LC-MS METABOLOMICS OF POLAR COMPOUNDS

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ABSTRACT

The metabolome is the complete set of small molecules coming from protein activity (anabolism and catabolism) in living systems. They have a broad range of chemical structures and physico-chemical properties and therefore different analytical methodologies are necessary. Highly polar metabolites, such as sugars and most amino acids are not retained by conventional reversed phase liquid (RP-LC) chromatography columns. Without sufficient retention, coelution may result in identification problems while the detection of compounds by mass spectrometry at low concentrations may also be problematic due to ion suppression. In order to retain compounds based on their hydrophilicity, polar stationary phases and hydrophilic interaction liquid chromatography (HILIC) provide a complementary tool to RP-LC for untargeted comprehensive metabolite fingerprinting. However, robustness of the methods is still limiting their applications. This review focuses on sample pre-treatment, stationary phases, analytical methods and applications for polar compound analysis in biological matrices.

INTRODUCTION

Metabolites are a record of chemical processes that have already happened in a biological system. This makes them better biomarkers than entities further upstream in the molecular biology of metabolism. Moreover, their concentration can control and modify the activity of proteins and genes and for those reasons metabolite analysis has growing interests in its different formats.

Classical bioanalysis is “targeted analysis”, that means that there is the previous knowledge of what a specific or group of specific compounds mean for the situation under study and their nature and, therefore, specific analytical techniques can be applied for their measurement. However, frequently markers of a situation are unknown and, looking for them requires a “non-targeted analysis”. Even more, unknown perturbed networks can be found with this methodology leading to new diagnosis, therapy or prevention. As an example, glucose is a well-known marker of diabetes that can be controlled with insulin, but pathophysiological changes leading to a higher risk of cardiovascular disease in those individuals are still unknown and could be found with non-targeted-analysis.

Regarding metabolites analysis, one main analytical difference lies in what is known about the parameters being measured. O. Fiehn [1] proposed a classification of analytical methods of metabolites according to these concepts:

1) Metabolite target analysis. That is the classical analysis looking for one or a few single compounds. Sophisticated methods for extractions, sample preparation, sample clean-ups and internal references may be used in this area, rendering target analysis

much more precise than other methods. This strategy is mainly used for analyses that need extreme sensitivity and accurate quantification. Applications are found widely in literature of both biology and analytical chemistry, and it would be out of scope to try to cover them appropriately.

2) Metabolic profiling. Increasing the complexity, for investigations of selected biochemical pathways the analytical procedure can be focused on a small number of pre-defined metabolites. Sample preparation and data acquisition can be focused on the chemical properties of these compounds with the chance to reduce matrix effects. For example, these pre-defined metabolites can be chosen based upon a class of compounds (such as amino acids, organic phosphates, fatty acids or carbohydrates), or based upon their association with a specific pathway. It is clear that sample preparation and quantification must undergo compromises if many different individual compounds are analysed simultaneously. For example, in metabolite profiling often, only crude sample fractionation and clean-up steps are carried out. Correspondingly, metabolite profiling is usually accompanied by rather focused biological questions and deep background knowledge, trying to cover all relevant compounds with authentic standards.

3) Metabolic fingerprinting. That is a rapid, high throughput global analysis developing signal patterns that, after applying chemometric tools, lead to a classification of samples according to their origin or their biological status. Signals obtained provide a kind of barcode to samples, that even without knowing their individual meaning permit their classification. Sometimes, metabolic fingerprints from different samples show differences capable to distinguish between individual signals that can then be

related to sample classification. It can be clearly distinguished from metabolite profiling because in any experiment it is tried to take all detectable peaks into account, including unknowns. However, it cannot be assumed that such techniques lead to the identification of the most important effects, because in many cases unknowns are also revealed to be statistically different in the biological situations under study.

4) Metabolomics. The small molecule inventory (SMI) or Metabolome is a pattern of molecules that reflects the cell's status. It is the totality of metabolic processes including anabolism and catabolism, as well as all the related cellular processes such as absorption, distribution, and detoxification of natural and xenobiotic materials, energy utilization, signal transduction, and regulation. Metabolome represents the current status of the cell or tissue.

Metabolomics has the ultimate goal of unbiased identification and quantification of all the metabolites present in a certain biological sample which was obtained from accurately defined experimental conditions. Since such an analysis reveals the metabolome of the biological system under study, this approach is called metabolomics, in analogy to genomics or proteomics. Both sample preparation and data acquisition must aim at including all classes of compounds, while at the same time assuring high recovery, and experimental robustness and reproducibility. The first requirement is to have available techniques that are as comprehensive as possible for metabolic analyses. As the chemistry of different metabolites is very heterogeneous, isolating and measuring them all together ('true metabolomics') is very hard, and most metabolic studies are really either "metabolic profiling" or "metabolic fingerprinting" of subsets of chemical classes.

Although in 2008 it seemed necessary to publish an illustrative review about the utility of separation science in metabolomic research [2], it is nowadays evident that chromatography plays a major role in such research [3]. Furthermore, liquid chromatography (LC) in reverse phase (RP) mode coupled to mass spectrometry (MS) could be considered as the "second gold-standard" for metabolomics, providing alternative, complementary information to NMR. The information derived from RPLC-MS analysis is probably less robust but much more sensitive and, therefore, informative.

There are several reasons for this choice: The metabolites that can be determined cover a broad range in molecular weights, polarities and physic-chemical properties. Moreover, the analytical conditions can be easily modified in order to achieve a more targeted analysis (profiling) or covering the broadest range of properties of the molecules i.e., types of metabolites (fingerprinting).

Moreover, RP-LC [4], is the technique of choice for many researchers in metabolomics. Stationary phase particle sizes above 2 µm (HPLC) are common. However, with the development of instrumentation capable of working at backpressures above 400 bar, stationary phase particles below 2 µm (ultra performance liquid chromatography - UPLC or ultra high performance liquid chromatography - UHPLC) have been introduced. This has resulted in large improvements in terms of analysis time (throughput), peak capacity and sensitivity.

The possibility of being coupled to a very powerful technique of structural elucidation such as MS is also a great advantage of this choice, and the mobile phases typically employed in RP-LC-MS (methanol or acetonitrile mixed with water plus volatile

additives such as formic or acetic acids or ammonium) are compatible with the detection. In addition, the sensitivity of the detection is also recognized as one of the powers of MS, although this property is strongly dependent of the type of detector employed, and the application of LC-MS to metabolomics is a matter for other reviews [5,6].

The type of information provided is without doubt a very important reason to make RP-LC-MS a technique with an increasing role in the field of metabolomics, however other technical issues are important as well: the high reliability and exchangeability of different instrumentation manufacturers; the robustness of the analytical conditions (from the chromatographic column to the mobile phases), and the reproducibility within the same brand (batch-to-batch) and between equivalent products from different suppliers.

Nevertheless, even though the advantages of RP-LC are readily accepted, the analysis of a large number of very important molecules in terms of metabolic information are a challenge: these include highly polar molecules (simple saccharides, amino acids, aminated bases and nucleic acids, short-chain organic acids, phosphated molecules, etc.), poorly retained in the typical RP stationary phases, and analytical strategies developed to overcome such problem (e.g. ionic-pairing) can be incompatible with MS.

Taking this in mind, it becomes evident that a type of liquid chromatography that can make use of all the advantages of instrumentation but can efficiently retain and separate highly polar compounds (hydrophilic) is of great interest for studies about metabolism, both for profiling and for fingerprinting. On the other hand, as will be mentioned afterwards, hydrophilic interaction liquid chromatography (HILIC) is able to

separate polar compounds with volatile buffers and high content of organic solvents in the mobile phase, which is optimal for desolvation. Therefore, the sensitivity of mass spectrometers can be theoretically increased when using desolvation in the ionization process, i.e. electrospray ionization (ESI).

Although the interest in HILIC is not new (in a very comprehensive 2008 review, papers from 1960's and 70´s were mentioned) [7], it is widely accepted that the take-off of such methodology was in 1990, with the successful coining of the term HILIC by Andrew Alpert [8] and the first attempts to establish the physicochemical mechanisms and interactions between analytes and mobile/stationary phases. Since then, HILIC has become a topic of increasing interest in the scientific literature, and papers describing new phases and applications have deserved the publications of several reviews [7,9-15], two special issues of the J. Sep. Sci. in 2008 and 2010 and one in the J. Chromatogr. in 2011.

One of the reasons for the relatively large number of general reviews in so short period of time is that under the term Hydrophilic very different types of phases and interactions can be included, and the results can be very different when applying the same conditions to different HILIC phases. In recent years new terms such as ERLIC (electrostatic-repulsion liquid chromatography)[16] or ANP (aqueous normal phase) [17] try to classify with more detail the types of chromatography taking into account the types of interactions. There are also other stationary phases and analytes that under specific conditions exhibit behaviors similar to those of HILIC even though the phase was not primarily designed for that separation.

HILIC mechanism involves different types of interactions: partition, ionic exchange, dipole-dipole interactions. It improves retention of highly polar analytes with mobile phases similar in composition to those of reversed-phase, although different proportions. HILIC conditions take place when a pseudostationary phase is formed with the support, the bonded phase and a water layer adsorbed onto this surface. This water must come from the mobile phase, and therefore mobile phases must contain a minimum amount of water, but the mobile phase must be mainly organic, with lower content of hydroxyl groups (90% acetonitrile, for example). High levels of organic contents in the mobile phase make polar compounds more highly retained in the water-rich liquid layer, whereas higher salt concentration would drive more solvated salt ions into the water-rich liquid layer, competing with analytes.

In fig. 1 a graphic summary of the interactions that can occur in HILIC is depicted. Essentially, they are the same mechanisms that govern separation in RP-LC, but with very different relevance, because the hydrophobic interactions are, in quantitative terms, the less important for the retention.

This complexity of mechanisms and the relatively low range of mobile phases to be used can be considered one of the drawbacks of the technique. Moreover, Ruta et al. [18] mentioned as drawbacks, too: high consumption of expensive acetonitrile, long equilibration times, peak shapes distorted, and lack of solubility of some compounds in high proportion of organic solvent.

In order to understand better the mechanisms that govern the separation, recent radar plots similar to those introduced used for RP-LC stationary phases [19] have been used to describe and compare 15 different HILIC stationary phases, in terms of

hydrophilicity, charge effects, structural selectivity and separation efficiency with twelve compounds as probes for quantitative estimation of the different mechanisms that may affect the chromatographic performance. In addition, a group of 21 compounds has been also tested, as probe pairs to test the interaction mode in HILIC [20]. In addition, two sulfobetaine-based zwitterionic columns have been evaluated with 76 model compounds and a generally applicable retention model was established [21]

The interest in such methodology and metabolomics sprang easily, and applications of HILIC to metabolomics have been specifically reviewed previously [22-24]. HILIC is still claimed as an attractive technique [24], but even though the possibilities look broad, the fact is that it is not used that far, and less than 60 papers have been published with the terms HILIC and metabo(l/n)omics in the title, the abstract or keywords, from 2002 to date.

It could be thought that HILIC should have gained users and applications in the metabolomics field, as the knowledge about such methodology increased, together with the increasing number of papers that described metabolomic applications with liquid chromatography. However, in 2010-2011, the number of papers about HILIC for metabolomics could be estimated in approximately 5% of the papers that described metabolomic approaches with liquid chromatography, and as the number of papers that describe LC and metabolomic applications is still increasing, the use of HILIC for that application is not.

Taking into account the advantages of RP-LC mentioned above, the reasons why HILIC has not been used so far, being theoretically so interesting for metabolomics and an

interest-growing technique, could be related to some real drawbacks, or to the lack of knowledge about the expected results that can be achieved, because HILIC can be a very diffuse term.

One of the most cited papers about HILIC from D. McCalley in 2007 wondered if HILIC with silica columns was a viable alternative to reversed-phase [25]. Maybe it is time to wonder if such an attractive methodology can really provide with the potential information, with the strict needs of metabolomics in terms of robustness, reproducibility and quality of the results.

The present review is not intended to solve questions regarding the real mechanisms that govern the separation in HILIC or other types of hydrophilic conditions, but to summarize the separations that have been developed under some common conditions: The main goal of the separation was the analysis (metabolic profiling or metabolomic fingerprinting) of highly polar compounds (out of reversed-phase possibilities) by means of LC-MS. In order to achieve that, commercially available stationary phases were used, whether sold as HILIC or not. The columns cited herein, together with some comments about the samples and/or metabolites studied, are summarized in table 1.

Sample pre-treatment

The first point arising from HILIC was that apparently sample matrix had a higher impact than in RP-LC and it should be less eluotropic than the mobile phase, therefore predominantly organic. This fact opposes the nature of compounds to be analyzed, mainly polar, which often are not completely soluble in a non-polar solvents. In that sense, urine samples are usually diluted with acetonitrile in different proportions [26-

30]. In some cases urine samples are lyophilized and reconstructed [31] with mixtures with different proportions as ACN/MeOH/formic acid (650:350:2, v/v/v).

Nevertheless, strategies for comprehensive analysis [32] do not consider that point and use solid phase extraction (SPE) and two complementary chromatographic methods with the aim of increasing the number of metabolites to be detected. The more polar fraction, i.e., the wash fraction from SPE, can be analyzed using the ZIC®-HILIC column, while the fraction eluted with an organic solvent, the less polar compounds, was analyzed using reversed phase technique. However, the efficiency obtained in HILIC was quite poor. Some authors just diluted urine with water [27] and they describe their method as an effective and repeatable method for urinary metabolite profiling.

Plasma samples need protein precipitation. For L-arginine metabolic pathways Brown et al. [33] selected isopropanol because it generated the greatest signal-to noise ratio and the best results in terms of precision compared with other precipitation reagents (acetonitrile, methanol, mixtures of all 3 solvents, and reagents with $MgSO_4$ added). Meanwhile, global serum profiles of renal cell carcinoma [34] were obtained after protein precipitation with methanol with both RP chromatography and HILIC separations.

It is important to keep in mind that the weakest point in the analytical process will limit the final outcome and, therefore, metabolites which are not totally soluble in the sample matrix will produce random results, independently of chromatographic conditions downstream. Hence, of critical importance are not only the

chromatographic and MS acquisition parameters, but also optimization of those variables that impact the sample treatment as well.

Stationary phases

If the goal is to retain and separate polar compounds, it is clear that that the stationary phase should be polar, and the first stationary phases where HILIC was tested were those used for normal phase (NP), i.e. bare silica, amino and cyano. Phases currently available are more numerous, and they are usually grouped within three categories: neutral, charged and zwitterionic phases. They are commonly bonded to silica supports, and that must be also taken into account, because the support is not inert under HILIC conditions. Although other materials could have been used for the support (Zr, Ti), there are no significant number of applications of such materials to the research of metabolism, and they will not be considered here.

Silica gel

HILIC mechanisms have been extensively investigated in silica gel, providing results that can be complementary to RP and therefore the data from two different separations of the same sample must be processed in order to achieve all the information required [4,35]. The interesting possibility of serial coupling with a RP column has also been developed with bare silica [36] which permitted the separation of the compounds not resolved in the front (solvent peak) of the RP-chromatogram, without significant loss of efficiency for the peaks retained by the RP column. The bare silica columns in table 1 are Ascentis® Si, Alltima™ HP HILIC, Atlantis® HILIC Silica, Daisogel SP-P, and Luna® Silica.

The introduction of ethylene bridged organic silica (BEH, for Acquity UPLC® and XBridge™ in table 1) improves the chemical resistance (especially at high pHs) and is suitable in the sub-two micron size that allows the development of UPLC or UHPLC applications. Cations such as carnitine and derivatives [28], small organic acids [26-28], neutral galactooligosaccharides [37] and amino acids and derivatives [28] have been determined with such materials

In addition, silica hydride material (type C silica) is an interesting chromatographic support that has recently been introduced for metabolomics. It has less than 5% of Si-OH, and may present (Cogent Diamond Hydride™) around 2% of bonded carbon on the surface, therefore less polar [17]. As mentioned earlier, the term aqueous normal phase (ANP) is applied to the mechanism in these columns, which is different to that in classical HILIC. The mobile phases used and analytes separated in these applications may be similar, but from the words of the authors, "with reproducible retention and faster equilibration" [29,38,39].

Neutral phases

The stationary phases with neutral groups for hydrophilic compounds include most of the bonded phases for HILIC but not all of the commercially available neutral HILIC phases have been used for metabolomic applications. Amide phases are one of the most popular (Acquity® BEH amide, TSKGel® Amide 80), and they have been applied to applications where strongly negatively charged metabolites (phosphated compounds) [40] were determined, together with amino acids and organic acids [40-42], and for the analysis of *Ganoderma* species it was found to give similar results to a charged phase (PolySULFOETHYL A™) and a zwitterionic one (ZIC®-HILIC) [43].

The cross-linked diol phase (Luna® HILIC) is another neutral phase that has been employed for metabolomic studies. In one exhaustive comparison of 10 HILIC phases it was found to give similar results to ZIC®-HILIC, but the latter was further optimized and used for the determination of metabolites in culture broths [44]. In the analysis of *Ganoderma* species previously mentioned, Luna® HILIC gave clustered results with a homemade phase that included sulfoxide and diol groups, and separated from the zwitterionic, charged or amide columns tested [43].

Pentafluorophenylpropyl (PFPP - Luna® PFP, Discovery® HS F5) phases have been used to obtain the widest range of metabolites in one single run, because they can retain polar compounds (aromatic amines, halogenated compounds and other polar aromatic compounds) under HILIC conditions [11], or RP-ionic exchange/hydrogen bonding/π- π interactions [45]. It is difficult to establish whether for a metabolomic application it is working under HILIC or non-HILIC conditions, but such phases have been applied for the classification of miso varieties [46] increasing the retention of polar metabolites such as organic acids and amino acids, including peaks corresponding to more non polar metabolites for the classification. Another application using PFPP compared its results with those from a HILIC amino column and found some advantages over PFPP, such as the resolution of some metabolic pairs and with similar structure, such as methylsuccinate/ethylmalonate, or malate/fumarate [31].

Charged phases

Aminopropyl phases (Inertsil NH2, Luna® NH2) can be considered charged phases, depending on the pH of the mobile phase. They have been described for metabolomic

studies with aqueous phases buffered with ammonium formate or acetate [47-49], and successfully applied to the identification of changes in organic acids or amino acids.

A study compared the performance of nine different chromatography approaches involving seven different column chemistries [50]. This study identified HILIC on an aminopropyl column as an effective method to separate a broad range of cellular metabolites including amino acids, nucleosides, nucleotides, coenzyme A derivatives, carboxylic acids, and sugar phosphates. By separating compounds into a 40-min positive ion run and a 50-min negative ion run, a total of ~140 compounds can be readily measured with a total running time of 90 min.

Zwitterionic phases

Zwitterionic phase, more specifically ZIC®-HILIC, is the phase most systematically evaluated, and with the best results in comparative studies for metabolomic applications (see table 1). It consists of groups with sulfobetaine bonded to silica, and therefore the possibilities for interaction include partition between the water layer on the surface, anion and cation exchange, and even non-polar interactions with the carbon backbone. The group of M. Lämmerhofer has identified the signals from more than 250 compounds using this column, and applied HILIC for targeted and non-targeted studies of fermentation broths [44,51], in acidic and neutral conditions. For the analysis of samples related to the leishmaniasis parasitic disease the group of C. Dujardin have also optimized the analytical conditions for working with such a column [52].

Future Perspectives

It can be accepted that HILIC is suitable to be implemented as a routine methodology from the clinical diagnosis perspective for some specific applications; however, much more work must be undertaken in order to give such an assessment from a more general point of view. For metabolomics, several researchers have successfully fulfilled the high requirements of the analysis in terms of efficiency/resolution and reproducibility, but the technique is far from being considered and accepted as a methodology for routine metabolic fingerprinting.

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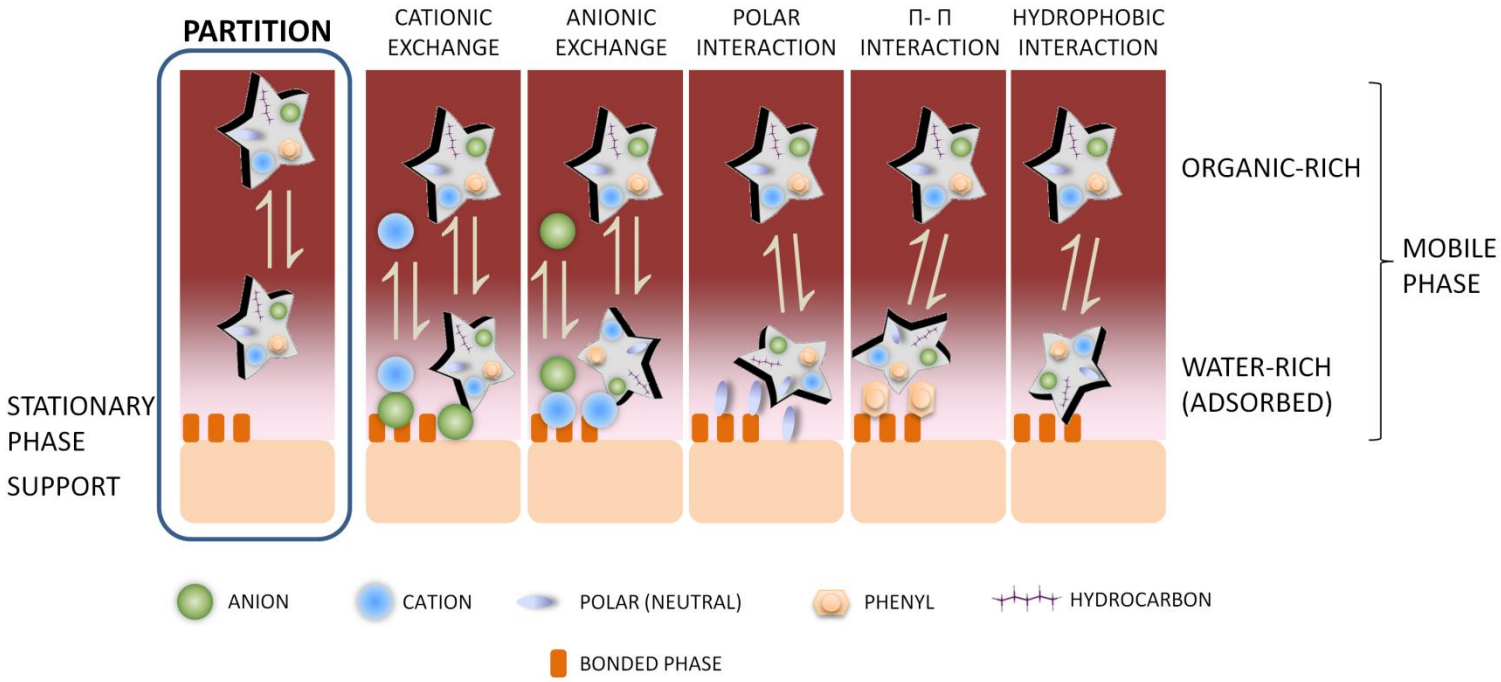
Column	Manufacturer	Target (T) or untarget (uT)	Sample	Disease	Analites (HILIC)	Comments	Reference
Ascentis Si	Supelco	T	Mix of ST		Arylamines and aminopyridines	Serial coupling RP-HILIC	[36]
Alltima HP HILIC	Alltech (Grace)	uT	Mesencephalon of mouse	Parkinson	Amino acids, small organic acids	RP and HILIC	[4]
Atlantis HILIC Silica	Waters	uT	Serum	Renal cell carcinome	Sphingomyelin, acetylphenylalanine, carnitine, glycerophosphory-choline	RP and HILIC	[34]
Atlantis HILIC Silica/ Luna Silica/ Ascentis Si	Waters/ Phenomenex/ Supelco	T	Mix of ST		p-XSA, caffeine, nortriptyline, benzylamine	Method development	[25]
Acquity UPLC BEH HILIC	Waters	uT	Human plasma			10 m/z	[53]
Acquity UPLC BEH HILIC	Waters	uT	Urine	Liver cancer	Carnitine and derivates, aminoacids and derivates, small organic acids	RP and HILIC	[28]
Acquity UPLC BEH HILIC	Waters	uT	Rat urine (Zucker)	Type II diabetes		RP and HILIC	[26]
Acquity UPLC BEH HILIC	Waters	uT	Rat urine	Hepatotoxicity of galN	Small organic acids		[27]
Xbridge BEH HILIC	Waters	T	Mix of ST		Galactooligosaccharides	Method development	[37]
Cogent Diamond	MicroSolv	T	Urine		Sugars, amino acids, organic acids	Method development	[38]

Hydride							
Cogent Diamond Hydride	MicroSolv	uT	Urine		Sugars, amino acids, organic acids	RP and HILIC. Focus on reproducibility	[29]
Cogent Diamond Hydride	MicroSolv	uT	*Staphylococcus aureus* and *Enterococcus faecium*		Nucleic bases, amino acids, polyamines, organic acids	Application of method	[39]
Luna HILIC/ PolySULFOETHYL A/ TSKGel Amide-80/ ZIC-HILIC/ Daisogel SP-P/ TGO	Phenomenex; PolyLC; Tosoh; Merck Sequant; Daiso; homemade	T	*Ganoderma* sp.		Not determined	RP and HILIC. Comparison (Hyerarchical clustering) between HILIC SP	[43]
Acquity BEH amide	Waters	uT	Liver	Steatotic liver	Amino acids, small organic acids, phosphated compounds	RP and HILIC	[40]
TSKGel amide-80	Tosoh	T	Fingerprint deposits		Creatinine	RP and HILIC (RP to lorazepam and 3-O-glucuronide)	[41]
TSKGel amide-80	Tosoh	T	*Arabidopsis thaliana*		Amino acids, small organic acids and sugars	RP and HILIC	[42]
TSKGel amide-80	Tosoh	uT	Urine	Lung cancer	Amino acids, small organic acids	RP and HILIC	[31]
ZIC-HILIC	Merck Sequant	T	*Arabidopsis thaliana*		Myo-inositol, sugars, sugar phosphates, sugar alcohols	Negative mode, ion trap	[54]
ZIC-HILIC	Merck Sequant	T	*Trypanosoma brucei*		Sugars, organic acids (energy metabolism	RP and HILIC	[55]

					pathways)		
ZIC-HILIC	Merck Sequant	uT	Human tears		Small organic acids, amino acids.	RP and HILIC	[56]
ZIC-HILIC	Merck Sequant	T	*Trypanosoma brucei*		127 ST broad range	Focus on QSRR	[57]
ZIC-HILIC	Merck Sequant	uT	Urine				[30]
ZIC-HILIC	Merck Sequant	uT	Rat urine		Amino acids, small organic acids		[32]
ZIC-HILIC	Merck Sequant	T	Fermentation broths		Amino acids, organic acids, β-lactam antibiotics (intermediates and degradation products), vitamins, and some endogenic amines	Systematic comparison of ten HILIC stationary phases. Final method Validated	[51]
ZIC-HILIC	Merck Sequant	T	Fermentation broths		258 ST	Study of β-lactam antibiotics production. PFPP and HILIC with QQQ.	[44]
ZIC-HILIC	Merck Sequant	uT	*Leishmania sp.*		Phospholipids, amino acids, fatty acids/fatty acyls, steroids, polypeptides, nucleotides	Method development	[52]
ZIC-HILIC	Merck Sequant	uT	*Leishmania donovani*	Sb resistance	Glycerophospholipids, sphingolipids, glycerolipids, sterol, amino acids, polypeptides, thiol compounds, carbohydrates, purines,	Method application	[58]

					polyamines.		
Luna NH2; Luna PFP	Phenomenex	T	*Methylobacterium extorquens*		Amino acids, organic acids,nucleotides, acyl-CoAs	Comparison NH2 and PFPP	[48]
Discovery HS F5	Supelco	uT, T	Miso varieties		Organic acids, amino acids	For polar and non-polar	[46]
Inertsil NH2	GLScience	T	Cerebellum of mouse		Gangliosides and sulfatides		[49]
Luna NH2	Phenomenex	T, uT	*Escherichia coli*		Amino acids, nucleotides, nucleosides, vitamins, redox-electron-carriers and CoAs	Method development. Comparison between several columns. RP and HILIC	[50]


PARTITION
CATIONIC EXCHANGE
ANIONIC EXCHANGE
POLAR INTERACTION
Π- Π INTERACTION
HYDROPHOBIC INTERACTION
ORGANIC-RICH
WATER-RICH (ADSORBED)
MOBILE PHASE
STATIONARY PHASE
SUPPORT
ANION
CATION
POLAR (NEUTRAL)
PHENYL
HYDROCARBON
BONDED PHASE

Reference List

1. O. Fiehn, Metabolomics--the link between genotypes and phenotypes. Plant Mol Biol, 48 (2002) 155-71.

2. H. J. Issaq, E. Abbott and T. D. Veenstra, Utility of separation science in metabolomic studies. J Sep Sci, 31 (2008) 1936-47.

3. G. J. Patti, Separation strategies for untargeted metabolomics. J Sep Sci, 34 (2011) 3460-9.

4. J. L. Liu, H. L. Wang, L. F. Zhang et al., Metabonomics study of brain-specific human S100B transgenic mice by using high-performance liquid chromatography coupled with quadrupole time of flight mass spectrometry. Biol Pharm Bull, 34 (2011) 871-6.

5. M. Mayr, A. Zampetaki, A. Sidibe et al., Proteomic and metabolomic analysis of smooth muscle cells derived from the arterial media and adventitial progenitors of apolipoprotein E-deficient mice. Circ Res, 102 (2008) 1046-56.

6. J. W. Allwood and R. Goodacre , An introduction to liquid chromatography-mass spectrometry instrumentation applied in plant metabolomic analyses. Phytochem Anal, 21 (2010) 33-47.

7. T. Ikegami, K. Tomomatsu, H. Takubo, K. Horie and N. Tanaka, Separation efficiencies in hydrophilic interaction chromatography. J Chromatogr A, 1184 (2008) 474-503.

8. A. J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J Chromatogr, 499 (1990) 177-96.

9. Z. Hao, B. Xiao and N. Weng, Impact of column temperature and mobile phase components on selectivity of hydrophilic interaction chromatography (HILIC). J Sep Sci, 31 (2008) 1449-64.

10. B. Dejaegher, D. Mangelings and Y. Vander Heyden, Method development for HILIC assays. J Sep Sci, 31 (2008) 1438-48.

11. P. Jandera, Stationary and mobile phases in hydrophilic interaction chromatography: a review. Anal Chim Acta, 692 (2011) 1-25.

12. H. P. Nguyen and K. A. Schug , The advantages of ESI-MS detection in conjunction with HILIC mode separations: Fundamentals and applications. J Sep Sci, 31 (2008) 1465-80.

13. Y. Hsieh, Potential of HILIC-MS in quantitative bioanalysis of drugs and drug metabolites. J Sep Sci, 31 (2008) 1481-91.

14. Y. Guo and S. Gaiki, Retention and selectivity of stationary phases for hydrophilic interaction chromatography. J Chromatogr A, 1218 (2011) 5920-38.

15. J. Bernal, A. M. Ares, J. Pol and S. K. Wiedmer, Hydrophilic interaction liquid chromatography in food analysis. J Chromatogr A, 1218 (2011) 7438-52.

16. A. J. Alpert, Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides. Anal Chem, 80 (2008) 62-76.

17. J. E. Sandoval and J. J. Pesek, Synthesis and characterization of a hydride-modified porous silica material as an intermediate in the preparation of chemically bonded chromatographic stationary phases. Anal Chem, 61 (1989) 2067-2075.

18. J. Ruta, S. Rudaz, D. V. McCalley, J. L. Veuthey and D. Guillarme, A systematic investigation of the effect of sample diluent on peak shape in hydrophilic interaction liquid chromatography. J Chromatogr A, 1217 (2010) 8230-40.

19. K. Kimata , K. Iwaguchi, S. Onishi et al., Chromatographic characterization of silica-c-18 packing materials - correlation between a preparation method and retention behavior of stationary phase. J Chromatogr Sci, 27 (1989) 721-728.

20. N. P. Dinh, T. Jonsson and K. Irgum, Probing the interaction mode in hydrophilic interaction chromatography. J Chromatogr A, 1218 (2011) 5880-91.

21. R. I. Chirita, C. West, S. Zubrzycki, A. L. Finaru and C. Elfakir, Investigations on the chromatographic behaviour of zwitterionic stationary phases used in hydrophilic interaction chromatography. J Chromatogr A, 1218 (2011) 5939-63.

22. Y. Iwasaki, Y. Ishii, R. Ito, K. Saito and H. Nakazawa, New approaches for analysis of metabolism compounds in hydrophilic interaction chromatography. J Liq Chromatogr R T, 30 (2007) 2117-2126.

23. S. Cubbon, C. Antonio, J. Wilson and J. Thomas-Oates, Metabolomic applications of HILIC-LC-MS. Mass Spectrom Rev, 29 (2010) 671-84.

24. K. Spagou, H. Tsoukali, N. Raikos et al., Hydrophilic interaction chromatography coupled to MS for metabonomic/metabolomic studies. J Sep Sci, 33 (2010) 716-27.

25. D. V. McCalley, Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds? J Chromatogr A, 1171 (2007) 46-55.

26. H. G. Gika, G. A. Theodoridis and I. D. Wilson, Hydrophilic interaction and reversed-phase ultra-performance liquid chromatography TOF-MS for metabonomic analysis of Zucker rat urine. J Sep Sci, 31 (2008) 1598-608.

27. K. Spagou, I. D. Wilson, P. Masson et al., HILIC-UPLC-MS for exploratory urinary metabolic profiling in toxicological studies. Anal Chem, 83 (2011) 382-90.

28. J. Chen, W. Wang, S. Lv et al., Metabonomics study of liver cancer based on ultra performance liquid chromatography coupled to mass spectrometry with HILIC and RPLC separations. Anal Chim Acta, 650 (2009) 3-9.

29. D. L. Callahan, D. De Souza, A. Bacic and U. Roessner, Profiling of polar metabolites in biological extracts using diamond hydride-based aqueous normal phase chromatography. J Sep Sci, 32 (2009) 2273-80.

30. S. Cubbon, T. Bradbury, J. Wilson and J. Thomas-Oates, Hydrophilic interaction chromatography for mass spectrometric metabonomic studies of urine. Anal Chem, 79 (2007) 8911-8.

31. Q. Yang, X. Shi, Y. Wang et al., Urinary metabonomic study of lung cancer by a fully automatic hyphenated hydrophilic interaction/RPLC-MS system. J Sep Sci, 33 (2010) 1495-1503.

32. H. Idborg, L. Zamani, P. O. Edlund, I. Schuppe-Koistinen and S. P. Jacobsson, Metabolic fingerprinting of rat urine by LC/MS Part 1. Analysis by hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci, 828 (2005) 9-13.

33. C. M. Brown, J. O. Becker, P. M. Wise and A. N. Hoofnagle, Simultaneous determination of 6 L-arginine metabolites in human and mouse plasma by using hydrophilic-interaction chromatography and electrospray tandem mass spectrometry. Clin Chem, 57 (2011) 701-9.

34. L. Lin, Z. Huang, Y. Gao et al., LC-MS based serum metabonomic analysis for renal cell carcinoma diagnosis, staging, and biomarker discovery. J Proteome Res, 10 (2011) 1396-405.

35. H. Lv, G. Palacios, K. Hartil and I. J. Kurland, Advantages of tandem LC-MS for the rapid assessment of tissue-specific metabolic complexity using a pentafluorophenylpropyl stationary phase. J Proteome Res, 10 (2011) 2104-12.

36. S. Louw, A. S. Pereira, F. Lynen, M. Hanna-Brown and P. Sandra, Serial coupling of reversed-phase and hydrophilic interaction liquid chromatography to broaden the elution window for the analysis of pharmaceutical compounds. J Chromatogr A, 1208 (2008) 90-4.

37. O. Hernandez-Hernandez, F. Montanes, A. Clemente, F. J. Moreno and M. L. Sanz, Characterization of galactooligosaccharides derived from lactulose. J Chromatogr A, 1218 (2011) 7691-6.

38. J. J. Pesek, M. T. Matyska, S. M. Fischer and T. R. Sana, Analysis of hydrophilic metabolites by high-performance liquid chromatography-mass spectrometry using a silica hydride-based stationary phase. J Chromatogr A, 1204 (2008) 48-55.

39. S. A. Weisenberg, T. R. Butterfield, S. M. Fischer and K. Y. Rhee, Suitability of silica hydride stationary phase, aqueous normal phase chromatography for untargeted metabolomic profiling of Enterococcus faecium and Staphylococcus aureus. J Sep Sci, 32 (2009) 2262-5.

40. J. C. Garcia-Canaveras, M. T. Donato, J. V. Castell and A. Lahoz, A comprehensive untargeted metabonomic analysis of human steatotic liver tissue by RP and HILIC chromatography coupled to mass spectrometry reveals important metabolic alterations. J Proteome Res, 10 (2011) 4825-34.

41. E. Goucher, A. Kicman, N. Smith and S. Jickells, The detection and quantification of lorazepam and its 3-O-glucuronide in fingerprint deposits by LC-MS/MS. J Sep Sci, 32 (2009) 2266-72.

42. R. t'Kindt, M. Storme, D. Deforce and J. Van Bocxlaer, Evaluation of hydrophilic interaction chromatography versus reversed-phase chromatography in a plant metabolomics perspective. J Sep Sci, 31 (2008) 1609-1614.

43. Y. Chen, W. Bicker, J. Wu, M. Y. Xie and W. Lindner, Ganoderma species discrimination by dual-mode chromatographic fingerprinting: a study on stationary phase effects in hydrophilic interaction chromatography and reduction of sample misclassification rate by additional use of reversed-phase chromatography. J Chromatogr A, 1217 (2010) 1255-65.

44. B. Preinerstorfer, S. Schiesel, M. Lammerhofer and W. Lindner, Metabolic profiling of intracellular metabolites in fermentation broths from beta-lactam antibiotics production by liquid chromatography-tandem mass spectrometry methods. J Chromatogr A, 1217 (2010) 312-28.

45. D. S. Bell, H. M. Cramer and A. D. Jones, Rational method development strategies on a fluorinated liquid chromatography stationary phase: mobile phase ion concentration and temperature effects on the separation of ephedrine alkaloids. J Chromatogr A, 1095 (2005) 113-8.

46. H. Yoshida, J. Yamazaki, S. Ozawa, T. Mizukoshi and H. Miyano, Advantage of LC-MS metabolomics methodology targeting hydrophilic compounds in the studies of fermented food samples. J Agric Food Chem, 57 (2009) 1119-26.

47. K. Ikeda, Y. Oike, T. Shimizu and R. Taguchi, Global analysis of triacylglycerols including oxidized molecular species by reverse-phase high resolution LC/ESI-QTOF MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci, 877 (2009) 2639-47.

48. S. Yang, M. Sadilek and M. E. Lidstrom, Streamlined pentafluorophenylpropyl column liquid chromatography-tandem quadrupole mass spectrometry and global (13)C-labeled internal standards improve performance for quantitative metabolomics in bacteria. J Chromatogr A, 1217 (2010) 7401-10.

49. K. Ikeda and R. Taguchi, Highly sensitive localization analysis of gangliosides and sulfatides including structural isomers in mouse cerebellum sections by combination of laser microdissection and hydrophilic interaction liquid chromatography/electrospray ionization mass spectrometry with theoretically expanded multiple reaction monitoring. Rapid Commun Mass Spectrom, 24 (2010) 2957-65.

50. S. U. Bajad, W. Lu, E. H. Kimball et al., Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. J Chromatogr A, 1125 (2006) 76-88.

51. S. Schiesel, M. Lammerhofer and W. Lindner, Multitarget quantitative metabolic profiling of hydrophilic metabolites in fermentation broths of beta-lactam antibiotics production by HILIC-ESI-MS/MS. Anal Bioanal Chem, 396 (2010) 1655-79.

52. R. t'Kindt, R. A. Scheltema, A. Jankevics et al., Metabolomics to unveil and understand

phenotypic diversity between pathogen populations. PLoS Negl Trop Dis, 4 (2010) e904.

53. X. Cai, L. Zou, J. Dong et al., Analysis of highly polar metabolites in human plasma by ultra-performance hydrophilic interaction liquid chromatography coupled with quadrupole-time of flight mass spectrometry. Anal Chim Acta, 650 (2009) 10-5.

54. C. Antonio, T. Larson, A. Gilday et al., Hydrophilic interaction chromatography/electrospray mass spectrometry analysis of carbohydrate-related metabolites from Arabidopsis thaliana leaf tissue. Rapid Commun Mass Spectrom, 22 (2008) 1399-407.

55. K. Burgess, D. Creek, P. Dewsbury, K. Cook and M. P. Barrett, Semi-targeted analysis of metabolites using capillary-flow ion chromatography coupled to high-resolution mass spectrometry. Rapid Commun Mass Spectrom, 25 (2011) 3447-52.

56. L. Chen, L. Zhou, E. C. Chan, J. Neo and R. W. Beuerman, Characterization of the human tear metabolome by LC-MS/MS. J Proteome Res, 10 (2011) 4876-82.

57. D. J. Creek, A. Jankevics, R. Breitling et al., Toward global metabolomics analysis with hydrophilic interaction liquid chromatography-mass spectrometry: improved metabolite identification by retention time prediction. Anal Chem, 83 (2011) 8703-10.

58. R. t'Kindt, A. Jankevics, R. A. Scheltema et al., Towards an unbiased metabolic profiling of protozoan parasites: optimisation of a Leishmania sampling protocol for HILIC-orbitrap analysis. Anal Bioanal Chem, 398 (2010) 2059-69.

Selected References

11. P. Jandera, Stationary and mobile phases in hydrophilic interaction chromatography: a review. Anal Chim Acta, 692 (2011) 1-25.

An updated review about HILIC and other stationary phases that can run under hydrophilic interaction mode, describing not only the chemistries but also the mechanisms that govern this separation mode.

34. L. Lin, Z. Huang, Y. Gao et al., LC-MS based serum metabonomic analysis for renal cell carcinoma diagnosis, staging, and biomarker discovery. J Proteome Res, 10 (2011) 1396-405.

A practical demonstration of the metabolomics workflow, from the metabolic fingerprinting to the biomarker elucidation. HILIC and RP have proven to be complementary; valuable data for discovering potential biomarkers were obtained from each chromatographic separation.

38. J. J. Pesek, M. T. Matyska, S. M. Fischer and T. R. Sana, Analysis of hydrophilic metabolites by high-performance liquid chromatography-mass spectrometry using a silica hydride-based stationary phase. J Chromatogr A, 1204 (2008) 48-55.

A practical demonstration of the suitability of Aqueous Normal Phase for polar metabolites. Chromatographic behavior was evaluated in terms of retention, peak shape, repeatability and temperature effects on cabohydrates, amino acids and other urine metabolites.

51. S. Schiesel, M. Lammerhofer and W. Lindner, Multitarget quantitative metabolic profiling of hydrophilic metabolites in fermentation broths of beta-lactam antibiotics production by HILIC-ESI-MS/MS. Anal Bioanal Chem, 396 (2010) 1655-79.

A large and comprehensive study full of valuable information. Analytical conditions were developed by testing more than 70 compounds, endogenous and antibiotic metabolites in 22 columns, 21 of them working under HILIC conditions. Final conditions were optimized and validated, and the method was successfully applied to real samples