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Novel Extraction Techniques Using ACQUITY UPLC with 2D Technology: Part IV – First Time Users

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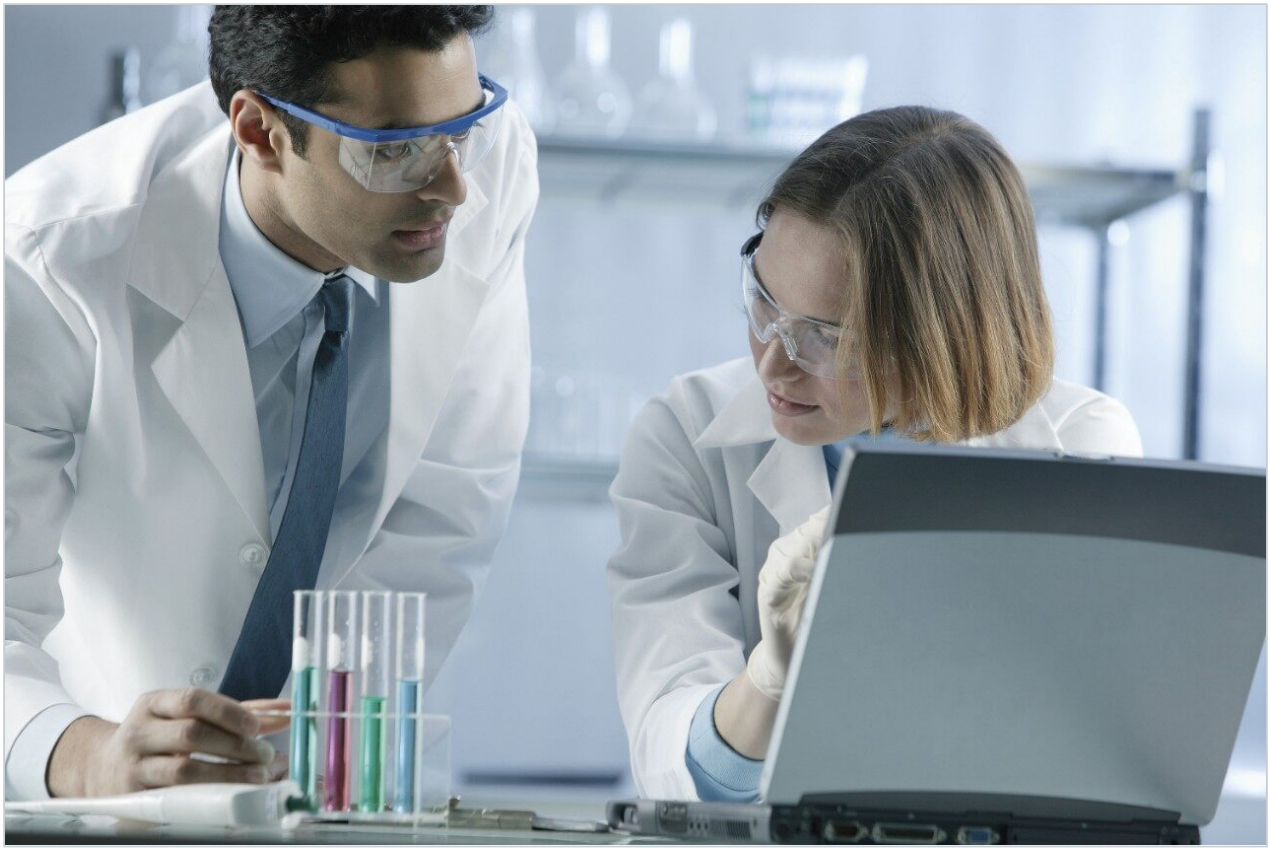

Abstract

This application note is on multidimensional chromatography, a technique inherently perceived as being difficult to operate and understand. Here we describe how it is possible for inexperienced users to produce quality results in a short amount of time.

Benefits

- Fast extraction protocol (45 min)
- Trace level detection (ppt)
- Increased separation powers

Introduction

PRELUDE

In the spring of 2016, after the acceptance of a co-authored research publication between two principal investigators from Boston University School of Medicine and Waters Corporation, a project to expand into a collaborative agreement was submitted to Waters Scientific Steering Committee. The goal of the agreement was to pool resources from both research teams. Five- to eight-month internships at Waters would be offered to graduate students from Boston University's department of Biomedical Forensics Sciences.

There was overwhelming interest from students. As part of the program, interns received daily one-on-one theoretical and hands-on training in mass spectrometry, 2D and 3D liquid chromatography, and sample preparation techniques. After mastering their laboratory skills, each intern was assigned to a research project that aligned directly with their thesis research for their M.S. degree. Project results were also made available in Waters application notes, peer-reviewed publications, and oral/poster presentations at select conferences.

In the spring of 2018, the project collaboration expanded to include a five-day, intensive, advanced chemistry laboratory class for five students (the first of its kind). After the first day of class, one student opted to intern at Waters that fall. By year-end, with a research project on microcystin analysis in urine by 2D LC-MS/MS nearing completion, the intern was offered a full-time scientist position at Waters for January 2019. In the spring of the same year, the collaboration hosted its second five-day advanced chemistry class with six

student interns (Figure 1).

Figure 1. Advanced chemistry class of 2019.

This application note is on multidimensional chromatography, a technique inherently perceived as being difficult to operate and understand. Here we describe how it is possible for inexperienced users to produce quality results in a short amount of time. The interns were challenged with this task and how to create a 2D LC-MS/MS protocol for the analysis of targeted molecules in a biological matrix. Each day had a main objective, and the day began with a one-hour lecture, leaving the remainder of the day for hands-on practice.

- Day one: Students were immersed in the theory of mass spectrometry and focused on how to select and optimize a multiple reaction monitoring (MRM) transition for each target analyte. By the end of the day, the 2D LC-MS/MS unit was properly set up with a 6 x 6 method development overnight run (up to 18 hr) for the next day's objective, choosing the optimum LC method.
- Day two: 2D LC-MS/MS results from the previous overnight run were reviewed and a final 2D LC-MS/MS method was then selected. The training continued with the creation of an eight-point-calibration curve for several organic solvents and additive variants. A second overnight run with the chosen LC method was set at the end of the day.
- Day three: Training continued with the creation and optimization of an extraction protocol for a targeted analyte in a biological matrix using a solid-phase extraction technique.

- Day four: Advance extraction techniques were covered (i.e., passive vs. captive) and a full eight-point-calibration curve for an un-extracted standard, a matrix-match extracted standard, and a matrix extracted sample were completed and launched for overnight analysis.
- Day five: Results were processed and quantified using software.

In a multi-task environment the students were trained on how to generate maximum results, and how to manage day-time workflow with day and overnight data acquisition. The raw results were tabulated in excel spreadsheets, MS spectrums and LC chromatograms provided in PowerPoint, and all data was made available for publication. This application note reflects the students' work and interprets the students' training during the five-day class.

When using LC-MS platforms, most users are confronted with analytical challenges that require very complex sample preparation protocols, thus producing complex extracts. In this case, the number of entities or analytes present in the final extract will largely exceed the separation power (peak capacity) of a single dimension chromatography system. Novel separation approaches, specific detection, and extraction chemistries can help, but those will usually produce limited performance. In recent years, many applications are coupling multiple layers of separation dimensions in the attempt to increase the separation power for the analysis of the complex mixture. Today, the concept of multidimensional chromatography is still perceived as a very difficult technique to master. By overlooking the perception of complexity, multidimensional chromatography simply adds extra components in order to achieve a specific workflow. Entry-level upgrades are 10–20% of the cost of a standard LC-MS/MS system. The return on investment produces an average 10-fold cost reduction in sample preparation protocols, analytical time, consumables, and resources.

The following are the intern's and student's thoughts on their experiences with the collaborative program and LC-MS/MS.

Malorie Mella, Boston University School of Medicine, Class of 2017: "My internship had an incredible impact on my career trajectory. The opportunity to build upon my fundamentals in chromatography and mass spectrometry with hands on experience such as setting up instruments, troubleshooting, and doing research for application notes was invaluable. Learning about the advantages and experimenting with 2D LC techniques truly cemented my working knowledge of how LC-MS could be applied in industry. I was also able to ace interviews and gain employment as an analytical chemist at a start-up pharmaceutical company where I single handedly developed several analytical methods for testing drug formulations in development using all the knowledge and techniques I learned during my internship. I am very thankful for my time there and owe my career to it."

Kayla Benvenuto, Boston University School of Medicine, Class of 2017: “My internship expanded my experience level, skill set and knowledge extensively. From sample preparation to method optimization, I was able to apply what I learned in the classroom and in textbooks hands on. I was given guidance and gained skill sets to be able to work independently. More importantly, I gained essential troubleshooting skills which have become imperative in my current employment position. Multidimensional chromatography was a major contributor to my skill set. Overall, I acquired extensive knowledge of chromatography.”

Brendan Scheitzer, Boston University School of Medicine, Class of 2017: “My internship exposed me to a large volume of hands-on experience. Overall, it was a great boon in understanding, focusing on practical instead of just theoretical, the complexities of chemical analysis using LC-MS/MS. I was paired to a daily one-on-one trainer ready and willing to share his wealth of knowledge with me. The experience I garnered there were unmatched and invaluable to me; it is hard to express just how much I learned from my time working at Waters Corporation.”

Robert Walsh, Boston University School of Medicine, Class of 2018: “My internship was incredibly useful and helpful both from a scientific learning and professional development perspective. On the scientific side, it provided more handson and in-depth experience with LC-MS/MS than could be obtained in any academic environment. It also allowed for me to learn multidimensional chromatography, a newer frontier in LC-MS/MS analyses that helps to improve LC performance and that is relatively simple to learn once you have a working understanding of LC. From a professional perspective, it gave me a taste of what working for private or industrial sector is like. Furthermore, it certainly helped my job applications to have industrial experience on my resume.”

Jacob Samuel, Boston University School of Medicine, Class of 2018: “As someone who was fascinated with instrumental analysis and wanted a deeper and more applicable education than what I had, this internship was perfect for me. Given that my project dealt with multiple classes of compounds, I was given ample practice and a variety of scenarios to learn and troubleshoot in sample preparation, chromatography and mass spectrometry. Additionally, I learned a good deal about innovation, not only in learning about and using multidimensional chromatography, but also pursuing better ways to meet one’s needs. On top of that, having a wide selection of tools available can really open your eyes to what is possible in method development. Ultimately, the industrial internship provided a great environment to learn and explore provided one is willing to put in the effort.”

Beatriz Renner, Boston University School of Medicine, Class of 2019: “The impact that my internship had on my career as a scientist was tantamount to having had industry experience. Once in my post as a Scientist in Waters Scientific Operations group, I was able to start hands on work immediately. All the skills I received

during my internship have not only been helpful but necessary to perform my current job duties as a scientist."

Devyani Bhandari, Boston University School of Medicine, Class of 2019: "My experience as an intern at Waters Corporation has been very enlightening. This internship has not only advanced my scientific knowledge but also prepared me to fit right into any industrial setting. It also has made me into a problem solving and self-motivated individual. The hands-on training provided me with hands on skills that are in demand across many fields like pharmaceuticals, environmental, forensics, and many more. I believe this internship is an asset to the BMFS program and has helped me standout from my competitors in terms of skills, knowledge, and experience."

Ketki Bagwe, Boston University School of Medicine, Class of 2019: "As I came from a background of biology, I had no experience with LC-MS/MS apart from some theoretical basics. Despite this, within a couple of weeks, I was able to handle the LC-MS instrument independently. The learning curve was steep, but it was an enjoyable and educational process. The internship is very hands on, which gives you the necessary skills required for a lab-based job and helps polish your knowledge of chemistry. I was given the opportunity to take ownership of my project, and this along with being able to work with and troubleshoot an LC-MS instrument on your own gives one a confidence as well as a skill boost. As this lab specializes in multi-dimensional liquid chromatography, it is a great place to learn about cutting-edge research projects. It proved to be a great learning experience and in addition gave me an exposure to industry as well as corporate culture."

Olivia George, Boston University School of Medicine, Class of 2020: "In attending the Advance Chemistry course at Waters Corporation, I was able to get a taste of the kinds of technologies used in the real world and have hands-on experience with cutting-edge instrumentation. Going through the process of understanding sample preparation, chromatography, and mass spectrometry gave me valuable insight and pushed me to want to take an internship at Waters. The work and life skills, such as time management and being able to work independently, learned here will be of paramount value in future job searches. These skills will allow me to be far ahead of my competitors in the job market."

Miranda Shaine, Boston University School of Medicine, Class of 2020: "Taking the Advanced Chemistry class at Waters Corporation was one of the most educational and eye-opening experiences I have had. The privilege of working hands on with the instrumentation that is used universally in many scientific fields taught me how to apply the knowledge I have learned directly to practical uses. The expertise and guidance of the teacher inspired me to take an internship at Waters Corporation, providing a one-on-one, hands on opportunity to learn the ins and outs of the application. I am confident that once I complete the internship, I will have the knowledge and ability to be a strong candidate for other professional opportunities."

Introduction

Synthetic cannabinoids belong to a class of drugs known as novel psychoactive substances. Such substances are manufactured to produce similar effects to illicit drugs but are currently unregulated. With changing confirmations and the rapid evolution of compounds, identification and quantification of these compounds can be difficult with routine screening and confirmatory tests. Developing a method for the rapid and sensitive screening and quantification of these compounds is useful in identifying the presence of these increasingly abused compounds.

Furthermore, antibiotics are used in the treatment of bacterial infections. In administering antibiotics, therapeutic monitoring of dosage is important as the incorrect dose can lead to bacterial resistance or excessive elimination of naturally endogenous beneficial bacteria.

This research sought to develop a method for the rapid and sensitive screening and quantification of several compound classes using two-dimensional, ultra-performance liquid chromatography (UPLC) with tandem mass spectrometry (MS-MS). Traditional HPLC columns are 4.6 µm × 100 mm and packed with a synthetic sorbent. Silica particle beads are often used to obtain unchanged normal-phase chromatography. A ligand, typically C_{18}, is added to create a non-polar stationary phase for reversed-phase chromatography. In UPLC there is higher pressure, however the flow rate drops compared to HPLC. A guard column is introduced to increase the life of the column and filter out the debris or contaminants.

Additionally, various matrices can complicate extraction processes, which makes it difficult to detect or quantify their analytes due to matrix effects. Many laboratories are using sample preparation techniques that include solvent evaporation followed by reconstitution. These steps are not only time-consuming, but also have the potential to lose trace amounts of the desired compounds. Two-dimensional liquid chromatography (2D-LC) allows for robust and reproducible methods that eliminate steps for evaporation and reconstitution. The elimination of these steps decreases sample preparation time without losing the quality of the results.

Experimental

MRM transition

The process began with optimizing a Multiple Reaction Monitoring (MRM) transition for several target analytes divided into two classes (Figures 2 and 3). Two MRM transitions for each compound were recorded for qualitative and quantitative purposes by selecting the most intense signals for the parent ion. MRM

transition data was obtained for each drug class at pH 3, 7, and 10 over a range of different Collision Induced Dissociation (CID) energies. This enabled the 2D-LC instrument to recognize the mass-to-charge ratios of interest in the specific method (Table 1).

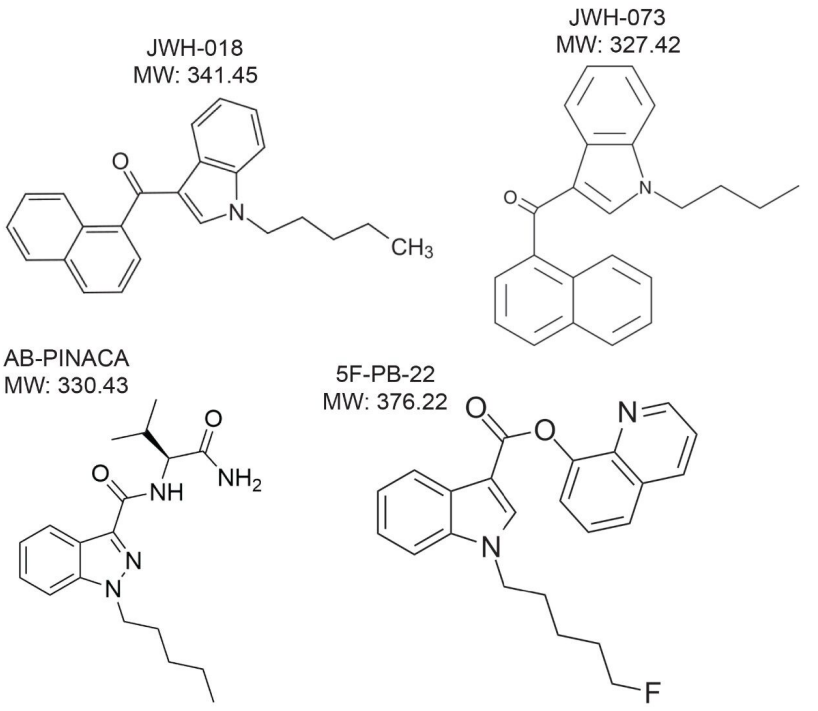

Figure 2. Chemical structure for cannabinoids.

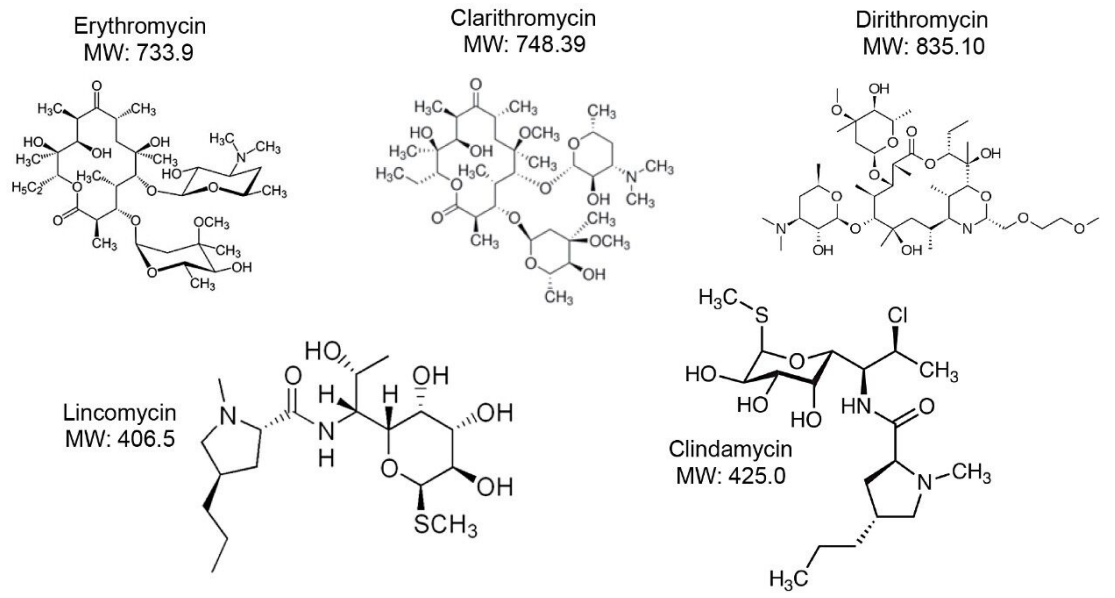

Figure 3. Chemical structure for macrolides.

	Precursor Ion	Quant	CID	Qual	CID	Cone
Cannabinoids						
JWH-018	342.3	155.1	25	127.1	40	30
JWH-073	328.3	155.1	25	127.1	40	30
AB-PINACA	331.3	145.1	35	215.2	25	30
5F-PB-22	377.3	144.1	30	232.2	30	30
Macrolides						
Clindamycin	425.2	126.3	20	377.4	30	30
Clarithromycin	748.5	158.3	25	590.6	20	30
Erythromcyin	734.5	158.2	30	576.5	25	30
Dirithromycin	835.6	158.2	35	677.6	25	30
Lincomycin	407.6	126.2	35	359.3	15	30

Table 1. MRM transition for cannabinoids and macrolides.

Separation

Upon selection of the analyte's MRM, the next phase was chromatographic condition optimization for each target analyte. Due to the wide range of chemical compositions and polarities of the target analytes, parameters must be established before analysis. The chromatographic conditions were tested on XBridge C_{18} and Oasis HLB trapping chemistries and BEH C_{18} separation chemistries. The HLB trap differs from the C_{18}

trap because a polymer is used instead of silica. The loading mobile phase (low pH, high pH, and neutral pH) and eluting mobile phases (MeOH + 0.5% ammonium hydroxide and ACN + 0.5% ammonium hydroxide) were also optimized. The best method for each drug class was selected based on the maximum signal intensity and the ability to display the best Gaussian chromatography peak shape for all the compounds within their respective drug classes.

Extraction

After selection of the best chromatographic method at the ideal pH, varying solutions (10 ppb in water, methanol, and acetonitrile) were tested to determine the best sample preparation of each compound class to elute for solid-phase extraction (SPE). A seven-point calibration curve was evaluated with concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/mL of each analyte for macrolides, with a resultant five-point calibration curve (0.5, 1.0, 2.0, 5.0, 10.0 ng/mL) being utilized for cannabinoids quantification. SPE was performed on concentrations 0.1, 1.0, and 10.0 ng/mL of each class in water and urine samples for comparison to the calibration curve. The SPE column was conditioned with 2 mL methanol, 2 mL water. Then 2 mL of the spiked sample was loaded. The column was then washed with 5% methanol solution and target analytes were eluted with 2 mL of low pH 3 or high pH 10 solvents, including acetonitrile and/or methanol. The extracted solutions and two methanol blanks were analyzed on instrumentation for quantitation. It should be noted that the 2D-UPLC-MS/MS technique does not require the analyst to evaporate the solution to dryness and reconstitute; thereby, decreasing the work time for this method.

Chromatography and MS-MS conditions

Loading conditions

Column:	Oasis HLB, 20 µm–40 mg (3.9 × 5 mm)
Loading:	MilliQ water (pH 7)
Flow rate:	2 mL/min
At-column dilution:	5% (0.1 mL/min loading pump, 2 mL/min diluting pump)

UPLC conditions

UPLC

System:	ACQUITY UPLC with 2D Technology configured for trap and elute with At-column dilution
Runtime:	10 min
Columns:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 × 50 mm (p/n: 186002350); Oasis HLB Direct Connect HP, 20 µm, 2.1 mm × 30 mm, (p/n: 186005231)
Column temp.:	60 °C
Mobile phase A:	Water + 0.5% ammonium hydroxide
Mobile phase B:	Acetonitrile + 0.5% ammonium hydroxide
Elution:	5-min linear gradient from 5% (B) to 95% (B)
Flow rate:	0.500 mL/min (elution pump)
Injection volume:	50 µL
Injection rate:	250 µL/min

MS conditions

System:	Xevo TQ-S
Ionization mode:	ESI+
Capillary voltage:	2.5 kV
Cone voltage:	30.0 V
Source offset:	30.0 V
Desolvation temp.:	30 °C
Desolvation gas:	650 L/hr
Cone gas:	50 L/hr

Results and Discussion

MRM Optimization

The target analytes for this application note were separated into two classes: synthetic cannabinoids and macrolide antibiotics.
Each class contained up to five target analytes sharing a common chemical backbone. The workflow began by creating 1.0 mg/mL
stock solution for each target analyte in either methanol or acetonitrile. From the stock concentration, an infusion solution at
1.0 µg/mL in 50/50 methanol/water was made at three different pHs; acidic (pH 3), neutral (pH 7), and basic (pH 10). Each target
analyte was infused at 10 µL/min under full scan ESI positive, to highlight which pH gave the highest intensity. By comparing
the full scan spectra at various pHs, the task at hand was to identify in which state (e.g., [M]+, [M+H]+, [M-H2O]+, [M+Na]+, etc.)

a target analyte will be present in chromatography conditions (Figures 4 and 5).

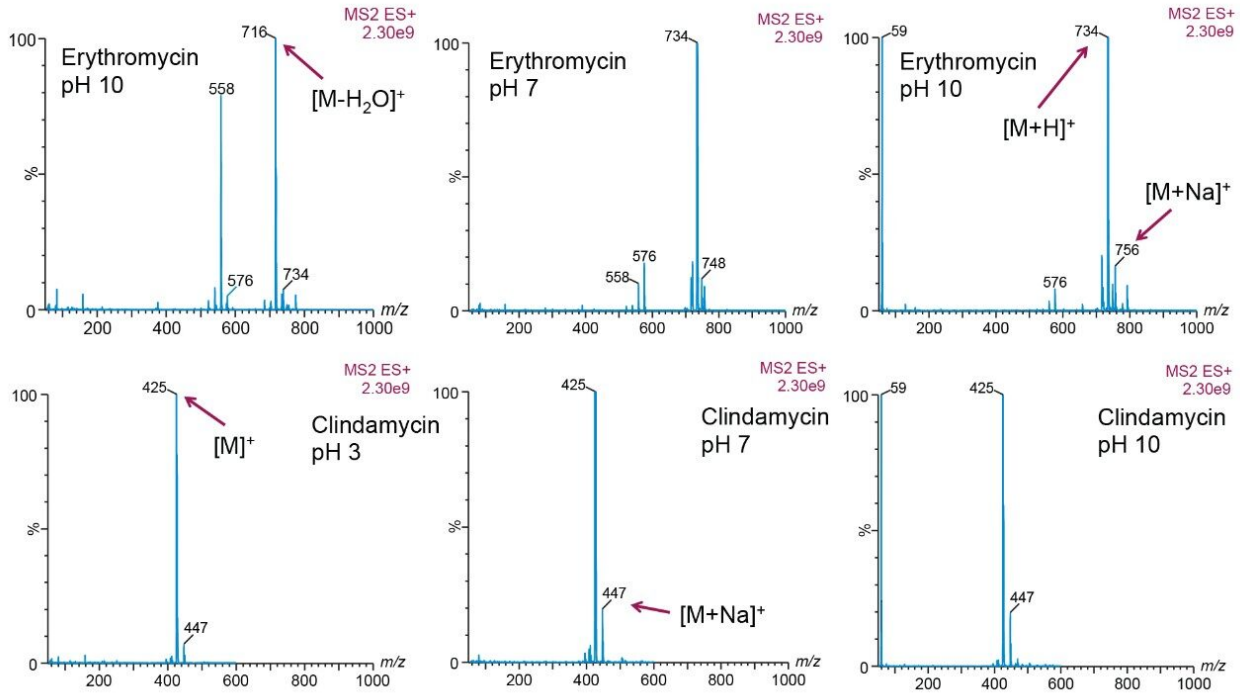

Figure 4. Full-scan spectra for erythromycin and clindamycin at pH 3, 7, and 10.

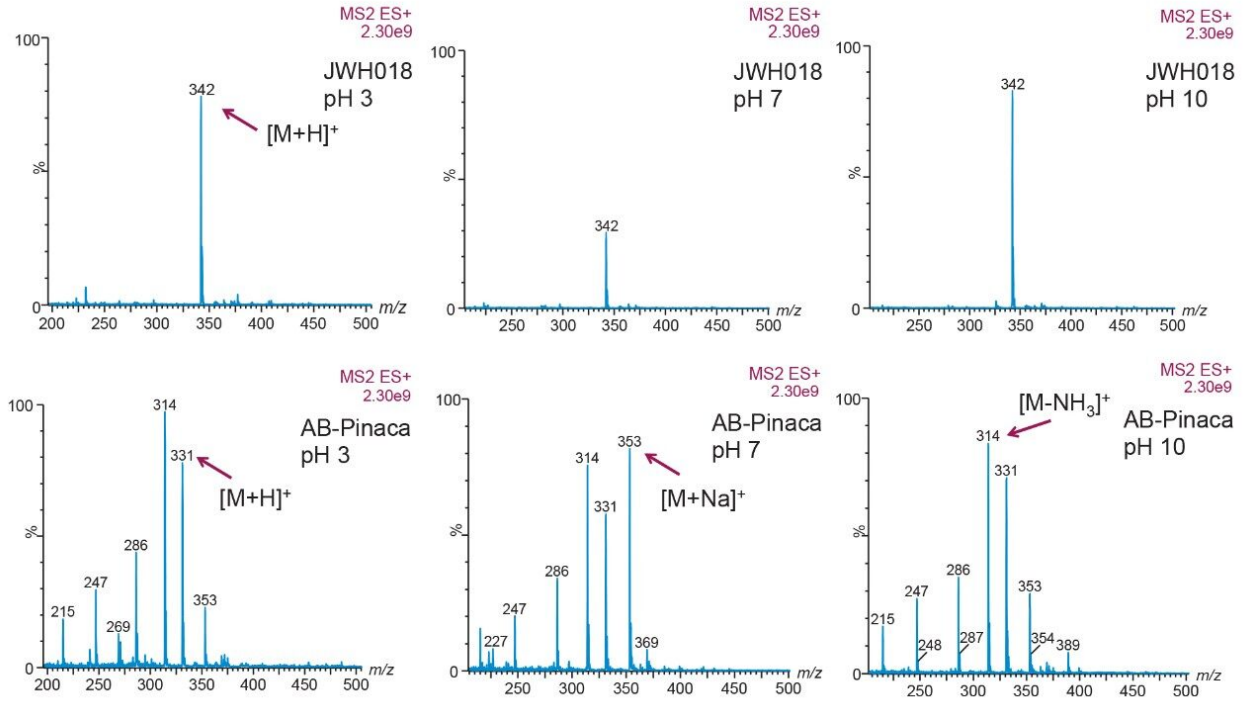

Figure 5. Full-scan spectra for JWH-018 and AB-Pinaca at pH 3, 7, and 10.

Adducts were observed on the spectra at times, suggesting that another entity connected to the molecule of interest thus increasing the weight of the target analyte. Often, a sodium atom could be attached, or the loss of a water molecule was observed. While this does not diminish the data, it is still important to recognize if it appears in the data.

The next phase was to optimize the fragmentation of the precursor ion into product ions by increasing the collision energy. Figure 6 shows a full-scan spectrum (bottom) for clindamycin (*m/z* 425) and two product ion spectrums at CID 10 and CID 25, while Figure 7 show a similar product ion spectrum for AB-Pinaca (*m/z* 331).

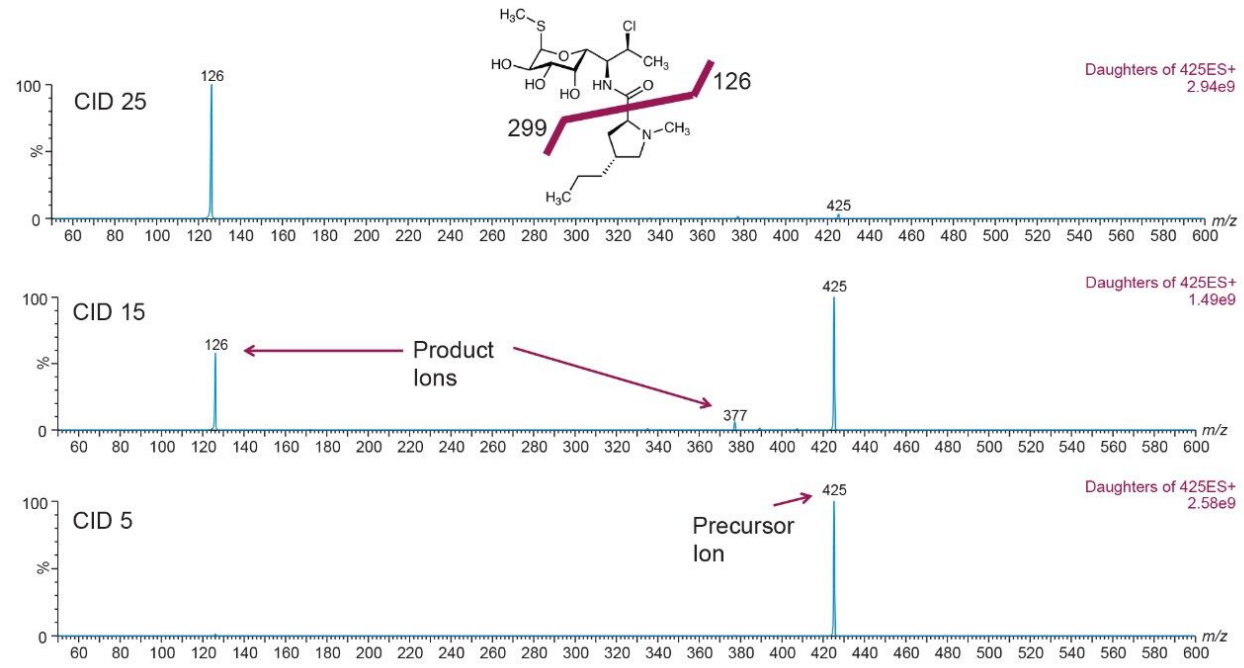

Figure 6. Daughter spectra for clindamycin at collision energy 5 V, 15 V, and 25 V.

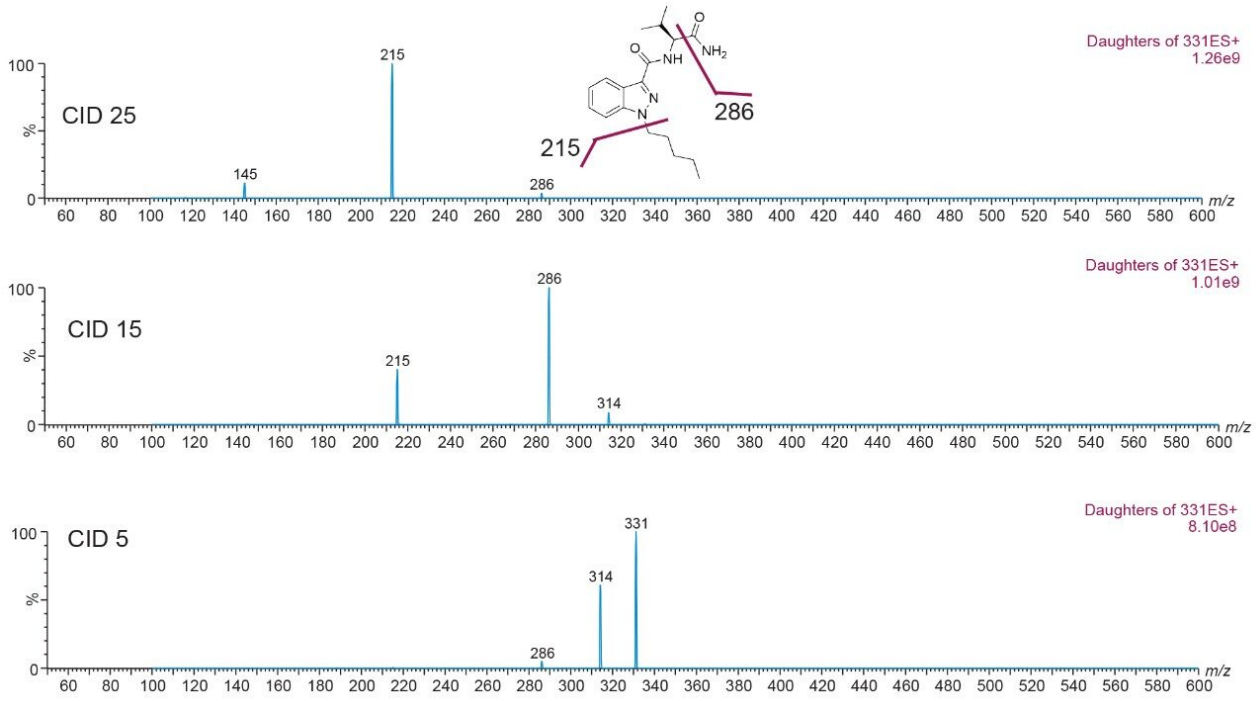

Figure 7. Daughter spectra for AB-Pinnaca at collision energy 5 V, 15 V, and 25 V.

LC Method Development

Prior to running urine samples, the 2D-UPLC-MS/MS conditions were optimized. The 2D LC set-up consisted of three pumps (loader, diluter, and eluter), and two columns (trap column with 10-µm particle sizes and an analytical column with 1.7 µm particle size). Two quaternary pumps were utilized for loading and diluting, and one binary pump was for eluting. The loader pump flow rate was set at 0.1 mL/min to carry samples from the autosampler into the 50 µL mixer. The diluter pump had a flow rate of 2 mL/min to reduce the organic content of samples (20:1 dilution ratio). Samples were retained in the trap column, then eluted and separated by the analytical column and eluter pump (Figure 8). The loading, trapping, and elution conditions of different 2D-LC methods were evaluated to determine which would best analyze, detect, and quantitate each target analyte. With the loading and eluting step, different solvents (methanol or acetonitrile) and pH levels (pH 3, 7, or 10) were considered. When trapping, different columns (i.e., C_{18}, C_8, or HLB) can affect the elution of the compounds based on their affinity to the column's functional groups. In total, up to 36 permutations can be selected (Figure 9). But, due to time constraint, quadrant two (acetonitrile elution at high pH) was used for the cannabinoids, and quadrant one (acetonitrile elution at low pH) for the macrolides (Tables 2 and 3). Tables 2 and 3 are color coded to show the different chromatographic evaluation results for the two different compound classes. Green boxes indicate Gaussian peaks in the chromatogram and the intensity of the corresponding signals. Yellow boxes represent any abnormal peak shapes such as leading, tailing, shouldering, or split peaks. Red boxes depict unacceptable chromatograms resulting from noise, elevated baseline, or poor sample retention. For the synthetic cannabinoids, the best was method 11 (Figure 10) which used a C_{18} trap and analytical column with an elution pH of 10. For the macrolide antibiotics, the optimal method was three which utilized HLB columns that had a pH 3 elution (Figure 11). Each method was chosen because they resulted in Gaussian peaks with high signal intensity for all compound classes (Figures 12 and 13).

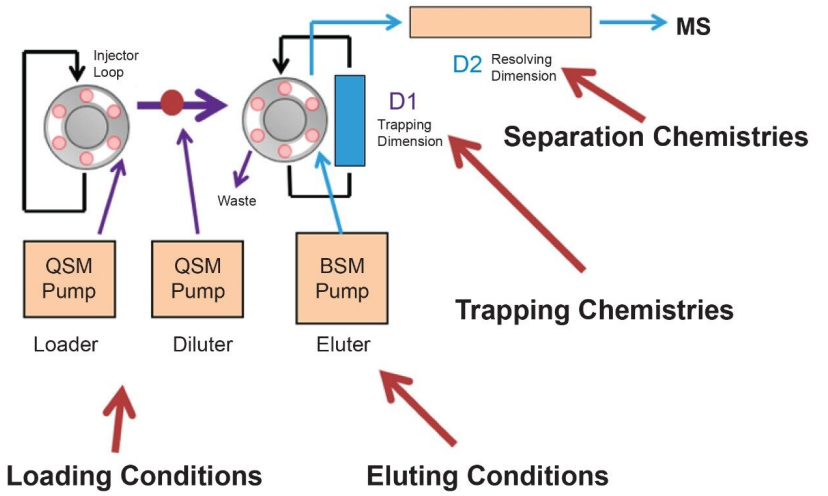

Figure 8. A 2D-LC, three-pump configuration.

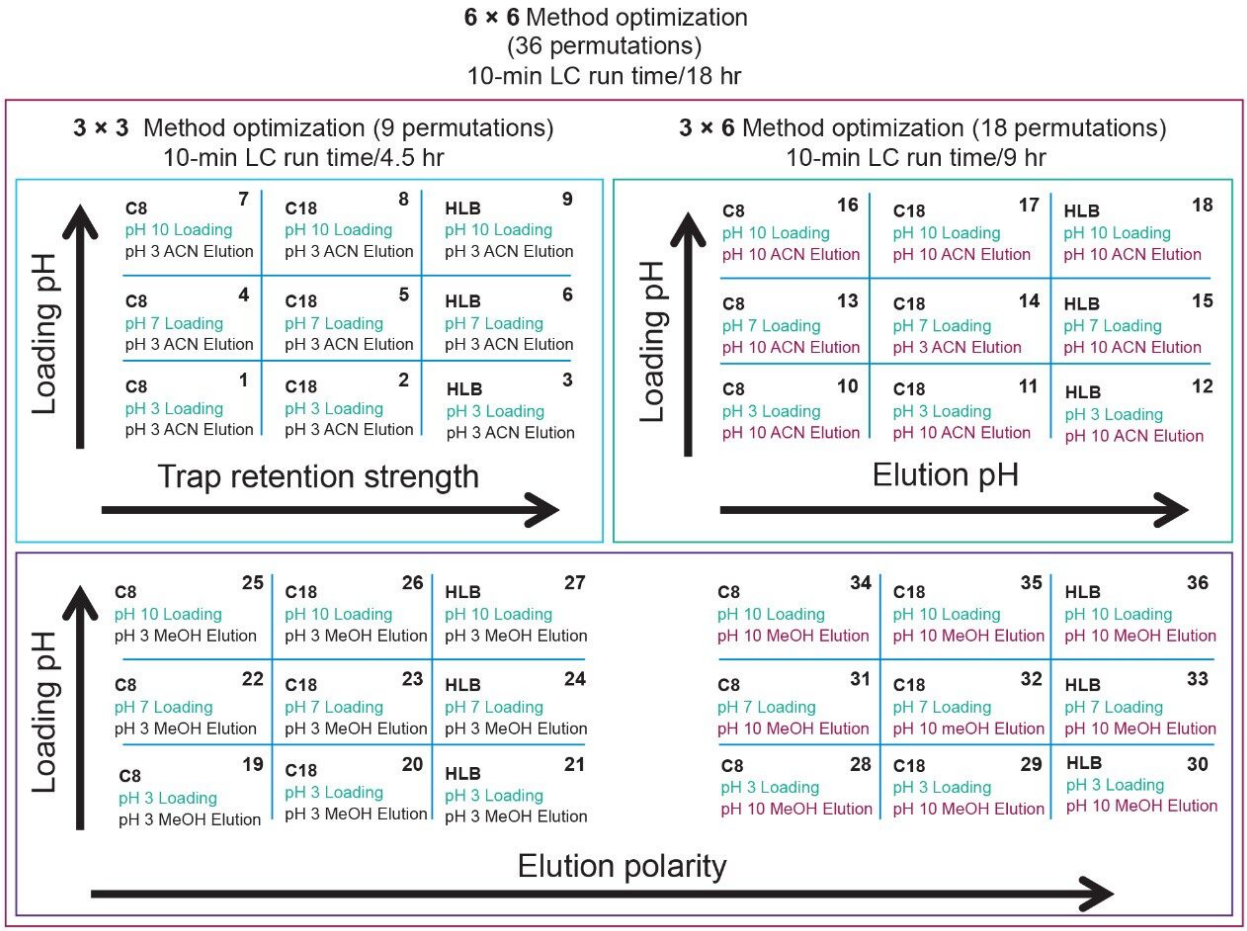

Figure 9. A 6 x 6 grid method development.

Method	10	11	12	13	14	15	16	17	18
JWH-073 H_20	e7	e6	e6	e7	e6	e5	e7	e5	e5
JWH-073 MeOH	e7	e8	e8	e7	e8	e8	e8	e8	e8
JWH-073 ACN	e8	e8	e8	e8	e8	e8	e8	e8	e8
AB-PINACA H_20	e6	e7	e7	e6	e7	e7	e6	e7	e7
AB-PINACA MeOH	e7	e7	e7	e7	e7	e7	e7	e7	e7
AB-PINACA ACN	e7	e7	e7	e7	e7	e7	e7	e7	e7
JWH-018 H_20	e6	e5	tail	e6	e6	tail	e6	e6	tail
JWH-018 MeOH	e6	e8	e8	e7	e8	e8	e7	e8	e8
JWH-018 ACN	e8	e8	tail	e8	e8	tail	e8	e8	tail
5F-PB-22 H_20	e8	e8	tail	e8	e7	tail	e8	e7	tail
5F-PB-22 MeOH	e8	e8	tail	e8	e8	tail	e8	e8	tail
5F-PB-22 ACN	e8	e8	tail	e8	e8	tail	e8	e8	tail

Table 2. Cannabinoids 6 x 6 grid results (Methods 10–18).

Method	1	2	3	4	5	6	7	8	9
Lincomycin H_20	e6	e6	e6	e5	e5	e6	e6	e6	e6
Lincomycin MeOH	e5	e5	e6	e6	e6	e6	e6	e6	
Lincomycin ACN	e4	e4	e4	e4	e4	e4	e5	e5	e4
Clindamycin H_20	e6	e6	e6	e5	e5	e5	e6	e6	e5
Clindamycin MeOH	e5	e5	e5	e6	e5	e5	e6	e6	e4
Clindamycin ACN	e4	e4	e4	e4	e4	e4	e5	e5	e4
Erythromycin H_20	e5	e5	e5	e5	e5	e5	e6	e6	e6
Erythromycin MeOH	e5	e5	e5	e5	e6	e6	e6	e6	e5
Erythromycin ACN	e4	e4	e4	e5	e5	e5	e5	e5	e5
Azithromycin H_20	e5	e5	e5	e5	e5	e4	e5	e5	e5
Azithromycin MeOH	e5	e5	e5	e5	e5	e6	e5	e5	e4
Azithromycin ACN	e4	e4	e4	e4	e5	e5	e4	e4	e4
Dirithromycin H_20		e5							
Dirithromycin MeOH	e5	e5	e5	e5	e5	e5	e5	e5	e4
Dirithromycin ACN	e4	e4	e4	e4	e4	e4	e4	e4	e4

Table 3. Macrolides 6 x 6 grid results (Methods 1–9).

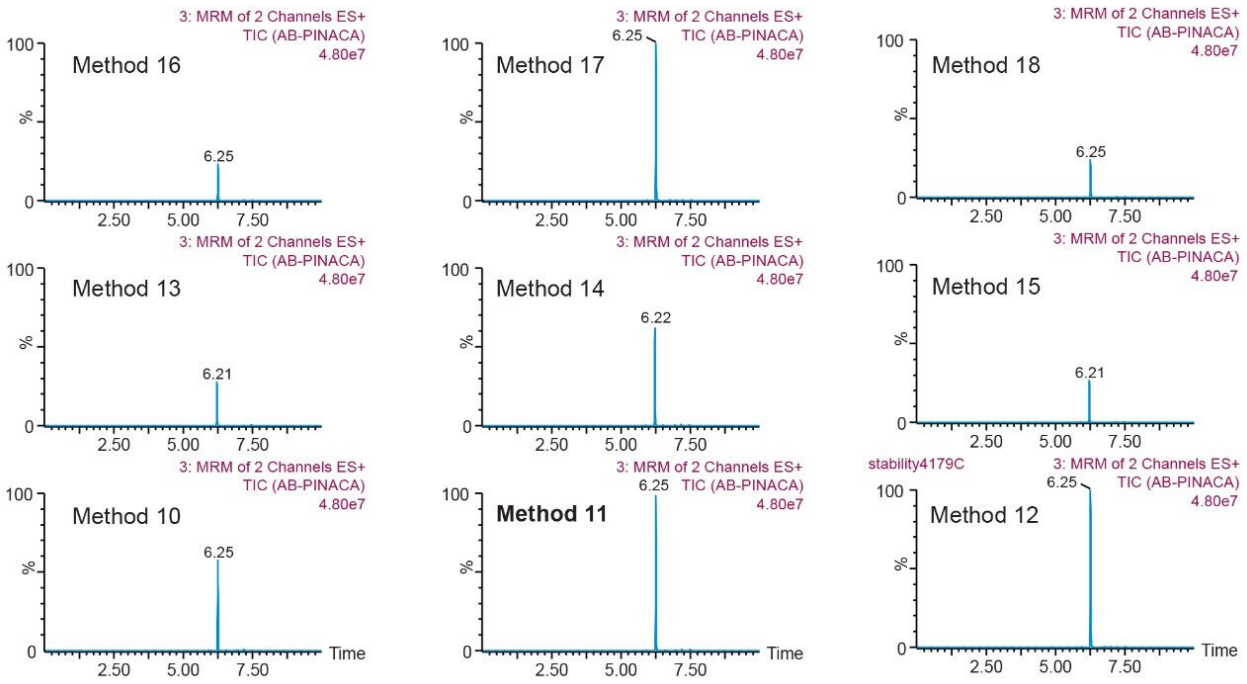

Figure 10. Second-grid results (ACN pH 10 elution) for AB-pinnaca.

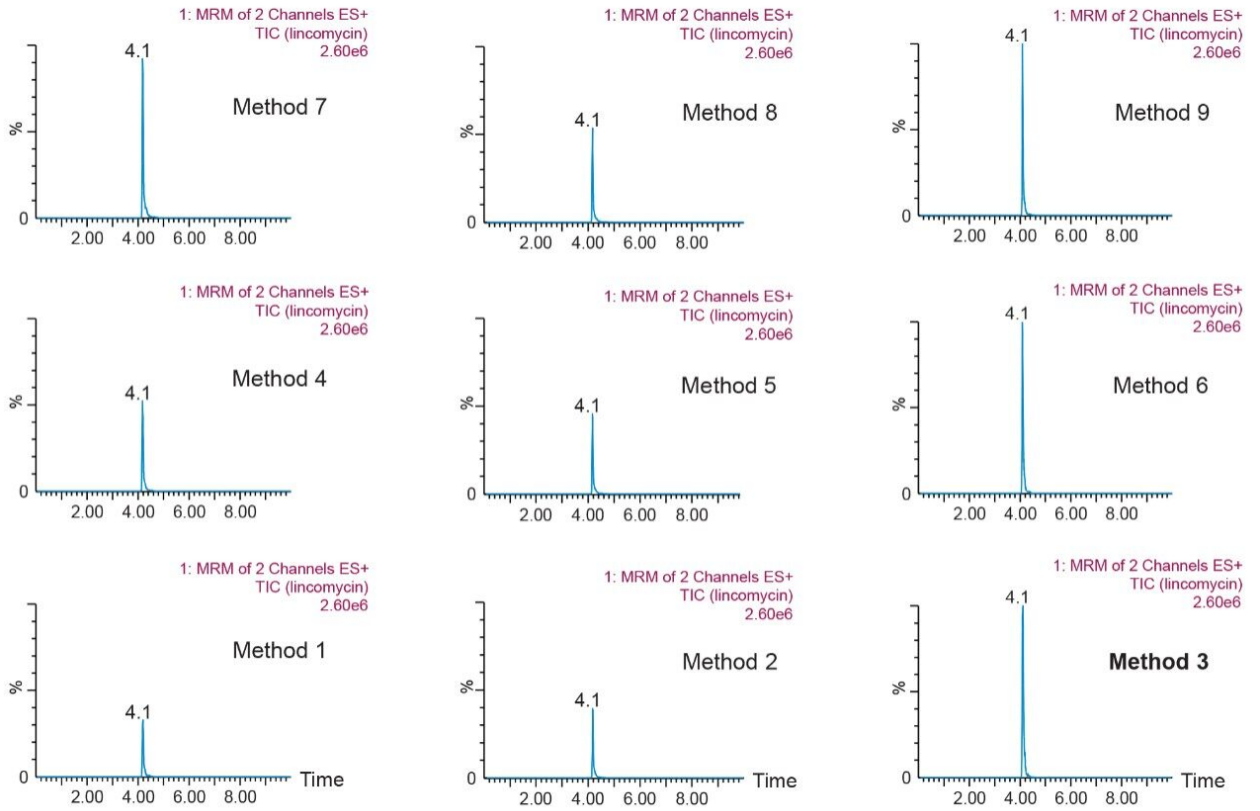

Figure 11. First-grid results (ACN pH 10 elution) for lincomycin.

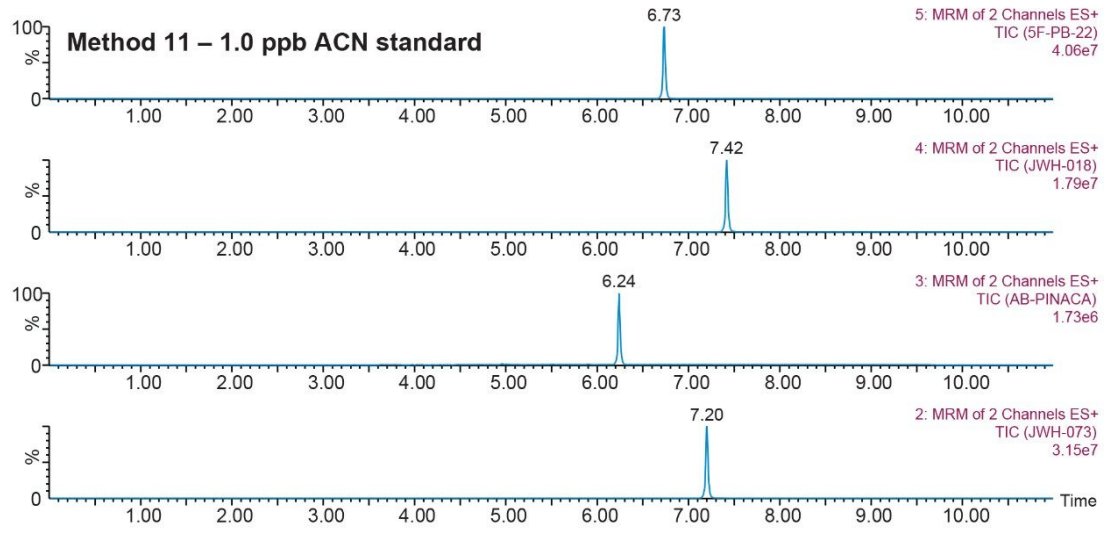

Figure 12. Method 11 final LC condition for cannabinoids.

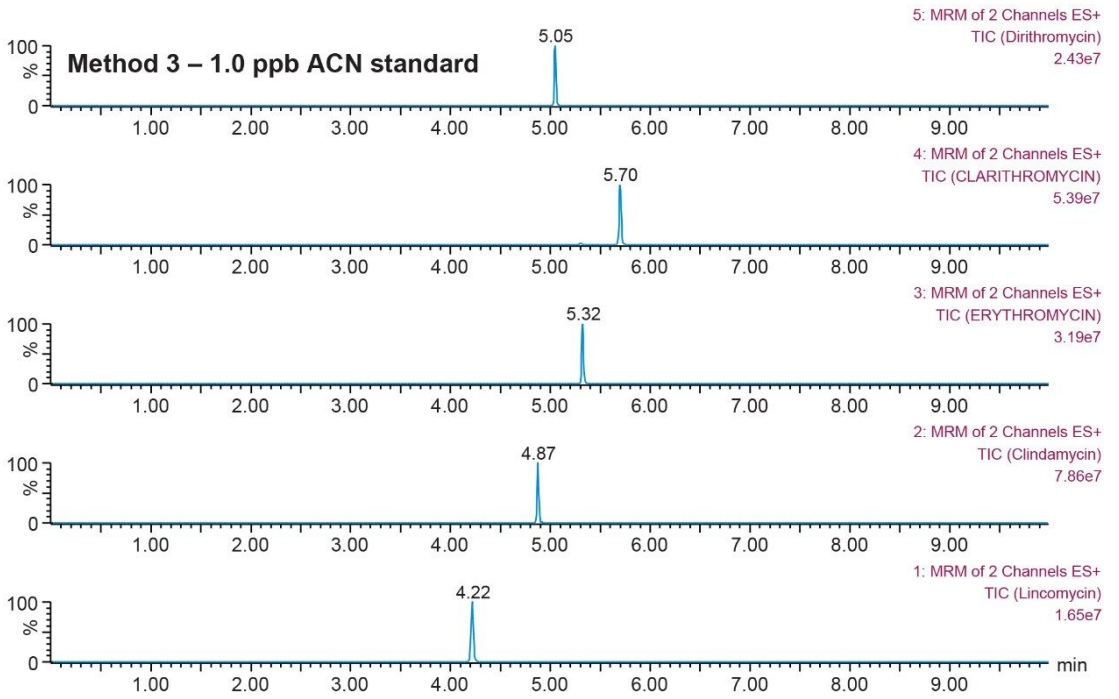

Figure 13. Method 3 final LC condition for macrolides.

SPE Evaluation

Solid-phase extraction (SPE) was optimized by loading various solutions through a column packed with a sorbent material. Compounds adsorb to the stationary phase based on their polarities and chemical interactions with the stationary phase and mobile phase. The protocol in Figure 14 showcases a four-step process: (i) condition, (ii) load, (iii) wash, and (iv) elution. Each step must be done in the correct sequence. A typical extraction protocol for a 1D LC method will require two additional steps, evaporation-to-dryness with nitrogen stream and reconstitution with compatible initial mobile-phase conditions. Those steps are necessary and very time consuming. However, since a 2D LC approach was utilized for this work, 100% organic solvents can be loaded without any risk of breakthrough. Both the evaporation-to-dryness and reconstitution steps are simply eliminated from the protocol.

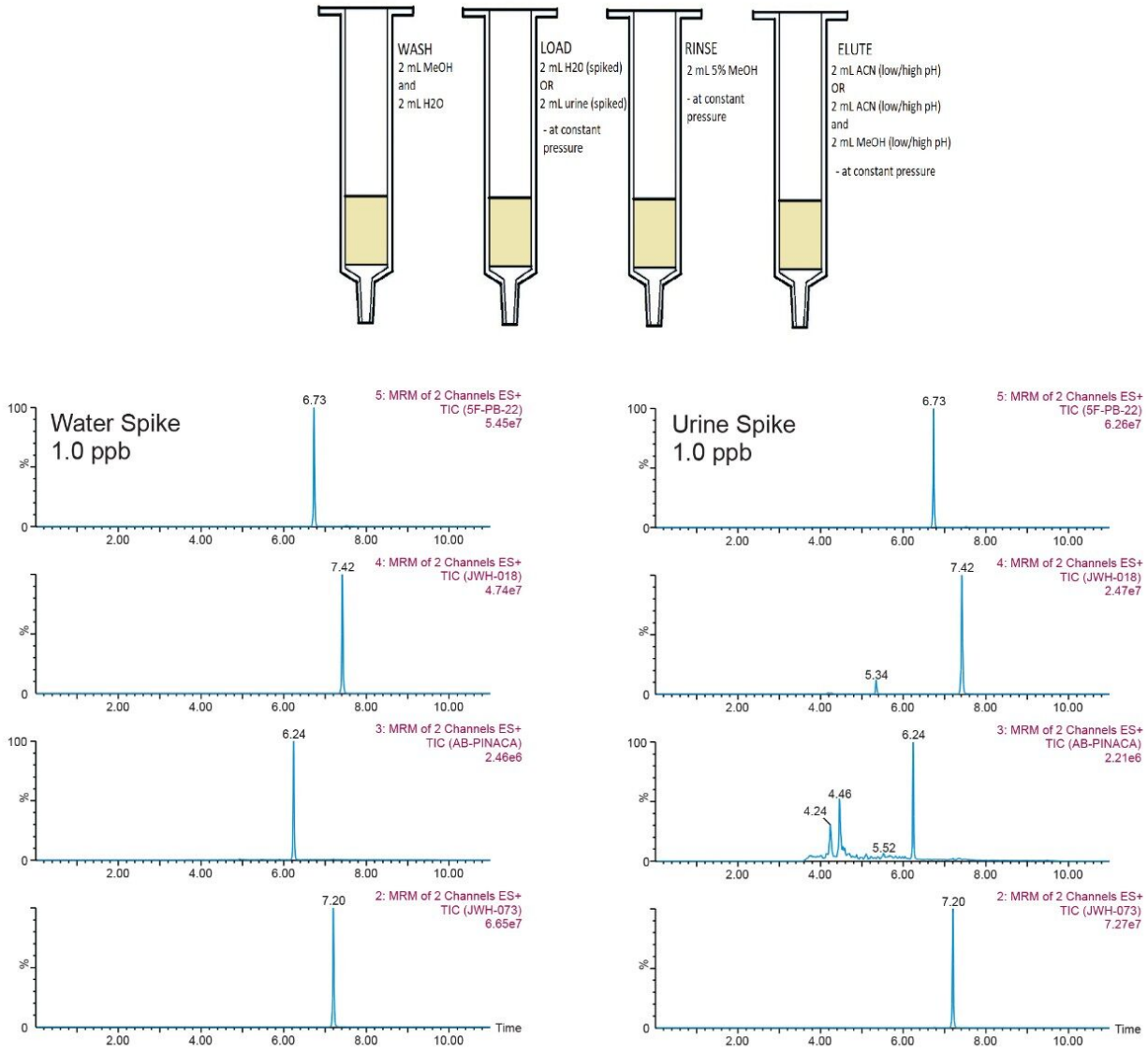

Figure 14. Oasis HLB extraction protocol. Water vs. urine spike at 1.0 ppb for cannabinoids.

The optimization continued with an unextracted seven-point calibration curve from 0.1 ng/mL to 10.0 ng/mL of each class in methanol standards (Table 4). Each concentration was injected as a triplicate injection. The results showed excellent linearity (r^2 value of 0.995 and higher) for all analytes over the three orders concentration range. The results showed good linearity for both the cannabinoids and macrolides. The 0.1, 1.0, and 10 ng/mL standards show a clear 10x signal increase, thus confirming that the calibration is well within the linear dynamic range of the ESI source. With higher concentrations, response signals will plateau due to multiplier saturation. With lower concentrations, it is a common trend to see response signals producing a similar flat profile. For the cannabinoids, JWH-018, JWH-073, and 5F-PB-22 are still giving an

intense signal suggesting that the detection limit could be pushed to another order of magnitude and reach 0.01 ng/mL. AB-Pinaca shows a weak response at 0.1 ng/mL. The macrolides also show the same intense signal at 0.1 ng/mL concentration and can have a lower detection limit at 0.01 ng/mL.

Cannabinoids

Concentration	JWH-018		JWH-073		AB-Pinaca		5F-PB-22	
0.1 ng/mL	42376		48365		3039		82737	
	50613		48829		3385		88272	
	41121	44703	47598	48264	3389	3271	81398	84136
0.2 ng/mL	82640		95238		6359		162056	
	84742		96283		6696		163993	
	85281	84221	98949	96823	6462	6506	163656	163235
0.5 ng/mL	211653		249639		16749		414454	
	213583		251707		16806		414613	
	221554	215597	254771	252039	16521	16692	422459	417175
1.0 ng/mL	400617		474504		30814		802991	
	395330		472456		32354		798936	
	409823	401923	491170	479377	30937	31368	808659	803529
2.0 ng/mL	827132		982615		66385		1619531	
	849708		1026665		68640		1632794	
	867593	848144	1015578	1008286	66568	67198	1624436	1625587
5.0 ng/mL	2118138		2545887		169581		3903371	
	2091037		2462942		169713		3888132	
	2137402	2115526	2527190	2512006	159866	166387	3938191	3909898
10.0 ng/mL	4517436		5092516		335098		8154014	
	4522630		5058708		332784		8189790	
	4594984	4545017	5077731	5076318	328927	332270	8230106	8191303

Macrolides

Concentration	Lincomycin		Clindamycin		Erythromcyin		Dirithromycin		Clarithromycin	
0.1 ng/mL	48732		139376		50779		29749		40742	
	47085		150785		51991		29650		41085	
	47390	47390	145694	145285	49699	50823	30464	29954	39280	40369
0.2 ng/mL	86 48		440515		144299		41538		81548	
	86146		446496		147782		40689		82146	
	88319	88319	435634	440882	107543	107543	40125	40784	80319	81337
0.5 ng/mL	172006		612553		206346		147555		172006	
	169912		609482		216855		128907		159912	
	168556	169104	603446	608494	216441	213214	123786	133416	159104	163674
1.0 ng/mL	497 425		1493268		517796		237496		397425	
	460859		1407556		549916		266505		460859	
	452807	452807	1356541	1419122	556999	541570	264644	256215	452807	437030
2.0 ng/mL	1220366		2886240		1070765		533954		1220366	
	1197894		2872810		1113874		553526		1197894	
	1217494	1217494	2906257	2888436	1090295	1091645	569369	555283	1217494	1211918
5.0 ng/mL	2229137		3631240		1853697		1011420		2229137	
	2185799		3538503		1966590		1067978		2185799	
	2190020	2181020	3654848	3608231	1969159	1929815	1056900	1045432	2190020	2201652
10.0 ng/mL	4746808		9836681		6468572		2736202		4746808	
	4764926		9814969		6753882		2754025		4764926	
	4621000	4621000	9942943	9864964	6782923	6001792	2628581	2706269	4621000	4710911

Table 4. Calibration standards for cannabinoids and macrolides.

Sample Quantification

Three calibration points (0.1, 1.0, and 10 ng/mL) were spiked in water and urine samples, representing an extracted standard curve and matrix-match-extracted curve, respectively (Tables 5 and 6). Two elution conditions were evaluated for the extraction protocol. Both the aqueous and urine spiked samples were eluted with 100% methanol at pH 3 (2% formic acid) and 100% methanol at pH 10 (2% ammonium hydroxide). The rational for the different pH values was to evaluate which elution condition (neutral or ionized) would produce the highest recoveries. The aqueous spike was used to calculate the extraction protocol recoveries against an un-extracted standard, without any sample matrix effects. The urine spike recoveries were calculated against an extracted standard giving a measurement of matrix effects in relation to the overall performance of the extraction protocol for intermediate complex samples. In Tables 5 and 6, the un-extracted standard for 0.1, 1.0, and 10.0 ng/mL values from Table 4 are listed in the first column for each

targeted analyte. The next set of values are the area counts for the methanol high pH and low pH for the aqueous spikes. The calculated recovery values showed a consistent >75% range for all analytes at pH 10, except for erythromycin at 50%. Because all analytes share a common basic functionality, the results suggest that most of the analytes were eluted under ionized conditions.

	MeOH Std	Water spike MeOH pH 10	Rec (%) Unextracted	Water spike MeOH pH 3	Rec (%) Unextracted	Urine Spike MeOH pH 10	Rec (%) Matrix match	Urine Spike MeOH pH 3	Rec (%) Matrix match
Lincomycin									
0.1 ng/mL	47390	40885		17926		NA		NA	
		45552		16813		NA		NA	
		46196	93.3	15602	35.4	NA		NA	
1.0 ng/mL	452807	441188		116032		26850		84892	
		450026		121886		25501		89252	
		436869	97.8	126295	26.8	24131	5.8	82043	70.3
10.0 ng/mL	4621000	4443201		1671245		436113		1165995	
		4522476		1578697		545868		1320507	
		4441750	96.7	1594858	34.9	467473	10.8	1306946	78.3
Erythromcyin									
0.1 ng/mL	50823	27483		8433		730		7688	
		30871		8855		159		7625	
		30733	58.4	8265	16.8	214	1.2	7459	89.1
1.0 ng/mL	541570	276472		58304		1885		46303	
		309588		56442		2530		46326	
		308027	55.0	53423	10.4	2711	0.8	46165	82.5
10.0 ng/mL	6001792	3635829		452483		22737		384812	
		3867455		472260		25124		396957	
		3930066	63.5	447452	7.6	24364	0.6	402262	86.3
Dirithromycin									
0.1 ng/mL	29954	27483		8433		730		7688	
		30871		8855		159		7625	
		30733	99.1	8265	28.4	214	1.2	7459	89.1
1.0 ng/mL	256215	246472		58304		1885		46303	
		259588		56442		2530		46326	
		248027	98.1	53423	21.9	2711	0.9	46165	82.5
10.0 ng/mL	2706269	2635829		452483		22737		384812	
		2867455		472260		25124		396957	
		2930066	103.9	447452	16.9	24364	0.9	402262	86.3
Clindamycin									
0.1 ng/mL	145285	106971		121230		28334		62802	
		109680		125175		25637		60259	
		109642	74.9	121727	84.5	27026	24.8	61465	50.1
1.0 ng/mL	1419122	1040599		1071958		311548		556745	
		1063648		1093206		338498		561963	
		1067858	74.5	1099673	76.7	340500	31.2	550564	51.1
10.0 ng/mL	9864964	7771114		6916064		3189764		4153474	
		7868466		7044741		3184155		4170394	
		7808003	79.2	7071084	71.1	3198315	40.8	4130499	59.2
Clarithromcyin									
0.1 ng/mL	56369	50896		58143		11783		17942	
		51515		57852		12099		18367	
		51951	91.3	54524	111.7	12116	23.3	15753	30.5
1.0 ng/mL	537030	492546		524920		148703		158401	
		501637		582565		160563		174531	
		500852	92.8	586397	114.6	162245	31.5	175437	30.0
10.0 ng/mL	4710911	4220907		4435334		1532587		1525855	
		4614376		4631597		1658556		1661427	
		4596810	95.0	4587986	107.8	1703403	36.4	1689200	35.7
JWH-073									
0.1 ng/mL	48264	47805		31145		44155		36407	
		47518		31168		46266		22290	
		46452	97.9	30514	64.1	44786	95.4	23806	88.9
1.0 ng/mL	479377	466522		284804		452617		281305	
		480167		280741		484731		285210	
		450179	97.1	264867	57.7	477035	101.3	290000	103.1
10.0 ng/mL	5076318	5020078		3265096		5057457		3109286	
		5056999		3225266		5063175		3014589	
		5082637	99.5	3103528	63.0	5015731	99.8	3092884	96.1
AB-Pinaca									
0.1 ng/mL	3271	3009		3159		3005		3151	
		3023		3087		3151		3102	
		3104	93.1	3027	94.5	3090	101.2	3080	100.6
1.0 ng/mL	31368	32502		31935		30909		32360	
		31899		31318		29287		31779	
		30367	100.7	32097	101.3	30191	95.4	30562	99.3
10.0 ng/mL	332270	337729		311327		308446		307251	
		328551		325797		299932		310693	
		318128	98.8	301192	94.1	301031	92.4	323742	100.4
JWH-018									
0.1 ng/mL	44703	42357		25579		41760		20904	
		41170		25206		40157		19677	
		40843	92.7	24868	56.4	39897	97.9	19912	80.0
1.0 ng/mL	401973	405110		215327		351821		171213	
		399564		206715		382821		170673	
		399656	99.9	200322	51.6	379177	92.5	174697	83.0
10.0 ng/mL	4545017	4429495		2380868		4203158		1902890	
		4380101		2351677		4247996		1961431	
		4288088	96.1	2275455	51.4	4238743	96.9	1910831	82.4
5F-PB-22									
0.1 ng/mL	84136	83074		71105		82623		69287	
		80938		71979		82100		65743	
		81452	97.2	71396	85.0	84202	101.4	67412	94.4
1.0 ng/mL	803529	810751		693319		781598		699077	
		709814		687008		734350		680855	
		815799	96.9	676508	85.3	754498	97.2	691291	100.7
10.0 ng/mL	8191303	7263341		5881725		6813359		5520238	
		7509062		5902296		6839270		5428217	
		7401062	90.2	5897431	72.0	6638412	91.5	5449536	92.7

Table 5. Water vs. urine recoveries for cannabinoids.

	MeOH Std	Water spike MeOH pH 10	Rec (%) Unextracted	Water spike MeOH pH 3	Rec (%) Unextracted	Urine Spike MeOH pH 10	Rec (%) Matrix match	Urine Spike MeOH pH 3	Rec (%) Matrix match
JWH-073									
0.1 ng/mL	48264	47805		31145		44155		36407	
		47518		31168		46266		22290	
		46452	97.9	30514	64.1	44786	95.4	23806	88.9
1.0 ng/mL	479377	466522		284804		452617		281305	
		480167		280741		484731		285210	
		450179	97.1	264867	57.7	477035	101.3	290000	103.1
10.0 ng/mL	5076318	5020078		3265096		5057457		3109286	
		5056999		3225266		5063175		3014589	
		5082637	99.5	3103528	63.0	5015731	99.8	3092884	96.1
AB-Pinaca									
0.1 ng/mL	3271	3009		3159		3005		3151	
		3023		3087		3151		3102	
		3104	93.1	3027	94.5	3090	101.2	3080	100.6
1.0 ng/mL	31368	32502		31935		30909		32360	
		31899		31318		29287		31779	
		30367	100.7	32097	101.3	30191	95.4	30562	99.3
10.0 ng/mL	332270	337729		311327		308446		307251	
		328551		325797		299932		310693	
		318128	98.8	301192	94.1	301031	92.4	323742	100.4
JWH-018									
0.1 ng/mL	44703	42357		25579		41760		20904	
		41170		25206		40157		19677	
		40843	92.7	24868	56.4	39897	97.9	19912	80.0
1.0 ng/mL	401973	405110		215327		351821		171213	
		399564		206715		382821		170673	
		399656	99.9	200322	51.6	379177	92.5	174697	83.0
10.0 ng/mL	4545017	4429495		2380868		4203158		1902890	
		4380101		2351677		4247996		1961431	
		4288088	96.1	2275455	51.4	4238743	96.9	1910831	82.4
5F-PB-22									
0.1 ng/mL	84136	83074		71105		82623		69287	
		80938		71979		82100		65743	
		81452	97.2	71396	85.0	84202	101.4	67412	94.4
1.0 ng/mL	803529	810751		693319		781598		699077	
		709814		687008		734350		680855	
		815799	96.9	676508	85.3	754498	97.2	691291	100.7
10.0 ng/mL	8191303	7263341		5881725		6813359		5520238	
		7509062		5902296		6839270		5428217	
		7401062	90.2	5897431	72.0	6638412	91.5	5449536	92.7

Table 6. Water vs. urine recoveries for macrolides.

For the urine spike, the low area counts in comparison to their aqueous spikes indicate strong matrix effects, predominantly suppression effects. One observation worth mentioning is the complete reversal of elution conditions. The results suggest for the urine spiked sample, a low-pH methanol elution yielded better recoveries for all analytes (>75% range), except for erythromycin and clindamycin at 50% and 30%, respectively. The area counts for urine and water spiked samples with low-pH methanol elution gave similar values.

The extraction protocol used in this work was designed for a generic screening approach, meaning that the wash step was as mild as possible so not to elute any crucial analyte (target or unknown) during the wash step. In this instance, the only wash step was a simple 5% methanol wash between the loading phase and elution phase. For intermediate and complex samples, the drawback of a single wash extraction protocol will be an increased signal on the background noise, usually visible by extra peaks and baseline distortion at the expected retention time of a target analyte. Here the urine extract showed a clean baseline at 1 ppb with an intense signal, suggesting the feasibility of a low pertrillion range detection (Figure 14). As for the cannabinoids, only AB-pinaca showed extra peaks and baseline distortion; a mild case and far away from the target analyte, which suggests that at the expected retention time of AB-pinaca there is no visible interferences (Figure 15).

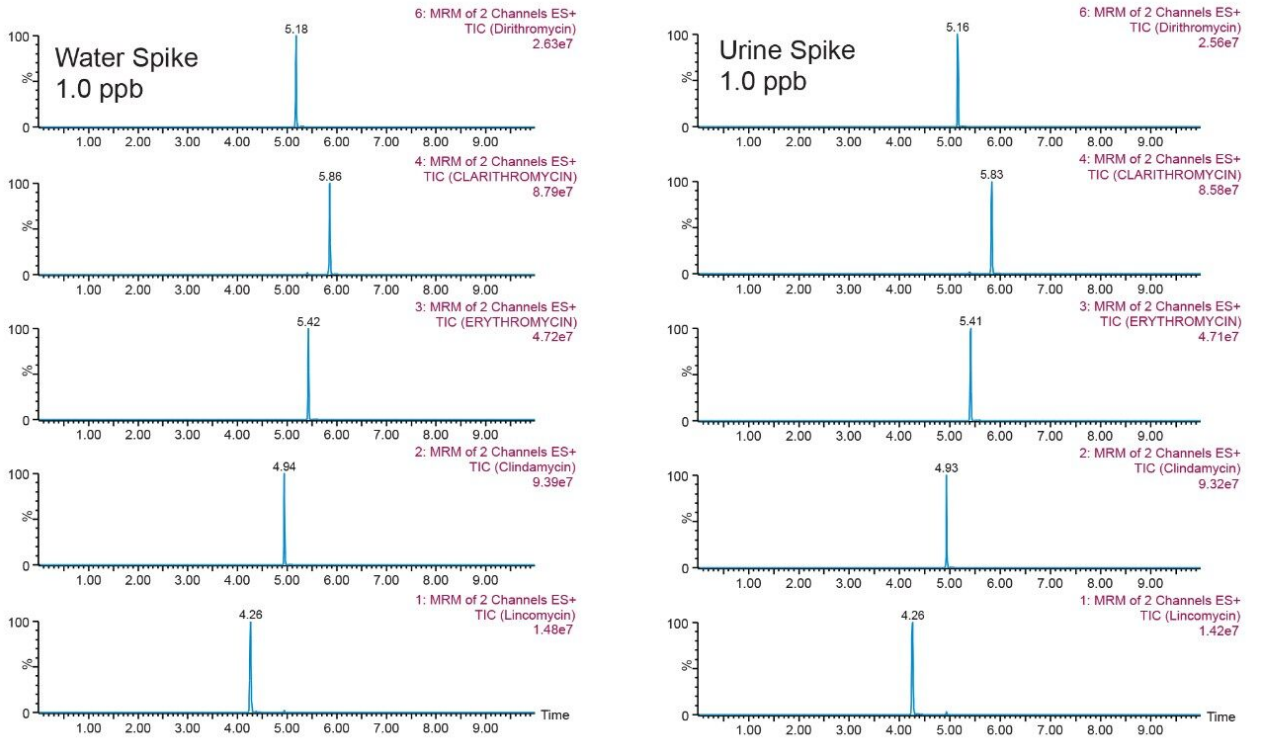

Figure 15. Waters vs. urine spike at 1.0 ppb for macrolides.

Conclusion

Overall, the use of a 2D-LC-MS/MS method made it possible to produce a successful, five-day method for the analysis of macrolides and cannabinoids. The workflow started with the infusion of the target analyte at three pH values to determine which pH would provide the best signal. The quick 3 × 3 LC-MS/MS overnight runs gave a clear chromatography map and made it possible to have a better understanding of the analytes' chromatographic behavior. Once the LC method was chosen, most of the evaluation time was focused on the optimization of the extraction protocol. For the cannabinoids, optimal LC conditions were found to include a C_{18} trap column with a pH 3 loading, and a C_{18} analytical column with an acetonitrile elution at pH 10 (Method 11). The SPE elution with acetonitrile at pH 3 yielded satisfactory results. The limit of detection was identified to be 0.1 ng/mL; however, for the macrolides, the optimal LC conditions included an HLB trap column with a pH 3 loading, and a C_{18} analytical column with an acetonitrile elution at pH 3 (Method 3). The SPE elution with methanol at pH 3 gave excellent results. The limit of detection was identified to be 0.1 ng/mL.

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