TECHNICAL NOTE 21998

Superior separation of nicotine and tobacco related alkaloids by utilizing the selectivity of hydrophilic interaction chromatography (HILIC)

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Goal

To develop an HPLC-MS/MS separation for the tobacco alkaloids nicotine, nornicotine, cotinine, norcotinine, trans-3'-hydroxycotinine, and anabasine.

Introduction

The analysis of tobacco-related alkaloids such as nicotine and its metabolites presents many challenges for the analyst. The highly polar nature of these compounds limits the retention on common reverse phases such as the C18 phase. One major challenge caused by this lack of retention is the chromatographic resolution of several of the isobaric compounds is insufficient for LC-MS analysis. One solution to this is through the use of a different column phase such as the Thermo Scientific™ Accucore™ HILIC analytical column.

Hydrophilic interaction chromatography (HILIC) is an increasingly popular technique for separating polar compounds, such as tobacco alkaloids, which do not retain well using reversed-phase chromatography. For HILIC, a more polar mobile phase is the stronger elution solvent with water being the strongest elution solvent. This allows better retention of polar analytes than possible with reversed-phase separation conditions (Figure 1).

This technical note describes the use of a Thermo Scientific™ Accucore™ HPLC column using Core Enhanced Technology to facilitate fast and high efficiency separations. The 2.6 µm diameter particle technology is not fully porous, but instead features a solid core and a porous outer layer. The tightly controlled 2.6 µm diameter of the Accucore particles provides much lower backpressure than seen with sub-2 µm materials and are therefore an alternative format to traditional UHPLC. Analyte properties that govern retention with Accucore HILIC are acidity/basicity, which determines hydrogen bonding, and polarizability, which determines dipole-dipole interactions.

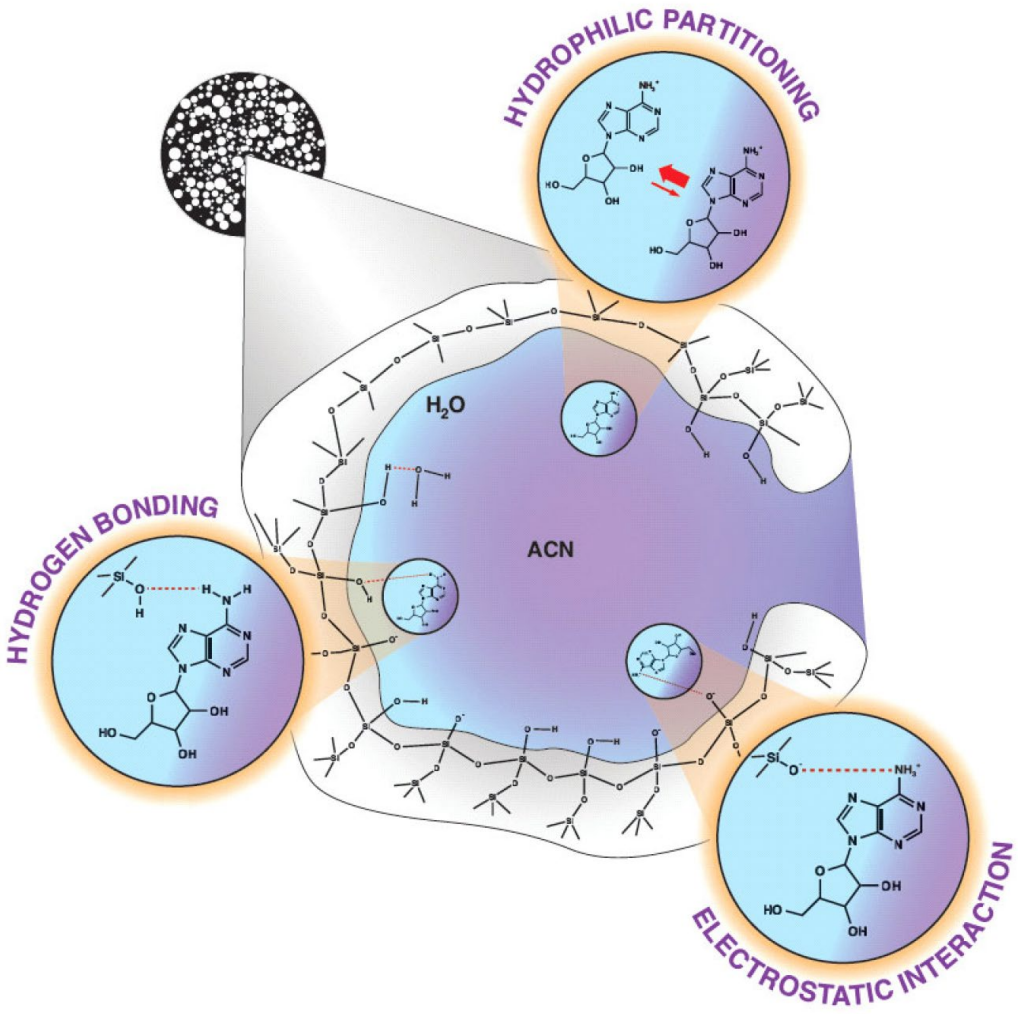

Figure 1. Diagram showing common HILIC retention mechanisms

Experimental

Instruments

Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of the following:	
• System base Vanquish Horizon	P/N VH-S01-A
• Binary Pump H	P/N VH-P10-A
• Split Sampler HT	P/N VH-A10-A
• Column Compartment H	P/N VH-C10-A
• Active pre-heater	P/N 6732.0110
Thermo Scientific™ TSQ Quantiva™ Triple-Stage Quadrupole Mass Spectrometer	Cat. no: IQLAAEGAAXFAOUMZZZ

Consumables

Thermo Scientific™ UHPLC-MS grade water	P/N W8-1
Thermo Scientific™ Optima™ UHPLC-MS grade acetonitrile	P/N A956-1
Fisher Chemical™ Optima™ UHPLC-MS grade ammonium formate	P/N A115-50
Fisher Chemical™ Optima™ UHPLC-MS grade formic acid	P/N 10596814
Accucore HILIC column 150 × 2.1 mm, 2.6 µm	P/N 17526-152130
Thermo Scientific™ Chromacol™ GOLD-Grade Inert Vial, 2 mL	P/N 2-SVWGK
Thermo Scientific™ 9mm Autosampler Vial Screw Thread Caps	P/N 9-SCK(B)-ST1

Standards preparation

Reference standard stock solutions were diluted in LC-MS acetonitrile to give the working standard solutions ranging from 120 ng/mL to 400 ng/mL (Table 1).

Table 1. Working standard solution concentrations

Analyte	Working concentration (ng/mL)
Trans-3'-hydroxycotinine	200
Nicotine	400
Nornicotine	400
Cotinine	200
Norcotinine	120
Anabasine	400

HILIC separation conditions

Table 2. HILIC conditions

Parameter	Setting
Injection volume	2 µL
Mobile phase A	20 mM ammonium formate (aq) adjusted to pH 3 with formic acid
Mobile phase B	Acetonitrile (LC-MS grade)
Gradient	Table 3

Table 3. Gradient ramp conditions

Time (min)	Flow (mL/min)	%B	Curve
0	0.75	98	5
1	0.75	98	5
4	0.75	45	5
4	0.75	98	5
7	0.75	98	5

*Curve value of 5 indicates linear change

Mass spectrometer conditions

Table 4. MS/MS source conditions

Parameter	Setting
Source	Thermo Scientific™ Ion Max source with HESI-II probe
Polarity	Positive ionization
Spray voltage	3500 V
Vaporizer temperature	450 °C
Sheath gas pressure	55 Arb
Aux gas pressure	18 Arb
Ion transfer tube temperature	365 °C
CID gas pressure	1.5 mTorr

Results and discussion

Separation

Isobaric compounds present a challenge for mass spectrometry as the compounds that are isobaric cannot be differentiated if they co-elute. This co-elution would make it difficult to determine the contribution from each analyte. As a result, the chromatographic separation is crucial in determining the accurate concentration of each analyte in the sample.

Chromatographic resolution of all isobaric compounds (norcotinine, anabasine, and nicotine) was achieved on the Accucore HILIC column (Figure 2). In comparison, the crucial components nicotine and anabasine remained unresolved on Competitor A column under the same conditions (Figure 3). These had excellent baseline resolution using the Accucore HILIC column, which is especially important in samples where the nicotine concentration is much higher than the anabasine concentration, making baseline separation more challenging.

Table 5. Compound transition details

Compound	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)
Nicotine	Positive	163.3	132.2	14.4
Nornicotine	Positive	149.4	80.2	22.1
Cotinine	Positive	177.2	98.2	20.9
Norcotinine	Positive	163.1	80.2	27.2
Trans-3'-hydroxycotinine	Positive	193.4	134.2	18.1
Anabasine	Positive	163.1	146.2	15.1

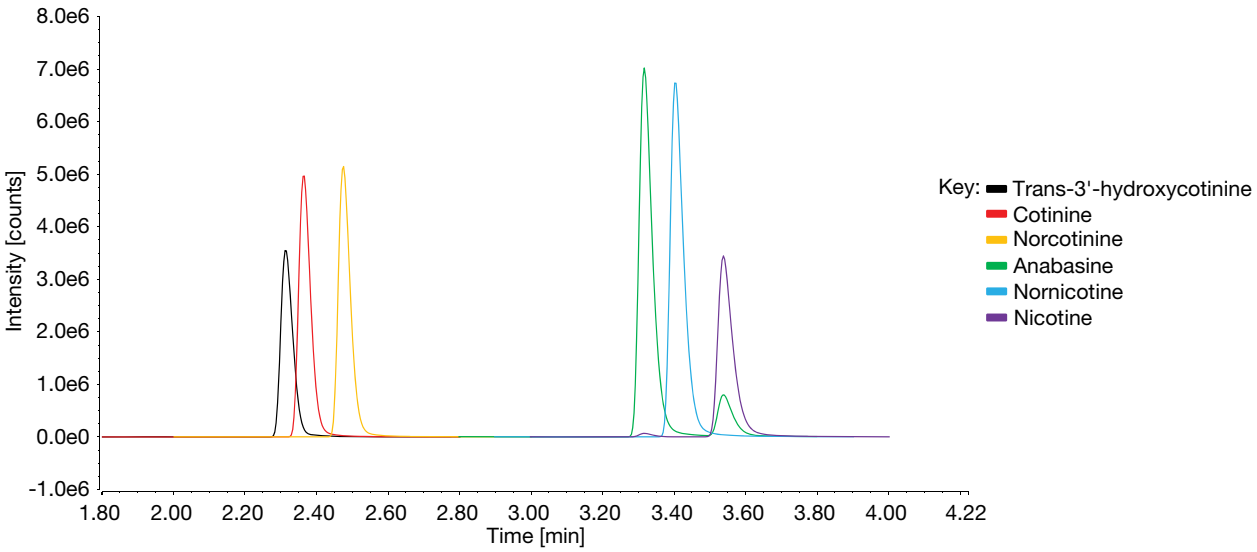

Figure 2. Chromatogram showing the separation provided by the Accucore HILIC column

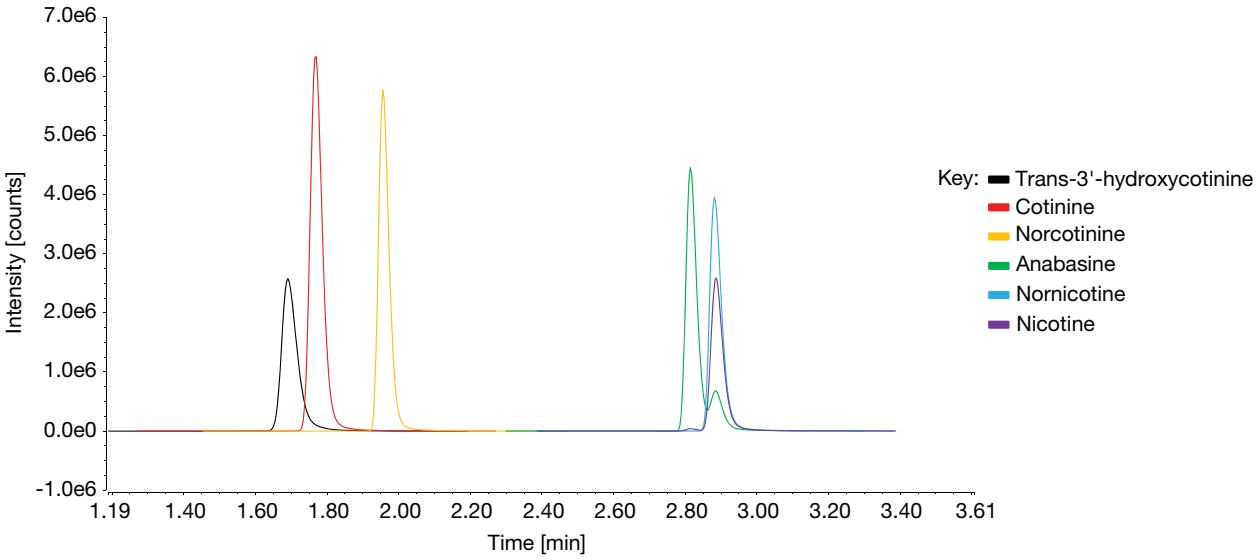

Figure 3. Chromatogram showing the Competitor A column separation

Figure 4 shows the extracted ion chromatogram for anabasine where interference from isobaric nicotine compound can be seen. Chromatographic resolution for Competitor A was 1.26, insufficient to mitigate against an impact on the data quality. Much higher resolution on the Accucore HILIC column was achieved (3.08) allowing more accurate and reproducible data as shown in Figure 5.

Analytical challenges

As tobacco use and nicotine products are still popular in many parts of the world, it is quite a challenge to create a blank sample that is not contaminated by target analytes. This is one of the more challenging aspects of this assay. A vaping colleague or solvents handled multiple times in the laboratory can prevent the assayer from receiving a clean blank sample. Small amounts of nicotine can also be found in food items from the nightshade family of plants such as tomatoes, potatoes, and green peppers.[1] Additionally, cross contamination from people and surfaces is a potential issue. It is therefore advised to allocate nicotine-free areas of the lab, which have been thoroughly cleaned prior to any analysis, as this was found to reduce background levels to permit greater sensitivity and reduce variability between analysis.

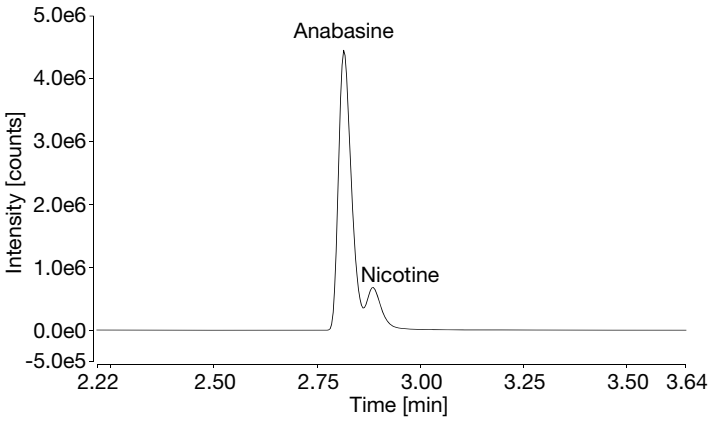

Figure 4. Anabasine component channel for the Competitor A column

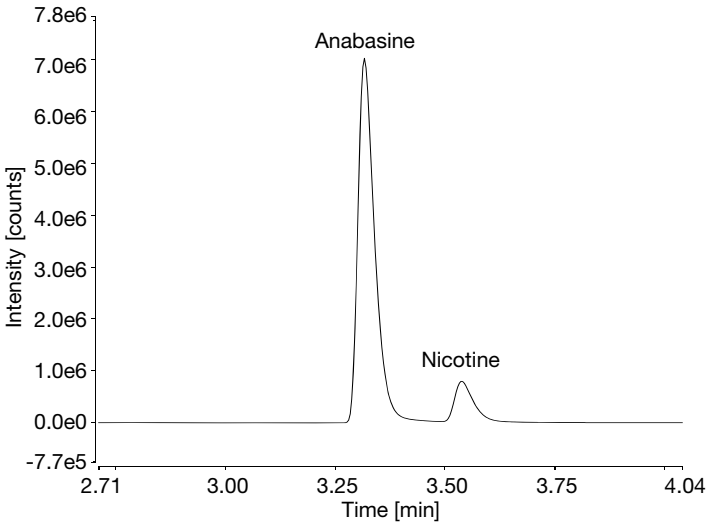

Figure 5. Anabasine component channel for Accucore HILIC

Accucore HILIC advantages

Using the Accucore HILIC column to analyze polar analytes such as nicotine has several additional benefits, beyond the excellent retention of polar analytes that are not retained well on the C18. These include the use of a high percentage of organic solvent that, when using a mass spectrometer, facilitates an increase in the ionization efficiency of the analyte in the ion source, directly improving the sensitivity of the assay.

Furthermore, the Accucore HILIC column demonstrates fantastic peak shape for all the analytes while also providing baseline separation in less than 4 minutes.

Conclusion

The superior baseline separation of the Accucore HILIC column for six nicotine-related alkaloids has been demonstrated compared to the separation provided by a competitor's HILIC column. Chromatographic separation is a key requirement for maintaining high levels of confidence in your analytical results.

Reference

1. Siegmund, B.; Leitner, E.; & Pfannhauser, W. (n.d.). Determination of the Nicotine Content of Various Edible Nightshades (Solanaceae) and Their Products and Estimation of the Associated Dietary Nicotine Intake. *J. Agric. Food Chem* **1999,** *47*(8), 3113–3120. Retrieved October 28, 2019, from https://pubs.acs.org/doi/abs/10.1021/jf990089w

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