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RESEARCH ARTICLE

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Stability of drugs of abuse in synthetic oral fluid investigated using a simple "dilute and inject" method of analysis

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Abstract

Human oral fluid is well established as a matrix for drug screening, particularly in the workplace. The need to synthesise synthetic oral fluid (SOF) has been recognised in order to overcome human oral fluid's composition variability. We have used SOF spiked with six common drugs of abuse or their primary metabolites: morphine, amfetamine, benzoylecgonine, cocaine, diazepam, and (−)-Δ^9-tetrahydrocannabinol (THC) in order to assess the suitability of this matrix for quality assurance purposes. For confirmation of a drug screening test, controls and spiked standards are normally required. All our analytes were detected by LC–MS/MS using a quick and easy "dilute and inject" sample preparation approach as opposed to relatively slower solid-phase extraction. The limit of detection (LOD) was 10 ng/ml for diazepam and THC and 5 ng/ml for morphine, amfetamine, benzoylecgonine and cocaine. Validation results showed good accuracy as well as inter- and intra-assay precision (CV [%] < 5). Our work highlighted the importance of adding Tween® 20 to the SOF and calibrants to reduce losses when handling THC. Furthermore, drug stability was tested at various temperatures (5°C, 20°C and 40°C), for a number of days or after freeze–thaw cycles. Recommendations regarding storage are provided, the spiked SOF being stable at 5°C for up to 1 week without significant drug concentration loss.

KEYWORDS
"dilute and inject", drug stability, LC–MS/MS, synthetic OF

1 | INTRODUCTION

Human oral fluid (OF) is a complex matrix that is produced mainly by the parotid, submandibular and sublingual salivary glands. It is a clear aqueous liquid that contains various anions and cations (e.g., sodium, potassium, calcium and magnesium), mucin, enzymes (e.g., amylase), immunoglobulins, urea and ammonia.[1,2] Its beneficial functions such as preparing food for digestion, anti-microbial activity and the protection of the oral cavity are physiologically very important roles.[3,4] The composition of OF and its variability have been described.[5] Numerous factors such as circadian rhythm, drug intake or various medical conditions influence OF composition.[6,7] The OF variability may also be due to factors such as xerostomia (dry mouth syndrome), hyposalivation or the use of psychotropic drugs.[8–10] Despite OF variability, there is evidence of its successful use to diagnose infectious diseases (e.g., malaria) or to identify biomarkers in OF to follow the development and prognosis of a disease.[11,12]

Urine and blood are common biological matrices utilised for drug testing, which provide comprehensive information regarding drug exposure. Blood concentrations accurately represent drug

concentrations at the time of sampling, while urine concentrations are more useful to reflect drug elimination. OF is a well-established qualitative simple alternative to urine or blood for the analysis of drugs of abuse in workplace drug screening being simple and non-invasive to collect.[13,14] OF road-side drug testing of drivers suspected of driving under the influence of psychoactive drugs is undertaken by police forces in a number of countries worldwide including the United Kingdom and may demonstrate recent drug consumption.[15–17]

Due to the variability in human OF composition, there is a clear need to be able to synthesise synthetic oral fluid (SOF) that would allow for its use as a reproducible reference material for the preparation of quality control samples for confirmatory or drug-screening programmes as a substitute for human OF.[18]

Enders and McIntire reported a "dilute and inject" LC–MS/MS method for the detection of opioids in oral fluid. The oral fluid was collected utilising a sampling device and diluted 1 in 10 prior to LC–MS/MS MRM (multiple reaction monitoring) with a run time of just below 3 min. Isomers such as codeine and hydromorphone were not separated, but these could be distinguished in the sample due to different ion transitions. Calibrants were prepared in synthetic saliva and covered a concentration range of 2.5 to 1000 ng/ml.[19] Similarly, a simple analytical method has been developed in our laboratory to quantify six target drugs in SOF by LC–MS/MS using a "dilute and inject" approach. To compensate for any ion suppression due to the matrix, a mixture of deuterium-labelled analogues of all our target drugs was employed as internal standards.[20,21] Nevertheless, our study included testing drug stability in spiked SOF at various temperatures or after freeze–thaw cycles.

This study explores the usefulness of SOF for the detection of a number of drugs of abuse spiked at concentrations relevant to the UK Section 5A drug-driving legislation 2014.[22] For the purpose of our work, we have synthesised SOF and prepared standard drug solutions containing morphine, amfetamine, benzoylecgonine (BZE), cocaine, diazepam and (−)-Δ^9-tetrahydrocannabinol (THC) at 100 ng/ml to comply with drug testing devices such as DrugWipe® 3S, which has been approved by the Home Office. Of note, apart from being classified as drugs of abuse, morphine, amfetamine and diazepam are also prescription medications.

2 | EXPERIMENTAL

2.1 | Chemicals

Cerilliant® certified reference material solutions (1 mg/ml of morphine, amfetamine, benzoylecgonine, diazepam and THC solutions in methanol; 1 mg/ml cocaine solution in acetonitrile) and deuterated analogues (100 μg/ml d_3-morphine, d_5-amfetamine, d_3-benzoylecgonine, d_5-diazepam and d_3-THC solutions in methanol; 100 μg/ml d_3-cocaine solution in acetonitrile) were purchased from Sigma Aldrich (Dorset, UK). The purity of all reference solutions was greater than 99%. Mucin (bovine mucin from submaxillary glands), potassium thiocyanate, calcium chloride dihydrate, magnesium chloride hexahydrate, Tetronic® 90R4 and Tween® 20 were purchased from Sigma Aldrich (Dorset, UK). Potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium azide, sodium hydrogen carbonate, urea, ammonium acetate and glacial acetic acid were purchased from Fisher Scientific (Loughborough, UK). All reagents were of analytical reagent grade with the exception of Tween® 20 which was BioXtra grade. LC–MS grade acetonitrile was purchased from VWR (Lutterworth, UK). Deionised water was obtained at 18 MΩ.cm^{-1} resistivity from Elga Purelab Flex water dispenser (High Wycombe, UK).

2.2 | LC–MS/MS analysis

The compounds were analysed using an Agilent 6460 triple quadrupole mass spectrometer with an electrospray ionisation (ESI) source and 1260 binary pump attached (Waldbronn, Germany) with a Waters Acquity® UPLC™ HSS T3 C_{18} (1.8 μm, 2.1 × 50 mm) column. The column temperature was 30°C. The mobile phase A consisted of 10 mM ammonium acetate and 0.1% glacial acetic acid in water, while mobile phase B consisted of 10 mM ammonium acetate, 0.1% glacial acetic acid and 10% water in acetonitrile. The flow rate was 0.4 ml/min. The gradient was programmed as 5% B (0 min), 35% B (4 min), 100% B (8 to 11.5 min), and 5% B (11.51 to 13.5 min). The injection volume was 10 μl. The mass spectrometer was operated in the positive ionisation and dynamic multiple reaction monitoring mode. The nitrogen gas temperature in the ESI source was set at 325°C, gas flow at 10 L/min, nebuliser at 55 psi, sheath gas temperature at 400°C, sheath gas flow at 12 L/min and the capillary voltage at 3500 V. The ion transitions included in the acquisition method and retention times for each analyte are presented in Table 1.

2.3 | SOF synthesis

The SOF composition is presented in Table 2 and was prepared as described in our previous paper.[18] SOF was prepared by dissolving the compounds in water taking extra care when handling mucin and Tween® 20 as both have the potential to foam.

2.4 | Calibrant preparation

A drug mix (10 μg/ml) containing morphine, amfetamine, benzoylecgonine, cocaine, diazepam and THC was prepared by diluting each stock solution (1 mg/ml) in Tween® 20 mobile phase (0.09% Tween® 20, 10 mM ammonium acetate, 0.1% glacial acetic acid and 5% acetonitrile in water). The Tween® 20 mobile phase was used to prepare a series of calibrants (10 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 500 ng/ml and 1000 ng/ml). SOF was spiked with the same mixture of the six drugs (10 μg/ml) to obtain 100 ng/ml of each for LC–MS/MS analysis.

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Rt range (min)
Morphine ($C_{17}H_{19}NO_3$)	286.1	201.4	1.88 ± 0.1
d_3-morphine	289.2	201.1	1.88 ± 0.1
Amfetamine ($C_9H_{13}N$)	136.1	91.1	3.14 ± 0.1
d_5-amfetamine	141.2	96.1	3.14 ± 0.1
Benzoylecgonine ($C_{16}H_{19}NO_4$)	290.2	168.2	3.83 ± 0.1
d_3-benzoylecgonine	293.1	171.0	3.83 ± 0.1
Cocaine ($C_{17}H_{21}NO_4$)	304.2	182.2	5.04 ± 0.1
d_3-cocaine	307.1	185.1	5.04 ± 0.1
Diazepam ($C_{16}H_{13}ClN_2O$)	285.1	193.1	7.40 ± 0.1
d_5-diazepam	290.0	198.0	7.40 ± 0.1
THC ($C_{21}H_{30}O_2$)	315.3	193.1	9.70 ± 0.1
d_3-THC	318.2	195.9	9.70 ± 0.1

TABLE 1 Ion transitions and retention times of six target drugs and their deuterated analogues analysed by LC–MS/MS in positive electrospray ionisation mode

TABLE 2 SOF composition

Component	Concentration
Potassium chloride	1360 mg/L
Bovine mucin (from sub-maxillary glands)	1300 mg/L
Potassium dihydrogen phosphate	950 mg/L
Sodium chloride	860 mg/L
Sodium azide	500 mg/L
Sodium hydrogen carbonate	440 mg/L
Potassium thiocyanate	250 mg/L
Calcium chloride[a]	210 mg/L
Urea	180 mg/L
Magnesium chloride[a]	60 mg/L
Tween® 20	0.09%

[a]Measured as hydrates.

2.5 | Internal standard solution preparation

Internal standard mix (100 ng/ml) containing the deuterated analogues of morphine, amfetamine, benzoylecgonine, cocaine, diazepam and THC was prepared by diluting each deuterated stock solution (100 μg/ml) with the Tween® 20 mobile phase.

2.6 | Method validation

A semi-quantitative method validation was performed on three different days by two different analysts. Calibrants as described above and a single calibrant spiked at 2000 ng/ml were analysed followed by the non-spiked calibrant to assess the limit of detection and carry-over. Alongside six spiked SOF samples at 100 ng/ml, six samples of Tween® 20 mobile phase spiked at 100 ng/ml with analytes of interest were analysed to assess matrix effect. The concentration of 100 ng/ml was chosen to comply with the operating concentrations for the drug testing devices (e.g., DrugWipe® 3S).

2.7 | Sample preparation for LC–MS/MS analysis

SOF (100 μl, spiked or non-spiked), internal standard mix (100 μl of 100 ng/ml) and mobile phase (800 μl) for sample preparation (10 mM ammonium acetate, 0.1% glacial acetic acid and 5% acetonitrile in water) were added to glass autosampler vials (2 ml), which were capped, gently vortexed and submitted to LC–MS/MS analysis.

2.8 | Testing drug stability

Freshly prepared SOF was spiked with all six drugs (100 ng/ml), divided into aliquots (5 ml), placed in glass containers and subjected to varying stability experiments. All samples were kept in the dark by wrapping each glass container in aluminium foil. The spiked SOF was stored at various temperatures 5°C (refrigerator), 20°C (workbench) or 40°C (incubator) for 1, 3, 7, 14 and 30 days. Furthermore, the spiked SOF was divided into six aliquots to conduct freeze–thaw experiments. The aliquots were frozen at −20°C (freezer). Once thawed, the aliquots were kept for 7 h at ambient temperature. There were six freeze–thaw cycles conducted within 2 weeks of SOF preparation. All aliquots were analysed with calibrants by LC–MS/MS as described above.

3 | RESULTS AND DISCUSSION

3.1 | LC–MS/MS method development

The preparation of the SOF and its subsequent characterisation for quality control purposes has been described previously by our group.[18] The SOF was spiked with six commonly misused drugs and their metabolites: morphine, amfetamine, benzoylecgonine, cocaine, diazepam and THC at 100 ng/ml.

Certain variables were investigated during the LC–MS/MS method development that could influence the analysis, such as the

composition of the mobile phase and various analytical columns. The conventional aqueous and organic mobile phase for the analysis of urine samples by LC–MS in our laboratory consists of 0.3% formic acid in water and 0.3% formic acid in acetonitrile, respectively, and has previously been shown to be important in obtaining reproducible retention times of various analytes extracted from human urine.[23] Our initial method development experiments were done with this mobile phase and a Waters Acquity® UPLC™ BEH C_{18} column (1.8 μm, 2.1 × 50 mm). This mobile phase enabled the separation of all compounds, but compound peak tailing was an issue with morphine, which was also poorly retained. Varying the composition of the mobile phase did not resolve the situation. However, adding ammonium acetate (10 mM) and acetic acid (0.1%) to both the aqueous and organic mobile phases retained morphine approximately 1 min longer, and it was decided to employ this mobile phase for further analysis.

To improve peak shape, experiments were performed with a variety of chromatography columns including Agilent Zorbax Eclipse plus C_{18} (3.5 μm, 4.6 mm × 100 mm), Agilent Zorbax Extend C_{18} (1.8 μm, 2.1 × 50 mm) or Waters XBridge C_{18} (3.5 μm, 4.6 mm × 150 mm) and Acquity® UPLC™ HSS T3 C_{18} column (1.7 μm, 2.1 × 50 mm). The Acquity® UPLC™ HSS T3 C_{18} column (1.8 μm, 2.1 × 50 mm) performed best and was used with aqueous and organic mobile phase containing ammonium acetate and acetic acid for all experiments.

Without compromising the analysis, lengthy sample preparation steps routinely employed in laboratories, such as solid phase extraction, were avoided by employing the "dilute and inject" approach, which has been regularly employed in anti-doping analysis, toxicology investigations and preclinical research.[24–26] We performed a 1 in 10 dilution of SOF spiked drug solutions with mobile phase for sample preparation (10 mM ammonium acetate, 0.1% glacial acetic acid and 5% acetonitrile in water) before LC–MS/MS analysis alongside

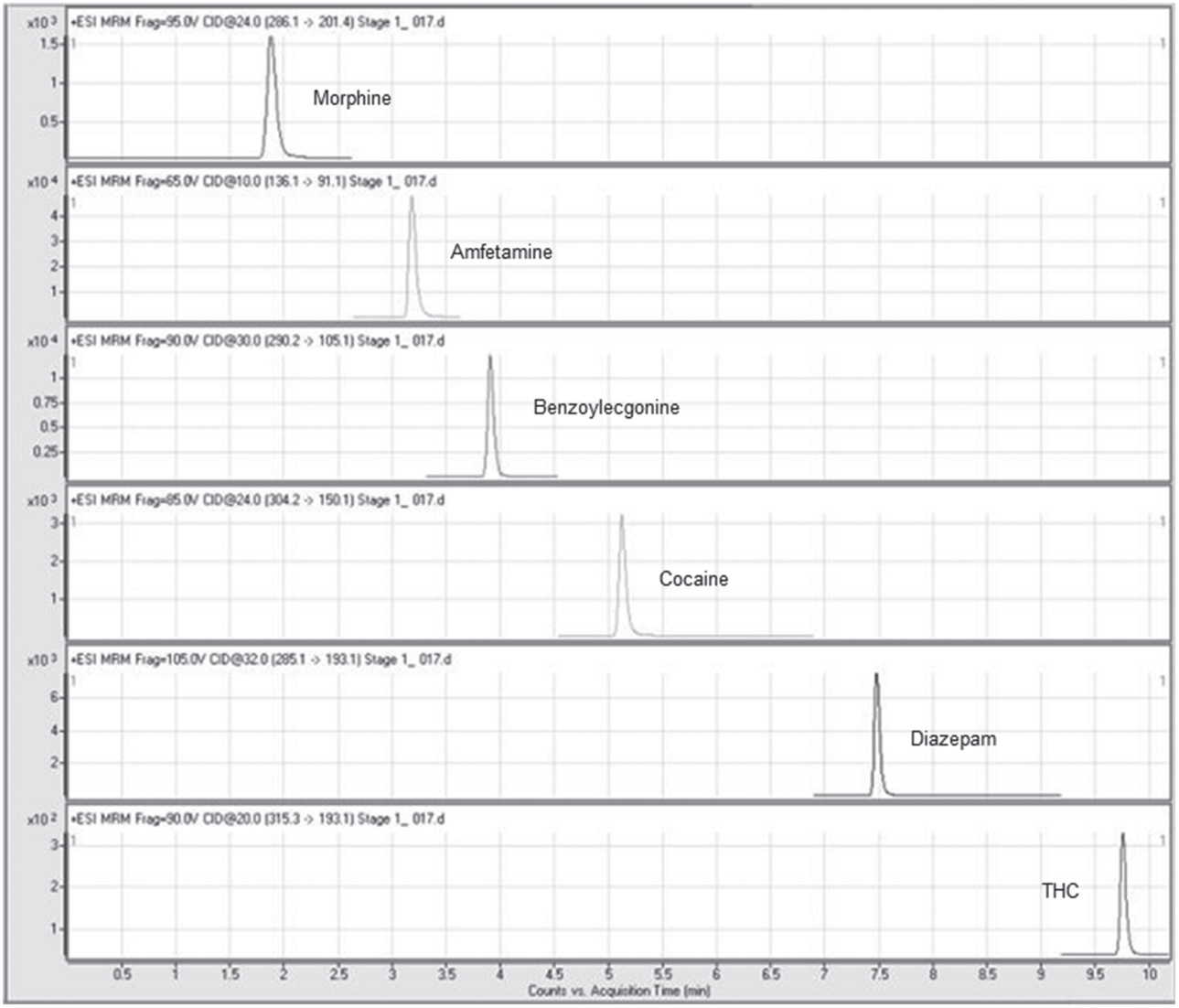

FIGURE 1 Representative chromatograms showing data obtained from the analysis of SOF spiked with a drug mix containing morphine, amfetamine, benzoylecgonine, cocaine, diazepam and THC each at 100 ng/ml

calibrants prepared in Tween® 20 mobile phase (0.09% Tween® 20, 10 mM ammonium acetate, 0.1% glacial acetic acid and 5% acetonitrile in water) rather than SOF. Our smallest calibrant was spiked at 10 ng/ml and our largest at 1000 ng/ml.

The different chemistries of our drugs result in divergent interactions between the mobile and stationary phases. Morphine elutes first with a retention time (Rt) 1.88 min, followed by amfetamine Rt 3.14 min, benzoylecgonine Rt 3.83 min, cocaine Rt 5.04 min, diazepam Rt 7.40 min and THC Rt 9.69 min. No shifts in retention times were observed when comparing retention times of the compounds in the spiked SOF, calibrants and as single standard solutions under the same LC–MS/MS conditions. Each selected ion transition of our compounds of interest appeared in the corresponding acquisition window as a single peak with a signal to noise ratio greater than 3:1. No background interference or presence of any other peak apart from our peak of interest was seen. Figure 1 shows the good chromatographic separation and peak shape with negligible background noise obtained.

3.2 | Method validation

Initial preparation of calibrants used a solution containing 10 mM ammonium acetate, 0.1% glacial acetic acid and 5% acetonitrile in water after which a calibration curve was run. For all our analytes with the exception of THC, the coefficient of determination (r^2) was greater than 0.995, which indicated losses of THC probably due to sticking or adsorbing onto the glass surface.[27] This phenomenon has been previously reported for THC urinary metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid indicating that the losses may have occurred.[28,29]

The addition of Tween® 20 to the solution of 10 mM ammonium acetate, 0.1% glacial acetic acid and 5% acetonitrile in water in the amount that corresponds to the amount of Tween® 20 in the SOF recipe, r^2 was greater than 0.995 for all our analytes including THC. Making calibrants with Tetronic® 90R4 in the same concentration instead of Tween® 20 was considered. However, it was discovered that Tetronic® 90R4 provided less consistent LC–MS/MS data than Tween® 20, which supported our decision to prepare our calibrants with Tween® 20. At the beginning of the study during the method development stage, SOF was spiked with drugs at low concentrations such as 5 ng/ml or 10 ng/ml. The intention was to get an approximate estimate of LOD and use this information to decide on the calibrant concentration range. To avoid unnecessary additional costs, calibrants were prepared as described and utilised for LOD estimation. Diazepam and THC showed a greater limit of detection of 10 ng/ml compared with other drugs, hence the use of 10 ng/ml as the lowest calibrant concentration. Morphine, amfetamine, benzoylecgonine and cocaine had lower limits of detection estimated, from the 10 ng/ml calibrant, to be approximately 5 ng/ml. We compared THC concentrations after storage in both silanised and non-silanised glassware before pursuing method validation. Since no difference was observed, non-silanised glassware was selected for solution storage.

To investigate any matrix effect and any potential ion suppression, SOF spiked with the solution containing all six drugs at 100 ng/ml was analysed alongside samples of Tween® 20 mobile phase spiked with the same solution at the same concentration. The matrix effect and subsequent ion suppression was most prominent for amfetamine and morphine. Amfetamine's and morphine's signal from the spiked SOF was about 43% and 50% lower than the amfetamine and morphine's signal from the Tween® 20 mobile phase. However, the peak counts for both drugs were still large enough to allow for the quantification. Amfetamine and morphine peaks were detected in the corresponding acquisition windows with a signal to noise ratio greater than 3:1. Matrix did not appear to affect the measured signal intensity of benzoylecgonine, cocaine and diazepam. THC losses were reduced with the addition of Tween® 20, and the matrix effect on the THC signal was negligible. None of the six drugs showed carryover when the single calibrant spiked at 2000 ng/ml was analysed followed by the non-spiked calibrant.

Table 3 shows the results obtained on validation day 1 for THC as a model compound. Data are presented for THC, since the quantification of this drug proved to be the most challenging. Calibrants 1–6

TABLE 3 Validation day 1 THC results ($n = 1$)

		THC			d_3-THC		
	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Rt (min)	Peak area	Accuracy (%)	Rt (min)	Peak area
Calibrant 1	10	10	9.80	150	101	9.80	1039
Calibrant 2	50	49	9.80	659	99	9.80	1017
Calibrant 3	100	98	9.80	1229	98	9.80	964
Calibrant 4	200	196	9.80	2650	98	9.80	1051
Calibrant 5	500	511	9.80	6819	102	9.80	1039
Calibrant 6	1000	995	9.80	13,512	100	9.80	1058
Average			9.80		**100**	**9.80**	**1028**
Sd					**2**		**34**
CV (%)					**2**		**3**

Abbreviations: CV [%], coefficient of variation; Sd, standard deviation.

were spiked with THC and other drugs at our concentration range (10 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 500 ng/ml and 1000 ng/ml). Even without internal standard adjustment, the instrument response was found to be proportional to the drug spiked concentration.

Table 4 represents the results obtained on validation day 2 for THC as a model compound. Tween® 20 (1–6) samples were Tween® 20 mobile phase samples spiked with THC (together with other drugs) at 100 ng/ml, while SOF (1–6) was spiked with THC (among other drugs) at the same concentration. The THC signal response was very similar for both Tween® 20 and SOF spiked samples, although the d_3-THC internal standard signal either decreases (samples 1–4) or slightly increases (samples 5 and 6) by approximately 20% in the spiked SOF samples compared with spiked Tween® 20 samples, which fortunately did not influence the precision of the concentration measurements.

Our acquisition method showed good inter- and intra-day precision according to CV (%) values as demonstrated in Table 5.

3.3 | Drug stability testing

The influence of two factors such as temperature and storage time was investigated in our study to determine whether any of these had any effect on the stability of drugs in the spiked SOF. The summary of measured drug concentrations in the spiked SOF after each storage condition is presented in Table 6. Figures 2, 3 and 4 represent plots for all drugs stored at 5°C, 20°C and 40°C, respectively.

TABLE 4 Day 2 THC validation results ($n = 1$)

	THC			d_3-THC	
	Concentration (ng/ml)	Rt (min)	Instrument response	Rt (min)	Instrument response
Tween® 20				9.69	1039
Tween® 20 (1)	96	9.69	1306	9.69	1030
Tween® 20 (2)	93	9.69	1358	9.69	1109
Tween® 20 (3)	97	9.69	1311	9.69	1024
Tween® 20 (4)	98	9.69	1394	9.69	1079
Tween® 20 (5)	95	9.69	1306	9.69	1045
Tween® 20 (6)	101	9.69	1244	9.69	933
Average	**96**	**9.69**	**1320**	**9.69**	**1036**
Sd	**3**		**51**		**60**
CV	**3**		**4**		**6**
SOF Blank				9.69	822
SOF (1)	107	9.69	1249	9.69	879
SOF (2)	103	9.69	1206	9.69	889
SOF (3)	105	9.71	1274	9.69	917
SOF (4)	107	9.69	1232	9.69	872
SOF (5)	85	9.69	1332	9.69	1180
SOF (6)	92	9.69	1395	9.69	1150
Average	**100**	**9.69**	**1281**	**9.69**	**981**
Sd	**9**		**70**		**144**
CV (%)	**9**		**5**		**15**

TABLE 5 Assay inter- and intra-day precision (BZE-benzoylecgonine)

	Concentration (ng/ml)								
	Morphine	Amfetamine	BZE	Cocaine	Diazepam	THC	Average	Sd	CV
Day 1	97	99	97	101	94	100	**98**	**3**	**3**
Day 2	94	100	95	100	92	102	**97**	**4**	**4**
Day 3	97	99	97	101	94	100	**98**	**3**	**3**
Average	**96**	**99**	**96**	**101**	**93**	**101**			
Sd	**2**	**1**	**1**	**1**	**1**	**1**			
CV (%)	**2**	**1**	**1**	**1**	**1**	**1**			

TABLE 6 A summary of drug concentration (ng/ml) measured in spiked SOF after each storage condition

		5°C	20°C	40°C
Amfetamine	Day 1	84	83	83
	Day 3	84	83	85
	Day 7	85	82	83
	Day 14	81	80	79
	Day 30	81	80	80
Benzoylecgonine	Day 1	88	89	104
	Day 3	88	94	147
	Day 7	90	100	151
	Day 14	88	108	156
	Day 30	93	127	156
Cocaine	Day 1	97	93	73
	Day 3	95	89	27
	Day 7	95	80	20
	Day 14	89	66	5
	Day 30	85	48	1
Diazepam	Day 1	85	85	85
	Day 3	88	87	87
	Day 7	88	87	86
	Day 14	83	84	83
	Day 30	85	85	84
Morphine	Day 1	81	79	77
	Day 3	81	78	79
	Day 7	81	79	77
	Day 14	77	76	74
	Day 30	78	77	74
THC	Day 1	108	100	89
	Day 3	107	96	77
	Day 7	98	100	56
	Day 14	89	84	40
	Day 30	87	76	22

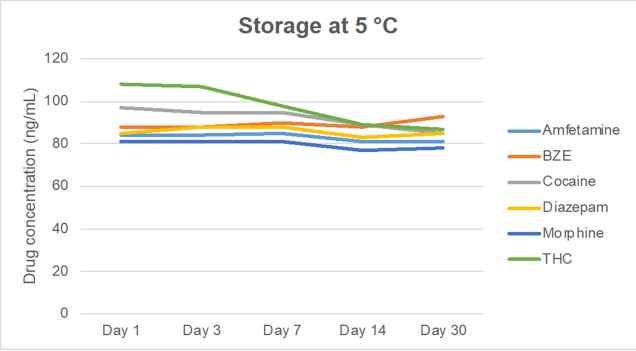

FIGURE 2 Drug concentrations after storage at 5°C [Colour figure can be viewed at wileyonlinelibrary.com]

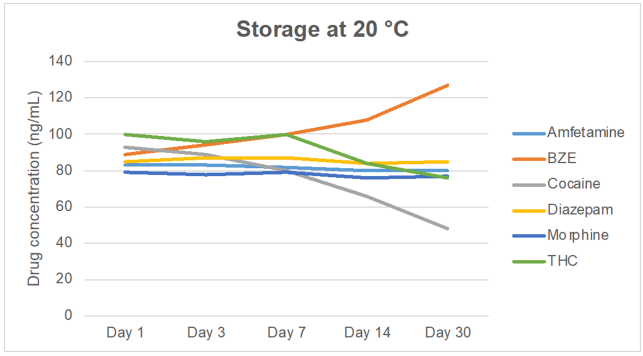

FIGURE 3 Drug concentrations after storage at 20°C [Colour figure can be viewed at wileyonlinelibrary.com]

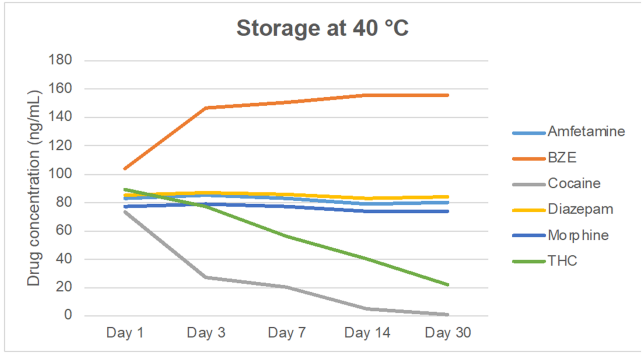

FIGURE 4 Drug concentrations after storage at 40°C [Colour figure can be viewed at wileyonlinelibrary.com]

Apart from THC, there was little effect on the concentration for any of the other five drugs when stored at 5°C for the 30-day experiment; no concentration decreased by more than 10%. The drugs were stable for up to 1 week at 5°C. After storage at 20°C, there was a decrease in concentration of cocaine, which was most affected (approximately 50%) and THC (approximately 20%). There was an expected increase in the concentration of benzoylecgonine since cocaine hydrolyses readily.[30] The storage of samples at 40°C acted as an accelerated degradation study. Significant decreases of approximately four times the rate of degradation for THC were observed at 40°C when compared with that at 20°C, while cocaine was completely degraded within 30 days when stored at 40°C.

THC seems to be the most challenging compound to work with and the scientific literature shows variable data regarding its storage under various conditions, containers and biological matrices. For instance, in brief, the blood of 16 volunteers suspected of cannabis smoking was stored in glass vials and polystyrene plastic tubes at room temperature for 4 days or at −20°C for 4 weeks. The THC amount remained unchanged in the glass vials, but almost all THC was lost when stored in the plastic containers.[31] Among other cannabinoids, THC stability was investigated by Lee et al., who collected OF with the Quantisal™ device from 10 volunteers. These were stored at

TABLE 7 Drug concentrations measured in spiked SOF after each freeze–thaw (F/T) cycle

Cycle	Amfetamine (ng/ml)	Benzoylecgonine (ng/ml)	Cocaine (ng/ml)	Diazepam (ng/ml)	Morphine (ng/ml)	THC (ng/ml)
No F/T	100	100	100	100	100	100
F/T 1	116	109	93	101	99	73
F/T 2	118	112	95	105	104	74
F/T 3	114	108	91	100	101	74
F/T 4	119	111	93	103	102	73
F/T 5	116	108	91	99	100	72
F/T 6	119	109	91	99	99	74

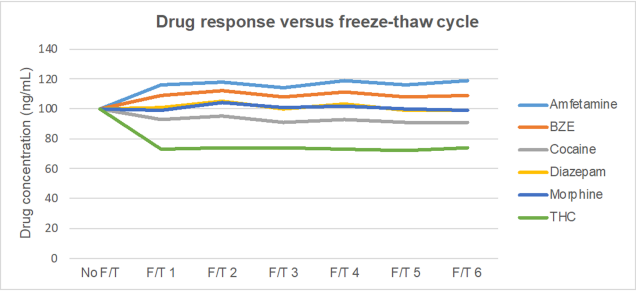

FIGURE 5 Drug response versus freeze–thaw cycle (F/T) [Colour figure can be viewed at wileyonlinelibrary.com]

4°C for 1 and 4 weeks and at −20°C for 4 and 24 weeks. THC remained stable for 1 week at 4°C, which matches our findings. After 4 weeks at 4°C, 4 and 24 weeks at −20°C, THC was stable in up to 80% of samples.[32] Molnar et al. investigated the recovery of spiked THC in oral fluid stored in polypropylene containers. They found that the surfactant Triton® X-100 significantly decreased the adherence of THC to the plastic tubes.[33] As discussed, Tween® 20 employed in our work similarly reduced the THC losses.

Table 7 and Figure 5 show observable changes for each of the drugs following the freeze–thaw experiments. THC shows the largest loss (approximately 25%) after the first freeze–thaw cycle, which may be partly due to adsorption onto glassware.[27] Benzoylecgonine, cocaine, diazepam and morphine showed less change, although there was an unexpected increase in drug concentration for amfetamine. This would suggest that these changes may either be due to inter-assay variability or could be due to variability in instrument response. There was no observable impact on the stability of drugs in SOF following repeated freezing and thawing. Even THC showed good consistency of results after repeated freeze–thaw cycles.

4 | CONCLUSION

Human oral fluid is a complex mixture composed of various mucins, amylases and mineral salts. It has gained attention as an alternative matrix to urine or blood for testing drugs of abuse at workplace or road-side drug driving. OF is easily collected, but the variability of its composition may not allow its use as a reproducible reference material. A SOF has the distinct advantage of consistency and is easily prepared simply by dissolving its components in water. Our work has shown that it appears to be a stable and reliable matrix for drug testing purposes.[18] We have developed a simple dilute and inject analytical method to detect six different drugs of abuse (morphine, amfetamine, cocaine, diazepam and THC) including the primary metabolite of cocaine, benzoylecgonine spiked in SOF. Spiked SOF was diluted with mobile phase (1:10) and directly injected into the LC–MS instrument for analysis. The limit of detection for diazepam and THC was 10 ng/ml, while the limit of detection was lower for morphine, amfetamine, benzoylecgonine and cocaine. Validation results showed good accuracy as well as inter- and intra-assay precision. Our work highlighted the importance of adding Tween® 20 to the SOF and calibrants to reduce THC losses most likely due to adsorption to glass surfaces. We have found that the spiked SOF can be stored at 5°C for up to 1 week without significant drug concentrations loss. It is mainly after the first freeze–thaw cycle that the drug concentrations were affected, while after repeated freeze–thaw cycles there was a good consistency among them. Additional work is needed to compare human OF with the SOF to translate further this work to analytical toxicology or forensic science.

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CONFLICT OF INTEREST

Authors have no conflicts of interest. Dr Alessandro Musenga contributed to this study while at the Drug Control Centre, King's College London, London, UK.

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