Trapping-Enrichment Multidimensional Liquid Chromatography with On-Line Deuterated Solvent Exchange for Streamlined Structure Elucidation at the Microgram Scale

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ABSTRACT: At the forefront of chemistry and biology research, development timelines are fast-paced and large quantities of pure targets are rarely available. Herein, we introduce a new framework, which is built upon an automated, online trapping-enrichment multi-dimensional liquid chromatography platform (TE-*Dt*-mDLC) that enables: 1) highly efficient separation of complex mixtures in a first dimension (^1D-UV); 2) automated peak trapping-enrichment and buffer removal achieved through a sequence of H_2O and D_2O washes using an independent pump setup; and 3) a second dimension separation (^2D-UV-MS) with fully deuterated mobile phases and fraction collection to minimize protic residues for immediate NMR analysis while bypassing tedious drying processes and minimizing analyte degradation. Diverse examples of target isolation and characterization from organic synthesis and natural product chemistry laboratories are illustrated, demonstrating recoveries above 90% using as little as a few micrograms of material.

Keywords: Analytical Methods; Liquid Chromatography; Mass Spectrometry; Multicomponent Isolation; Structure Elucidation

INTRODUCTION

The need to identify and characterize target components in complex mixtures is crucial at nearly every stage of chemical and biological research (*e.g.* drug discovery and development, natural products, agriculture and food sciences, metabolomics, biosynthesis, high-throughput screening, *etc*.). Innovations in analytical technologies over the past two decades have enabled demanding synthetic and biosynthetic processes for more complex therapeutic targets.[1-6] Among these, enhancements in coupling chromatographic techniques with spectroscopy/spectrometry have been transformative for impurity profiling, multicomponent detection, and high-throughput analyses.[7-11] However, in many instances, unambiguous structure elucidation *via* NMR and chemical characterization requires multiple tedious purification steps including double-pass or multi-pass column separation.[12-14]

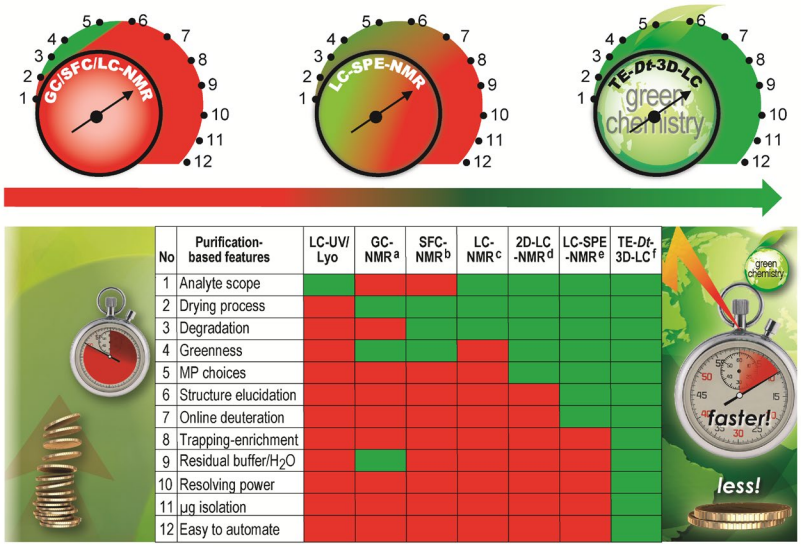
Numerous examples of one-dimensional (^{1}D) separation techniques hyphenated with NMR for structure elucidation have been introduced,[15-17] including GC-NMR,[18] SFC-NMR,[19] LC-NMR and LC-MS-NMR.[20-25] Combining NMR and solid-phase extraction (SPE) with LC as a desalting step, has proven valuable for metabolite identification.[26-31] While numerous 1D-LC approaches exist to purify and characterize analytes in complex mixtures, automated multi-dimensional separation techniques with integrated fraction collection and MS acquisition is an underdeveloped area.

Traditional separation bottlenecks have been overcome by widespread deployment of multi-dimensional chromatographic capabilities for complex mixtures.[3, 32-34] Notably, the use of two-dimensional liquid chromatography (2D-LC) combined with computer-assisted simulation provided an incremental advance in delivering improved peak capacity and selectivity.[32, 35-39] However, purification laboratories have historically not benefited from these advances, which have the potential to streamline isolation and characterization of target components. Only a few papers have been published on 2D-LC/NMR; for instance, the research groups of Hiller and Brüll demonstrated its practicality for online separation and monitoring of polymers.[40-41] The authors outlined some of the limitations of this setup, including analyte solubility, solvent incompatibility between LC dimensions, poor sensitivity and signal interferences from the mobile phase solvents, buffers and organic

modifiers. Moreover, this workflow was not focused on enabling purification, structure elucidation or a full characterization footprint.

Purification laboratories are often viewed as expensive operations, wherein method development and optimization are substantially more costly than their analytical counterparts, especially at the semi/preparative scale. Purification activities involve additional challenges including analyte solubility, loading optimization, component stability upon fraction collection, waste accumulation and disposal, and tedious drying processes. Despite the great value that online 2D-LC combined with NMR might have for targeted purifications, the development of an automated online multi-dimensional separation platform incorporating efficient separation of complex mixtures and solvent exchange (to deuterated solvents in preparation for NMR) remains a tremendous challenge.

Ideally, 2D-LC-NMR should integrate several critical features to overcome limitations of currently available technologies, thus securing its viability as a mainstay for industrial applications. This includes the need for automation-friendly setups that deliver higher resolution separations beyond LC-SPE for microgram quantities of target compounds, while bypassing tedious drying processes and component manipulation prior to NMR analysis (Figure 1).



No	Purification-based features	LC-UV/ Lyo	GC-NMR[a]	SFC-NMR[b]	LC-NMR[c]	2D-LC-NMR[d]	LC-SPE-NMR[e]	TE-*Dt*-3D-LC[f]
1	Analyte scope							
2	Drying process							
3	Degradation							
4	Greenness							
5	MP choices							
6	Structure elucidation							
7	Online deuteration							
8	Trapping-enrichment							
9	Residual buffer/H_2O							
10	Resolving power							
11	µg isolation							
12	Easy to automate							

Figure 1. Evolution of purification technologies to enable efficient target identification. (a) Gas chromatography coupled to NMR;[18] (b) Sub/super critical fluid chromatography coupled to NMR;[19] (c) LC coupled to NMR;[20-22] (d) 2D-LC coupled to NMR;[40-41] (e) LC coupled to solid phase extraction (SPE) and NMR;[26-31] (f) This paper: multi-dimensional liquid chromatography with trapping-enrichment deuterated solvent exchange (TE-*Dt*-mDLC).

To address these challenges, we developed an online approach for multi-component isolation that streamlines microgram-scale structure elucidation enabled *via* automated multi-dimensional liquid chromatography with trapping, enrichment, and solvent exchange (TE-*Dt*-mDLC) combined with mass spectrometry (MS) and diode array detectors (DAD). To facilitate its widespread implementation, we have established fundamental aspects of primary importance across all three dimensions, including proper combinations of column chemistries and dimensions, recovery optimization, system cleaning details, flow rate and combinations of deuterated solvents. In addition, this concept is illustrated with diverse applications in the context of target characterization and analysis of complex multi-component reaction mixtures from modern synthetic and natural product chemistry laboratories.

RESULTS AND DISCUSSION

Recent developments in the field of organic synthesis are leading to increasingly complex mixtures of closely related species that often prove challenging for analysis and target characterization. In many instances, a high purity isolation requires multiple purification passes and adequate conditions upon fraction collection and drying to reduce compound degradation, which in our experience has proven to be time-consuming. To circumvent these challenges, a new approach (TE-*Dt*-mDLC) is introduced (Figure 2a) enabling highly efficient multi-component separations together with the delivery of enriched fractions of target analytes in deuterated solvents ready for NMR analyses.

Specifically, the first dimension (^{1}D) employs a quaternary pump that allows the screening of different mobile phase combinations (organic and aqueous eluents with non-volatile or volatile salts; acidic, neutral, or basic pH). As soon as the optimized ^{1}D method is deployed, analytes of interest can be accumulated onto a trapping column. To increase the amount of compound isolated, multiple ^{1}D separations can be performed, each time trapping the target analyte on the same column before releasing all of the accumulated material to the second dimension. Then, a second pump can deliver water to the trapping column to ensure the removal of salts and buffers from the ^{1}D effluent, followed by D_2O to remove protic solvents. The target analyte, conditioned in D_2O on the trap, is subsequently eluted into the

^{2}D column using D_2O, CD_3CN and CD_3OD-based mobile phases delivered by a third pump. This ^{2}D separation is hyphenated with UV and/or MS detection, as well as an automated fraction collector module that enables peak isolation and spectrometric data acquisition. The second dimension enables further separation of species that are not resolved by the ^{1}D separation, provides potential for focusing target analytes to increase their concentrations, and simultaneously reduces the level of protic solvent interferences in the isolated fractions for convenient NMR analyses (see Figure 2b).

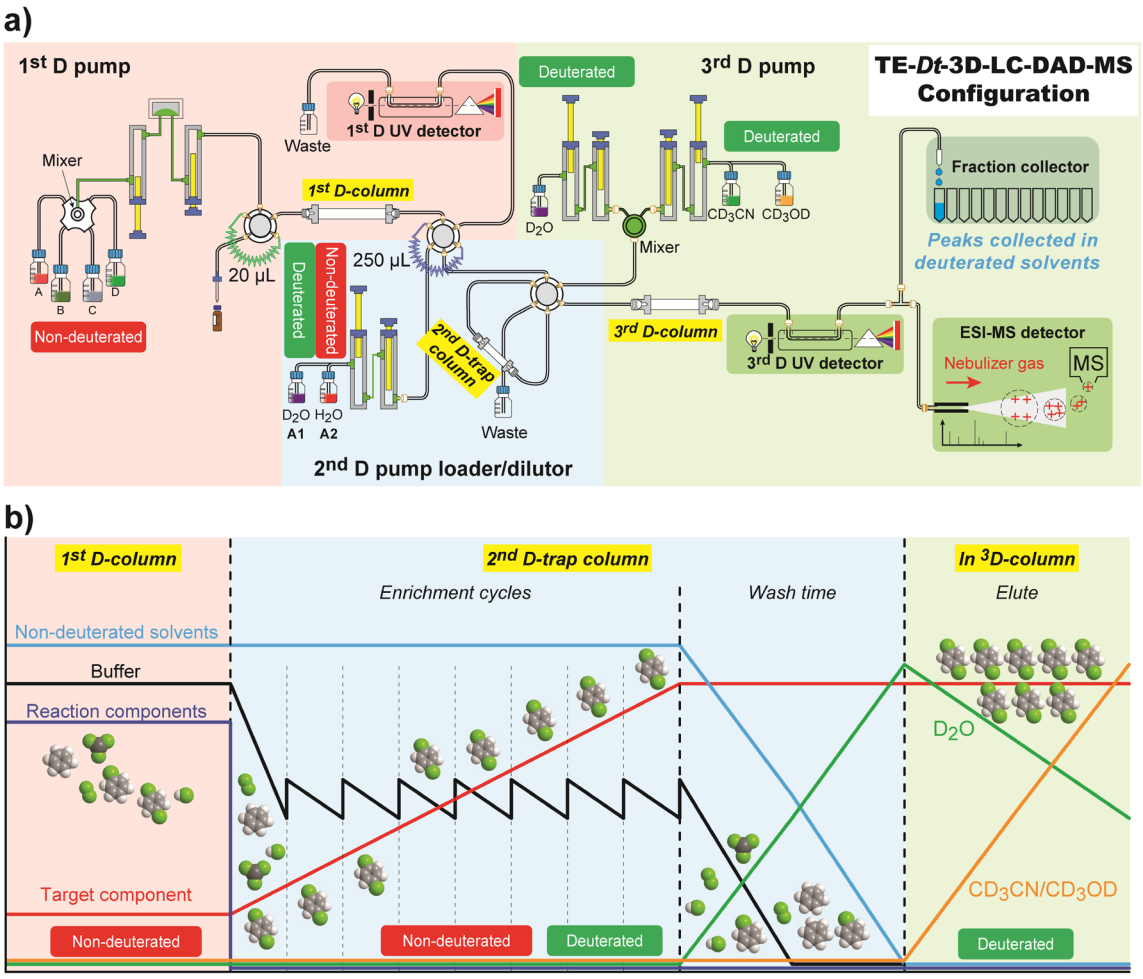


Figure 2. a) Illustration of instrument setup for TE-Dt-mDLC with fraction collection. b) Illustration of changes in mixture components across the entire workflow. Further details regarding instrument components and stationary phases are discussed in the Supplemental Information.

An experimental result demonstrating the potential of this concept is shown in Figure 3. To understand the capabilities of this new approach, a mixture containing a series of indole-based compounds was studied. As illustrated in Fig. 3a, the indoles selected possess

different polarity values, covering the widest gradient retention profile possible (low, medium, and high retention). Therefore, the mixture was first separated in the ^{1}D stage, taking advantage of the ^{1}D setup that enables baseline resolution of all mixture components. Subsequently, the isolation, trapping, and enrichment steps were tested with a focus on one particular indole compound (indole-4). During the enrichment process, the ^{1}D chromatogram region containing indole-4 is enriched by using a trap column located upstream from the ^{1}D-UV detector. Thus, the target compound will not be observed in the ^{1}D chromatogram during the trapping step. The efficacy of the trapping and enrichment steps, as well as the collection step following ^{2}D separation, were verified by re-injecting the collected fraction back into the ^{1}D column (blue trace in Figure 3a), which confirmed a high purity level for indole-4.

To characterize the overall performance of the system using analytes covering a range of polarity, the weakly retained indole-2, moderately retained indole-7, and strongly retained indole-10 were selected as the main targets for isolation and enrichment (figures 3b and 3c); in these experiments we also aimed to demonstrate the ability to prevent carryover through sufficient cleaning steps. Using these indoles, the effects of stationary phase chemistries (Oasis HLB vs. Xbridge C18) and column geometries were evaluated in terms of the recovery, linearity and purity levels achieved throughout the trapping-enrichment cycle. The comparison between the Oasis HLB and Xbridge C18 materials clearly demonstrates the importance of the choice of stationary phase chemistry for the trapping material. The HLB material enables repeated trapping of analyte from the first dimension with high recovery (> 90%) for all three analytes. On the contrary, the recovery of the more hydrophilic indoles (2 and 7) was poor when using the Xbridge C18 material, which only delivered an acceptable recovery for the most hydrophobic indole (indole-10).

The HLB (hydrophilic lipophilic balance) material is well-adapted for a wide range of compounds having diverse polarities, thus providing good retention of hydrophilic and lipophilic compounds during the different wash cycles, while the C18 stationary phase provides good results only with relatively hydrophobic compounds. Therefore, with the correct choice of cartridge chemistry, recovery values above 90% were obtained across the entire range of indoles. Furthermore, no other impurity peaks or carryover were observed

in the ^{2}D chromatograms (Fig. 3c), indicating a good chromatographic performance across all steps including isolation, trapping and enrichment of target analytes.

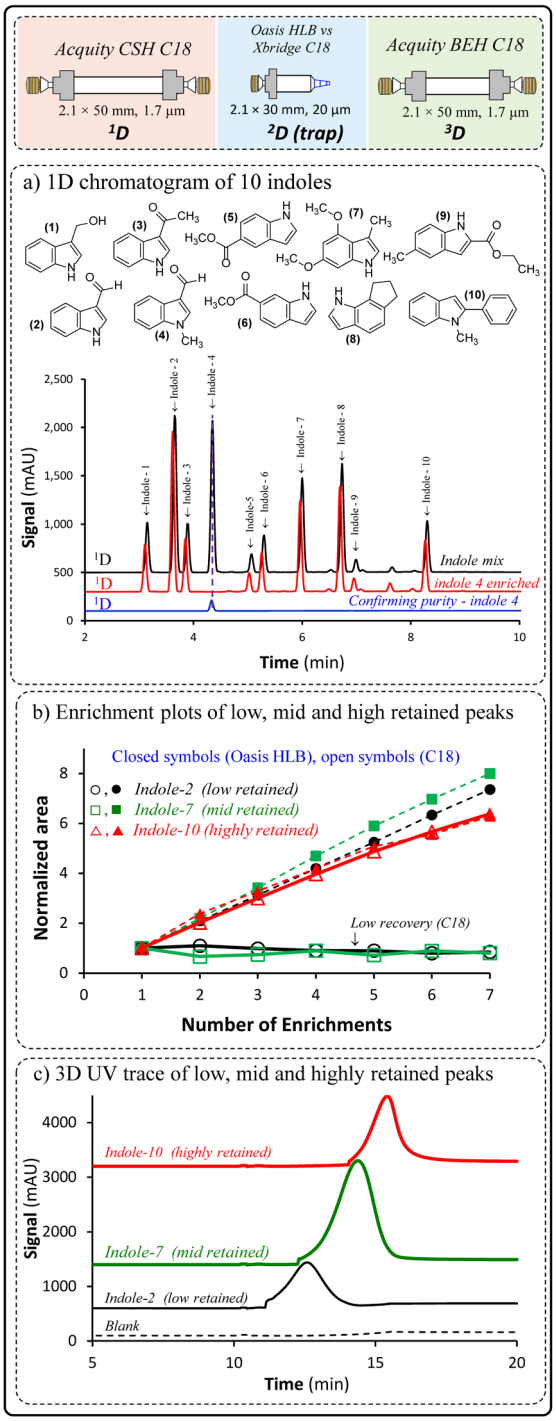


Figure 3. a) Representative ^{1}D chromatogram of 10 indoles. Indole-4 is enriched on the trapping column and isolated with a fraction collector following elution from the ^{2}D dimension column. ^{1}D chromatogram confirming the isolation of indole-4 is shown in blue. b) Enrichment linearity results for low (indole-2), mid (indole-7) and high (indole-10) hydrophobic analytes. Normalized area was calculated as the area obtained after n enrichment cycles relative to the area obtained after one enrichment cycle. Closed symbols correspond

to results obtained from enrichment using Oasis HLB direct connect HP column (2.1 x 30 mm, 20 µm) while open symbols correspond to results obtained from enrichment using a Xbridge C18 direct connect HP column (2.1 x 30 mm, 10 µm). c) ^{2}D chromatograms of indoles 2, 7, and 10 after seven enrichment cycles on Oasis HLB direct connect HP column. ^{1}D conditions: Waters Acquity UPLC CSH C18 (2.1 x 100 mm, 1.7 µm), Temperature: 40 °C. Detection: UV 260 nm. Sample: 3 µL of 1.0 mg/mL of each component in H2O. Flow rate: 1.0 mL/min. Mobile phase: 0.1 % HCOOH in H_2O (A) and CH_3CN (B). Gradient: 5-95% B (10min), 95%B (5min), 95%B-5%B (0.1min), 5%B (3min). Trapping conditions: Oasis HLB direct connect HP, Temperature: 25 °C. Flow rate: 2.0 mL/min. Mobile phase: 100% H_2O. Loop volume: 250 µL. ^{2}D conditions: Waters Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm), Temperature: 40 °C. Detection: UV 260 nm. Flow rate: 1.0 mL/min. Mobile phase: D_2O (A) and CD_3OD (B). Gradient: 25-95% B (10min), 95%B (5min), 95%B-5%B (0.1min), 5%B (3min).

Multi-component reaction mixtures are often generated during the early stages of organic synthesis methodology development. As reaction optimization progresses, substantially better outcomes are achieved in terms of both yield and purity. Meanwhile, efficient analytical and preparative methods are required to guide process optimization, component characterization and batch release. Separation, analysis, and characterization of new fluorination chemistries can be notoriously difficult, where in addition to the difficulty of resolving closely related constitutional isomers, the separation of proteo- and fluoro-species can often be challenging.[42-49]

Figure 4a illustrates an example of a remote C–H fluorination reaction, wherein carbon radicals (generated by hydrogen atom transfer from distal alkoxy radical species) are quenched with a fluorinating reagent to forge new carbon−fluorine bonds at remote positions in the molecule.[50] A reaction mixture containing the main target component and several closely related byproducts (Figure 4b) is produced (conditions described in ESI). Having arrived at a satisfactory TE-*Dt*-mDLC workflow in this study, characterization and identification of reaction components can be achieved with minimal effort as depicted in Figure 4b,c. An RPLC-UV method using Waters Acquity UPLC CSH C18 (2.1 x 100 mm, 1.7 µm) column and formic acid-based mobile phase was rapidly deployed in the first dimension to separate all species, enabling straightforward visualization of reaction yield and impurity profile. Major target components were trapped and enriched (90% desired product: 7 cycles, 5% impurity: 14 cycles) using an Oasis HLB direct connect HP (2.1 x 30 mm, 20 µm) column combined with a sequence of H_2O followed by D_2O washes to remove buffer and minimize H_2O levels from the ^{1}D effluent. In the second dimension (UV and MS detection), a Waters Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm) column with

CD_3CN-based eluent was used for multi-component isolation via integrated fraction collection while simultaneously generating ESI-MS (+) spectra for each peak ($[M-H]^+$ shown in Figure 4c).

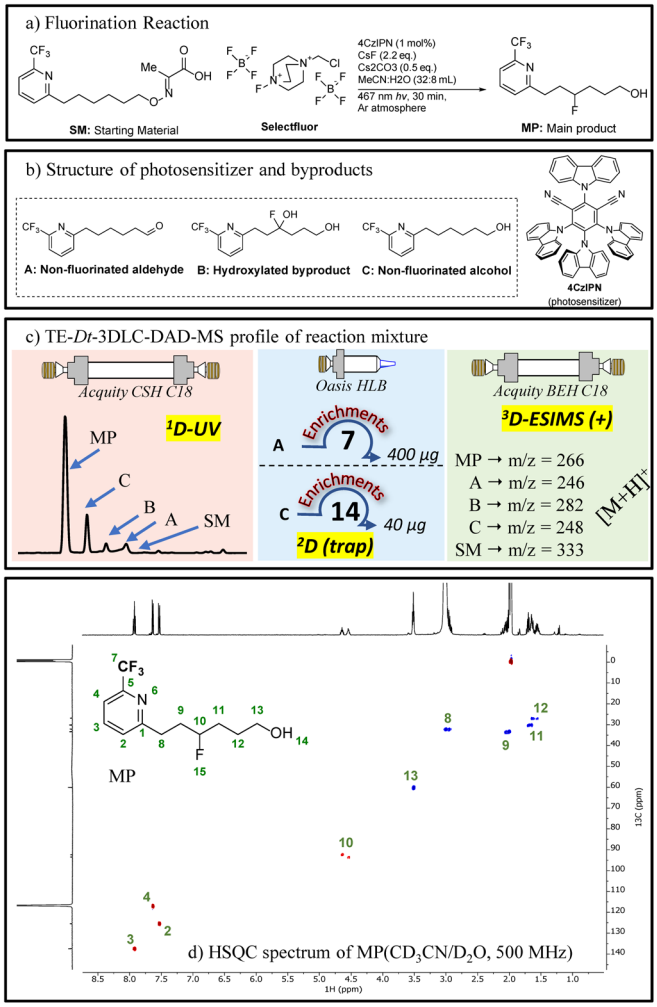


Figure 4. a) C–H fluorination reaction. b) Structure of photosensitizer and byproducts. c) Purification and tentative identification of reaction components *via* TE-*Dt*-mDLC-DAD-MS. Chromatographic conditions are the same as in Figure 2, except ^{2}D solvent was: CD_3CN instead of CD_3OD. d) HSQC spectrum of main product.

This automated online setup was used to yield 400 µg of desired product and 40 µg of non-fluorinated alcohol impurity in deuterated solvent for direct NMR analysis within just a few hours (recovery ≈ 91%), streamlining the characterization of both components by 1D and 2D NMR analysis (HSQC spectrum in Figure 4d) without the need to employ water-suppression pulse sequences (see full chemical characterization data in ESI: ^{1}H, ^{13}C, ^{19}F, COSY, HSQC and HMBC spectra in Figures S1-S10). It is important to point out that, in addition to generating pure components free of buffers in deuterated solvent for direct

NMR analysis, this workflow allows for other convenient features including: 1) LC-UV area % analysis to monitor reaction conversion based on the 1D separation, and 2) MS detection following the 2D separation for structure elucidation purposes. In this case, UV and ESI-MS (+) data were convenient to monitor and identify other minor impurities in the reaction mixture including a non-fluorinated aldehyde (3%, m/z = 246) and a hydroxylated product (1.5%, m/z = 282).

A second example related to natural product synthesis[45, 51] is provided in Figure 5a. Here, a tetraol intermediate obtained from a tetra-boronic ester precursor yields a reaction mixture with the desired product but also other byproduct components. In this case, an enantioselective RPLC-UV method on a column packed with a chiral stationary phase (Phenomenex Lux Cellulose-4) using an acidic mobile phase (0.1% phosphoric acid in water) enables separation of closely related components in the first dimension. UV data reveals the relative amount of all reaction components (MP: 95%, A: 2.4% and B: 2.6%), while MS detection allows confirmation of product formation along with the presence of other minor diastereoisomers (figure 5b). The desired component was then trapped and enriched using the Xbridge C18 direct connect HP column enabling efficient removal of phosphoric acid, ACN and water residues from the ^{1}D separation. Then, a fully deuterated ^{2}D separation using another chiral column (Chiracel OD-R) with D_2O/CD_3CN-based eluent enabled purity upgrade (figure 5b) while also generating a pure component in deuterated solvent for direct NMR analysis (recovery ≈ 90%). It is important to highlight that this approach allowed unambiguous structure elucidation and fast-paced characterization of the main product using 100 µg of material as illustrated in the HSQC spectrum (figure 5c) as well as other NMR data (ESI).

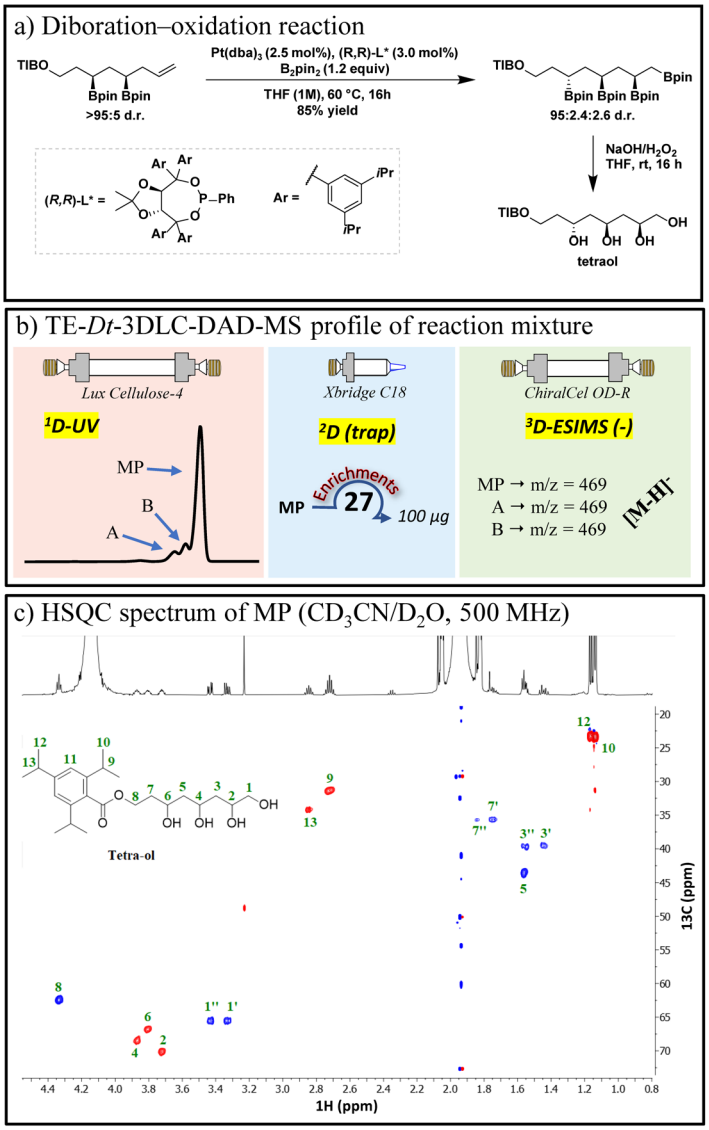


Figure 5. a) Tetra-ol intermediate reaction. b) Purification and tentative identification of reaction components via TE-*Dt*-mDLC-DAD-MS. ^{1}D conditions: Phenomenex Lux Cellulose-4 (4.6 x 150 mm, 3.0 µm), Temperature: 40 °C. Detection: UV 210 nm. Sample: 3 µL of 1.0 mg/mL of each component in H_2O. Flow rate: 1.0 mL/min. Mobile phase: 0.1 % H_3PO_4 in H_2O (A) and CH_3CN (B). Gradient: 20-50% B (15min), 50%B (1min), 50%B-20%B (0.1min), 20%B (5min). Trapping conditions: Xbridge C18 direct connect HP, Temperature: 25 °C. Flow rate: 2.0 mL/min. Mobile phase: 100% H_2O. Loop volume: 500 µL. ^{2}D conditions: ChiralCel OR-R (4.6 x 150 mm, 3.0 µm), Temperature: 40 °C. Detection: UV 210 nm. Flow rate: 1.0 mL/min. Mobile phase: D_2O (A) and CD_3OD (B). Gradient: 25-95% B (10min), 95%B (5min), 95%B-5%B (0.1min), 5%B (3min). c) HSQC spectrum of main product.

CONCLUSIONS

In summary, we have introduced a fully automated approach that enables multi-dimensional liquid chromatography separation incorporating trapping and enrichment of target analytes, solvent exchange with deuterated solvents (TE-*Dt*-mDLC), coupled to

different detectors (UV and MS) to enable fast-paced structure elucidation at the microgram scale from multi-component mixtures. Target components can be conveniently isolated, enriched, and collected in deuterated solvent without manual intervention while bypassing tedious drying processes. By deployment of this concept, complex reaction mixtures (*e.g.* modern synthetic and natural product chemistry) can be characterized efficiently *via* MS and NMR while minimizing labor and reducing degradation of target compounds. We expect that this new workflow will enable a diverse range of next-generation multi-dimensional purification capabilities to be developed across academic and industrial laboratories, especially for those concentrating on novel organic syntheses and new chemical modalities. An expansion of the applications reported herein can be envisaged including biopharmaceutical processes, which can often require pre-treatment prior to analysis. This approach also enables the combination of different separation modes across ^{1}D and ^{2}D dimensions beyond RPLC (*e.g.* size exclusion, ion exchange, hydrophobic interactions, *etc*.).

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Entry for the Table of Contents

Trapping-Enrichment Deuterated Multidimensional-Liquid Chromatography (TE-*Dt*-mDLC) for rapid microgram-scale structure elucidation by NMR is introduced. This concept enables a streamlined chemical characterization of multicomponent mixtures (*e.g.* catalysis, natural products) while bypassing tedious drying processes with recoveries above 90% using as little as a few micrograms of material.