

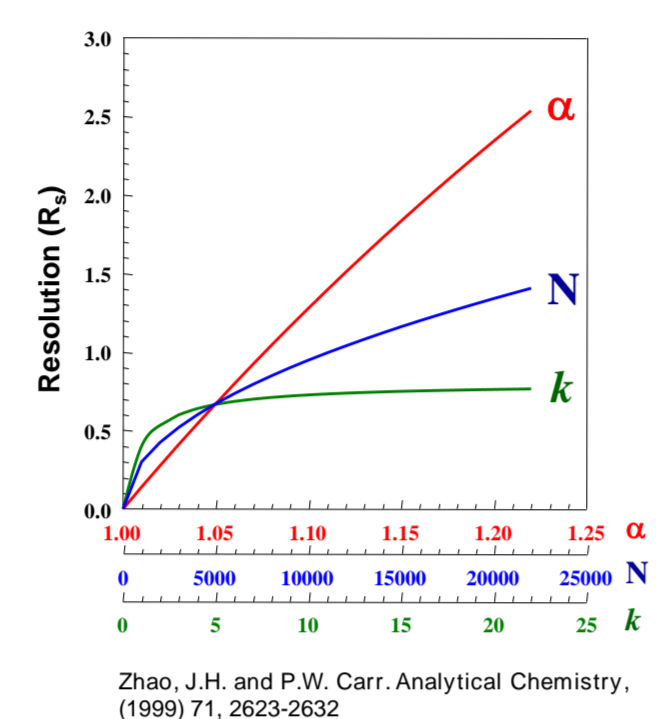
# A Practical, Selectivity Based Hydrophilic Interaction Liquid Chromatography (HILIC) Method Development Protocol

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## 1. Introduction

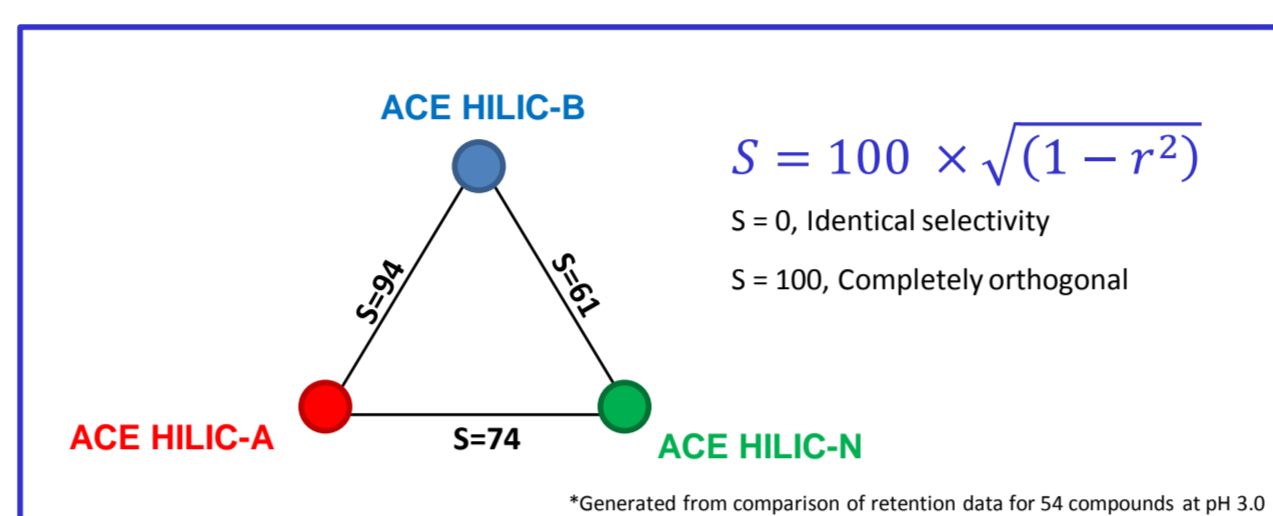
- Exploring **chromatographic selectivity** is a **powerful** approach for LC method development.
- Efficient **method development** requires a **logical** exploration of the key chromatographic parameters affecting selectivity.
- Rationally designed method development strategies **assess key parameters** 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**.



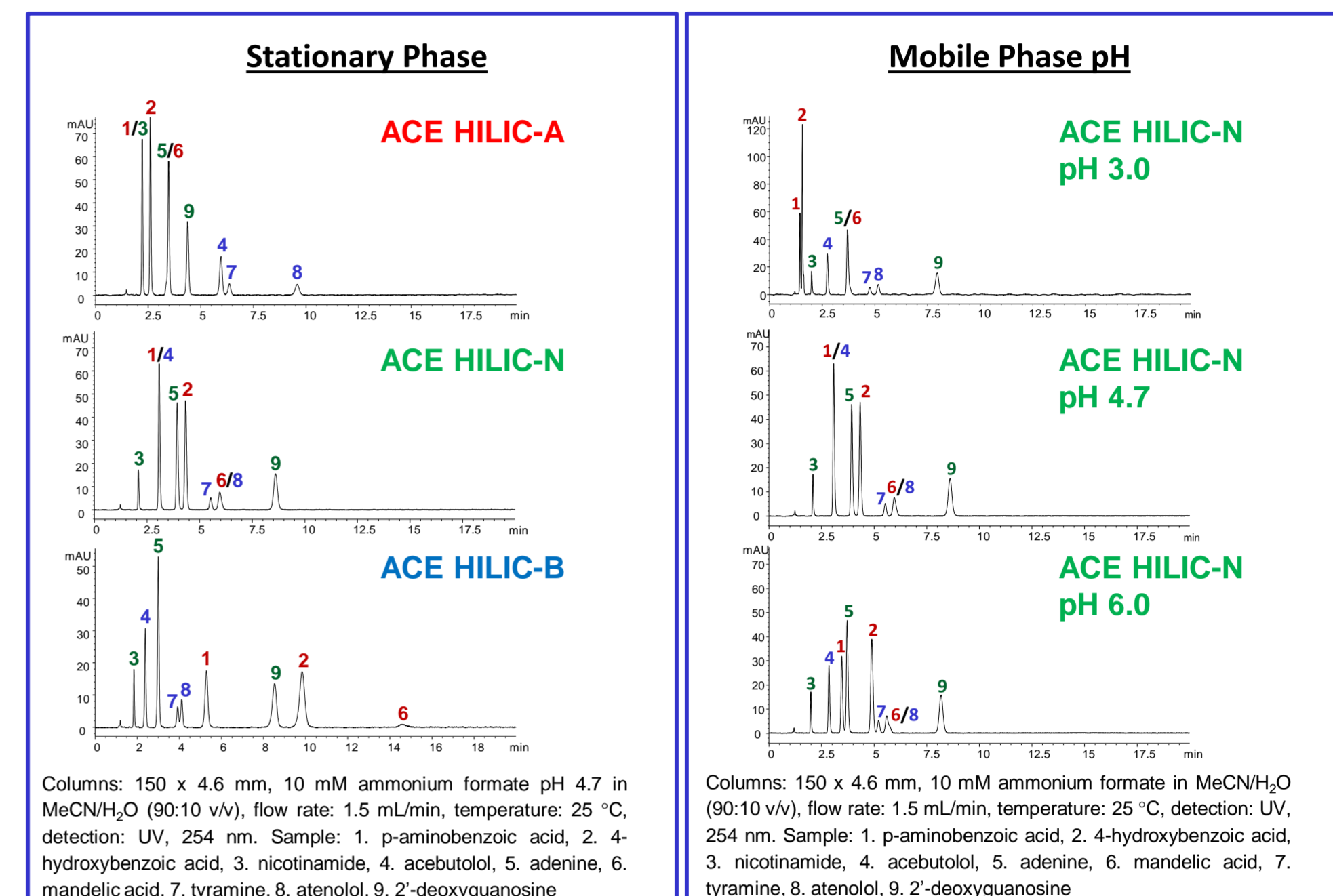
Zhao, J.H. and P.W. Carr: Analytical Chemistry, (1999) 71, 2623-2632

## 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**



## 3. Effect of stationary phase and pH



Columns: 150 x 4.6 mm, 10 mM ammonium formate pH 4.7 in MeCN/H<sub>2</sub>O (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. acetabutool, 5. adenine, 6. mandelic acid, 7. tyramine, 8. atenolol, 9. 2'-deoxyguanosine

## 4. ACE MD Protocol



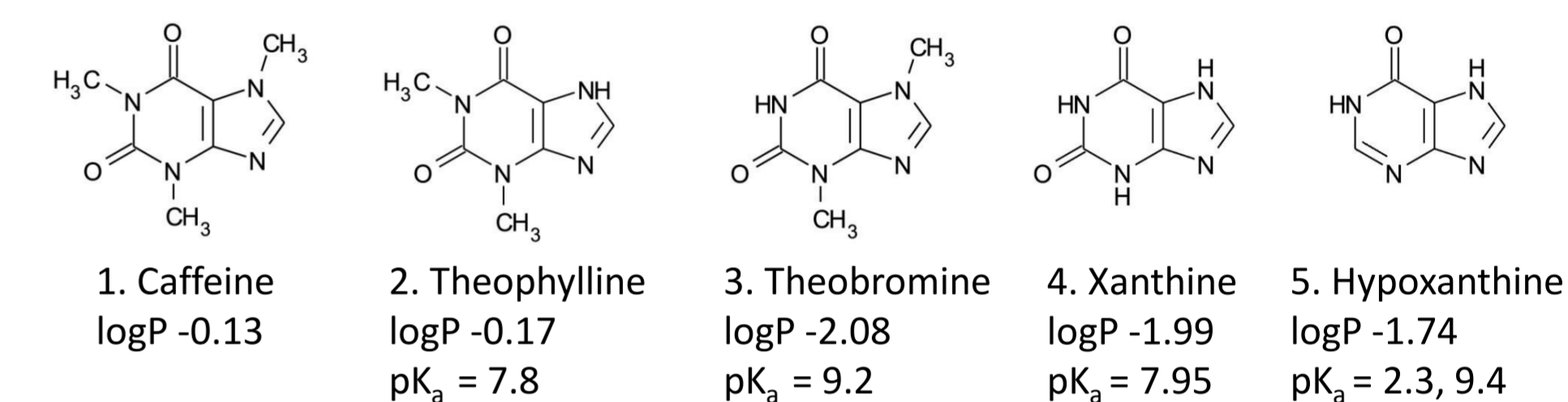
## 5. Screening Conditions

- Isocratic** and **gradient** screening is performed as follows:

Table 1	ACE HILIC-A, ACE HILIC-B and ACE HILIC-N, 150 x 4.6 mm
<b>Column</b>	ACE HILIC-A, ACE HILIC-B and ACE HILIC-N, 150 x 4.6 mm
<b>Isocratic screening</b>	10 mM ammonium formate in MeCN/H <sub>2</sub> O (90:10 v/v)
<b>Gradient screening</b>	Ammonium formate at pH 3.0, 4.7 or 6.0. Line A: 10 mM ammonium formate in MeCN/H <sub>2</sub> O (94:6 v/v) Line B: 10 mM ammonium formate in MeCN/H <sub>2</sub> O (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
<b>Flow rate</b>	1.5 mL/min
<b>Temperature</b>	25 °C
<b>Detection</b>	Dependent on sample

## 6. Example 1: Caffeine and Related Substances

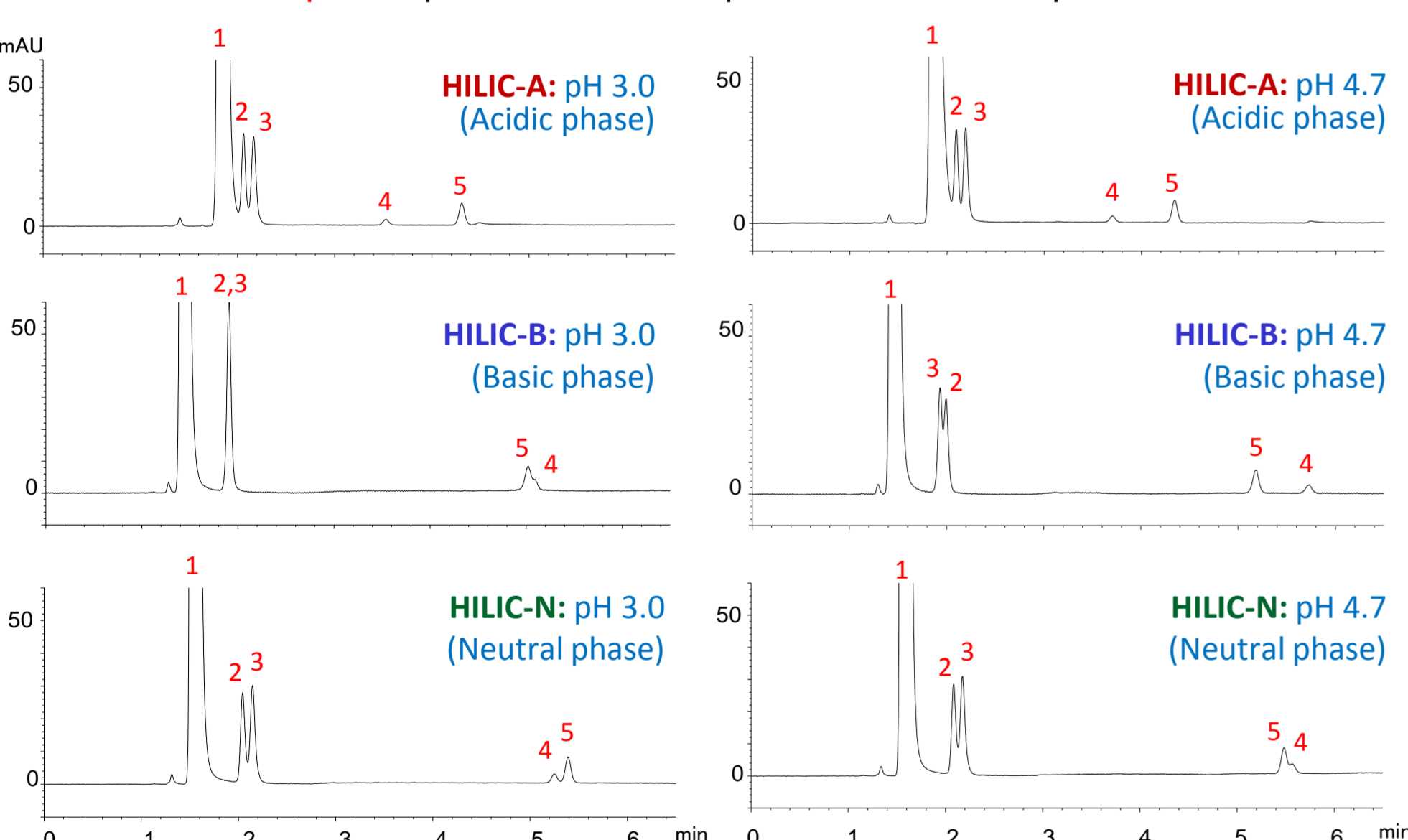
- The **screening protocol** was used to develop a **HILIC method** for caffeine and related substances.
- All analytes are **polar neutral** with **negative logP values**.



- 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.

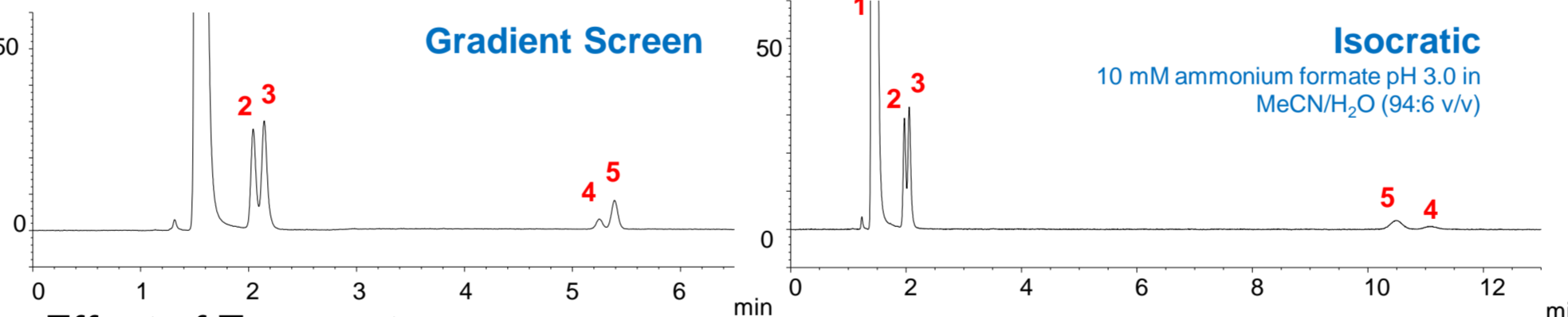


Gradient screen of caffeine and related compounds at pH 3.0 and 4.7. Conditions as per table 1. Sample: 1. Caffeine, 2. Theophylline, 3. Theobromine, 4. Xanthine, 5. Hypoxanthine

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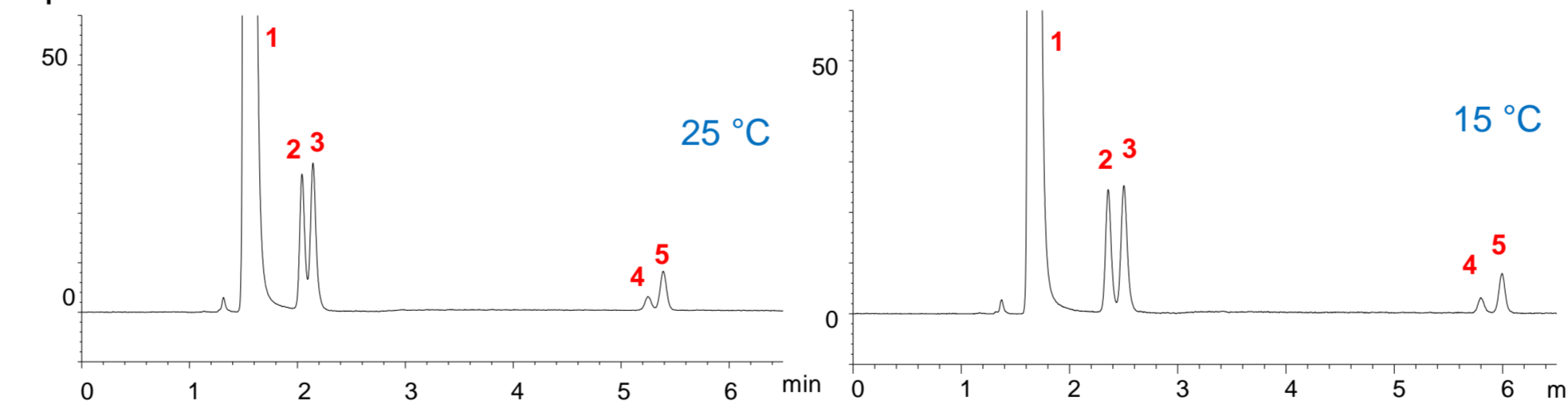
## 8. Caffeine and Related Substances: Optimisation

- 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.



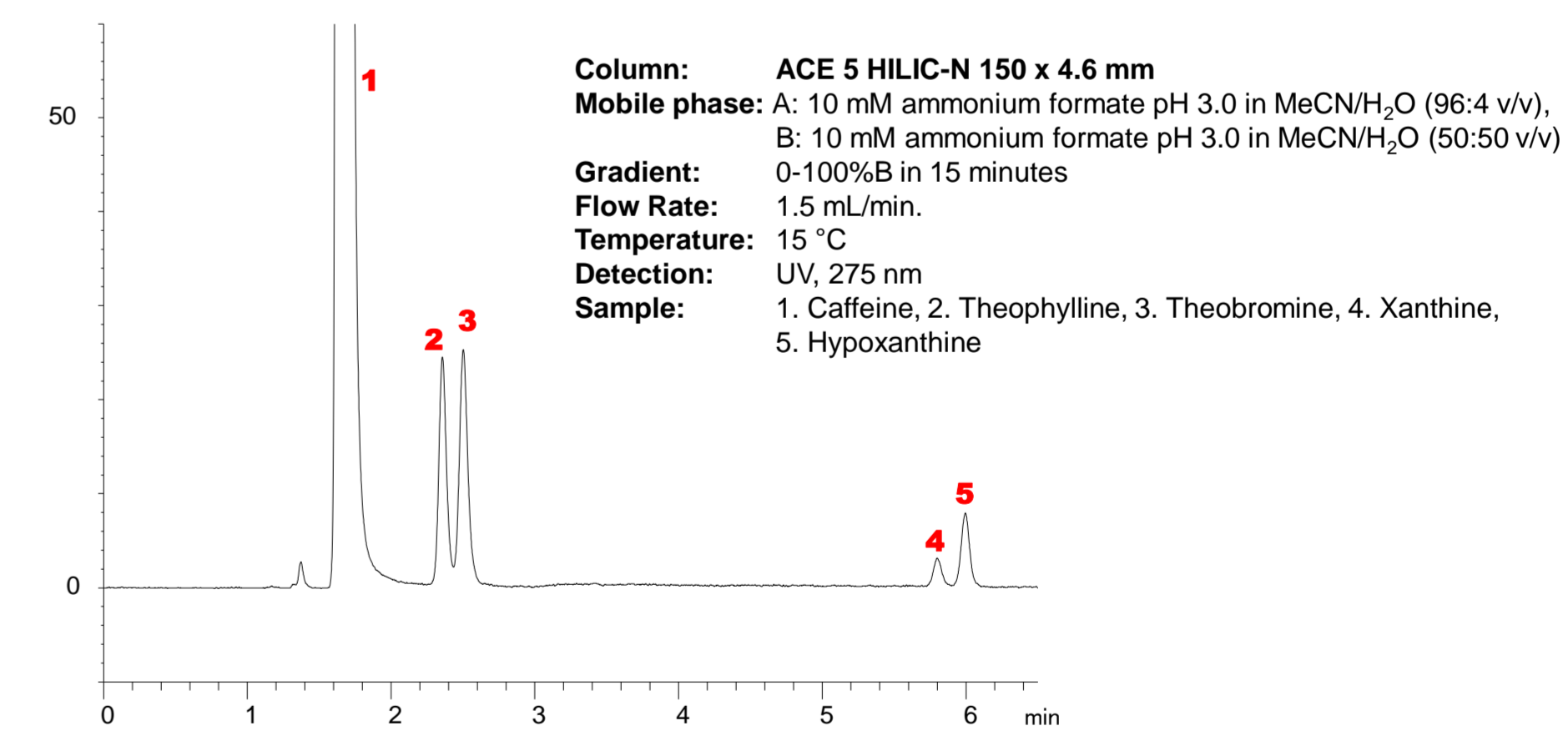
### Effect of Temperature

- 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

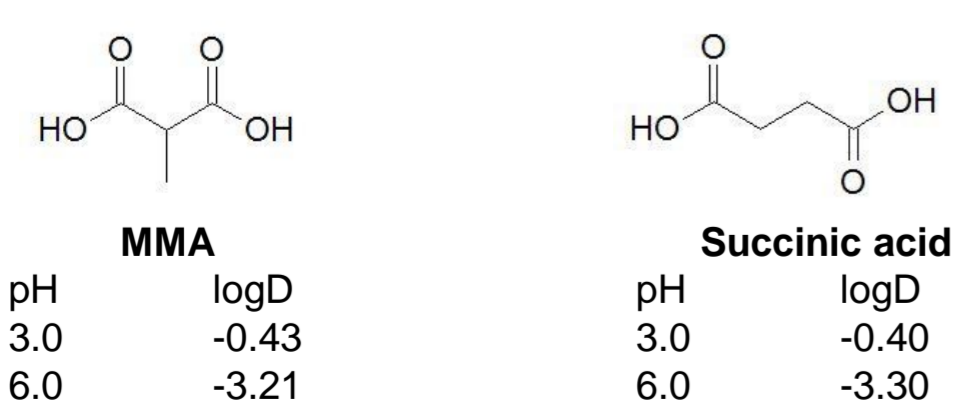


Column: ACE 5 HILIC-N 150 x 4.6 mm  
Mobile phase: A: 10 mM ammonium formate pH 3.0 in MeCN/H<sub>2</sub>O (96:4 v/v), B: 10 mM ammonium formate pH 3.0 in MeCN/H<sub>2</sub>O (50:50 v/v)  
Gradient: 0-100%B in 15 minutes  
Flow Rate: 1.5 mL/min  
Temperature: 15 °C  
Detection: UV, 275 nm  
Sample: 1.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

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## 10. Example 2: MMA and Succinic Acid

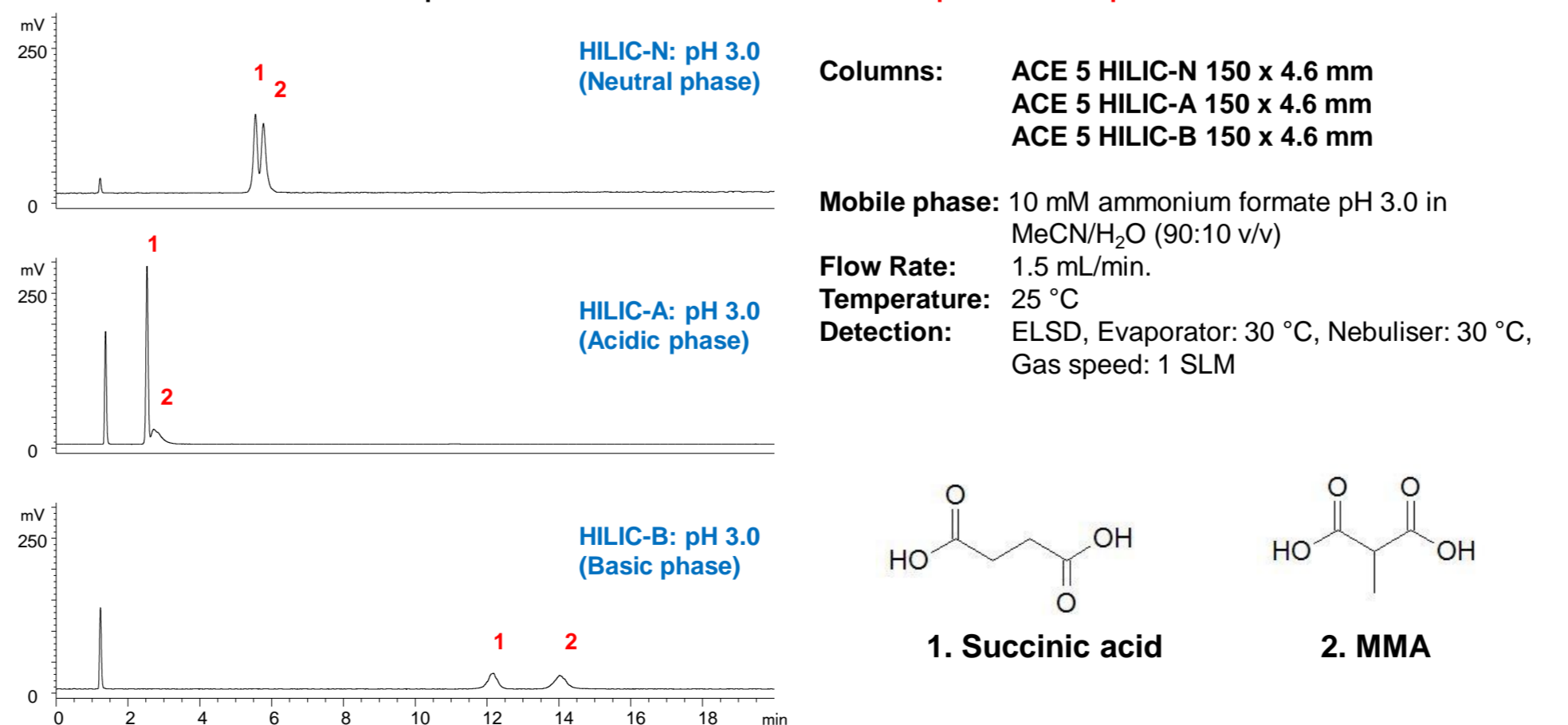
- Elevated levels of methylmalonic acid (MMA)** in blood/urine is routinely tested for as an **indicator of vitamin B12 deficiency**.
- The **isobaric species succinic acid** may be present in biomatrices at **concentrations up to 50x that of MMA**.
- Chromatographic **separation** of these two components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



- 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

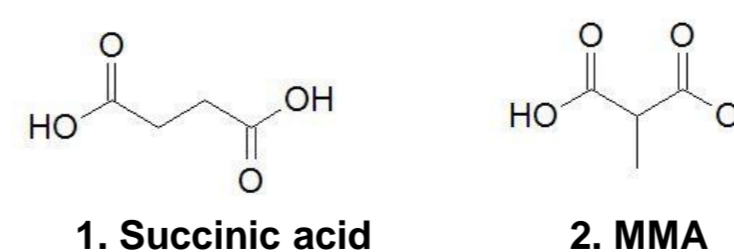
## 11. Example 2: MMA and Succinic Acid

- 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<sub>2</sub>O (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



## 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 **complementary 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**.

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