Applikationsbericht

Rapid Analysis of Pharmaceuticals in Human Tissues Using the ACQUITY UPLC with 2D Technology

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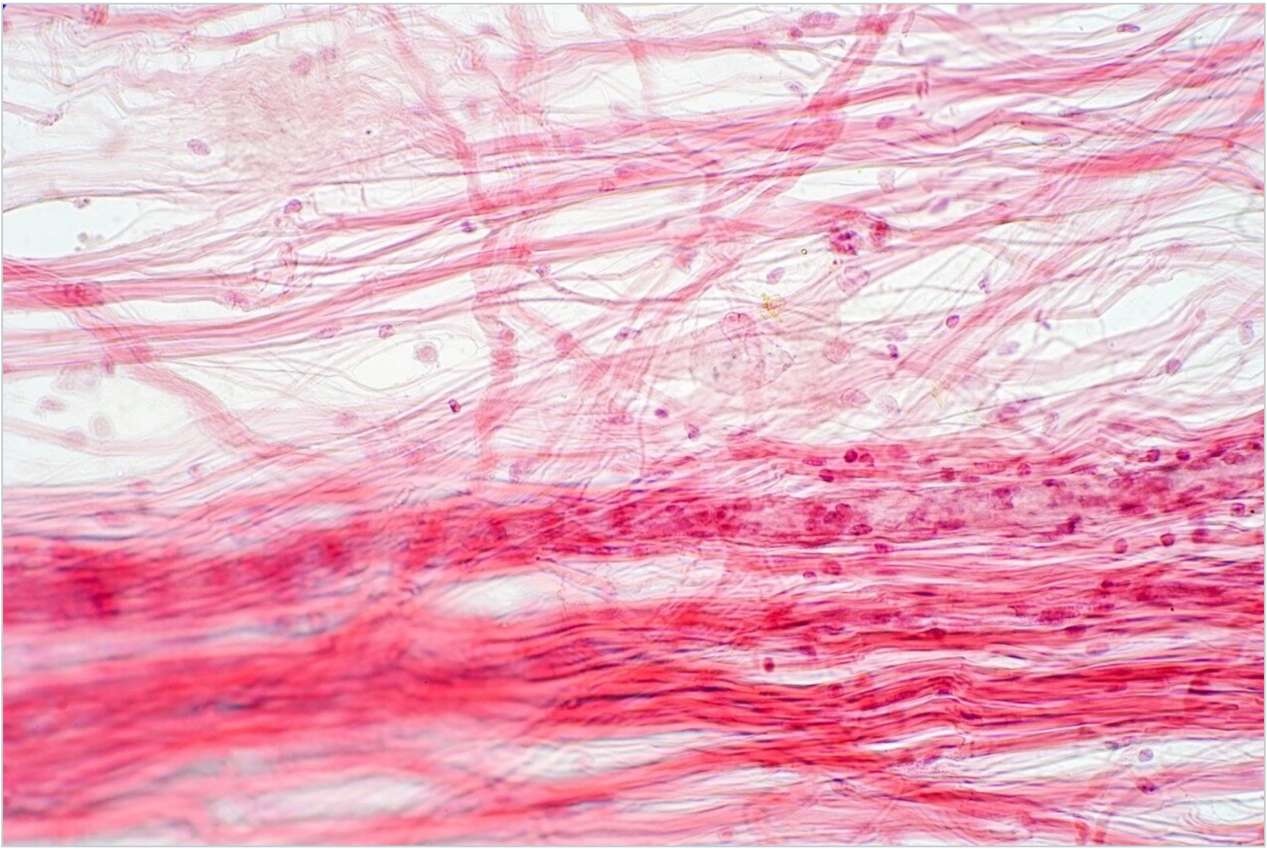

For forensic toxicology use only.

Abstract

This application demonstrated the automated and fast method development capability of the ACQUITY UPLC with 2D Technology for the analysis of pharmaceuticals in human tissue samples.

Benefits

- Fast extraction protocol (45 min)
- Trace level detection (ppt)
- 90 sec homogenization

Introduction

According to the Scientific Working Group for Forensic Toxicology (SWGTOX), the field of forensic toxicology handles the analysis of drugs or chemicals in biological materials, and the interpretation of those results for medico-legal purposes.[1] In this field, forensic toxicologists often work with medical examiners to perform postmortem toxicological analyses on blood or biological tissues of deceased individuals in order to determine cause and manner of death.[1] Because these results are relied upon in a court of law, validity, reliability, accuracy and precision of the analytical techniques used to perform these analyses are essential.

The core focus of a forensic toxicology laboratory is the accurate identification and quantitation suspected drugs or chemicals in biological samples. The target matrix can vary between blood, plasma, urine, saliva, vitreous fluid, hair, nails, and organs such as brain, heart, lung, liver, kidney, spleen, and stomach contents. The Forensic Toxicology Research Team at the Federal Aviation Administration performs such analyses on samples from victims of fatal aircraft accidents to provide insight to the analysis of accident causation.[2] Aircraft accidents and crashes are often brutal enough to severely impair any human remains, which is why the toxicologists must rely on more complex biological tissues for analysis, i.e. brain, heart, lung, liver, kidney, spleen, etc. Additionally, they must have the ability to detect and measure many substances, from drugs and alcohol, to toxic gases and industrial chemicals.[2] Therefore, there is a need to develop multi-residue analyses and efficient sample preparation methods in order to analyze samples in a timely manner.

The analytical techniques currently available are divided into two categories, some platforms are used for screening methods (qualitative) and other solutions are used for confirmation methods (quantitative). Most laboratories are usually equipped with gas chromatography (GC) or liquid chromatography (LC) hyphenated to a mass spectrometer (MS). For several decades, GC-MS was the tool of choice for bio-analysis. With the introduction of atmospheric pressure ionization technique, LC-MS is now the most popular technique in the field of forensic toxicology.

Detection and quantification of drugs in complex matrices is difficult to accomplish due to time-consuming extraction processes, and the difficulty to detect an analyte at trace levels. A robust extraction and clean up methodology, in which a homogenization step precedes, is a must in order to reach a target limit of detection (LOD) and to maintain instrument performance. The use of advanced hyphenated instrumentation platforms, such as UPLC-MS/MS has allowed analysts to detect trace levels of analytes. Traditional extraction techniques used in most laboratories are decades old and do not have the robustness to produce quality results. A micro extraction protocol combined with a multi-dimensional chromatography (2D LC-MS/MS) can decrease sample preparation time without sacrificing the quality seen with current single dimension chromatography techniques.[3,4,5]

Experimental

Two MRM transitions (quantification and confirmation) for all drugs were selected and optimized. The MRM conditions are listed in Table 1. All human biological specimens used for this study were provided by the Federal Aviation Administration (FAA).

	Ion Mode	Precursor ion	Cone	Product ion	CE
Zolpidem	ESI+	308.3	40	235.3	35
				263.3	25
Citolapram	ESI+	325.4	30	109.1	25
				262.4	20
Norbuprenorphine	ESI+	414.6	30	101.2	45
				57.2	50
Oxycodone	ESI+	316.2	30	298.2	20
				241.2	30
Normeperidine	ESI+	234.2	30	160.2	15
				56.2	25
Dextrorphan	ESI+	258.3	30	157.3	35
				133.1	30
Dextromethorphan	ESI+	272.2	30	147.2	30
				215.2	25
Diazapam	ESI+	285.1	30	154.1	25
				105.1	25
Diltiazem	ESI+	415.3	30	178.2	25
				370.2	15
Quetiapine	ESI+	384.5	30	253.3	25
				221.3	30
Diphenydramine	ESI+	256.4	30	167.2	10
				152.2	30
Buprenorphine	ESI+	468.4	30	55.3	45
				84.3	40
Promethazine	ESI+	285.4	30	86.2	20
				198.2	20
Dihydrocodeine	ESI+	302.3	30	171.1	40
				199.2	35
Doxylamine	ESI+	271.3	30	182.2	15
				167.2	25
Flecainide	ESI+	415.3	30	98.1	25
				398.4	25
Hydromorphone	ESI+	286.2	30	157.3	40
				185.1	30
Nordiazepam	ESI+	271.1	30	140.2	25
				[illegible]	[illegible]

Loading conditions

Loading:	MilliQ Water (pH 7)
Flow rate:	2 mL/min
AT-column dilution:	5% (0.1 mL/min Loading pump and 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
Column:	ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Mobile phase A:	Water + 0.5% formic acid
Mobile phase B:	Acetonitrile + 0.5 % formic acid
Elution:	5 minute linear gradient from 5% (B) to 95% (B)
Flow Rate:	0.500 mL/min (Elution pump)
Injection volume:	100 µL

MS conditions

MS System:	Xevo Q-ST TQ-S

Ionization mode:	ESI Positive
Capillary voltage:	3.0 kV
Cone voltage:	90.0 V
Source temp.:	150 °C
Desolvation temp.:	550 °C
Desolvation gas:	1100 L/hr
Cone gas:	50 L/hr

Results and Discussion

2D LC method development

The analysis of started with the chromatography optimization of the 2D LC-MS/MS. The 2D LC-MS/MS is setup as depicted in Figure 3. This configuration was constructed with two quaternary pumps and one binary pump. The binary pump was set for gradient elution and the quaternary pumps were plumbed for "AT-column dilution" to create two distinct streams (loader and dilutor). The loader pump was set 0.1 mL/min for loading the extracts from the injection loop onto a 50 µL mixer, while the dilutor pump was set at 2 mL/min flow rate for dilution following a re-focusing effect on the trap column. From the chemical structures of the target analytes, a high retention strength sorbent material (Oasis HLB, 40mg) was selected for the trap column, while a high XBridge Hybrid C_{18} sorbent (BEH C_{18}) was chosen for the analytical column. The next phase of the optimization was to select the trapping and elution conditions. As seen in previous publications, a 6x6 2D LC evaluation grid gives an excellent starting point to provide an overview of the chromatographic behavior for a target analyte. For this application, the 2D LC optimization process focused with methods 3, 6, 9, 12, 15, and 18. The results are tabulated in Table 2. The color coded chart was created to identify which analytical conditions give the best chromatographic profile with a quick visual survey. The green box depicts a Gaussian peak shape for quantification analysis. The yellow box was used to flag chromatography issues, such as peak split, tailing, shoulder or leading profiles. Finally, the red box indicates an absence of signal, most likely due to breakthrough effect during loading phase on the trap column or poor elution from the trap onto the analytical column. Additional parameters can be adjusted to ensure proper mass transfer during loading and elution phase. One parameter in particular is the sorbent bed mass on the first dimension. Two sorbent bed masses (40 mg vs 80 mg) were evaluated for the retention and elution of the target analytes. As shown in Table 2, method 9 using an HLB 80 mg bed mass and method 6 using HLB 40 or 80 mg provided the best chromatography performance for all 21 target analytes.

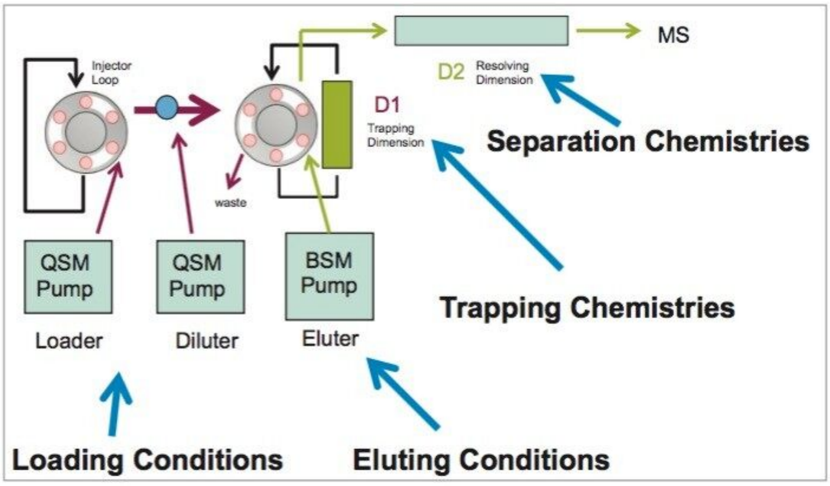

Figure 3. 2D LC configuration with AT-column dilution (3 pumps design).

	Meth 3	Meth 3	Meth 6	Meth 6	Meth 9	Meth 9	Meth 12	Meth 12	Meth 15	Meth 15	Meth 18	Meth 18
	40 mg HLB	80 mg HLB	40 mg HLB	80 mg HLB	40 mg HLB	80 mg HLB	40 mg HLB	80 mg HLB	40 mg HLB	80 mg HLB	40 mg HLB	80 mg HLB
	pH 3	pH 3	pH 7	pH 7	pH 10	pH 10	pH 3	pH 3	pH 7	pH 7	pH 10	pH 10
Normeperidine (water)	e7	e7	e7	e7	e7	e7	e6	e6 tail	e7	e6 tail	e7	e7
Normeperidine (MeOH)	e7	e7	e7	e7	e7	e7	e6 tail	e6 tail	e7	e6 tail	e7	e7
Normeperidine (ACN)	e7	e6	e7	e6	e7	e6	e6	e5 tail	e6 tail	e6 tail	e6	e6
Dextrorphan (water)	e7	e7	e7	e7	e7	e7	e6 tail	e6 tail	e6	e6 tail	e7	e6 tail
Dextrorphan(MeOH)	e7	e7	e7	e7	e7	e7	e6 tail	e6 tail	e7	e6 tail	e7	e7 tail
Dextrorphan (ACN)	e6	e6	e7	e6	e7	e6	e6	e5 tail	e6 tail	e6 tail	e6	e6 tail
Nordiazepam (water)	e7	e6	e7	e6	e7	e6	e7	e7	e7	e7	e7	e7
Nordiazepam (MeOH)	e7	e6	e7	e6	e7	e6	e7	e7	e7	e7	e7	e7
Nordiazepam (ACN)	e7	e6	e7	e6	e7	e6	e7	e7	e7	e7	e7	e7
Dextromethorphan (water)	e7	e7	e6	e6	e7	e7	e6	e6 tail	e6	mp e6 tail	e6	e6
Dextromethorphan (MeOH)	e7	e7	e7	e7	e7	e7	e7	e7 tail	e7	e7 tail	e7	e7
Dextromethorphan (ACN)	e7	e7	e7	e7	e7	e7	e6	e6 tail	e7	e6 tail	e6	e6
Diazepam (water)	mp e7	mp e6	e7	e6	e7	e6	mp e7	mp e7	e7	e7	e7	e7
Diazepam (MeOH)	mp e7	mp e6	mp e7	mp e6	mp e7	mp e6	mp e7	mp e7	e7	e6	e7	e7
Diazepam (ACN)	mp e6	mp e6	e7	mp e6	e7	e6	mp e7	mp e7	e7	e6	e7	e7
Promethazine (water)	e7	e7	e7	e6	e7	e7	e7	e6	e6	e6	e6	e6
Promethazine (MeOH)	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7
Promethazine (ACN)	e7	e6	e7	e6	e7	e6	e6	e6	e6	e6	e6	mp e6
Oxazepam (water)	e7	mp e6	e7	e6	e7	e6	mp e7	mp e7	e7	mp e7	e7	e7
Oxazepam (MeOH)	e7	mp e6	e7	e6	e7	e6	mp e7	mp e7	e7	e7	e7	e7
Oxazepam (ACN)	e7	mp e6	e7	e6	e7	e6	mp e7	mp e7	e7	e7	e7	e7
Termazepam (water)	e7	e7	e7	e6	e7	e6	e7	e7	e7	e7	e7	e7
Termazepam (MeOH)	e7	mp e6	e7	mp e6	e7	mp e6	mp e7	mp e7	e7	e7	e7	e7
Termazepam (ACN)	e7	mp e6	e7	mp e6	e7	mp e6	mp e7	mp e7	e7	e7	e7	e7
Flecainide (water)	e7	e7	e7	e7	e7	e7	e7	e7	e7	e6	e7	e7
Flecainide (MeOH)	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7
Flecainide (ACN)	e7	e7	e7	e7	e7	e7	e7	e6	e7	e7	e7	e7
Diphenhydramine (water)	e6	e7	e6	e6	e6	e6	e6	e6	e6	e6	e6	e6
Diphenhydramine (MeOH)	e7	e7	e7	e7	e7	e7	e7	e6	e7	e6	e7	e7
Diphenhydramine (ACN)	e7	e7	e7	e7	e7	e7	e6	e6	e7	e6	e7	e6
Hydromorphone (water)	mp e5	e5	e6	e6	e6	e6	e5	e5	e7	e6	e6	e6
Hydromorphone (MeOH)	sp e5	e5	e6	e6	e6 tail	e6	e5	e5	e7	e7	e6	e6
Hydromorphone (ACN)			e6	e6	sp e5	e5			e6	e6	e6	e6
Dihydrocodeine (water)	e6	e6	e6	e6	e6	e6	e6	e6	e7	e6	e7	e6
Dihydrocodeine (MeOH)	e6	e6	e6	e6	e7	e7	e6	e6	e7	e7	e7	e7
Dihydrocodeine (ACN)	e4	mp e4	e6	e6	e6	e6			e6	e6	e6	e6
Zolpidem (water)	e7	e7	e7	e7	e7	e7	e7 tail	e7	e7	e7	e7	e7
Zolpidem (MeOH)	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7
Zolpidem (ACN)	e7	e7	e7	e7	e7	e7	e7 tail	e7	e7	e7	e7	e7
N Desmethyl citolapram (water)	mp e7	mp e7	mp e6	mp e6	e7	mp e7	mp e6 tail	mp e6	mp e6 tail	mp e6	mp e6 tail	mp e6 tail
N Desmethyl citolapram (MeOH)	mp e7	mp e7	e7	e7	e7	e7	mp e6 tail	mp e6	e7 tail	mp e6	e7 tail	mp e6 tail
N Desmethyl citolapram (ACN)	mp e7	mp e7	e7	e7	e7	e7	mp e6 tail	mp e6	e7 tail	mp e6	mp e7 tail	mp e6 tail
Oxycodone (water)	e6	mp e6	e6	e6	e7	mp e7	mp e6	mp e6 tail	e6	mp e6	mp e7	mp e7
Oxycodone (MeOH)	e6	mp e6	e6	e6	e7	e7	mp e6	mp e6 tail	e7	e6	mp e7	mp e7
Oxycodone (ACN)	mp e5	mp e6	e6	e6	e7	mp e7	mp e5	mp e6 lead	e6	e6	mp e7	mp e7
Citolapram (water)	mp e7	mp e7	e6	mp e6	e7	e7	mp e7	mp e6	e6	mp e6	e7	e7
Citolapram (MeOH)	mp e7	mp e7	e7	e7	e7	e7	mp e7	mp e7	e7	e7	e7	e7
Citolapram (ACN)	mp e7	mp e7	e7	e7	e7	e7	mp e7	mp e7	e7	e7	e7	e7
Quetiapine (water)	e7	e7	e7	e6	e7	e7	e7	e7	e7	e7	e7	e7
Quetiapine (MeOH)	e7	e6	e7	e6	e7	e7	e7	e7	e7	e7	e7	e7
Quetiapine (ACN)	e6	e6	e6	e6	e7	e6	e6	e6	e7	e6	e7	e6
Norbuprenorpine (water)	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e4	mp e5	mp e5
Norbuprenorpine (MeOH)	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5
Norbuprenorpine (ACN)	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5	mp e5
Diltiazem (water)	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7	e7
Diltiazem (MeOH)	e8	e7	e8	e7	e8	e7	e7	e7	e8	e7	e7	e7
Diltiazem (ACN)	e7	e7	e7	e7	e8	e7	e7	e7	e7	e7	e7	e7
Buprenorphine (water)	e6	e6	e6	e5	e6	e6	e6	e6	e5	e5	e5	e6
Buprenorphine (MeOH)	e6	e6	e6	e6	e6	e6	e6	e6	e6	e6	e6	e6
Buprenorphine (ACN)	e6	e6	e6	e6	e6	e6	e6	e6	e6	e6	e6	e6

Table 2. 6x6 grid results.

The rationale behind the selection of Method 6 related to the fact that the loading conditions for the target analytes on the trap column can be done at pH 7, while Method 9 utilize a high pH additive (NH_4OH). Therefore, as cost saving measures, the final protocol will use a pH 7 loading onto an 40 mg HLB on the first dimension, followed by an elution with acetonitrile at pH 3 onto a BEH C_{18} analytical column (See Figure 4).

The final separation showed excellent Gaussian peak shapes for all analytes. However water spikes exhibited lower intensities, which is expected due to secondary interactions with the active sites, most likely due to ion exchange retention with the glass vial surface. The ionic interaction can be eliminated by simply changing the diluent. In this case, methanol and acetonitrile diluents both gave higher intensities (See Figure 5).

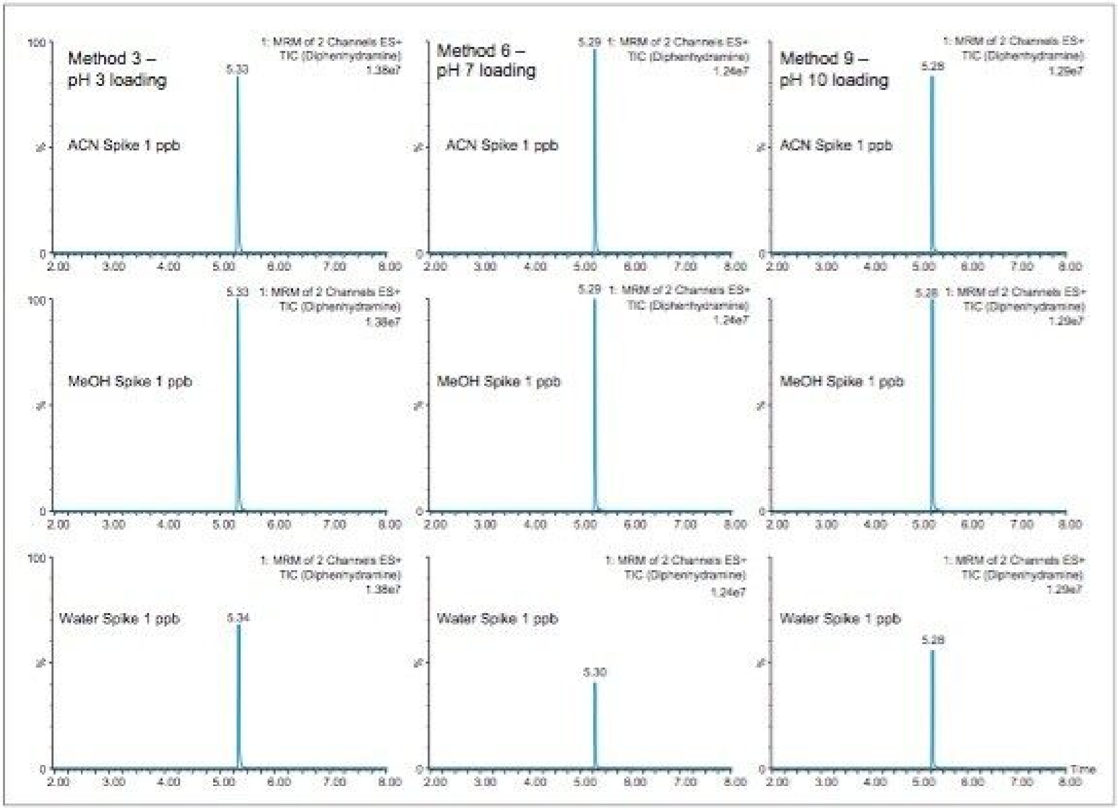

Figure 4. Method 6 chromatogram at 1 ppb in acetonitrile.

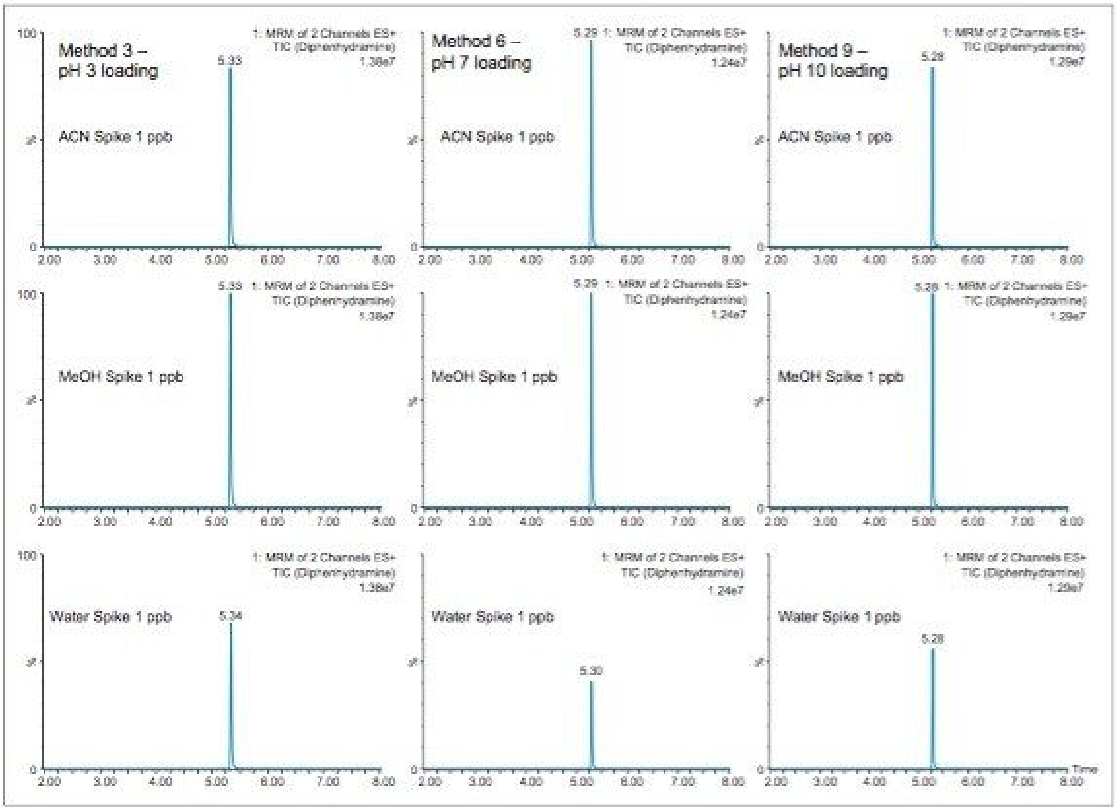

Figure 5. Results for method 3, 6, and 9 with 40 mg HLB bed mass for Diphenhydramine.

SPE extraction evaluation

After selecting the optimum 2D LC conditions, the work focused on the extraction optimization. The first step of the process targeted the choice of the sorbent. In this scenario, a mixed mode sorbent (Reversed Phased/Cation Exchange, Oasis MCX) was selected since all target analytes contain an amine functionality in their chemical structures. Hence, the evaluation started with two sorbent masses (60 and 150 mg) as presented in Table 3. The workflow started by loading a 2 mL water spike at 1 ppb and proceeded with a pH 3 water wash to ionize the basic compounds so they are captured onto the cation exchange portion of the sorbent. With target basic analyte secured, the reversed phase portion of the sorbent was eluted with a pH 3 high organic solvent wash. In this instance, a 100% Methanol with 2% formic acid was used for the secondwash. The elution of the basic analyte was performed with 100% acetonitrile with 2% ammonium hydroxide. The high pH value neutralizes the amine functionality, thus releasing all basic analytes from the cation exchange sorbent. The last wash and the final elution were collected to monitor if all analytes were in fact retained as predicted. As seen in table 2, the 60 mg sorbent bed showed signs of breakthrough for oxazepam, temazepam and N Desmethylcitolapram. When compared to a 150 mg sorbent bed, oxazepam and temazepam exhibited no breakthrough during the Methanol wash. However at this point in the evaluation, it was clear that several analytes exhibited poor recovery with the MCX cartridge. These issues were resolved by selecting a mixed mode sorbent with a reversed phased portion and a weak cation exchange portion (Oasis WCX). The methodology is similar, but the wash step and elution are governed by pH to ionize or neutralize the weak cation exchanger on the sorbent, as opposed to the analyte itself. The side by side comparison between MCX and WCX is presented in Table 4. As shown, Normeperidine, Dextrorphan, Dextromethorphan, N-Desmethylcitolapram and Norbuprenorphine, all show poor recoveries when using a strong cation exchanger. For two analytes, the results show a 10x signal difference between MCX and WCX. For those problem analytes, a dual methodology was crafted and two target analytes were used as markers (Citolapram and Diphenhydramine) for recovery evaluation purpose.

	MCX 60 mg		MCX 60 mg		MCX 150 mg		MCX 150 mg	
	Wash		Elution		Wash		Elution	
	MeOH 2% FA	Mean	ACN 2% NH_4OH	Mean	MeOH 2% FA	Mean	ACN 2% NH_4OH	Mean
	Area		Area		Area		Area	
Normeperidine	5441		282621		3217		114004	
	4525		352711		4399		142777	
	5545	5170	348768	328033	2475	3364	142185	132989
		1.57%				2.47%		low Rec
Dextrorphan	1922		228514		4663		17365	
	1976		280310		5321		21185	
	1880	1926	284213	264346	4103	4696	21618	20056
		0.72%				18.97%		low Rec
Nordiazepam	453		203376		627		227462	
	594		252200		452		246626	
	477	508	255220	236932	604	561	258576	244221
		0.20%				0.23%		
Doxylamine	5196		668535		5848		654529	
	6600		773747		5998		765944	
	6939	6245	762365	734882	4881	5576	777176	732550
		0.84%				0.76%		
Dextromethorphan	5994		406573		5046		295028	
	5225		551406		4873		395256	
	4944	5388	561553	506511	5144	5021	419907	370064
		1.06%				1.35%		low Rec
Diazepam	1978		177922		1325		203456	
	1539		223835		1871		224448	
	1476	1664	227167	209641	1672	1623	228516	218807
		0.79%				0.74%		
Promethazine	7934		451453		8713		461876	
	7232		642717		9263		642419	
	8153	7773	677627	590599	9520	9165	688151	597482
		1.31%				1.53%		
Oxazepam	51897		122881		4089		264526	
	40530		121777		3542		312994	
	49072	47166	124248	122969	4709	4113	327211	301577
	Breakthrough	27.28%		low Rec		1.35%		
Temazepam	56319		74337		3587		510018	
	58663		74699		3263		551183	
	53763	56248	75706	74914	3212	3354	548403	536535
	Breakthrough	42.88%		low Rec		5.88%		
Flecaninide	3530		564321		3978		593246	
	4794		735545		3331		812857	
	3264	3863	767391	689086	3855	3721	852158	752754
		0.53%				0.49%		
Diphenhydramine	1347		130365		6193		126349	
	1174		165624		7741		166090	
	1626	1382	169623	155204	6314	6749	173075	155171
		0.89%				4.17%		
Hydromorphone	347		42595		150		78233	
	210		44585		132		80272	
	154	237	45313	44164	164	149	80441	79649
		0.53%				0.19%		
Dihydrocodeine	451		193420		446		161234	
	417		202722		415		172063	
	314	394	209123	201755	196	352	171609	168302
		0.19%				0.20%		low Rec
Zolpidem	12152		1299467		2123		1267340	
	13871		1389542		2869		1366250	
	10986	12336	1419628	1369546	2672	2555	1380714	1338101
		0.89%				0.19%		
N DesmethylCitolapram	67930		261488		45163		25522	
	75121		356963		49196		35525	
	72058	71703	388218	335556	47818	47392	35001	32016
	Breakthrough	17.61%			Breakthrough	59.69%		low Rec
Oxycodone	539		397220		1316		370819	
	534		420253		1238		398264	
	530	534	427808	415094	1629	1394	402835	390639
		0.13%				0.36%		
Citolapram	5931		550657		4589		521151	
	6539		731095		5966		702231	
	6803	6424	773777	685176	6610	5722	734192	652525
		0.93%				0.87%		
Quetiapine	9066		516308		12078		450202	
	10734		617433		11410		533633	
	13784	11195	659231	597657	15261	12916	540583	508139
		1.84%				2.48%		
Norbuprenorphine	253		13998		152		8774	
	217		16520		188		11343	
	274	248	17578	16032	150	163	11129	10415
		1.52%				1.54%		low Rec
Diltiazem	19983		1701151		19237		1692454	
	19711		2107899		20532		2087306	
	19746	19813	2134585	1981212	20085	19951	2127205	1968988
		0.99%				1.00%		
Buprenorphine	1310		46300		195		43476	
	1338		61701		144		58034	
	1491	1380	62986	56996	186	175	58454	53321
		2.36%				0.33%		

Table 3. Recovery values for MCX 60 mg versus MCX 150 mg cartridge.

	WCX 100 mL extracts wash – MeOH + FA	WCX 100 mL extracts elution – ACN + FA	MCX 100 mL extracts wash – MeOH + FA	MCX 100 mL extracts elution – ACN + NH_4OH
Normeperidine	10043	472271	1085	143796
Dextrorphan	72	256835	178	12510
Nordiazepam	129486	15397	204	196527
Doxylamine	1049	129629	56	298312
Dextromethorphan	149	348720	729	278889
Diazepam	124873	25360	212	336645
Promethazine	610	539304	1817	718461
Oxazepam	134574	12057	522	113356
Temazepam	11833	32516	0	525964
Flecainide	1020	444358	598	549411
Diphenhydramine*	0	377237	4084	458358
Hydromorphone	0	4882	0	6504
Dihydrocodeine	0	21500	0	23872
Zolpidem	31687	692233	135	1291959
Ndesmethylcitalopram	0	409467	0	60843
Oxycodone	0	172149	0	181488
Citalopram*	1492	796273	4150	1447050
Quetiapine	652	430552	0	497057
Norbuprenorphine	134	11497	0	8789
Dilitazem	51	2726724	27332	3279491
Buprenorphine	1430	18819	933	41542

Table 4. Recovery values for WCX 150 mg vs MCX 150 mg cartridge.

The next phase of the application was to optimize the solid-liquid extraction of the solid sample (tissue) and evaluate the proper loading condition onto the mixed mode SPE sorbent. Store-bought calf liver was used for the sample preparation optimization, in order to preserve the human tissue specimens. When analyzing tissue samples, the homogenization process is typically performed with a common kitchen blender or a hand-held homogenizer (ex: Polytron). Those techniques can be cumbersome and are difficult to apply to small mass samples. In recent years, novel developments with ceramic or stainless steel ball bearings in combination with high speed orbital shakers have shown the ability to reach complete cell membrane breakdown in less than 60 seconds. With variable cycle speed, this novel homogenization protocol can process sample sizes from 0.1 to 5 grams. In this application, the mass range of tissue sample was set at 1.0 grams with to 4 mL extraction solvent ratio. In Figure 6, various organic solvents (acetonitrile, methanol, acetone) and pH range (2,7 and 10) were evaluated to measure which extraction conditions give maximum recovery percentage. In this application, the extraction of tissue with acetonitrile with no additives gave the highest signal.

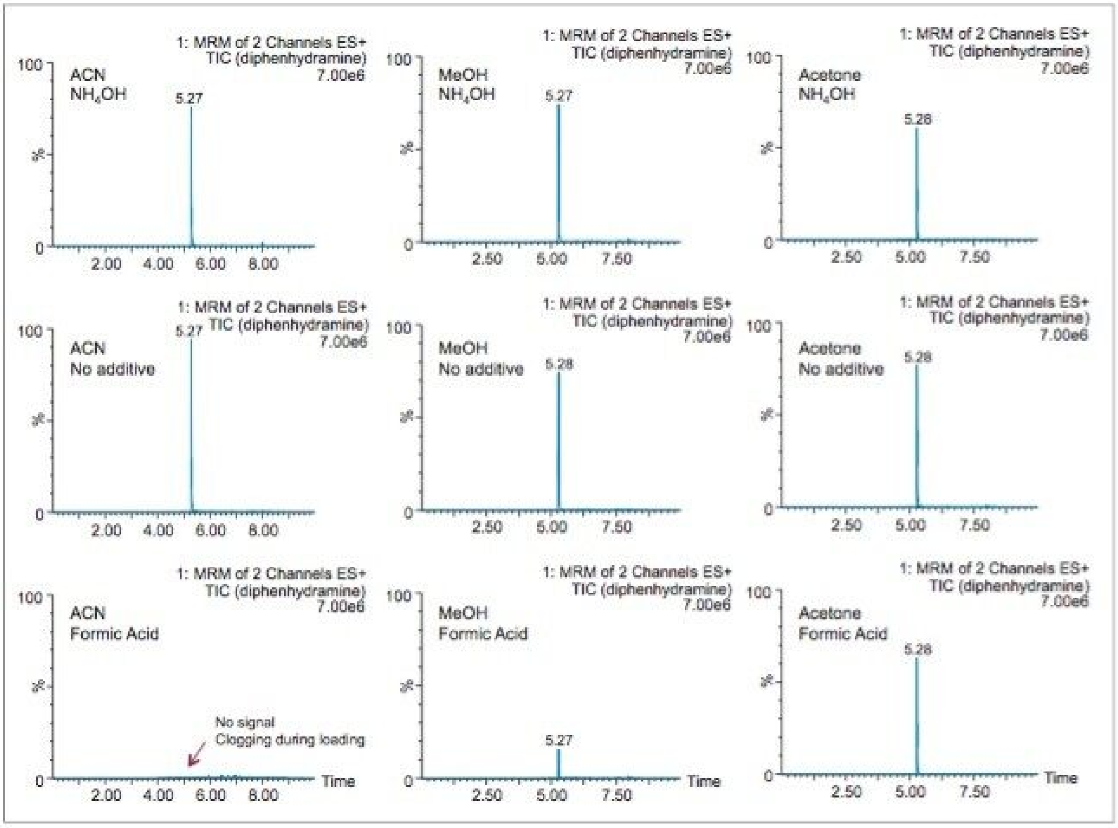

Figure 6. Optimization of the solid-liquid homogenization process.

Once the tissue sample was completely homogenized, it was centrifuged which produced a solid pellet on the bottom of the tube with the organic supernatant above. The organic supernatant was then filtered and decanted. Depending on the extraction conditions (pH and polarity), the target analyte is expected to be in solution and un-bound in the extraction solvent. In some applications, this crude extract can be used directly for quantification, however there is a high risk the raw sample extract will seriously reduce the robustness of the LC-MS/MS performance after a few injections. In traditional SPE protocols, when the target analyte is dissolved in a high percentage of organic solvent, the supernatant is usually evaporated to dryness and reconstituted in an aqueous diluent for further clean up. In instances where an evaporation-to-dryness step is needed, there is a risk of evaporative loss or possible re-dissolution issues. An effective way to avoid this lengthy step is to simply dilute the organic supernatant in a large aqueous volume at an organic/water ratio of less than 5%. A water volume between 100 and 200 mL is more than adequate to reach low organic ratio without any risk of breakthrough on the trapping column during loading phase. It may be perceived as a drawback, since the loading volume is quite large. However, with a loading flow rate at 10 mL/min using a large bore SPE barrel (6 cc with 150 mg bed mass), a 100 mL sample can be concentrated in 10 min, while evaporating to dryness can take several hours to complete.

The chromatograms in Figure 7 show the chromatography profile for an acetonitrile standard, water extracted standard and a spiked liver sample at 1 ppb level using the finalized extraction protocol. It is worth mentioning the stable baseline in both the water and liver extract, which is an indication that the extraction protocol, completed in 30 minutes, is producing a very clean extract. Table 5 depicts the overall recovery ratio for a liver tissue sample. Results demonstrated that 18 analytes have recovery values, measured against a post spiked deuterated internal standard (liver ion ratio recovery), within an acceptable range of 75% to 110%. The other analytes still show recovery ratio above 50%. The overall performance of both extraction methods gave an excellent linearity range as shown in Table 6. The R^2 values for all analytes ranged from 0.995 to 0.999 values. The limit of detection (LOD) for all analytes was set at 0.001 ng/mL (3x Sigma value).

Figure 7. MCX vs WCX Chromatogram for MeOH std, water extracted std water, and matrix match extracted std for Diphenhydramine.

Compound	Water std	Water ion ratio	Liver std	Liver ion ratio
MCX method	recovery %	recovery %	recovery %	recovery %
Nordiazepam	90.9	94.7	90.6	103.8
Doxylamine	117.4	100.5	80.8	67.5
Diazepam	91.8	95.6	70.6	80.9
Promethazine	62.7	62.0	80.3	84.3
Oxazepam	57.1	56.5	74.9	78.6
Temazepam	82.4	81.5	76.5	80.3
Flecainide	82.3	70.5	92.6	77.3
Diphenhydramine	107.6	106.2	93.2	97.9
Hydromorphone	54.1	79.4	57.6	63.4
Dihydrocodeine	63.8	78.9	64.9	59.4
Zolpidem	98.6	97.6	88.0	92.4
Oxycodone	70.6	69.8	67.9	71.3
Citalopram	84.9	84.0	75.5	79.2
Quetiapine	69.1	68.4	104.1	109.2
Diltiazem	94.9	93.9	90.8	95.3
Buprenorphine	87.6	108.5	81.1	74.2
WCX method				
Normeperidine	100.2	101.9	70.6	106.9
Dextrorphan	61.7	67.7	31.0	47.0
Dextromethorphan	65.8	66.8	49.1	74.3
Diphenhydramine	108.7	110.4	55.6	84.1
Ndesmethylcitalopram	67.2	75.5	52.5	68.7
Citalopram	66.3	67.3	55.2	83.6
Norbuprenorphine	73.5	82.6	50.8	66.6

Table 5. Recovery values for water extract vs calf liver extract.

Compound – MCX	IS	Linearity	Weighting	range (ng/mL)	R^2	LOD (ng/mL)
Nordiazepam	nordiazepam-d5	quadratic	1/x	0.025 - 10	0.997	0.001
Doxylamine	doxylamine-d5	quadratic	1/x	0.025 - 10	0.996	0.001
Diazepam	nordiazepam-d5	quadratic	1/x	0.025 - 10	0.999	0.001
Promethazine	temazepam-d5	quadratic	1/x	0.05 - 10	0.997	0.001
Oxazepam	temazepam-d5	quadratic	1/x	0.05 - 10	0.995	0.001
Temazepam	temazepam-d5	quadratic	1/x	0.05 - 10	0.998	0.001
Flecainide	doxylamine-d5	quadratic	1/x	0.050 - 10	0.998	0.001
Diphenhydramine	temazepam-d5	quadratic	1/x	0.025 - 10	0.996	0.001
Hydromorphone	hydromorphone-d5	quadratic	1/x	0.1 - 10	0.997	0.001
Dihydrocodeine	dihydrocodeiene-d6	quadratic	1/x	0.025 - 10	0.995	0.001
Zolpidem	temazepam-d5	quadratic	1/x	0.050 - 10	0.998	0.001
Oxycodone	temazepam-d5	quadratic	1/x	0.05 - 10	0.999	0.001
Citalopram	temazepam-d5	quadratic	1/x	0.050 - 10	0.997	0.001
Quetiapine	temazepam-d5	quadratic	1/x	0.050 - 10	0.996	0.001
Diltiazem	temazepam-d5	quadratic	1/x	0.025 - 1	0.996	0.001
Buprenorphine	dihydrocodeiene-d6	quadratic	1/x	0.05 - 10	0.996	0.001
Compound - WCX						
Normeperidine	temazepam-d5	quadratic	1/x	0.05 - 10	0.999	0.001
Dextrorphan	temazepam-d5	quadratic	1/x	0.05 - 10	0.996	0.001
Dextromethorphan	temazepam-d5	quadratic	1/x	0.010 - 10	0.999	0.001
Diphenhydramine	temazepam-d5	quadratic	1/x	0.05 - 10	0.999	0.001
Ndesmethylcitalopram	ndesmethylcitalopram-d5	quadratic	1/x	0.025-10	0.999	0.001
Citalopram	temazepam-d5	quadratic	1/x	0.05 - 10	0.998	0.001
Norbuprenorphine	ndesmethylcitalopram-d5	quadratic	1/x	0.05 - 10	0.999	0.001

Table 6. Linear range and detection limits.

Sample quantification

When analyzing highly complex sample types (class C matrix or solid samples), extraction recoveries are most often overwhelmed by matrix effects, which can lead to either suppression or enhancement in the MS detector. These effects are related to the inability of the sample clean up protocol to fully remove interferences from the raw sample.

In this work, the extraction protocol relied heavily on the use of a mixed mode sorbent using two trapping mechanisms. In this application, the Oasis MCX and WCX both have a reverse phase and cation exchange ligands to fractionate target basic analytes from neutral and acidic interferences. As seen in Figures 8 and 9, the MCX and WCX extracts for citolapram in various human tissue sample showed outstanding clean chromatograms at concentration values between 1.0 and 0.05 ppb.

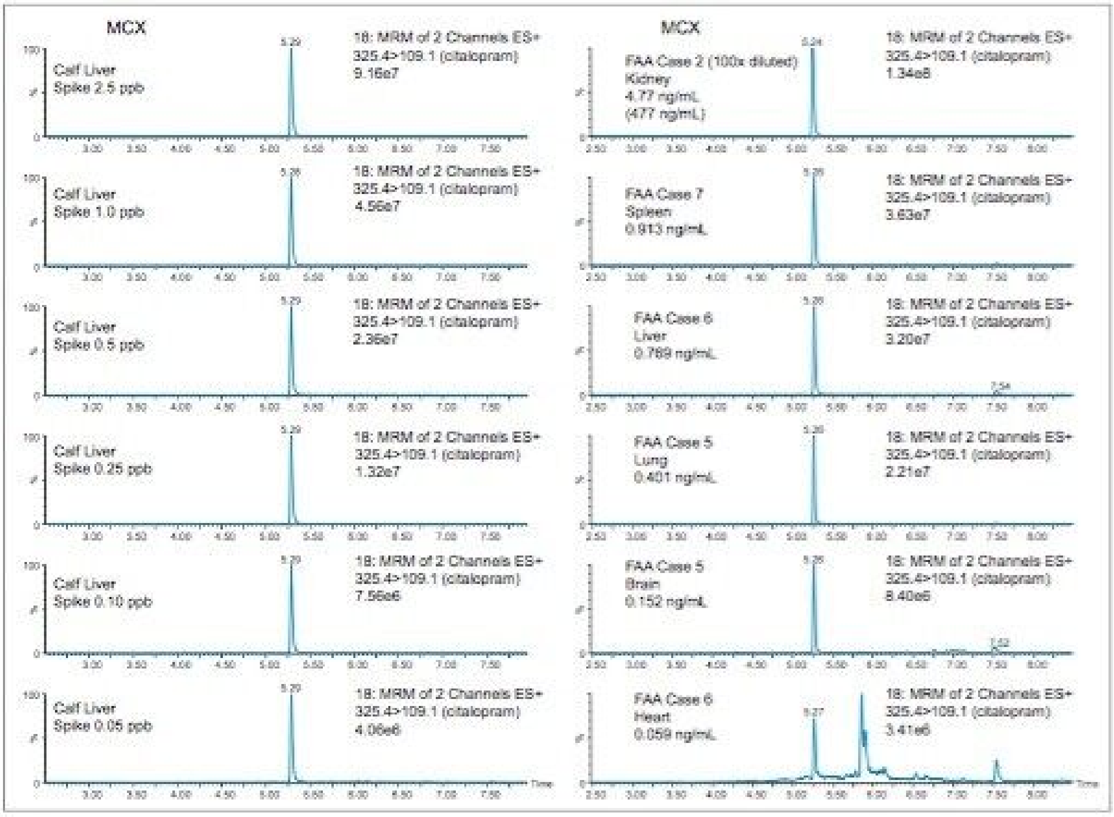

Figure 8. MCX chromatograms for tissue samples.

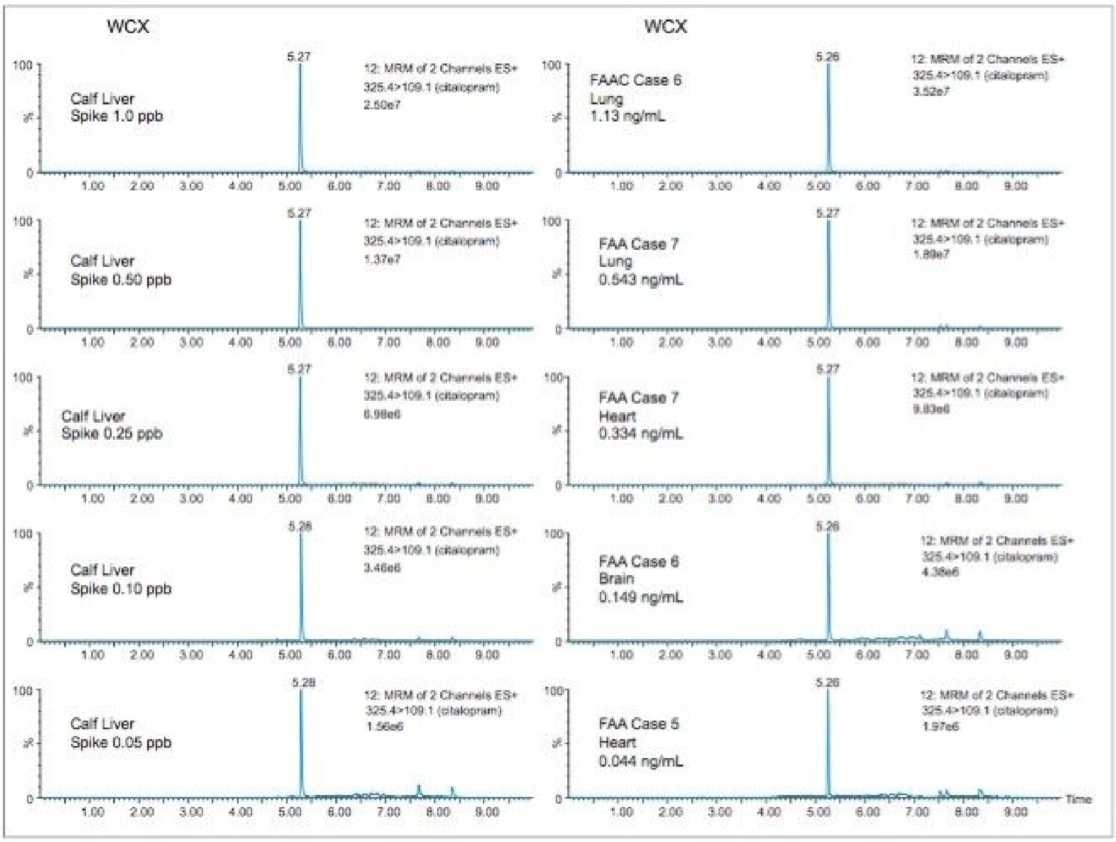

Figure 9. WCX chromatograms for tissue samples.

The results for the analyses of biological specimens (heart, brain, lung, liver, kidney, and spleen) are presented in Table 7. The extracts were quantified against a matrix match standard (calf liver) with a corresponding deuterated internal standard. The results were quantified within the linear range of 0.01 to 10 ng/mL. Therefore, some tissue extracts were subjected to a 100:1 dilution step before injection, to avoid flat top peak shape due to detector saturation. From the case studies in this application, case 7 tested positive for dextromethorphan (cough suppressant) and case 5 tested positive for flecainide (antiarrythmic agent). Also, case 2 tested positive for citolapram (antidepressant). As seen, since citolapram was selected as an efficiency marker for the MCX and WCX protocols, the results show comparable and precise performances for a variety tissue samples. The column chemistries used for this application gave an excellent performance analyzing well over 1000 sample injections.

Quantifcation (ng/mL)	WCX extracts					MCX EXTRACTS												
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Case 2 Heart	ND	0.08	1.23	493.0	669.0	ND	TLD	ND	0.03	5.29	0.01	0.73	9.15	690.0	96.7	ND	0.32	ND
Case 2 Lung	ND	0.02	0.36	973.0	740.0	ND	0.05	ND	0.03	2.82	0.02	0.76	7.25	966.0	76.0	ND	0.37	ND
Case 2 Liver	ND	0.02	0.18	1690.0	1070.0	ND	0.03	ND	0.16	1.70	0.02	3.10	9.90	1012.0	327.0	ND	0.56	ND
Case 2 Kidney	ND	TLD	2.15	544.0	504.0	ND	TLD	ND	0.40	10.8	0.05	1.33	6.05	477.0	221.0	ND	0.18	ND
Case 2 Spleen	ND	0.00	1.69	445.0	643.0	ND	TLD	ND	TLD	8.83	0.02	1.54	4.40	616.0	90.0	ND	0.22	ND
Case 2 Brain	0.01	0.05	2.72	115.0	525.0	ND	TLD	ND	TLD	8.78	0.01	0.60	3.65	587.0	82.90	ND	0.21	ND
Case 5 Heart	ND	0.17	TLD	0.03	0.04	ND	ND	ND	830.0	ND	ND	ND	ND	0.18	TLD	44.6	0.57	ND
Case 5 Lung	ND	0.23	TLD	0.02	0.02	ND	ND	ND	500.0	ND	ND	ND	ND	0.40	0.013	20.9	0.34	ND
Case 5 Liver	ND	0.09	0.01	0.11	0.28	ND	ND	ND	970.0	ND	ND	ND	ND	0.07	TLD	117.0	0.97	ND
Case 5 Kidney	ND	0.07	TLD	0.13	0.17	ND	ND	ND	588.0	ND	ND	ND	ND	0.07	TLD	35.3	0.45	ND
Case 5 Spleen	ND	0.06	TLD	0.51	1.00	ND	ND	ND	423.0	ND	ND	ND	ND	0.03	TLD	48.0	0.68	ND
Case 5 Brain	ND	0.01	TLD	0.40	0.60	ND	ND	ND	138.0	ND	ND	ND	ND	0.15	TLD	10.4	0.14	ND
Case 6 Heart	ND	0.06	0.03	0.20	0.43	0.04	TLD	ND	0.23	TLD	ND	ND	ND	0.06	0.03	ND	ND	ND
Case 6 Lung	ND	0.40	0.10	0.61	1.13	0.05	TLD	ND	0.04	0.08	ND	ND	ND	1.17	0.16	ND	ND	ND
Case 6 Liver	ND	0.21	0.03	0.31	0.43	0.08	TLD	0.008	TLD	0.09	ND	ND	ND	0.77	0.11	ND	ND	ND
Case 6 Kidney	0.81	0.06	0.16	0.38	0.40	0.24	0.04	0.053	TLD	0.35	ND	ND	ND	3.63	0.12	ND	ND	0.04
Case 6 Spleen	ND	0.35	0.04	0.75	1.45	0.14	TLD	0.032	TLD	0.08	ND	ND	ND	0.43	TLD	ND	ND	ND
Case 6 Brain	ND	0.14	0.02	0.06	0.15	0.02	TLD	ND	TLD	0.01	ND	ND	ND	0.56	0.023	ND	ND	ND
Case 7 Heart	0.38	448.0	0.38	0.13	0.33	ND	0.26	ND	ND	0.62	ND	ND	ND	0.41	TLD	ND	ND	ND
Case 7 Lung	2.23	1684.0	4.26	0.25	0.54	ND	3.06	ND	ND	5.30	ND	ND	ND	0.84	0.16	ND	ND	ND
Case 7 Liver	1.18	1258.0	1.61	0.32	0.57	ND	0.81	ND	ND	2.90	ND	ND	ND	0.13	TLD	ND	ND	ND
Case 7 Kidney	2.38	365.0	0.67	0.13	0.17	ND	0.41	ND	ND	0.98	ND	ND	ND	0.62	0.05	ND	ND	ND
Case 7 Spleen	0.73	554.0	0.47	0.25	0.59	ND	0.48	ND	ND	1.07	ND	ND	ND	0.91	0.07	ND	ND	ND
Case 7 Brain	0.25	391.0	0.31	0.27	0.40	ND	0.15	ND	ND	0.46	ND	ND	ND	0.73	0.16	ND	ND	ND

WCX extracts (Detected)

A: Dextrorphan
B: Dextromethorphan
C: Diphenhydramine
D: ndesmethylcitalopram
E: citalopram

MCX extracts (Detected)

F: Nordiazepam
G: Doxylamine
H: Diazepam
I: Flecainide
J: Diphenhydramine
K: Hydromorphone
L: Dihydrocodeine
M: Zolpidem
N: Citalopram
O: Quetiapine
P: Diltiazem
Q: Buprenorphine
R: Oxycodone

WCX extracts (Un-Detected)

Normeperidine

MCX extracts (Un-Detected)

Promethazine
Oxazepam
Temazepam

Trace level Detection (TLD) <0.01 ng/mL

Not Detected (ND)

Table 7. Quantification values for human tissue samples.

Conclusion

This application demonstrated the automated and fast method development capability of the ACQUITY UPLC with 2D Technology for the analysis of pharmaceuticals in human tissue samples. The quantification limit was set at 10 ppt using a 1.0 g of sample. The micro extraction protocol offered the option to evaluate several elution parameters in a short time period. The elution optimization was completed within a 4 hrs hands-on work and the 2D LC results were analyzed using an over-night run using a multi-methods sample list (18 hrs). With the extraction protocol optimized, the final protocol produced a clean extract in 30 minutes without any evaporation to dryness and reconstitution into initial mobile phase conditions.

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