Practical Considerations Using Quantisal® Oral Fluid Collection Devices and SPE Method Development by Polymeric Mixed-Mode Cation Exchange

A White Paper from Biotage

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Abstract

Oral fluid is of increasing importance as a biological matrix in drugs of abuse and related analyte testing because of the ease of sample collection, and difficulty in adulteration of samples, particularly when compared with urine.

Oral fluid for drug testing is usually collected using proprietary collection devices such as the Quantisal® device (Immunalysis), which allow oral fluid to be collected and stored without degradation before analysis. Collection devices contain various components such as buffer salts and surfactants which along with typical endogenous oral fluid constituents present particular challenges in sample preparation for LC-MS/MS.

In this white paper we examine recovery and matrix effects for 85 common DOAs, when collected using the Quantisal device. Utilizing water, synthetic oral fluid and patient samples as matrixes, the impact of various wash solvents on analyte recovery and matrix effects from the Quantisal buffer, in polymeric mixed-mode cation exchange SPE is investigated.

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Introduction

Oral Fluid Background

Oral fluid represents a complex, heterogeneous biological fluid primarily produced by the parotid, submandibular, and sublingual salivary glands. Together, these glands make up the majority of saliva, which is excreted into the oral cavity through a collective network of striated ducts. Although only the major glands possess a collective secretive orifice, all salivary glands produce a secrete that vary in complexity. Healthy adults can produce up to 0.5 to 1.5 liters of saliva per day or between 0 to 6 mL/min.[1] The volume and composition vary, either due to stimulation or attenuation, or because of the circadian rhythm, which also alters its ionic concentration throughout the day and night. Regardless of an individual's wellbeing, when salivating, their oral fluid is primarily composed of water, which is rendered hypotonic (compared to serum) once it enters the oral cavity.[2]

The remaining contents include mucins for lubrication, along with amylases, proteases, and lipases for digestion and various antimicrobial functioning proteins (IgA, lysozyme, etc.). The electrolytic content of saliva is greatest with sodium, potassium, chloride, and bicarbonate, with calcium, magnesium, and phosphate to a lesser extent – all of which originate from serum and are actively transported across networks of capillaries into salivary ducts.[3] It is at this excretion interface where new frontiers in diagnostic applications have focused. Assays designed to monitor both nucleic acid and protein biomarkers for the prophylactic detection of breast, pancreatic, and ovarian cancers have recently been examined. [4-6] However, the past decade has seen a pronounced rise in monitoring drugs of abuse (DOA) using oral fluids.[7-10]

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Oral Fluid Collection Devices

As oral fluid offers applications for the detection of malignancies, it is also highly suitable biological matrix for testing DOA within drug treatment programs, criminal justice settings, pre-employment screening, and driving under the influence of drugs (DUID). Within these settings, oral fluids offers a unique opportunity due to the ease of sample collection and the difficulty surrounding sample adulteration as compared to urine. Devices used to collect saliva for DOA diagnostic applications include collection through absorbent cellulose pads, buccal swabs, and direct collection of expectorant. However, the use of applicators or pads is ostensibly favored in the U.S. market place. These devices are validated among the clinical and forensic communities and include, but are not limited to: Quest Diagnostics' FDA approved Oral-Eze device, Immunalysis' Quantisal® ih2 device, and OraSure Technologies Intercept collection device.

Each device includes an applicator or pad which the subject places their tongue under or over for a prescribed amount of time or until the device indicates salivary saturation through the implementation of a color indicator. The applicator is then stored in a buffer, and saturated with components designed to preserve the oral fluid sample. Many of these buffers possess proprietary components and preservatives. Fortunately, most possess overlapping contents, e.g. multiple salts added for pH buffering, including bicarbonate, and mono/dibasic sodium phosphate or citrate. Broad-spectrum antimicrobial agents are also present, usually Proclin 300 or 950, both of which are toxic. However, the most challenging agent that may be present is an excipient or emulsifying agent like the polysorbate Tween 20 or long chain polyethylene glycol (PEG). These agents are generally disruptive to the purification process of oral fluids as they act as a chemical bridge between the biphasic layers under liquid-liquid extractions (LLE). Moreover, isolation of analytes from oral fluid using SPE becomes more complicated as the choice of sorbent wash and analyte capture must be carefully considered in order to successfully remove the emulsifying agent without disrupting analyte complexation with SPE sorbent.

In this white paper, we describe the relationship between 85 analytes and their subsequent response to the recovery and matrix effects of Immunalysis' Quantisal® buffer as used with water as a surrogate oral fluid, synthetic oral fluid from UTAK, and patient submitted oral fluid. Moreover, we examine the impact upon recovery and matrix effects upon varying solvent polarity of the organic wash to improve analyte detection and SPE method ruggedness upon a large and diverse panel of analytes.

Sample Preparation

EVOLUTE® EXPRESS CX Extraction Protocol Using PRESSURE+ 48 Positive Pressure Manifold

Reagents & Materials

All standards were purchased from Cerilliant (Round Rock, TX). HPLC grade water, methanol (MeOH), and acetonitrile (ACN), tetrahydrofuran (THF), Acetone, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and methyl tert-butyl ether (MTBE) were purchased from Sigma Aldrich (St. Louis, MO) in addition to reagent grade isopropyl alcohol (IPA), formic acid, and ammonium hydroxide (NH_4OH). EVOLUTE® EXPRESS CX 60 mg/3 mL tabless SPE columns (611-0006-BXG), Biotage® PRESSURE+ 48 positive pressure manifold (PPM-48), and TurboVap® LV (415000) were supplied by Biotage.

Standards & Sample Pretreatment

Standards were pooled in multiple stocks at a concentration of 10 µg/mL in methanol and stored at –20 °C. After reaching room temperature, working stock solutions were spiked directly into surrogate oral fluid (HPLC grade water), synthetic oral fluid (generously donated by UTAK, P/N: 43049), or into the Quantisal® device (P/N:QS-0025) followed by acid pretreatment with formic acid. Surrogate and synthetic oral fluids were combined with Immunalysis Quantisal buffer (P/N: EXTBUF-1000) at a 1:3 ratio, per manufacturer's instructions. Immunalysis Quantisal oral fluid collection device and accessories were generously donated by Immunalysis (P/N: QS-0025 and 6212-FS-416).

Extraction Parameters

Analytes were isolated using a EVOLUTE® EXPRESS CX 60 mg/3 mL column using a Biotage® PRESSURE+ 48 positive pressure manifold. Prior to sample loading (1.0 mL, unless otherwise stated) the sorbent was conditioned and equilibrated with 1.0 mL of methanol and 4% formic acid, respectively. Interferences were removed with 2.0 mL of 4% formic acid followed by 2.0 mL of various organic solvents with water ranging from 0 to 100% organic solvent (Tables 1 and 2).

Table 1. EVOLUTE® EXPRESS CX SPE Extraction Parameters for Quantisal® Oral Fluids.

Step	Volume (mL)	Solvent(s)	Pressure (psi)	Time (min.)
Condition	1.0	MeOH	≤0.5	
Equilibration	1.0	4% FA	≤0.5	
Load	1.0	Sample	≤0.5	
Wash #1	2.0	4% FA	0.5	
Wash #2	2.0	S1-S13	≤0.5	
Dry			40	5.0
Elution	2.0	E1	Gravity	
Dry		Quick Pulse	40	2x

Table 2. Organic Wash Solvent #2 Physical constants and aqueous distribution.

Solvent ID	Solvent	% Aqueous [v/v]	BP† (°C)
S1	MeOH	50	N/A
S2	MeOH	0	64.7
S3	MeCN	50	N/A
S4	MeCN	0	76.1
S5	IPA	50	N/A
S6	IPA	0	80.3
S7	MTBE	5	N/A
S8	MTBE	0	55.2
S9	THF	50	65.0
S10	Acetone	0	56.1
S11	Acetone	50	N/A
S12	DMSO	50	189
S13	DMF	50	154-6
E1	DCM/IPA/NH_4OH	[78:20:2]	N/A

N/A: not applicable. †Denotes boiling point of the neat solvent.

Table 3. Sciex 4000QTRAP electrospray ionization source parameters for target analytes.

Ionization mode	Positive and Negative
Curtain (V)	30
CAD	Medium
IonSpray Voltage (kV)	+1.5
Source Temperature (°C)	600
GS1 (psi)	50
GS2 (psi)	70
sMRM window	45 seconds
Target Scan Time	2.581 seconds

After drying the sorbent for 5 min under 40 psi of nitrogen, analytes were eluted by gravity into 100 µL of 50 mM HCl in methanol with 2 mL of DCM/MeOH/NH_4OH [78:20:2]. The elution solvent evaporated under a stream of nitrogen at 2.0 L/min at 40°C using a New TurboVap® LV from Biotage (P/N 415000). Unless otherwise mentioned, all extracts were reconstituted with 100 µL of 10% methanol in 0.1% formic acid and immediately analyzed via LC/MS-MS.

Chromatography and Mass Spectrometry

HPLC and Mass Spectrometry Parameters for 85 Analytes

HPLC Parameters

Analytes were chromatographically separated on an Agilent 1260 Infinity HPLC (Agilent, Santa Clara, CA) using a 50 x 3.0, 2.7 µm Restek Raptor Biphenyl column (Restek, Bellefonte, PA) with a flow rate of 0.5 mL/min. Sample injection volume was 10 µL. Analytes were eluted over a 5 minute gradient elution using 0.1% formic acid in methanol from 10% to 90% at 40 °C.

Post Column Infusion (PIC) Parameters

All 85 analytes were infused at 20 ng/mL using a Harvard Appartus infusion pump at 20 mL/min, post-LC. Sample were prepped according to table 13 without standards using UTAK synthetic oral fluid. All samples were subsequently extracted accoriding to table 14 using 50% aqueous washes for MeOH, MeCN, IPA, Acetone and their respective neat solutions. Full scan data was collected from 150–2000 m/z using source parameters outlined in table 3.

MS Parameters

A Sciex 4000QTRAP triple quadrupole mass spectrometer (Sciex, Foster City, CA) equipped with a Turbo Ionspray® interface for mass analysis was used for direct injection/infusion and extracted oral fluid analyses, respectively. Experimentally determined transitions were acquired under scheduled Multiple Reaction Monitoring (sMRM) mode and their corresponding optic voltages and gas metrics were collected under ESI positive and negative ionization conditions. Samples consisted of synthetic opioids & opiates (26), benzodiazepines (13), stimulants (13), TCAs (6), anticonvulsants (4), antipsychotics (6), SSRIs (4), SNRIs (2), carbamates (2), z-drugs (2), anesthetics (2), cannabinoids (1), NDRI (1), SARI (1), and two plant alkaloids (Tables 3 and 4).

Table 4. Sciex 4000QTRAP sMRM transition parameters for target analytes.

Compound	Class	Parent (m/z)	Quant (m/z)	Qual (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)
7-Aminoclonazepam	Benzo	286.00	121.00	222.20	3.75	56	43/35	20/18
α-OH-alprazolam	Benzo	325.08	297.00	216.00	4.90	91	37/55	18/16
α-OH-midazolam	Benzo	342.06	324.00	203.00	4.50	96	31/39	24/16
Alprazolam	Benzo	309.15	281.10	205.00	5.09	91	37/59	22/14
Chlordiazepoxide	Benzo	300.12	283.10	227.00	4.24	81	19/31	24/18
Clonazepam	Benzo	316.05	270.00	214.10	4.80	81	35/51	22/16
Diazepam	Benzo	285.07	193.00	88.90	5.24	101	47/85	32/14
Lorazepam	Benzo	321.10	275.10	229.10	4.71	50	50/40	14/14
Midazolam	Benzo	326.13	291.10	249.10	4.35	116	39/51	22/20
Nordiazepam	Benzo	271.12	140.00	165.00	4.92	111	41/41	8/10
Oxazepam	Benzo	287.12	241.00	269.10	4.78	100	30/20	14/14
Amino-Flunitrazepam	Benzo	284.14	135.10	93.00	4.13	96	39/75	22/14
6-Acetylmorphine	Opiate	328.14	165.00	211.20	3.03	30	60/30	10/14
Buprenorphine	Opiate	468.26	55.00	83.40	4.23	151	87/67	8/14
Codeine	Opiate	300.21	165.10	115.10	2.99	111	51/95	12/4
Dihydrocodeine	Opiate	302.20	199.20	128.10	2.95	86	45/83	16/20
Fentanyl	Opiate	337.19	188.20	105.10	4.22	86	31/55	14/18
Hydromorphone	Opiate	286.15	185.00	157.10	2.44	121	41/57	30/26
Meperidine	Opiate	248.13	220.20	174.10	3.72	111	29/29	18/14
Methadone	Opiate	310.32	265.00	105.00	4.64	66	21/37	22/6
Temazepam	Benzo	301.20	255.10	177.10	5.05	150	50/60	12/12
EDDP	Opiate	278.22	234.10	249.20	4.46	56	43/33	18/14
N-Desmethyltapentadol	Opiate	208.16	107.10	121.10	3.42	100	50/20	14/12
Norcodeine	Opiate	286.15	152.20	165.20	2.68	101	83/53	10/12
Norketamine	Anesthetic	224.08	207.00	125.00	3.46	66	19/31	16/10
Naloxone	Opiate	328.12	310.10	235.20	2.89	71	27/37	16/14
Norbuprenorphine	Opiate	414.26	55.00	83.20	3.96	141	97/71	8/12
Norfentanyl	Opiate	233.13	84.10	55.00	3.49	71	27/53	6/8
Hydrocodone	Opiate	300.19	199.20	128.00	3.18	106	41/81	16/22
Oxycodone	Opiate	316.20	298.10	241.10	3.11	76	25/37	6/18
Oxymorphone	Opiate	302.22	284.10	226.90	2.19	66	27/37	14/18
Tapentadol	Opiate	222.15	106.90	121.00	3.51	86	33/31	18/8
Tramadol	Opiate	264.19	57.90	42.10	3.56	71	41/123	10/6
Propoxyphene	Opiate	340.20	58.10	266.20	4.35	51	43/13	8/22
Normeperidine	Opiate	234.16	160.10	188.10	3.71	71	23/20	14/10
Amphetamine	Stimulant	136.07	91.20	199.00	2.34	41	21/13	14/8
Benzoylecgonine	Stimulant	290.16	168.10	76.90	3.69	71	27/79	14/12
Cocaethylene	Stimulant	318.18	196.20	81.90	4.01	66	29/51	34/6
MDA	Stimulant	180.11	163.10	163.00	2.84	36	13/13	14/12
MDEA	Stimulant	208.16	163.10	105.10	3.33	61	19/35	14/18
MDMA	Stimulant	194.11	163.00	105.10	3.10	66	17/35	12/18
Phentermine	Stimulant	150.09	90.90	133.00	2.89	46	25/13	16/8
Methamphetamine	Stimulant	150.09	91.00	119.00	2.78	66	23/15	16/10
Methylphenidate	Stimulant	234.13	84.10	55.90	3.66	81	29/73	4/8
Phencyclidine	Stimulant	244.16	86.00	91.00	4.30	61	17/47	6/16
Cocaine	Stimulant	304.24	182.10	77.00	3.80	56	27/70	14/10
Ritalinic Acid	Stimulant	220.11	84.00	56.10	3.42	66	27/70	14/8
Carbamazepine	Anticonv.	237.15	179.10	165.00	4.60	26	47/61	12/12
Clozapine	Antipsyc.	327.00	270.10	296.10	3.95	100	34/36	10/12
Gabapentin	Anticonv.	172.14	154.00	137.10	2.47	61	19/23	26/8
Haloperidol	Antipsyc.	376.10	165.10	123.00	4.23	66	33/55	12/10
Lamotrigine	Anticonv.	257.99	43.00	213.00	3.38	91	71/37	6/16
Olanzapine	Antipsyc.	313.21	256.10	83.90	2.76	86	31/33	14/14
Pregabalin	Anticonv.	160.20	142.20	55.00	1.84	20	15/35	10/10
Quetiapine	Antipsyc.	384.11	253.10	221.00	4.32	96	31/49	20/16
Risperidone	Antipsyc.	411.40	191.00	109.90	4.20	81	41/71	14/4
Ziprasidone	Antipsyc.	413.08	194.10	130.00	4.37	81	39/95	16/10
Amitriptyline	TCA	278.18	91.00	117.00	4.50	81	33/33	14/8
Bupropion	NDRI	240.00	184.00	131.00	3.72	71	19/37	14/10
Carisoprodol	Carbamate	261.15	97.20	176.20	4.38	30	20/10	12/12

Compound	Class	Parent (m/z)	Quant (m/z)	Qual (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)
Cyclobenzaprine	TCA	276.19	216.00	215.00	4.46	81	33/55	16/16
Duloxetine	SNRI	298.14	153.90	44.10	4.45	41	9/29	26/6
Fluoxetine	SSRI	310.14	44.10	148.00	4.26	66	41/13	0/12
Imipramine	TCA	281.00	85.90	58.10	4.44	66	25/61	14/8
Ketamine	Anesthetic	238.15	125.00	207.20	3.63	66	39/21	8/16
Lurasidone	SSRI	493.23	166.10	67.00	4.94	146	61/109	8/10
Meprobamate	Carbamate	219.20	158.20	97.10	3.79	26	11/19	8/6
Nortriptyline	TCA	264.13	233.10	91.20	4.48	81	21/35	18/6
Paroxetine	SSRI	330.16	70.00	192.10	4.45	106	47/29	10/16
Cotinine	Alkaloid	177.12	80.00	98.00	1.34	81	37/29	12/6
Nicotine	Alkaloid	163.13	129.90	117.00	0.68	66	27/29	10/8
Dextromethorphan	Opiate	272.19	215.10	147.10	4.29	86	37/43	16/24
Δ9-THC	Cannabinoid	315.22	193.10	123.20	5.65	40	30/43	10/10
Sertraline	SSRI	305.90	274.90	159.10	4.63	31	17/33	10/10
Zolpidem	Z-drug	308.17	235.10	263.00	4.09	96	49/37	10/10
Trazodone	SARI	372.15	176.10	148.20	4.20	96	35/49	14/8
Normorphine	Opiate	272.21	165.30	76.90	0.86	101	55/93	12/12
Noroxycodone	Opiate	302.20	284.10	187.20	2.93	91	23/33	16/16
O-desmethyl-cis-tramadol	Opiate	250.18	58.00	42.00	2.95	46	37/109	8/6
MDPV	Stimulant	276.19	126.20	135.10	3.86	86	39/37	8/10
Mirtazapine	TCA	266.22	195.10	72.00	3.89	76	37/37	16/12
N-Desmethylcyclobenzaprine	TCA	262.19	231.10	216.10	4.42	81	23/31	12/14
Zaleplon	Z-drug	306.16	264.10	236.20	4.96	101	31/39	20/14
Norhydrocodone	Opiate	286.17	199.00	128.10	2.99	101	37/75	16/8
Venlafaxine	SNRI	278.21	58.00	260.20	3.87	71	49/17	8/22

Results and Discussion

Water as Surrogate: Matrix Effects

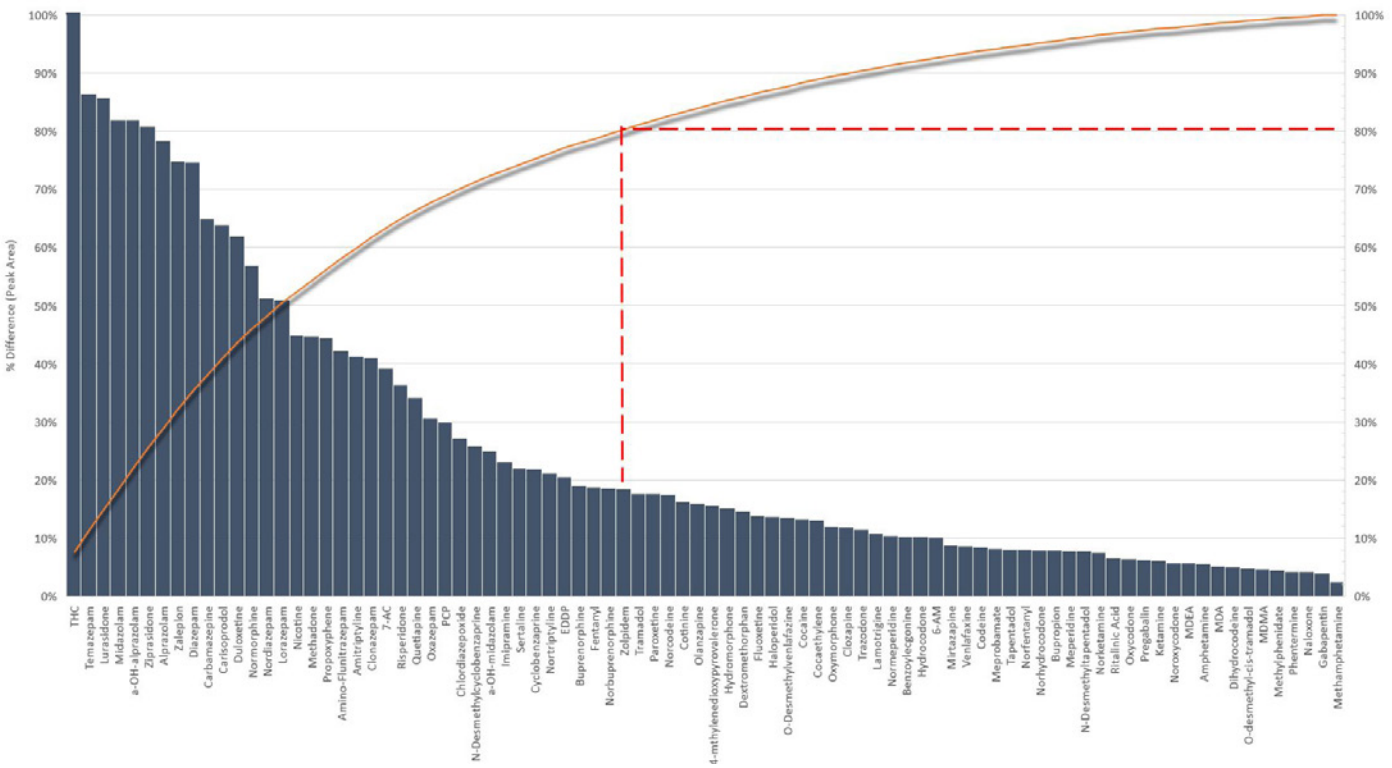


Figure 1. Frequency distribution chart highlighting the cumulative effect of wash solvents S1-S13 upon the peak area of each DOA analyzed using water as a surrogate oral fluid by EVOLUTE® EXPRESS CX mixed-mode cation.

In order to determine the relationship between the Quantisal® buffer and the various classes of drugs in table 4, we used water as an oral fluid surrogate and monitored peak area response using the protocol outlined in table 1, with E1 as the elution system. Analytes (100 ng/mL) were spiked into a pooled solution of Quantisal buffer and water at 3:1. The percent difference between maximum and the minimum peak area (n = 3) produced among all thirteen wash solvents was calculated for each individual analyte and used to evaluate analyte response against wash solvent effects using Quantisal buffer (figure 1). Here, the frequency distribution chart highlights that analytes are influenced the greatest by the thirteen differing wash solvents. Each individual analyte is represented in descending order (right to left) according to frequency where the wash solvent impacts peak area, e.g. Δ9-THC returned peak areas that yielded the greatest percent difference among the all wash solvents, whereas methamphetamine remained less sensitive to all thirteen wash solvents, producing little variation in peak area.

The right vertical axis, with corresponding orange line, illustrates the cumulative relative frequency of the total number of analytes measured. The algorithm used to generate this concave representation reveals the threshold where analytes begin to respond to the thirteen wash solvents as measured by percent difference. The general assumption that analytes with peak areas greater than or equal to 20% difference are likely more responsive to the individual characteristics of certain wash solvents (figure 1). Hence, the vast majority of the DOAs used in this experiment are insensitive to the individual physical characteristics of the organic wash solvents examined by mixed-mode cation exchange SPE.

Benzodiazepines

All benzodiazepines demonstrated variable differences among all wash solvents when analyzing the matrix effects of the Quantisal buffer. As a class, the core of benzodiazepine consist of a diazepine heterocyclic ring system with two nitrogen atoms along with a fused benzene ring, however, with different functional moieties and side chains, they can possess enough intrinsic dissimilarity to yield disparate matrix effects among the wash solvents (figure 2a). Figure 2a shows both the upper and lower boundaries of generally acceptable matrix effects of (+/-) 15%. The box-and-whisker plots show the mean, upper/lower bounds, and quartiles for the matrix effects of all benzodiazepines for each wash solvent (figure 2a). Within this plot, the solvents remaining within the acceptable margins consist of 50% acetonitrile (MeCN), 50% isopropyl alcohol (IPA), 50% acetone, 50% tetrahydrofuran (THF), and perhaps 100% IPA and methanol (MeOH). Thus, these solvents appear to offer excellent (low) to marginal matrix effects when solely considering the interference of the Quantisal buffer. The composition of the benzodiazepines analyzed in this experiment were the following:

diazepam, nordiazepam, clonazepam, 7-aminoclonazepam, alprazolam, α-hydroxyalprazolam, midazolam, α-hydroxymidazolam, lorazepam, oxazepam, temazepam, amino-flunitrazepam, and chlordiazepoxide.

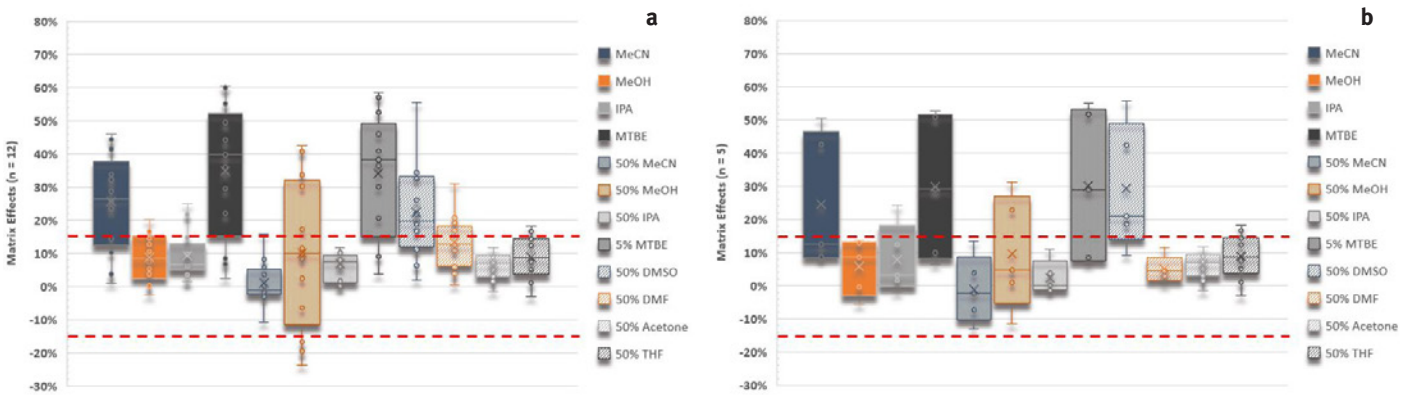


Figure 2. Box and Whisker plots of matrix effects for representative (a) benzodiazepines and (b) antipsychotics. All analytes were extracted using water as a surrogate oral fluid and Quantisal® buffer by EVOLUTE® EXPRESS CX mixed-mode cation exchange SPE.

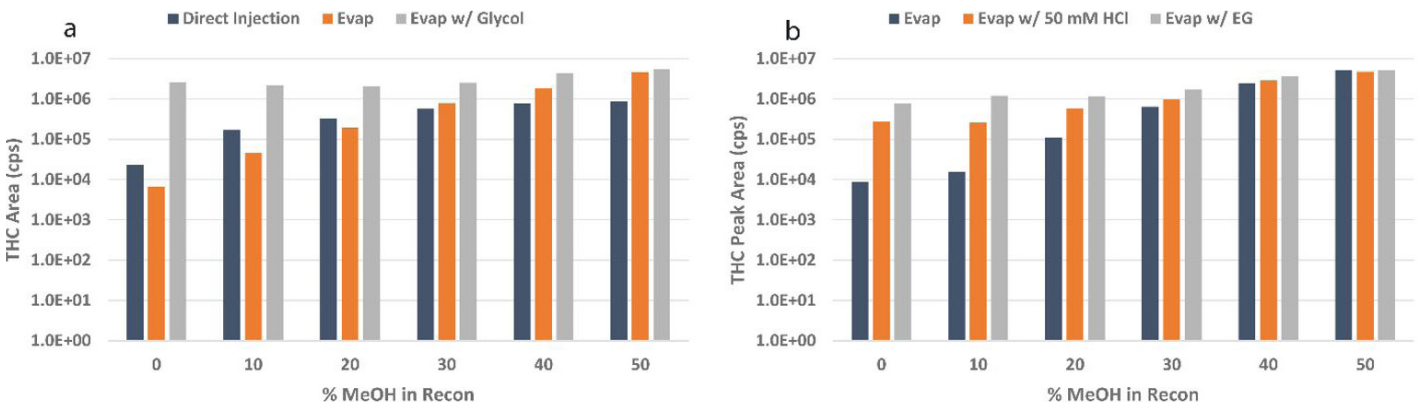


Figure 3. Effect of percent methanol in Mobile Phase B upon the reconstitution of 100 ng/mL of Δ9-THC to (a) the direct injection and evaporation, with and without, ethylene glycol (EG) and (b) the same concentration of all 85 analytes combined using both 50 mM methanolic HCl and EG evaporation additives.

Antipsychotics

The antipsychotics *carbamazepine*, *lurasidone*, *quetiapine*, *risperidone*, and *ziprasidone* displayed similar trends as the benzodiazepines, as the core structure of the latter four analytes possess matching internal piperazine (pK_a 7.2) or piperidine (pK_a 11.2) heterocycles along with either a benzoisothiazole (pK_a 2.0) or benzoisoxazole (pK_a -0.51) upon their termini. Neither solution of methyl tert-butyl ether (MTBE) prevented these antipsychotics from displaying strong signal enhancement (figure 2b). Washes with MeOH (neat), 50% MeCN, 50% IPA, and 50% acetone fell within acceptable thresholds with 50% dimethylformamide (DMF) demonstrating its capacity to reduce matrix effects for antipsychotics (figure 2b). It should be noted, both DMF and dimethyl sulfoxide (DMSO) wash solutions required an extra wash step and elution. This was necessary to prevent potential elution of either wash solvent as their boiling points are 153–4 °C and 189 °C, respectively, and will not evaporate without a high vacuum system. Therefore, they were discontinued following the surrogate analysis.

A point of interest regarding the Quantisal buffer: the only wash solutions that did not remove the blue dye during the organic solvent wash were those with MTBE along with neat MeCN, acetone, and IPA. Although methadone and propoxyphene yielded divergent matrix effects for MTBE washes, both possessed the blue dye in their reconstitution solutions, whereas the remaining wash solvents were clear on collection, as were their reconstitution volumes. This finding indicates that the dye's ability to contribute to matrix suppression might be likely. Moreover, the addition of other additives within the buffers chemical landscape, or other analytes, could also play a stronger role in signal suppression or enhancement.

Cannabinoids and Alkaloids

Nicotine's matrix effects were lowest when washing with either 50% acetone (4%), THF (5%), MTBE [neat] (5%), or 50% MeCN (6%). Conversely, both IPA neat and 50% IPA yielded ›25% matrix effects. Both DMSO and DMF yielded matrix effects at 17% and 18%, respectively. Interestingly, cotinine, a major metabolite of nicotine, was resistant to all wash solvents, yielding a modest 16% difference between the maximum and minimum peak area compared to nicotine's 45%. The cannabinoid trans-Δ9-tetrahydrocannabinol (Δ9-THC) responded well to 50% MeCN, 50% MeOH, and acetone washes (data not shown). Neat wash solutions of MeOH and IPA yielded approximately 50–150% less peak area response (data not shown).

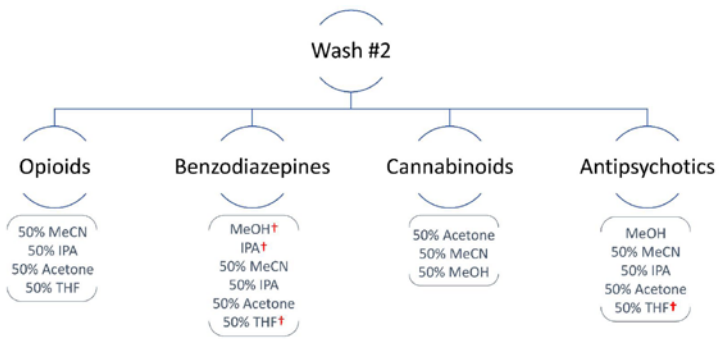


Figure 4. Recommended wash solvent systems that maintain matrix effects within the industry standard of ± 15% for DOA classes that remain responsive to variations in solvent wash polarity.

† Denotes caution be taken when applying this solvent as some analytes. produced matrix effects at the border of acceptable limits (±15%).

Failure to maintain Δ9-THC retention under high organic wash systems is consistent with its lack of ionizable groups capable of complexing with the negative charge of the sulfonate moiety on the EVOLUTE® EXPRESS CX sorbent. This also, however, presents an issue with its reconstitution as indicated by low peak area for under high aqueous conditions (figure 3). Under the current 10% mobile phase B (MPB) reconstitution conditions Δ9-THC showed peak areas five times lower than those extracted under optimum conditions. Demonstrably, variation of reconstitution additives versus final organic solvent content showed the relative impact of each upon Δ9-THC's peak area response when extracted as a neat sample (figure 3a).

To realize the effects of solvent polarity and, perhaps, the benefits of evaporation additives upon Δ9-THC, 100 ng/mL of the cannabinoid was directly injected and compared to the evaporation of elution solvent E1 both with and without 2 µL of ethylene glycol (EG) across varying concentrations of MPB (figure 3a). It can quickly be determined that EG possesses the intrinsic ability to maintain large peak areas, whereas both direct injection and evaporation samples appear to only benefit from increasing the organic nature of the reconstitution volume, sometimes by 1.5 orders of magnitude.

To determine if any of the accompanying 85 analytes had an effect upon Δ9-THC reconstitution, the same analysis was performed, however, 50 mM methanolic HCl was also analyzed (figure 3b). Again, EG proved to be stable and showed the best results, improving as the amount of organic solvent increases above 20%. Evaporation without any additives showed dramatic improvement as the MeOH percentage increased above 20% (figure 4b). In this case, we also monitored analyte peak shape for any distortion that might occur from an injection volume mismatch. We found at 40–50% MeOH, *hydromorphone, oxymorphone,* and *normorphine* began to tail excessively with both *pregabalin* and *gabapentin* exhibiting peak broadening and *amphetamine* experiencing splitting (data not shown). However, ethylene glycol did not have any observable effect.

Water as Surrogate: Matrix Effects Summary

Using water as a surrogate allowed for the direct examination of potential matrix effects produced by Quantisal buffer when considering an analyte panel. Special attention is required for the aforementioned drug classes and their respective analytes. Figure 4 illustrates which organic wash systems are appropriate for specific classes of drugs when using Quantisal for the detection of analytes in oral fluids. The figure provides an investigator with a method development roadmap, where they can apply the proper organic wash solvent(s) that will potentially minimize or remove matrix effects from their panel of analytes, thus tailoring the SPE method based on their analyte panel. Fr equency distribution analysis of the water surrogate SPE extraction demonstrated that 44% of the 85-member panel responded best when applying the aqueous based organic wash systems for MeOH, MeCN, IPA, and Acetone along with their corresponding neat solvents. The remaining 54% of the analytes were indifferent to all wash systems.

Detergent Analysis: Matrix Effects Via Post-Column Infusion

As demonstrated by the surrogate analysis, benzodiazepines, antipsychotics, cannabinoids, and plant alkaloids appeared to show more sensitivity with both aqueous and neat solvent systems: acetone (S10, S11), acetonitrile (S3, S4), IPA (S5, S6), and methanol (S1, S2). As these solvent wash systems generally provided reduced matrix effects for all drug classes, their direct affects upon the detector signal was monitored by post column infusion (PCI) analysis. Here, we evaluated all analyte classes under same LC/MS and extraction conditions (Tables 1, 3, and 4), using a 20 ng/mL methanol solution of all analytes (Table 4). Figure 5a shows the both the total ion chromatogram (TIC) for a mobile phase blank (orange trace, figure 5a) and the TIC from the injection of the Quantisal buffer (blue trace, figure 5a). Notably, the Quantisal signal produced an increase in signal intensity within the first minute of elution and rapidly increased between 3 and 5.5 minutes when compared to the blank injection. Examining the mass spectra within this retention time showed the presence of an unknown polyglycol system with C_2H_4O fragments increasing in mass by +44 amu from 350 to 1350 m/z (figure 5b, blue spectra).

Using PCI, additional investigative efforts focused on the employment of each solvent system at both 0% and 50% aqueous solutions. Using 50% aqueous wash conditions, the PCI experiment exhibited a significant decrease in signal for all four-wash systems in the first minute of elution and within the expected polyglycol window of elution (figure 6a).

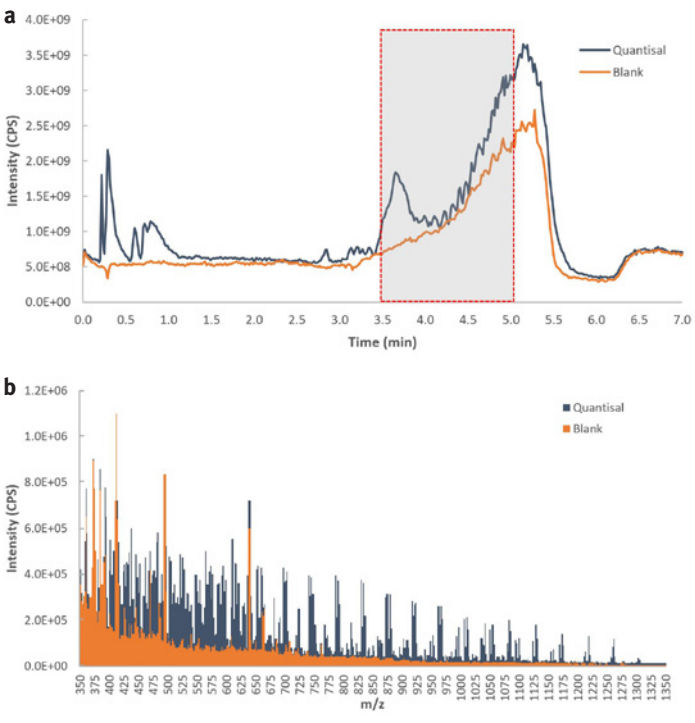


Figure 5. Representative TIC (a) and mass spectra (b) for PCI data of Quantisal and mobile phase blank full scan analysis from 150–2000 m/z under tables 3 & 4 LC/MS conditions. PCI flow rate was set at 20 µL/min.

The mass spectral data in figure 6b superimposes the 50% MeOH (S1) trace over 50% MeCN (S3), 50% acetone (S10), and 50% IPA (S5), and are therefore considered qualitatively similar. When compared to the Quantisal trace, this data further supports the absence of any polyglycol oligomers and the use of these four 50% aqueous wash systems (figure 6). Conversely, evaluation of neat organic washes for the same solvents showed variable results. When compared to Quantisal, MeCN (S4), acetone (S11), and IPA (S6) neat washes showed similar responses within the polyglycol window of elution and the absence of peaks at 1.0 and 3.7 minutes (figure 6). Inspection of the mass spectra within the polyglycol window for these wash systems shows the presence of polyglycol oligomers, and strongly suggests avoiding the use of these neat wash solvents for the removal of surfactants (figure 6). Interestingly, the use of neat MeOH (S2) demonstrated the opposite effect and a decrease in signal intensity within the polygylcol elution window was realized within both the TIC and mass spectra (figure 6, orange TIC and figure 6, blue spectra).

Synthetic Oral Fluid

Based on cumulative data from the surrogate analysis and PCI data, all subsequent analyses focused upon the 50% aqueous wash systems for methanol, acetonitrile, acetone and IPA. Application of these wash systems were examined at analyte concentrations more readily accepted by SAMHSA (Substance Abuse and Mental Health Services Administration) cutoff's (HYPERLINK "https://www.federalregister.gov/documents/2015/05/15/2015-11523/mandatory-guidelines-for-federal-workplace-drug-testing-programs" \l "h-8" 80 FR 28053, 5/15/15) for analyses of oral fluids. Extraction took place under the same parameters as table 1 using 50% aqueous MeOH, MeCN, IPA and Acetone. A 1:3 ratio of UTAK synthetic oral fluid (100 µL oral fluid) to Quantisal buffer (200 µL) was diluted in 100 µL of 4% formic acid and spiked with 100 mL of 20 ng/mL analyte standards in table 4. As can be seen in table 5, variability for each benzodiazepine exists among individual solvent wash systems for both recovery and matrix effects. General recoveries for benzodiazepines were best with 50% MeOH with the remaining solutions faring moderate to excellent. However, the matrix effects were generally poor, lying outside the acceptable ±15% range.

Similarly, the variable response of the antipsychotics, SNRIs, Z-drugs, and alkaloids demonstrated their intrinsic molecular properties were likely more influential than the wash conditions applied (Table 6 and 7). Zaleplon has a pK_a of < 1, making it more susceptible to changes in polarity as it has no other ionizable groups capable of complexing with the CX sorbent. As a result, zaleplon shows poor recovery with the aprotic solvents, whereas zolpidem retains its positive charge and is complexed to the sorbent, thus producing enhanced recoveries.

With exception to the carbamates, the remaining analytes, shown in table 8, averaged excellent recoveries and marginal matrix effects. Loss of the carbamates was not unanticipated since their main mode of interaction relies solely on their reverse phase characteristics. Hence, the application of moderate to high levels of organic wash will disrupt the intermolecular interaction of these class of drugs, ultimately releasing them into the wash.

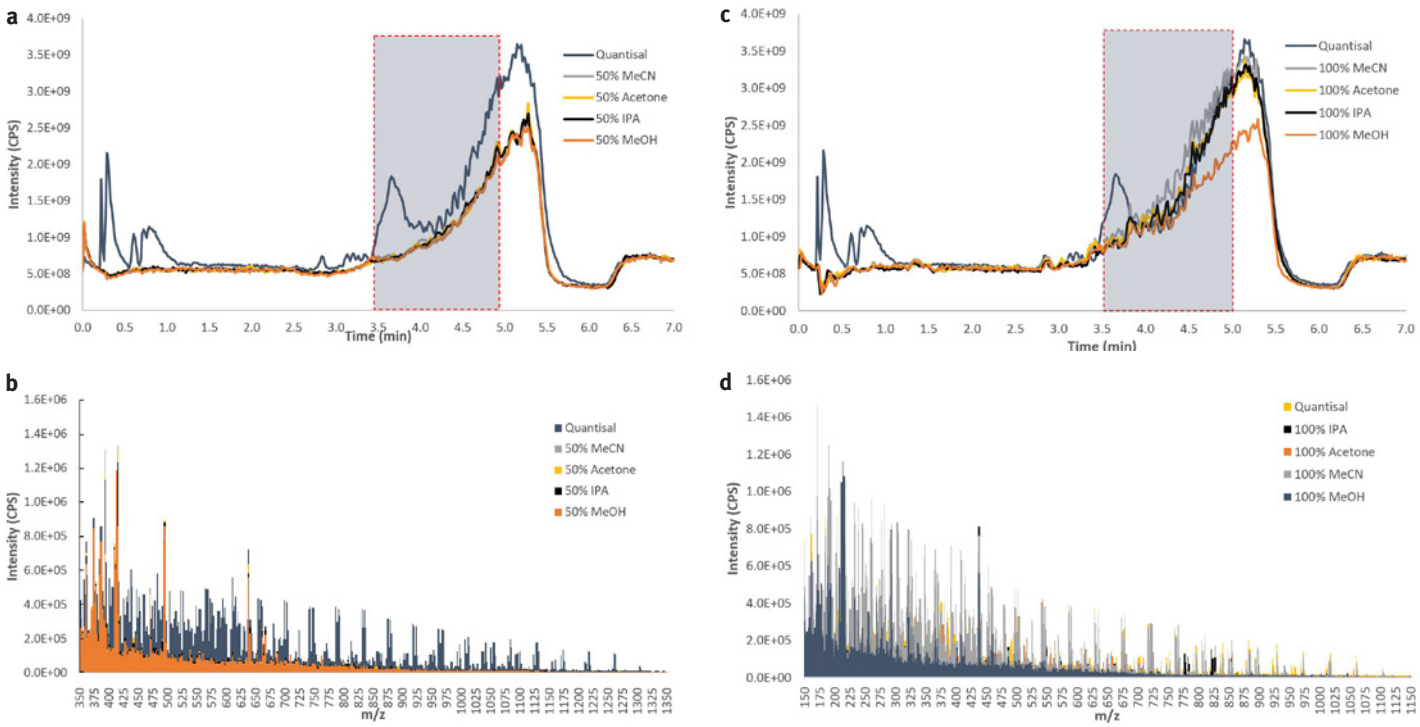


Figure 6. Representative TICs (a) and mass spectra (b) for PCI data of Quantisal extraction using 50% MeOH, 50% MeCN, 50% acetone, and 50% IPA (aq). Evaluation of TIC's (d) and mass spectra (d) for neat MeOH, MeCN, acetone, and IPA wash systems. Full scan analysis from 150–2000 m/z under tables 3 and 4 LC/MS conditions. PCI flow rate was set at 20 µL/min.

Synthetic Oral Fluid: Summary

On average, the 50% MeOH and IPA wash yielded the best recoveries, and to a lesser extent, the matrix effect. However, the matrix effects were still inconsistent across all analytes. This is likely to be an artifact from the synergistic effect of lower analyte signal produced at 20 ng/mL and the concomitant effect of residual matrix detritus. Notably, the matrix effects for the same analytes improves with increased analyte signal from 100 ng/mL extractions of 250 µL of synthetic oral fluid under the same conditions (data not shown). As no sustained trend among the DOA analytes and solvent polarity (aprotic vs protic) was derived, it is likely that each analytes response is directly correlated to combination of their direct intermolecular interaction with the EVOLUTE® EXPRESS sorbent and the individual wash solvents.

Table 5.
Percent recovery and matrix effects of benzodiazepines extracted from Quantisal® buffer using UTAK synthetic oral fluid. All extractions were prepped using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol (n = 3).

	Analyte(s)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
Benzodiazephines	Diazepam	115%	69%	86%	104%	-13%	-23%	-20%	-18%
	Nordiazepam	99%	98%	115%	98%	-17%	-21%	-21%	-31%
	Clonazepam	123%	44%	37%	59%	-41%	-66%	-50%	-30%
	7-Aminoclonazepam	80%	62%	84%	97%	-42%	-53%	-42%	-5%
	Alprazolam	91%	87%	89%	93%	17%	24%	17%	7%
	α-Hydroxyalprazolam	90%	101%	92%	90%	-75%	-39%	-55%	-62%
	Midazolam	69%	105%	96%	79%	-57%	-33%	-50%	-69%
	α-Hydroxymidazolam	72%	104%	96%	85%	-61%	-39%	-48%	-64%
	Lorazepam	129%	16%	18%	41%	-4%	-37%	-7%	8%
	Oxazepam	149%	87%	62%	48%	1%	-14%	1%	-7%
	Temazepam	93%	82%	41%	42%	-28%	-13%	-21%	-31%
	Aminoflunitrazepam	100%	74%	94%	114%	23%	2%	15%	39%
	Chlordiazepoxide	84%	129%	117%	101%	12%	22%	24%	13%

Table 6.
Percent recovery and matrix effects of antipsychotics extracted from Quantisal buffer using UTAK synthetic oral fluid. All extractions were prepped using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol (n = 3).

	Analyte(s)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
Antipychotics	Clozapine	81%	151%	118%	77%	24%	31%	24%	12%
	Haloperidol	63%	131%	111%	86%	-29%	-15%	-27%	-38%
	Olanzapine	101%	84%	91%	76%	47%	18%	15%	44%
	Quetiapine	139%	83%	113%	111%	6%	-2%	13%	-5%
	Risperidone	68%	107%	115%	101%	-86%	-78%	-72%	-77%
	Ziprasidone	129%	65%	107%	122%	1%	-23%	4%	15%

Quantisal Device

Data from the synthetic oral fluid provided, on average, excellent recoveries, however, most of the matrix effects were outside acceptable levels regardless of the solvent choice. While this was generally attributed to the low levels of analyte versus the impact of matrix residue, the comparison of synthetic oral fluid to patient was explored using the same solvent systems. Therefore, extraction of patient oral fluids using the Quantisal® device was performed. Patients were instructed on the use of the device per manufacturer's instructions and were declared as negative controls to allow for the direct analysis of all panel analytes in table 4. From the device, 300 µL was removed (100 µL of oral fluid) and combined with 100 µL of 4% formic acid and 100 µL of 20 ng/mL standards. The total volume of 0.5 mL was loaded and extracted under the same parameters as synthetic oral fluids.

Table 7.
Percent recovery and matrix effects of SNRI's, Z-drugs, and plant alkaloids extracted from Quantisal ®buffer using UTAK synthetic oral fluid. All extractions were prepped using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol (n = 3).

	Analyte(s)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
SNRI	Duloxetine	47%	116%	103%	68%	27%	54%	42%	38%
	Venlafaxine	122%	95%	98%	102%	2%	-4%	-2%	-6%
Z-Drugs	Zaleplon	83%	1%	3%	34%	-46%	-45%	-51%	-62%
	Zolpidem	72%	181%	113%	74%	-3%	20%	-10%	-36%
Alkaloids	Cotinine	87%	93%	121%	107%	4%	11%	23%	7%
	Nicotine	86%	88%	125%	107%	65%	82%	90%	87%

Table 8.
Average percent recovery and matrix effects for remaining drug classes extracted from Quantisal buffer using UTAK synthetic oral fluid. Value within parentheses indicates number of analytes in drug class and therefore each value is an average recovery or matrix effect (n=3). All extractions were prepped using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol. N/A = not applicable.

	Drug Class (# of analytes)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
Miscellaneous Drug Classes	TCA's (6)	77%	122%	99%	79%	-63%	-43%	-68%	-84%
	Stimulants (13)	95%	99%	102%	101%	-33%	-27%	-26%	-31%
	Anticonvulsants (4)	86%	76%	79%	79%	-31%	-20%	-13%	79%
	SSRI (4)	136%	101%	112%	109%	-33%	-17%	-31%	-47%
	SARI/NDRI (2)	110%	111%	119%	101%	42%	47%	45%	38%
	Cannabinoid (1)	96%	71%	66%	73%	N/A	N/A	N/A	N/A
	Anesthetics (2)	85%	108%	109%	102%	-9%	9%	7%	16%
	Syn Opioids and Opiates (26)	92%	98%	105%	105%	-46%	-36%	-32%	-35%
	Carbamates (2)	16%	1%	1%	2%	-24%	7%	5%	-17%

Table 9.
Percent recovery and matrix effects of benzodiazepines extracted from Quantisal device using patient submitted oral fluids. All extracts were prepared using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol (n = 3).

	Analyte(s)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
Benzodiazepines	Diazepam	90%	81%	76%	92%	11%	4%	-16%	5%
	Nordiazepam	104%	105%	98%	103%	0%	-7%	-12%	-20%
	Clonazepam	86%	65%	41%	50%	-27%	-34%	-50%	-14%
	7-Aminoclonazepam	86%	83%	82%	98%	-70%	-114%	-111%	-73%
	Alprazolam	111%	104%	99%	98%	18%	21%	6%	8%
	α-Hydroxyalprazolam	98%	92%	92%	97%	-40%	-25%	-30%	-34%
	Midazolam	101%	104%	107%	95%	-27%	-28%	-15%	-55%
	α-Hydryoxymidazolam	102%	101%	111%	93%	-8%	-8%	6%	-22%
	Lorazepam	78%	18%	16%	30%	-34%	-43%	-55%	-25%
	Oxazepam	95%	72%	50%	50%	-41%	-57%	-63%	-24%
	Temazepam	98%	77%	46%	47%	-22%	-33%	-55%	-24%
	Aminoflunitrazepam	99%	96%	92%	92%	3%	-14%	-10%	-5%
	Chlordiazepoxide	115%	121%	116%	111%	-11%	-6%	-4%	-30%

Here, we see that patient extracted oral fluids yield excellent recoveries for all benzodiazepine analytes under both protic wash solvents, 50% MeOH (S1) and 50% IPA (S5), (Table 9). Again, however, while the recoveries hold within acceptable limits, the matrix effects are irregular both within a wash solvent category and among individual analytes. Similar trends follow for the antipsychotics (table 10) and more modestly for the alkaloids, Z-drugs, and SNRI, and remaining analytes (table 11 and 12).

Table 10.
Percent recovery and matrix effects of antipsychotics extracted from Quantisal device using patient submitted oral fluids. All extracts were prepared using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol (n = 3).

	Analyte(s)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
Antipsychotics	Clozapine	123%	126%	118%	119%	41%	31%	33%	24%
	Haloperidol	124%	128%	129%	107%	27%	21%	32%	-5%
	Olanzapine	144%	124%	108%	105%	41%	24%	27%	16%
	Quetiapine	97%	103%	90%	114%	3%	-11%	-22%	5%
	Risperidone	124%	125%	118%	117%	-24%	-44%	-35%	-70%
	Ziprasidone	115%	120%	106%	135%	5%	-8%	-15%	7%

Table 11.
Percent recovery and matrix effects of SNRIs, Z-drugs, and plant alkaloids extracted from Quantisal device using patient submitted oral fluids. All extracts were prepared using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol (n = 3).

	Analyte(s)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
SNRI	Duloxetine	123%	111%	115%	80%	37%	35%	48%	12%
	Venlafaxine	120%	108%	123%	95%	1%	-19%	1%	-21%
Z-Drugs	Zaleplon	98%	1%	4%	35%	4%	-17%	-21%	-15%
	Zolpidem	107%	107%	129%	92%	0%	-6%	12%	-35%
Alkaloids	Cotinine	101%	103%	103%	111%	-31%	-14%	-10%	-11%
	Nicotine	102%	87%	103%	99%	39%	69%	79%	59%

Table 12.
Average percent recovery and matrix effects for remaining drug classes extracted from Quantisal device using patient submitted oral fluids. Value within parentheses indicates number of analytes in drug class and therefore each value is an average recovery or matrix effect (n=3). All extractions were prepped using EVOLUTE® EXPRESS CX 60 mg cartridges and washed under four different conditions: 50% aqueous acetone, acetonitrile, IPA, and methanol. N/A = not applicable.

	Drug Class (# of analytes)	% Recovery				% Matrix Effects			
		50% MeOH	50% MeCN	50% Acetone	50% IPA	50% MeOH	50% MeCN	50% Acetone	50% IPA
Miscellaneous Drug Classes	TCA's (6)	111%	113%	122%	100%	2%	-11%	3%	-39%
	Stimulants (13)	101%	100%	101%	105%	-22%	-31%	-28%	-21%
	Anticonvulsants (4)	88%	75%	76%	85%	-39%	-45%	-43%	85%
	SSRI (4)	104%	117%	103%	117%	5%	-2%	-6%	-15%
	SARI/NDRI (2)	109%	97%	112%	110%	36%	33%	42%	38%
	Cannabinoid (1)	107%	64%	61%	66%	N/A	N/A	N/A	N/A
	Anesthetics (2)	98%	100%	102%	114%	-11%	-16%	-18%	8%
	Syn Opioids & Opiates (26)	98%	98%	101%	104%	-29%	-37%	-35%	-23%
	Carbamates (2)	18%	1%	1%	4%	-81%	-72%	-67%	-44%

Quantisal Device Summary

As with the synthetic oral fluid analysis, on average, the 50% MeOH and IPA wash yielded the best recoveries with the majority of matrix effect remaining inconstant with acceptable levels. Both carbamates were predictably absent and Δ9-THC mirroring recovery results from the synthetic analysis using the 50% aqueous protic wash solvent, methanol and a slight decrease (~10%) among all other wash systems. Matrix effects were not recorded due to lack of appropriate additives that secure the solvation of Δ9-THC upon reconstitution. Zaleplon also behaved similarly with low recoveries for both aprotic wash systems and ›90% for aprotic aqueous based MeOH and ›35% for aqueous based IPA. Overall, the 50% MeOH wash system provided superior global recoveries for the analytes, which is in good agreement with extraction metrics produced from synthetic oral fluid.

Summary

The frequency distribution analysis using water surrogate for oral fluid narrowed the organic solvent wash landscape using varying degrees of solvent polarity. This allowed for the examination of the direct relationship between the Quantisal buffer, all analytes, and the mixed-mode EVOULTE® Express CX sorbent. Although most analytes were well suited for this sorbent, approximately 45% showed variability. Among the thirteen different combinations of solvents, the entirety of the 85 analytes panel routinely responded to four wash systems (55%), two polar protic (50% MeOH and 50% IPA), two polar aprotic (50% acetone and 50% MeCN) and neat methanol. The four aqueous based solvent wash systems were examined for their ability to provide detergent free extracts, yeilding sound recoveries (85–115%).

Detergent Considerations

Infusion studies overwhelmingly demonstrated washing with any of the four aqueous based wash solvents yields mass spectra devoid of any detergent or polyglycol signal. Moreover, washing with neat MeOH also produced clean mass spectra, whereas washing with neat MeCN, acetone, or IPA had little effect on the reduction of polyglycol signal. The lack of solvation between the detergent and neat organic solvents is evidence of poor solvation effects between the sorbent bed, glycopolymer, and solvent. Furthermore, when examining the chromatography during LC/MS analyses, the suspect polyglycol does not begin to elute until he organic content of mobile phase B breaches the 50% threshold around 3.5 minutes.
Hence, it would be plausible that marginal increases in the organic portion of wash step #2 might have a positive impact in matrix effects where detergents are concerned. As such, a variety of aqueous based wash systems can be used to prevent or suppress detergent signals, ensuring analyte detection toward the lower limits of detection.

Synthetic Oral Fluid and Patient Oral Fluid from Quantisal Device

Experiments using 100 µL (20 ng/mL DOA) of oral fluid, demonstrated analogous recoveries when comparing results from patient (Quantisal device) oral fluid and UTAK synthetic oral fluid among any of the aqueous based wash solvents. While the use of 50% MeOH generally yielded superior recoveries for both studies, however, matrix effects suffered regardless of the aqueous based or neat wash system employed. Conversely, SPE analysis with all four wash systems using synthetic oral fluid at 100 ng/mL (250 µL oral fluid) produced both recovery and matrix effects within acceptable tolerances (data not shown). The discrepancy between matrix effects of the 100 mL and 250 mL synthetic oral fluid experiments along with the PCI data diminishes the theory that detergents act as the main constituent of signal suppression, and therefore, matrix effects in oral fluid buffers. This is further realized when 50% MeOH is employed at wash step #2 and generally results in superior recoveries, but poor matrix effects when using 100 mL of synthetic/patient oral fluid and 200 mL Quantisal buffer. However, this assumes the collection device presents the appropriate ratio of oral fluid to Quantisal buffer. A discrepancy in true oral fluid volume collected and/or inconsistent pre-loaded buffer volumes within the device will contribute to arbitrary recoveries and matrix effects.[11] Moreover, since drug collection is dependent upon dilution, either of the aforementioned discrepancies could affect analyte signal, directly influencing analyte-droplet formation within the source due to interfering species that have co-eluted[12].
Hence, high concentrations of interferent(s) relative to analyte(s) would potentiate the surface tension and viscosity of droplets formed via the ESI process diluting analyte signal.[13] However, this phenomenon could be mitigated using deuterated internal standards.[14] Although this should be determined empirically and is not considered a permanent solution to the matrix effects of all analyte and biological matrix combinations.

Although at face value the SPE results did not temper the removal of all matrix components, one can modulate the level of aqueous methanol (or other solvents) to achieve a level of sample cleanliness capable of removing both detergents and interferent. As such, the tables 13 and 14 present the best approach when using either synthetic oral fluid from UTAK or Immunoanalysis' oral fluid device.

Table 13. Sample preparation parameters for both UTAK synthetic oral fluids and Quantisal® Device.

UTAK Synthetic Oral Fluid		Quantisal® Oral Fluid Device	
Solvent ID	**Volume (µL)**	**Sample ID**	**Volume (µL)**
Synthetic Oral Fluid	100	Quantisal Device	300
Quantisal Buffer	200	N/A	
Standard(s)	Up to 100	Standard(s)	Up to 100
4% Formic Acid (aq)	100	4% Formic Acid (aq)	100
Total Volume Loaded	**500**	**Total Volume Loaded**	**500**

Table 14. 60 mg EVOLUTE® Express CX SPE Sample Processing Parameters for oral fluids.

Step	Volume (mL)	Solvent(s)	Pressure (psi)	Time (min.)
Condition	1.0	MeOH	≤0.5	
Equilibration	1.0	4% Formic Acid (aq)	≤0.5	
Load	0.5	Sample [1]	≤0.5	
Wash #1	2.0	4% Formic Acid (aq)	0.5	
Wash #2	2.0	50% MeOH [2,3]	≤0.5	
Dry #1			40	5.0
Elution	2.0	DCM/MeOH/NH_4OH (4,5) [78:20:2]	Gravity	
Dry #2			40	1-2x
		Quick Pulse [6]		

1. Load up to 1.0 mL of total sample including buffer, standards(s) and 4% formic acid when using 60 mg EVOLUTE® Express CX.
2. To remove residual matrices at low analyte concentrations, e.g. <20 ng/mL, consider increasing organic constituent in 5% increments and/or split the 2.0 mL wash into two, 1 mL aliquots and monitor recovery, matrix effects, and polyglycol removal.
3. Fractionation of wash step #2 using two different 50% organic washes can also be applied, e.g. wash with 1 mL of 50% MeOH followed by 1 mL of 50% IPA (analyte(s) dependent).
4. To improve upon recoveries, consider splitting the 2.0 mL wash into two, 1 mL aliquots and monitor recovery.
5. Consider replacing DCM with MeCN, however, do not go below 20% MeOH otherwise neither pregabalin nor gabapentin will elute.
6. Place a quick pulse of nitrogen to remove any residual solvent in the luer tips. Conversely, consider a 2–3 minute dry step.

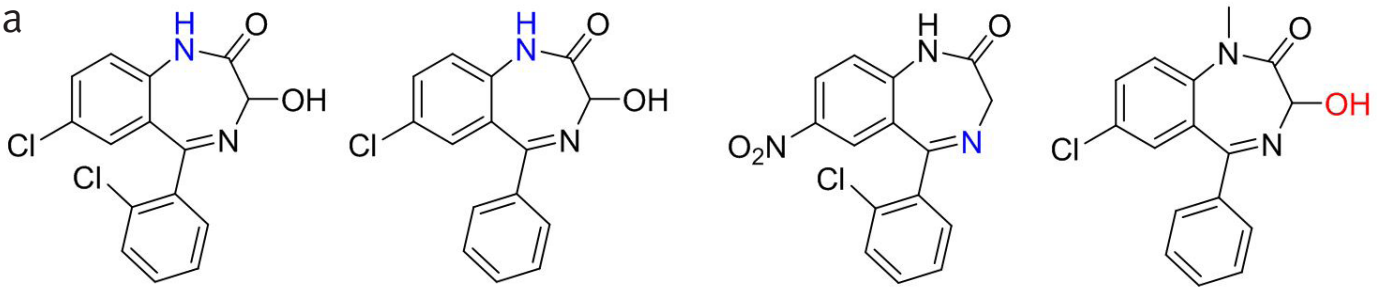
Caveats When Selecting SPE Wash(s)

The experiments reported in this white paper surveyed a broad chemical landscape composed of structurally diverse analytes and their general response to washes of differing polarity. Unfortunately, it fails to secure all 85 analytes within the general criteria for recovery and matrix effects (±15%) for a single wash solvent. However, within a class of analytes, the juxtaposition of appropriate wash solvents may engender a successful outcome for both recovery and matrix effects. Below in figure 7, we examine the utility of different wash solvents as they pertain to specific analytes within a class of drugs. When examining the percent recovery in figure 7b, we see that four specific benzodiazepines possess a range of recoveries among different wash systems when compared the remaining nine. Clonazepam, lorazepam, oxazepam, and temazepam all show distinct attenuation of their recoveries when 50% MeCN, acetone, and IPA are used as wash solvents. These four analytes demonstrate sensitivity to altering the polarity of wash solvents. However, at a pH around 2.0, only clonazepam would yield a positive charged capable of complexation (figure 7a). Additionally, roughly half of the ionized state of clonazepam would be committed to complexation since the pH of the pre-treatment solution nearly mirrors the imines pKa.

Conversely, the structures and pKa of lorazepam, oxazepam, and temazepam, highly suggests they are incapable of complexation with the exchange mechanism of the EVOLUTE® Express CX sorbent. Rather, their respective phenyl or chloro-phenyl moieties are likely the dominating factor for analyte retention via the reverse phase character of the mixed-mode sorbent, yet remain resistant to solubilization of 50% MeOH. When comparing the recovery of each analyte using neat MeOH as a wash solvent, the recovery of all four analytes is less than 45% (including clonazepam) with matrix effects at 3–22%. The remaining nine analytes maintain recoveries ≥ 75% under the same conditions (data not shown).

For these four analytes, we see the opportunity cost of switching from 50% MeOH (recoveries › 80%, matrix effects ranging -22 to -41%) to neat MeOH (recoveries 13-45%, matrix effects ranging from 3–22%). While both wash solvents effectively remove the surfactant from the Quantisal buffer, it is clear from the example that even among a structurally similar group of analytes a compromise between recovery and matrix effects be considered.

a



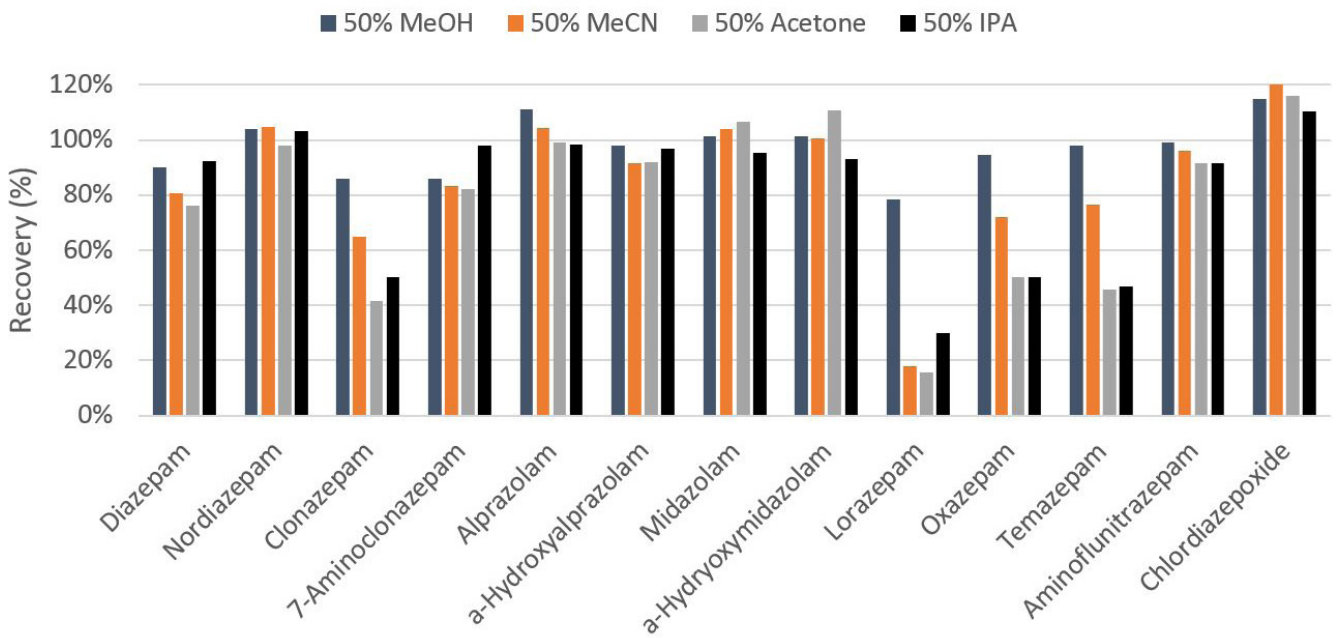
Lorazepam
$Pk_a = 12.6$

Oxazepam
$Pk_a = 12.6$

Clonazepam
$Pk_a = 1.9$

Temazepam
$Pk_a = 10.7$

b



c

	Analyte(s)		% Matrix Effects			
		MeOH	50% MeOH	50% MeCN	50% Acetone	50% IPA
Benzodiazepines	Diazepam	30%	11%	4%	-16%	5%
	Nordiazepam	-9%	0%	-7%	-12%	-20%
	Clonazepam	22%	-27%	-34%	-50%	-14%
	7-Aminoclonazepam	-51%	-70%	-114%	-111%	-73%
	Alprazolam	33%	18%	21%	6%	8%
	α–Hydroxyalprazolam	5%	-40%	-25%	-30%	-34%
	Midazolam	-19%	-27%	-28%	-15%	-55%
	α–Hydryoxymidazolam	-17%	-8%	-8%	6%	-22%
	Lorazepam	19%	-34%	-43%	-55%	-25%
	Oxazepam	3%	-41%	-57%	-63%	-24%
	Temazepam	11%	-22%	-33%	-55%	-24%
	Aminoflunitrazepam	15%	3%	-14%	-10%	-5%
	Chlordiazepoxide	22%	-11%	-6%	-4%	-30%

Figure 7. Four of thirteen benzodiazepines examined using Quantisal device in patient oral fluid (a) Percent recovery under specific wash solutions administered in Wash #2 (b) and corresponding matrix effects for each analyte (c). All samples were extracted using the parameters in tables 13 (Quantisal) and 14 (n=3).

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