Automated Multicolumn Screening Workflow in Ultra-High Pressure Hydrophilic Interaction Chromatography for Streamlined Method Development of Polar Analytes

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ABSTRACT:

The pharmaceutical industry is rapidly advancing toward new drug modalities, necessitating the development of advanced analytical strategies for effective, meaningful, and reliable assays. Hydrophilic Interaction Chromatography (HILIC) is a powerful technique for the analysis of polar analytes. Despite being a well-established technique, HILIC method development can be laborious owing to the multiple factors that affect the separation mechanism, such as the selection of stationary phase chemistry, mobile phase eluents, and optimization of column equilibration time. Herein, we introduce a new automated multicolumn and multi-eluent screening workflow that streamlines the development of new HILIC assays, circumventing the existing tedious 'hit-or-miss' approach. A total of 12 complementary columns packed with sub-2 µm fully porous and 2.7 µm superficially porous particles operated on readily available ultra-high pressure liquid chromatography (UHPLC) instrumentation across a diverse set of commercially available polar stationary phases were investigated. Different mobile phases with pH ranging from pH 3 to 9 were evaluated using different organic modifier. The gradient and column re-equilibration were judiciously set to ensure a reliable assay screening framework yielding straightforward separation conditions for subsequent optimization and method deployment in fast-paced laboratory settings. This UHPLC screening system is coupled with a diode array and charged aerosol detectors (DAD and CAD) to ensure versatile detection for a variety of compounds. This fast-screening platform lays the foundation for a convenient generic workflow, accelerating the pace of HILIC method development and transfer across both academic and industrial sectors.

Keywords:

Hydrophilic interaction chromatography; Assay Screening; Method development; Liquid Chromatography; Polar Compounds, Charged aerosol detector.

Highlights:

- Systematic HILIC assay development of polar analytes remains a challenge
- A new, automated HILIC-DAD-CAD method development workflow for polar analytes
- Screening of 12 columns with sub-2.7 µm fully and superficially porous particles
- Evaluation of different eluent pH and column equilibration time
- Diverse compounds and real-life pharmaceutical examples were explored

1. INTRODUCTION

The introduction of new analytical enabling technologies capable of streamlining assay development plays a crucial role in advancing novel products across both pharmaceutical and industrial sectors [1-3]. Liquid chromatography (LC) is a widespread analytical technique that significantly enhances our ability to identify, quantify, and characterize compounds and materials, leading to advancements in medicine, environmental monitoring, food safety, and forensic analysis. LC reliability and robustness in conjunction with an unmatched versatility in terms of detection choices, have gradually cemented its supremacy for enabling a broad spectrum of modern early and late development pharmaceutical processes [4]. Furthermore, LC can be operated in various separation modes including reversed-phase (RP), normal-phase (NP), ion-exchange (IEX), size exclusion chromatography (SEC), and others, each offering distinct separation mechanisms and selectivity for specific types of analytes across a wide polarity range [5].

The chromatographic separation of complex mixtures of polar analytes can often be challenging. Polar hydrophilic compounds traditionally encountered in modern pharmaceutical processes exhibit strong interactions with NPLC stationary phases and limited solubility in the mobile phase, something that has been partially circumvented with the evolution of sub/supercritical fluid chromatography (SFC) using unconventional additives in water-rich modifiers [6]. RPLC, which is considered the gold standard for the separation of apolar to moderately polar compounds, often delivers sub-optimal results when dealing with highly hydrophilic analytes commonly referred to as “fast flyers” across RP stationary phases, resulting in poor retention and reduced separation efficiency. IEX is widely used for the separation of organic salts and biopharmaceuticals due to its unique selectivity and non-denaturing characteristics, but it is not suitable for achieving chromatographic resolution across the vast spectrum of small hydrophilic substances. Unified

chromatography (UC) [7], and the recently introduced Dual Gradient Unified Chromatography (DGUC)[8] are rapidly emerging as a powerful multimode universal separation technique in the analytical toolbox covering the widest polarity range for complex multicomponent mixtures. However, although this approach is poised to have a significant impact on the field, it is still considered "unconventional" and unlike RPLC it has not yet become commonplace across late-stage pharmaceutical development laboratories and highly regulated environments following good manufacturing practices (GMP).

Hydrophilic Interaction Liquid Chromatography (HILIC) is a key alternative to traditional RPLC analysis, facilitating the separation of highly polar analytes that quite often exhibit poor retention in RP mode. First described by Alpert in 1990 [9], separation in HILIC occurs by hydrophilic analyte retention through the partition between an aqueous layer formed at the surface of the polar stationary phase and the organic-rich mobile phase [10]. HILIC enables the separation of highly polar analyte classes such as nucleotides [11], carbohydrates [12], peptides [13], organic acids [14] and biogenic amines [15], which are challenging to separate using other chromatographic techniques. In addition to its unique separation performance, HILIC also displays advantageous features over other chromatographic techniques, such as readily MS-compatible solvents [16], alternative selectivity, low operating backpressure, and possible coupling to RPLC for two-dimensional LC applications [17].

Currently, pharmaceutical applications of HILIC are rapidly proliferating due in part to increased biotherapeutic research[18]. HILIC-MS has long been the gold standard technique for released glycan analysis, a critical quality attribute for mAbs monitored during GMP batch release [19]. New drug modalities such as oligonucleotide have also benefited from the implementation of HILIC separations as well [20]. Furthermore, HILIC-MS approaches for metabolite profiling have

enabled a better understanding of drug-induced metabolic changes *in vitro* and *in vivo* [21], and improved bioreactor process control during the production of new therapeutic modalities [22].

Timely development of robust HILIC separations can provide critical insight into drug structure, purity, and stability from discovery to large-scale manufacturing [23]. However, HILIC method development is uniquely difficult, as multiple retention mechanisms occur within a single separation which must be individually tuned to achieve optimal analyte retention and resolution. Therefore, optimizing separation performance in HILIC requires systematic scouting of a diverse set of stationary and mobile phases as well as lengthy column equilibration times between runs to ensure method reproducibility [24, 25]. This development process is highly manual and very labor-intensive, especially when developing separations for complex multicomponent mixtures [26]. Recently, new automated approaches to chromatographic screening have accelerated method development across multiple separation modes such as IEX, hydrophobic interaction chromatography (HIC), SFC, chiral separations, and multidimensional separations [27-32]. However, the benefits of automated column and mobile phase screening have not been realized for HILIC separations.

In this manuscript, a new automated multicolumn ultra-high pressure HILIC screening was coupled to DAD and CAD for streamlined method development. This workflow combines 12 columns and 6 different mobile phases that can be run overnight without manual intervention. Tangible outcomes from this HILC-DAD-CAD screening workflow include the development of a new analytical assay for multicomponent mixtures of a wide spectrum of polar hydrophilic analytes, including sugars, amino acids, peptides, nucleotides, and vitamins.

2. MATERIALS AND METHODS

2.1. Instrumentation

This work was performed on an Agilent 1290 Infinity II UHPLC (Agilent Technologies, Santa Clara, CA). This UHPLC system was equipped with an autosampler (model G4226A), quaternary pump (model G4204A), thermostated column compartment (model G1316C), column switching valve (model 5067-4273) and valve drives (model G1170A) for column and mobile phase screening. The system is also equipped with Diode Array Detector (DAD) module with 600 nL flow cell (model G7117B) and Corona charged aerosol detector (Veo RS) CAD detector from (Thermo Scientific, Waltham, MA). For the CAD detector, nitrogen was used at 60 psi pressure; 35°C nebulizer temperature, 5 Hz data acquisition rate; and 1 power function. ChemStation C.01.07 SR3 (Agilent Technologies, Santa Clara, CA) was used for data acquisition and instrument control. A Waters SQ Detector 2 quadrupole mass spectrometer and diode array (PDA) detector (Waters Corp., Milford, MA) coupled with a quaternary Ultra-Performance Liquid Chromatography (UPLC) system was used. Electrospray ionization source (ESI) conditions were as follows: polarity, positive and negative; Mass range, 50 to 1000 m/z; Capillary voltage, 3.00 kV; Cone voltage 30 V; Source temperature, 150 °C; Desolvation temperature, 350 °C; Cone gas, 1 L/h; Desolvation gas, 650 L/h.

2.2. Chemicals and reagents

Trifluoroacetic Acid (TFA), formic acid, ammonium acetate, ammonium formate was purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). Methanol was obtained from Fisher (Fair Lawn, NJ). Acetic acid (99.7%) was purchased from Acros (West Chester, PA). Optima grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Hydrochloric acid 37% solution was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water produced from a MilliQ system (model IQ-7000) was used for mobile phase preparations. Mixture of chemicals (**Fig. 1**) amino acids (*i.e.* arginine, lysine, asparagine, histidine, proline, glutamine, glutamic acid, aspartic

acid), nucleotides mixture (*i.e.* adenosine 5' monophosphate, uridine 5' monophosphate, cytidine 5' monophosphate, thymidine 5' monophosphate, adenosine 5' diphosphate, adenosine 5' diphosphate), sugar mixture (*i.e.* glucose, fructose, galactose, n-acetyl glucosamine, n-acetyl galactosamine, fucose, maltose, lactose, sucrose), vitamins mixture (i.e. riboflavin, vitamin B12, pyridoxine, niacin, biotin) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Peptides mixture (*i.e.* leu-enkephalin, met-enkephalin, Val-Tyr-Val, Gly-Tyr, angiotensin II) were purchased from Bachem Americas, (CA, USA). Columns (HILIC-A, HILIC-B, HILIC-N) were purchased from Mac-mod (PA, USA). Atlantis premier BEH Z-HILIC, Acquity premier BEH Amide, and Acquity UPLC BEH HILIC were purchased from Waters (Milford, MA, USA). InfinityLab Poroshell 120 HILIC, Poroshell 120 HILIC-OH5 were purchased from Agilent Technologies (Santa Clara, CA). Raptor Fluorophenyl was obtained from Restek Corporation (Bellefonte, PA, USA). Kinetex HILIC column was purchased from Phenomenex (Torrance, CA, USA). Fortis HILIC diol was obtained from Fortis Technologies Ltd (Cheshire, UK). Syncronis™ HILIC column was purchased from Thermo Scientific™ (Waltham, MA, USA).

2.3. HILIC screening conditions

Twelve HILIC columns were used in this screening study (**Table 1**). Column and mobile phase selector valves enabled automated screening. Different mobile phases were also screened for all the columns operated at the same temperature (**Table 2**).

Mobile phases (A) were composed of mixtures of 95:5 (*v/v*) acetonitrile and 20 mM ammonium formate solution, pH 3 adjusted with formic acid, 95:5 (*v/v*) acetonitrile and 20 mM ammonium acetate solution, pH 6, and pH 9 adjusted with ammonium hydroxide. Mobile phases (B) were composed of mixtures of 50:50 (*v/v*) acetonitrile and 20 mM ammonium formate solution, pH 3 adjusted with formic acid, 50:50 (*v/v*) acetonitrile and 20 mM ammonium acetate solution, pH 6, pH 9 ammonium hydroxide. A generic gradient elution program was applied starting at 100% A

for 1.0 min, followed by a linear gradient elution to 100% B from 1- 6.0 min, and then held at 100% A from 6.0 to 10 min for column equilibration. The flow rate was 0.5 mL/min, and the column temperature was set at 40 °C. Gradient separations were performed with compound mixtures, and the injection volume was 1-5 µL.

2.4. Sample preparation for HILIC analysis

For amino acids analysis, a stock solution at 5.0 mg/mL of each individual compound (arginine, lysine, asparagine, histidine, proline, glutamine, glutamic acid, aspartic acid) was prepared in pure water. A mixture of amino acids was prepared from the individual stock solutions at a concentration of 100 µg/ml in acetonitrile/ water (90:10%, *v/v*) for UHPLC analysis. For nucleotides analysis, a stock solution of each individual nucleotide (adenosine 5' monophosphate, uridine 5' monophosphate, cytidine 5' monophosphate, thymidine 5' monophosphate, adenosine 5' diphosphate, adenosine 5' diphosphate) was prepared at 5 mg/mL in acetonitrile/ water (90:10%, *v/v*) with 0.1% TFA.

A mixture of five nucleotides was prepared from the individual stock solutions at a concentration of 100 mg/ml in acetonitrile/ water (90:10%, *v/v*) with 0.1% TFA for analysis. For sugar analysis, a stock solution of each individual sugar (glucose, fructose, galactose n-acetyl glucosamine, n-acetyl galactosamine, fucose, maltose, lactose, and sucrose) was prepared at 50 mg/mL in pure water. A mixture of nine sugars was prepared from the individual stock solutions at a concentration of 1.0 mg/ml in acetonitrile/ water (90:10% *v/v*).

For vitamins analysis, a stock solution of riboflavin and niacin vitamins was prepared at the concentration of 5.0 mg/ml in pure water, while vitamin b12, pyridoxine, biotin at 5.0 mg/mL was prepared in water with ammonium hydroxide (28%). A mixture of five vitamins was prepared

from the individual stock solutions at a concentration of 100 µg/ml in acetonitrile/ water (90:10% *v/v*). For peptide analysis, a stock solution of each individual peptide (leu-enkephalin, met-enkephalin, Val-Tyr-Val, Gly-Tyr, angiotensin II) was prepared at 5 mg/mL in acetonitrile/ water (90:10% *v/v*) with 0.1% TFA. A mixture of five nucleotides was prepared from the individual stock solutions at a concentration of 150 mg/ml in acetonitrile/ water (90:10% *v/v*) with 0.1% TFA for analysis.

2.5. Forced degradation of ribavirin

A mixture of 1 mL of 30% hydrogen peroxide and 9 mL of ribavirin solution (1.0 mg/mL in water), was prepared and heated to 80 °C for 90 minutes. Aliquots were collected and diluted with acetonitrile before analysis.

3. RESULTS AND DISCUSSION

3.1 UHP-HILIC-DAD-CAD screening platform

Since its introduction, HILIC has been associated with lengthy method development, especially when compared to RPLC. Due to its unique and complex retention mechanism, coupled with the proliferation of different HILIC column chemistries, the initial stages of any HILIC method development are often seen as particularly challenging. In this work, we propose an automated HILIC screening platform to enable a facile selection of stationary and mobile phases (**Fig. 2**). The setup described in **Figure 2** allows a comprehensive coverage of different and orthogonal choices of column chemistries, mobile phase buffers and pH values. The screening system heavily exploits the use of multicolumn and multi solvent valves to install, onto the same system, a choice of three mobile phases and up to twelve columns. These selection valves also enable a high degree of automation, which in turn limits the manual intervention from analysts. The advantage of this

approach is the reduced time needed to obtain full coverage of all conditions (up to 36 combinations of mobile and stationary phases). Furthermore, UHPLC equipment is fundamental to allow the use of the latest generation of HILIC columns, which all exploit sub-2.0 μm fully porous silica particles (FPP) and sub-3.0 μm superficially porous silica particles (SPP). Recent generation HILIC columns are becoming progressively popular thanks to their higher efficiency, shorter run times and overall good reproducibility, all characteristics which are of fundamental importance in the current drug discovery and development landscape to tackle increasingly complex chemical entities.

To test the powerfulness of the above-mentioned UHP-HILIC screening system, a selection of 33 biomolecules, categorized into five different classes (amino acids, peptides, nucleotides, sugars, and vitamins mixtures) were used (**Table S1**). The analytes were chosen to cover a wide spectrum of polarity and chemical properties (**Table S3**), as well as to represent the different categories of molecules currently under development and study in pharmaceutical R&D.

As previously mentioned, due to the increasing number of HILIC stationary phases currently available on the market, the column selection represents a key step in the initial HILIC method development. The HILIC columns employed in this screening system ensure a vast coverage of the most conventional (bare and hybrid silica, amide and amino, diol), as well as less common column chemistries (*e.g.*, Fluorophenyl, zwitterionic columns) (**Table 1**).

Another important consideration in HILIC separation is the mobile phase buffer and pH selection. Thanks to this setup's ability to simultaneously install a high number of mobile phases, we can quickly cover a wide range of pH and buffer concentration strengths (**Table 2**). The chosen buffers, namely ammonium formate, ammonium acetate and their concentration provide high buffer capacity, maximize the selectivity differences for the different mixtures herein tested, high solubility in acetonitrile and high compatibility with additional detectors, such as CAD and MS.

The samples were screened using generic gradient conditions, capable to ensure: i) retention and ii) elution of all compounds used in the test mixtures. The system was also equipped with multiple detectors, such as DAD and CAD; this choice was to guarantee the detection of UV visible compounds and chemical species (e.g., sugars), which are invisible to DAD.

Finally, particular care was put into a crucial step of any HILIC analysis, the column equilibration time. Due to its unique retention mechanism, it is vital to allow enough time for an optimal equilibration of any HILIC column to ensure the best reproducibility of any HILIC separation. In this work, a four-minute equilibration time was found to be enough for stable and reproducible HILIC separation across all columns present on the UHP-HILIC screening platform (**Table S2**).

3.2 HILIC screening of polar analyte mixtures

After establishing the screening system, the five mixtures of amino acids, nucleotides, peptides, vitamins, and sugars were tested. A scorecard was built to summarize and visualize the screening results for all combinations of columns and mobile phases per mixture (**Fig. 3**). In the scorecard, green-colored cells represent combinations with baseline separation of all mixture components (value = 1), while the progressive change from green to red depicts all scenarios with incomplete ($0 < \text{values} < 1$) or no separation (value = 0). Cells with a star mark indicate the best combination of mobile phase and column chemistry for each mixture, delivering a baseline separation.

Amino acids, in particular those showing high polarity as well as acid/base properties (e.g., aspartic acid, lysine, *etc*.), can be difficult to analyze with RPLC, especially if an MS and CAD-friendly method is required. Indeed, non-volatile ion-pairing agents (e.g., phosphoric acid, sodium perchlorate, pentafluoropropionic acid, triethylamine, and heptafluorobutyric acid) are required to obtain good performance under RPLC conditions with highly polar analytes. The advantage of HILIC lies exactly in its ability to overcome these shortcomings. For amino acids, the screening

stage highlighted the combination of an acidic mobile phase (pH = 3) and a zwitterionic column (BEH Z-HILIC) as the best condition for the separation of polar neutral and ionized amino acids (**Fig. 3** and **4e**). This could be attributed to the peculiar nature of zwitterionic columns, as they incorporate moieties with negative and positive charge (1:1 molar ratio), yielding a net zero surface charge. This provides more flexible and wider HILIC separations for a variety of amino acids, with additional interaction such as ionic or ion-exchange retention mechanisms to provide further complementarity in the overall separation profile compared to bare silica columns. In this example (**Fig. 3** and **4e**), at pH = 3, the retention order is as follows: amino acids with overall no net charge (proline, glutamine, asparagine, glutamic and aspartic acid), followed by those with a net positive charge (histidine, arginine, lysine). The zwitterionic selector used on BEH Z-HILIC (sulfoalkylbetaine) presents a permanent negative charge ($-SO_3^-$) placed on the outside extreme of the selector, and a permanent positive charge ($-NR_4^+$) located more internally, embedded in the selector itself. Zwitterionic columns may exhibit localized ion-exchange properties, which can help explain the elution order seen for amino acids. Basic amino acids, which show a net positive charge (**Table S3**) at pH = 3, will be more retained by the stationary phase as they may interact with the negative charge placed at the end of the sulfoalkylbetaine selector following an ion-exchange mechanism in addition to the hydrophilic partitioning already in place. Among the amino acids with no overall net charge, those traditionally classified as polar neutral (proline, glutamine and asparagine) elute earlier than those categorized as acidic (glutamic and aspartic acid). This might be due to their stronger polar character, increasing the hydrophilic partitioning.

For peptides, the scorecard shows that good separation can be achieved on a greater number of columns at pH = 3 and, to a lower extent at pH = 6 (**Fig. 3**), while shifting to more basic conditions (pH = 9) causes a progressive loss in separation performance across all columns usable at this pH

value on the screening setup (column 1-3, **Table 1**). This trend is correlated to the overall acidic nature of the peptides present in the mixture (**Table S3**), as they show an increased net negative charge since the mobile phase pH is higher than the average pI of the peptides present in this mixture [33]. At high pH, a lower number of HILIC stationary phases will be capable of ensuring good separation of the five peptides as they will be progressively more ionized, with the only column still generating sufficient resolution (although without baseline separation) being the BEH Z-HILIC, thanks to its additional ability to generate separation based on ionic interactions. The best conditions for peptide separation were found on a bare silica column at acidic pH (**Fig. 3** and **4b**).

For nucleotides mixture, nucleosides with different nucleobases and same number of phosphate groups were selected (adenosine 5' monophosphate, uridine 5' monophosphate, cytidine 5' monophosphate, thymidine 5' monophosphate), as well as nucleotides with same nucleoside but different phosphorylation degree (adenosine 5' diphosphate, adenosine 5' triphosphate), to increase the complexity of this mixture (**Fig. 4**). In general, the polyhydroxy fructan and polyhydroxy columns (Poroshell 120 HILIC-OH5, and HILIC-N) showed the highest selectivity. The best separation was achieved on a polyhydroxy fructan column (Poroshell 120 HILIC-OH5) at acidic pH (**Fig. 4a**), showing good results for different degree of phosphorylation (elution order: adenosine monophosphate < adenosine diphosphate < adenosine triphosphate), as well as for nucleotides with same number of phosphate groups in their structures.

Sugars are challenging to analyze by RPLC due to their high polarity and need for volatile mobile phases to enable the use of alternative detectors such as CAD and MS. In addition, many mono- and di-saccharides are also isomers, thus complicating even further the chromatographic separation of these closely related structures (**Fig. 1** and **Table S3**). This complication with sugar separation can also be seen under HILIC conditions (Fig. 3). From the scorecard, it can be seen

that, unlike the previous mixtures of peptides and nucleotides, only a handful set of experimental conditions delivered acceptable performance. Nonetheless, the HILIC screening setup was able to quickly identify ideal conditions for the baseline separation of this challenging mixture, highlighting once more the extended coverage of separation conditions that this screening setup can successfully deliver. In this example, the BEH Z-HILIC column delivered satisfactory results at all pH values here considered, with basic conditions (pH 9) yielding the best separation overall of sugars (**Fig. 4d**). Once again, this zwitterionic column seems to provide additional resolution power thanks to its ability to generate ionic interactions with samples. In the example of sugars, this proved fundamental to separate isomeric mono- and di-saccharides (fructose – glucose – galactose; sucrose – maltose – lactose). However, further work may be needed to support this statement.

Finally, water-soluble vitamins including vitamin B2 (riboflavin), B3 (niacin), B6 (pyridoxine), B7 (biotin), and B12 (cyanocobalamin), were also screened. These compounds are relatively polar due to the presence of different functional groups that can be ionizable. Therefore, vitamins can exist as anions, cations, or zwitterions in the range of pH considered here (pH = 3–9). Despite the great complexity of the sample's nature, the HILIC screening system rapidly identified several combinations yielding overall good separation **(Fig. 3**). In general, vitamins showed weak retention behavior at acidic conditions (data not shown) despite different columns being able to generate acceptable resolution at pH = 3. The best results were found on the Acquity BEH amide column at pH 6 and pH 9 (**Fig. 4c**) for the vitamins mixture.

3.3. HILIC application in pharmaceutical reaction analysis

3.3.1 HILIC-CAD for the separation of diastereomeric building blocks from enzymatic catalysis

After having discussed the fundamental aspects of the UHP-HILIC screening platform, a real-life case was tested to verify its usefulness. To do so, an example from a reaction mixture exploiting enzymes to deliver products with a high stereoselectivity is given in **Fig. 5a**. In drug development, biocatalysis is utilized for highly stereoselective reactions to produce intermediates for macromolecule synthesis in the pharmaceutical industry. In the example described here, a method capable of delivering the separation of mesaconic acid (starting material) from the two diastereomeric product intermediates (2S,3S-methyl aspartic acid and 2S,3R-methyl aspartic acid) used in the synthesis of a pipeline macrocyclic peptide was needed. RPLC conditions were first tested (**Fig. 5c**) using CAD as a detector due to the poor UV signal of the species present in this mixture. Because of the high polarity of the analytes in this reaction mixture, RPLC-CAD failed to deliver retention and, more importantly, the separation between all components (**Fig. 5c**). Indeed, RPLC can deliver sub-optimal results when volatile mobile phases are needed for good compatibility with detectors such as CAD. In this context, exploiting the orthogonality of HILIC can be extremely advantageous.

Purified standard materials for the targeted diastereomers were injected and screened on the HILIC systems (**Figure S1**). The system was able to identify the best conditions to separate the isomer mixture and the reaction products from the starting material (mesaconic acid). The combination of a BEH HILIC column with acetonitrile /20 mM ammonium formate at pH 3 provided the best selectivity while ensuring retention of all analytes (**Fig. 5b**). This successful HILIC application for diastereomers separation enabled the use of an analytical method to allow the selection of enzyme

variants that can produce the desired methyl aspartic acid (2S, 3R) product instead of the native methyl aspartic acid (2S, 3S) product.

3.3.2 HILIC-MS for forced degradation studies on drug substance

Another pharmaceutical application where HILIC can provide a key advantage and deliver improved results over the more established RPLC is forced degradation studies (FDS) for candidate drug substances. FDS is crucial to determine the intrinsic stability of active pharmaceutical ingredients (APIs), whether as is (drug substance) or in a complete formulation (drug product). FDS are also used to understand the degradation pathway(s) of APIs, knowledge that is essential during the entire drug development process. RPLC-UV represents the established state-of-the-art chromatographic methods used in FDS analyses. In recent years, there has been a marked increase in highly polar and ionizable small molecule APIs on the market, acting against a multitude of diseases. These highly polar small APIs can be problematic to analyze with RPLC, especially if volatile conditions are needed to enable hyphenation to MS for tentative structure elucidation of the discovered degradation products.

In this study, ribavirin, a commercially available antiviral utilized against hepatitis C as well as RSV infections, was exposed to oxidation stress conditions and, subsequently, analyzed via RPLC-MS (**Fig. 6b**). With RPLC-MS, ribavirin and its degradation products were not retained by the column, thus they eluted within the column void volume. This is also confirmed by the *m/z* values found for the single peak present in the RPLC chromatogram (**Fig. 6b**). Therefore, HILIC was considered a valid alternative to develop a method capable of retaining, identifying, and separating ribavirin and its main degradation species. The HILIC screening platform quickly allows us to find out the best conditions, with a zwitterionic column (BEH Z-HILIC) at pH 3, delivering the best results. Thanks to the intrinsic volatile nature of the HILIC mobile phases used by the HILIC screening setup, the method was immediately ready to be used on a UHPLC-MS system for the

identification of all peaks (**Fig. 6a**). The developed HILIC method successfully separates the API ($[M+H]^+$ = *m/z* 245.1) from all oxidation degradation impurities generated during the stress procedure, while simultaneously collecting vital data for initial structure elucidation of the degradation species thanks to the optimal compatibility of the HILIC method with MS detection.

CONCLUSION

Developing reliable HILIC assays can often be challenging, requiring systematic scouting of multiple columns and mobile phase combinations. To overcome this tedious task, the strategy outlined in this work relies on the introduction of an automated multicolumn HILIC screening comprising 12 state-of-the-art columns and six mobile phases with different additives and pH values. The availability of both DAD and CAD allowed the detection of all analytes tested including molecules lacking UV chromophore. As illustrated herein, this approach enables efficient and fast development and deployment of new assays for the analysis of different (bio)pharmaceutical modalities, including amino acids, sugars, nucleotides, peptides, and vitamins using convenient, readily available LC technology that can be leveraged across different stages of pharmaceutical development.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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LIST OF TABLES

Table 1. UHPLC-HILIC columns used in this study.

	Column	Dimension (I.D.) (mm) x L (mm)	Particle size (µm)	Phase	pH range
1	Acquity UPLC BEH	2.1 x 50	1.7	HILIC	1.0 - 9.0
2	Acquity Premier BEH	2.1 x 50	1.7	Amide	2.0 -11.0
3	Atlantis Premier BEH Z-HILIC	2.1 x 50	1.7	Sulfobetaine	2.0 - 10.0
4	Kinetex HILIC	2.1 x 50	1.7	Unbonded silica	2.0 - 7.5
5	Fortis HILIC DIOL	2.1 x 50	1.7	Diol	2.0 - 8.0
6	Poroshell 120 HILIC-OH5	2.1 x 50	2.7	Polyhydroxy fructan	1.0 - 7.0
7	Infinity Poroshell 120 HILIC	2.1 x 50	1.9	Bare silica	0.0 - 8.0
8	Raptor Fluorophenyl	2.1 x 50	1.8	Fluorophenyl	2.0 - 8.0
9	Syncronis™ HILIC	2.1 x 50	1.7	Zwitterionic	2.0 - 8.0
10	HILIC-A	2.1 x 50	1.7	Silica	2.0 - 7.0
11	HILIC-B	2.1 x 50	1.7	Aminopropyl	2.0 - 7.0
12	HILIC-N	2.1 x 50	1.7	Polyhydroxy	2.0 - 7.0

Table 2. Mobile phases used in this study

	Mobile phases	pH
A1	20 mM ammonium formate in 95:5% (*v/v*) ACN: H_2O	3.0
A2	20 mM ammonium acetate in 95:5% (*v/v*) ACN: H_2O	6.0
A3	20 mM ammonium acetate in 95:5% (*v/v*) ACN: H_2O with 0.04% NH_4OH	9.0
B1	20 mM ammonium formate in 50:50% (*v/v*) ACN: H_2O	3.0
B2	20 mM ammonium acetate in 50:50% (*v/v*) ACN: H_2O	6.0
B3	20 mM ammonium acetate in 50:50% (*v/v*) ACN: H_2O with 0.04% NH_4OH	9.0

FIGURES CAPTIONS

Fig. 1. Structures of thirty-three compound families, including nucleotides, amino acids, sugars, vitamins, and peptide mixtures that were used in this study.

Fig. 2. UHP-HILIC-DAD-CAD screening system with twelve different columns and six mobile phases with different additives.

Fig. 3. Scorecard showing the combination of columns and mobile phases screening for all the mixtures. Star symbols represent the most promising conditions for further chromatographic optimization.

Fig. 4. Representative chromatograms from HILIC screening of different mixtures: (a) sugars were separated on Atlantis premier BEH Z-HILIC column at pH 3; (b) vitamins were separated on Acquity Premier BEH amide at pH 6; (c) nucleotides were separated on Poroshell 120 HILIC-OH5 column at pH 3; (d) peptides were separated on infinity poroshell 120 HILIC column at pH 3; (e) amino acids were separated on Atlantis premier BEH Z-HILIC column at pH 9.

Fig. 5. Application of HILIC vs. RPLC to separate polar starting materials and products in a biocatalytic reaction. (a) Schematic of the biocatalytic reaction converting mesaconic acid to 2S,3R methyl aspartic acid, and 2S,3S methyl aspartic acid. (b) HILIC separation, eluent (A) 95% acetonitrile /20 mM ammonium formate, eluent (B) 50% acetonitrile / 20 mM ammonium formate, isocratic: 0 – 6 min at 100 % A, Acquity UPLC BEH HILIC (2.1× 50 mm I.D., 1.7 μm) column, flow rate was 0.5 mL/ min, and column temperature: 40 °C. (c) RPLC separation, eluent (A) 20 mM ammonium formate, eluent (B) 90% acetonitrile / 20 mM ammonium formate (pH 3.5),

gradient: 0 – 5 min 5- 95 % B, Acquity BEH C18 (2.1× 50 mm I.D., 1.7 µm) column, flow rate was 0.5 mL/min, and column temperature: 40 °C.

Fig. 6. Application of HILIC vs. RPLC in forced degradation studies (oxidation) of Ribavirin to separate its degradation products. (a) HILIC separation, eluent (A) 95% acetonitrile /20 mM ammonium formate, eluent (B) 50% acetonitrile / 20 mM ammonium formate, isocratic: 0 – 1 min at 100 % A, B 100 at 6.0 min, Atlantis Premier BEH Z-HILIC (2.1× 50 mm I.D., 1.7 µm) column, flow rate was 0.5 mL/ min, and column temperature: 40 °C. (b) RPLC separation, eluent (A) 0.1% FA, eluent (B) acetonitrile, gradient: 0 – 0.5 min 5% B, 0.5 – 10 min 5- 95 % B, Acquity BEH C18 (2.1× 50 mm I.D., 1.7 µm) column, flow rate was 0.5 mL/min, and the column temperature: 40 °C.

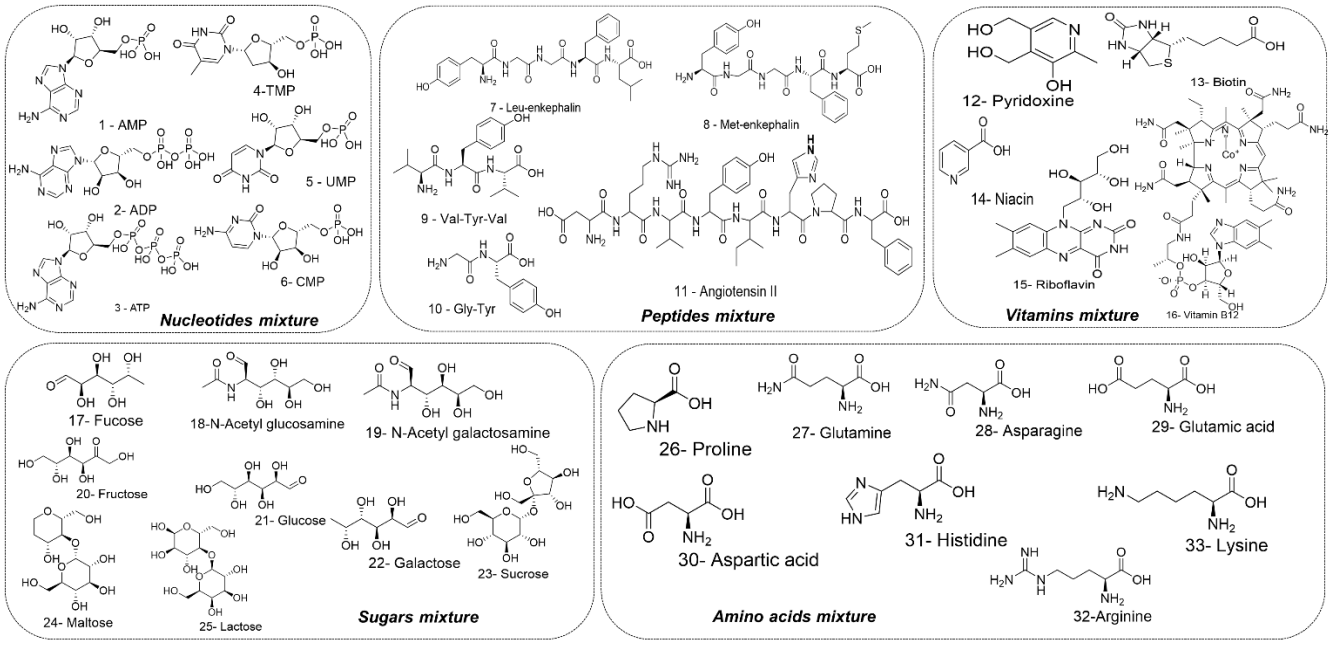

Figure 1

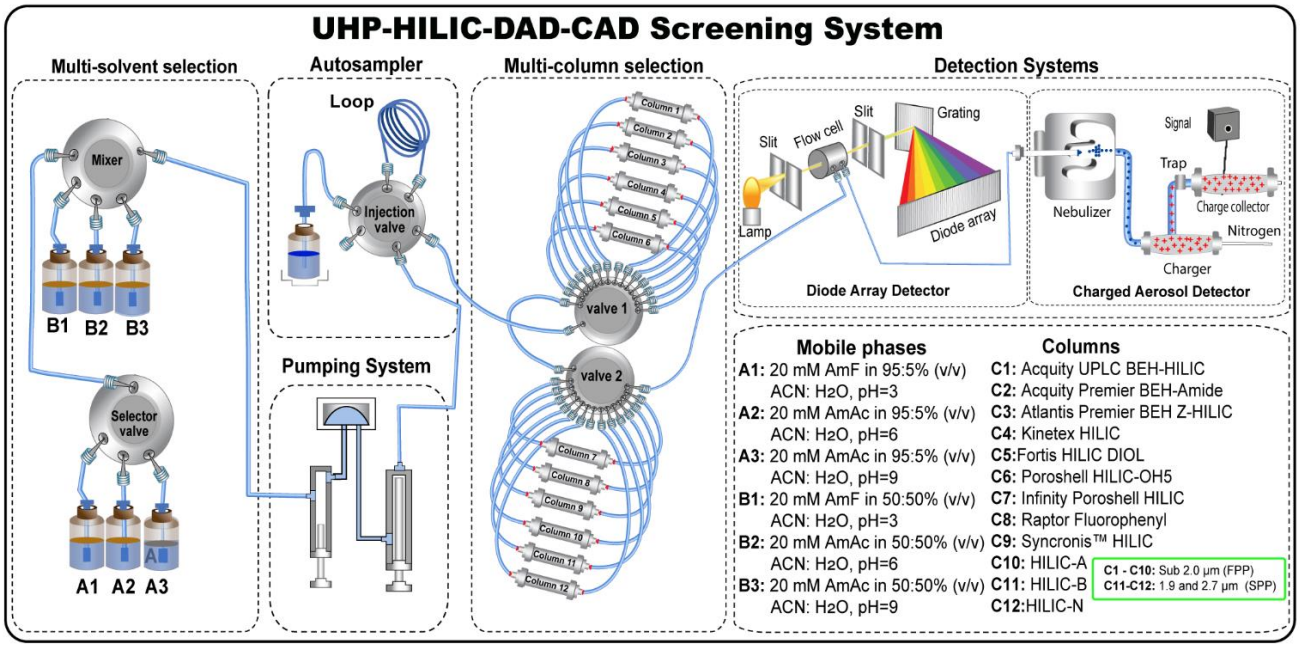

Figure 2

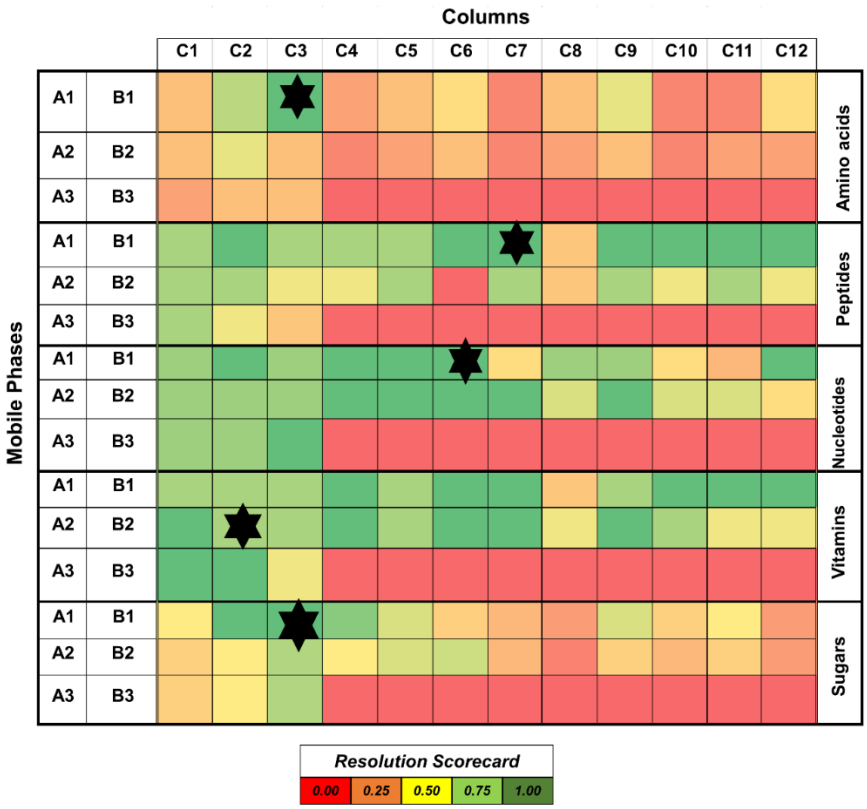

Figure 3

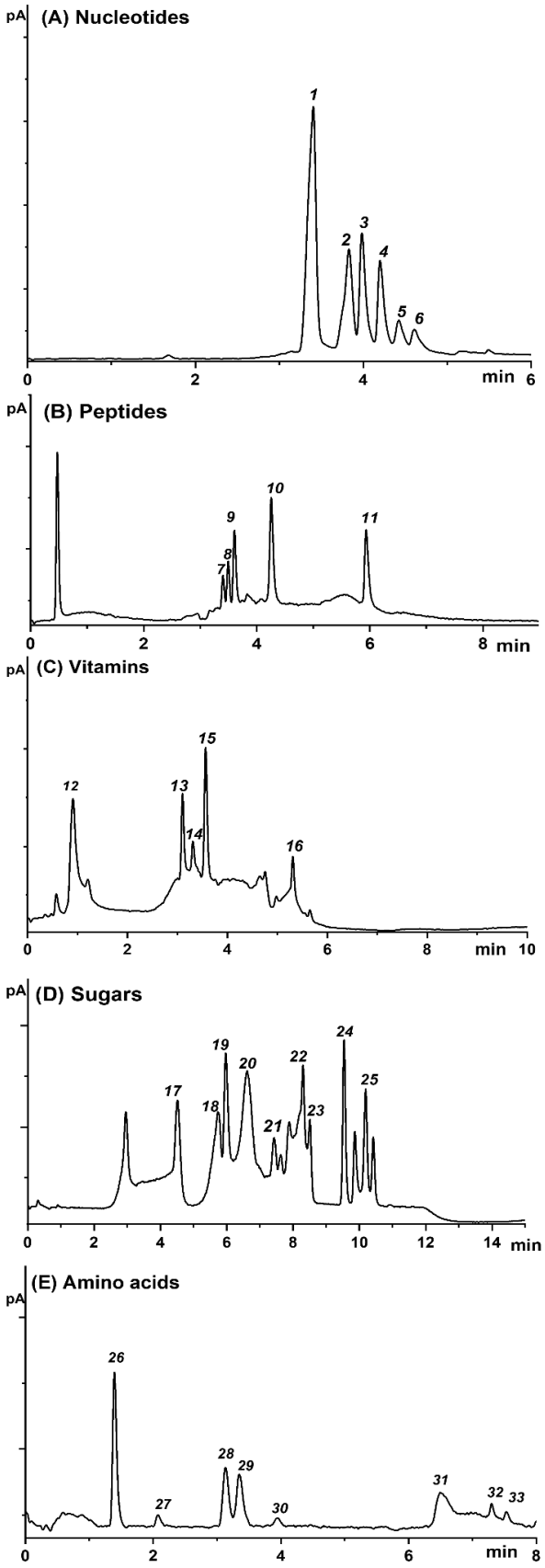

Figure 4

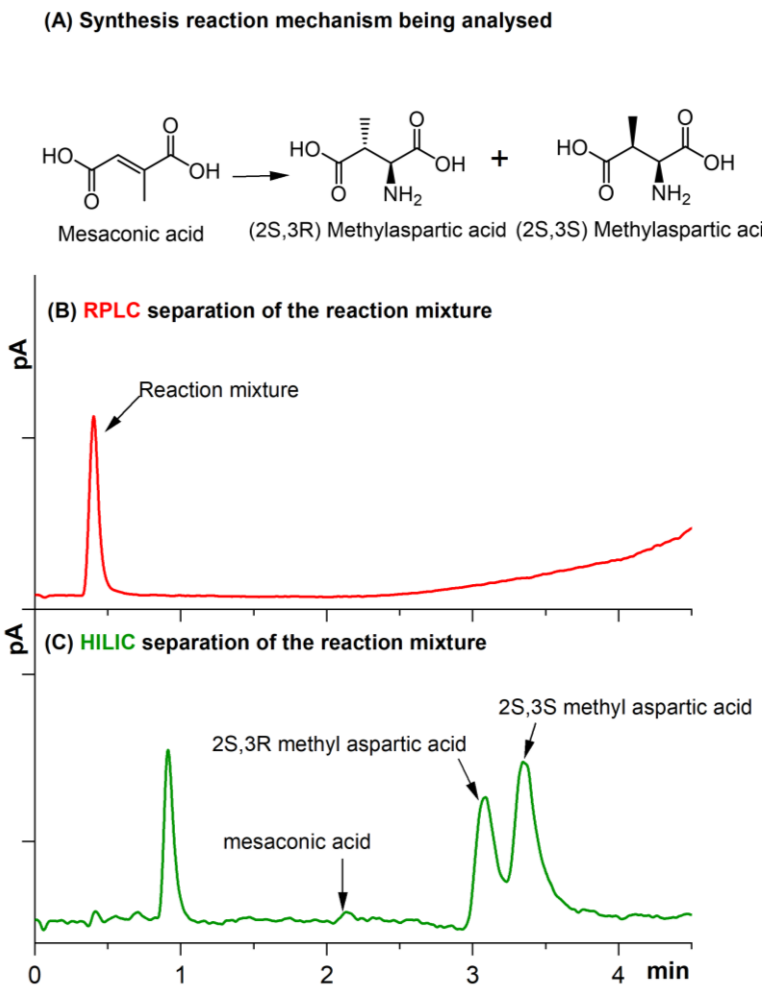

Figure 5

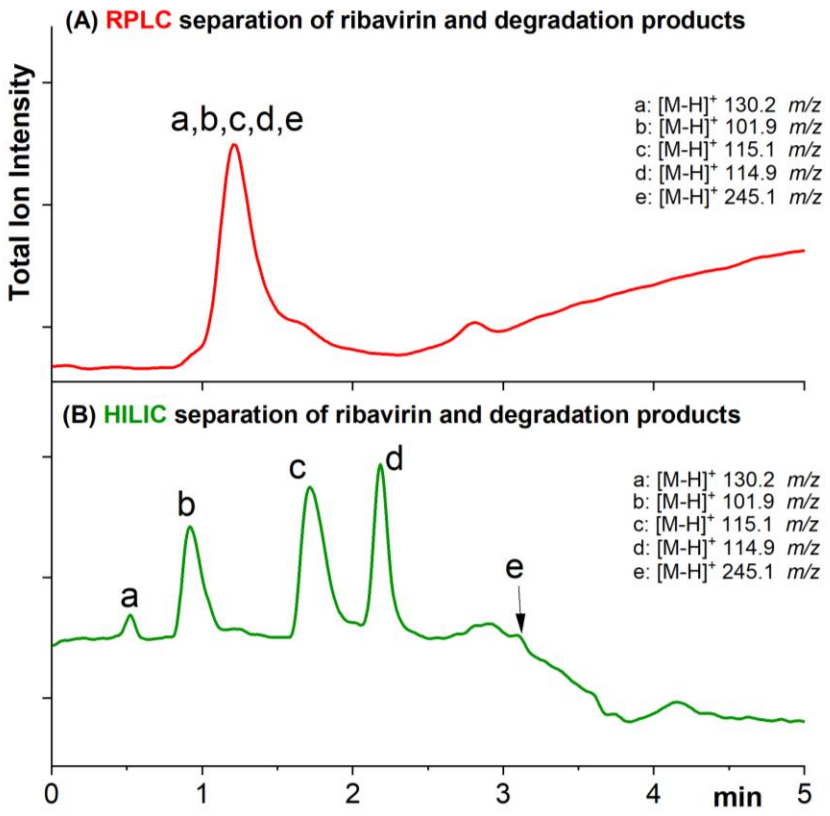

Figure 6