# Two-Dimensional LC/MS/MS to Reduce Ion Suppression in the Determination of Cannabinoids in Blood Plasma

## Application Note

Forensic Toxicology


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### Abstract

Cannabinoids in plasma were analyzed after minimal sample pretreatment with an Agilent 6495 Triple Quadrupole LC/MS. Significant loss of signal compared to analysis of standard solutions was observed in plasma samples. Matrix-related ion suppression could be overcome by using the Agilent 1290 Infinity II 2D-LC solution with high-resolution sampling. The setup was tuned to enable simultaneous analysis of the psychoactive THC (Δ-9 tetrahydrocannabinol) and metabolites carboxy-THC (THC-COOH) and hydroxyl-THC (THC-OH) in blood plasma.


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## Introduction

The analysis of cannabinoids in biological matrices such as saliva, urine, hair, and blood is of utmost importance in forensic toxicology laboratories. Cannabis, or marijuana, is one of the most frequently used illegal drug substances. Detection of Δ-9 tetrahydrocannabinol (THC) or metabolites in plasma is indicative of recent usage, and can can be benificial in driving under-the-influence or professional screening campaigns[1,2].

THC is the principal psychoactive component of cannabis, and is quickly metabolized and cleared from blood. The main metabolites are carboxy-THC (THC-COOH) and hydroxyl-THC (THC-OH). Both can be detected in biological matrices over a longer period. Detection of THC in plasma indicates recent use, whereas detection of metabolites can be an indication of past use[3].

Analysis of these substances in whole blood and plasma often requires extensive sample preparation to reduce the amount, or remove proteins, lipids and phospholipids, and other potential interfering compounds. Therefore, protein precipitation, liquid-liquid extraction, solid-phase-extraction, and their combinations are needed prior to analysis with LC/MS/MS. Sample preparation is a time-consuming and exhaustive part of the analytical scheme, and ways to bypass this are sought after. The main problem for biological matrices such as blood plasma, is that a substantial amount of matrix material enters the ionization source and influences the ionization process. In most cases, this results in reduced analytical sensitivity, known as ion suppression. Analytical sensitivity is also reduced in the long term due to fouling of the source and the MS entrance.

Performing a heart-cut on the chromatographic region of interest and analyzing the cut(s) on a second-dimension column with orthogonal selectivity can strongly reduce or even eliminate ionization suppression. Using the Agilent 1290 Infinity II 2D-LC solution to transfer small fractions of a first-dimension separation, in which the THC signal was nearly completely suppressed by plasma matrix constituents, onto a second dimension, reduced ionization suppression to such an extent that accurate quantification of THC together with THC-OH and THC-COOH in blood plasma was obtained after minimal sample pretreatment.

## Experimental

### Chemicals, samples, and sample preparation

Acetonitrile was HPLC-S quality, and water and methanol were ULC/MS quality. Solvents were purchased from Biosolve (Valkenswaard, The Netherlands) Ammonium acetate and formic acid were from Sigma-Aldrich (Bornem, Belgium).

Stock solutions in methanol of THC, THC-COOH, and THC-OH. Their respective labeled internal standards THC-d3, THC-COOH-d3, and THC-OH-d3 were from Sigma-Aldrich (Bornem, Belgium). These were mixed and diluted to the appropriate concentration (0 to 100 ng/mL for standards, and 20 ng/mL for the internal standards) in 0.2 % formic acid in acetonitrile. A portion of this solution was then mixed with an equal portion of water to obtain the standard for injection in water/acetonitrile containing 0.1 % formic acid and 10 ng/mL of the internal standards.

Plasma samples were prepared by mixing 100 µL plasma with 100 µL of 0.2 % formic acid in acetonitrile containing 20 ng/mL of the internal standards (and standards for spiking experiments). The acetonitrile phase was added in four 25 µL aliquots, and after each addition, the sample was vortexed for 10 seconds. The complete mixture was then centrifuged for 4 minutes at 10,000 g. The clear upper phase was transferred to an autosampler vial, and injected for analysis.

### Instrumentation

An Agilent 1290 Infinity II 2D-LC solution was used. The configuration is described below.

#### LC/MS configuration for one-dimensional analyses

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with 100 µL analytical head and 100 µL loop
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with 2-position/6-port valve head (G4231B) and divider assembly (G7116-60006)
- Agilent low dispersion kit for Agilent 1290 Infinity II LC (5067-5963)
- Agilent 6495 Triple Quadrupole LC/MS (G6495A) with Jet Stream technology source

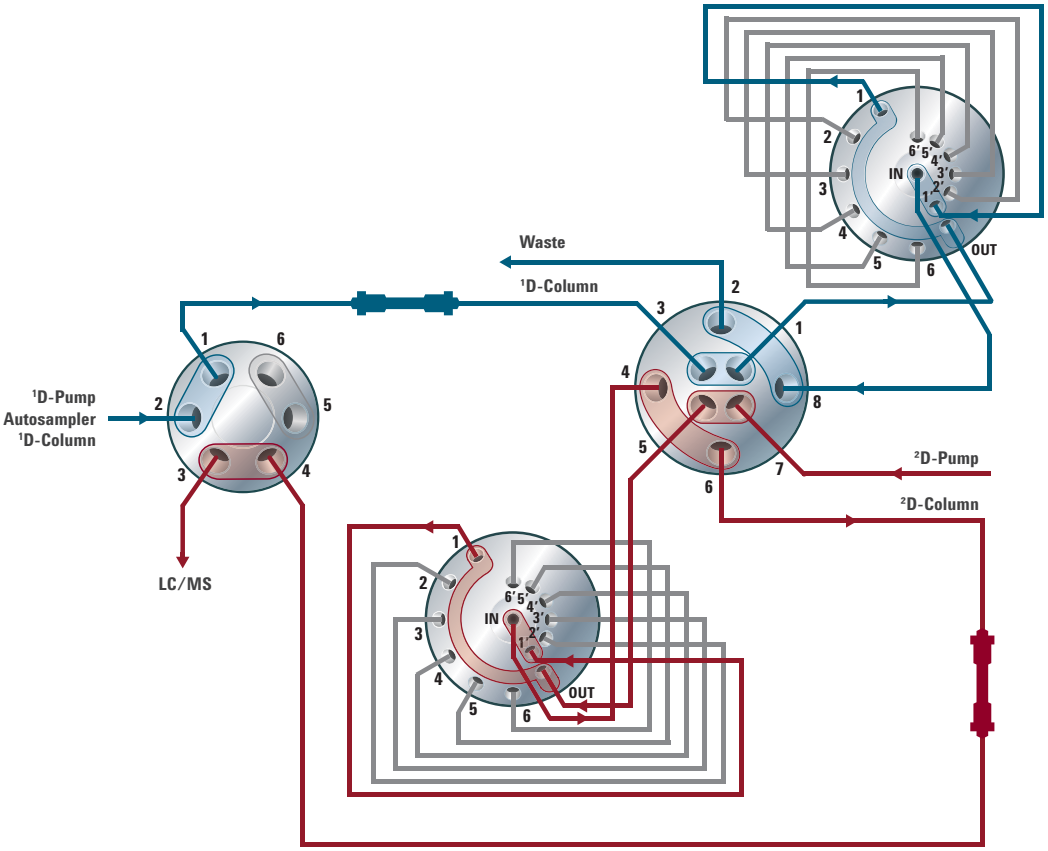
#### Additional modules for two-dimensional analyses

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity Valve Drives (3x G1170A) with 2D-LC valve (2-position/4-port duo-valve, G4236A) and multiple heart-cutting valves (2x G4242-64000) equipped with 40 µL loops

Figure 1 shows the configuration of the valves for multiple heart-cutting with the Agilent 1290 Infinity II 2D-LC solution.

### Software

Agilent OpenLab CDS ChemStation Edition software, version C.01.07 [27] with Agilent 1290 Infinity 2D-LC acquisition software, version A.01.02 [24] was used for control of the 2D-LC system, and Agilent MassHunter acquisition software B.08.00 for the triple quadrupole LC/MS system.



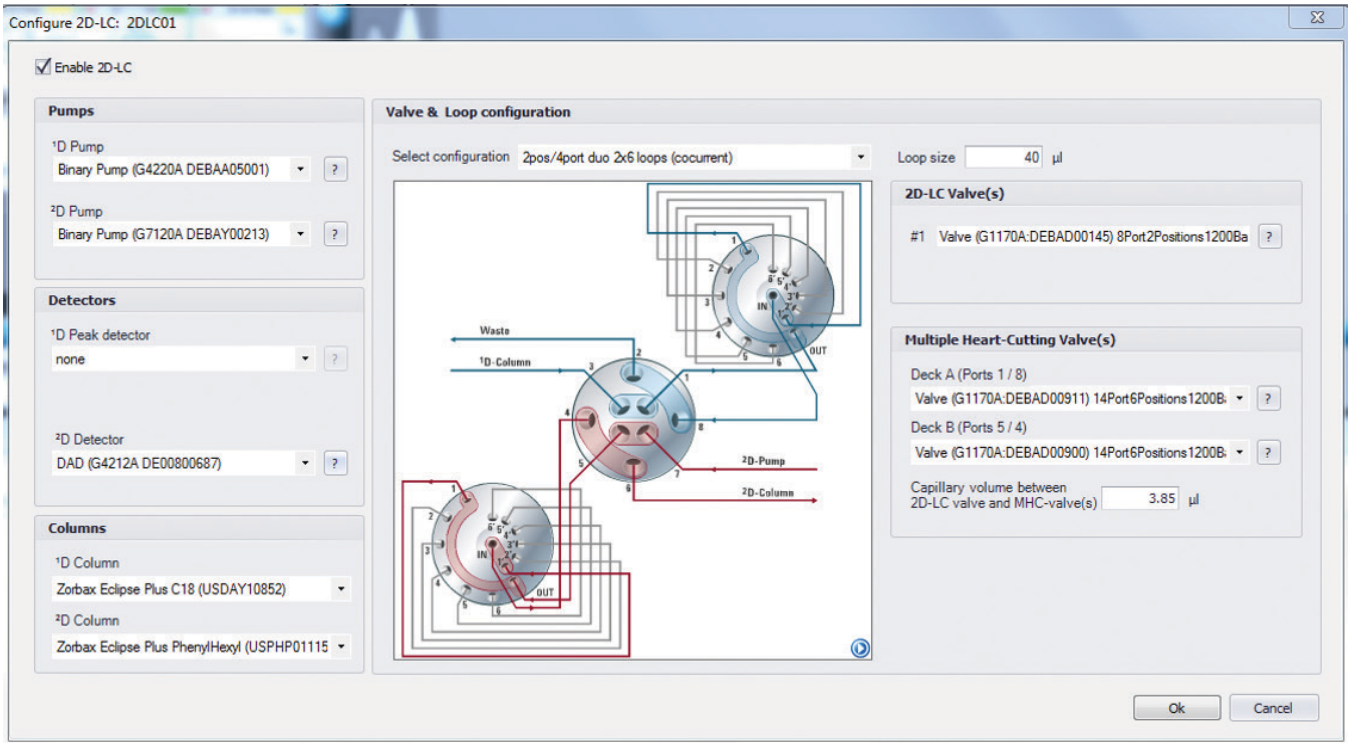




Figure 1. Instrument and valve configuration for multiple heart-cutting analysis.

## Method for one-dimensional and first-dimension analysis

| Parameter | Value |
|---|---|
| Column | Agilent ZORBAX Eclipse Plus C18 RRHD, 2.1 × 50 mm, 1.8 µm (p/n 959757-902) |
| Prefilter | Agilent 1290 Infinity inline filter, 0.3 µm, (p/n 5067-4638) |
| Mobile phase A | 5 mM Ammonium acetate in water |
| Mobile phase B | Methanol |
| Flow rate | 0.4 mL/min |
| Gradient | 0 to 1 minute: 55 %B<br>1 to 9 minutes: 55 to 100 %B |
| Column temperature | Left, 30 °C<br>For two-dimensional analysis, the valve was switched from Position 1 to Position 2 after:<br>4.50 minutes (THC-COOH)<br>5.80 minutes (THC-OH)<br>7.40 minutes (THC) |
| Injection | Injection program with needle wash (flush port, 3 seconds, methanol)<br>Draw 40 µL sample<br>Draw 20 µL 0.1 % formic acid in water<br>Inject |
| Injector temperature | 8 °C |

## Second-dimension analysis with high-resolution sampling

| Parameter | Value |
|---|---|
| Column | Agilent ZORBAX Eclipse Plus Phenyl-Hexyl RRHT, 3.0 × 50 mm, 1.8 µm, (p/n 959941-312) |
| Mobile phase A | 0.1 % formic acid in water |
| Mobile phase B | Methanol |
| Flow rate | 0.6 mL/min (idle flow 0.2 mL/min) |
| Gradient | 0 to 1.2 minutes: 50 to 100 %B<br>1.2 to 1.9 minutes: 100 %B<br>1.9 to 2.3 minutes: 50 %B |
| Cycle time | 2.3 minutes |
| $^{1}$D Sampling | 4 seconds (67 % loop filling) |
| Column temperature | Right, 50 °C |

## MS source parameters

| Parameter | Value |
|---|---|
| Ionization | Agilent Jet Stream technology source |
| Drying gas temperature | 140 °C |
| Drying gas flow | 15 L/min |
| Nebulizer pressure | 40 psig |
| Sheath gas temperature | 385 °C |
| Sheath gas flow | 12 L/min |
| Capillary voltage | 3,000/3,000 V (±) |
| Nozzle voltage | 300/1,700 V (±) |
| Ion funnel high-pressure RF | 150/190 V (±) |
| Ion funnel low-pressure RF | 60/140 V (±) |

## MS acquisition parameters

| Segment 1: 0 to 4.5 minutes, to waste | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Segment 2 (THC-COOH): 4.5 to 5.9 minutes, to MS | | | | | | | | |
| Compound | Precursor ion | MS1 Res | Product ion | MS2 Res | Dwell* (ms) | Collision energy (V) | Cell accelerator voltage (V) | Polarity |
| THC-COOH | 343.2 | Unit | 299.1 | Unit | 20/35 | 22 | 2.5 | Negative |
| THC-COOH | 343.2 | Unit | 245.1 | Unit | 20/35 | 32 | 4 | Negative |
| THC-COOH | 343.2 | Unit | 191.1 | Unit | 20/35 | 30 | 2 | Negative |
| THC-COOH d3 | 346.2 | Unit | 248.1 | Unit | 20/35 | 32 | 4 | Negative |
| THC-COOH d3 | 346.2 | Unit | 302.1 | Unit | 20/35 | 22 | 2 | Negative |
| Segment 3 (THC-OH): 5.9 to 7.4 minutes, to MS | | | | | | | | |
| Compound | Precursor ion | MS1 Res | Product ion | MS2 Res | Dwell (ms) | Collision energy (V) | Cell accelerator voltage (V) | Polarity |
| THC-OH | 331.2 | Unit | 313.2 | Unit | 20/35 | 10 | 2.5 | Positive |
| THC-OH | 331.2 | Unit | 201.2 | Unit | 20/35 | 26 | 6 | Positive |
| THC-OH | 331.2 | Unit | 193.1 | Unit | 20/35 | 20 | 6 | Positive |
| THC-OH d3 | 334.2 | Unit | 196.1 | Unit | 20/35 | 20 | 6 | Positive |
| THC-OH d3 | 334.2 | Unit | 201.2 | Unit | 20/35 | 26 | 6 | Positive |
| Segment 4 (THC): 7.4 minutes, to MS | | | | | | | | |
| Compound | Precursor ion | MS1 Res | Product ion | MS2 Res | Dwell (ms) | Collision energy (V) | Cell accelerator voltage (V) | Polarity |
| THC | 315.2 | Unit | 259.1 | Unit | 20/35 | 20 | 4 | Positive |
| THC | 315.2 | Unit | 193.1 | Unit | 20/35 | 25 | 5 | Positive |
| THC | 315.2 | Unit | 123 | Unit | 20/35 | 40 | 2 | Positive |
| THC d3 | 318.2 | Unit | 123 | Unit | 20/35 | 40 | 2 | Positive |
| THC d3 | 318.2 | Unit | 196.1 | Unit | 20/35 | 25 | 5 | Positive |

*Dwell time was set at 20 ms for $^{2}$D acquisition, at 35 ms for $^{1}$D acquisition.

## Results and Discussion

### One-dimensional analysis

Because the extracts contain approximately 50 % acetonitrile, and relatively large volumes are injected, an injection program was mandatory to focus the analytes on the column. THC-COOH particularly suffers from excessive band broadening and peak splitting when injected in an overly strong solvent. Injecting a 20 µL plug of 0.1 % formic acid in water helps improve the peak shape by diluting the acetonitrile, and neutralizing THC-COOH. As a result, injection of 40 µL of the sample was made possible without significant peak broadening.

Following the injection of a standard solution, the three cannabinoids were well separated and clearly detected. When injecting a spiked plasma extract, some interferences and ionization suppression hinder the analysis. Figure 2 shows examples of such analyses. The injection was first carried out on a 50 mm long column. In this case, the signal for THC was completely suppressed, and the signal for THC-OH showed approximately 74 % suppression (based on comparison of peak areas). One way to reduce ionization suppression is to increase the chromatographic resolution. The most obvious way to do this is to use a longer column. A 100-mm column was installed, and the analysis of standard and sample was repeated. The bottom part of Figure 2 shows that this reduced the suppression for the THC-OH peak from 74 to 58 %, and that a small signal was detected for THC. However, the response for THC in the plasma was too small to be used for quantitative purposes. As THC-OH and THC-COOH are detected on both columns, this strategy can be applied to quantify them by isotope dilution after addition of labeled internal standards. Analysis of a nonspiked plasma sample revealed the presence of THC-COOH and THC-OH, and indicated that the donor had used cannabis.

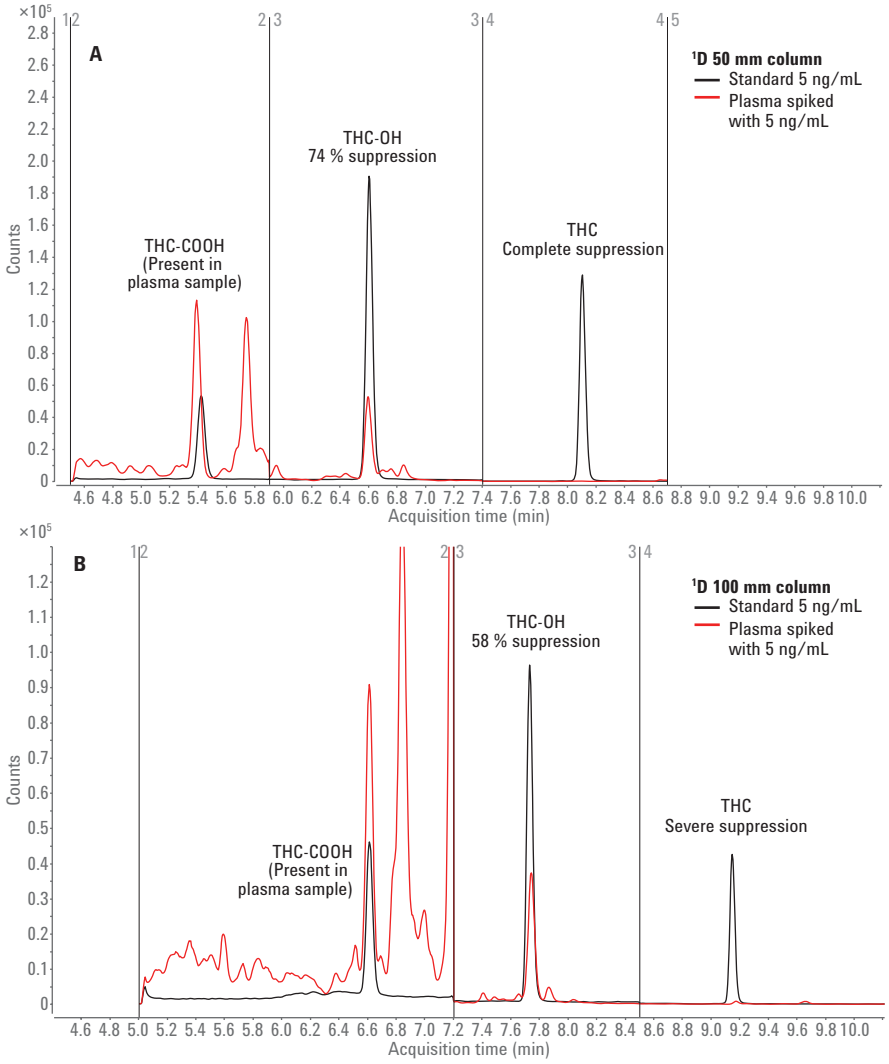



Figure 2. Total ion current for the 1D analysis of a standard solution and plasma extract (both solutions contain 5 ng/mL of each analyte and no internal standards) on a 50 mm (A) and 100 mm (B) column. The gradient was identical for both analyses (see Experimental section).

A solution for the loss in signal for the THC peak can be found in an exhaustive sample preparation step to purify the extract further and fractionate THC better. In this case, it was likely that ionization suppression was caused by the phospholipids present in the plasma[4]. Another option is to collect the peak (or elution window) of interest, and reanalyze this fraction on a second column/mobile phase combination with different selectivity compared to the original analysis. This could separate the target from the abundant matrix component(s) and reduce or even eliminate the suppression.

## Two-dimensional analysis

Offline collecting and reinjecting fractions is time-consuming, and there is a considerable risk of sample loss or contamination in intermediate steps (for example, drying and redissolving). The procedure can be performed online with the Agilent 1290 Infinity II 2D-LC solution and MHC option. The THC peak can be sampled in small fractions, and each of these fractions can then be analyzed automatically on a second-dimension column. In this study, a phenyl-hexyl stationary phase with a formic acid and methanol mobile phase was selected.

Although THC-COOH and THC-OH could be analyzed in the 1D-LC analyses, some 2D-LC tests were carried out to have a better understanding of the impact of sample matrix on these compounds as well. High-resolution sampling on the THC-COOH peak was done with positive and negative ionization. In positive ionization, significant amounts of interferences with the same transition but different ratios were detected. These were separated from the THC-COOH in the second dimension due to the orthogonal separation behavior, as shown in Figure 3.

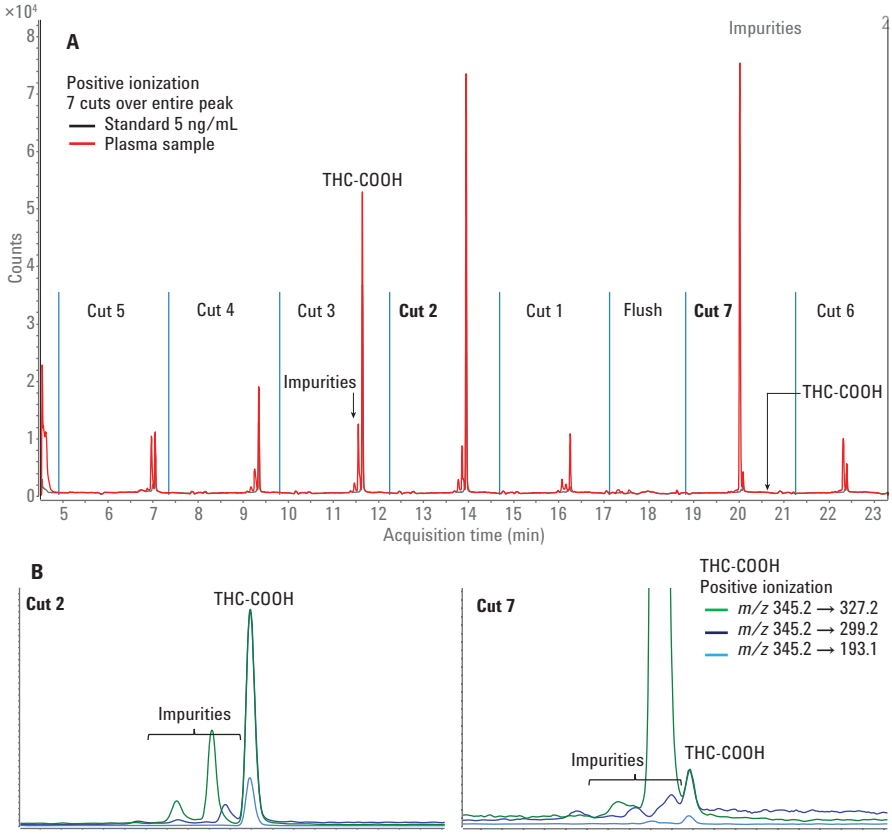



Figure 3. Positive ionization MHC analysis of THC-COOH of a standard solution (5 ng/mL of each analyte and internal standards) and nonspiked plasma extract. A) Total ion current of the complete analysis of the seven heart-cuts. B) THC-COOH transitions in detail of Cut 2 (contains mainly THC-COOH) and Cut 7 (contains mainly the interference).

The relative abundance of the second-dimension peaks changes between different fractions or cuts. This indicates that the peak detected in the first dimension using positive ionization is not corresponding to one product. The interference is not detected with negative ionization, and as THC-COOH is acidic, negative ionization gives excellent analytical sensitivity for this compound on the 6495A Triple Quadrupole MS, and was consequently selected as polar for 1D-LC analysis of this compound. The MHC method also confirmed the presence of THC-COOH in the nonspiked plasma sample.

The same approach was applied to the THC-OH peak, where some suppression was observed in 1D-LC analyses. No significant interferences were detected with MHC, but signal suppression was reduced 2- to 3-fold (from 74 to only 28 %) in comparison with the original separation on the 50-mm column.

The main problem in the one-dimensional separation of samples was the (nearly) complete suppression of the signal for THC. The application of high-resolution sampling on this peak resulted in radical improvement of the signal. Not all signal suppression was removed, but further optimization of second-dimension stationary and mobile phase could be done to improve this. The recovery was satisfactory, and the detected peaks can be used to quantify the THC in plasma extracts. Figure 4 shows an example of such an analysis and an overlay of Cut 2 from five consecutive injections of a spiked plasma sample.

It is clear that the procedure is repeatable, and the method was evaluated for repeatability, linearity, and accuracy. A set of standard solutions, sample, and spiked samples was prepared and injected with the final method. Single injections of standard solutions with concentration ranging from 0.1 to 50 ng/mL were used to evaluate linearity, and the calibration curve was used to determine the concentration of the analytes in the nonspiked plasma sample.

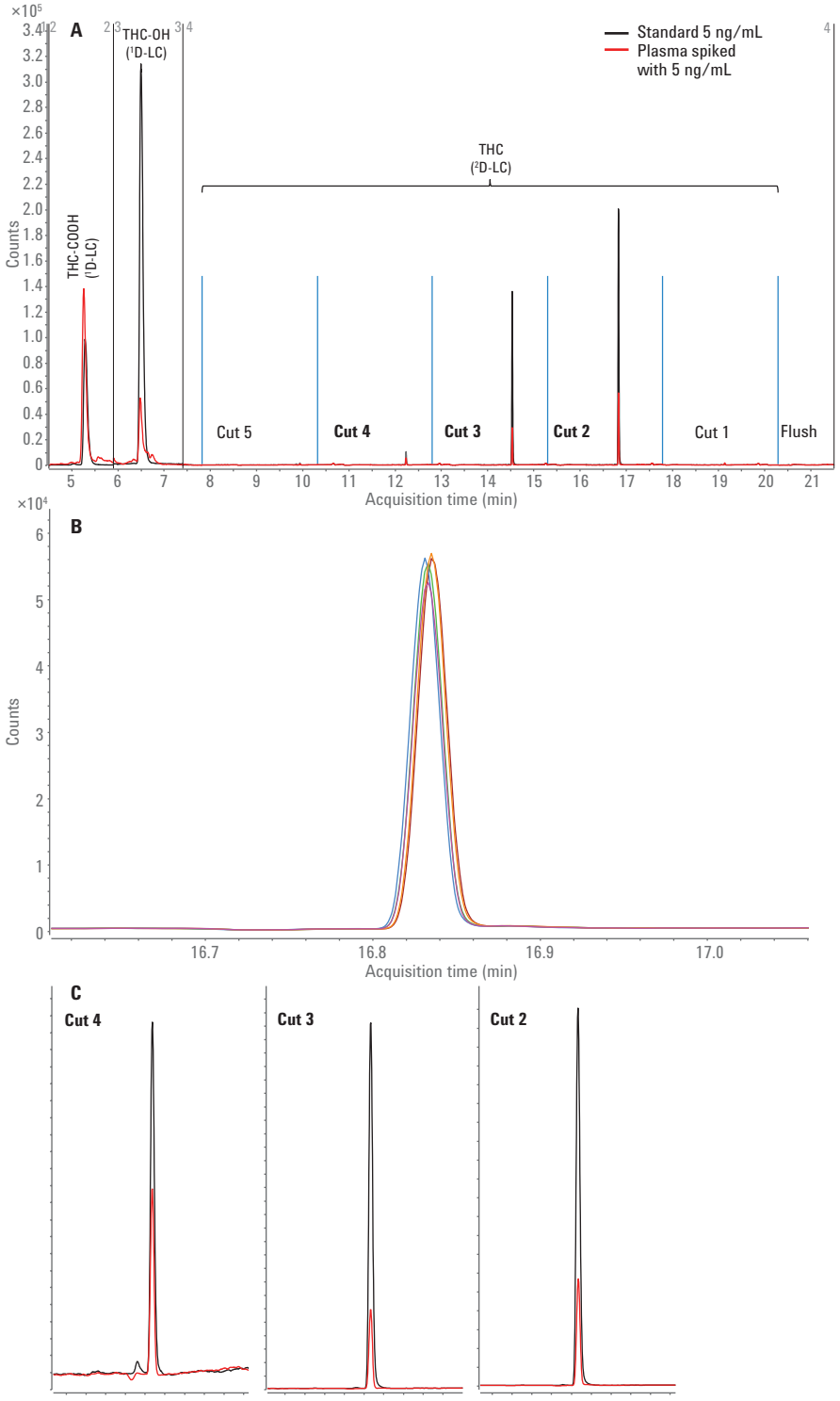



Figure 4. MHC analysis of a standard solution and spiked plasma extract (both solutions contain 5 ng/mL of each analyte and internal standards). A) Total ion current of the complete analysis (THC-COOH and THC-OH in 1D-LC, THC in 2D-LC). Five cuts were taken. B) TIC overlay for Cut 2 from five consecutive injections of spiked plasma. C) Detail of TIC for Cuts 2 to 4.

Spiked samples were prepared at the same concentrations as the standard solutions to calculate the accuracy of the method, and injection precision was determined by five consecutive injections of both standard solutions and spiked samples (5 ng/mL). Detection limits in standard solutions were below 0.1 ng/mL for all compounds. Figure 5 shows an example of low-level standard solutions and plasma sample, and Table 1 summarizes the main results for method evaluation.

## Conclusion

The Agilent 1290 Infinity II 2D-LC solution with the Agilent 6495 Triple Quadrupole LC/MS enables simultaneous quantification of THC, THC-COOH, and THC-OH in plasma samples after minimal sample preparation. THC-COOH and THC-OH were analyzed after one-dimensional chromatography, while THC was only detected in plasma samples after high-resolution sampling to reduce ionization suppression caused by the matrix. The developed method showed good linearity and precision. The method can be highly useful to quantify compounds in difficult matrices originating from various fields.

## References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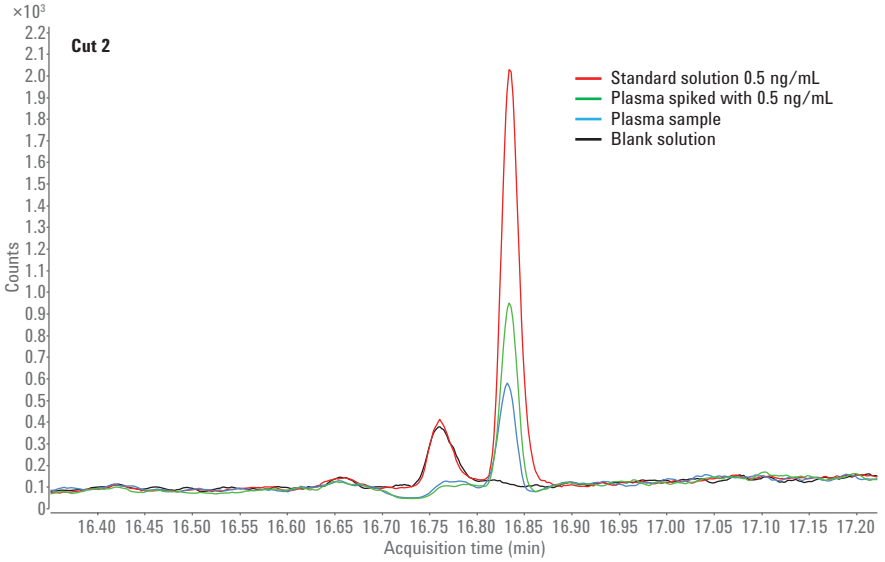


Figure 5. Quantifier transition for analysis of Cut 2 of THC peak in blank solution, 0.5 ng/mL standard solution, plasma extract and spiked plasma extract (0.5 ng/mL).

Table 1. Method evaluation results.

| | | Standards | | Plasma | | | |
|---|---|---|---|---|---|---|---|
| | | $R^2$ [a] | Area RSD% (n = 5, 5 ng/mL) | Area RSD% (n = 5, 5 ng/mL) | Concentration (ng/mL) | Concentration spike 1 ng/mL (Recovery%) | Concentration spike 10 ng/mL (Recovery%) |
| $^1$D | THC-COOH | 0.9976 | 1.12 | 1.45 | 14.670 | 16.722 (205.2) | 24.669 (100.0) |
| | THC-OH | 0.9966 | 1.29 | 2.29 | 0.180 | 1.031 (85.1) | 9.208 (90.3) |
| $^2$D | THC Cut 2 | 0.9935 | 4.78 | 7.81 | | | |
| | THC Cut 3 | 0.9957 | 5.49 | 8.51 | | | |
| | THC Cut 4 | 0.9962 | 14.66 | 9.00 | | | |
| | THC Cut 2–4 | | 3.15[b] | 1.78[b] | 0.433[c] | 1.451[c] (101.8) | 9.484[c] (90.5) |

[a] Calibration levels: 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng/mL
[b] RSD% calculated on the sum of the peak areas for Cuts 2–4
[c] Average concentration from Cuts 2–4

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