

# Agilent LC Triple Quadrupole Clinical Research Compendium

Application Compendium



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# Introduction

This application compendium contains a collection of clinical research application notes demonstrating the use of the Agilent Triple Quadrupole LC/MS systems and presents the productivity advantages of the Agilent systems over traditional LC/MS analyses. Routine applications for hormones analyses, Therapeutic Drug Monitoring (TDM), and Vitamin D analyses are included, as well as applications for proteomics and metabolomics studies.

The Triple Quadrupole LC/MS works in conjunction with a number of Agilent systems suited to meet specific needs. For example, in the studies presented, analyses were performed using the Agilent Ultivo system, the Agilent SimpliQ WCX SPE, Agilent StreamSelect, and Agilent Captiva NDLipids, BondElut PBA SPE, to name a few.

The application notes in this compendium are abridged. For the complete text containing all data, figures, and method details, refer to the article reference at the end of each note.

# Progesterone Metabolism in Serum

## Abstract

Progesterone and its metabolites play an important role in the female body. Therefore, it is often used in hormone replacement therapies. However, current research has shown that progestins—synthetic progesterone—can play a role in various diseases, especially breast cancer.

The LC/MS/MS method presented here is for the analysis of progesterone and relevant, dependent hormones. These are progesterone, 3 $\alpha$ -, 5 $\alpha$ -, and 20 $\alpha$ -dihydroprogesterone as well as allopregnanolone, pregnanolone, and deoxycorticosterone.

## Experimental

- **LC system:** Agilent 1290 Infinity II LC
- **MS:** 6495 triple quadrupole LC/MS
- **Column:** BEH C18 100  $\times$  2.1 mm, 1.7  $\mu$ m (Waters Corporation)
- **Sample prep:** Liquid-liquid extraction was used for extracting progesterone metabolites in serum samples, calibrators (in acetonitrile), or quality control (in 0.1%BSA) followed by derivatization using hydroxylamine solution.
- **Software:** Agilent MassHunter Workstation Software

## Results and discussion

The LC/MS/MS method was able to separate all progesterone metabolites, as shown in an example serum sample in Figure 1. The relevant separation of the isomers was also successful.

The analytical validation showed satisfactory values in intra- and inter-assay precision, with all values below 10%. Precision data are shown in Table 1, in addition to limit of quantification (LOQ) and linearity data.

To test the LC/MS/MS method, serum samples from men and postmenopausal women were processed and measured. The results for both sexes show similar values for the relevant progesterone-related hormones and the ratio (Table 2).

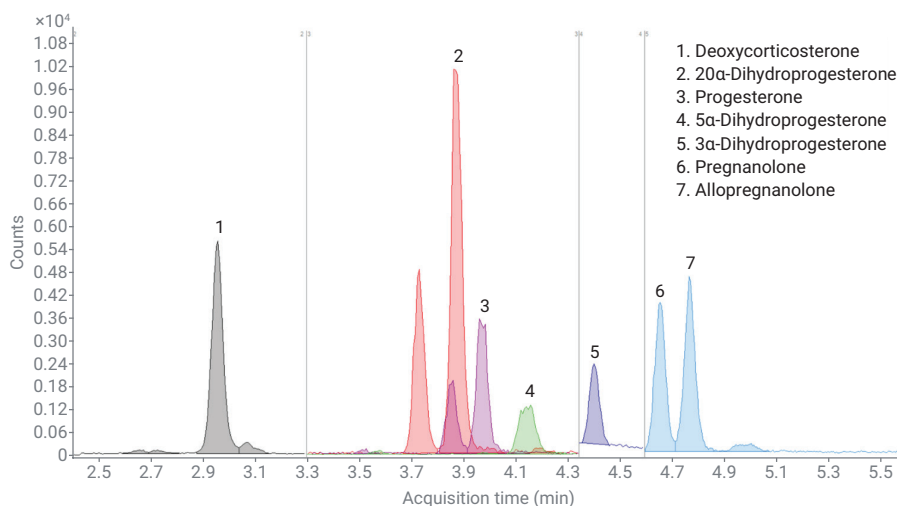


Figure 1. Chromatogram of an example serum sample. The gray lines separate the time windows in which only the MRM transitions of the selected analytes are measured to achieve a better dwell time.

Table 1. Analytical validation data consisting of inter-assay, intra-assay, limit of quantification (LOQ), and linearity.

Analyte	Intra-Assay (%)	Inter-Assay (%)	LOQ (ng/L)	Linearity (ng/L)
Deoxycorticosterone	4.3	8.6	1.00	5 to 1,000
20α-Dihydroprogesterone	5.6	8.5	4.10	
Progesterone	5.2	9.4	2.48	
5α-Dihydroprogesterone	4.7	7.7	0.80	
3α-Dihydroprogesterone	6.8	5.4	0.80	
Pregnanolone	4.4	8.6	1.00	
Allopregnanolone	6.8	8.6	0.80	

Table 2. Median (ng/L) of relevant progesterone-related hormones and the ratio of 5α- to 3α-dihydroprogesterone by sex.

Analyte	Men (n = 64)		Postmenopausal Women (n = 54)	
	Median	CI 95%	Median	CI 95%
20α-Dihydroprogesterone	58.3	26.8 to 211.4	40.0	8.5 to 127.6
Progesterone	48.2	24.8 to 154.2	27.9	10.5 to 132.6
5α-Dihydroprogesterone	26.4	7.7 to 148.2	16.3	3.1 to 202.4
3α-Dihydroprogesterone	2.0	1.0 to 5.7	1.7	0.9 to 8.4
5α/3α Ratio	13.6	3.4 to 29.9	8.2	2.2 to 68.5

CI = confidence interval

## Conclusion

The LC/MS/MS method presented in this application note offers the possibility to reliably measure progesterone and relevant metabolites. The LOQ achieved also allows the measurement of these hormones in men and postmenopausal women, who naturally have lower levels of progesterone.

**Note:** For specific test operations and parameters, please refer to application note: 5994-2784EN [Progesterone Metabolism in Serum](#).

# Analytical Determination of Testosterone in Human Serum Using an Agilent Ultivo Triple Quadrupole LC/MS

## Abstract

This research study develops a robust, sensitive, and relatively fast analytical method for the quantitation of free testosterone in serum using a miniature Agilent Ultivo Triple Quadrupole LC/MS. Lower limits of quantitation, chromatographic precision, calibration linearity, range, and accuracy are outlined. Ultivo reduces the need for user intervention for system maintenance, making the system operation and maintenance manageable for nonexpert MS users.

## Experimental

- **LC:** Agilent 1290 Infinity II LC
- **MS:** Agilent Ultivo Triple Quadrupole Mass Spectrometer
- **Column:** Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm (part number 699775-902)
- **Sample prep:** Eleven levels of calibrators were prepared by serial dilutions of highest concentrations of testosterone in spiked human serum. Protein precipitation method was applied and supernatant was then diluted in water for injection.
- **Software:** Agilent MassHunter Workstation Software

## Results and discussion

### Linearity

The calibration concentrations ranged from 1 pg/mL to 100 ng/mL for the testosterone analyte.  $R^2$  values were greater than 0.999,  $n = 3$ , with the testosterone compound displaying linear responses throughout the concentration range with a  $1/x$  weighting factor applied. Precision data observed over the three batches resulted in a %RSD variation of <5% across all calibration levels.

### Analytical sensitivity

Negative serum sample blanks show measurable amounts of endogenous testosterone present. Figure 2A shows a small testosterone response in blank serum,  $n = 3$ . Figure 2B illustrates negative serum spiked with 1 pg/mL testosterone. A significant difference in area count and signal-to-noise ratio can be seen from these calibrators over the respective matrix blanks; therefore, the calibration curves were created, and calculations undertaken using a blank-offset feature.

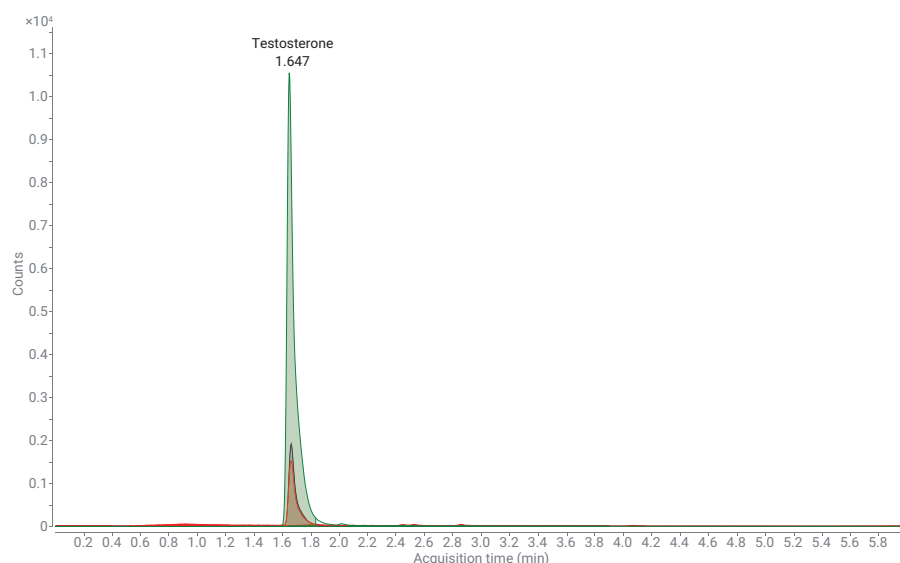


Figure 1. Overlaid MRM chromatograms, showing elution of testosterone (500 pg/mL) and the d3 internal standard (25 ng/mL).

### Conclusion

This research project demonstrates that the Agilent Ultivo Triple Quadrupole LC/MS produces excellent linearity, precision, and analytical sensitivity across a range of 1 pg/mL through 100 ng/mL for free testosterone in human serum in a 6-minute analysis cycle time.

**Note:** For specific test operations and parameters, please refer to application note: 5991-8847EN [Analytical Determination of Testosterone in Human Serum Using an Agilent Ultivo Triple Quadrupole LC/MS](#).

[5991-8847ZHCN.pdf \(agilent.com.cn\)](#)

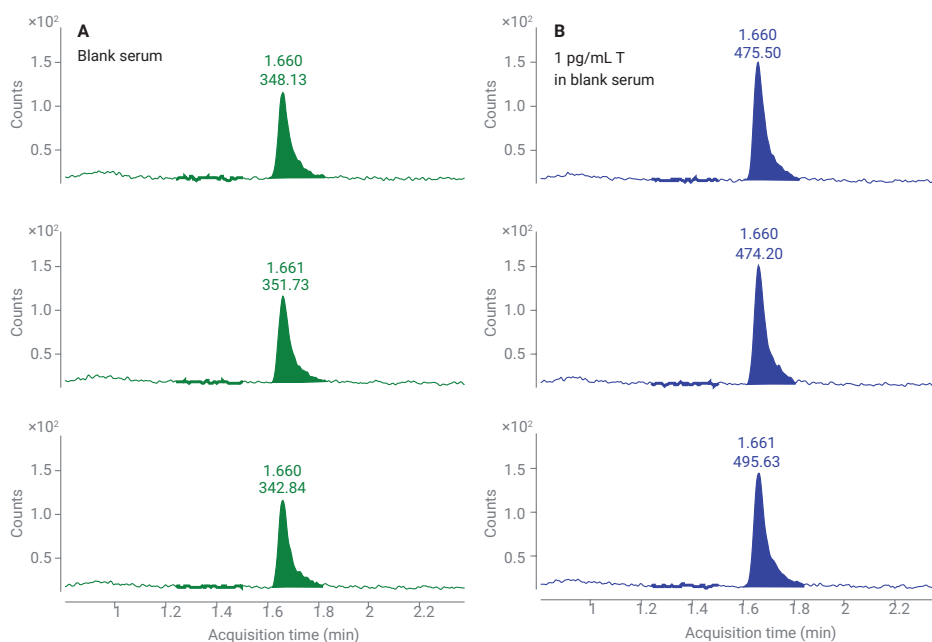


Figure 2. Triplicate injection of blank serum (A) and blank serum spiked with 1 pg/mL testosterone (T) (B).



# Plasma Catecholamines by LC/MS/MS Using Agilent Captiva ND<sup>Lipids</sup>, BondElut PBA SPE, 1290 Infinity LC, and 6460 Triple Quadrupole LC/MS

## Abstract

We developed a highly sensitive and specific LC/MS/MS method to quantitate norepinephrine, epinephrine, and dopamine in plasma. An Agilent Captiva ND<sup>Lipids</sup> filtration and a selective solid phase extraction procedure was used to clean up plasma. The method achieved the required functional sensitivity and quantitated analytes over a sufficiently wide dynamic range. Reproducibility was excellent for all compounds (CV <5%). All calibration curves displayed excellent linearity, with  $R^2 > 0.9997$ .

## Experimental

- **LC system:** Agilent 1290 Infinity LC
- **MS:** 6460 triple quadrupole LC/MS
- **Column:** Agilent Pursuit PFP, 2 × 150 mm, 3 μm (p/n A3051150X020), Agilent BondElut PBA cartridge (100 mg, 3 mL, p/n 12102127), Agilent Captiva NDLipids cartridge (3 mL, p/n A5300635)
- **Sample prep:** Samples, calibrators in plasma matrix, and controls were extracted via protein precipitation and lipid removal method followed by SPE for further sample cleaning.
- **Software:** Agilent MassHunter Workstation software

## Results and discussion

Chromatographic separation of all analytes was achieved with a PFP column, which eliminated interferences from compounds that share common fragments (Figure 1).

Matrix effects varied from 42 to 119%, and recovery efficiencies varied from 56 and 59% (Table 1). Therefore, matrix effects were observed, but were compensated for by the internal standards, and gave acceptable recovery efficiencies as demonstrated in Tables 2 and 3.

Calibration standards were extracted over a series of three days to establish inter-day precision and accuracy. All three analytes had acceptable accuracies, and the coefficient of variation values were less than 5% for all concentrations within the linear range (Table 2). ChromSystems controls were extracted over a series of three days, and three times during one day to establish inter- and intraday, and coefficient of variation values were less than 4% (Table 3). The method had excellent linearity within the measured range of 5 to 2,500 pg/mL, with an  $R^2$  value greater than 0.9997.

## Conclusion

We developed a robust analytical method for quantifying epinephrine, norepinephrine, and dopamine in plasma. All three analytes were extracted simultaneously with good recoveries using an Agilent Captiva ND<sup>Lipids</sup> filtration and solid phase extraction. Chromatographic separation of the analytes using conditions compatible with LC/MS/MS was also developed.

**Note:** For specific test operations and parameters, please refer to application note: 5991-6530EN [Plasma Catecholamines by LC/MS/MS Using Agilent Captiva ND<sup>Lipids</sup>, BondElut PBA SPE, 1290 Infinity LC, and 6460 Triple Quadrupole LC/MS](#).

[5991-6530CHCN.pdf \(agilent.com.cn\)](#)

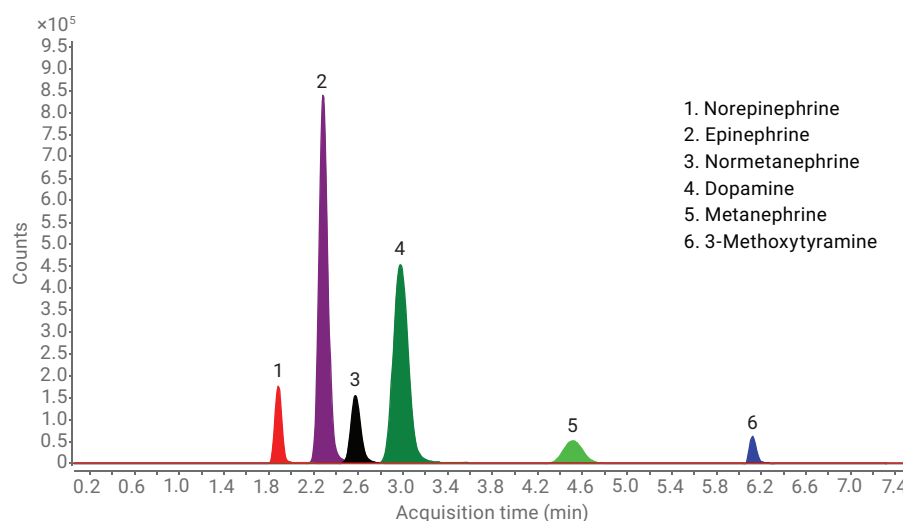


Figure 1. Chromatography of catecholamines, metanephrines, and 3-methoxytyramine.

Table 1. Matrix effects and recovery efficiencies of the SPE procedure.

Compound	Matrix effect % (n = 3)		Recovery efficiency % (n = 3)	
	Average	SD	Average	SD
Norepinephrine	42.3	1.9	56.3	6.3
Epinephrine	70.1	6.6	56.5	2.4
Dopamine	118.5	21.5	58.7	4.3

Table 2. Summary of the analyte performance.

Compound	$R^2$ (n = 3)	Concentration (pg/mL)	Concentration (nmol/L)	Accuracy % (n = 3)	Inter-day CV % (n = 3)
Norepinephrine	0.9999	5	0.03	107.6	4.7
		20	0.12	95.4	1.2
		250	1.5	98.6	1.4
		2,500	14.8	100.2	0.3
Epinephrine	0.9998	5	0.03	108.4	2.1
		20	0.11	96.5	1.1
		250	1.4	97.5	1.6
		2,500	13.6	100.6	0.3
Dopamine	0.9997	5	0.03	108.7	3.2
		20	0.13	98.8	2.6
		250	1.6	98.1	0.9
		2,500	16.3	99.6	1.1

Table 3. Results of ChromSystems controls by LC/MS/MS.

Compound	QC level	Measured value intra-day (n = 3)		Intra-day CV % (n = 3)	Measured value inter-day (n = 3)		Inter-day CV % (n = 3)
		pg/mL	nmol/L		pg/mL	nmol/L	
Norepinephrine	0010	240	1.42	3.6	242	1.43	3.0
	0020	1,756	10.4	0.7	1,767	10.4	0.8
Epinephrine	0010	93.4	0.51	1.6	92.3	0.50	1.2
	0020	451	2.46	0.7	449	2.45	0.4
Dopamine	0010	164	1.07	0.9	162	1.06	0.8
	0020	595	3.88	0.5	597	3.90	0.9

# Determination of Hormones in Serum by LC/MS/MS Using Agilent Bond Elut Plexa SPE

## Abstract

A method for the determination of 13 hormones and their respective internal standards in serum was developed. This application note demonstrates the ease-of-use and effectiveness of Bond Elut Plexa SPE cartridges for hormone determination in serum.

## Experimental

- **LC system:** Agilent 1290 Infinity LC
- **MS:** Agilent 6460A triple quadrupole LC/MS/MS system
- **Column:** Agilent InfinityLab Poroshell HPH-C8, 2.1 mm × 50 mm, 2.7 µm (part number 699775–706)
- **Sample prep:** Agilent Bond Elut Plexa Solid Phase Extraction (SPE) cartridges
- **Software:** Agilent MassHunter workstation software

## Results and discussion

### SPE optimization

SPE method was optimized to achieve best recoveries and maximum interference removal. Eventually, 30% MeOH in water and 100% MeOH were chosen as optimal wash solvent and elution solvent, respectively.

### LC/MS/MS optimization

An Agilent InfinityLab Poroshell 120 EC-C8 column (2.1 × 50 mm, 2.7 µm) was used in the initial method with shorter retention times compared to an Agilent Poroshell 120 EC C18 column.

Formic acid (0.1%), ammonium hydroxide (0.02%, pH 10.5), and ammonium fluoride (pH 6.2) mobile phases were also evaluated.

The Poroshell HPH-C8 column is stable up to pH 11, and provides similar selectivity to a Poroshell 120 EC-C8 column. The Poroshell HPH-C8 column was used for method optimization with both ammonium hydroxide and ammonium fluoride mobile phases, and for the final analytical method. Ammonium fluoride (1 mM) mobile phase yielded the highest analyte response, and was used for the final method (see Figure 1).

## Recovery and reproducibility

Recovery and reproducibility were evaluated by extracting prespiked and blank serum samples using the SPE protocol (see Table 1).

## Conclusion

A method was developed for the determination of 13 hormones in serum. Analyte response in both positive and negative mode was enhanced with the addition of 1 mM ammonium fluoride. An Agilent InfinityLab Poroshell HPH-C8 (2.1 mm × 50 mm, 2.7 µm) column yielded fast run times and baseline separation of three isobaric pairs, and was compatible with wide pH range of mobile phase. The method provided excellent recoveries (80 to 105%) and low RSD values (2.8 to 5.8%). Agilent Bond Elut Plexa SPE products require little method development and are simple to use, making them the ideal choice for biological analysis.

**Note:** For specific test operations and parameters, please refer to application note: 5991-8042EN [Plasma Catecholamines by Determination of Hormones in Serum by LC/MS/MS Using Agilent Bond Elut Plexa SPE](#). 5991-8042ZHCN.pdf ([agilent.com.cn](http://agilent.com.cn))

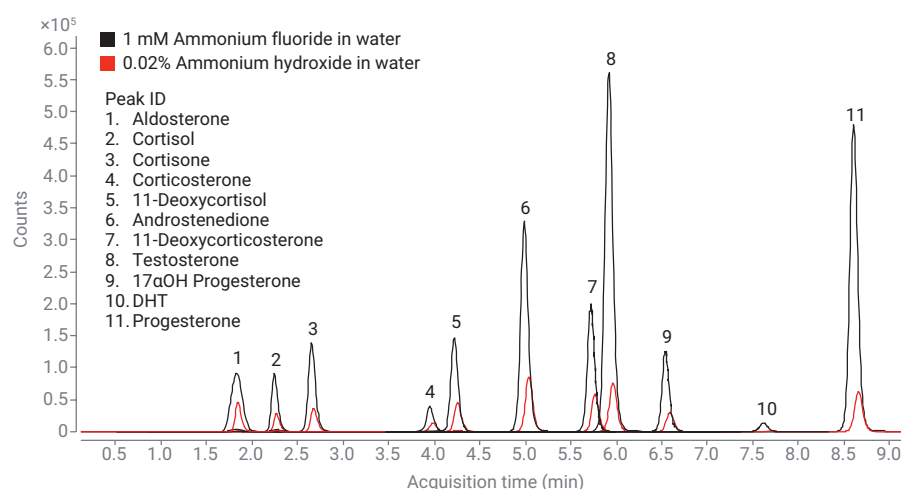


Figure 1. Total ion chromatogram for a 100 ng/mL hormone sample with 1 mM ammonium fluoride in water (black trace), and 0.02% ammonium hydroxide in water (red trace). An Agilent InfinityLab Poroshell HPH-C8, 2.1 mm × 50 mm, 2.7 µm was used. The LC/MS/MS gradient was kept the same for comparison purposes. β-Estradiol and estrone were added to the method after these chromatograms were generated, and are not included.

Table 1. Percent recovery and RSD values (shown in parentheses) are provided (n = 5).

Analyte	Fortified concentration (ng/mL)	% Recovery (RSD)
Aldosterone	10	94 (5.8)
Cortisol	10	102 (5.4)
Cortisone	10	99 (5.7)
Corticosterone	10	97 (5.1)
11-Deoxycortisol	10	97 (3.9)
β-Estradiol	100	81 (5.3)
Testosterone	10	86 (3.3)
11-Deoxycorticosterone	10	91 (4.6)
Androstenedione	10	92 (4.7)
Estrone	100	80 (3.9)
17αOH Progesterone	10	105 (4.2)
DHT	100	88 (2.8)
Progesterone	10	90 (4.0)

# A Fast Analytical LC/MS/MS Method for the Simultaneous Analysis of Barbiturates and 11-nor-9-carboxy- $\Delta^9$ -Tetrahydrocannabinol (THC-A) in Urine Using ESI Negative Ionization Mode and Alternating Column Regeneration

## Abstract

This work developed a fast analytical method combining eight barbiturates and THC-A in a single analysis using alternating column regeneration (ACR) to increase sample throughput. The simple sample preparation techniques used provided rapid analysis, good analytical sensitivity, and quantitation over a wide dynamic range.

## Experimental

- **LC system:** Agilent 1290 Infinity II HPLC
- **MS:** 6470 triple quadrupole LC/MS
- **Columns:**  $\times 2$  Agilent Poroshell 120 EC-C18, 2.1  $\times$  100 mm, 1.9  $\mu\text{m}$  (p/n 695775-902)
- **Sample prep:** Calibrators were prepared by spiking standards in drug-free human urine solution. 10:1 dilute and inject.
- **Software:** Agilent MassHunter Acquisition (B.08.00)

## Results and discussion

### Chromatography

The main emphasis of this work was increased throughput. Therefore, under these chromatographic conditions, the isobars amobarbital and pentobarbital did not separate, and are reported as a single peak. If separation between amobarbital and pentobarbital is required, adjust the gradient and the run time to achieve baseline separation between those isobars (see Figure 1).

### Calibration curves

All calibration curves were linear, and a 1/x weighting factor was used. Examples are shown in Figure 2.

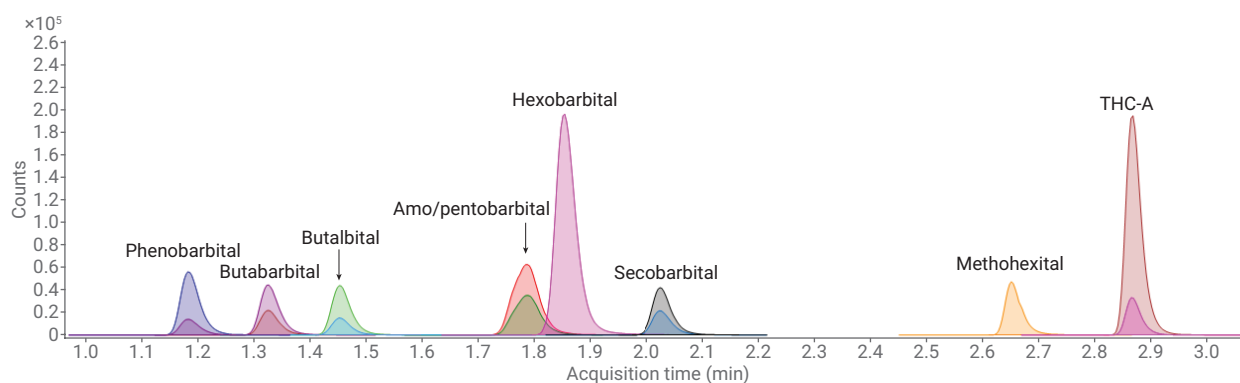


Figure 1. dMRM Chromatogram showing elution of the nine compounds at 500 ng/mL.

## Quantitation results

We created a 10-point calibration curve ranging from 5 ng/mL to 5,000 ng/mL for all compounds except amo/pentobarbital and THC-A, which ranged from 0.5 ng/mL to 500 ng/mL. All compounds were analyzed down to 1 ng/mL (0.1 ng/mL for amo/pentobarbital and THC-A), and showed a signal-to-noise ratio of 5 or better.

## Conclusion

This work combined detection of barbiturates and THC-A into a single analytical method. The addition of ACR reduced the analysis runtime to 3.7 minutes injection to injection, which translates to a 26% improvement in throughput, although there was no separation between amobarbital and pentobarbital. Calibration curves for all compounds were linear, with correlations of 0.99 or better. LLOQs for urine-spiked amo/pentobarbital and THC-A were 0.5 ng/mL or better and for others the LLOQs were 5.0 ng/mL or better.

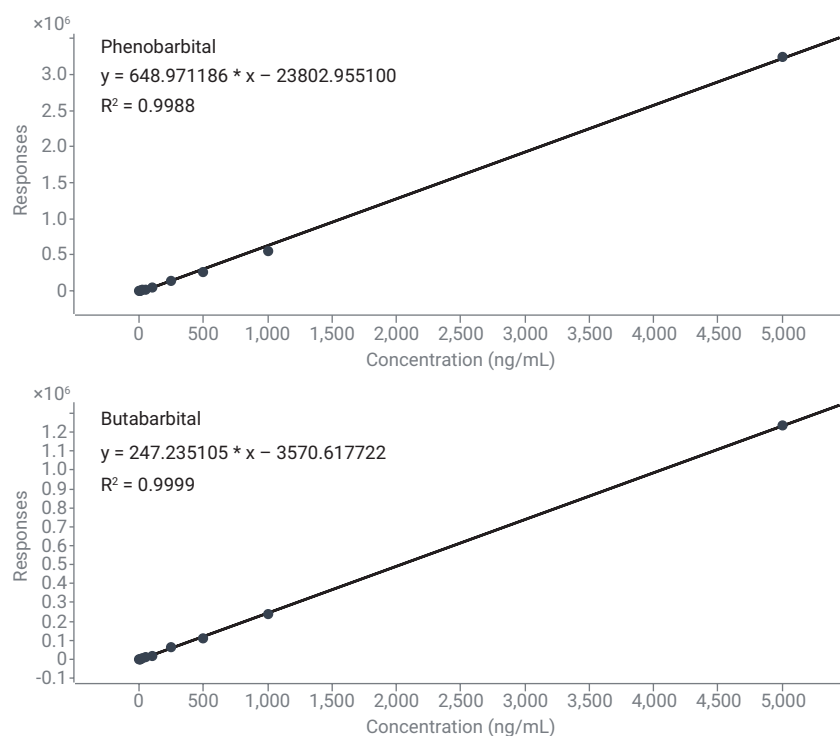


Figure 2. Example calibration curves.

**Note:** For specific test operations and parameters, please refer to application note: 5991-8981EN A [Fast Analytical LC/MS/MS Method for the Simultaneous Analysis of Barbiturates and 11-Nor-9-Carboxy- \$\Delta^9\$ -Tetrahydrocannabinol \(THC-A\) in Urine.](https://www.agilent.com.cn/cs/library/applications/5991-8981ZHCN_LCMSMS_Application.pdf)  
[https://www.agilent.com.cn/cs/library/applications/5991-8981ZHCN\\_LCMSMS\\_Application.pdf](https://www.agilent.com.cn/cs/library/applications/5991-8981ZHCN_LCMSMS_Application.pdf)

# Quick and Routine Research Quantification of Melatonin in Plasma with the Agilent Ultivo LC/TQ



Figure 1. Ultivo LC/TQ with standard ESI ion source.

## Abstract

This Application Note demonstrates the research quantification of melatonin (MEL) with an Agilent 1260 Infinity II Prime LC system coupled to an Agilent Ultivo triple quadrupole LC/MS with an ESI source. The optimized LC/MS method achieves a lower limit of quantification (LLOQ) of 10 pg/mL melatonin-D4 (MEL-D4) spiked into plasma. This sensitive, fast research method can quantify low levels of endogenous MEL from a complex human plasma extract prepared with a simple protein-precipitation procedure.

## Experimental

- **LC system:** Agilent 1260 Infinity II Prime LC
- **MS:** Agilent Ultivo triple quadrupole LC/MS with an ESI source
- **Column:** Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9  $\mu$ m (p/n 699675-902)
- **Sample prep:** Protein precipitation method was applied to plasma.
- **Software:** Agilent MassHunter LC/MS Data Acquisition for Ultivo 1.1

## Results and discussion

### Method optimization

MassHunter Optimizer software was used to optimize MRM transitions for MEL and MEL-D4 (Table 1).

Table 1. Optimized MRM transition parameters.

Compound	Type	Precursor ( $m/z$ )	Product ( $m/z$ )	Fragmentor (V)	CE (V)
MEL	Quantifier	233.1	174.1	87	12
MEL	Qualifier	233.1	159.0	87	32
MEL-D4	Quantifier	237.2	178.1	87	12
MEL-D4	Qualifier	237.2	163.1	87	32

Optimized fragmentor and collision energy (CE) voltages were similar to previously obtained values using MassHunter Optimizer software with an Agilent 6470 triple quadrupole LC/MS. This is a typical example illustrating that MRM methods can confidently be migrated across Agilent LC/TQ instruments.

### Method sensitivity, precision, and linearity

Due to the presence of endogenous MEL in plasma, MEL-D4 spiked into plasma extract was used to determine the limit of detection (LOD), LLOQ, and the upper limit of quantitation (ULOQ) of MEL.

Excellent assay precision (RSD% <10%) as well as average accuracy (95 to 105%) were obtained for all levels. The correlation coefficient ( $R^2$ ) for the calibration curve was 0.998 over four orders of dynamic range. Excellent retention time precision was observed (RSD% = 0.07%) for the 78 injections (see Figure 1).

### Quantification of endogenous MEL

A simple protein precipitation procedure was evaluated for extraction recovery of MEL from plasma. MEL-D4 was used as a surrogate for MEL for recovery % calculations. Average recovery was 95.0% ( $\pm 6.0\%$ , 1 SD).

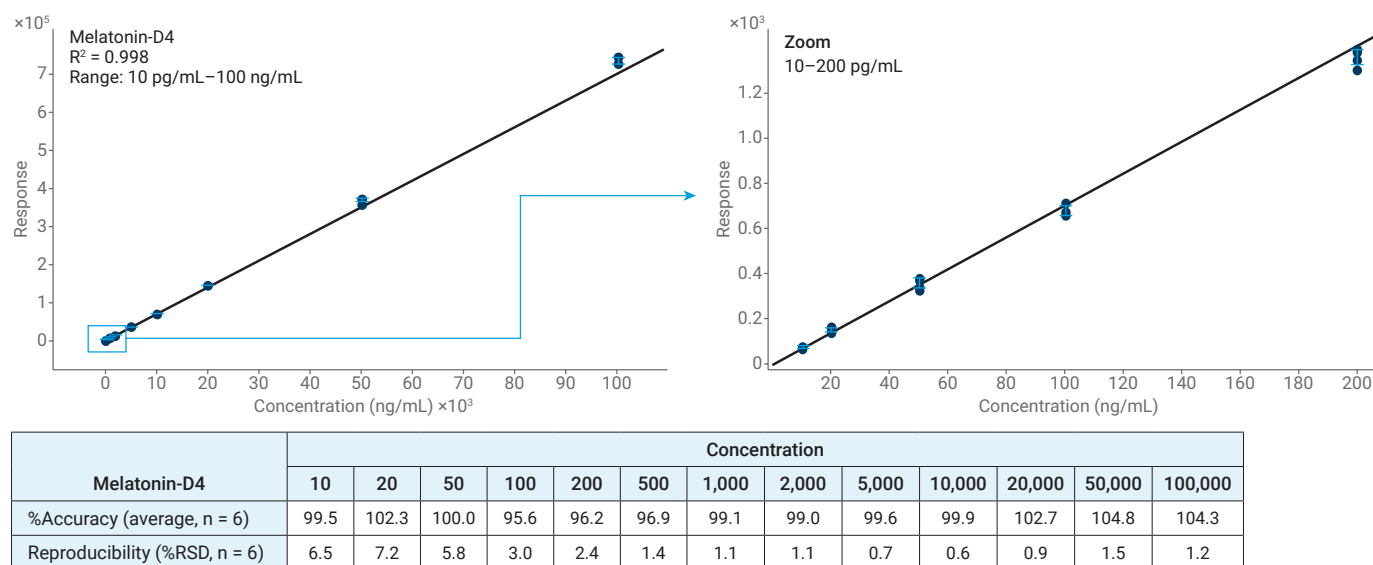
Endogenous MEL in the NIST SRM 1950 plasma was calculated from the external calibration curve of MEL-D4 spiked into plasma extract. To account for losses in the sample preparation, the calculated concentrations were corrected with the observed-versus-expected MRM peak areas ratio from prespiked MEL-D4. Average concentration of endogenous MEL in human plasma was 40.8 pg/mL.

### Conclusion

This Application Note demonstrates that the Ultivo-ESI inherits the outstanding performance of the standard Ultivo system. It achieves the required analytical sensitivity for low ppt level research quantification of melatonin in plasma. The system is also an economical and fit-for-purpose instrument for quantifying low levels of endogenous melatonin from plasma with minimal sample preparation.

**Note:** For specific test operations and parameters, please refer to application note: 5994-0533EN [Quick and Routine Research Quantification in Melatonin in Plasma with Agilent Ultivo LC/TQ](#).

[application-melatonin in plasma-5994-0533zh-cn-agilent.pdf](#)



**Figure 1.** Calibration curve for MEL-D4 spiked into plasma extract. Average accuracies and precision (%RSD) for each level are provided in the table.



# Rapid Analysis of Cyclosporine A, Everolimus, Sirolimus, and Tacrolimus Drugs in Whole Blood Using an Agilent Triple Quadrupole LC/MS/MS System with Automated Online Sample Cleanup

## Abstract

A highly sensitive and specific analytical method has been developed for quantitation of a panel of Cyclosporin A (CsA), Everolimus (Eve), Sirolimus (Sir), and Tacrolimus (Tac). This method has a run time of 2 minutes and is suitable for the simultaneous quantification of all four analytes in whole blood.

## Experimental

- **MS:** Agilent 6460/6470 triple quadrupole MS equipped with an Agilent Jet Stream source
- **Columns:** Trapping: Agilent ZORBAX Eclipse Plus C18, 2.1 × 12.5 mm, 5 µm (p/n 821125-936)  
Analytical: Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm (p/n 699975-302)
- **Sample prep:** All calibrators in whole blood matrix, QCs, and samples were prepared using a simple protein precipitation method.
- **Software:** Agilent MassHunter Quantitative Software B.07.00

## Results and discussion

Excellent linearity was observed for all analytes on both instruments, with  $R^2$  values >0.995 including all 11 concentration levels tested. Consistent retention times for each analyte guaranteed the reproducibility of the method. Sufficient analyte response at low linearity levels assures accurate quantitation down to the lowest concentrations tested.

Interday injections were performed with commercially available calibrators (ChromSystems) and QC samples (BioRad) to evaluate the accuracy and robustness of this method. Data was acquired over 14 days by four different operators. The observed accuracies for each level of QC are tabulated in Table 1.

Table 1. BioRad QC results.

Compound	Target (ng/mL)	Mean (ng/mL)	Accuracy (%)	CV (%)
CsA	95.6	95.6	100.0	6.3
	187.0	197.6	105.7	4.9
	307.0	321.6	104.8	4.8
Sir	5.1	4.8	94.1	13.9
	8.5	8.6	101.2	11.5
	17.3	17.9	103.5	10.4
Tac	4.2	4.5	107.1	7.4
	7.6	7.7	101.3	6.6
	12.5	13.1	104.8	7.9

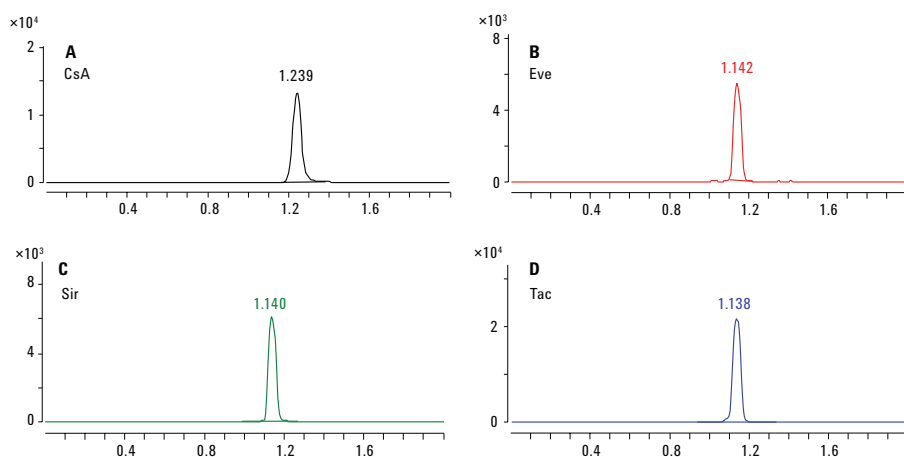


Figure 3. Chromatograms of quantifier MRM transitions for Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D).

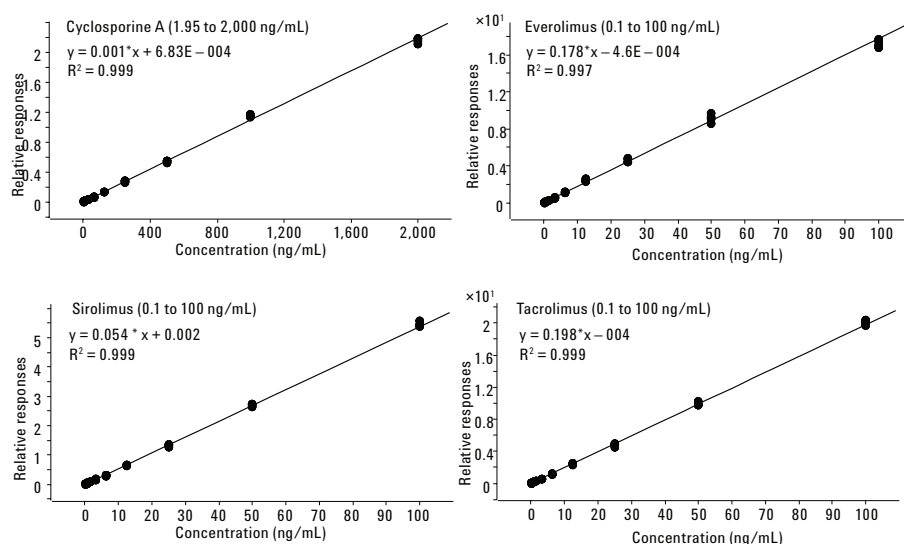


Figure 4. Calibration curves of Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D) 11 levels, 44 points, (type: linear, origin: ignore, weight: 1/x).

## Conclusion

A high-throughput, 2 minute analytical method for the quantitation of Cyclosporin A, Everolimus, Sirolimus, and Tacrolimus has been developed using an Agilent 6460 or 6470 Triple Quadrupole Mass Spectrometer. A simple protein precipitation followed by automated online sample cleanup minimized the matrix effect and ion suppression due to biological compounds present in blood. Using this method, reliable and quick quantitation of all four analytes in whole blood matrix was demonstrated. Excellent linearity of all analytes has been confirmed over the desired ranges.

**Note:** For specific test operations and parameters, please refer to application note: 5991-3344EN [Rapid Analysis of Cyclosporine A, Everolimus, Sirolimus, and Tacrolimus Drugs in Whole Blood Using an Agilent Triple Quadrupole LC/MS/MS System with Automated Online Sample Cleanup](#).

5991-3344CHCN.pdf ([agilent.com.cn](http://agilent.com.cn))

# Vitamin D Metabolite Analysis in Biological Samples Using Agilent Captiva EMR—Lipid

## Abstract

A rugged and reliable sample preparation method has been developed to test vitamin D metabolites in biological samples using Agilent Captiva EMR—Lipid for 96-well plate based *in situ* protein precipitation and pass-through lipid cleanup. Significant improvements in analytical sensitivity and robustness relative to protein precipitation were attributed to high lipid removal, and was demonstrated using postcolumn infusion, matrix effect, and phospholipid analysis experiments.

## Experimental design

**LC system:** Agilent 1290 Infinity II LC

**MS:** Agilent 6460 Triple Quadrupole LC/MS with Agilent Jet Stream

**Column:** Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 μm LC column (699775-902)

**Sample prep:** Agilent Captiva EMR-Lipid

**Software:** Agilent MassHunter Workstation Software

## Results and discussion

### Linearity

The data were processed with MassHunter quantification software. Calibration curves gave  $R^2$  values between 0.992 and 0.997 for 25-OH D2 and D3 over the 10 to 750 ng/mL range using linear regression fit and  $1/x^2$  weighting. The accuracy of all calibrators were within ±10% of expected values.

### Accuracy and precision results

The study produced outstanding accuracy and precision results from both intra-day and inter-day investigation. Accuracy for all QCs were 90 to 110 % and %RSD <10. Absolute recoveries of QC without internal standard correction were between 89 to 106 % for all levels with %RSD <15, which demonstrates that the Captiva EMR—Lipid does not retain 25-OH vitamin D. Carryover was not detected in any blank or double blank samples.

### Monitoring phospholipid removal by LC/MS/MS

Phospholipid removal analysis used MS/MS precursor ion scan for  $m/z$  = 184, as shown in Figure 1. The matrix removal from Captiva EMR—Lipid was calculated at 99.53%.

## Matrix effects

Plasma samples were postspiked with 25-OH D2 and D3 at 50 ng/mL following the Captiva EMR–Lipid workflow (A) and following PPT (B) to compare response reproducibility and relative peak area. The PPT samples gave analyte responses up to 80% lower than Captiva EMR–Lipid-treated samples, and gave variable peak areas with %RSD >25.

## Standard postcolumn infusion

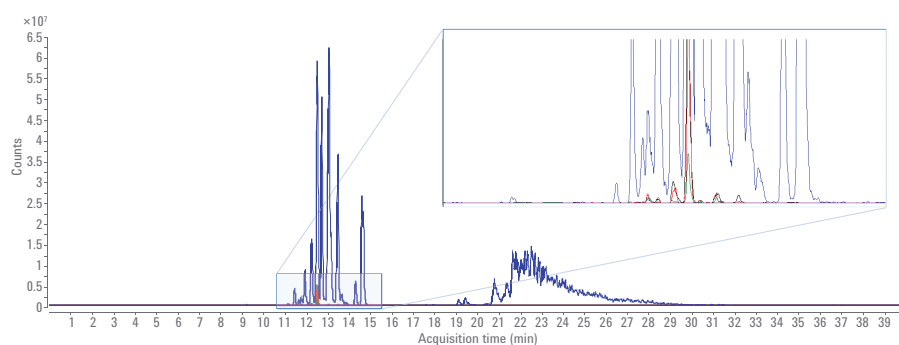
A 50 ng/mL solution of 25-OH D2 and D3 was pumped through a T-in after the LC column. Blank plasma samples were then injected. Figure 2B shows that 25-OH D2 and D3 coelute with a suppression zone. The Figure 2A overlay shows the removal of the suppression signals with Captiva EMR–Lipid, significantly improving analyte response.

## Sample preparation with Agilent Captiva EMR–Lipid

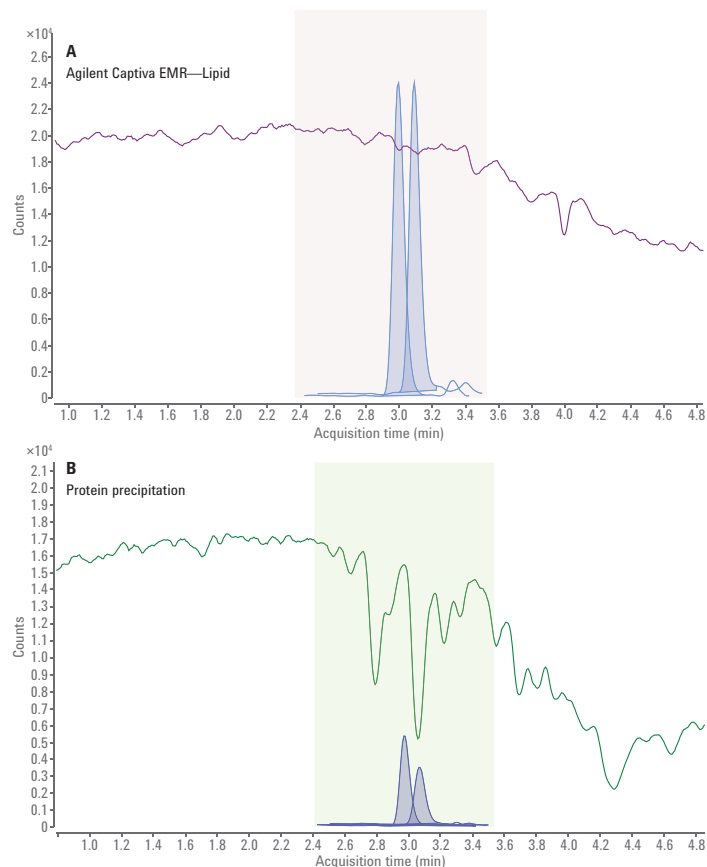
The EMR–Lipid plates were simple to use, provided efficient cleanup, and gave high analyte recovery and precision for vitamin D metabolites. Proteins were efficiently filtered without clogging, consistently giving clear eluents ready for injection. Matrix removal for all major lipid classes was consistently high for a wide variety of sample types, some of which extend beyond the scope of this work.

## Conclusion

Excellent accuracy (90 to 110 %), precision (<10% RSD), and cleanup results (>99% phospholipid removal) were achieved using the Agilent Captiva EMR–Lipid 96-well plate for the multiday method verification of vitamin D metabolites. Proteins were effectively filtered from plasma and serum samples using in situ protein



**Figure 1.** Phospholipid analysis product ion scan overlay of  $m/z = 184$ . Blue trace = PPT. Green, red, and black traces = Agilent Captiva EMR–Lipid ( $n = 3$ ).



**Figure 2.** Chromatogram overlays of a postcolumn infusion trace at 50 ng/mL. PPT only (B). Agilent Captiva EMR–Lipid (A).

precipitation without clogging, while lipids were captured with the newly developed EMR–Lipid sorbent. The method is simple to perform, fast, and provides superior matrix removal ensuring maximum analytical sensitivity, minimal carryover, and high reproducibility.

**Note:** For specific test operations and parameters, please refer to application note: 5991-7956EN [Vitamin D Metabolite Analysis in Biological Samples Using Agilent Captiva EMR–Lipid](#). [5991-7956CHCN.pdf \(agilent.com.cn\)](#)

## Peptide Quantification in Plasma Using the Agilent 6495 Triple Quadrupole LC/MS Coupled with the Agilent 1290 Infinity II LC System

### Abstract

This application note showcases the quantitative sensitivity, precision, and accuracy of multiple reaction monitoring (MRM)-based LC/MS analysis of peptides derived from protein biomarkers in human plasma using the Agilent 1290 Infinity II LC system coupled to the Agilent 6495 triple quadrupole LC/MS with Jet Stream ionization source. Results demonstrated outstanding performance of peptide quantification in human plasma using this standard flow-based LC/TQ system.

### Experimental

- **LC:** Agilent 1290 Infinity II LC system
- **MS:** Agilent 6495C triple quadrupole LC/MS system with Jet Stream source
- **Column:** Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (part number 959759-902)
- **Sample prep:** Human plasma was digested with trypsin, then spiked with stable-isotope standard (SIS) peptide mixture at nine different concentrations.
- **Software:** Agilent MassHunter workstation Quantitative Analysis (v10.1)

### Results and discussion

The 1290 Infinity II LC system gives excellent chromatography. Distribution of peptide retention time versus its corresponding RSD shows the RSD ranges from 0.01 to 0.42%, with a median RSD of 0.13%.

To assess the quantitative sensitivity, precision, and accuracy for peptide quant, human plasma digests were spiked with SIS peptide mixture at nine different concentrations, then used to produce standard curves for 99 SIS peptides. Figure 1 and Table 1 show the excellent quantitative performance of one of the SIS peptides – AFLTPR from apolipoprotein M in plasma.<sup>4</sup>

## Conclusion

This application note describes the overall analytical sensitivity of the 6495 triple quadrupole LC/MS for peptide quantification in human plasma. Using a commercially available biomarker assessment kit from a third-party vendor, the outstanding performance of the 6495 LC/TQ coupled with the 1290 Infinity II LC system and Jet Stream ionization source was demonstrated.

## Reference

1. Robust and Reproducible Protein Quantification in Plasma using the Evosep One and the Agilent 6495 Triple Quadrupole LC/MS. *Agilent Technologies application note*, publication number 5994-1928EN, 2020.

**Note:** For specific test operations and parameters, please refer to application note: 5994-2285EN [Peptide Quantification in Plasma Using the Agilent 6495 Triple Quadrupole LC/MS coupled with the Agilent 1290 Infinity II LC System](#).

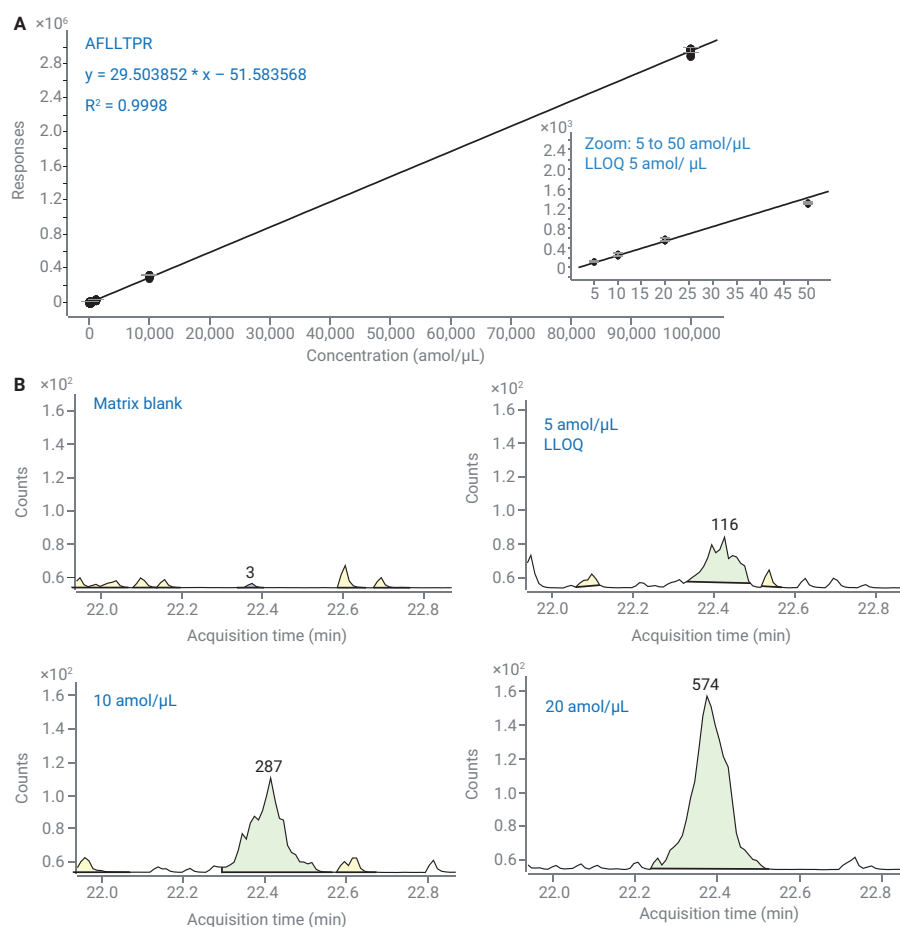


Figure 1. Quantitative performance of the SIS peptide AFLTPR from apolipoprotein M in plasma. (A) standard curve of SIS peptide AFLTPR for the range from 5 amol/μL to 100 fmol/μL in 1 μg/μL plasma digest with inset showing detail for the range of the curve from 5 to 50 amol/μL. (B) extracted ion chromatograms for peptide AFLTPR showing the LLOQ.

Table 1. Precision and accuracy for the Agilent 6495 triple quadrupole LC/MS analysis of the SIS peptide standard AFLTPR in 1 μg/μL plasma digest.

Level (amol/μL)	Average Response	RSD (%) (n = 5)	Accuracy (%) (n = 5)
Matrix blank	3	NA	NA
5	117	9.3	114.2
10	251	9.7	102.4
20	562	4.6	104.0
50	1,295	1.6	91.3
100	2,925	4.1	100.9
200	5,174	3.3	88.6
1,000	28,396	1.3	96.4
10,000	302,313	2.5	102.5
100,000	2,944,790	1.1	99.8

# From Nanoflow to Standard Flow LC/MS for Routine Quantitative Plasma Proteomics in Diabetic Kidney Disease Research

## Abstract

This application note explores the feasibility of transferring the nanoflow LC/MS-based PromarkerD method to a standard flow LC/MS platform. The results demonstrate overall equivalent data on the Agilent standard flow system compared to a nanoflow counterpart system. Successful detection of the four Promarker proteins in whole plasma comparable to depleted plasma analysis highlights the analytical sensitivity offered by the 6495 LC/TQ for routine protein measurement in biomarker research.

## Experimental

- **LC:** Agilent 1290 Infinity II LC system
- **MS:** Agilent 6495B triple quadrupole LC/MS system
- **Column:** Agilent ZORBAX Eclipse Plus Rapid Resolution C18, 50 × 2.1 mm, 1.8 µm in size (part number 959757-902)
- **Software:** Skyline

## Results and discussion

### Nanoflow to standard flow comparison

Twelve plasma samples were prepared by Proteomics International (PI), and one aliquot set was analyzed in PI using a non-Agilent nanoflow LC/TQ platform, then another aliquot set was sent to Atturos and analyzed using the Agilent standard-flow 6495 LC/TQ. Overall, a consistent trend was observed (Figure 1). Manual checking on the deviated results confirm good data quality obtained from the standard-flow platform.

### Depleted plasma versus whole plasma on the 6495 LC/TQ

The four PromarkerD biomarkers in whole reference plasma and reference plasma depleted of the top 14 serum proteins were measured using the Agilent 6495 LC/TQ. All the four protein biomarkers could be detected in the whole reference plasma with at least one peptide.

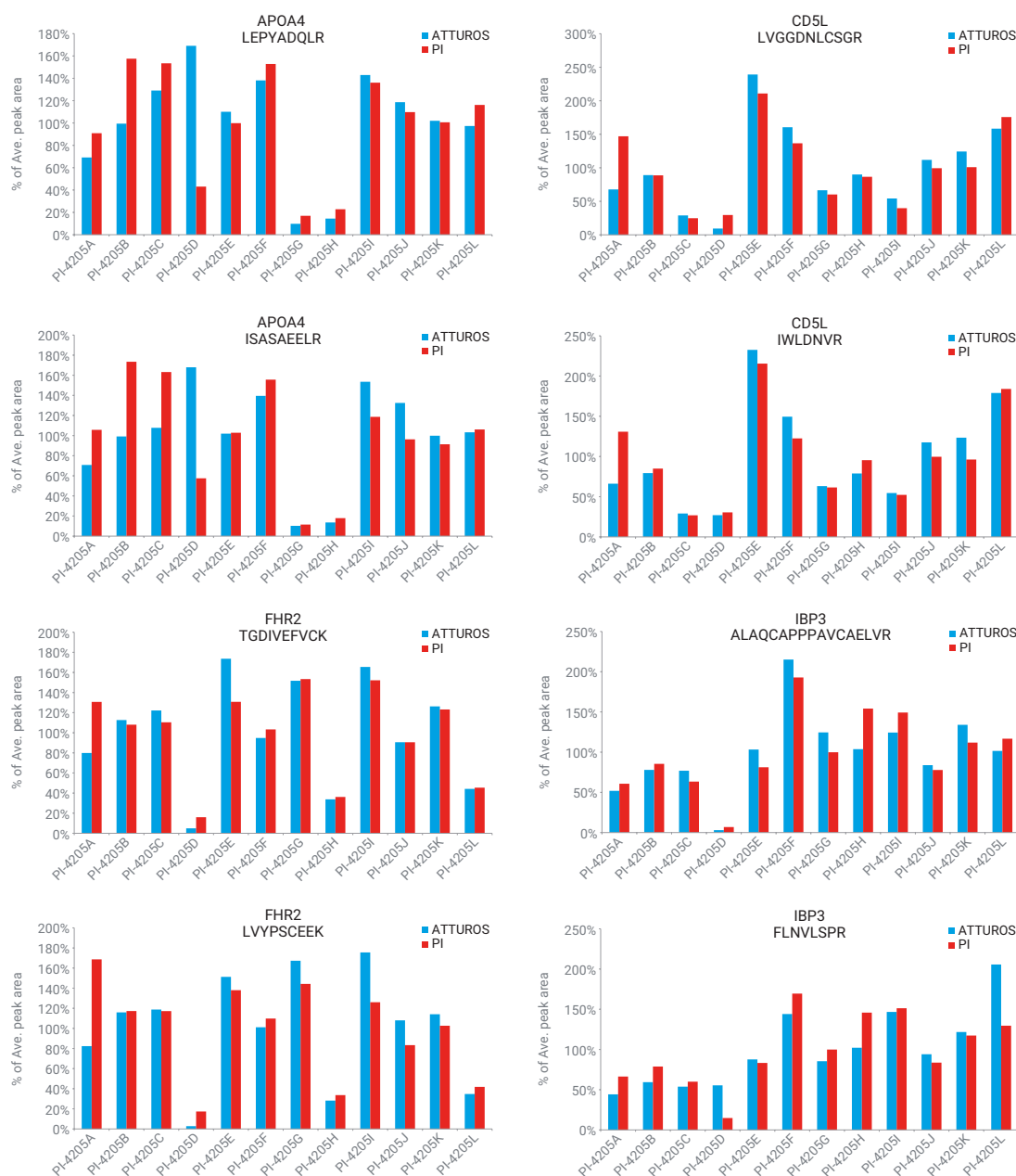


Figure 1. Comparison of eight peptides from four selected proteins (APOA4, CD5L, FHR2, and IBP3) across 12 plasma samples analyzed by nanoflow LC/TQ in PI (red bars) and standard flow LC/TQ in Atturos (blue bars). The individual subject's peak area for a given peptide is expressed as a percentage of the average peak area for that peptide across the 12 plasma samples.

## Conclusion

This application note demonstrated that it is feasible to transfer a dynamic MRM method for peptide quantification in plasma from nanoflow to standard flow LC/TQ platforms. The analytical sensitivity offered by the Agilent 6495 LC/TQ demonstrated that it is possible to use this standard-flow LC/TQ system

on routine protein measurement in complex matrices for potential clinical research assay development in the future.

## Reference

1. Peters, K. E. *et al.* Validation of a Protein Biomarker Test for Predicting Renal Decline in Type 2 Diabetes: The Fremantle Diabetes

Study Phase II. *J. Diabetes Complications* **2019** Aug 27, 107406.

**Note:** For specific test operations and parameters, please refer to application note: 5994-2381EN *From Nanoflow to Standard Flow LC/MS for Routine Quantitative Plasma Proteomics in Diabetic Kidney Disease Research.*



# Measurement of D- and L-2-Hydroxyglutarate Enantiomers and *Alpha* Ketoglutaric Acid by LC/MS/MS

## Abstract

Literature reports have indicated the ratio of 2-hydroxyglutaric acid (2-HGA) to alpha ketoglutarate ( $\alpha$ KG) correlated with tumor phenotype. Derivatization of 2-HGA with (+)-o,o-diacetyl-L-tartaric anhydride (DATAN) permitted separation of the two resulting diastereoisomers without a chiral stationary phase and the underivatized  $\alpha$ KG on a C18 column using an Agilent 1290 Infinity II LC connected to an Agilent 6490 triple quadrupole LC/MS with an Agilent Jet Stream ion source. This method produced linear standard curves over the range 0.34 to 135.04  $\mu$ M with  $R^2$  values  $>0.9$  and low matrix effects.

## Experimental

- **LC:** Agilent 1290 Infinity II LC
- **MS:** Agilent 6495 triple quadrupole LC/MS with Agilent Jet Stream ion source
- **Column:** Agilent ZORBAX SB-C18, 4.6  $\times$  150 mm, 5  $\mu$ m
- **Software:** Agilent MassHunter quantitative analysis

## Results and discussion

The method was adequately tested, and demonstrated good precision and recovery values with a limit of quantitation (LOQ) of 0.20  $\mu$ M in collected samples.

The assay precision was estimated based on five replicates for any given level in a single run on a single day.

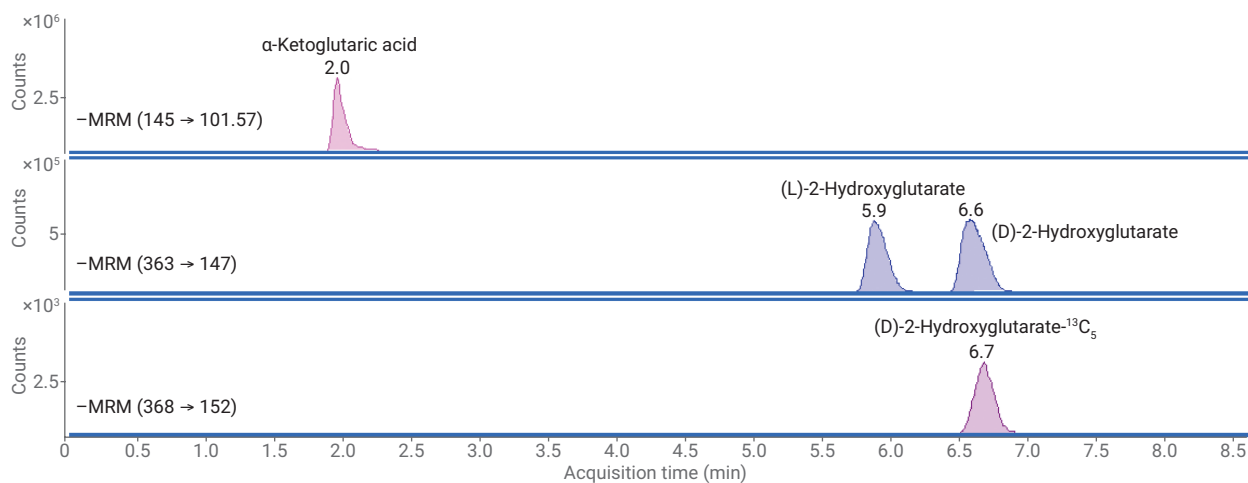


Figure 1. Standard MRM chromatograms representing the separation of (D) and (L)2-HGA, D-2-HGA- $^{13}\text{C}_5$ , and  $\alpha\text{KG}$ .

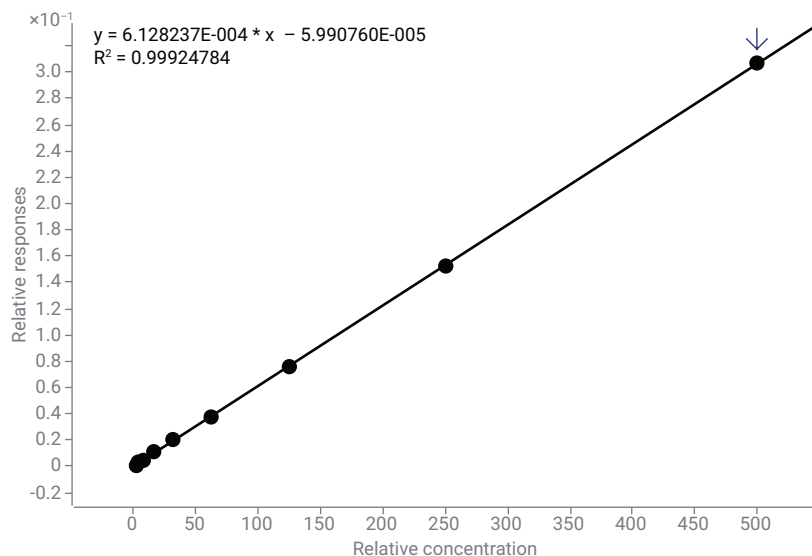


Figure 2. Calibration curve of  $\alpha\text{KG}$ .

## Conclusion

This single LC/MS/MS method, developed after a derivatization procedure with DATAN, allows the quantification of both (L) and (D)2-HGA and  $\alpha\text{KG}$  without derivatization in biological fluids (serum and plasma). This method can be used for clinical research as a tool and predictive biomarker for translational research.

The results generated can be used to calculate the ratio of 2-HGA relative to the amount of  $\alpha\text{KG}$  present, which will relate to the phenotype. Our LC/MS/MS method showed rapid separation with good sensitivity for the determination of both enantiomers without a chiral column. In addition, the LOQ values obtained were comparable to those obtained using previously described methods.

**Note:** For specific test operations and parameters, please refer to application note: 5994-0341EN [Measurement of D- and L-2-Hydroxyglutarate Enantiomers and  \$\alpha\text{KG}\$  Acid by LC/MS/MS](#).

## Maximizing Triple Quadrupole Mass Spectrometry Productivity with the Agilent StreamSelect LC/MS System

### Abstract

This Application Note demonstrates how the Agilent StreamSelect LC/MS system increases the throughput of traditional LC/MS analyses. By mirroring an LC method on two HPLC systems and coordinating the use of a single triple quadrupole mass spectrometer, up to twice the throughput can be achieved.

Agilent MassHunter StreamSelect software requires minimal user input, and is compatible with existing LC/MS methods. Based on the parameters provided, the software determines the most efficient way of injecting and analyzing samples automatically.

A previously developed analytical method for the analysis of 25-OH vitamin D2 and D3 by LC/MS/MS used a single high-performance liquid chromatography (HPLC) stream.<sup>1</sup> To demonstrate an increase in LC/MS productivity while maintaining excellent robustness and reliability, the method was implemented on a StreamSelect LC/MS system.

### Experimental

- **LC:** Agilent StreamSelect LC/MS system
- **MS:** 6460 triple quadrupole mass spectrometer equipped with Jet Stream
- **Columns:**  
Trapping: Agilent Eclipse Plus C18 Guard column, 2.1 × 12.5 mm, 5 µm  
Analytical: Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm
- **Software:** Agilent MassHunter StreamSelect software

### Results and discussion

In the previously developed analytical method, which used a single HPLC stream, it was observed that more than 50% of the data collected by the mass spectrometer was not needed since the analytes of interest eluted during a narrow retention time window. The StreamSelect system mirrors components of a single-stream system to provide a second stream, operating in parallel to the first. By loading the standard LC/MS method and specifying when the analytes of interest elute, the MassHunter StreamSelect software is able to determine the most efficient method of injecting and analyzing a batch of samples. By staggering injections on parallel streams and switching between the two streams at the appropriate time, the StreamSelect system can achieve up to twice the throughput of a standard LC/MS system (Figure 1).

The two parallel LC systems displayed excellent agreement when comparing quantitative results (Figure 2) with an  $R^2 > 0.999$  for both 25-OH vitamin D2 and D3. Deviations in retention time between the two streams were also minimal—well below 10% RSD.

Since both LC systems and the MS device were controlled by a single piece of integrated automation software, excellent reliability and robustness were also observed. If a system error occurs while a batch is running, the system will automatically reschedule samples to run on a single LC system without losing any samples.

## Conclusion

The StreamSelect LC/MS system was evaluated using a previously developed analytical method for the measurement of 25-OH vitamin D2 and D3 in serum. MassHunter StreamSelect software coordinates a completely integrated LC/MS system consisting of two parallel HPLC streams and a single triple quadrupole mass spectrometer.

No special method development is required as the user selects a standard LC/MS method, defines when the analytes of interest elute, and the software automatically coordinates the analysis by setting all necessary timing. The use of the StreamSelect LC/MS system not only provides equivalent sensitivity, linearity, and reproducibility compared to the previously developed method, but also increases the productivity of a single triple quadrupole mass spectrometer. This Application Note illustrates the potential of the StreamSelect LC/MS system as a solution for enhanced productivity in a wide range of LC/MS analyses

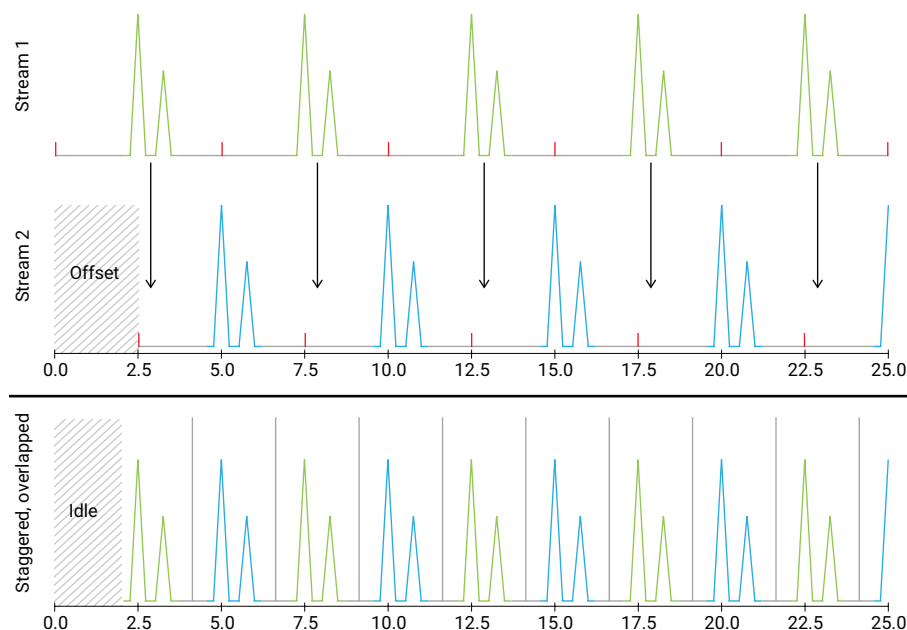


Figure 1. The StreamSelect LC/MS system maximizes MS usage by running staggered analyses on two parallel LC systems, and automatically coordinating MS analysis.

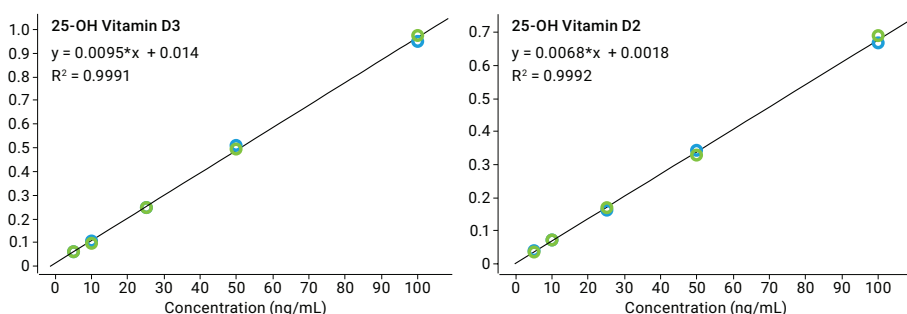


Figure 2. Combined calibration curves for 25-OH vitamin D2 and D3 across both LC systems: Stream 1 (green) and Stream 2 (blue).

## Reference

1. Doyle, R.; Szczesniowski, A.; McCann, K. Rapid Analysis of 25-OH Vitamin D in Serum Using an Agilent Triple Quadrupole LC/MS System with Automated Online Sample Cleanup, *Agilent Technologies*, publication number 5991-2035EN, **2013**.

**Note:** For specific test operations and parameters, please refer to application note: 5991-2900EN [Maximizing Triple Quadrupole Mass Spectrometry Productivity with the Agilent StreamSelect LC/MS System](#). 5991-2900CHCN.pdf ([agilent.com.cn](http://agilent.com.cn))

# Analysis of Cannabinoids and Their Metabolites in Urine Using the MassHunter StreamSelect LC/MS System

## Abstract

A highly selective analytical method has been developed for the analysis of six cannabinoids using liquid chromatography with triple quadrupole mass spectrometry (LC/MS/MS). A six-minute chromatographic method separates cannabidiol (CBD), cannabidiolic acid (CBDA), cannabinol (CBN), tetrahydrocannabinol (THC), nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THC-COOH), and 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (THC-OH). Sample throughput was nearly quadrupled by running four simultaneous, staggered chromatographic analyses on a single mass spectrometer using Agilent MassHunter StreamSelect LC/MS software.

## Experimental

- **LC:** Agilent StreamSelect LC/MS system
- **MS:** Agilent 6470 triple quadrupole mass spectrometer with Jet Stream
- **Columns:** Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7  $\mu$ m
- **Sample prep:** Urine samples were diluted 1:10 with 70% methanol containing labeled internal standards.
- **Software:** Agilent MassHunter StreamSelect software

## Results and discussion

StreamSelect acquired data for 894 samples over a period of 24 hours, which equates to 97 seconds per analysis. Compared to a six-minute run time for the same analysis using traditional LC/MS, this results in a 3.7x increase in sample throughput.

Chromatography remained robust and reproducible within and across streams (Figures 1 and 2). Over the course of nearly 27 hours, 1,000 identical urine samples containing 100 ng/mL of each of the six analytes were run across the four LC streams. Retention time %RSDs were excellent, ranging from 0.43 to 1.54%.

Whether calibrated individually or combined, all streams showed excellent quantitative results with linearity from 5 to 5,000 ng/mL (Figure 3; THC is representative of all results achieved). Replicate injections of 200 ng/mL samples resulted in accuracies ranging from 95.3 to 109.1% and %RSDs ranging from 0.47 to 2.79% across all analytes.

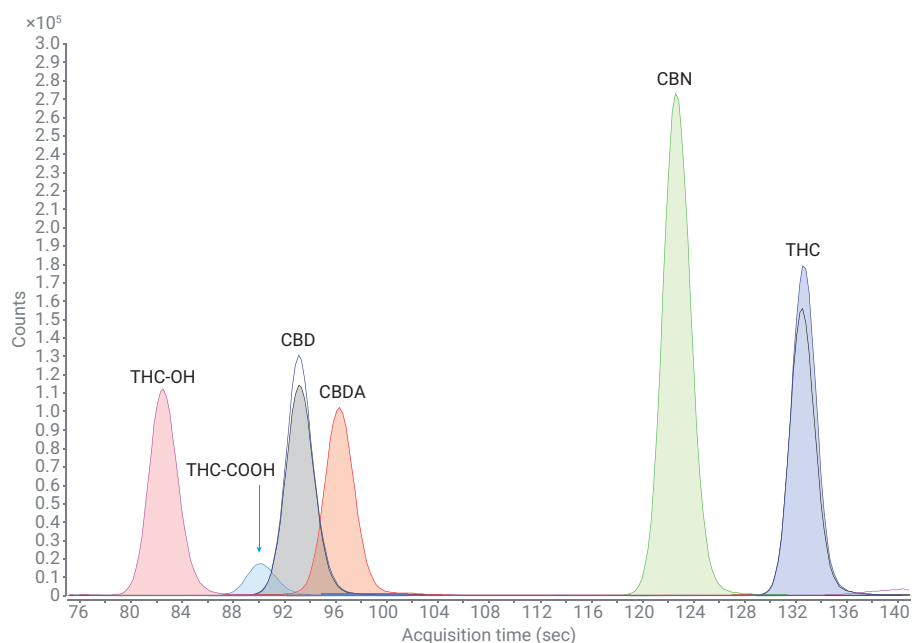


Figure 1. Chromatographic separation of six cannabinoid metabolites.

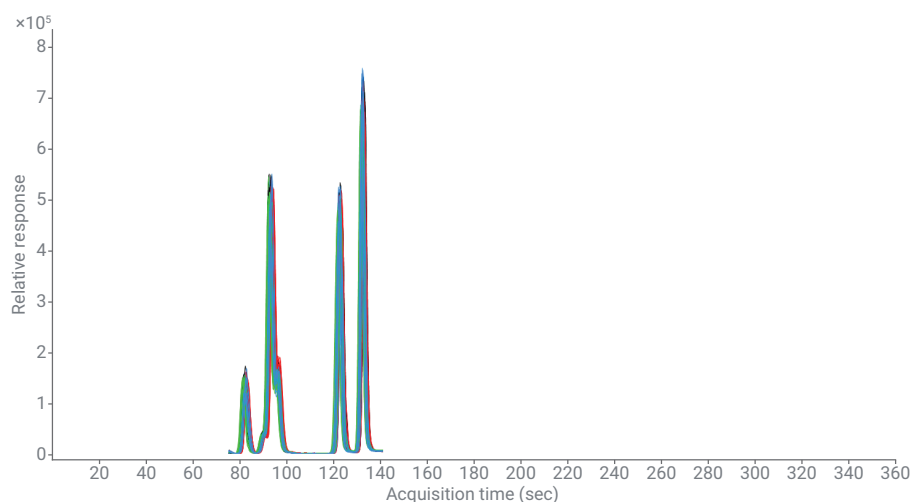


Figure 2. Overlaid chromatograms from streams one (black), two (red), three (green), and four (blue).

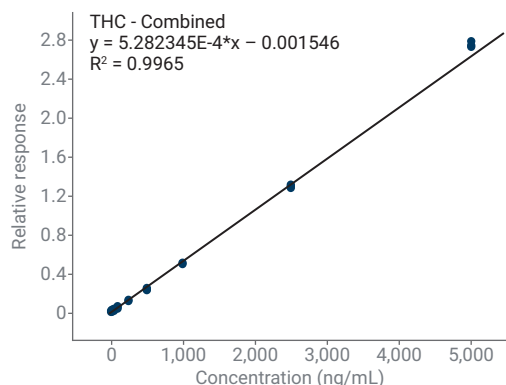


Figure 3. Combined calibration curve for THC, composed of calibrators from all four streams.

## Conclusion

A robust and accurate LC/MS/MS method has been developed for the analysis of six cannabinoids. By running this method on a four-stream StreamSelect instrument, data acquisition has been reduced to 1.5 minutes per sample. Quantitative results and retention times remain highly reproducible across all four streams, meaning reliable results will be achieved regardless of which stream is used to analyze a sample. Furthermore, calibration curves from each stream show excellent agreement, and resulted in equivalent quantitative results when treated individually or combined into a single calibration curve.

**Note:** For specific test operations and parameters, please refer to application note: 5994-0879EN [Analysis of Cannabinoids and Their Metabolites in Urine Using the MassHunter StreamSelect LC/MS System](#).

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