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Determination of Urea and Allantoin in Cosmetics Using the Acclaim Mixed-Mode HILIC Column

INTRODUCTION

Reversed-phase (RP) silica columns (e.g., C18 and C8) are widely used for separating small molecules. However, these columns are unsuitable for retaining and separating highly polar compounds. Some modified RP columns, such as the Acclaim® PolarAdvantage (PA) column, a type of polar-embedded column, retain some polar compounds. However, chromatographers often need to use a buffer, which usually impairs MS detection and results in insufficient retention of some highly polar compounds. Hydrophilic interaction chromatography (HILIC), termed by Alpert in 1990, is a technique capable of retaining these highly polar compounds with additional benefits, including complementary selectivity compared to RP columns, enhanced sensitivity for MS detection, and simplified sample preparation.¹ However, due to the low-hydrophobicity surfaces associated with traditional HILIC columns (i.e., silica-, cyano-, amino-, and diol- phases), small molecules cannot be separated via hydrophobic interaction. Mixed-mode hydrophilic interaction-cation-exchange chromatography (HILIC-CEX) promotes hydrophilic interactions overlaid on ionic interactions with a cation-exchange matrix and this high-performance technique has the potential for peptide separations.²⁻⁵

The Acclaim Mixed-Mode HILIC column is a new stationary phase that combines both HILIC and RP characteristics.⁶ The new phase is based on high purity and spherical silica functionalized with a silyl ligand containing both hydrophilic and hydrophobic functionalities. This packing material can be used in either HILIC mode (in high organic conditions) or RP mode (in high aqueous conditions). The optimal balance between the hydrophilic and hydrophobic moieties on the silica surface provides unique chromatographic properties that make this new phase useful for many applications, including determination of hydrophobe distribution and degree of ethoxylation (EO number) in a broad variety of ethoxylated surfactants.⁶

In this application note, we investigate the chromatographic behavior of highly polar compounds on the Acclaim Mixed-Mode HILIC column using allantoin and urea as test compounds. We discuss the influence of different sample diluents, and different concentrations and pH values of the buffer in the mobile phases on HILIC. After method optimization, we determined the concentrations of allantoin and urea in cosmetic products.

EQUIPMENT

UltiMate® 3000 HPLC system:

HPG 3400A pump with SRD 3400 Solvent Rack with degasser

TCC-3000 thermostatted column compartment

WPS-3000TSL autosampler

VWD-3400 UV-Vis detector

Chromeleon® 6.80 SP2b Chromatography Workstation

Anke TDL80-2B centrifuge, Anting Scientific Instrumental Factory, Shanghai, China

85-2 magnetic stirrer, Hongpu Instrumental Factory, Minhang, Shanghai, China

PH 140A constant temperature oven, Yiheng Science and Technique Ltd., Shanghai, China

REAGENTS AND STANDARDS

Deionized (DI) water from a Milli-Q® Gradient A10 (Millipore)

Acetonitrile (CH₃CN), HPLC grade, Fisher

Allantoin, purum, ≥ 98%, Fluka

Urea, analytical grade, SCRC, China

n-Heptane, analytical grade, SCRC, China

Acetic acid (HAc), analytical grade, SCRC, China

Ammonium acetate, analytical grade, SCRC, China

Diatomaceous earth, Hyflo Super Cel®, Sigma-Aldrich

CHROMATOGRAPHIC CONDITIONS

Column: Acclaim Mixed-Mode HILIC column, 5 μm, 4.6 × 150 mm (P/N: 066843)

Mobile Phase: Premix of 97% CH₃CN : 3% H₂O (v/v, using premixed mobile phase yields a more stable base-line at the 200 nm detection wavelength)

Flow Rate: 1.0 mL/min

Temperature: 30 °C

Inj. Volume: 5 μL

Detection: Absorbance at 200 nm

PREPARATION OF STANDARDS

Stock Standard Solutions

The concentrations of stock standard solutions were 1000 mg/L for allantoin and 10,000 mg/L for urea. They were prepared with DI water.

Mixed Working Standard Solutions

The mixed stock standard solution was diluted with a solution of 90% acetonitrile, 10% DI H₂O (v/v) to prepare the mixed working standard solutions used for calibration. The concentrations of each analyte in the mixed working standard solutions are shown in Table 1.

Table 1. Concentration of the Mixed Working Standard Solutions

Analyte	Concentration (mg/L)					
	# 1	# 2	# 3	# 4	# 5	# 6
Allantoin	3.125	6.25	12.5	25.00	50.00	125.0
Urea	25.00	50.00	100.0	200.0	400.0	1000

SAMPLE PREPARATION

Two cosmetic products (samples 1 and 2) were purchased from a local market. About 0.5 g of sample and 0.5 g diatomaceous earth were placed into a 50 mL beaker. 10 mL n-heptane was added, and the mixture was stirred for 5 min using a magnetic stirrer. The organic phase was discarded, and the inorganic phase was extracted two more times. The residue was dried completely at 60 °C in a constant temperature oven. Three milliliters of water and 7 mL acetonitrile were added to the dried residue, stirred for 3 min, allowed to stand for 10 min, and then the solution layer was moved to a 10 mL centrifuge tube and centrifuged at 3000 rpm for 10 min. The same sample was extracted two more times with water and acetonitrile. The solution layer of all three extracts was moved to a 100 mL glass flask and diluted to the mark with acetonitrile. Prior to injection, the extract was filtered through a 0.45 μm filter.

RESULTS AND DISCUSSION

Comparison of the Retention of Highly Polar Compounds on Acclaim Mixed-Mode HILIC, Acclaim 120 C18, and Acclaim PA Columns

Allantoin and urea (structures shown in Figure 1) are compounds with high polarity and therefore are good candidates for HILIC. Typical RP silica columns such as the Acclaim 120 C18 are unable to retain these compounds, as shown by chromatogram B in Figure 2. Reducing the CH₃CN to 30% does not result in retention of either compound. These compounds are retained on the Acclaim PA column (a polar-embedded phase, chromatogram C in Figure 2) under conditions that should yield the maximum retention of polar compounds (no CH₃CN), but resolution is poor. Chromatogram A of Figure 2 shows that allantoin and urea are well retained and resolved on the Acclaim Mixed-Mode HILIC column.

Influence of Sample Diluent on Acclaim Mixed-Mode HILIC Column Chromatography

As in RP-HPLC, sample diluent can strongly influence peak shape and sample solubility during HILIC chromatography. Here, three solvents commonly used in HPLC, differing in solvent strength in the order CH₃CN > CH₃OH > water, were used to prepare standard solutions of the polar compounds allantoin and urea. The ideal sample diluent should be 100% CH₃CN or as close to initial mobile phase conditions as possible. As shown in Figure 3, the best peak shapes for allantoin and urea are obtained using 100% CH₃CN as the sample solvent. However, highly polar analytes often have low solubilities in organic solvents, making some samples difficult to run on a HILIC column. Although 100% water can dissolve polar samples better, it is not suitable for injecting on a HILIC column because of the resulting poor peak shape (chromatogram A, Figure 3).

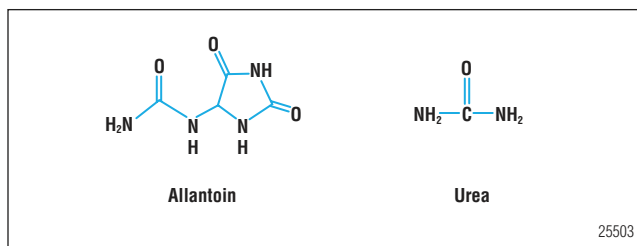


Figure 1. Structures of allantoin and urea.

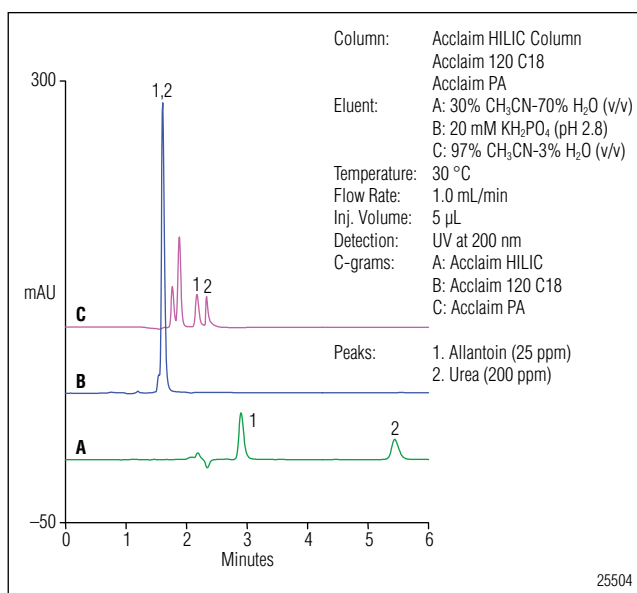


Figure 2. Chromatograms of allantoin and urea on A) Acclaim Mixed-Mode HILIC, B) 120 C18, and C) PA columns.

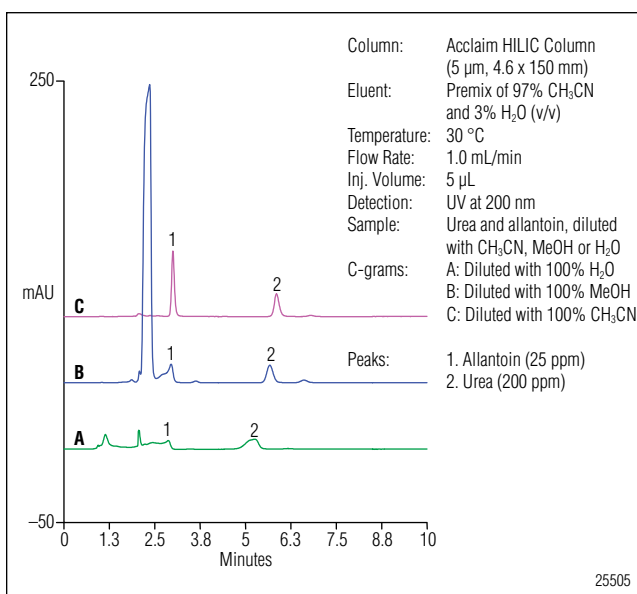


Figure 3. Chromatograms of allantoin and urea diluted with A) water, B) methanol, and C) acetonitrile.

Therefore, the influence of using methanol/water and CH₃CN/water diluents with different proportions on peak shapes of allantoin and urea were investigated. Figure 4 shows chromatograms of allantoin and urea using three different methanol/water mixtures as diluents, and none yielded acceptable peak shapes. Using CH₃CN/water mixtures as diluents, peak shapes are acceptable at 75% CH₃CN and higher (Figure 5). When the proportion of CH₃CN increases to 90%, sharp and symmetric peak shapes are observed. Most polar analytes are soluble in 90% CH₃CN/10% water.

Influence of Mobile Phase Buffer on HILIC Chromatography

Phosphate buffers are not recommended due to precipitation in the highly organic mobile phases commonly used in HILIC. The buffers usually used with HILIC columns are ammonium formate, ammonium acetate, formic acid, and phosphoric acid. We evaluated the influences of the pH value and concentration of an acetic acid-ammonium acetate buffer on HILIC chromatography. The pH value of the acetic acid-ammonium acetate buffer was adjusted by changing the proportion of acetic acid and ammonium acetate. As shown in Figure 6, although the retention time of allantoin

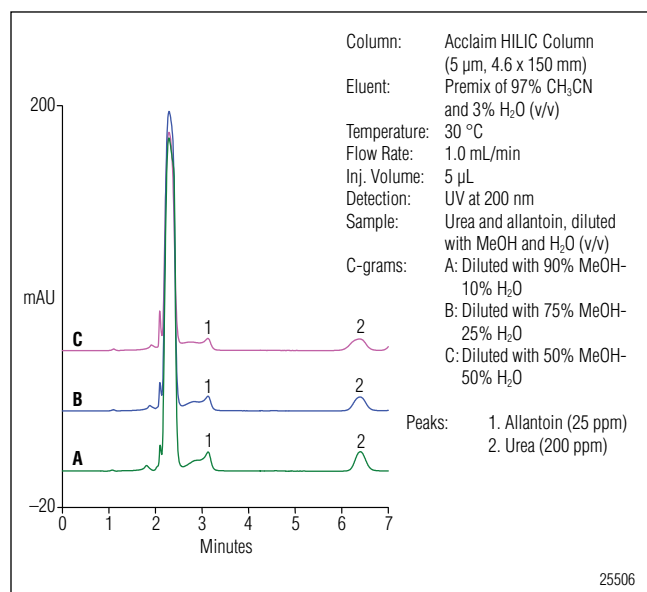


Figure 4. Chromatograms of allantoin and urea diluted with methanol/water with differing proportions.

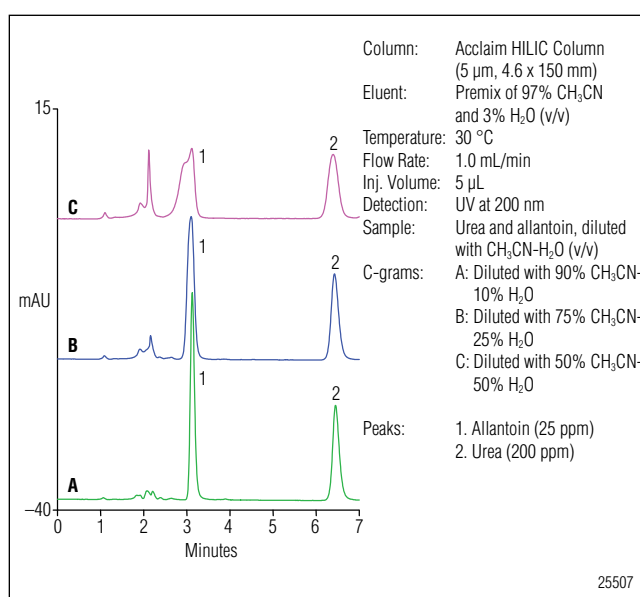


Figure 5. Chromatograms of allantoin and urea diluted with acetonitrile/water with differing proportions.

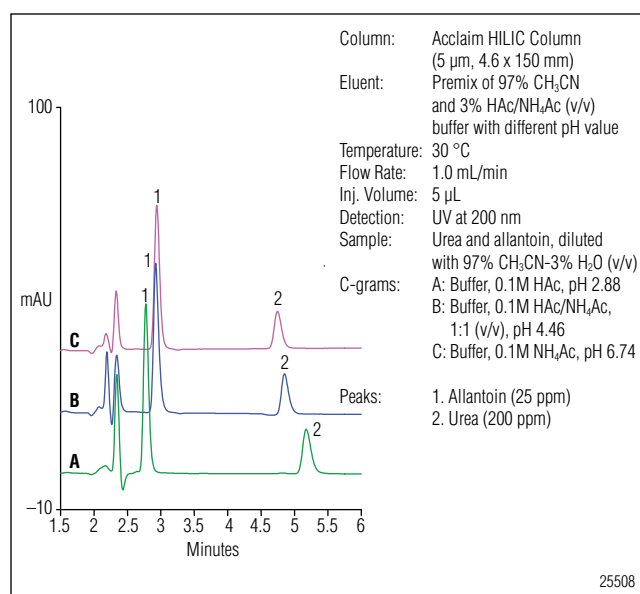


Figure 6. Chromatograms of allantoin and urea when the buffers in mobile phase are at different pH values.

increases and that of urea decreases with increasing buffer pH, their peak shapes are maintained. Figure 7 shows chromatograms of allantoin and urea when the ammonium acetate concentration in the mobile phase changes from 100 to 0 mM (100% H₂O). Peak shapes are also maintained with changes in the buffer concentration, even in the absence of buffer. However, for some ionic compounds, e.g., benzoate, a buffer in the mobile phase is needed for good peak shape and retention (Figure 8).

Reproducibility, Linearity and Detection Limits

Prior to sample analysis, we estimated the reproducibility by making seven replicate injections of a mixed standard solution with concentrations of 25 mg/L for allantoin and 200 mg/L for urea. The RSDs for retention time were both 0.000, and the RSDs for peak area were 0.178 for allantoin and 0.379 for urea.

Calibration linearity for allantoin and urea was investigated by making replicate injections of a mixed standard prepared at six different concentrations. The external standard method was used to calculate the calibration curve and to quantify these compounds in samples. Table 2 shows the data from the calibration as calculated in Chromeleon. The single-sided Student's test method was used for estimating method detection limits (MDL). These data are also reported in Table 2.

Table 2. Calibration Data as Reported by Chromeleon and MDLs for the Two Analytes

Analyte	Equation	r	RSD	MDL (mg/L)
Allantoin	$A = 0.1362 c + 0.0163$	0.9998	1.6408	0.66
Urea	$A = 0.0111 c + 0.0039$	0.9990	2.9740	11.6

Note: The single-sided Student's test method (at the 99% confidence limit) was used for determining MDL, where the standard deviation (SD) of the peak area of seven injections is multiplied by 3.14 to yield the MDL.

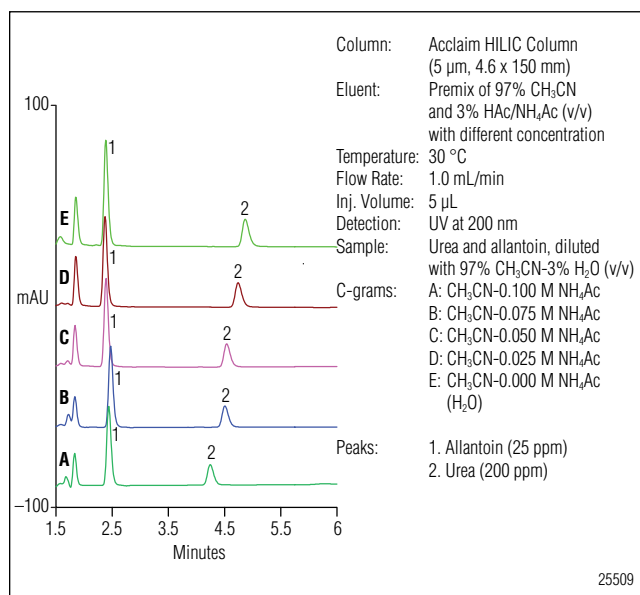


Figure 7. Chromatograms of allantoin and urea diluted when changing buffer (NH₄Ac) concentration in mobile phase

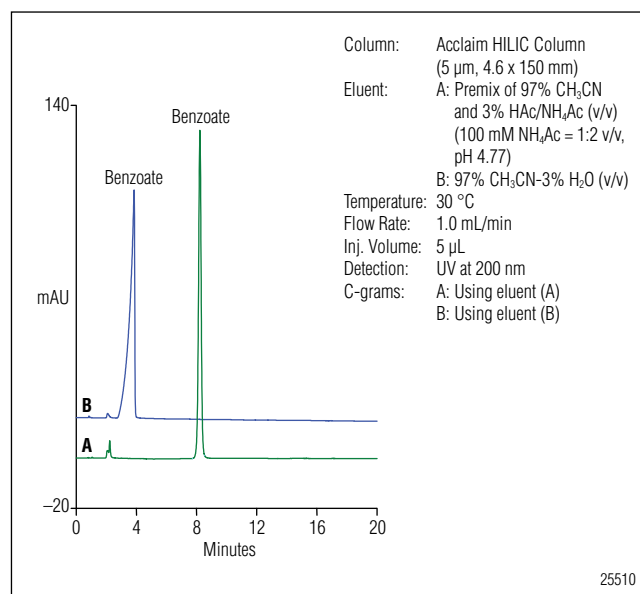


Figure 8. Chromatograms of benzoate on the Acclaim Mixed-Mode HILIC column using different mobile phases.

Sample Analysis

Allantoin and urea are added to cosmetic products for skin protection and regeneration, especially for the treatment of dry skin. We analyzed two different cream samples. Figure 9 shows chromatograms of a blank, sample 2, and the same sample spiked with standards. The amounts of allantoin and urea in each sample and the spike recovery from sample 2 are summarized in Table 3. Urea was found in both of the samples, and allantoin was found in sample 2.

CONCLUSION

For HILIC, sharp symmetric peaks for polar compounds are obtained using 90% acetonitrile, 10% water as the sample diluent. Our recommended buffer for HILIC mobile phases is ammonium acetate. For the determination of urea and allantoin, the pH value of the buffer does not have a significant influence on peak shape. However, keeping a certain buffer concentration in the mobile phase can yield better peak shape for some ionic polar compounds (e.g., for benzoate). Our experiments demonstrate that the Acclaim Mixed-Mode HILIC column is suitable for separation of highly polar compounds such as allantoin and urea and their determination in cosmetic products.

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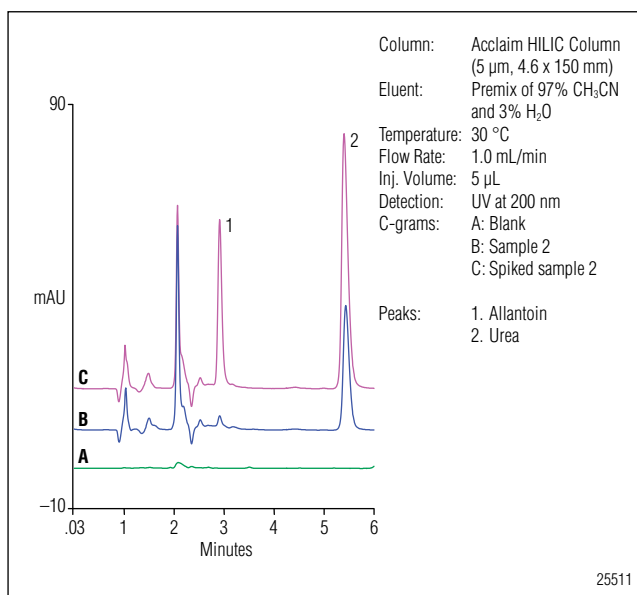


Figure 9. Overlay of chromatograms of the blank, sample 2 and spiked sample 2.

Table 3. Analysis Results for Cosmetic Products

Analyte	Sample 1	Sample 2			
	Detected (g/Kg)	Detected (g/Kg)	Added (g/Kg)	Found (g/Kg)	Recovery (g/Kg)
Allantoin	ND	0.37	5.00	4.92	98.4
Urea	53.5	76.6	90.0	87.6	97.3

- Note: 1. One sample and spiked sample were prepared, respectively, and 3 injections were made for each.
 2. Detected = Measured Value of sample x Diluted fold
 3. Found = Measured value of spiked sample — Measure value of sample
 4. ND = "not detected"

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