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NEW SOLUTIONS APPLIED IN ORAL FLUID DRUG TESTING: FINE-TUNING AND OPTIMIZATION OF THE SPME-LC-MS METHOD

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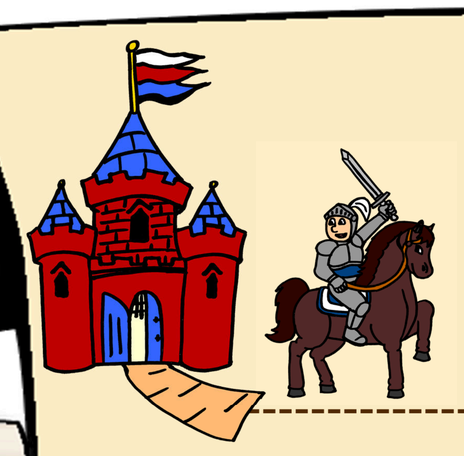
THY QUEST IS TO DEVELOP LC-MS/MS BASED ANALYTICAL PROTOCOL FOR ORAL FLUID DRUG TESTING...



MATERIALS AND METHODS

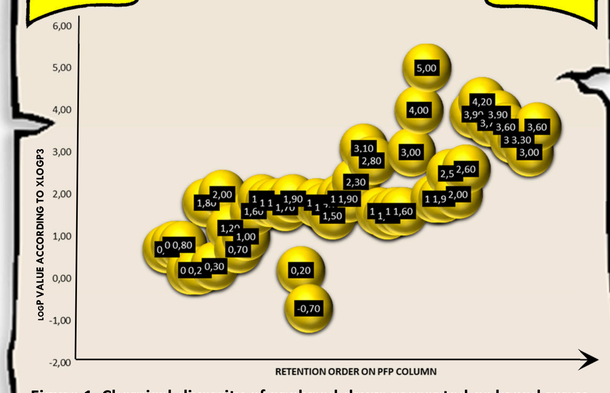


Figure 1. Chemical diversity of analysed drugs presented as broad range of partition coefficient (logP) values. Drugs arranged by retention order on PFP column.

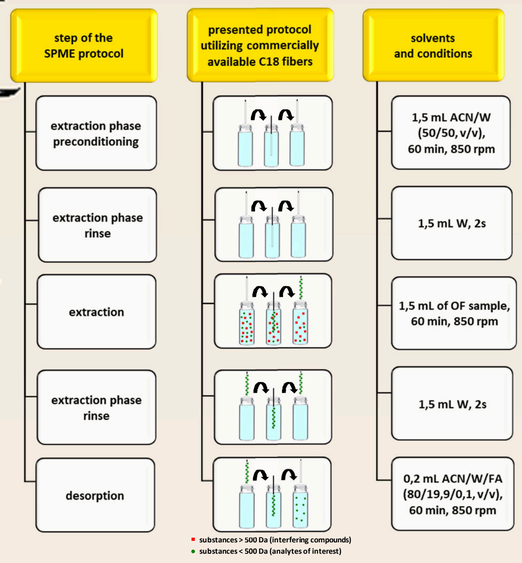


Figure 2. Extraction protocol utilizing commercially available SPME fibers with 1,5 cm C_{18} coating (MilliporeSigma/Supelco, Bellefonte, PA, USA).

parameter		value
mobile phase	phase A:	water/formic acid (99,9/0,1 v/v)
	phase B:	acetonitrile /formic acid (99,9/0,1 v/v)
	total flow rate:	300 µL/min
stationary phase	PFP column:	Kinetex® 2.6 µm F5 100Å 100x3 mm (Phenomenex, Torrance, CA, USA)
	injection volume:	10 µL
gradient	0 - 0,5 min:	10% acetonitrile
	0,5 - 14 min:	linear increase of acetonitrile from 10 to 57,65%
	14 - 15 min:	linear increase of acetonitrile from 57,65 to 100%
	14,5 - 17,5 min:	100% acetonitrile
	17,5 - 23 min:	10% acetonitrile

Table 1. Chromatographic conditions.

STEP 1: SAMPLE COLLECTION

Passive drool collection method has less significant impact on analyte concentration, therefore is utilised in presented protocol.

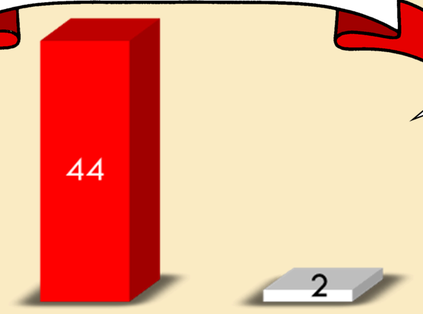
Figure 3. Measured amount of drugs with different sample collection methods: use of absorbent swabs *vs* passive drool (to polypropylene tubes). Chart represents number of drugs in each segment.

STEP 2: SAMPLE PREPARATION

$$ME(\%) = \frac{signal\ for\ analyte\ spiked\ into\ matrix\ after\ extraction}{signal\ for\ analyte} \times 100\%$$

Figure 4. Matrix effect (ME) for analysed drugs with different extraction methods: solvent protein precipitation (SPP) *vs* solid-phase microextraction (SPME). Chart represents number of drugs in each segment.

STEP 3: SPME CONDITIONS



Acetonitrile/water provides more efficient preconditioning of the extraction phase.

Matrix effect is less significant with SPME, what indicates that measured values are much closer to actual drug concentrations due to the superior sample clean-up.

Figure 5. Impact of different solvents for extraction phase preconditioning on extraction process efficiency. Chart represents number of drugs with increased extraction efficiency with each mixture.

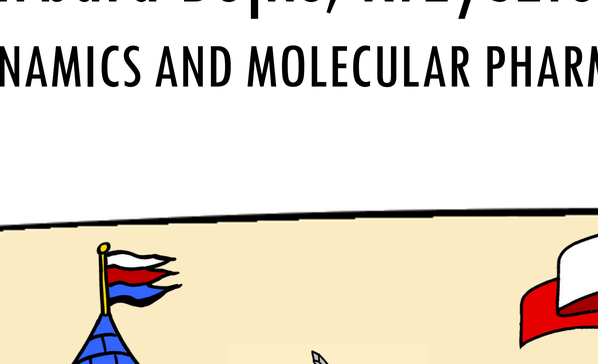


Figure 6. Impact of sample pH value on extraction efficiency. Number of drugs with best extraction efficiency from sample of certain pH value.

STEP 4: LC-MS/MS ANALYSIS

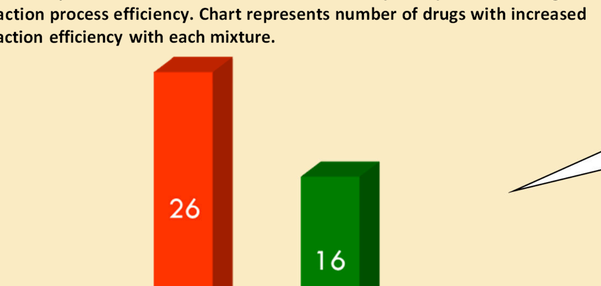
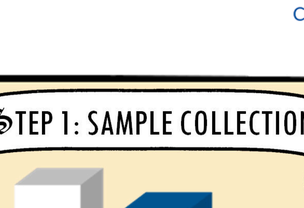


Figure 10. Shimadzu LCMS-8060 triple quadrupole utilised in this research.

pH values of 6 and 7 are optimal for SPME efficiency and are within physiological range for oral fluid.

Acetonitrile/water/formic acid as desorption solvent provides best desorption efficiency from C_{18} fibers.



Lowest carry-over effect with acetonitrile/water/formic acid enables fibre re-use after brief cleaning procedure.

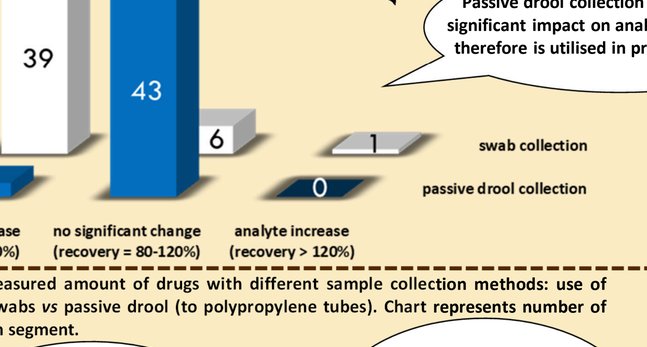


Figure 9. Selection of desorption solvent: part 3 – chromatographic peak shapes. Peak shapes for sotalol. Color code: ACN/W/FA (80/19,9/0,1, v/v) – red; MeOH/W/FA (80/19,9/0,1, v/v) – blue; ACN/MeOH/W/FA (40/40/19,9/0,1, v/v) – green; ACN/MeOH/IPA/W/FA (30/25/25/19,9/0,1, v/v) – black.

Figure 7. Selection of desorption solvent: part 1 – efficiency. Number of drugs with best desorption efficiency with each mixture.

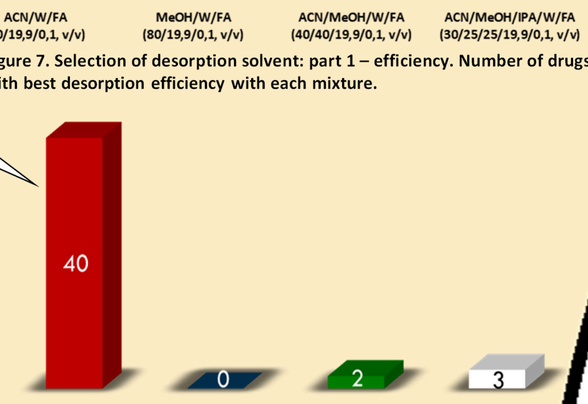
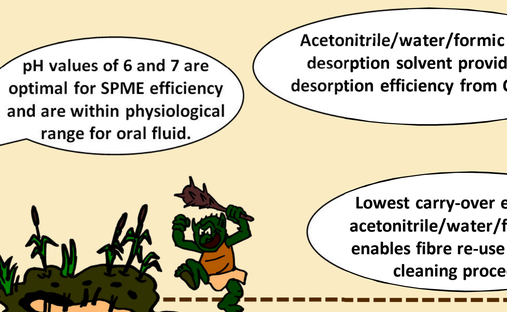


Figure 8. Selection of desorption solvent: part 2 – carry-over effect. Number of drugs with lowest carry-over effect (after performing second desorption of same fiber) with each mixture.

Tandem mass spectrometry provides excellent selectivity and sensitivity.



METHOD VALIDATION

substance	R^2	LLOQ of method
6-acetylcodeine	0,9981	25 pg/mL
11-deoxycortisol	0,9825	25 pg/mL
acebutolol	0,9982	25 pg/mL
amphetamine	0,9985	5 ng/mL
androstenedione	0,9958	1 ng/mL
atenolol	0,9722	1 ng/mL
betaxolol	0,9991	10 pg/mL
bisoprolol	0,9862	2,5 pg/mL
buprenorphine	0,9991	10 pg/mL
carteolol	0,9972	25 pg/mL
carvedilol	0,9823	25 pg/mL
cocaine	0,9869	2,5 pg/mL
corticosterone	0,9865	1 ng/mL
cortisol	0,9926	5 ng/mL
cortisone	0,9876	5 ng/mL
dexamethasone	0,9912	25 pg/mL
epitestosterone	0,9955	10 pg/mL
esmolol	0,9997	10 pg/mL
fenoterol	0,9981	25 pg/mL
fentanyl	0,9872	2,5 pg/mL
formoterol	0,9838	10 pg/mL
furosemide	0,981	1 ng/mL
heroine	0,997	25 pg/mL
labetalol	0,9826	25 pg/mL
methadone	0,9892	1 pg/mL
methandienone	0,958	25 pg/mL
methylphenidate	0,9931	10 pg/mL
metoprolol	0,9991	100 pg/mL
morphine	0,9938	1 ng/mL
nadolol	0,996	100 pg/mL
nandrolone	0,9974	25 pg/mL
nebivolol	0,9811	1 pg/mL
nikethamide	0,9849	25 pg/mL
norfentanyl	0,9952	100 pg/mL
orciprenaline	0,9943	1 ng/mL
oxandrolone	0,9968	100 pg/mL
oxycodone	0,9915	100 pg/mL
pindolol	0,998	25 pg/mL
prednisolone	0,9961	1 ng/mL
propranolol	0,9857	2,5 pg/mL
remifentanil acid	0,9993	100 pg/mL
salmeterol	0,9787	1 pg/mL
sotalol	0,9922	1 ng/mL
strychnine	0,9985	1 ng/mL
testosterone	0,9532	1 ng/mL
timolol	0,9987	25 pg/mL

Table 2. Selected results from method validation: coefficient of determination (R^2) and lower limit of quantification (LLOQ) values for analysed drugs.

APPLICATION 1: WORKPLACE/ROADSIDE TESTING

Figure 11. Commercial immunochromatographic oral fluid drug test. Evaluated device detected 2 substances – cocaine (LOD = 20 ng/mL) and morphine (LOD = 40 ng/mL) – in concentrations both higher and lower than LOD stated by product specification leaflet, however it failed to detect amphetamine (LOD = 50 ng/mL) in 2 separate tests. Presented method established drug concentrations as 14,21 ± 0,07 and 27,77 ± 0,27 ng/mL for cocaine; 23,42 ± 0,27 and 41,17 ± 0,88 ng/mL for morphine; and 55,07 ± 1,27 and 98,96 ± 1,89 ng/mL for amphetamine.

APPLICATION 2: ANTI-DOPING CONTROL

substance	WADA class [1]	WADA MRPL [2, 3]	LOD required by WADA [2, 3]	LLOQ of presented method	
		substance concentration in ng/mL (ppb)			
androstenedione	S1 ANABOLIC AGENTS	2	1	1	✓
epitestosterone		2	1	0,01	✓
methandienone		2	1	0,025	✓
nandrolone		2	1	0,025	✓
oxandrolone		5	2,5	0,1	✓
testosterone		2	1	1	✓
fenoterol	S3 BETA-2 AGONISTS	20	10	0,025	✓
formoterol		6	3	0,01	✓
orciprenaline		20	10	1	✓
salmeterol		20	10	0,001	✓
furosemide	S5 DIURETICS	200	100	1	✓
amphetamine	S6 STIMULANTS	100	50	5	✓
cocaine		100	50	0,0025	✓
nikethamide		100	50	0,025	✓
methylphenidate		100	50	0,01	✓
strychnine		100	50	1	✓
buprenorphine	S7 NARCOTICS	5	2,5	0,01	✓
fentanyl		2	1	0,0025	✓
heroine		50	25	0,025	✓
methadone		50	25	0,001	✓
morphine		50	25	1	✓
norfentanyl		2	1	0,1	✓
oxycodone		50	25	0,1	✓
remifentanil acid		2	1	0,1	✓

substance	WADA class [1]	WADA MRPL [2, 3]	LOD required by WADA [2, 3]	LLOQ of presented method	
		substance concentration in ng/mL (ppb)			
11-deoxycortisol	S9 GLUCO-CORTICOIDS	30	15	0,025	✓
corticosterone		30	15	1	✓
cortisol		30	15	5	✓
cortisone		30	15	5	✓
dexamethasone		30	15	0,025	✓
prednisolone		30	15	1	✓
acebutolol	P1 BETA-BLOCKERS	100	50	0,025	✓
atenolol		100	50	1	✓
betaxolol		100	50	0,01	✓
bisoprolol		100	50	0,0025	✓
carteolol		100	50	0,025	✓
carvedilol		100	50	0,025	✓
esmolol		100	50	0,01	✓
labetalol		100	50	0,025	✓
metoprolol		100	50	0,1	✓
nadolol		100	50	0,1	✓
nebivolol		100	50	0,001	✓
pindolol		100	50	0,025	✓
propranolol		100	50	0,0025	✓
sotalol		100	50	1	✓
timolol		100	50	0,025	✓

Table 3. Comparison of the World Anti-Doping Agency's requirements and performance of presented method.

APPLICATION 3: THERAPEUTIC DRUG MONITORING

Rapid drug penetration from plasma to oral fluid enables its use as a matrice for non-invasive therapeutic drug monitoring. However ephasis needs to be put on establishing corelation of drug binding to proteins betwen both matrices.

substance	drug binding to:	
	oral fluid proteins	blood proteins [4]
acebutolol	80%	10-26%
amphetamine	34%	15-40%
atenolol	42%	6-16%
betaxolol	79%	50%
bisoprolol	72%	30%
buprenorphine	14%	92-96%
dexamethasone	79%	70%
esmolol	74%	55%
fentanyl	19%	80-85%
formoterol	74%	61-64%
furosemide	82%	95-99%
heroine	89%	0%
carvedilol	31%	95-98%
cortisol	87%	95%
labetalol	65%	50%

substance	drug binding to:	
	oral fluid proteins	blood proteins [4]
methadone	11%	85-90%
metoprolol	69%	12%
metylphenidate	34%	10-33%
morphine	54%	30-40%
nadolol	64%	30%
nandrolone	64%	58%
nebivolol	44%	93-98%
oxycodone	64%	45%
pindolol	7%	40-70%
prednisolone	91%	more than 90%
propranolol	44%	more than 90%
salmeterol	21%	96%
sotalol	26%	0%
testosterone	63%	98%
timolol	65%	10%

Table 4. Comparison of drug binding to oral fluid proteins (established during research) and values reported for blood proteins.

ILLUSTRATIONS BY Ł.SOBCZAK

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REFERENCES:
[1] PROHIBITED LIST (HTTPS://WWW.WADA-AMA.ORG/SITES/DEFAULT/FILES/WADA_2019_ENGLISH_PROHIBITED_LIST.PDF).
[2] MINIMUM REQUIRED PERFORMANCE LEVELS FOR DETECTION AND IDENTIFICATION OF NON-THRESHOLD SUBSTANCES (HTTPS://WWW.WADA-AMA.ORG/SITES/DEFAULT/FILES/RESOURCES/FILES/TD2019MRPL_ENG.PDF).
[3] DECISION LIMITSFOR THE CONFIRMATORY QUANTIFICATION OF THRESHOLD SUBSTANCES (HTTPS://WWW.WADA-AMA.ORG/SITES/DEFAULT/FILES/TD2019DL_FINAL_ENG_CLEAN.PDF).
[4] DRUGBANK DATABASE (HTTPS://WWW.DRUGBANK.CA).

ABBREVIATIONS USED:
ACN – ACETONITRILE
C_{18} – OCTADECYL GROUP
FA – FORMIC ACID
IPA – ISOPROPANOL
LC-MS(/MS) – HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY
LOD – LIMIT OF DETECTION
MeOH – METHANOL
MRPL – MINIMUM REQUIRED PERFORMANCE LEVELS
OF – ORAL FLUID
PFP – PENTAFLUOROPHENYL GROUP
SPME – SOLID-PHASE MICROEXTRACTION
W – WATER
WADA – THE WORLD ANTI-DOPING AGENCY