A Practical, Selectivity Based Hydrophilic Interaction Liquid Chromatography (HILIC) Method Development Protocol

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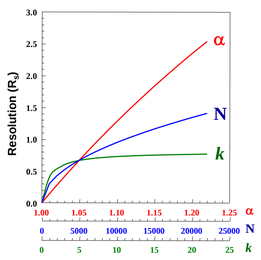
1. Introduction

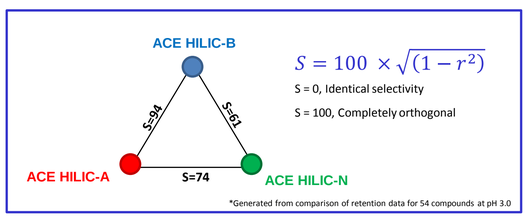
- Exploring chromatographic selectivity is a powerful approach for LC method development.
- Efficient method development requires a logical exploration of the key chromatographic parameters affecting selectivity.
- Rationally designed method development strategies assess key parameters and allow well informed decision making leading to robust stationary phase / mobile phase selection.
- Method development strategies based on column / mobile phase screening and optimisation are commonly utilised for reversed phase.
- For HILIC, such strategies are less common / less well defined.
- This poster demonstrates a simple, step-by-step approach to HILIC method development, based on the concept of exploring column selectivity.



Zhao, J.H. and P.W. Carr. Analytical Chemistry, (1999) 71, 2623-2632

2. Selectivity in HILIC

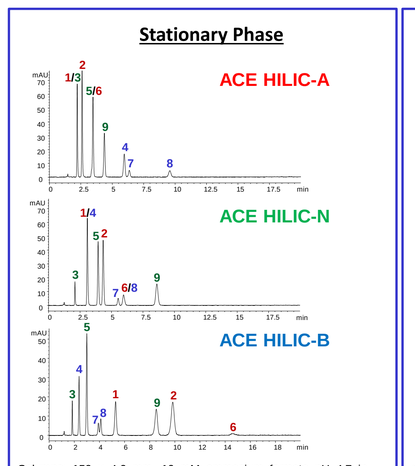
- In HILIC, the column stationary phase has a significant effect on chromatographic selectivity.
- The ACE HILIC range consists of three complementary phases specifically designed to offer maximum selectivity differences – ideal for method development:
  - **ACE HILIC-A**
  - **ACE HILIC-B**
  - **ACE HILIC-N**



*Generated from comparison of retention data for 54 compounds at pH 3.0

- Mobile phase pH is also a powerful parameter and can affect ionisation of analytes and the stationary phase itself.
- Method development strategies based on screening different stationary phases and mobile phase pH are therefore the optimum choice.
- Buffer concentration and temperature are less influential, however can be used to fine-tune methods.

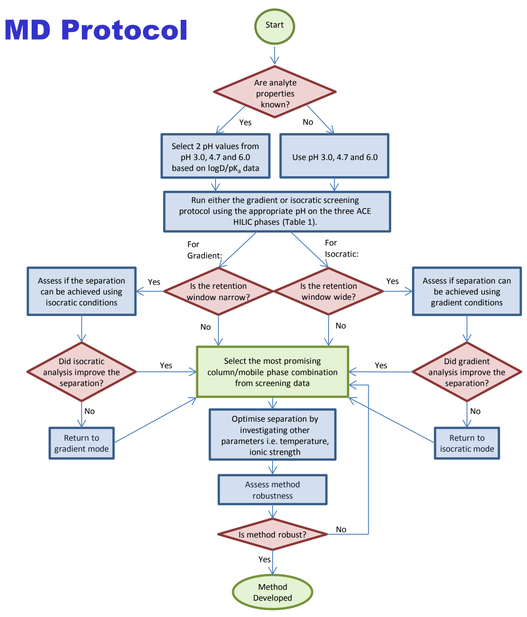
3. Effect of stationary phase and pH



Columns: 150 x 4.6 mm, 10 mM ammonium formate pH 4.7 in MeCN/H_2O (90:10 v/v), flow rate: 1.5 mL/min, temperature: 25 °C, detection: UV, 254 nm. Sample: 1. p-aminobenzoic acid, 2. 4-hydroxybenzoic acid, 3. nicotinamide, 4. acebutolol, 5. adenine, 6. mandelic acid, 7. tyramine, 8. atenolol, 9. 2'-deoxyguanosine

Columns: 150 x 4.6 mm, 10 mM ammonium formate in MeCN/H_2O (90:10 v/v), flow rate: 1.5 mL/min, temperature: 25 °C, detection: UV, 254 nm. Sample: 1. p-aminobenzoic acid, 2. 4-hydroxybenzoic acid, 3. nicotinamide, 4. acebutolol, 5. adenine, 6. mandelic acid, 7. tyramine, 8. atenolol, 9. 2'-deoxyguanosine

4. ACE MD Protocol



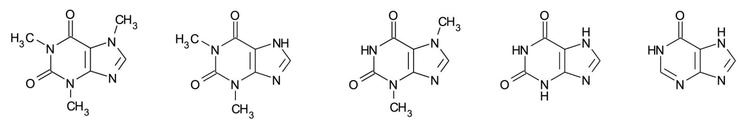
5. Screening Conditions

- Isocratic and gradient screening is performed as follows:

Table 1	
Column	ACE HILIC-A, ACE HILIC-B and ACE HILIC-N, 150 x 4.6 mm
Isocratic screening	10 mM ammonium formate in MeCN/H_2O (90:10 v/v) Ammonium formate at pH 3.0, 4.7 or 6.0.
Gradient screening	Line A: 10 mM ammonium formate in MeCN/H_2O (94:6 v/v) Line B: 10 mM ammonium formate in MeCN/H_2O (50:50 v/v) Ammonium formate at pH 3.0, 4.7 or 6.0. Gradient: **Time (mins.) / %B** 0 / 0 15 / 100 20 / 100 21 / 0 41 / 0
Flow rate	1.5 mL/min
Temperature	25 °C
Detection	Dependent on sample

6. Example 1: Caffeine and Related Substances

- The screening protocol was used to develop a HILIC method for caffeine and related substances.
- All analytes are polar neutral with negative logP values.

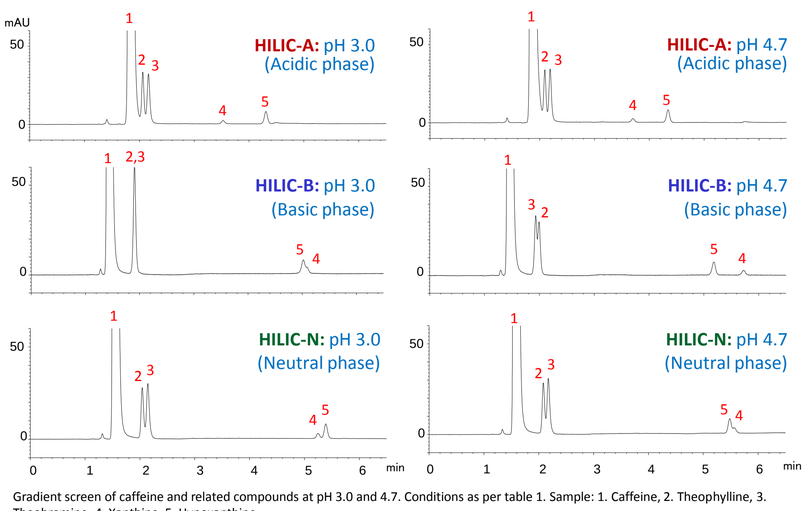


1. Caffeine logP -0.13
2. Theophylline logP -0.17 pK_a = 7.8
3. Theobromine logP -2.08 pK_a = 9.2
4. Xanthine logP -1.99 pK_a = 7.95
5. Hypoxanthine logP -1.74 pK_a = 2.3, 9.4

- The mixture was screened on the ACE HILIC-A, HILIC-B and HILIC-N phases at pH 3.0 and 4.7.

7. Caffeine and Related Substances: Gradient Screen

- The HILIC-N at pH 3.0 provided the most potential for a full separation.



Gradient screen of caffeine and related compounds at pH 3.0 and 4.7. Conditions as per table 1. Sample: 1. Caffeine, 2. Theophylline, 3. Theobromine, 4. Xanthine, 5. Hypoxanthine

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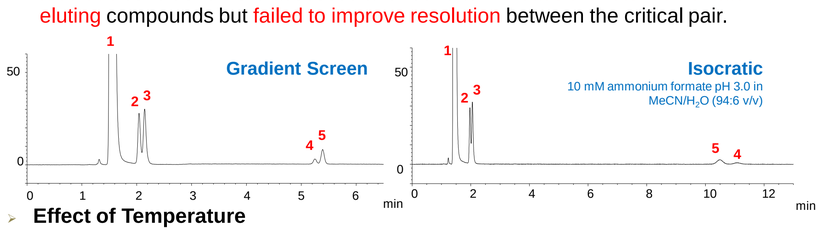
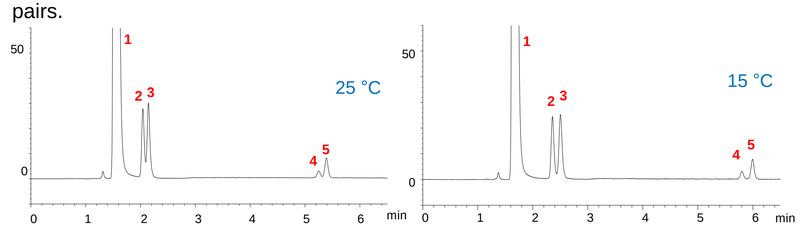
8. Caffeine and Related Substances: Optimisation

- **Isocratic or gradient mode**
- Isocratic conditions were assessed and found to increase the retention of later eluting compounds but failed to improve resolution between the critical pair.

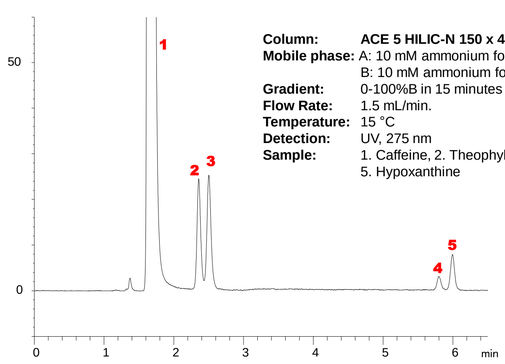


- **Effect of Temperature**
- Reducing the temperature was found to improve the resolution of both impurity peak pairs.



9. Caffeine and Related Substances: Final Method

- Reduced temperature was utilised to achieve separation of the mixture on the ACE 5 HILIC-N at pH 3.0.
- A small increase in acetonitrile in the gradient starting conditions was also found to be beneficial



Column: ACE 5 HILIC-N 150 x 4.6 mm
Mobile phase: A: 10 mM ammonium formate pH 3.0 in MeCN/H_2O (96:4 v/v), B: 10 mM ammonium formate pH 3.0 in MeCN/H_2O (50:50 v/v)
Gradient: 0-100%B in 15 minutes
Flow Rate: 1.5 mL/min.
Temperature: 15 °C
Detection: UV, 275 nm
Sample: 1. Caffeine, 2. Theophylline, 3. Theobromine, 4. Xanthine, 5. Hypoxanthine

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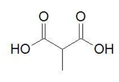
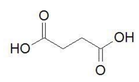
10. Example 2: MMA and Succinic Acid

- Elevated levels of methylmalonic acid (MMA) in blood/urine is routinely tested for as an indicator of vitamin B12 deficiency.
- The isobaric species succinic acid may be present in biomatrices at concentrations up to 50x that of MMA.
- Chromatographic separation of these two components with good retention to eliminate matrix interference is therefore essential for accurate determination.
- Physico-chemical properties are similar and suitable for HILIC mode



MMA

pH	logD
3.0	-0.43
6.0	-3.21



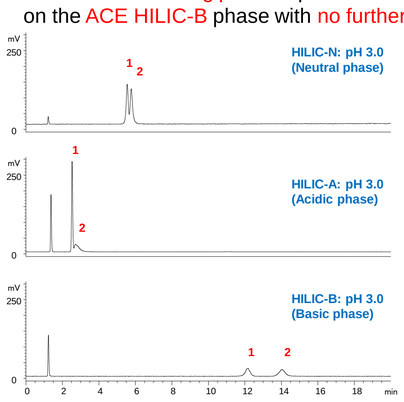
Succinic acid

pH	logD
3.0	-0.40
6.0	-3.30

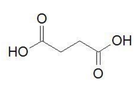
- The two compounds were screened on the three ACE HILIC phases.
- The two acids were predicted to possess a single negative charge at pH 3.0 and a double negative charge at pH 6.0, therefore these two mobile phase pH's were selected for screening

11. Example 2: MMA and Succinic Acid

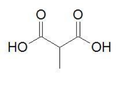
- Very strong retention was observed at pH 6.0 on all phases (data not shown).
- At pH 3.0, the analytes proved difficult to separate on the ACE HILIC-N and ACE HILIC-A phases due to similar analyte properties.
- The ACE HILIC-B provided additional retention and selectivity for the two analytes.
- Use of the screening protocol provided a separation of these challenging analytes on the ACE HILIC-B phase with no further development required.



Columns: ACE 5 HILIC-N 150 x 4.6 mm, ACE 5 HILIC-A 150 x 4.6 mm, ACE 5 HILIC-B 150 x 4.6 mm
Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O (90:10 v/v)
Flow Rate: 1.5 mL/min.
Temperature: 25 °C
Detection: ELSD, Evaporator: 30 °C, Nebuliser: 30 °C, Gas speed: 1 SLM



1. Succinic acid



2. MMA

12. Summary

- A systematic and rationally designed method development strategy can aid in streamlining the method development process.
- In HILIC, column stationary phase and mobile phase pH are the most critical parameters affecting selectivity.
- The step-by-step method development strategy proposed in this poster therefore provides a powerful means by which to probe selectivity of a new application.
- The ACE HILIC-A, HILIC-B and HILIC-N phases have been shown to provide complimentary selectivity, ideal for method development.
- Screening an analyte mixture on these three phases has been demonstrated as an effective method development strategy for selecting an appropriate stationary phase/mobile phase combination.
- Optimisation can be achieved by altering parameters such as ionic strength, % organic and temperature.