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Efficient and Clean Extraction of a Multi-Drug Panel with Oasis PRiME MCX for Clinical Research

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APPLICATION BENEFITS

- Simple, 3- or 4-step SPE procedures
- Minimal residual phospholipids compared to reversed-phase or traditional mixed-mode cation exchange techniques
- High and reproducible recoveries for basic analytes

WATERS SOLUTIONS

Oasis PRiME HLB 96-well µElution Plates

Oasis™ PRiME MCX µElution Plates

Oasis MCX µElution Plates

ACQUITY™ BEH C_{18}, 1.7 µm, 2.1 x 100 mm Column

Xevo™ TQ-S micro Mass Spectrometer

ACQUITY UPLC™ I-Class System (FTN)

MassLynx™ Software v4.1

TargetLynx™ XS

KEYWORDS

Solid Phase-Extraction (SPE), MCX, mixed-mode cation-exchange, phospholipid removal

INTRODUCTION

Mixed-mode solid-phase extraction (SPE) has long been used for bioanalytical sample preparation due to its ability to produce clean extracts using LC-MS friendly solvents. Nevertheless, it is often perceived as complicated compared to other techniques and strong cation exchange sorbents often fail to remove relatively high concentrations of residual phospholipids compared to other mixed-mode techniques.[1] Oasis PRiME HLB, a novel reversed-phase sorbent developed in 2015,[2,3] has been shown to successfully remove phospholipids from biological samples, but does not have the specificity of mixed-mode sorbents. In order to combine the specificity of mixed-mode ion exchange with the cleanliness of Oasis PRiME HLB, Waters® has recently developed a new product, Oasis PRiME MCX that is based on the Oasis MCX Mixed-mode strong Cation eXchange sorbent. Oasis PRiME MCX is designed to produce even cleaner extracts than conventional ion-exchange protocols while using simple 3- or 4-step SPE methods. This application note highlights the use of Oasis PRiME MCX using a multi-drug panel containing opioids, benzodiazepines, stimulants, anti-epileptics, synthetic cathinones, and other compounds to demonstrate the simplicity and cleanliness achieved with this product for clinical research. Comparisons were also made to extractions using Oasis MCX and the reversed-phase sorbent, Oasis PRiME HLB.

EXPERIMENTAL

Reagent grade chemicals were obtained from Fisher Scientific. Target analyte stock solutions were acquired from Cerilliant (Round Rock, TX). Human plasma was obtained from Lampire Biological laboratories (Pipersville, PA).

Sample preparation

Combined stock solutions were prepared by diluting high concentration stock solutions in methanol to create a working stock solution with analyte concentrations of 2, 10, and 25 µg/mL. Daily working solutions were prepared by diluting the working stock solution 1:10 in MilliQ water. This daily working solution was added to blank plasma to produce fortified plasma.

Oasis PRiME HLB Protocol: 100 µL of plasma was diluted with 100 µL of 5% strong ammonia (Fisher 28–30%) and loaded directly onto the wells of an Oasis PRiME HLB µElution Plate. Samples were washed with 200 µL of 95:5 water:MeOH. Samples were eluted with 2 x 25 µL aliquots of 90:10, 80:20, or 50:50 ACN:MeOH. All extracted samples were diluted with 150 µL of 97:2:1 Water:ACN:formic acid prior to injection on the LC-MS/MS system.

Oasis MCX Protocol: 100 µL of plasma was diluted with 100 µL of 4% H_3PO_4 and loaded directly onto the wells of the Oasis MCX µElution Plate. All samples were washed with 200 µL of 2% formic acid, followed by 200 µL MeOH. Samples were eluted with 2 x 25 µL aliquots of 50:50 ACN:MeOH containing 5% strong ammonia (Fisher 28–30%). All extracted samples were diluted with 150 µL of 97:2:1 Water:ACN:formic acid prior to injection on the LC-MS/MS system.

4-Step Oasis PRiME MCX Protocol: 100 µL of plasma was diluted with 100 µL of 4% H_3PO_4 and loaded directly onto the wells of the Oasis PRiME MCX µElution Plate. All samples were washed with 200 µL of 100 mM ammonium formate containing 2% formic acid, followed by 200 µL MeOH. Samples were eluted with 2 x 25 µL aliquots of 50:50 ACN:MeOH containing 5% strong ammonia (Fisher 28–30%). All extracted samples were diluted with 150 µL of 97:2:1 Water:ACN:formic acid prior to injection on the LC-MS/MS system.

3-Step Oasis PRiME MCX Protocol: 100 µL of plasma was diluted with 100 µL of 200 mM ammonium formate:4% H_3PO_4 and loaded directly onto the wells of the Oasis PRiME MCX µElution Plate. Samples were washed with 200 µL MeOH and eluted with 2 x 25 µL aliquots of 50:50 ACN:MeOH containing 5% strong ammonia (Fisher 28–30%). All extracted samples were diluted with 150 µL of 97:2:1 Water:ACN:formic acid prior to injection on the LC-MS/MS system. All 3 mixed-mode protocols are shown in Figure 1.

Oasis MCX 4-Step Method	Oasis PRiME MCX 4-Step Method	Oasis PRiME MCX 3-Step Method
100 µL plasma + 100 µL 4% H_3PO_4	100 µL plasma + 100 µL 4% H_3PO_4	100 µL plasma + 100 µL 200 mM NH_4COOH/4% H_3PO_4
Load sample onto sorbent	**Load sample onto sorbent**	**Load sample onto sorbent**
Wash 1 200 µL 2% formic acid	**Wash 1** 200 µL 100 mM NH_4COOH/2% formic acid	**Wash 1** 200 µL MeOH
Wash 2 200 µL MeOH	**Wash 2** 200 µL MeOH	**Elute** 2 x 25 µL (50:50 ACN:MeOH + 5% NH_4OH)
Elute 2 x 25 µL (50:50 ACN:MeOH + 5% NH_4OH)	**Elute** 2 x 25 µL (50:50 ACN:MeOH + 5% NH_4OH)	**Dilute** Add 150 µL 2% ACN/1% FA
Dilute Add 150 µL 2% ACN/1% FA	**Dilute** Add 150 µL 2% ACN/1% FA	

Figure 1. Mixed-mode extraction protocols used in this application. The Oasis MCX 4-step is a traditional Oasis MCX extraction protocol without conditioning or equilibration. Oasis PRiME MCX 4-step modifies the Oasis MCX protocol by adding 100 mM ammonium formate to the first wash step. The Oasis PRiME MCX 3-step protocol adds 200 mM ammonium formate to the sample diluents and eliminates the aqueous wash step (Wash 1).

Method conditions

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY BEH C_{18}, 1.7 µm, 2.1 x 100 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection vol.:	2 µL
Flow rate:	0.6 mL/min
Mobile phase A:	0.1% Formic acid in MilliQ water
Mobile phase B:	0.1% Formic acid in acetonitrile (ACN)
Purge solvent:	50:50 MeOH:H_2O
Wash solvent:	25:25:25:25 MeOH:H_2O:IPA:ACN

Table 1 shows the LC gradient program.

Table 1. UPLC gradient program.

Time (min)	Flow (mL/min)	% MPA	% MPB
0.0	0.6	98	2
3.33	0.6	33	67
4.0	0.6	10	90
5.5	0.6	10	90
6.0	0.6	98	2
7.0	0.6	98	2

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI positive
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/hr
Cone gas flow:	150 L/hr
Acquisition range:	MRM transitions optimized for individual compounds
Capillary voltage:	1.0 kV
Collision energy:	Optimized for individual compounds
Cone voltage:	Optimized for individual compounds

Data management

MS software:	MassLynx v4.1
Quantification software:	TargetLynx XS

Phospholipid levels were quantitated by performing a parent ion scan of the common fragment ion, *m/z* 184.

RESULTS AND DISCUSSION

A variety of compounds were extracted using reversed-phase SPE (Oasis PRiME HLB) and mixed-mode SPE (Oasis MCX and Oasis PRiME MCX). A number of parameters were investigated, including extraction recovery, matrix effects and cleanliness, as measured by residual phospholipids. Table 2 lists all target analytes and their respective retention times and predicted LogP values. Figure 2 shows the chromatography of all target analytes with selected compounds labeled.

Table 2. Target Analytes, LogP values and Retention Times. LogP values are from Chemicalize.[4]

Name	RT	Log P	Name	RT	Log P
Morphine	0.86	0.9	BZE	1.52	-0.59
Oxymorphone	0.91	0.78	7-aminoclonazepam	1.51	
Hydromorphone	0.98	1.62	N-desmethyl Zopiclone	1.58	
Dihydrocodeine	1.15	1.55	Zopiclone	1.61	0.81
Naloxone	1.15	1.62	Tramadol	1.68	2.45
Codeine	1.17	1.34	N-desmethyl Tramadol	1.69	
Pregabalin	1.20	-1.35	Methylphenidate	1.70	2.25
Gabapentin	1.20	-1.27	Tapentadol	1.71	2.96
Methylone	1.21	1.23	alpha-PVP	1.77	
Noroxycodone	1.25		7-aminoflunitrazepam	1.69	
6-beta Naltrexol	1.26		Cocaine	1.81	2.28
Naltrexone	1.26	1.36	Normeperidine	1.82	
Amphetamine	1.28	1.8	Meperidine	1.83	2.46
Oxycodone	1.28	1.03	Zolpidem	1.85	3.02
6-MAM	1.28	1.09	alpha-PVP metabolite 1	1.88	
MDA	1.30	1.86	Norbuprenorphine	1.90	2.3
Norhydrocodone	1.31	1.86	Chlordiazepoxide	1.93	3.05
Ethylone	1.32	1.59	Trazodone	1.99	3.13
O-desmethyl Tramadol	1.32	1.72	Cocaethylene	2.01	2.64
Methedrone	1.33	1.45	Fenfluramine	2.03	3.47
Hydrocodone	1.34	1.96	PCP	2.09	4.49
Dehydronorketamine	1.33	2.91	Fentanyl	2.15	3.82
Methamphetamine	1.36	2.24	alpha-OH Midazolam	2.13	
MDMA	1.37	1.86	Midazolam	2.17	3.97
m-OH BZE	1.34		Flurazepam	2.23	3.95
Butylone	1.41	1.75	Buprenorphine	2.27	3.55
Phentermine	1.43	2.08	EDDP	2.29	4.63
Mephedrone	1.47	2.12	Verapamil	2.52	5.04
Norketamine	1.47	2.91	Propoxyphene	2.56	4.90
MDEA	1.48	2.22	Methadone	2.60	5.01
Ritalinic acid	1.48	-0.36	alpha-OH Alprazolam	2.51	
Ketamine	1.52	3.35	Alprazolam	2.68	3.02
Norfentanyl	1.54	1.42			

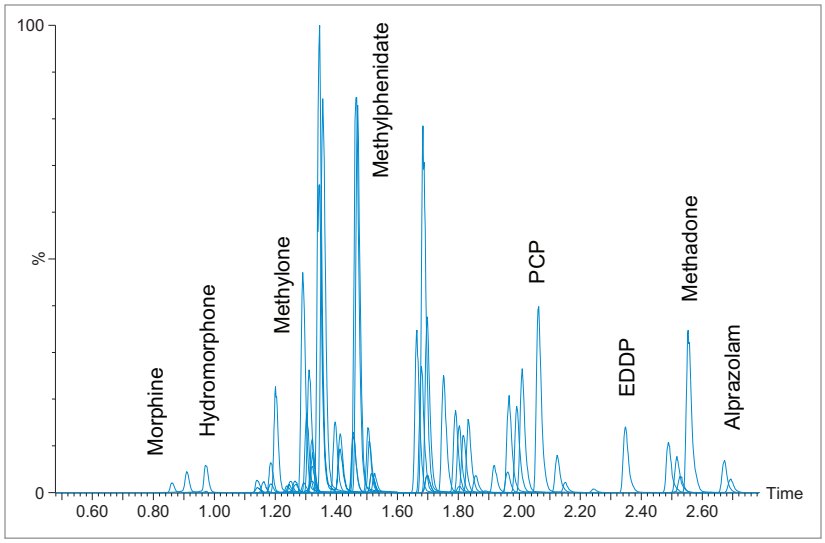

Figure 2. Chromatography of all the compounds evaluated in this application. Selected compounds are labeled.

Figure 3A shows the recoveries for all analytes using Oasis PRiME HLB. Many demonstrate poor recovery using the recommended starting elution of 90:10 ACN:MeOH, which is designed to minimize residual phospholipids. While recoveries of most compounds can be significantly improved by increasing the proportion of methanol in the elution solvent, certain compounds, suchas pregabalin and gabapentin, opiates such as norhydrocodone, norfentanyl, and normeperedine, and stimulants such as methamphetamine and MDMA are still recovered at less than 20%. Recoveries for many others remain under 50%. This is a key example of the limitations of reversed-phase SPE for polar, ionizable molecules. Matrix effects for the three conditions are shown in Figure 3B.

Figure 3A. Extraction recovery results using Oasis PRiME HLB. The extraction protocols are described in the Materials and Methods section. Recovery after elution with 90:10, 80:20 and 50:50 ACN:MeOH are shown in red, blue and green, respectively. Figure 3B. Matrix effects results using Oasis PRiME HLB. Color coding is the same as Figure 3A (N=4 for each).

In order to specifically extract basic compounds, while maintaining or even improving the cleanliness seen with Oasis PRiME HLB, Waters has developed Oasis PRiME MCX, a mixed-mode solid-phase extraction product based on Oasis MCX Technology. Oasis PRiME MCX is designed with protocols to specifically extract basic molecules, with the added benefit of removing residual phospholipids. Two new extraction procedures, a 3-step procedure and a 4-step procedure have been developed to maximize recovery of basic compounds while minimizing residual phospholipids.

Figure 4 demonstrates the recoveries the target compounds using Oasis MCX, as well as Oasis PRiME MCX, in both 3- and 4-step procedures. It is evident that there is virtually no difference in the extraction efficiency for the 3-step procedure, the 4-step procedure or the traditional Oasis MCX Procedure. Matrix effects for the mixed-mode extractions are seen in figure 4B. With the exception of verapamil, most matrix effects were less than 20%.

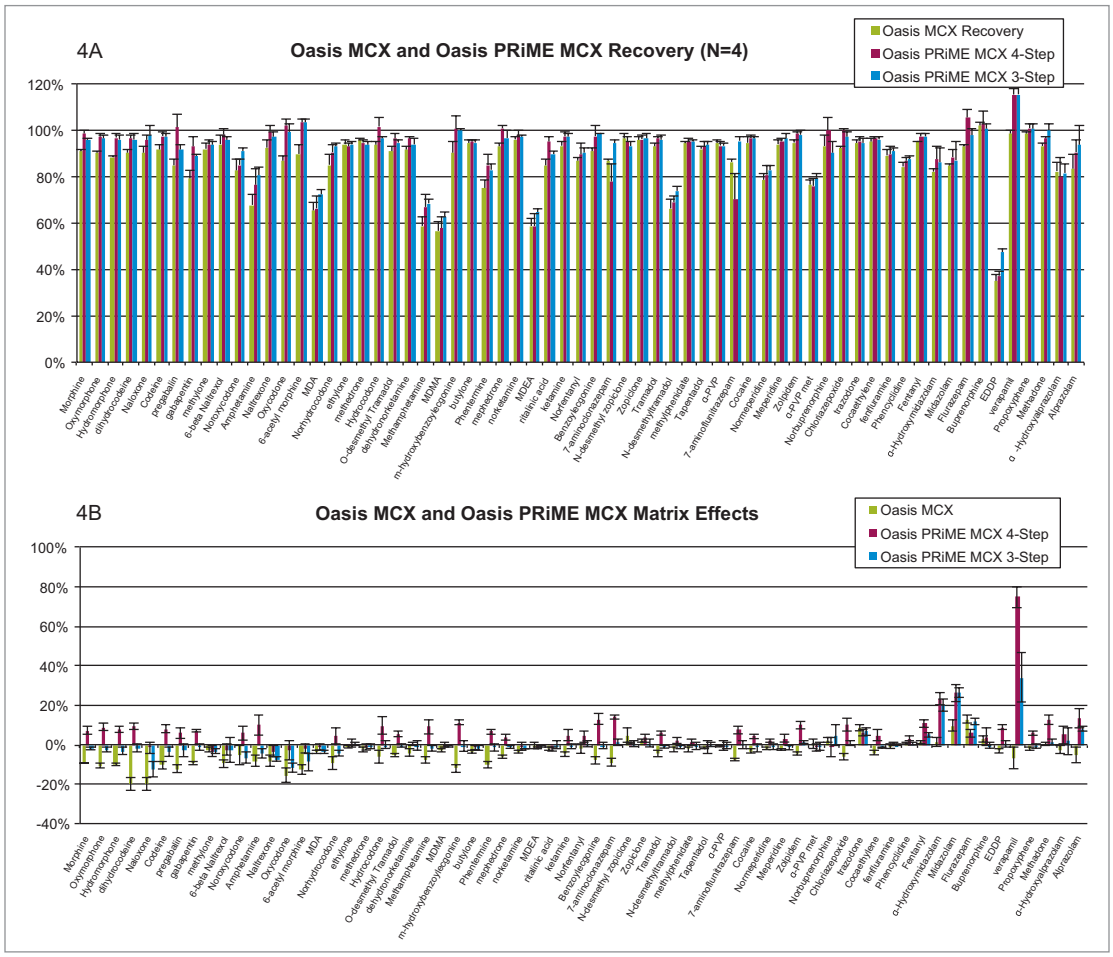

Figure 4A. Extraction recovery results using Oasis MCX and Oasis PRiME MCX extraction protocols. The extraction protocols are described in the Materials and Methods section and shown in Figure 1. Figure 4B. Matrix effects results using Oasis MCX and Oasis PRiME MCX extraction protocols. Color coding is the same as Figure 4A. (N=4 for each).

Comparative phospholipid levels in the sample extracts from the protocols in Figure 1 are shown in Figures 5 and 6. Figure 5A shows chromatographic traces of residual phospholipids from the 4-step and 3-step Oasis PRiME MCX protocols and the traditional Oasis MCX protocol. The summed areas are shown in Figure 5B. Normalizing the areas reveal that the 3- and 4-step Oasis MCX PRiME protocols remove 95% and 98% of phospholipids, respectively when compared to the traditional Oasis MCX extraction protocol. Comparisons were also made with Oasis PRiME HLB, which had been specifically designed to remove phospholipids from biological samples. As shown in Figure 6, phospholipid levels using the 3-step Oasis PRiME MCX protocol were comparable to those seen with Oasis PRiME HLB, while those seen using the 4-step Oasis PRiME MCX protocol were significantly reduced compared to the recommended Oasis PRiME HLB elution protocol of 90:10 ACN:MeOH. Not surprisingly, the increase in methanol required to improve the recovery of many of the target compounds from Oasis PRiME HLB resulted in a significant increase in residual phospholipids.

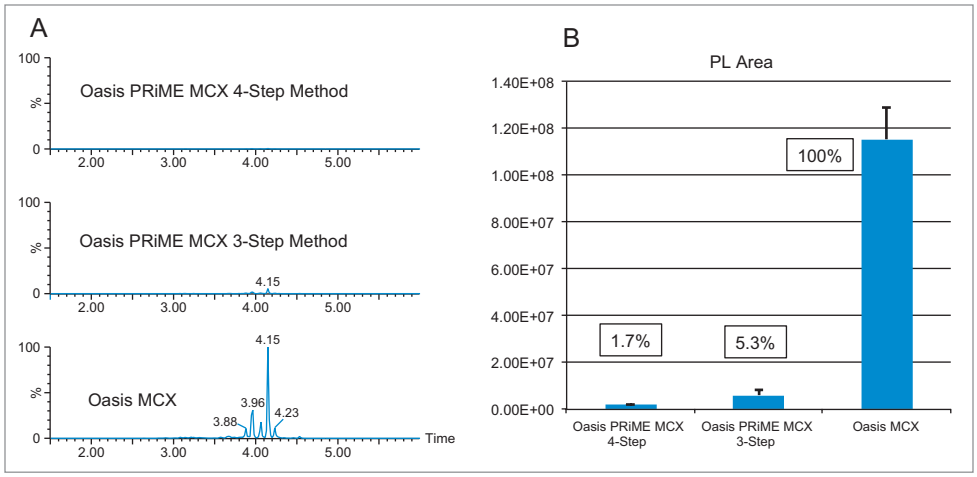

Figure 5A. Residual phospholipid traces from Oasis MCX and Oasis PRiME MCX extraction protocols. All three traces are shown at the same scale. Figure 5B. Relative areas of residual phospholipids shown in Figure 5A. The relative abundances of the phospholipid areas are shown in labels next to the area bars (N=4).

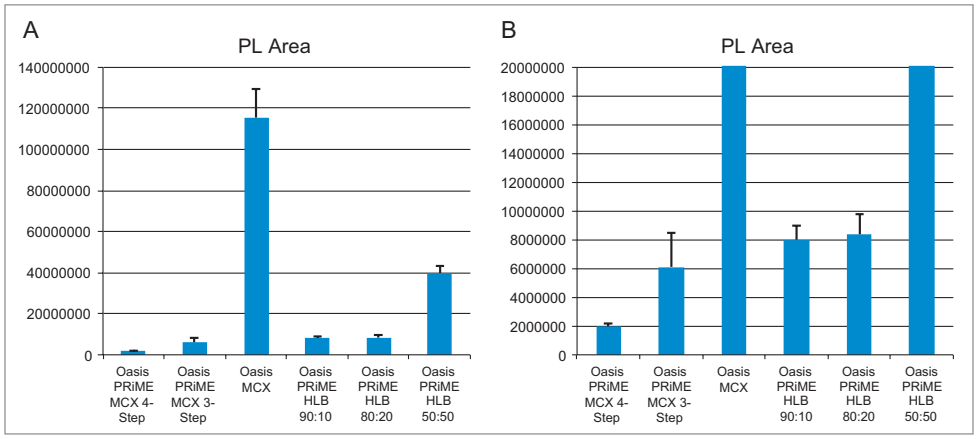

Figure 6A. Relative phospholipid areas from the Oasis MCX and Oasis PRiME MCX protocols and from the Oasis PRiME HLB protocols. Figure 6A shows the mean phospholipid area of each protocol. Figure 6B has been zoomed in to highlight the lower abundance areas of Oasis PRiME MCX and Oasis PRiME HLB. The numbers for Oasis PRiME HLB refer to the proportions of acetonitrile and methanol, respectively used for the extraction.

CONCLUSIONS

Oasis PRiME MCX continues the development of simpler and faster SPE workflows and products that also provide cleaner extracts. While Oasis PRiME HLB is effective in miminizing residual phospholipids, it may not be the optimal choice for extracting polar, ionizable bases such as the ones in this application as shown in Figure 3A. By contrast, traditional mixed-mode ion-exchange sorbents/protocols can result in efficient and reproducible recoveries of these compounds (Figure 4A) but can result in high levels of residual phospholipids. Using Oasis PRiME MCX, efficient and reproducible recovery of bases from biological samples can be achieved that is equivalent to Oasis MCX for clinical research. At the same time, residual phospholipids can be reduced by 98% compared to conventional Oasis MCX protocols and can even be lower than those achieved with Oasis PRiME HLB. Thus, the advantages of phospholipid removal are combined with the specificity and selectivity of a mixed-mode polymeric SPE sorbent.

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