# Rapid Analysis of 17 Bile Acids in Human Plasma by LC-MS/MS

## Optimizing Selectivity and Sensitivity in C18 Chemistries and Inert Hardware

By Haley Berkland (Restek Corporation) and Andrew Percy, PhD (Cambridge Isotope Laboratories, Inc.)

**Abstract**

The quantitative analysis of bile acids in plasma is critical for diagnosing many structural liver diseases. Accurate reporting can be difficult because of analyte characteristics, matrix effects, and other factors. In this work, we describe a robust, selective LC-MS/MS method for the analysis of 17 bile acids in under 10 minutes, including three isomer groups, with special attention given to a matrix interference observed in routine human plasma analysis.

**Introduction**

Bile acids are a group of major catabolic products of cholesterol, and they are critical signaling molecules that regulate cholesterol and glucose. The analysis of bile acids in human plasma is an important diagnostic tool as bile acids are biomarkers of liver disease and are also used as indicators of potentially harmful side effects of new drugs. There are two main types of bile acids based upon their functional groups: unconjugated (or free) and conjugated, primarily with glycine- or taurine-based residues (Figure 1, Table I). Quantitation of bile acids in matrix can be very challenging due to several factors. These include structural similarities, varying polarity and stereochemistry, the presence of isomers, limited fragmentation of unconjugated bile acids in a mass spectrometer, high endogenous levels, and matrix effects caused by phospholipids or triglycerides.

**Related Products**

- *Raptor Inert ARC-18 column, 2.7 µm, 100 x 2.1 mm (cat.# 9314A12-T)*
- *Raptor Inert ARC-18 EXP guard column cartridge, 2.7 µm, 5 x 2.1 mm (9314A0252-T)*
- *Amber vial, 2 mL (cat.# 21143)*
- *Vial insert, glass (cat.# 21776)*
- *Vial cap (cat.# 24498)*
- *EXP direct connect holder for EXP guard cartridges (cat.# 25808)*

**Figure 1:** Bile Acids Base Chemical Structure

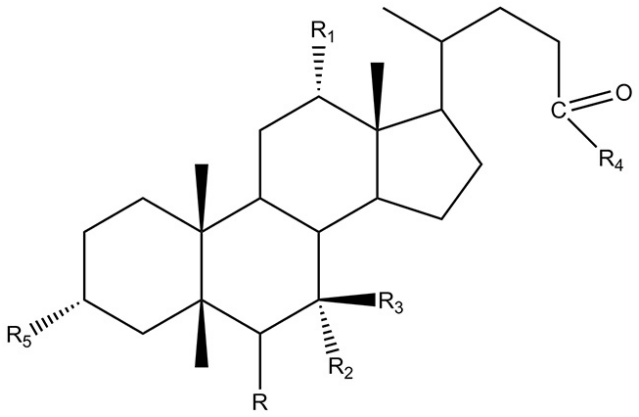


**Table I:** R Group Identifications for Figure 1

| Compound Name | R | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|---|
| Ursodeoxycholic acid (UDCA) | H | H | H | OH | OH | OH |
| Hyodeoxycholic acid (HDCA) | OH | H | H | H | OH | OH |
| Cholic acid (CA) | H | OH | H | OH | OH | OH |
| Chenodeoxycholic acid (CDCA) | H | H | H | OH | OH | OH |
| Deoxycholic acid (DCA) | H | OH | H | H | OH | OH |
| Dehydrolithocholic acid (DHLCA) | H | H | H | H | OH | =O |
| Lithocholic acid (LCA) | H | H | H | H | OH | OH |
| Glycoursodeoxycholic acid (GUDCA) | H | H | H | OH | $NHCH_2CO_2H$ | OH |
| Glycocholic acid (GCA) | H | OH | H | OH | $NHCH_2CO_2H$ | OH |
| Glycochenodeoxycholic acid (GCDCA) | H | H | H | OH | $NHCH_2CO_2H$ | OH |
| Glycodeoxycholic acid (GDCA) | H | OH | H | H | $NHCH_2CO_2H$ | OH |
| Glycolithocholic acid (GLCA) | H | H | H | H | $NHCH_2CO_2H$ | OH |
| Tauroursodeoxycholic acid (TUDCA) | H | H | H | OH | $NHCH_2CH_2SO_3H$ | OH |
| Taurocholic acid (TCA) | H | OH | H | OH | $NHCH_2CH_2SO_3H$ | OH |
| Taurochenodeoxycholic acid (TCDCA) | H | H | H | H | $NHCH_2CH_2SO_3H$ | OH |
| Taurodeoxycholic acid (TDCA) | H | OH | H | H | $NHCH_2CH_2SO_3H$ | OH |
| Taurolithocholic acid (TLCA) | H | H | H | H | $NHCH_2CH_2SO_3H$ | OH |

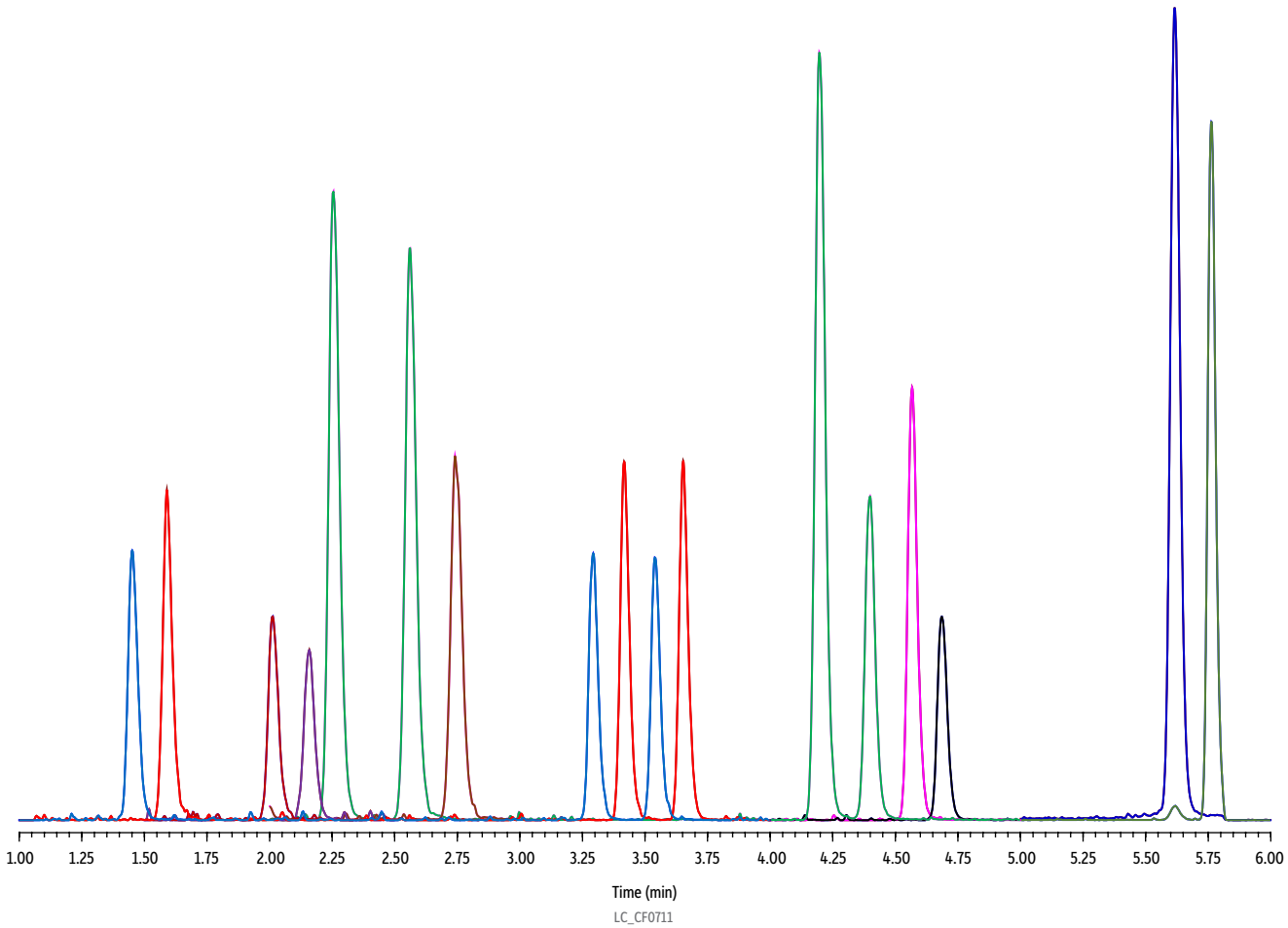
As mentioned above, matrix effects are a commonly encountered issue in bile acids analysis where interferences with the analytes of interest are caused by the sample matrix or other substances that are added to the sample. Matrix interferences may present as a partially or fully coeluting peak with one of the analytes or internal standards, or as a reduction in the MS signal, which may impact the limit of detection and method accuracy and precision for a particular analyte(s). As a result, matrix interferences can cause issues with identification and quantitation of the analytes of interest. Pinpointing the source of matrix interferences can be difficult as they can be caused by many factors. Patient medications and lifestyle/diet factors; sample preservatives or anticoagulants; contamination during sample processing; or compounds endogenous to the matrix all could result in matrix interference. Because matrix interferences can have such an impact on the performance of an LC-MS/MS assay, it is extremely important that any potential interferences are identified and explored during method development and validation.

As many method developers know, it can be difficult to address all the potential matrix interferences that may be present in authentic patient samples when using a surrogate matrix during routine validation. Several years ago, Restek released an application note for the analysis of 17 bile acids in plasma by LC-MS/MS. Utilizing a Raptor C18 column, this method effectively separated 17 bile acids, including three sets of isomers, in an 8.5-minute runtime. When further studies were performed with this method, it was discovered that a matrix interference present in clinical patient samples was partially coeluting with one of the deuterated bile acid internal standards, resulting in inconsistent internal standard response. To address this issue, a new analytical method was developed herein. The objectives of the revised method were to resolve the matrix interference while still achieving respectable separation of the 17 target bile acids, including the three sets of isomers, for subsequent qualification and quantification LC-MS/MS studies.

### *Original Bile Acids Method and Matrix Interference*

The original application was developed on a Raptor C18 column, and the method parameters are shown in Figure 2.

**Figure 2:** 17 Bile Acids in Human Plasma on Raptor C18 (Original Method)




| | |
|---|---|
| **Column** | Raptor C18 (cat.# 9304252) |
| Dimensions: | 50 mm x 2.1 mm ID |
| Particle Size: | 1.8 µm |
| Pore Size: | 90 Å |
| Guard Column: | UltraShield UHPLC precolumn filter 0.2 µm (cat.# 25810) |
| Temp.: | 60 °C |
| **Sample** | |
| Diluent: | 70:30 Water:methanol |
| Inj. Vol.: | 3 µL |
| **Mobile Phase** | |
| A: | 5 mM Ammonium acetate in water |
| B: | 50:50 Acetonitrile:methanol |

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.5 | 65 | 35 |
| 2.00 | 0.5 | 60 | 40 |
| 2.50 | 0.5 | 55 | 45 |
| 3.50 | 0.5 | 50 | 50 |
| 4.60 | 0.5 | 45 | 55 |
| 5.70 | 0.5 | 20 | 80 |
| 5.90 | 0.8* | 5 | 95 |
| 6.50 | 0.8* | 5 | 95 |
| 6.51 | 0.5 | 65 | 35 |
| 8.50 | 0.5 | 65 | 35 |

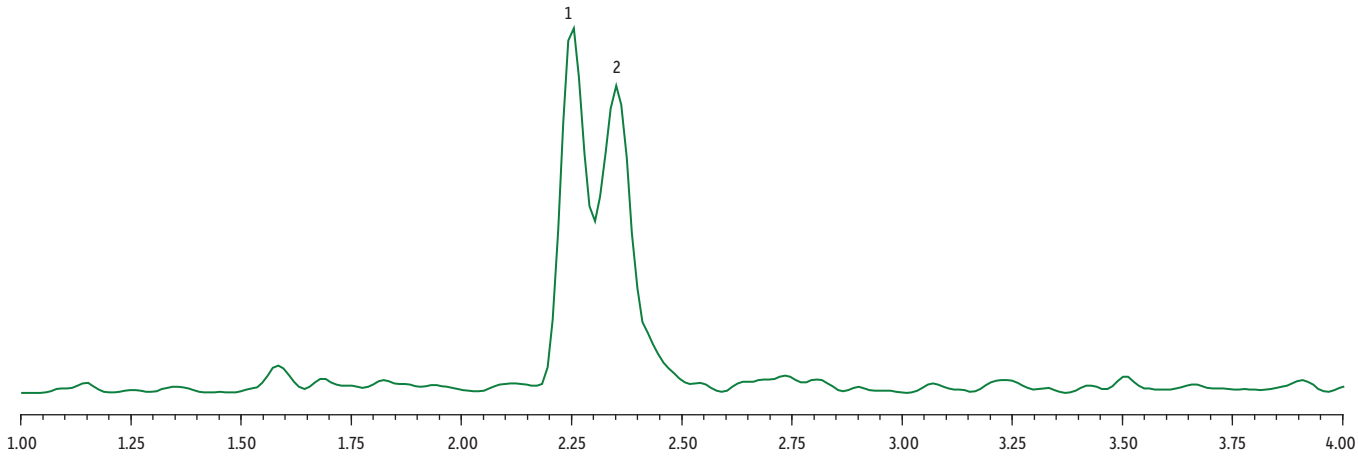
| | |
|---|---|
| **Detector** | MS/MS |
| Ion Mode: | ESI- |
| Mode: | MRM |
| **Instrument** | UHPLC |
| **Notes** | *The flow rate was increased to 0.8 mL/min to more thoroughly flush phospholipids from the analytical column, thereby reducing matrix effects. |

Want even better performance when analyzing metal-sensitive compounds? Check out Inert LC columns at www.restek.com/inert.

| Peaks | $t_R$ (min) | Conc. (ng/mL) | Precursor Ion | Product Ion |
|---|---|---|---|---|
| 1. Glycoursodeoxycholic acid (GUDCA) | 1.45 | 540 | 448.4 | 74.1 |
| 2. Tauroursodeoxycholic acid (TUDCA) | 1.59 | 1,080 | 498.4 | 80.1 |
| 3. Glycocholic acid (GCA) | 2.01 | 540 | 464.3 | 74.2 |
| 4. Taurocholic acid (TCA) | 2.16 | 1,080 | 514.4 | 80.0 |
| 5. Ursodeoxycholic acid (UDCA) | 2.25 | 360 | 391.4 | 391.4 |
| 6. Hyodeoxycholic acid (HDCA) | 2.56 | 360 | 391.4 | 391.4 |
| 7. Cholic acid (CA) | 2.74 | 90 | 407.3 | 407.2 |
| 8. Glycochenodeoxycholic acid (GCDCA) | 3.29 | 540 | 448.4 | 74.1 |
| 9. Taurochenodeoxycholic acid (TCDCA) | 3.42 | 1,080 | 498.4 | 80.1 |
| 10. Glycodeoxycholic acid (GDCA) | 3.54 | 540 | 448.4 | 74.1 |
| 11. Taurodeoxycholic acid (TDCA) | 3.65 | 1,080 | 498.4 | 80.1 |
| 12. Chenodeoxycholic acid (CDCA) | 4.20 | 360 | 391.4 | 391.4 |
| 13. Deoxycholic acid (DCA) | 4.40 | 90 | 391.4 | 391.4 |
| 14. Glycolithocholic acid (GLCA) | 4.57 | 540 | 432.3 | 74.0 |
| 15. Taurolithocholic acid (TLCA) | 4.69 | 1,080 | 482.4 | 80.0 |
| 16. Dehydrolithocholic acid (DHLCA) | 5.62 | 90 | 373.3 | 373.3 |
| 17. Lithocholic acid (LCA) | 5.76 | 180 | 375.5 | 375.3 |

When patient samples were later analyzed with the original method described in Figure 2, a matrix interference was found to be partially coeluting with $D_4$ UDCA, the deuterated internal standard used for quantitation of UDCA. The matrix interference present when analyzed with the original method is shown in Figure 3.

**Figure 3:** Matrix Interference Partially Coeluting with $D_4$ UDCA on Raptor C18 (Original Method)




| Peaks | $t_R$ (min) | Precursor Ion | Product Ion |
|---|---|---|---|
| 1. Matrix interference | 2.25 | - | - |
| 2. $D_4$ UDCA | 2.35 | 395.40 | 395.40 |

| | |
|---|---|
| **Column** | Raptor C18 (cat.# 9304252) |
| Dimensions: | 50 mm x 2.1 mm ID |
| Particle Size: | 1.8 µm |
| Pore Size: | 90 Å |
| Guard Column: | UltraShield UHPLC precolumn filter 0.2 µm (cat.# 25810) |
| Temp.: | 60 °C |
| **Standard/Sample** | |
| Diluent: | 65:35 Water:methanol |
| Inj. Vol.: | 3 µL |
| **Mobile Phase** | |
| A: | 5 mM ammonium acetate in water, pH unadjusted |
| B: | Methanol:acetonitrile (v/v, 50:50) |

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.5 | 65 | 35 |
| 2.00 | 0.5 | 60 | 40 |
| 2.50 | 0.5 | 55 | 45 |
| 3.50 | 0.5 | 50 | 50 |
| 4.60 | 0.5 | 45 | 55 |
| 5.70 | 0.5 | 20 | 80 |
| 5.90 | 0.8* | 5 | 95 |
| 6.50 | 0.8* | 5 | 95 |
| 6.51 | 0.5 | 65 | 35 |
| 8.50 | 0.5 | 65 | 35 |

| | |
|---|---|
| **Max Pressure:** | 275 bar |
| **Detector** | Shimadzu LCMS-8045 in ESI- mode |
| **Instrument** | Shimadzu Nexera X2 |
| **Sample Preparation** | A 50 µL aliquot of patient sample was added to a microcentrifuge tube. Fifty microliters of internal standards was added to the tube and vortexed. The samples were protein precipitated using 800 µL of ice-cold acetonitrile. After vortexing and centrifugation at 4200 rpm for 10 min, the supernatant was transferred to a new vial and dried down at 60 °C under nitrogen. All samples were reconstituted in 200 µL of 35% methanol in water. |
| **Notes** | *The flow rate was increased to 0.8 mL/min to more thoroughly flush phospholipids from the analytical column, thereby reducing matrix effects.<br><br>The flow was diverted to waste before 1 minute and after 6 minutes to protect the mass spectrometer. |

In some cases of matrix interferences, the simplest option to remove the interference is to choose an alternative product ion that is not affected. Because unconjugated bile acids exhibit limited fragmentation in a mass spectrometer, $D_4$ UDCA uses the parent mass as both the precursor and product ions, meaning that choosing a different product ion is not an option for this compound.

*Method Requirements*

The primary requirement of the redeveloped method was to chromatographically separate the matrix interference from $D_4$ UDCA. Additionally, the analyte list contained three sets of isomeric compounds: taurine conjugated isomers (TUDCA, TCDCA, TDCA); glycine conjugated isomers (GUDCA, GCDCA, GDCA); and unconjugated isomers (UDCA, HDCA, CDCA, and DCA). The method was also required to adequately resolve the isomers within these sets.

*Method Optimization*

A Raptor Inert ARC-18 100 x 2.1 mm, 2.7 µm analytical column (cat.# 9314A12-T) was selected for development of the new method. A Raptor Inert ARC-18 5 x 2.1 mm, 2.7 µm EXP guard column (cat.# 9314A0252-T) was also installed to protect the analytical column. The elution gradient, column temperature, flow rate, and injection volume were optimized to provide adequate resolution of all analytes and the matrix interference.

## Experimental

*Calibration Standards and Quality Control Samples*

Bile acid standard mixtures were obtained from Cambridge Isotope Laboratories. The stable, isotope-labeled bile acids mixes (unconjugated, cat.# MSK-BA1; conjugated, cat.# MSK-BA2) were used as internal standards. $D_4$ hyodeoxycholic acid (cat.# DLM-11626-0) was also obtained from Cambridge Isotope Laboratories as a separate standard. Unlabeled bile acids mixes (unconjugated, cat.# MSK-BA1-US; conjugated, cat.# MSK-BA2-US) were used as primary reference standards. Reference standards for hyodeoxycholic acid (HDCA) and dehydrolithocholic acid (DHLCA) were obtained separately.

Calibration standards and fortified QC samples were prepared at the concentrations shown in Table II. An internal standard working solution comprising the 17 isotopically labeled bile acids was prepared to a concentration of 2 µM.

**Table II:** Calibration Standard and Quality Control Sample Concentrations in Plasma (µM) for All Analytes

| Cal F | Cal E | Cal D | Cal C | Cal B | Cal A | LLOQ | Low QC | Medium QC | High QC |
|---|---|---|---|---|---|---|---|---|---|
| 0.05 | 0.1 | 0.2 | 0.5 | 1 | 5 | 0.05 | 0.1 | 0.5 | 1 |

*Sample Preparation*

For control samples, a 90 µL aliquot of 2x charcoal-stripped plasma ($K_2$·EDTA) was spiked with 10 µL of calibrator/QC material and vortexed. Internal standards (10 µL) were added and vortexed. Samples were protein precipitated using 400 µL of ice-cold acetonitrile. After vortexing and centrifugation at 4200 rpm for 15 minutes, the supernatant was transferred to a glass test tube and dried down under nitrogen. All samples were reconstituted in 200 µL of 60:40 water:mobile phase B (MPB). MPB was 50:50 methanol:acetonitrile (v/v).

*Instrument Conditions*

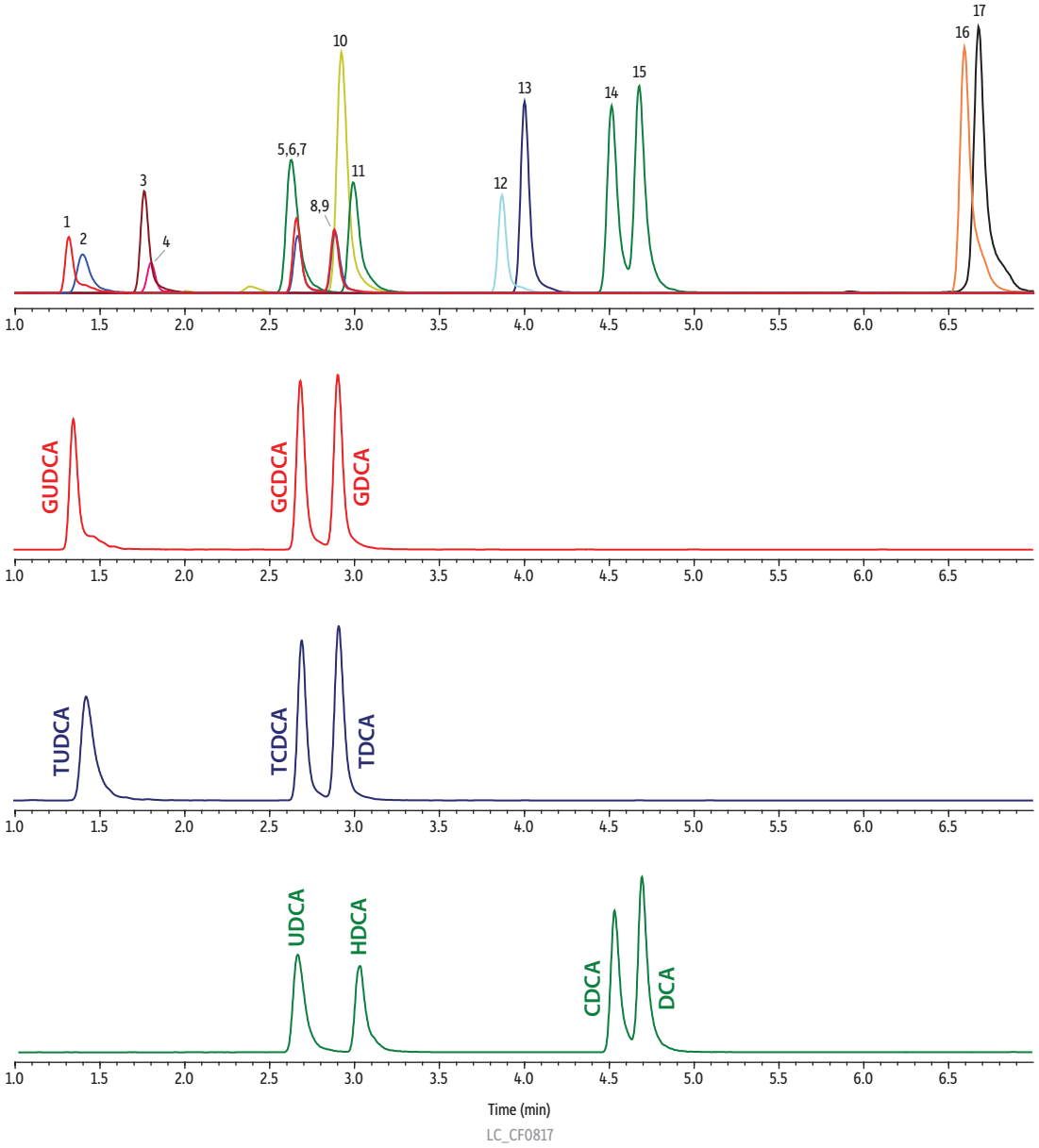
LC-MS/MS analysis of bile acids in human plasma was performed on a Shimadzu Nexera UHPLC with a Shimadzu LCMS-8045 MS/MS in negative ESI mode. Instrument conditions and analyte transitions for the new method are provided in Figure 4.

## Results and Discussion

*Chromatographic Performance*

The 17 bile acids, including three sets of isomers, were well separated over 9.5 minutes (total run time). The matrix interference was also appreciably separated from $D_4$ UDCA (Figure 5).

**Figure 4:** Bile Acids with Isomer Separation in Human Plasma on Raptor Inert ARC-18 (New Method)




| Peaks | tR (min) | Precursor Ion | Product Ion |
|---|---|---|---|
| 1. GUDCA | 1.32 | 448.40 | 74.15 |
| 2. TUDCA | 1.40 | 498.10 | 80.05 |
| 3. GCA | 1.78 | 464.10 | 74.15 |
| 4. TCA | 1.80 | 514.20 | 80.05 |
| 5. UDCA | 2.63 | 391.50 | 391.50 |
| 6. GCDCA | 2.66 | 448.40 | 74.15 |
| 7. TCDCA | 2.66 | 498.10 | 80.05 |
| 8. GDCA | 2.88 | 448.40 | 74.15 |
| 9. TDCA | 2.88 | 498.10 | 80.05 |
| 10. CA | 2.92 | 407.20 | 407.20 |
| 11. HDCA | 2.99 | 391.50 | 391.50 |
| 12. TLCA | 3.87 | 482.10 | 80.05 |
| 13. GLCA | 4.00 | 432.20 | 74.15 |
| 14. CDCA | 4.51 | 391.50 | 391.50 |
| 15. DCA | 4.68 | 391.50 | 391.50 |
| 16. LCA | 6.59 | 375.40 | 375.40 |
| 17. DHLCA | 6.68 | 373.20 | 373.20 |

**Column**
Dimensions: 100 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Inert ARC-18 EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9314A0252-T)
Temp.: 50 °C

**Standard/Sample**
Diluent: 60:40 Water:mobile phase B
Conc.: 5 µM
Inj. Vol.: 5 µL

**Mobile Phase**
A: 5 mM Ammonium acetate in water, pH unadjusted
B: Methanol:acetonitrile (v/v, 50:50)

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.5 | 60 | 40 |
| 6.00 | 0.5 | 30 | 70 |
| 7.00 | 0.5 | 20 | 80 |
| 7.50 | 0.8* | 0 | 100 |
| 8.10 | 0.8* | 0 | 100 |
| 8.20 | 0.5 | 60 | 40 |
| 9.50 | 0.5 | 60 | 40 |

Max Pressure: 365 bar

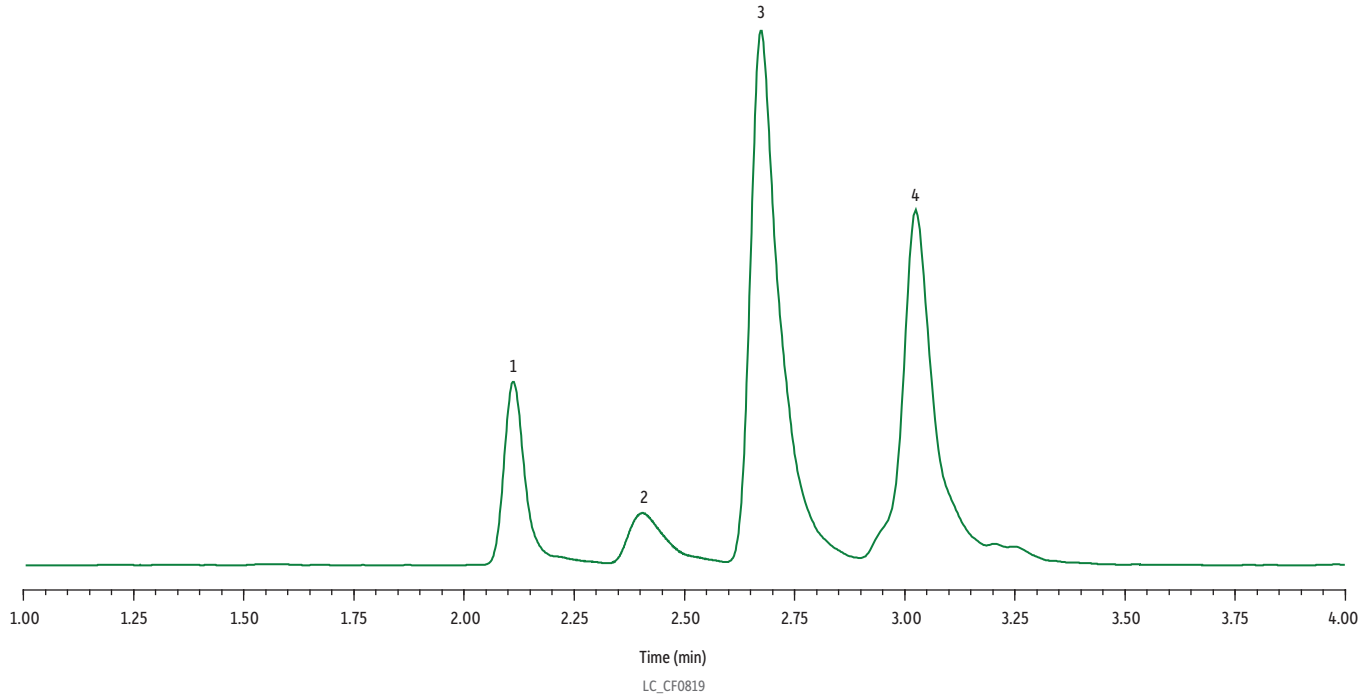
**Detector** Shimadzu LCMS-8045 in ESI- mode
**Instrument** Shimadzu Nexera X2

**Sample Preparation** For control samples, a 90 µL aliquot of 2x charcoal-stripped plasma ($K_2$EDTA) was added to a microcentrifuge tube and spiked with 10 µL of calibrator/QC material and vortexed. Ten microliters of internal standards was added and vortexed. Samples were protein precipitated using 400 µL of ice-cold acetonitrile. After vortexing and centrifugation at 4200 rpm for 15 minutes, the supernatant was transferred to a glass test tube and dried down under nitrogen. All samples were reconstituted in 200 µL of 60:40 water:mobile phase B. The sample was transferred to a clean 2 mL screw-thread vial (cat.# 21143) with a glass insert (cat.# 21776) and capped with short-cap, screw-vial closures (cat.# 24498).

**Notes** *The flow rate was increased to 0.8 mL/min to more thoroughly flush phospholipids from the analytical column, thereby reducing matrix effects.

The flow was diverted to waste before 1 minute and after 7 minutes to protect the mass spectrometer.

**Figure 5:** Plasma Matrix Interference Separated from $D_4$ UDCA on Raptor Inert ARC-18




| Peaks | $t_R$ (min) | Precursor Ion | Product Ion |
|---|---|---|---|
| 1. Matrix interference | 2.11 | - | - |
| 2. Matrix interference | 2.41 | - | - |
| 3. $D_4$ UDCA | 2.67 | 395.40 | 395.40 |
| 4. $D_4$ HDCA | 3.02 | 395.40 | 395.40 |

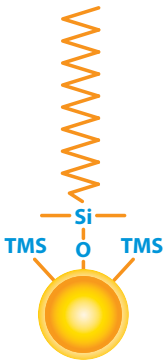
| | |
|---|---|
| **Column** | Raptor Inert ARC-18 (cat.# 9314A12-T) |
| Dimensions: | 100 mm x 2.1 mm ID |
| Particle Size: | 2.7 µm |
| Pore Size: | 90 Å |
| Guard Column: | Raptor Inert ARC-18 EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9314A0252-T) |
| Temp.: | 50 °C |
| Standard/Sample Diluent: | 60:40 Water:MPB |
| Inj. Vol.: | 5 µL |
| Mobile Phase | |
| A: | 5 mM ammonium acetate in water, pH unadjusted |
| B: | Methanol:acetonitrile (v/v, 50:50) |

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.5 | 60 | 40 |
| 6.00 | 0.5 | 30 | 70 |
| 7.00 | 0.5 | 20 | 80 |
| 7.50 | 0.8* | 0 | 100 |
| 8.10 | 0.8* | 0 | 100 |
| 8.20 | 0.5 | 60 | 40 |
| 9.50 | 0.5 | 60 | 40 |

| | |
|---|---|
| **Max Pressure:** | 365 bar |
| **Detector** | Shimadzu LCMS-8045 in ESI- mode |
| **Instrument** | Shimadzu Nexera X2 |
| **Sample Preparation** | For control samples, a 100 µL aliquot of patient sample was added to a microcentrifuge tube. Ten microliters of internal standards was added and vortexed. Samples were protein precipitated using 400 µL of ice-cold acetonitrile. After vortexing and centrifugation at 4200 rpm for 15 minutes, the supernatant was transferred to a glass test tube and dried down under nitrogen. All samples were reconstituted in 200 µL of 60:40 water:MPB. The sample was transferred to a clean 2 mL screw-thread vial (cat.# 21143) with a glass insert (cat.# 21776) and capped with short-cap, screw-vial closures (cat.# 24498). |
| **Notes** | The flow rate was increased to 0.8 mL/min to more thoroughly flush phospholipids from the analytical column, thereby reducing matrix effects.<br><br>The flow was diverted to waste before 1 minute and after 7 minutes to protect the mass spectrometer. |

The new method developed here utilized a Raptor ARC-18 column, whereas the original method used a Raptor C18 column. Both the Raptor ARC-18 and Raptor C18 columns are C18 stationary phases and show similar selectivity for the three sets of isomers, allowing for adequate separation of these analytes. The difference between these columns is that the Raptor C18 is a traditional C18 ligand containing methyl side chains with an end-capped surface, while the Raptor ARC-18 is a sterically protected C18 ligand containing isobutyl side chains (Figure 6). Stationary phase differences, such as end capping and carbon load, can influence the retention and selectivity of a stationary phase. In this instance, the difference in selectivity allowed the Raptor ARC-18 column to resolve all analytes as well as the matrix interference, which was not possible on the Raptor C18 column.

**Figure 6:** Retention Properties of Raptor C18 (Left) and Raptor ARC-18 (Right)



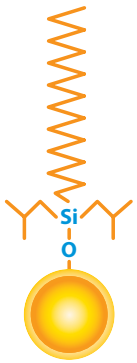

**Stationary Phase Category:**
C18, octadecylsilane (L1)

**Ligand Type:**
End-capped C18

**Particle:**
1.8 µm, 2.7 µm, or 5 µm superficially porous particle (SPP or "core-shell" particle) silica

**Pore Size:**
90 Å

**Surface Area:**
125 $m^2/g$ (1.8 µm), 130 $m^2/g$ (2.7 µm), or 100 $m^2/g$ (5 µm)




**Stationary Phase Category:**
C18, octadecylsilane (L1)

**Ligand Type:**
Sterically protected C18

**Particle:**
1.8 µm, 2.7 µm, or 5 µm superficially porous particle (SPP or "core-shell" particle) silica

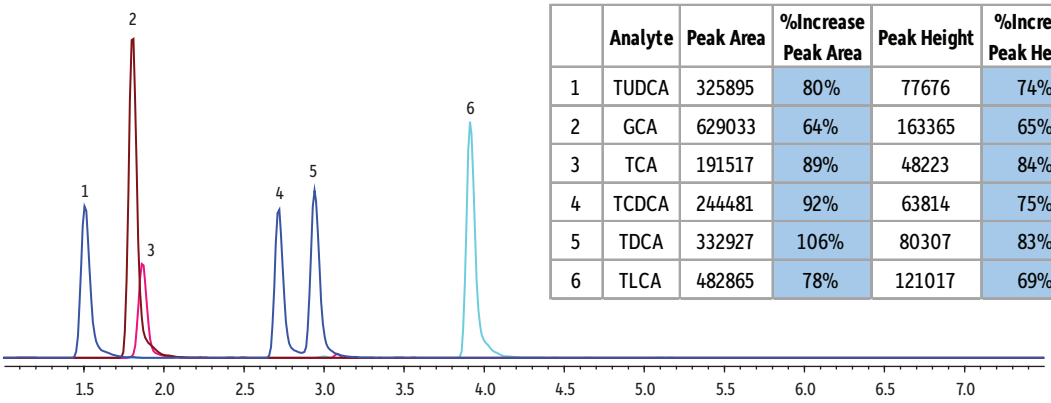
**Pore Size:**
90 Å

**Surface Area:**
125 $m^2/g$ (1.8 µm), 130 $m^2/g$ (2.7 µm), or 100 $m^2/g$ (5 µm)

For this revised method, a column with inert hardware was chosen over one with the traditional stainless-steel hardware. These columns have a premium inert coating applied to the stainless-steel surface of the LC column that is designed to reduce nonspecific binding of chelating analytes. As bile acids are acidic in nature, some chelation with metal surfaces may occur, resulting in undesirable peak shapes and loss of sensitivity. When analyzed with an inert column, lower detection limits may be achieved for this group of analytes. In Figure 7 below, the performance of a select number of bile acids are compared when analyzed on an inert versus an untreated (non-inert) column.

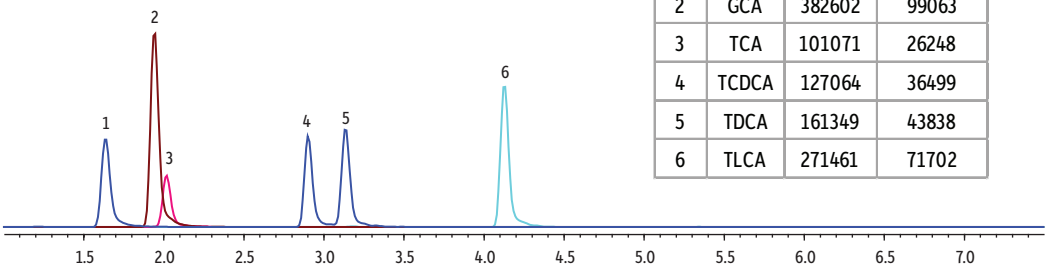
**Figure 7:** Comparison of Peak Response for Bile Acids on Raptor Inert ARC-18 and Raptor ARC-18 Columns

**Raptor Inert ARC-18**




| | Analyte | Peak Area | %Increase Peak Area | Peak Height | %Increase Peak Height |
|---|---|---|---|---|---|
| 1 | TUDCA | 325895 | 80% | 77676 | 74% |
| 2 | GCA | 629033 | 64% | 163365 | 65% |
| 3 | TCA | 191517 | 89% | 48223 | 84% |
| 4 | TCDCA | 244481 | 92% | 63814 | 75% |
| 5 | TDCA | 332927 | 106% | 80307 | 83% |
| 6 | TLCA | 482865 | 78% | 121017 | 69% |

**Raptor ARC-18**




| Peak | Analyte | Peak Area | Peak Height |
|---|---|---|---|
| 1 | TUDCA | 181245 | 44626 |
| 2 | GCA | 382602 | 99063 |
| 3 | TCA | 101071 | 26248 |
| 4 | TCDCA | 127064 | 36499 |
| 5 | TDCA | 161349 | 43838 |
| 6 | TLCA | 271461 | 71702 |

Time (min)

LC_CF0820

| Peaks | Precursor Ion | Product Ion |
|---|---|---|
| 1. TUDCA | 498.10 | 80.05 |
| 2. GCA | 464.10 | 74.15 |
| 3. TCA | 514.20 | 80.05 |
| 4. TCDCA | 498.10 | 80.05 |
| 5. TDCA | 498.10 | 80.05 |
| 6. TLCA | 482.10 | 80.05 |

**Column** See notes.
Dimensions: 100 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Temp.: 50 °C

**Standard/Sample**
Diluent: 60:40 Water:mobile phase B
Conc.: 0.25 µM
Inj. Vol.: 5 µL

**Mobile Phase**
A: 5 mM ammonium acetate in water, pH unadjusted
B: Methanol:acetonitrile (v/v, 50:50)

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.5 | 60 | 40 |
| 6.00 | 0.5 | 30 | 70 |
| 7.00 | 0.5 | 20 | 80 |
| 7.50 | 0.8* | 0 | 100 |
| 8.10 | 0.8* | 0 | 100 |
| 8.20 | 0.5 | 60 | 40 |
| 9.50 | 0.5 | 60 | 40 |

**Max Pressure:** 300 bar
**Detector** Shimadzu 8045-LCMS in ESI- mode
**Instrument** Shimadzu Nexera X2
**Sample Preparation** For control samples, a 100 µL aliquot of patient sample was added to a microcentrifuge tube. Ten microliters of internal standards were added and vortexed. Samples were protein precipitated using 400 µL of ice-cold acetonitrile. After vortexing and centrifugation at 4200 rpm for 15 minutes, the supernatant was transferred to a glass test tube and dried down under nitrogen. All samples were reconstituted in 200 µL of 60:40 water:mobile phase B. The sample was transferred to a clean 2 mL screw-thread vial (cat.# 21143) with a glass insert (cat.# 21776) and capped with short-cap, screw-vial closures (cat.# 24498).

**Notes** *The flow rate was increased to 0.8 mL/min to more thoroughly flush phospholipids from the analytical column, thereby reducing matrix effects.

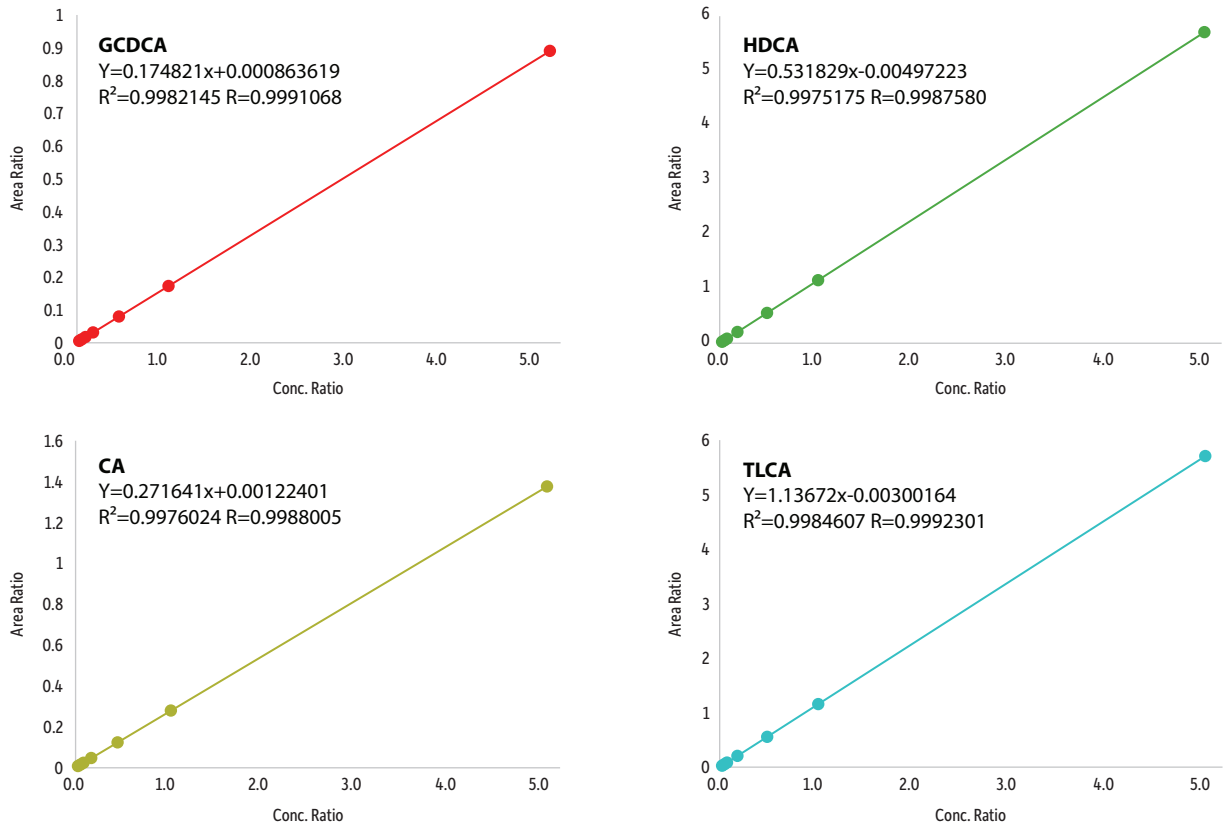
The flow was diverted to waste before 1 minute and after 7 minutes to protect the mass spectrometer.

Columns are:
- Raptor Inert ARC-18 (cat.# 9314A12-T)
- Raptor ARC-18 (cat.# 9314A12)

*Linearity*

Method linearity was verified by analysis of a six-point calibration curve (n=3). A 1/x weighted linear regression was used for all compounds. Representative calibration curves are shown in Figure 8. The method has a dynamic range of 0.05–5 µM for all analytes and excellent correlation coefficients (>0.99). The calibration range was selected to represent typical bile acids concentrations in human plasma samples.

**Figure 8:** Representative Calibration Curves




*Accuracy and Precision*

Accuracy and precision were assessed at four different concentrations (LLOQ, low QC, medium QC, high QC; see Table II for details) and evaluated both intraday and as an average of three days (n=9). Blank matrix samples were extracted and analyzed to confirm that endogenous levels of bile acids in the charcoal stripped plasma were below the detection limit. Method accuracy was assessed as the percentage of the measured concentration relative to the fortified concentration. The interday precision of the method was assessed using percent relative standard deviation (%RSD). These results, shown in Table III, demonstrate that the method is accurate and precise over the range studied for the quantitative analysis of bile acids in human plasma.

**Table III:** Method Accuracy and Precision Results in Plasma (Interday)

| Analyte | LLOQ (0.05 µM) | | Low QC (0.1 µM) | | Medium QC (0.5 µM) | | High QC (1 µM) | |
|---|---|---|---|---|---|---|---|---|
| | %Recovery | %RSD | %Recovery | %RSD | %Recovery | %RSD | %Recovery | %RSD |
| GUDCA | 81.6% | 7.77% | 91.8% | 7.75% | 94.3% | 5.73% | 95.2% | 6.31% |
| GCA | 84.4% | 2.84% | 90.1% | 9.36% | 93.8% | 7.66% | 95.1% | 5.55% |
| TCA | 87.8% | 6.20% | 88.1% | 9.20% | 91.9% | 7.20% | 93.3% | 8.58% |
| UDCA | 81.3% | 4.21% | 92.0% | 9.50% | 94.2% | 5.25% | 95.0% | 6.21% |
| GCDCA | 87.3% | 2.94% | 92.2% | 8.71% | 93.9% | 5.80% | 85.1% | 7.16% |
| TCDCA | 88.4% | 6.45% | 84.4% | 10.08% | 94.6% | 5.82% | 93.0% | 8.59% |
| CA | 84.7% | 6.83% | 92.0% | 10.22% | 94.3% | 6.92% | 95.3% | 6.18% |
| GDCA | 87.6% | 9.33% | 90.6% | 11.74% | 93.7% | 7.80% | 94.1% | 6.99% |
| HDCA | 84.9% | 7.73% | 88.7% | 11.71% | 92.5% | 8.07% | 94.7% | 7.58% |
| TDCA | 86.7% | 9.66% | 88.0% | 10.61% | 94.8% | 3.48% | 95.4% | 6.41% |
| TUDCA | 82.2% | 7.47% | 91.0% | 9.94% | 94.1% | 6.43% | 92.3% | 9.07% |
| TLCA | 86.4% | 7.02% | 87.8% | 10.38% | 93.3% | 8.51% | 95.6% | 5.90% |
| GLCA | 82.9% | 5.61% | 91.8% | 7.66% | 93.9% | 6.29% | 93.6% | 7.76% |
| CDCA | 84.4% | 8.25% | 93.6% | 5.88% | 95.7% | 4.83% | 95.7% | 5.91% |
| DCA | 87.1% | 5.54% | 89.0% | 9.77% | 94.3% | 6.40% | 95.7% | 6.03% |
| LCA | 83.3% | 2.84% | 92.1% | 7.97% | 93.4% | 6.75% | 95.2% | 6.36% |
| DHLCA | 83.6% | 3.72% | 91.1% | 8.62% | 94.4% | 5.28% | 95.1% | 6.19% |

## Conclusions

A robust, selective LC-MS/MS method was developed and verified for the analysis of 17 bile acids in human plasma using a Raptor Inert ARC-18 column. The method was paired with a simple protein precipitation sample preparation step. Standard curves demonstrated excellent linearity and precision/accuracy over routine intraday and interday analyses.

This work highlights the importance of alternative column chemistries for the resolution of isomers and matrix interferences. The Raptor C18 and Raptor Inert ARC-18 are both C18-based stationary phases, but each have a unique selectivity resulting from differences, such as end capping and carbon load. In this study, this difference in selectivity allowed for a critical matrix interference to be resolved while still maintaining the separation of the isomers. In addition, the use of an inert LC column in this work resulted in increased peak responses. The improved analyte sensitivity this afforded, makes it well suited for future applications of experimental test samples, as would be the case in microbiome research.

*This method has been developed for research use only; it is not suitable for use in diagnostic procedures without further evaluation.*

## Raptor Inert ARC-18 HPLC Columns

| Catalog No. | Product Name | Units |
|---|---|---|
| 9314A12-T | Raptor Inert ARC-18 100 x 2.1 mm, 2.7 µm column | ea. |


## Raptor Inert ARC-18 EXP Guard Column Cartridge

| Catalog No. | Product Name | Units |
|---|---|---|
| 9314A0252-T | Raptor Inert ARC-18 EXP Guard Column Cartridge, 2.7 µm, 5 x 2.1 mm | 3-pk. |

## EXP Direct Connect Holder

| Catalog No. | Product Name | Units |
|---|---|---|
| 25808 | EXP Direct Connect Holder for EXP Guard Cartridges, Includes Fitting & Ferrules | ea. |


## Short-Cap Vial with Grad Marking Spot

| Catalog No. | Product Name | Units |
|---|---|---|
| 21143 | Short-Cap Vial with Grad Marking Spot, 9-425 Screw-Thread, 2.0 mL, 9 mm, 12 x 32 (vial only) | 1000-pk. |

## Vial Inserts

| Catalog No. | Product Name | Units |
|---|---|---|
| 21776 | Vial Inserts, Glass, Big Mouth w/Bottom Spring, 250 µL | 100-pk. |

## Vial Caps

| Catalog No. | Product Name | Units |
|---|---|---|
| 24498 | Short Screw Cap, Polypropylene, Screw-Thread, PTFE/Silicone/PTFE Septa, Blue, Preassembled, 2.0 mL, 9 mm | 1000-pk. |