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HPLC-CAD

Evaluating LC methods for enhanced charged aerosol detector response: a case study using underivatized amino acids

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Keywords

Branched chain amino acids (BCAA), charged aerosol detector (CAD), ion pair chromatography (IPC), hydrophilic interaction chromatography (HILIC), impurity profiling

Application benefits

- Measurement of underivatized amino acids using a Thermo Scientific™ Vanquish™ Flex UHPLC system.
- Comparison of IPC and HILIC approaches for determining impurity profiles of branched-chain amino acids (BCAA).

Goal

This application note examines the impact of fundamental differences between IPC and HILIC on CAD performance illustrated by measurement of BCAA impurity profiles.

Introduction

Measurement of low-molecular weight, charged polar molecules, such as amino acids, can be challenging. Reversed phase chromatography, a common HPLC approach, cannot be used for the separation of these hydrophilic molecules due to their poor retention. However, this issue can be overcome by the inclusion of ion pairing reagents of opposite charge in the mobile phase.[1] An alternate approach for separation of hydrophilic compounds is HILIC, which uses highly polar stationary phases and highly organic mobile phases.[2]

Quantitation of amino acids is also a challenge as many do not possess a chromophore so they show poor response with a UV/Vis detector. Therefore, the European Pharmacopeia makes use of derivatization with ninhydrin by the “amino acid analyzer” (AAA). In general, derivatization leads to higher validation costs and often to a loss of robustness. AAAs will not measure impurities that do not react with the derivatizing agent and quantitative accuracy for some impurities may depend on their derivatization efficiency. As an alternative detector, the CAD can detect amino acids without derivatization of the analytes. The CAD is a universal detector, and therefore capable of measuring any non-volatile and many semi-volatile compounds. All non-volatile compounds give uniform response independent of an analyte’s physicochemical properties. Additionally, a chromophore is not mandatory for an analyte to be detected.[3] CAD therefore enables a simpler, more robust alternative to AAA with the added capability to simultaneously measure common impurities such as organic acids, which are not derivatized with ninhydrin. However, the mobile phase used with the CAD must be volatile. Mobile phase quality and composition can significantly impact detector performance (e.g., response and noise).[4,5] The mobile phase compositions used with IPC and HILIC differ significantly. Although there are several publications using either IPC[6] or HILIC[7] with CAD for analysis of amino acids, only one directly compares CAD performance using these two approaches.[8]

This application note explores the impact of IPC and HILIC mobile phase compositions on CAD performance demonstrated by the measurement of the impurity profiles of the BCAAs leucine, isoleucine, and valine (Figure 1). The performance of the HILIC method[8] is evaluated compared to a published IPC method proposed as a suitable replacement for the derivatization approach—“amino acid analyzer”—used in The European Pharmacopoeia.[9–11, 13]

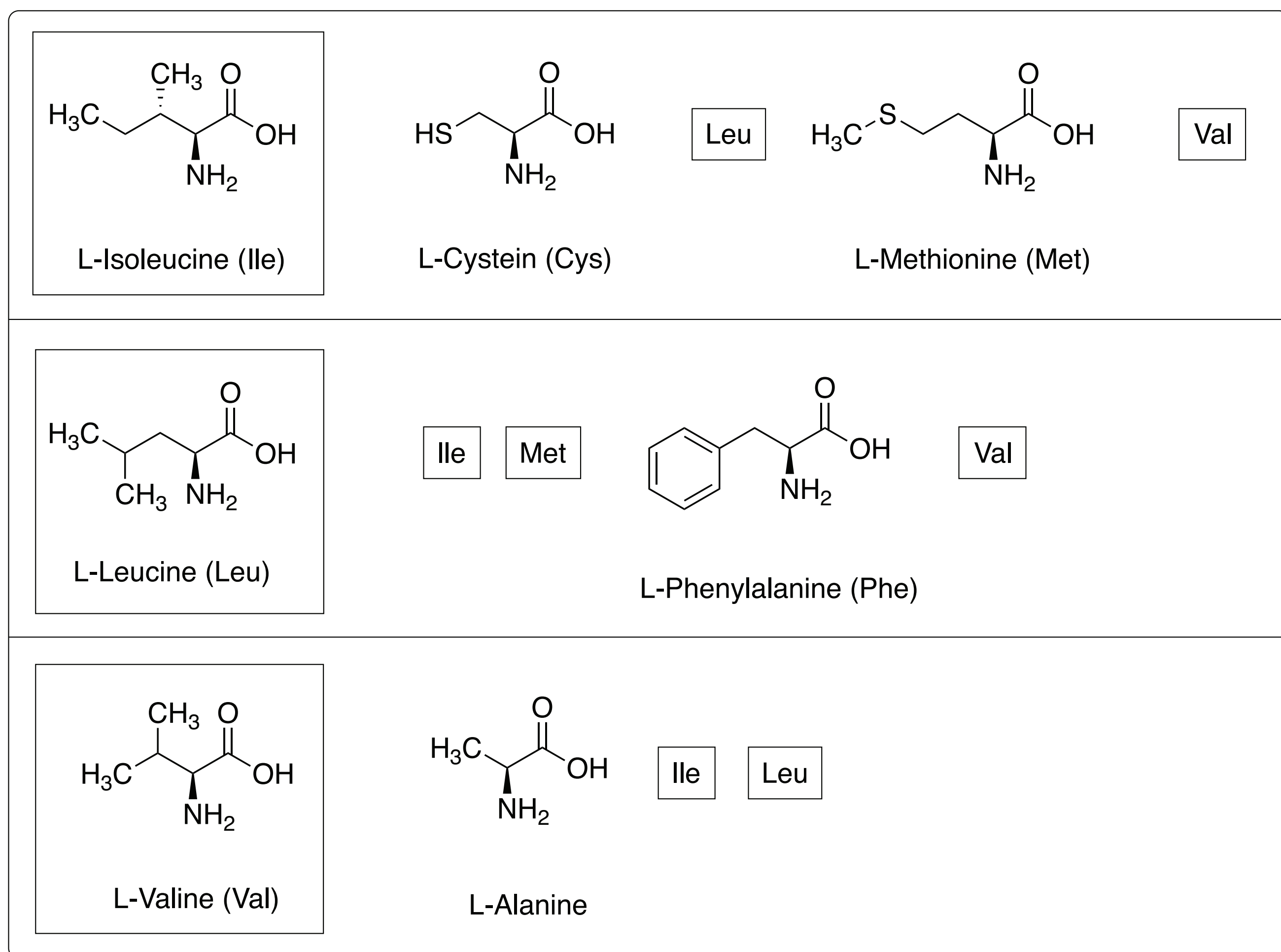

Figure 1. Chemical structures of the BCAA and their respective impurities. For clarity, when a BCAA is an impurity, it is shown as its abbreviation.

Experimental

Table 1. Chemicals

Chemical name	Part number
Deionized water, 18.2 MΩ·cm resistivity or higher	N/A
Acetonitrile (ACN), Optima™ LC/MS grade	A955-212
Ammonium formate, Optima™ LC/MS grade	A11550
Formic acid, 99.0+%, Optima™ LC/MS grade	A117-50
Heptafluorobutyric acid (HFBA) ≥ 99.5%	Sigma-Aldrich (Steinheim, Germany)
Trifluoroacetic acid (TFA) ≥ 99%	Sigma-Aldrich (Steinheim, Germany)
L-Alanine (Ala) ≥ 98%	Sigma-Aldrich (Steinheim, Germany)
L-Cysteine (Cys) ≥ 97%	Sigma-Aldrich (Steinheim, Germany)
L-Isoleucine (Ile) ≥ 98%	Sigma-Aldrich (Steinheim, Germany)
L-Leucine (Leu) ≥ 98%	Sigma-Aldrich (Steinheim, Germany)
L-Methionine (Met) ≥ 98%	Sigma-Aldrich (Steinheim, Germany)
L-Phenylalanine (Phe) ≥ 98%	Sigma-Aldrich (Steinheim, Germany)
L-Valine (Val) ≥ 98%	Sigma-Aldrich (Steinheim, Germany)

Table 2. Sample handling

Item name	Part number
Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer	14-955-152
Vials (amber, 2 mL), Fisher Scientific	03-391-6
Thermo Scientific™ 11 mm Autosampler Snap-It Caps	13-622-292

Standard preparation

Standard solutions of the amino acids were prepared by dissolving 10.0 mg of the respective substance in 10.0 mL water. For the HILIC method, the standard solutions were diluted to the required concentration in the HILIC mobile phase consisting of a mixture of 50 mM aqueous ammonium formate (pH 2.8) and ACN (20:80, v/v). For the IPC method, the standard solutions were diluted with water. All standard solutions were stored at 8 °C.

Sample solution

Sample solutions of Ile, Leu, and Val were prepared daily by weighing of 100 mg of the respective sample, with each dissolved in 10.0 mL water. For the IPC method, the samples were injected without further dilution. For the HILIC method, the sample solutions were diluted with a mixture of ACN/water resulting in a sample concentration of 2.5 mg/mL and an ACN proportion of 70% (v/v).

Table 3. Instrumentation

Module	Part number
Vanquish Flex UHPLC system consisting of:	
Vanquish System Base	VH-S01-A
Vanquish Binary Pump F	VF-P10-A-01
Vanquish Split Sampler FT	VF-A10-A
Vanquish Column Compartment H with passive pre-heater	VH-C10-A-02
Vanquish Charged Aerosol Detector H	VH-D20-A
Corona 1010 Nitrogen generator	6295.0200

Chromatography Data System

Thermo Scientific™ Chromeleon™ Chromatography Data system (CDS) was used for data acquisition and data analysis.

Table 4. IPC and HILIC methods

	IPC method		HILIC method
Column	Thermo Scientific™ Acclaim™ Polar Advantage II (150 × 4.6 mm, 3 µm), **P/N 063191**		Thermo Scientific Accucore™ 150 Amide HILIC (150 × 4.6 mm, 2.6 µm), **P/N 16726-154630**
Mobile phase	A: 11.5 mM HFBA and 6.5 mM TFA in water B: ACN		50 mM aqueous ammonium formate (pH 2.8)/ACN (20:80, v/v)
Gradient	**Time (min)**	**%B**	Isocratic
	0–2	2	
	2–3	2–14	
	3–15	14	
	15–16	2	
	16–20	2	
Run time	20 min		16 min
Flow rate	0.8 mL/min		0.6 mL/min
Column temperature	25 °C		25 °C
Autosampler temperature	8 °C		8 °C
Autosampler wash solvent	No wash		No wash
Injection volume	10 µL		15 µL
CAD settings	Evaporation temperature: 50 °C Power function value: 1.0 Filter constant: 5 s Data collection rate: 10 Hz		Evaporation temperature: 50 °C Power function value: 1.0 Filter constant: 5 s Data collection rate: 10 Hz

Results and discussion

For validation, the performance of both methods was compared with regards to selectivity, LOQ, and linearity. Both approaches are sufficient to resolve all BCAA and impurities in less than 16 min. Figure 2 shows the use of both IPC and HILIC for the measurement of BCAA and their key impurities, with impurities spiked at levels expected to be found in samples.

As CAD behaves as a mass-flow dependent device, sensitivity of the two methods was compared based on mass injected on column. For 5 ng, each of the 7 amino acids studied, S/N was approximately 3 times higher, estimated LOQ was approximately 2 times lower, and better precision (lower % RSD) was obtained with the HILIC method. The linearity range for the IPC and HILIC method was 15–120 ng on column ($R^2 \geq 0.990$) and 10–80 ng on column ($R^2 \geq 0.991$), respectively. These ranges met the requirement of a compendial method covering 0.03–0.24% impurity concentrations with respect to the main substance.[12] More validation details can be found in Reference 8.

The composition of the mobile phase can have a major impact on CAD response.[4] The higher the proportion of organic content in the mobile phase, as is typical for HILIC approaches, the greater the response. This becomes evident when comparing the S/N and limits of quantitation (LOQ) between the two approaches, with HILIC producing twice the response and approximately half the LOQ of the IPC method (see Reference 8 for greater detail). Increasing ACN content beyond 80% led to improved analyte resolution but negatively impacted sensitivity due to peak broadening, lower column efficiency, and increased background current (data not shown). The latter could be a result of column bleed. Depending on the chemical modification of silica columns, differences in the stationary phase stability can be observed, and, in the worst case, lead to increased background currents and noise. These issues are more prominent with HILIC columns. A more stable silica-based column with amide groups bonded to the solid core particles, such as the Accucore 150 Amide HILIC column, is preferred.

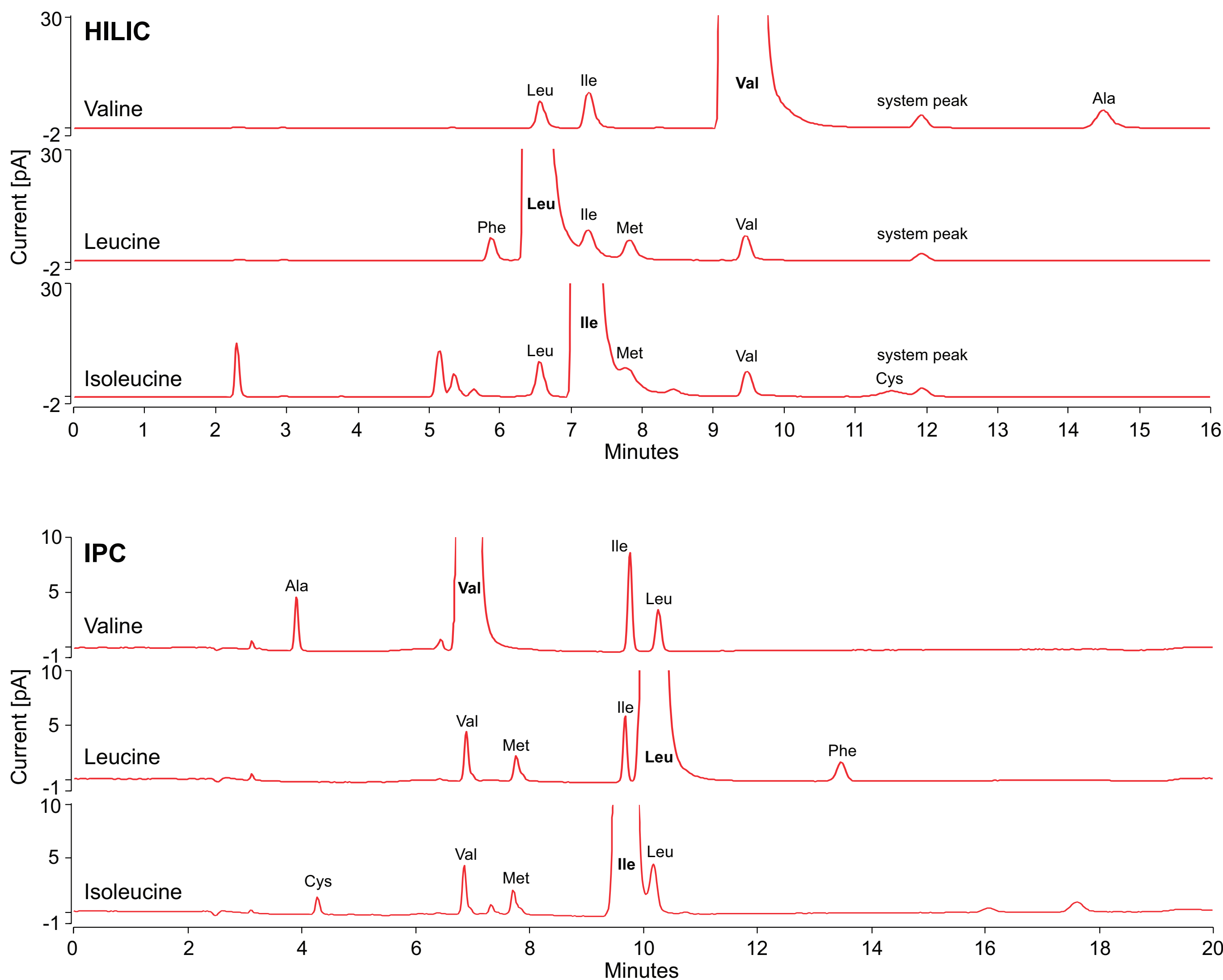

Figure 2. Comparison of zoomed chromatograms for impurity profiling of the BCAAs obtained using IPC (bottom) or HILIC (top). Individual impurities were spiked at 0.1% (m/m). Unlabeled peaks represent unknown impurities.

Unlike the isocratic conditions used with the HILIC method, the IPC approach required the use of a gradient to improve the retention times and peak shapes of late eluting, hydrophobic amino acids. It should be noted that ion pair levels are affected by the organic content of the mobile phase and thus will change as the column is exposed to a gradient. Sufficient time must be allowed during each run to establish ion pairing agent equilibrium or variation in analyte retention time may be observed. With the CAD it is essential to consider the effects of the ion pair type and concentration on chromatographic separation and detection. During method development a variety of ion pairs were evaluated at different concentrations (data not shown).[8] A mixture of HFBA at 11.5 mM with TFA at 6.5 mM was found to give optimal analyte separation and was used for all studies presented in this application note. Another factor to consider when using a CAD is that like all mobile phase components, the ion pairing agent must be volatile. The ion pairing ion can form less volatile salts with other ionic species of opposite charge (e.g., analytes, other additives, impurities, or sample matrix components) that coexist within a given aerosol droplet, leading to increased detector noise.[4] Finally, the purity of ion pair agents can vary from vendor to vendor with contaminants adversely affecting CAD performance.

To strengthen the power of the described HILIC method, the performance of the HILIC method was evaluated against a published IPC method.[13] Both methods achieved comparable performance. However, the IPC method needs a sample concentration four times higher than the HILIC method due to the lower sensitivity.

Conclusion

- Both IPC and HILIC methods can be used for direct measurement of amino acids using the CAD.
- The HILIC method outperformed an IPC method proposed for compendial implementation.[8] Furthermore, the HILIC method is more straightforward, selective, and unlike current compendial methods, does not require derivatization.
- The Accucore 150 Amide HILIC column is the preferred stationary phase due its stability against column bleeding and thus avoiding method performance loss by increased background noise.

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