Artificial oral fluid characterisation: Potential for use as a reference matrix in drug testing

Ivana Gavrilović | Alessandro Musenga | David Cowan | Alison Woffendin | Andrew Smart | Fan Gong | Duncan Harding | Kim Wolff

1Drug Control Centre, King’s Forensics, King’s College London, London, UK
2Laboratoire Suisse d’Analyse du Dopage, Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Lausanne, Switzerland
3Department of Analytical, Environmental and Forensic Sciences, King’s College London, London, UK
4Home Office, Centre for Applied Science and Technology (CAST), London, UK

Correspondence
Ivana Gavrilović, Drug Control Centre, King’s Forensics, King’s College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH, UK.
Email: ivana.gavrilovic@kcl.ac.uk

Funding information
Home Office Centre for Applied Science and Technology, Grant/Award Number: HOS/13/038

Abstract
Quality assurance schemes for drug-screening programmes require access to large quantities of biological matrices for reference or control samples. This presents problems when the availability of a matrix, such as oral fluid (OF) for screening or for confirmatory purposes, limits the collection of large volumes. In such cases, synthetic alternatives of OF may provide a solution. The preparation of an artificial (synthetic) oral fluid (AOF) was conducted by dissolving its components (salts, surfactant, antimicrobial agent and mucin) in water. We characterised the physical properties of AOF to determine its suitability as a matrix for quality assurance purposes. The evaluation of pH, specific gravity (SG), conductivity (mS cm$^{-1}$), freezing point depression ($^\circ$C), light-scattering and kinematic viscosity (mm$^2$ s$^{-1}$) showed AOF to be a stable, reliable matrix. Synthetic OF was prepared using components (mucin, surfactants and so on) obtained from different suppliers and a comparison was performed. Our results suggest that AOF is a feasible matrix for the preparation of quality assurance samples for confirmatory or drug screening programmes.

KEYWORDS
AOF, drug screening, quality assurance samples, reference matrix

1 | INTRODUCTION

Oral fluid (OF) has gained a considerable hold as a biological matrix for drug-testing purposes in recent years$^{1-4}$ and is a convenient alternative to blood because it does not require invasive collection procedures or complex professional skills for sample collection. There is a preponderance of evidence that commends the usefulness of OF for the detection of both illicit drugs$^5$ and prescribed medication,$^6$ the basic premise being that a drug present in blood may be detected when it passes through the oral mucosa into the oral cavity. There has also been considerable interest in the efficacy of devices for the collection, transportation, handling and storage of OF.$^7$ Particular interest has been shown in the development of POCT (point of collection drug-screening devices) for OF, recently reviewed by Wolff et al.$^8,9$ OF has been identified as the most accessible matrix for road-side screening of drugs in drivers apprehended and suspected of driving under the influence of psychoactive drugs because OF is usually easy to obtain and may be indicative of recent consumption$^{10}$ and, for example, has been used in Australia for confirmatory testing.

However, human OF is a complex mixture of fluids originating from different salivary glands and gingival crevicular fluid. Secretions from the parotid, submandibular, sublingual and minor mucous glands are the primary contributors to this mixture$^{11}$ along with glycoproteins known as mucins, which form mucus when dissolved. It has been reported that mucins contribute to the rheological properties of OF as a result of their unique chemical and structural characteristics.$^{12}$
Mucins not only demonstrate antibacterial activity but also act as a protective surfactant to cover biomaterials to suppress an immunological response. As lubricants, mucins can also produce oral surface covering substitutes in the absence of saliva. Mucus itself can cause problems in handling when transferring small volumes by pipette. Ease of handling may also be related to the elasticity and viscosity (spinnbarkeit) of OF. Handling human OF in the laboratory can be complicated by the presence of cellular debris, nasal secretions, bacteria and residues of ingested fluids and foodstuffs, materials that need to be removed to prevent interference with assay procedures.

The preparation of quality assurance samples for OF drug screening programmes requires laboratory assay and handling, storage and preservation studies under carefully controlled circumstances. Organisations such as the U.S. Food and Drug Administration (FDA) require rigorous approval processes whilst routine assays require periodic testing for quality assurance. All such testing requires large quantities of OF with consistent composition. Human OF is difficult to obtain in large volumes, and the composition of unstimulated whole human OF is not consistent showing differences between the sexes, in the concentrations of sodium and chloride, in the circadian rhythm and hence flow rate, and in relation to the donor’s state of health.

In the general population, collecting human OF for evaluation and/or quality control purposes may be difficult because of the variability in ‘normal’ salivary secretion rates, obviously volunteers with ‘dry mouth’ syndrome (xerostomia) or hyposalivation would be unsuitable. While it is theoretically possible to collect large volumes of pooled human OF from a large number of volunteers in order to make a uniform reference sample and indeed has been used by NIST for the testing of devices in the US, no reference material is currently available from any of the organisations (such as NIST or NIBSC) who prepare uniform international reference samples and materials. Exogenous factors such as other drug use may impact on salivation because both prescribed medication and illicit drug use have been reported to inhibit salivation, for example, cocaine and morphine. Indeed, many prescription and nonprescription drugs list dry mouth as a major side effect including benzodiazepines and narcotic analgesics, glucocorticoids and antipsychotics among others. A high prevalence of buccal dryness has also been reported with elderly populations (aged 65 to 95 years), because the aging process is associated with reduced salivary flow. In addition, the variability of collection devices used in vivo and the need for evaluation of analytical processes predicates the need for a more dependable source of this matrix.

Preparation of artificial OF (AOF) is not new, and a patent was filed for ‘synthetic oral fluid standards’ with the United States Patent Office (Patent No 5,736,322) in 1998 by Goldstein for testing, calibration and standardisation of devices using OF; the OF standard contained a mucin and a protease inhibitor. In 2012, as part of the ‘Guides to Type Approval’ document for screening devices, the U.K. Home Office Centre for Applied Science and Technology (CAST) created an AOF as a matrix for drug driving tests. Artificial saliva has been used during numerous studies in odontology and has been manufactured as a saliva substitute to serve as mouth and throat lubricants in many instances. The complex nature of mucin has prevented the chemical synthesis of truly traceable material, and hence, this is a potentially important consideration in the preparation of synthetic OF. Mucin is however available from well-known sources and have traceable batch references, which can be used instead, but the material needs to be characterised as suggested by Pillai et al.

We used the Guides to Type Approval formulation (Home Office, 2012) and sought to characterise AOF for use as a suitable reference material and investigate its usefulness as a quality control matrix for approval of devices to be used at the road-side to screen for drug-driving. Synthetic OF could also be used for method development of confirmatory tests. Physical measurements selected as relevant to the intended purpose of the AOF were: pH, kinematic viscosity ($\text{mm}^2 \text{s}^{-1}$), specific gravity (SG), conductivity (mS cm$^{-1}$), freezing point depression (°C) and light-scattering (average particle size [z average]), the distribution of particle sizes (polydispersity index—PDI) and the number of particles present (derived count rate—DCR). The intention was to select a range of physicochemical measurements to determine which, if any, might be affected by changes in the composition of the AOF including any batch to batch variability and storage conditions. This paper indicates the value of the measurements selected some of which, for example, pH and perhaps also SG and conductivity, can be readily reproduced (at low cost) in most analytical laboratories. We wished however also to evaluate some of the more sophisticated approaches such as light scattering to evaluate any lack of stability of the AOF and, especially, its tendency to flocculate.

## EXPERIMENTAL

### 2.1 Preparation of AOF

Bovine mucin from submaxillary glands was obtained from two suppliers Sigma-Aldrich (Dorset, UK) and Merck Millipore (Watford, UK). Potassium thiocyanate, calcium chloride dihydrate, magnesium chloride hexahydrate, ProClin™ 300, Tetronic® 90R4 and Tween® 20 were purchased from Sigma-Aldrich (Dorset, UK). Potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium azide, sodium hydrogen carbonate and urea were purchased from Fisher Scientific (Loughborough, UK). All reagents were of analytical reagent grade apart from Tween® 20, which was BioXtra grade. The AOF was synthesised according to the composition given in Table 1 using analytical balances calibrated to the ISO 17025 standard.

AOF stock solutions (100 ml) were prepared by dissolving weighed amounts of all components with 75-ml ultra-pure water (Elga Maxima, resistivity 18.2 MΩ cm$^{-1}$) in a volumetric flask and then making up to volume with ultra-pure water. For all experiments, AOF solution was transferred to amber glassware and stored upright, for up to 7 days at 4°C, in the dark to minimise any algae formation or microbial growth. Baseline measurements were undertaken to determine the physical properties of the AOF for each experiment.
described below. One operator made up the solutions to avoid an operator effect. Recorded data were stored for comparison with further preparations of AOF in order to assess interbatch consistency. Hanwell radio sensors configured in a Radiolog software system via USB interface helped ensure standardised environmental conditions (Hanwell Solutions Ltd, Letchworth, UK).

### 2.2 Choice of mucin

Two sources of bovine mucin from submaxillary glands were investigated: one from Sigma-Aldrich (Dorset, UK) produced by recombinant DNA technology and one batch from Merck Millipore, Nottingham, UK (lyophilised bovine mucin isolated from cattle). The Sigma-Aldrich material had a declared molecular weight (MW) of 400 kDa and was a white-yellowish powder with ≤2.5% free and 9%–24% bound sialic acid and was prepared using a modified method described by Tettamanti and Pigman.37 The material purchased from Merck Millipore was a flaky, red-pinkish, lyophilised hygroscopic powder stated to contain 74% protein, 12.8% sialic acid, 8.1% galactosamine and 3.4% glucosamine (product information sheet) prepared using the method of Nisizawa and Pigman.38,39

Four batches of 100 ml of AOF containing surfactant and bovine mucin (from each supplier) were prepared (solution 1—Sigma-Aldrich mucin plus Tetronic® 90R4; solution 2—Sigma-Aldrich mucin plus Tween® 20; solution 3—Merck Millipore mucin plus Tetronic® 90R4; solution 4—Merck Millipore mucin plus Tween® 20) (see Table 2).

### 2.3 Choice of surfactant

Two surfactants Tween® 20 (Figure 1) and Tetronic® 90R4 (Figure 2) were explored using the purest available variety of each. Three batches of 100 ml of AOF containing Sigma-Aldrich mucin were prepared with either Tetronic® 90R4 (solution A), Tween® 20 (solution B) or no surfactant (solution C) (see Table 3).

### 2.4 Choice of antimicrobial agent

The efficacy of two antimicrobial agents, sodium azide, primarily active against Gram-negative bacterial species,40 and ProClin™ 300 a broad spectrum microbial inhibitor41 and potentially safer antibacterial

#### TABLE 1

Composition of artificial oral fluid (AOF) (Home Office, 2012)

| Component                                      | Concentration (mg/L) |
|------------------------------------------------|----------------------|
| Potassium chloride                             | 1360                 |
| Bovine mucin (from submaxillary glands)        | 1300                 |
| Sigma-Aldrich® or Merck Millipore®            |                      |
| Potassium dihydrogen phosphate                | 950                  |
| Sodium chloride                                | 860                  |
| Sodium azide® or ProClin™ 300®                | 500 or 20            |
| Sodium hydrogen carbonate                      | 440                  |
| Potassium thiocyanate                          | 250                  |
| Calcium chlorideb                              | 210                  |
| Urea                                           | 180                  |
| Magnesium chlorideb                            | 60                   |
| Tween® 20® or Tetronic® 90R4®                 | 0.09%                |

*aSee methods for specific details.
*bMeasured as hydrates.

#### TABLE 2

Measurements of the physical properties for each solution

| Physical property                  | Normal human saliva | Solution 1 | Solution 2 | Solution 3 | Solution 4 |
|-----------------------------------|---------------------|------------|------------|------------|------------|
| pH                                | 6.60                | 6.71       | 6.76       | 6.76       | 6.67       |
| Specific gravity                  | 1.002–1.012         | 1.004      | 1.004      | 1.004      | 1.004      |
| Specific conductivity (mS cm⁻¹)   | 1.14                | 6.78       | 6.77       | 6.81       | 6.61       |
| Freezing point depression (°C)    | −0.07 to −0.70      | −1.07      | −0.99      | −1.16      | −0.67      |
| Kinematic viscosity (mm² s⁻¹)     | 1.01–1.79           | 1.165      | 1.168      | 1.069      | 1.068      |
| Light scattering³                  | 0.456               | 0.419      | 0.492      | 1.000      |
| z average (nm)                    | 143                 | 144        | 148        | 356        |
| DCR (kcps)                        | 40 500              | 44 400     | 43 200     | 22 700     |

³Light scattering measurements and units: dispersity (D)-polydispersity index PDI, particle size-z average (nm), derived count rate (DCR)-kilocounts per second (kcps).
agent was investigated. AOF containing either sodium azide (500 mg/L), ProClin™ 300 (20 mg/L) or no antimicrobial agent was inoculated with one of five microbial species, namely, two fungal species—Candida albicans (ATCC no. 10231) and Aspergillus brasiliensis (ATCC no. 16404) and three bacterial species—Escherichia coli (ATCC no. 8739), Pseudomonas aeruginosa (ATCC no. 9027) and Staphylococcus aureus (ATCC no. 6538). Fungal and bacterial species were purchased from LGC Standards (Teddington, UK). The choice of microorganisms and the procedure used was in accordance with the "Antimicrobial Effectiveness Testing" as described in the Pharmaceutical Microbiology Manual (2014) issued by the Food and Drug Administration. Initial plate counts were analysed after inoculation.

### 2.5 Characterisation of AOF

The following physical properties of each batch of AOF were determined in singlicate:

- **Appearance, SG and pH measurement**: a 10-ml volume of AOF was withdrawn from fresh 100-ml stock into a glass vial. The appearance (e.g., colour) was noted against a white background and SG, and pH recorded using an automated instrument (Mettler Toledo SC30, Leicester, UK).

- **Specific conductivity** was measured using a Jenway 4510 bench conductivity meter (Jenway, Stowe, UK) with 20-m fresh AOF. The metre was calibrated against 0.01-M potassium chloride solution conductance standard B according to ISO 7888 (Sigma-Aldrich, Dorset, UK).

- **Freezing point depression** was measured using DSC 2920 differential scanning calorimeter (TA Instruments, Elstree, UK) with 10 μL of AOF placed into aluminium hermetic pans (TA Instruments, Elstree, UK), which were sealed by crimping. Ultra-pure water was used as the reference solvent.

| Day   | Characterisation measurement | Solution A  | Solution B  | Solution C |
|-------|------------------------------|-------------|-------------|------------|
|       |                              | Tetronic® 90R4 | Tween® 20 | No surfactant |
| Day 1 | pH                           | 6.69        | 6.74        | 6.72       |
| Day 7 |                              | 6.75        | 6.79        | 6.81       |
| Day 1 | Specific gravity             | 1.004       | 1.004       | 1.004      |
| Day 7 |                              | 1.004       | 1.004       | 1.004      |
| Day 1 | Specific conductivity (mS cm⁻¹) | 6.91         | 6.95        | 6.85       |
| Day 7 |                              | 6.71        | 6.74        | 6.82       |
| Day 1 | Freezing point depression (°C) | −1.21       | −1.18       | −1.23      |
| Day 7 |                              | −1.36       | −1.05       | −0.78      |
| Day 1 | Kinematic viscosity (mm² s⁻¹) | 1.128       | 1.139       | 1.138      |
| Day 7 |                              | 1.063       | 1.105       | 1.056      |
| Day 1 | Light scattering               | 0.803       | 0.982       | 0.826      |
| Day 7 | a Dispersity (b)-PDI          | 0.680       | 0.753       | 0.690      |
| Day 1 | z average (nm)                | 1440        | 1260        | 1280       |
| Day 7 |                              | 2230        | 2760        | 1480       |
| Day 1 | DCR (kcps)                    | 33 200      | 14 600      | 24 600     |
| Day 7 |                              | 17 800      | 11 900      | 19 800     |

*aLight scattering measurements and units: dispersity (b)-polydispersity index PDI, particle size-z average (nm), derived count rate (DCR)-kilocounts per second (kcps).*
Light scattering, measurement of the average particle size (z average), the distribution of particle sizes (PDI) and the DCR, which is a measure of particle size and number, was performed using a Malvern Zetasizer (Malvern Instruments, Malvern, UK) instrument with a He-Ne laser of 677-nm wavelength. A fixed angle of detection of 90° was used to measure the heterogeneity of sizes of particles in AOF\textsuperscript{33} with 1-ml fresh AOF. A technical change to the instrument during servicing led to a recalibration of equipment between experiments. Only data sets within each experiment were compared.

Kinematic viscosity: A calibrated Ostwald (U-tube) viscometer was used to measure kinematic viscosity (mm\textsuperscript{2} s\textsuperscript{-1}) according to ISO 3104 and the British Pharmacopoeia 2001 (incorporating the requirements of the 3rd Edition of the European Pharmacopoeia 1997) using ultra-pure water (kinematic viscosity 0.8926 mm. s\textsuperscript{-1}) as a reference solution with 30-ml fresh AOF.

2.6 | Stability testing

A new batch of AOF was prepared using the surfactant Tween\textsuperscript{®} 20 and the antimicrobial agent ProClin\textsuperscript{™} 300. The physical properties of the AOF were evaluated at room temperature (RT), 4°C and −20°C. The stability testing was undertaken on single samples. AOF was stored for 1 month at each temperature, and pH, SG, specific conductivity, freezing point depression, light scattering and kinematic viscosity measurements were made at days 7, 14, 21 and 28. Samples stored at −20°C were thawed only once for each measurement. An additional batch of AOF stored at −20°C also underwent four freeze-thaw cycles, and a further batch was stored for 3 months at −20°C (no freeze-thaw cycles). All solutions were allowed to adjust to RT before measuring.

2.7 | LC–MS/MS analysis

Cerilliant\textsuperscript{®}-Certified Reference Material THC (1 mg/ml) and d\textsubscript{3}-THC ((6aR,10aR)-6a,7,8,10a-tetrahydro-6,6,9-trimethyl-3-(pentyl-d\textsubscript{3})-6H-dibenzol[b,d]pyran-1-ol. 100 μg/ml) as internal standard, both in methanol, were purchased from Sigma Aldrich (Dorset, UK). Ammonium acetate and glacial acetic acid were purchased from Fisher Scientific (Loughborough, UK), whereas liquid chromatography–mass spectrometry (LC–MS) grade acetonitrile was purchased from VWR (Lutterworth, UK). To illustrate the performance of the AOF, a batch of AOF was spiked with THC, to a concentration of 50 ng/ml. This solution was added immediately before diluting (one in 10 with aqueous solution containing 10-mM ammonium acetate, 0.1% glacial acetic acid and 5% acetonitrile) each aliquot (100 μl) of stored sample for to LC–MS/MS analysis.

An Agilent 6460 triple quadrupole mass spectrometer with an electrospray ionisation (ESI) source and 1260 binary pump attached (Waldbron, Germany) with a Waters Acquity\textsuperscript{®} UPLC\textsuperscript{®} HSS T3 C\textsubscript{18} (1.8 μm, 2.1 × 50 mm) column was used for the analysis. The solvent conditions were mobile phase A (10-mM ammonium acetate and 0.1% glacial acetic acid in water) and B (10-mM ammonium acetate, 0.1% glacial acetic acid and 10% water in acetonitrile) at a flow rate of 0.4 ml/min. The gradient was programmed as 5% B (0 min), 35% B (4 min), 100% B (8 to 11.5 min), 5% B (11.51 to 13.5 min). The injection volume was 10 μl. The mass spectrometer was operated in the positive ionisation multiple reaction monitoring mode (THC, m/z 315–193, d\textsubscript{3}-THC m/z 318–196). 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.

3 | RESULTS AND DISCUSSION

3.1 | Preparation of AOF

AOF is easily prepared by dissolving its components (salts, surfactant, antimicrobial agent and mucin) in water. Visual observations revealed that AOF solutions 1 and 2 appeared as whitish, translucent suspensions, whereas solutions 3 and 4 appeared red-pinkish in colour. Solution 4 was less transparent than solution 3. Solutions 1 and 2 were odourless, whereas solutions 3 and 4 had a ‘raw flesh-like’ odour. The degree of colouration of each of the solutions depended on the source of mucin used. All solutions appeared to be translucent as discussed in the Section 3.2.

Data from the temperature logging device confirmed that temperatures within the laboratory remained at 22.5°C ± 2.5°C.

3.2 | Choice of mucin

Human OF can be weakly alkaline to weakly acid, the pH ranging from approximately 6.0 to 8.0,\textsuperscript{44} with an optimum pH of 6.6,\textsuperscript{45} although results vary (pH 5.3 to 7.8) according to the method of sample collection and the state of stimulation.\textsuperscript{46} Measurements of the baseline pH of each AOF solution (Table 2) demonstrate that the different solutions of AOF were within the known range for human OF. The SG of human OF has been reported to be between 1.002 and 1.012,\textsuperscript{45} and the SG of the AOF matched that for normal human OF (Table 2).

The viscosity of human OF is due mainly to the presence of mucin. Human OF mucin consists of two main types: a high MW mucin, MUC5B (MW 2-40 MDa) and a low MW mucin, MUC7 (MW approx. 150 kDa). MUC5B is responsible for the viscosity of OF. Although the viscosity of stimulated and unstimulated whole human OF has been evaluated many times, no consensus has been
reached on an agreed value. Foglio-Bonda et al. measured kinematic viscosity of human saliva collected from healthy young volunteers and found it to be 1.40 ± 0.39 mm² s⁻¹ (Relative Standard Deviation (RSD) % = 27.81), which was within the range of our AOF, although at the lower end perhaps due to the use of bovine mucin. There appears to be no clear agreement with respect to the MW of mucin in bovine submaxillary glands. Sandberg et al. determined the average MW of bovine submaxillary mucin to be 0.8–4.2 MDa measured by light scattering experiments but pointed out that this depended on how the material was purified. Tettamanti and Pigman have reported the MW of the major mucin after extraction and purification from fresh bovine submaxillary glands (the portion not adsorbed to the hydroxyapatite gel) to be 400 kDa, which may explain the smaller kinematic viscosity observed by us. There were also differences in the kinematic viscosity of the AOF solutions with Merck Millipore mucin forming less viscous solutions than those using Sigma-Aldrich mucin.

Although data are limited, one report records the specific conductivity for human saliva, on six volunteers, as 1.14 mS cm⁻¹. The specific conductivity of AOF designed for orthodontic research varied between 3 and 27 mS cm⁻¹. The specific conductivity of our AOF had an average value of 6.74 mS cm⁻¹ within the reported range for orthodontic AOF and showed general consistency across the solutions (see Table 2).

Baseline measurements for the freezing point depression (°C) were varied. Solutions containing Tween® 20 showed less variability than those containing Tetronic® 90R4. The freezing point depression of the solutions was lower than that of ultra-pure water, which is to be expected because solutions are always less than that of the pure solvent and directly proportional to the molality of the solute. The reported freezing point depression of human saliva ranges from −0.15°C to −0.33°C dependent on the rate of salivary flow per unit time⁰⁰ whereas Lentner reported it to be between −0.07 and −0.70°C. Solution 4 best represented this range (Table 2).

At baseline, the light scattering measurements (z average and PDI) of solutions 1, 2 and 3 indicated that aggregates were not being measured due to poly-dispersal (high DCR values). Solution 2 (Sigma-Aldrich mucin + Tween® 20) became more opaque after 1 week. The opalescence observed may indicate physical instability of the formulation because of the presence of aggregates. The larger values (more than twice that of other solutions) obtained from the light scattering measurements of solution 4 were most probably due to the aggregation of the particles as evidenced by the smaller DCR value for the Merck Millipore mucin and surfactant Tween® 20 combination (Table 2). Subsequent experiments used AOF with mucin from Sigma-Aldrich because this material best matched the profile of human saliva.

3.3 | Choice of surfactant

Tween® 20 was the preferred surfactant over Tetronic® 90R4. It was found that Tween® 20 provided more consistent data than Tetronic® 90R4 when drugs spiked into AOF were analysed by LC–MS, which will be described in more detail in our next publication.

3.4 | Choice of antimicrobial agent

In general, a smaller number of colonies was observed with AOF containing ProClin™ 300 than with the AOF containing sodium azide. ProClin™ 300 was found to be more effective in preventing the growth of E. coli and S. aureus, but not P. aeruginosa. This matches documented literature. However, P. aeruginosa is not normally found in the oral cavity, but was chosen as an example of a representative Gram negative bacterium. The antimicrobial agents were less successful in limiting growth of the fungi C. albicans and A. brasiliensis.

3.5 | Characterisation of AOF

Three batches of AOF containing Sigma-Aldrich mucin were prepared with either Tetronic® 90R4, Tween® 20 or no surfactant (solutions A, B and C). Overall, the measurements of the physical properties of each solution (Table 3) obtained on day 1 appeared to be very similar to the baseline measurements (Table 2) demonstrating the reproducibility of the AOF preparation process. Upon preparation (day 1), all solutions appeared pale white in colour with no observable particulate. Table 3 shows the physical properties measurements of AOF containing Sigma-Aldrich mucin with either Tetronic® 90R4 (solution A), Tween® 20 (solution B) or no surfactant (solution C) for day 1 and day 7.

At day 7, in two solutions (A and B), a fine opaque film was observed at the bottom of the containers but not in the surfactant-free solution (solution C). The film did not appear to redissolve on shaking. The slight increase in pH may be indicative of solution degradation but was universal across the three solutions. Similarly, there were negligible differences in the conductivity measurements between solutions A and B containing surfactants after 7 days; the fall in conductivity was very small in the solution without surfactant. This is suggestive of a saturated solution with solutes making it unstable.

In the solution without surfactant, changes in the freezing point depression of AOF was most pronounced after 7 days, falling by 45% to −0.78°C compared with values for human saliva (−0.15 to −0.33°C). Tween® 20 was least affected after storage for 7 days. The baseline measurements for freezing point depression were more pronounced in the second experiment (Tween® 20 −1.18°C and Tetronic® 90R4 −1.21°C) when compared with the initial experiment (Tween® −0.99°C and Tetronic® 90R4 −1.07°C).

Similarly, there were differences in light scattering measurements. The average particle size (z average) was larger on day 7 in both solutions containing surfactants (solutions A and B) increasing by approximately, 50% after day 7 (see z average values), although this was less marked in the solution with no surfactant. The DCR decreased over
time in all three solutions suggesting particle flocculation. The increase in particle size (aggregates) and decrease in the number of particles after 7 days supported these results (Table 3).

In unstimulated whole saliva, kinematic viscosity has been reported to be $1.40 \pm 0.39 \text{ mm}^2 \text{s}^{-1}$ (RSD % = 27.81) and in healthy volunteers viscosity decreased exponentially as a function of time after sampling, reaching a plateau around $1.12 \text{ mm}^2 \text{s}^{-1}$ (1.12 cSt).\(^4\) Our solutions responded in a similar way, although solution B behaved most like human saliva. We deduced that the preferred combination of surfactant and mucin for our AOF was solution B containing Tween® 20 (Table 3).

### 3.6 Stability testing

#### 3.6.1 Stability of the physical properties of AOF over time using Sigma-Aldrich mucin and Tween® 20 surfactant with ProClin™ 300 antimicrobial agent and at three different temperatures

The physical properties of AOF prepared using the surfactant Tween® 20 at a concentration of 900 mg/L with the antimicrobial agent ProClin™ 300 at 20 mg/L and stored at three different temperatures for 28 days are shown in Table 4.

| Temperature | Measurement timeline | % CV |
|-------------|---------------------|------|
|             | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 |
| **pH**      |       |       |        |        |        |
| RT          | 6.71  | 6.83  | 6.88   | 6.87   | 6.89   | 1.1   |
| 4°C         | 6.90  | 6.97  | 6.95   | 6.89   |         | 0.6   |
| −20°C       | 6.76  | 6.60  | 6.74   | 6.60   |         | 1.3   |
| **Specific gravity** |       |       |        |        |        |
| RT          | 1.004 | 1.003 | 1.003  | 1.003  | 1.003  | 0.0   |
| 4°C         | 1.004 | 1.003 | 1.003  | 1.003  | 1.003  | 0.0   |
| −20°C       | 1.004 | 1.004 | 1.004  | 1.004  | 1.004  | 0.0   |
| **Kinematic viscosity (mm² s⁻¹)** |       |       |        |        |        |
| RT          | 1.085 | 1.095 | 0.975  | 0.999  | 0.987  | 5.6   |
| 4°C         | 1.047 | 1.014 | 0.984  | 0.975  |         | 3.2   |
| −20°C       | 0.975 | 0.966 | 0.984  | 0.978  |         | 0.8   |
| **Specific conductivity (mS cm⁻¹)** |       |       |        |        |        |
| RT          | 6.10  | 5.70  | 5.83   | 5.55   | 6.82   | 7.5   |
| 4°C         | 5.73  | 5.90  | 5.55   | 6.23   |         | 4.9   |
| −20°C       | 6.47  | 6.00  | 5.63   | 5.80   |         | 6.1   |
| **Light scattering** |       |       |        |        |        |
| Dispersity (D)-PDI |       |       |        |        |        |
| RT          | 0.869 | 0.644 | 0.709  | 0.838  | 0.859  | 12.9  |
| 4°C         | 0.649 | 0.695 | 0.697  | 0.584  |         | 8.1   |
| −20°C       | 0.688 | 0.881 | 0.831  | 0.801  |         | 10.2  |
| z average (nm) |       |       |        |        |        |
| RT          | 449   | 701   | 634    | 1070   | 1690   | 54.1  |
| 4°C         | 716   | 690   | 556    | 911    |         | 26.3  |
| −20°C       | 599   | 864   | 1040   | 707    |         | 32.7  |
| DCR (kcps)  |       |       |        |        |        |
| RT          | 7540  | 7170  | 6140   | 7750   | 8440   | 11.4  |
| 4°C         | 7330  | 6870  | 7070   | 7590   |         | 4.3   |
| −20°C       | 1340  | 1340  | 1500   | 1280   |         | 6.9   |

Note: RT = Room temperature 22.5 ± 2.5°C. Light scattering measurements and units: dispersity (D)-polydispersity index (PDI), particle size-z average (nm), derived count rate (DCR)-kilocounts per second (kcps). Variability in light scattering measurements is thought to be in large part due to a technical change to the Malvern Zetasizer Instrument during servicing that occurred while our experiments were conducted. Nevertheless, visual observation clearly showed where particulates were present and confirmed by these light scattering measurements.
3.6.2 | Stability over time

There were marginal differences in pH (increasing at RT and 4°C; decreasing at −20°C) and SG over the 28-day study period. Kinematic viscosity diminished marginally over time, and the AOF was always less viscous than the estimated value for human unstimulated whole saliva (1.40 ± 0.39 mm² s⁻¹, RSD % = 27.81).⁴⁷ Specific conductivity increased over time and was greater after 28 days storage at RT compared with lower temperatures.

Light scattering data also demonstrated small changes over time; the average particle size (z average) increased after 28 days compared with day 0 at all temperatures. Over time, the distribution of particle sizes (PDI) changed less and the number of particles present (DCR) in the AOF was most stable when stored at −20°C (Table 4). Our results indicate that the storage conditions of AOF may be important in terms of impact on particle dispersal. Storage at different temperatures did not have an impact on the stability of the AOF over time for pH, SG or kinematic viscosity (mm² s⁻¹), although the viscosity of AOF was greater at all time points when stored at −20°C compared with that at RT. After storage at −20°C differences were observed for light scattering measures (see z average values) and to a lesser extent at 4°C. The DCR decreased over time suggesting particle aggregation (Table 4). Aggregates were visible in all samples stored at −20°C after thawing, which is consistent with the fall in DCR.

3.6.3 | Stability of the physical properties of AOF over time using Sigma-Aldrich mucin and Tween® 20 surfactant with ProClin™ 300 following four freeze–thaw cycles at −20°C

A separate batch of AOF stock stored at −20°C underwent four freeze–thaw cycles and the physical properties were compared with the physical properties of AOF at day 0 (Table 5). The freeze–thaw process did not appear to affect SG, specific conductivity or kinematic viscosity. The freeze–thaw process however had a noticeable effect on the light scattering measurements (Table 5). The average particle size (z average) reduced from 1650 to 289 nm after the first freeze–thaw cycle and remained low (248) at 28 days suggesting dispersal of particulates. The distribution of particle sizes also changed (PDI fell by Table 5 Physical properties of artificial oral fluid (AOF) following four freeze–thaw cycles over 28 days using Sigma-Aldrich mucin and Tween® 20 surfactant

| Temperature | Measurement timeline | % CV |
|-------------|----------------------|------|
|             | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 |     |
| pH          |       |       |       |       |       |     |
| RT          | 6.71  | 6.83  | 6.88  | 6.87  | 6.89  | 1.1 |
| Freeze–thaw | 6.79  | 6.83  | 6.76  | 6.88  | 0.8   |     |
| Specific gravity |       |       |       |       |       |     |
| RT          | 1.004 | 1.003 | 1.003 | 1.003 | 1.003 | 0.0 |
| Freeze–thaw | 1.003 | 1.003 | 1.003 | 1.003 | 0.0   |     |
| Kinematic viscosity (mm² s⁻¹) |       |       |       |       |       |     |
| RT          | 1.085 | 1.095 | 0.975 | 0.999 | 0.987 | 5.6 |
| Freeze–thaw | 1.050 | 0.975 | 0.972 | 0.990 | 3.6   |     |
| Specific conductivity (mS cm⁻¹) |       |       |       |       |       |     |
| RT          | 6.10  | 5.70  | 5.83  | 5.55  | 6.82  | 8.3 |
| Freeze–thaw | 5.73  | 5.81  | 5.48  | 6.26  | 5.6   |     |
| Light scattering |     |       |       |       |       |     |
| Dispersity (Đ)-PDI |       |       |       |       |       |     |
| RT          | 0.869 | 0.644 | 0.709 | 0.838 | 0.859 | 12.9|
| Freeze–thaw | 1.000 | 0.515 | 0.588 | 0.511 | 35.8  |     |
| z average (nm) |       |       |       |       |       |     |
| RT          | 449   | 701   | 634   | 1070  | 1690  | 54.1|
| Freeze–thaw | 1650  | 289   | 111   | 248   | 125.5 |     |
| DCR (kcps) |       |       |       |       |       |     |
| RT          | 7537  | 7170  | 6140  | 7750  | 8440  | 11.4|
| Freeze–thaw | 5100  | 1280  | 1200  | 1410  | 84.7  |     |

Note: RT = Room temperature 22.5 ± 2.5°C. Light scattering measurements and units: dispersity (Đ)-polydispersity index (PDI), particle size-z average (nm), derived count rate (DCR)-kilocounts per second (kcps). Variability in light scattering measurements is thought to be in large part due to a technical change to the Malvern Zetasizer Instrument during servicing that occurred while our experiments were conducted. Nevertheless, visual observation clearly showed where particulates were present and confirmed by these light scattering measurements.
50%) after the first cycle and then remained stable during future freeze–thaw cycles. The DCR decreased to about a quarter of the original number of particles suggesting particle flocculation due to the freeze–thaw cycles (Table 5). Whembolua et al.\textsuperscript{54} suggests that human OF should be refrigerated immediately at 4°C if freezing is not possible to minimise degradation of any unstable analytes and to prevent bacterial growth but that storage at this temperature should be for no longer than necessary before freezing at or below –20°C. Although in our study the freeze–thaw cycles induced the particle flocculation, a subsequent centrifugation step afterwards may allow easier handling.

### 3.6.4 The physical properties of AOF over time (storage for 3 months at –20°C) using Sigma-Aldrich mucin and Tween\textsuperscript{®} 20 surfactant with ProClin\textsuperscript{™} 300

In order to investigate whether long-term storage of prepared material was possible, we analysed a batch of AOF stored for 3 months at –20°C (Table 6). There was little difference in the pH, SG and specific conductivity after 3 months. We observed a slight decrease in kinematic viscosity and changes in light scattering measurements (Table 6).

### 3.7 LC–MS/MS analysis

The results of the LC–MS/MS analysis of the AOF spiked at 50 ng/ml with THC and stored at different temperatures and time points are shown in Figure 3 relative to the initial measured concentration.

These initial data show that THC appears to be stable in the AOF for 7 days at 4°C, but perhaps less so at RT or at –20°C. Further evaluation of the stability of THC and other drugs of abuse in AOF at different storage temperatures and times is the subject of a further communication.

### 4 CONCLUSIONS

We investigated the physical properties of AOF to determine the usefulness of AOF as a traceable matrix for drug testing purposes. Evaluation of pH, SG, specific conductivity (mS cm\textsuperscript{−1}), freezing point depression (°C), light-scattering and kinematic viscosity (mm\textsuperscript{2} s\textsuperscript{−1}) showed our AOF to be a stable and reliable matrix. Two different types of mucin were investigated and mucin (produced by recombinant DNA technology) from Sigma-Aldrich better reflected the characteristics of human OF in terms of viscosity (spinnbarkeit) and particulate distribution when compared with Merck Millipore mucin; the user should take care that different batches give consistent results. The use of a surfactant was important to stabilise the viscosity of the AOF. Tween\textsuperscript{®} 20 maintained particle size and uniformity more consistently than Tetronic\textsuperscript{®} 90R4.

The revised AOF formulation incorporated Tween\textsuperscript{®} 20 alongside the antibacterial ProClin\textsuperscript{™} 300. We recommend using the AOF formulation with Sigma-Aldrich mucin, Tween\textsuperscript{®} 20 and ProClin\textsuperscript{™} 300. Tween\textsuperscript{®} 20 was found to provide more consistent data than Tetronic\textsuperscript{®} 90R4 when drugs spiked into AOF were analysed by

---

**TABLE 6** Physical properties of AOF following storage at –20°C for 3 months (Sigma-Aldrich mucin and Tween\textsuperscript{®} 20 surfactant)

| Measurement               | Day of AOF preparation | 3 months (–20°C) |
|---------------------------|------------------------|------------------|
| pH                        | 6.70                   | 6.73             |
| Specific gravity          | 1.0036                 | 1.0035           |
| Specific conductivity     | 5.69                   | 6.75             |
| Kinematic viscosity       | 0.981                  | 0.975            |
| Light scattering\textsuperscript{a} | 0.972                  | 0.918            |
| Dispersity (Đ)-PDI        | 230                    | 1339             |
| z average (nm)            | 4200                   | 1621             |
| DCR (kcps)                |                        |                  |

Note: Particle size-z average (nm), derived count rate (DCR)-kilocounts per second (kcps).

Abbreviation: AOF, artificial oral fluid.

\textsuperscript{a}Light scattering measurements and units: dispersity (Đ)-polydispersity index PDI.

**FIGURE 3** Liquid chromatography with tandem mass spectrometry (LC–MS/MS) normalised (relative to day 0) data for artificial oral fluid (AOF) spiked with THC at 50 ng/ml and stored at room temperature (RT), 4°C or –20°C for 7 days [Colour figure can be viewed at wileyonlinelibrary.com]
LC–MS. ProClin™ 300 was the preferred choice over the more toxic antibacterial sodium azide. We have established that freezing AOF at or below −20°C immediately after preparation helps preserve sample integrity. However, the flocculation of the particles was noticed after storage at −20°C.

This study provides evidence of the suitability of AOF for quality assurance purposes using Sigma-Aldrich mucin and Tween® 20 such as would be needed when large quantities of OF were required for widescale drug testing purposes such as for a national road-side drug testing scheme. No comparison (with reproducibility in mind) with human OF has been made, which would in any case be difficult given the variability of human OF. This is clearly a scientific weakness that needs to be kept in mind when considering the use of human OF in place drug testing programmes and roadside drug tests will be carried out.

ACKNOWLEDGEMENTS
Funding was received from the Home Office Centre for Applied Science and Technology, HOS/13/038: Stability Testing of Drug Solutions. We are grateful to the Institute of Pharmaceutical Sciences, School of Cancer and Pharmaceutical Sciences, King’s College London for their support with equipment and expertise.

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

ORCID
Ivana Gavrilović https://orcid.org/0000-0003-4942-6398

REFERENCES
1. Choo RE, Huestis MA. Oral fluid as a diagnostic tool. Clin Chem Lab Med. 2004;42(11):1273-1287.
2. Drummer OH, Verstraete A. Special edition of forensic science international detection of drugs in oral fluid. Forensic Sci Int. 2005;150 (2–3):117-117.
3. Drummer OH. Introduction and review of collection techniques and applications of drug testing of oral fluid. Ther Drug Monit. 2008;30(2): 203-206.
4. Vindenes V, Lund HME, Andresen W, et al. Detection of drugs of abuse in simultaneously collected oral fluid, urine and blood from Norwegian drug drivers. Forensic Sci Int. 2012;219(1–3):165-171.
5. Dyer KR, Wilkinson C. The detection of illicit drugs in oral fluid: another potential strategy to reduce illicit drug-related harm. Drug Alcohol Rev. 2008;27(1):99-107.
6. Mehta N, Kunef K, Shaparin N, Stripp R, Borg D, Fey E. Can oral fluid replace urine and blood drug testing as the gold standard in the pain management industry? J Pain. 2015;16(4):57-57.
7. Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta. 2007;383(1–2):30-40.
8. Wolf K, Agombar R, Clatworthy A, et al. Expert Panel on drug driving: Alternative matrices for confirmatory testing. Department for Transport. In: SB-2988 RR, ed2017. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/624915/expert-panel-report.pdf. Accessed July 17, 2020.
9. Crouch DJ, Walsh JM, Flegel R, Canganelli L, Baudys J, Atkins R. An evaluation of selected oral fluid point-of-collection drug-testing devices. J Anal Tox. 2005;29(4):244-248.
10. Gjerde H, Langel L, Favretto D, Verstraete AG. Detection of illicit drugs in oral fluid from drivers as biomarker for drugs in blood. Forensic Sci Int. 2015;256:42-45.
11. Milanowski M, Pomstowski P, Ligor T, Buszewski B. Saliva—volatile biomarkers and profiles. Crit Rev Anal Chem. 2017;47(3):251-266.
12. Veerman ECI, Valenti-jbenz M, Amerongen AVN. Viscosity of human salivary mucins—effect of pH and ionic-strength and role of sialic acid. J Biol Buccale. 1989;17(4):297-306.
13. Sandberg T, Carlsson J, Ott MK. Interactions between human neutrophils and mucin-coated surfaces. J Mater Sci-Mater. M. 2009;20(2):621-631.
14. Berg CH, Lindh L, Amebrant T. Intraoral lubrication of PRP-1, statherin and mucin as studied by AFM. Biofouling. 2004;20(1):65-70.
15. Gohara K, Ansai T, Koseki T, et al. A new automatic device for measuring the spininbarkeit of saliva: the Neva Meter. J Dent. 2004;32(4):335-338.
16. Food and Drug Administration Office of Regulatory Affairs ORA Laboratory Manual Volume II. https://www.fda.gov/media/73979/download. Accessed July 14, 2020.
17. Li-Hui W, Chuan-Quan L, Long Y, Ru-Liu L, Long-Hui C, Wei-Wen C. Gender differences in the saliva of young healthy subjects before and after citric acid stimulation. Clin Chim Acta. 2016;460:142-145.
18. Dawes C. Circadian-rhythms in human salivary flow-rate and composition. J Physiol-London. 1972;220(3):529-545.
19. Dawes C, Jenkins GN. Effects of different stimuli on composition of saliva in man. J Physiol-London. 1964;170(1):86-100.
20. Proctor GB. The physiology of salivary secretion. Periodontol 2000;2000. 2016;70(1):11-25.
21. Hopcraft MS, Tan C. Xerostomia: an update for clinicians. J Pain. 2015;16(3):117–117.
22. Lopez-Pintor RM, Casanas E, Gonzalez-Serrano J, et al. Xerostomia, hyposalivation, and salivary flow in diabetes patients. J Diabetes Res. 2016;1–15.
23. Quock RL. Xerostomia: current streams of investigation. Or Surg or Med or pa. 2016;122(1):53-60.
24. Sniegoski LT, Waddell J, Welch MJ, Fatah AA. Evaluation of Oral Fluid Testing Devices https://tsapps.nist.gov/publication/get_pdf.cfm?pub_id=902366. Accessed July 13, 2020.
25. Fratto G, Manzon L. Use of psychotropic drugs and associated dental diseases. Int J Psychiat Med. 2014;48(3):185-197.
26. Glare P, Walsh D, Sheehan D. The adverse effects of morphine: a prospective survey of common symptoms during repeated dosing for chronic cancer pain. Am J Hosp Palliat Care. 2006;23(3):229-235.
27. Thomson WM, Poulton R, Broadbent JM, Al-Kubaisy S. Xerostomia and medications among 32-year-olds. Acta Odontol Scand. 2006;64 (4):249-254.
28. Smidt D, Torpet LA, Nauntofte B, Heegaard KM, Pedersen AML. Associations between oral and ocular dryness, labial and whole salivary flow rates, systemic diseases and medications in a sample of older people. Commun Dent Oral Health. 2011;33(3):276-288.
29. Affoo RH, Foley N, Garrick R, Siqueira WL, Martin RE. Meta-analysis of salivary flow rates in young and older adults. J Am Geriatr Soc. 2015;63(10):2142-2151.
30. Centre for Applied Science and Technology. Preliminary Drug Testing Devices: A Guide to Type Approval Procedures for Drug Testing Devices Used for Transport Law Enforcement in Great Britain. Test Solution Matrix. 2012. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/190174/Guide_to_Type_Approval_Preliminary_Drug_Testing_Devices_2.pdf. Accessed July 17, 2020.

31. Gal JY, Fovet Y, Adib-Yadzi M. About a synthetic saliva for in vitro studies. Talanta. 2001;53(6):1103-1115.

32. Manosroi A, Pattamapun K, Khositsuntiwong N, et al. Physicochemical properties and biological activities of Thai plant mucilages for artificial saliva preparation. Pharm Biol. 2015;53(11):1653-1660.

33. Lapiedra RC, Gomez GE, Sanchez BP, Pereda AA, Turner MD. The liquid-liquid phase separation in protein solutions. Mol Pharm. 2016;13(3):1431-1444.

34. Skrinjar I, Vucicevic Boras V, Bakale I, et al. Comparison between three different saliva substitutes in patients with hyposalivation. J Maxillofac Oral Surg. 2015;14(3):653-658.

35. Mystkowska J, Car H, Dabrowski JR, Romanowska J, Klekotka M, Milewska AJ. Artificial mucin-based saliva preparation—physicochemical and tribological properties. Oral Health Prev Dent. 2018;16(2):183-193.

36. Pillai K, Akhter J, Mekkawy A, Chua TC, Morris DL. Physical and chemical characteristics of mucin secreted by pseudomyxoma peritonei (PMP). Int J Med Sci. 2017;14(1):18-28.

37. Tettamanti G, Pigman W. Purification and characterization of bovine and ovine submaxillary mucins. Arch Biochem Biophys. 1968;124(1-3):41-50.

38. Nisizawa K, Pigman W. Purification of a glycoprotein from bovine-submaxillary glands. Biochem J. 1960;75:293-298.

39. Nisizawa K, Pigman W. The composition and properties of the mucin clot from cattle submaxillary glands. Arch Oral Biol. 1959;1(2):161-170.

40. Lichtstein HC, Soule MH. Studies of the effect of sodium azide on microbial growth and respiration I the action of sodium azide on microbial growth. J Bacteriol. 1944;47(3):221-230.

41. https://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html? TablePage=111264510. Accessed July 15, 2020.

42. FDA. Pharmaceutical Microbiology Manual 2014 ORA ORA.007. Version 1.2. https://www.fda.gov/files/about%20fda/published/Pharmaceutical-Microbiology-Manual.pdf. Accessed July 17, 2020.

43. Stepto RFT. Dispersity in polymer science. Pure Appl Chem. 2009;81(2):351-353.

44. Guyton AC, Hall JE. Textbook of Medical Physiology. 13th ed. Philadelphia: W.B. Saunders; 2015.

45. Rai B. Oral fluid in toxicology. Intern J Toxicol. 2006;3(2):1-6.

46. Gittings S, Turnbull N, Henry B, Roberts CJ, Gershkovich P. Characterisation of human saliva as a platform for oral dissolution medium development. Eur J Pharm Biopharm. 2015;91:16-24.

47. Foglio-Bonda A, Pattarino F, Foglio-Bonda PL. Kinematic viscosity of unstimulated whole saliva in healthy young adults. Eur Rev Med Pharmacol. 2014;18(20):2988-2994.

48. Sandberg T, Blom H, Caldwell KD. Potential use of mucins as biophysical coatings. I. Fractionation, characterization, and model adsorption of bovine, porcine, and human mucins. J Biomed Mater Res a. 2009;91A(3):762-772.

49. Echipare L, Harju Z. Libre Texts https://chem.libretexts.org/Biochemical-Products.html? TablePage=111264510. Accessed July 17, 2020.

50. Yoshimura H, Matsumoto S, Matsumoto F, Inoue T. Mineral composition in resting saliva as compared with that in saliva secreted by stimulation: I. Physiol Soc Jap. 1963;25(9):441-450.

51. Lentner C. Thermal Properties of Tissues. In: Diem K, Ciba-Geigy LC, eds. Geigy Scientific Tables. Vol.7 Switzerland: Basel; 1975:36.

52. Raut AS, Kalonia DS. Pharmaceutical perspective on opalescence and microbic growth and respiration I the action of sodium azide on microbial growth. J Bacteriol. 1944;47(3):221-230.

53. Winder CL, Al-Adham ISI, Malek S, Buultjens TEJ, Horrocks AJ, Collier PJ. Outer membrane protein shifts in biocide-resistant Pseudomonas aeruginosa PAO1. J Appl Microbiol. 2000;89(2):289-295.

54. Whembolua GLS, Granger DA, Singer S, Kivlighan KT, Marguin JA. Bacteria in the oral and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. Horm Behav. 2006;49(4):478-483.

How to cite this article: Gavrilović I, Musenga A, Cowan D, et al. Artificial oral fluid characterisation: Potential for use as a reference matrix in drug testing. Drug Test Anal. 2021;13: 709–719. https://doi.org/10.1002/dta.2938