPC) and all Countercurrent Separation apparatus provide chemists with efficient ways to work with complex matrices, especially in the domain of Natural Products. However, despite the great advances provided by these techniques, more efficient ways of analyzing the output flow would bring further enhancement. This study describes a hyphenated approach made by coupling NMR with CPC through a hybrid-indirect coupling made possible by using a SPE apparatus intended for HPLC-NMR hyphenation. Some hardware changes were needed to adapt the incompatible flow-rates and a reverse-engineering approach that led to the specific software required to control the apparatus. 1D ¹H NMR and ¹H-¹H COSY spectra were acquired in reasonable time without the need for any solvent-suppression method thanks to the SPE nitrogen drying step. The reduced usage of expensive deuterated solvents from several hundreds of milliliters to the milliliter order is the major improvement of this approach compared to the previously published ones.

The wide variety of biphasic solvents systems available for the solid-support free Liquid-liquid chromatography (LLC) provide, for the time of the experiment, both the stationary and the mobile phases. Despite their lower theoretical plates when compared to solid-phase-based chromatography, their selectivity can be greater, leading to wider opportunities of separating constituents of a mixture. Their other major advantage is the theoretical 100% yield as no irreversible neither slowly reversible interactions have been described to our knowledge. These higher yields and reduced sample loss can greatly reduce the variability seen in bioassay data during bioguided-fractionation approaches.

LLC use for natural and synthetic compound separation is growing, both in use and coverage of structural families. This technology gets continuously improved on many of its aspects, to cite a few: machine design, solvent systems, industrial applicability or chiral separations. Another important field is the development or the adaptation of appropriate detectors. Along with a better characterization of the separated samples, they can also provide a reduction of fraction manipulation, thus reducing errors, degradation or loss of the fractions.

Nuclear Magnetic Resonance (NMR) has proved to be a universal detector for compounds containing non-zero spin nucleus. It provides extensive structural information and allows for quantification of one or more compounds of a mixture and is non-destructive.

Hyphenation of preparative and analytic techniques with diverse detectors is a common research tool. The greatest challenge with these coupled approaches is the transformation of the flux or a part of it coming from the chromatographic step into something suitable to the detector. Flow-rate, concentration and solvent composition are the main factors to be considered for these approaches to be effective. An extensive review of hyphenated methods for LLC has recently been published, depicting the growing interest in this field. NMR hyphenation in natural products research has been reviewed extensively and can provide insights to the reader about the use of LC-NMR approach in Natural Product research.

Many detectors have been successfully used with LLC, sometimes in a Direct (D) approach like UV-Visible spectroscopy or in Indirect (I) couplings allowing solvent susceptible detection like human taste. The existing mixed approaches can be split into two main categories. The first one, Hybrid-Direct (HD) consists of in-flow analysis with dilution and flow adaptation similar to LLC hyphenation with mass-spectrometry. The second one,
for which an example application is described in this article, is the Hybrid-Indirect (HI) coupling. In this case, automated or semi-automated sample collection, treatment and distribution to detectors are realized. Some HI couplings have been developed for LLC in the past, but HI coupling involving NMR seems to have never been reported. LLC hyphenation with NMR is not new per se, but the D approach previously reported required important amount of deuterated solvent, which additionally to their associated cost may have an influence on the separation.

Main limitations of NMR as a chromatographic detector are the low-sensitivity, especially compared to techniques like MS, UV or fluorescence spectroscopy. The use of non-deuterated solvent has a negative impact on dynamic range and then sensitivity and selectivity of the technique. Some of these limits are addressed by stop-flow approaches, specific sequences for solvent suppression but these are less easily applicable for mixtures of solvents and often require manual adjustment for each composition.

This work describes the realization of an HI coupling using a modified commercial LC-SPE-NMR interface and its application for the rapid separation and identification of molecules of interest. The hardware modifications involved a precise sampling of the LLC effluent through a calibrated loop and its distribution into the SPE sampling system using an integrated high-pressure valve.

These modifications required manual controlling of the valves position. No documentation about communication protocols could be shared by the manufacturer. For the purpose of interoperability of our LLC with the SPE interface and a control by a GNU/Linux powered computer, the serial protocol was reverse-engineered. The details of serial protocol reverse-engineering experiments and the development of a simulator to prove the guessed protocol will be detailed together with the development of the software. Such adaptation, reverse-engineering and improvement work is able to greatly enhance the innovation by allowing the users of any technology to design and apply their own ideas. For that, we favor Free (for an explanation of Free with an uppercase, see http://www.fsf.org) software and hardware. As they give the opportunity to understand, enhance and adapt the equipment to our needs as well as allowing others to reproduce or build over our work through the distribution of modifications.

**EXPERIMENTAL SECTION**

**Solvents**

All organic solvents used for CPC purification were HPLC grade except for the n-heptane which was synthesis grade. Ethyl acetate (EtOAc) and n-heptane were purchased from Scharlau (Barcelona, Spain) and the methanol (MeOH) from Carlo Erba (Rodano, Italy). LC–MS grade acetonitrile was purchased from Scharlau (Barcelona, Spain). Water for HPLC–MS analyses was purified using Elga (Bucks, UK) water purification system with a resistivity of at least 21 MΩ/cm. HPLC–MS solvents and CPC–MS auxiliary ethanol were acidified with 0.1% formic acid purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade ethanol for CPC–MS experiments was purchased from VWR (Fontenay-sous-Bois, France). SPE-NMR experiments were performed with acetonitrile-d6 purchased from Euriso-top (Gif-sur-Yvette, France).

**Centrifugal Partition Chromatography**

The CPC used is a FCPC 200 from Kromaton Technologies (Sainte-Gemmes-sur-Loire, France). It is composed of a 200 mL hydrostatic column connected to a Gilson 321-Hi binary pump controlled manually and is equipped with a 20 mL loop. Rotation speed was set at 1000 rpm.

**Analytic HPLC-MS**

HPLC–UV–MS analyses were performed on an Esquire 3000 Plus ion trap mass spectrometer (Bruker-Daltonics, Billerica, MA) using its ESI source. The LC part is an Agilent 1200 (Agilent Technologies, Santa Clara, CA) with binary pump, inline degasser, DAD, autosampler, column heater, and a ProntoSil C18 250 mm × 4.0 mm, 5 μm column (Bischoff Chromatography, Leonberg, Germany) equipped with the same phase guard column. The column temperature was kept constant at 25 °C and the flow rate was set at 1 mL/min. The mobile phases were A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid). The gradient was 0 min, 17% B; 2 min, 17% B; 22 min, 30% B; 37 min, 38% B; 45 min, 100% B; 50 min, 100% B. The HPLC output was split with an approximate 1:100 ratio into the ESI source.

**Reynoutria multiflora extract**

Reynoutria multiflora (Thunb.) Moldenke (=Fallopia multiflora, IPEN CH-o-TAL-20030306W ex Shanghai Botanical Garden) was identified and cultivated in the Jardin Botanique de Talence (France) by Dr Alain Badoc who also collected the leaves, stems and tuberous roots. The different parts were extracted separately with methanol, water was added and after evaporation of methanol using rotary evaporator, the extract was deposed on Amberlite®XAD-7HP column (Sigma-Aldrich Co, St Louis, MO, USA), rinsed with 10 volumes of water and eluted with methanol until the eluate is clear and shows no further UV absorbing compounds on silica TLC. The resulting methanol fraction for each different part were diluted in water, evaporated and freeze-dried. No yield determination was done for these extractions. The different extracts are referred to “Reynoutria multiflora-plantpart> extract”.

**Gnetum africanum extract**

Gnetum africanum Welw. roots have been harvested by Dr Gilbert Deccaux in July 2007 in Yaoundé (Cameroon). The identification was provided by M Victor Nana and registered in the national Cameroon herbarium under the reference 2165/SFRK.

Grinding and extraction have been executed by Dr Pierre Waffo-Téguo and Gérard Fondeville. 300 g of dried roots have been grinned using a Retsch SM-100 apparatus.
Material was then washed 2 times with 1.5 L of toluene (manufacturer not recorded) for 24 hours followed by lixiviation. Resulting marc was extracted twice with 2 L of an acetone/water (60:40 v/v) mixture for 24 hours. Acetone was then evaporated to yield 900 mL of aqueous extract that was further extracted with 6 L of methyl tert-butyl ether in a separatory funnel. The organic phase was then dried, dissolved with methanol and water for freeze-drying. While the methanol was evaporated in a rotary evaporator, a sticky gum not soluble in water formed (dried weight 22.9 g). The soluble fraction was freeze-dried yielding 2.7 g of an off-white powder called here “Gnetum africanum extract”.

Vitis vinifera extract

Vitis vinifera (cv. Merlot) stems have been harvested by the Domaine de Merlet (Léognan, France) owners. 9 kg of stems dried for 2 weeks in a 40°C oven were coarsely ground using a Bosch AXT22D garden-waste grinder then more finely with a Retsch SM-100 using a 6 mm square mesh. This crush material was then dried again at 40°C for one week then kept in air and light tight containers at -20°C.

The material was extracted in 2.5 kg batches in a percolation apparatus with 2 times 5 L acetone/water (60:40 v/v) mixture for 12 hours each time. Most of the acetone was then removed using a rotary evaporator and the distillated acetone weighted to assess its water content and reused for subsequent extractions and next steps.

The concentrated extract was then pre-purified on a XAD-7HP cleaned with 1 N NaOH, rinsed with water, 1 N HCl and multiple water rinses until the pH returns to neutral. After extract deposit, 15 liters of water were used to wash non-retained compounds and the retained compounds were eluted with an acetone/water (90:10 v/v) mixture. After evaporation of the acetone, the mainly aqueous fraction was freeze-dried. The powder obtained was then washed with dry ethyl acetate in a glass column. The ethyl acetate fraction after drying was diluted in a small amount of methanol and water was added to freeze-dry it. The resulting shiny brown powder is what is referred to “Vitis vinifera extract”.

SPE apparatus

The SPE-NMR interface used is a Prospekt 2 (Spark Holland, Emmen, Netherlands) customized by Bruker Biospin (Rheinstetten, Germany). It consists in a series of modules: the automated cartridge-exchange (ACE) controlling two clamps and a robotic arm for handling 2 plates of 96 cartridge together with 4 valves for liquid dispensing. The other module is the high-pressure dispenser (HPD) controlling two 2-mL high-pressure syringes and two valves for dispensing to ACE or aspirating different solvents. A schematic version of the apparatus is shown in Figure 1. The Prospekt 2 is controlled by serial RS-232 ports, which are converted by a Serial-to-Ethernet converter NPort Server Lite DE-304 (Moxa, Unterschleissheim, Germany). We have two of these modules in our different buildings, one is the modified version according to the procedure described in this work and is close to the CPC, the other unmodified one is connected to the NMR probe in another building.

The Prospekt-2 is assisted by a Knauer K-120 auxiliary pump (Berlin, Germany) controlled by RS-232 and connected to the Moxa box. No reverse-engineering was necessary for the Knauer pump as the manufacturer detailed all commands in the manual.

Serial monitoring

Serial monitoring was made using Serial Port Monitor (Eltima, Frankfurt, Germany) on the Windows machine running the Prospekt manual control program provided by Bruker.

Software development

Python 3 (http://www.python.org) development was made using GNU Emacs (http://www.gnu.org/s/emacs/) on an ArchLinux system (http://www.archlinux.org). The graphical user interface (GUI) was developed using PyQt4 (http://www.riverbankcomputing.co.uk/software/pyqt) a binding for Qt 4 (http://qt.digia.com). The software is made available as a GNU GPL v3 (http://www.gnu.org) software on GitHub (http://www.github.com/bjonnh/prosper)

SPE columns

The first trials were made with a sampler rack containing CN, C2, C8 EC-SE, C18, C18 HD, GP resin and SH resins from Spark (Emmen, Netherlands), the ion-exchange columns on the rack were not used for this study. Demonstration Oasis HLB cartridges used for preliminary experiments were graciously provided by Waters. The final experiments were made with a purchased 96-plate of the same model (Waters, Milford, USA).

Selection of SPE column

Vitis vinifera extract was used as a test sample to determine the best suited phase for the SPE columns. It was diluted at 1 mg/mL in a mixture of Water/Methanol/Ethyl acetate (50:35:15 v/v/v) aimed to mimic an ARIZONA-L system lower phase.

This sample was injected manually in the loop 100 μL at a time and pushed with 900 μL of a Water/Formic acid (99:0.1 v/v) mixture to the column at a flow-rate of 1 mL/min by the auxiliary pump. The 100/900 μL switch between sampling was made manually by running the pump for 54 seconds.

The SPE-column loading and valve switching was controlled manually with our software.

Oasis HLB retention testing

1 mg of the Gnetum africanum extract was used as a test sample for retention of HLB. The sample was dissolved in 1 mg/mL of the Water/Methanol/Ethyl acetate solution and injected following the previous protocol. The untreated sample was prepared by dissolving 1 mg of the extract in 1 mL of a mixture of Water and Acetonitrile (80:20 v/v). The eluate of the SPE column previously dried using nitrogen was obtained by pushing 500 μL of
Acetonitrile with the HPD. This eluate was then diluted with 500 μL of water for HPLC/UV comparison with the untreated sample.

**NMR**

The spectrometer used is a Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with 1H–13C inverse-detection LC probe (cell of 4 mm with an active volume of 120 μL). All acquisitions are made using the Bruker Topspin 2.0 software. For 1H 1D proton acquisitions, pulse width was determined as 6.5 μs by complete inversion method and a 90° pulse was used for acquisition. Homonuclear 1H–1H COSY was acquired using the cosypqpf pulse program with NS=2, TD=4096. 1H–13C HSQC was acquired with TD=4096, NS=64. The 1H 1D proton spectrum displayed in the figures has been zero-filled to 128K (from 32K) and an exponential line-broadening of 0.3 Hz.

**CPC-MS-SPE-NMR**

200 mg of each Reynoutria multiflora extract were separated by CPC using the ARIZONA-C solvent system in descending mode with a flow-rate of 6 mL/min and a rotation speed of 1000 rpm. The sample was diluted in 10 mL of an equivolumic mixture of each phase. The CPC was hyphenated with mass spectrometry and the Prospekt was not used in the LC-SPE configuration. By plugging-in Acetonitrile with the HPD. This eluate was then diluted with 500 μL of water for HPLC/UV comparison with the untreated sample. The volume of the loop used for the experiments described here is 100 μL, to reduce the number of switching steps required to sample enough material.

**RESULTS AND DISCUSSION**

Mechanical adaptation

One of the valves (ACE-V1 in Figure 1) of the Prospekt was not used in the LC-SPE configuration. By plugging-in a loop between ports 3 and 8 and using a tee between 4 and 6, it is possible to use this valve as an automated sampling loop. The volume of the loop used for the experiments described here is 100 μL, to reduce the
HPD-specific registers together with a generic register giving the current state of the module and 6 registers giving information about the module (like version or connected sub-modules). All the details regarding these commands and registers are given in supplementary information.

Software development

By using all the information gathered about the protocols, a Python application called Prosper was made to allow manual control of all the equipment from a Linux-based computer. An interface based on Qt was chosen for its ease of development and its extensive support by the community. The other main advantage of Python and Qt is that they allow the application to be ported easily to any other common operating system. The only required change being the serial port name. A modular approach was adopted for the conception of the application. The main modules are the Communicator, which is in charge of the interfac ing between serial data and the rest of the program and the Worker which is responsible for remembering the states, sending the Actions and managing the responses from the Prospekt. This modular architecture allows to easily add automated approaches if required. A graphical management of the cartridges allows the user to see which cartridge must be loaded and the interface provides a visual return of the current state of the machine (valves positions, pressures, volumes loaded in the syringes, gas states...). A screenshot of the software is provided in Figure 5.

Choice of cartridge

As the hydraulic system and the software were ready, the next step was to find appropriate cartridge for the studied compounds. We used extracts previously obtained in our laboratory to test the feasibility of the system using samples more complex than the model sample Reynoutria multiflora and solvent systems with a lower-phase having a greater elution strength than the ARIZONA-C used on it. The first sample was a 1 mg/mL solution of a Vitis vinifera cv. Merlot extract diluted in a mixture of solvents mimicking the ARIZONA-L solvent system lower phase. This allowed us to assert the ability of the 8 different phases contained on a sampling rack to retain the compounds of the extract. By connecting the UV detector of the HPLC-MS directly after the column we were able to follow in real-time if compounds were retained efficiently on the column during the injection phase. The sample was injected using a syringe in the loop through the port 1 of ACE-V1. The valve was then switched, and the solvent (in this case water with 0.1% formic acid) delivered by the auxiliary pump. This allowed to push the content of the loop on the column and elute the non-retained compounds. The columns were then eluted with pure acetonitrile. Before their use all the columns have been subjected to a cleaning cycle using acetonitrile and equilibration with 1 mL of the acidified water mixture. The resulting eluted sample and non-retained fraction were then dried and compared to the extract by either weight analysis. The best column was the SH-Resin with a retention of 200 μg over the 1 mg injected. All the others showed less than 100 μg of retention so they were not used for the rest of the study. Samples of Oasis HLB Prospekt columns containing 10.4 mg of the phase were also tested using the same approach and showed a negligible non-retained fraction together with a retention of more than 800 μg. This column type was then selected for more extensive selectivity and retention capacity assays.

To assess the retention capacity of these HLB cartridges, we used an extract of Gnetum africanaum containing a wider polarity range of stilbenoids (from poly-glycosylated to per-methylated) than the other plants of this study. The same protocol than previously was used and the eluted fraction was compared using HPLC-UV with the crude extract. The results are presented in Table 1, the chromatograms are presented as supplementary information. While the retention proved to be low for the more apolar compounds (between 39 and 44 minutes), the retention was considered sufficient to prove the concept of this study. The solvent used for dilution may be responsible for the poor retention of the more apolar compounds, but this hypothesis was not tested. Also the peaks in the 39-44 minutes region are highly overlapped, the integration is not expected to be accurate over that range with such a gradient.

Reynoutria multiflora separation and analysis

CPC-SPE-UV-MS hyphenation was realized by connecting the modified Prospekt on the outlet of the CPC and connecting the outlet of the Prospekt to an active splitter allowing recovery of the eluted solvent and analysis by UV-MS of a small portion of it. Three extracts of Reynoutria multiflora (leaves, stems and roots) were subjected to a separation in the 200 mL CPC apparatus using the ARIZONA-C system and hyphenated with the Prospekt. Shake-flask analysis by HPLC of the partition coefficient of THSG gave a value of 1.1 for ARIZONA-C, 2.1 for ARIZONA-D and 3.5 for ARIZONA-G. ARIZONA-C was determined as the model system for its highest water containing lower phase and convenient partition coefficient for THSG. Systems ARIZONA A and B with lower partition coefficient were not chosen as the unknown compound with a mass of 581 (visible on the Supporting information CPC-MS of the roots) was co-eluting with our model compound. The stationary phase retention measured by ejected volume during equilibration was in each case of 65% (70 mL ejected). And no noticeable stationary phase loss was noticed during separation apart from the volume injected with the sample (equivalent to 5 mL).

The compound of interest in these extracts, 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucopyranoside (THSG), was followed through the hyphenation with UV-MS after the Prospekt through an active splitter. This compound has also been used as the target compound for the CPC solvent system optimization. The mass followed in positive mode was 429 corresponding to the sodium adduct [M+Na]⁺ of this molecule as this signal was far
more stronger than the \([\text{M+H}]^+\) one. We noted that during CPC-MS experiments, the sodium adduct is the more abundant while in LC-MS its intensity is closer to the pseudo-molecular ion. The hypothesis here is that the CPC solvents and the ethanol used in the active splitter may contribute sodium ions in the MS inlet flow.

The weight of the fractions containing the THSG was of 50.5 mg for the roots, 7.5 mg for the stems and only trace amount (< 0.1 mg) in the leaves. Yield of the collected fractions, from 0 min to 80 min, was comprised between 74 and 81%. The extracted-ion-chromatogram traces for THSG shows intense signals in each case, showing the high sensitivity of this hyphenation.

During this experiment, we sampled 3 cartridges each with 10 cycles of loading/injection using ACE-V1 during the elution of the THSG. The cartridges were used to sample the beginning, top and end of the peaks. After nitrogen and over-night drying at reduced pressure, we subjected these cartridges to NMR analysis using our unmodified Prospekt connected to the NMR.

At first, the roots cartridge were used to ensure a sufficient amount of THSG. As the resulting proton NMR spectrum (Figure 6 left) had an excellent S/N ratio, we decided to try 2D experiments on the sample still in the probe. 'H-'H-COSY and 'H-'C-HSQC experiments yielded good spectra in reasonable amount of time as the COSY took approximately 5 minutes and HSQC 25 minutes.

CONCLUSIONS
A Direct coupling\(^\text{25}\) can, in the case of analytical-scale LLC, be more appropriate. However, this implies using a flow-rate compatible with the probe and a column/coil able to handle the additional back-pressure of it. Such an approach requires using partially deuterated LLC solvents and the use of solvent suppression sequences, or the use of exclusively deuterated solvents. In both these cases, the spectra obtained with an eventual parallel MS detection would have to account for partially or totally deuterated masses depending on the exchange rate of the molecules and the ratio of deuterated solvents. Another limitation of such an approach is the requirement of quick NMR acquisitions, or the use of stopped-flow LLC which can reduce the separation efficiency through degradation of solvents (hydrolysis of ethyl acetate in water) or the analytes or impossible due to limitations of certain types of rotary seals.

Using a Hybrid-Indirect approach reduce such issues at the price of a more complex equipment requirement. The use of a robotic SPE cartridge handler (such as the Prospekt used in this work) allows the combination of preparative and analytical techniques having highly differing requirements in term of analyte concentration, solvent type and fluidic characteristics such as viscosity and flow-rate. Beyond the convenience of having an automatized NMR acquisition of an important number of samples (192 without user intervention on the Prospekt), the volume and repeatability of the sampling on the preparative side is also an advantage of such an approach over manual SPE in an Indirect approach.

Reverse-engineering of the communication protocol of the Prospekt machine allowed us to develop a control software of our own. By using it we have been able to use this device beyond its initial capacities in order to hyphenate CPC with NMR. The recoveries and amount collected on the SPE cartridges was enough to run 1D proton, COSY and HSQC experiments. While our approach used a LC-NMR probe, it is possible for laboratories without such an equipment to simply collect the desorbed compounds from the SPE directly in an NMR tube. This could possibly provide a great improvement on field homogeneity and sample homogeneity which are some of the limiting factors of the probe we used.

Some improvements are still required for this approach to be fully workable in the case of complex samples and to enhance compatibility with all kinds of plant metabolites. While our hardware implementation allowed us to use the CPC in descending mode with the SPE columns used, it is now necessary to develop an approach able to cope with ascending mode mobile phases which are mostly composed of non-polar solvents. Some preliminary assays using a T junction and a split (see supplementary information for a hardware implementation) allowed us to obtain some results by using a continuous dilution with water-based phases, but selection of more suitable SPE phases would be more advisable.

Finding a suitable SPE phase is simultaneously one of the major limitations of the SPE approach as it requires extensive development and one of its major advantage such as when using Molecularly Imprinted Polymers phases and other highly analyte selective materials.

While the retention of the compounds proved to be sufficient for most of the analytes for a qualitative analysis, it does not allow quantitative analysis yet. One of the conceivable improvement would be to use a serial arrangement of two different SPE columns allowing the non-trapped compounds in the first one to be trapped on the second one, but this would lead to an additional dilution of the analytes.
Figure 1. Full schematic of the Prospekt and the subsequent modifications as green dotted lines.

Figure 2. Scheme of the experimental setup showing the integration of the Prospekt in the CPC/UV/MS chain. Two different machines have been used for this study, the one with an * is the modified one.

Figure 3. Use of the valve ACE-V1 for sampling a small amount of the CPC effluent and sending it in the SPE flow.

Figure 4. An example of a communication. Our software, Prosper, asks the Prospekt to change the position of the ACE-V1 valve, requests the general state of the machine, then the state of the valve.

Figure 5. A screenshot of the main window and the cartridge selection window of Prosper.
Figure 6. NMR 1D and 2D spectra obtained with THSG sampled on a SPE cartridge from the Reynoutria multiflora root extract CPC separation.
Table 1. Retention of the *Gnetum africanum* extract components on the HLB column.

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ASSOCIATED CONTENT

Supporting Information
Chromatograms and tables of all the Prospekt commands are available as supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions
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REFERENCES


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