Potential and limitations of solid phase microextraction coupled to high sensitive LC-MS/MS system in analysis of prohibited substances from saliva

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ENVIRONMENTAL/FOOD | SAMPLE PREPARATION | MASS SPECTROMETRY

OVERVIEW

Saliva as alternative specimen for drugs detection in forensic and clinical chemistry

Advantages:

✓**Saliva sample collection is easy, non-invasive and doesn't require any special training compare to blood sampling.**

✓**Saliva is available at any time and can be collected at the public view to prevent adulteration or sample substitution and which supervision during collection would not compromise the privacy of the tested athletes.**

✓**Saliva is the direct filtration of blood, therefore it reflects blood concentration of substances.**

✓**Shorter time windows of drug detection compare with an alternative matrices; specifically addressed to drugs banned only in competition (S6-S9).**

✓**Saliva offers the possibility to measure the free fraction of the drug, which is biologically active form.**

Disadvantages:

✓**Very low concentrations of drugs present in saliva.**

✓**Dry mouth reduces saliva volume, on the other hand stimulation can affect test results.**

✓**Possible instrumentation problems due to complex macromolecular matrix of saliva.**

Potential and limitations of SPME in analysis of prohibited substances from saliva

Advantages:

✓ **in vivo sampling with SPME avoids hydrophobic compound losses, stability and volume collection issue**

✓**SPME offers good sample clean-up compare with traditional sample preparation method (e.g. LLE), preventing matrix effect**

✓**Thin-Film format of SPME offers enough sensitivity for simultaneous quantification of 46 prohibited substances with limits of detection at pg/mL levels**

✓**SPME simplifying the entire sample preparation process and eliminating several complicated and costly stages of conventional sample analysis**

Disadvantages:

✓**Necessity of careful optimization including pH and ionic strenght sample adjustment before use the final protocol in specific application**

✓**Non-exhaustive extraction method require using very sensitive mass spectrometer due to very low concentrations of drugs present in saliva.**

RESULTS and DISCUSSION

Tab.1: Analytical performance of 46 prohibited compounds

Compound name	WADA Class	logP*	RT	Linearity (R^2)	S/N**
6-acetylcodeine	Narcotics	2.08	6.4	0.9545	5.32
Acebutolol	Beta blocker	1.71	9.1	0.9386	293.66
Alprenolol	Beta blocker	3.10	8.5	0.9515	153.29
Aminegluthemide	Hormones	0.82	5.1	0.9766	111.67
Androstenedione	Steroids	2.75	12.3	0.8765	70.27
Atenolol	Beta blocker	0.16	5.9	0.9710	13.85
Bambuterol	Beta agonist	1.49	6.8	0.9674	323.64
Benzphetamine	Stimulant	4.14	8.9	0.9118	127.10
Betaxolol	Beta blocker	2.81	8.2	0.9599	104.57
Boldenone	Steroids	3.05	12.4	0.8794	52.23
Budesonide	Glucocorticosteroids	2.18	11.9	0.9183	2.25
Cannabidiol	Cannabinoids	5.79	9.7	0.7346	3.09
Cannabinol	Cannabinoids	6.23	11.1	0.9302	70.41
Clenbuterol	Other anabolic	2.33	6.3	0.9461	44.15
Clostebol	Steroids	3.76	13.0	0.9288	36.23
Epitestosterone	Steroids	3.32	11.9	0.8871	2.67
Esmolol	Beta blocker	1.70	6.7	0.9608	106.91
Exemestane	Hormones	3.70	12.7	0.8835	4.10
Fentanyl	Narcotics	4.05	9.1	0.9418	275.59
Fluoxymesterone	Steroids	2.38	5.7	0.9714	199.02
Formoterol	Beta agonist	2.20	6.4	0.9710	167.82
Fulvestrant	Hormones	8.90	16.6	0.9317	25.56
Heroin	Narcotics	1.58	6.5	0.9254	58.13
Labetalol	Beta blocker	3.09	7.8	0.9508	296.86
Methadone	Narcotics	3.93	11.1	0.9249	82.91
Methandrostenone	Steroids	3.51	11.3	0.9547	1.11
Metoprolol	Beta blocker	1.60	5.9	0.9676	12.12
Nadolol	Beta blocker	0.81	11.1	0.9249	35.87
Nandrolone	Steroids	2.62	10.6	0.9372	11.92
Nikethamide	Stimulant	0.33	4.1	0.9491	20.41
Norphentanyl	Narcotics	1.67	5.1	0.9744	101.62
Oxprenolol	Beta blocker	2.10	7.2	0.9617	307.5
Phendimetrazine	Stimulant	1.70	4.6	0.9678	12.70
Pindolol	Beta blocker	1.75	5.3	0.8620	1.95
Propranolol	Beta blocker	3.48	8.7	0.9481	144.4
Prostanozol	Steroids	5.70	16.1	0.7971	3.11
Ritalin	Stimulant	0.20	5.1	0.9739	33.85
Salbutamol	Beta agonist	0.64	8.8	0.9067	161.58
Seleginine	Stimulant	2.70	5.8	0.9307	3.47
Sotalol	Beta blocker	0.24	3.2	0.9314	7.96
Stanozolol	Steroids	3.81	7.8	0.9507	52.88
Strychnine	Stimulant	1.93	5.3	0.9419	19.83
Terbutaline	Beta agonist	0.90	2.7	0.9587	5.01
Testosterone	Steroids	3.32	11.4	0.7891	12.22
Timolol	Beta blocker	1.83	5.7	0.9807	137.97
Zilpaterol	Other anabolic	1.26	2.5	0.9592	2.98

** Value from Syracuse Research Corporation, PhysProp Database, accessed June 2016*
***Signal-to-noise ratio for 10 ppt fortified sample (lowest points in extraction method)*

SPME-LC/MS in presented protocol showed good linearity from low-ppt levels with high S/N ratio!

Fig. Matrix-matched calibration curve for fulvestrant (left) and timolol (right) log P 8.9 and 1.2, respectively. (10 pg/mL up to 200 pg/mL)

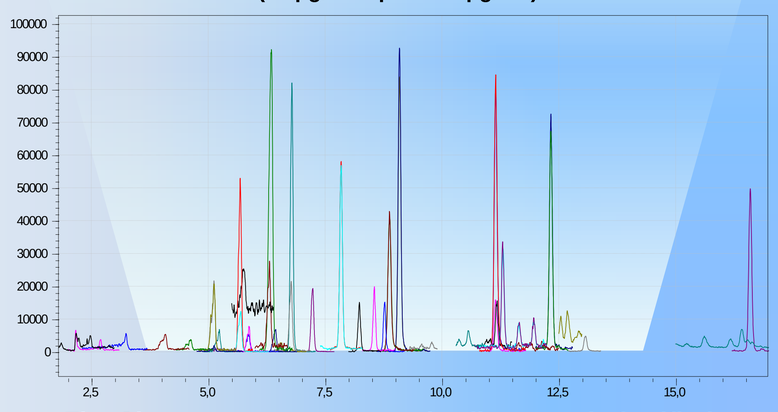


Fig. Chromatogram presented saliva sample fortified to 10 ppt level (SPME used for sample preparation)

Optimized procedure:

Oral fluid samples were collected from one individual by expectoration in 10 mL glass vial

↓

Matrix-matched calibration with 46 standards was prepared by direct dilution of analyte stock standards of various concentrations in oral fluid in the concentration range of 10 - 200 pg mL^{-1}

↓

Conditioning	Extraction	Rinsing	Desorption
30 min	90 min	10 sec	60 min
1500 rpm	1500 rpm	1500 rpm	1200 rpm
MeOH/H_2O (50/50 v/v)	Saliva	H_2O	ACN/MeOH/H_2O (40/40/20 v/v) + 0.1% FA
1500 µL	1200 µL	1500 µL	1200 µL

↓

Evaporation and reconstitution in 200 µL MeOH/ACN/W/FA 40/40/19.9/0.1

↓

Transfer in 250 µL inserts and place the inserts in the well plate

↓

Evaporation-reconstitution in 50 µL MeOH/ACN/W/FA 40/40/19.9/0.1

↓

Injected (10µl) into high-sensitive LCMS8060 system

↓

Possibility to inject 0.1µl in Nexera system with excellent reproducibility and still good S/N ratio as well as sensitivity of injected samples (10ppt level)

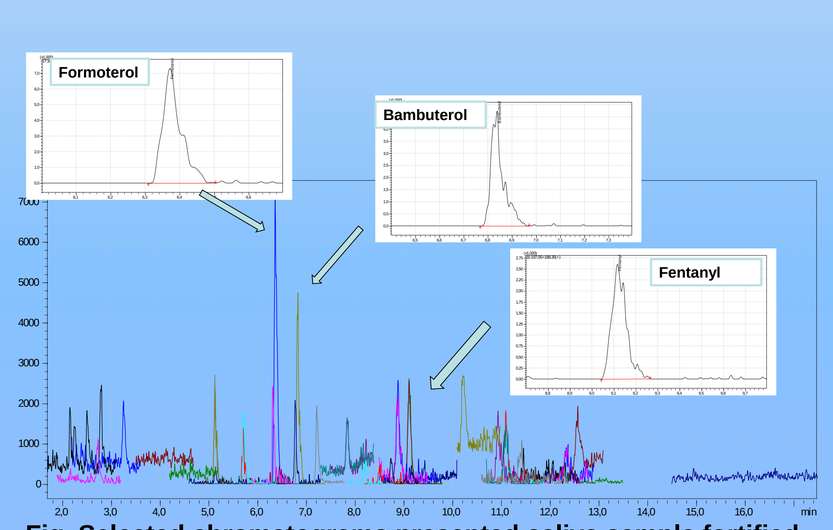
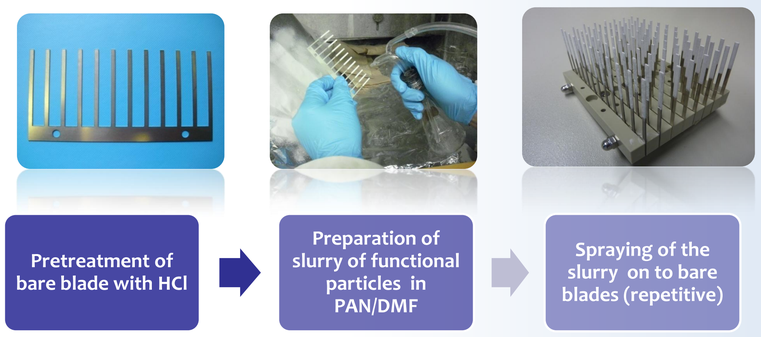


Fig. Selected chromatograms presented saliva sample fortified to 10 ppt level with 0.1 µl of injection volume (need to be confirmed if equal results will give 0.1ppt of 10 µl inj.)

EXPERIMENTAL

Thin film microextraction coatings preparation



•HLB particles (60µm, average particle diameter) were immobilized on bare metal blades using spraying method [1].
•Extraction phase (thin-film) has thickness of ca. 165µm

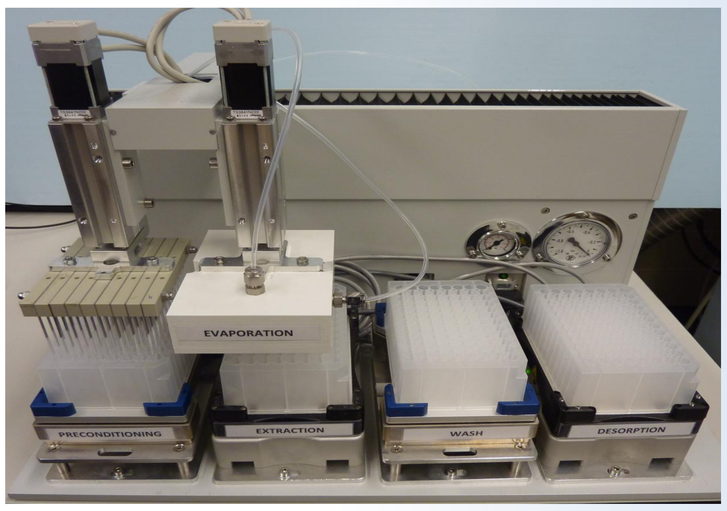


Fig. 1: Automated Concept 96-blade system (PAS Technology) University of Waterloo, Canada

Fig. 2: Shimadzu LCMS-8060 with LC Nexera system (Nicolaus Copernicus Univ, Collegium Medicum, Poland)

✓Triple quadrupole mass spectrometer with the highest sensitivity

✓Fully Automated MRM Optimization

✓Only 40µL (1mg/mL) of mix of substances was necessary for full MRM optimization of 46 compounds

✓ High scan speed (more data points over a peak) without loss of mass accuracy or quantitative precision

Instrumental conditions:

✓LC method: Shimadzu Nexera LC system; 10 µL inj. volume; PFP column (Kinetex Phenomenex,100x2.1mm, 3.0 µm); column temp. 40^{0}C; mobile phase A: 0.1% formic acid in water/acetonitrile (89.9/10 v/v); mobile phase B:0.1% formic acid in acetonitrile; gradient 35 min

✓ Mass spectrometry method LCMSS8060: positive ionization mode; Nebulizing Gas Flow: 3L/min; Heating Gas Flow: 10L/min; Interface Temp: 325^{0}C; DL Temp: 275^{0}C; Heat Block Temp: 300^{0}C; Drying Gas Flow: 10L/min.

SUMMARY

Advantages of SPME technique in saliva analysis

✓TF-SPME provides higher sensitivity and shortest extraction time, compared to SPME fiber, and high-throughput sample preparation when combined with the automated Concept 96-blade system

✓short analysis time per sample (3.7 min/sample)

✓Using TF-SPME, matrix effect ranged from 86 to 107%, while using liquid-liquid extraction matrix effect ranged from 15 to 133% [2]

✓ability to direct an analysis of complex samples as saliva but also whole blood, urine, tissue, plasma

✓ To improve data quality Internal Standard is recommended, especially when evaporation-reconstituton step is applied

✓High sensitivity of instruments will allow remove preconcentration step and improve precision of method

REFERENCES

1. **Mirnaghi F.S. et al. Anal. Chem. 2011, 83, 6018-6025.**
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ACKNOWLEDGMENT

The authors would like to acknowledge the Natural Sciences and Engineering Research Council of Canada Industrial Research program, the Canada Research Chairs program, Shimadzu Europa GmbH for supplying the LCMS-8060 Triple Quadrupole LC/MS/MS and Shimpol A.M. Borzymowski Company for technical assistance. This work was supported by the National Centre of Science of Poland (grant no. 2013/11/N/ST4/01017).