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Current technologies and considerations for drug bioanalysis in oral fluid

Drug oral fluid analysis was first used almost 30 years ago for the purpose of therapeutic drug monitoring. Since then, oral fluid bioanalysis has become more popular, mainly in the fields of pharmacokinetics, workplace drug testing, criminal justice, driving under the influence testing and therapeutic drug monitoring. In fact, oral fluid can provide a readily available and noninvasive medium, without any privacy loss by the examinee, which occurs, for instance, during the collection of urine samples. It is believed that drug concentrations in oral fluid may parallel those measured in blood. This feature makes oral fluid an alternative analytical specimen to blood, which assumes particular importance in roadside testing, the most published application of this sample. Great improvements in the development of accurate and reliable methods for sample collection, *in situ* detection devices (on-site drug detection kits), and highly sensitive and specific analytical methods for oral fluid testing of drugs have been observed in the last few years. However, without mass spectrometry-based analytical methods, such as liquid chromatography coupled to mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS), the desired sensitivity would not be met, due to the low amounts of sample usually available for analysis. This review will discuss a series of published papers on the applicability of oral fluid in the field of analytical, clinical and forensic toxicology, with a special focus on its advantages and drawbacks over the normally used biological specimens and the main technological advances over the last decade, which have made oral fluid analysis of drugs possible.

Drug detection in **oral fluid** specimens was used for the first time in the late 1970s, for the purpose of therapeutic drug monitoring [1]. Since then, oral fluid analysis has increased enormously, as can be seen by the increase in the number of publications on this matter over the past few years. This coincided with the development of more sensitive and reliable analytical equipment. However, these advances in technological resources were not solely responsible for this situation, as will be discussed further. Indeed, the unique properties of oral fluid and its advantages over other biological matrices played a very important role.

Advantages of oral fluid testing include the fact that the sample is collected noninvasively under direct supervision and without loss of privacy, reducing the risk of an invalid specimen being provided or sample adulteration and/or substitution [2]. Furthermore, it is thought that saliva drug concentrations can be related to free drug concentrations in plasma and, consequently, to the pharmacological effects of drugs [3].

On the other hand, drugs that are ingested orally, as well as those that can be smoked, may be detected in high concentrations in oral fluid following recent use, due to residual amounts

of drug remaining in the oral cavity [4–8]. Therefore, results for these substances may not be accurate because the drug concentration found in the oral fluid may not reflect the blood drug concentration.

Another important disadvantage of studying oral fluid is that examinees are sometimes unable to produce sufficient amounts of material for analysis [2].

For a better understanding of the increasing importance of this biological specimen on bioanalysis, as well as the need for sensitive techniques, this review will briefly discuss oral fluid physiology and the underlying mechanisms of drug incorporation. In addition, collection devices, detected substances and fields of application will be reviewed. Finally, the available analytical techniques for drug detection in oral fluid will be outlined, since their application represents the main goal of this paper.

Physiology & mechanisms of drug incorporation

The terms oral fluid and saliva are often used in an interchangeable manner to designate the same biological specimen. However, oral fluid is the liquid sample obtained from the oral cavity, and constitutes a number of secretions from several

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ORAL FLUID

Alternative biological specimen, extremely useful for the assessment of drug-impaired driving

sources [8], including saliva. In fact, three pairs of major salivary glands (parotid, submaxillary and sublingual), as well as an estimated 450–750 minor accessory salivary glands (situated on the tongue, the buccal mucosae and the palate) and oro-naso-pharyngeal secretions, contribute to some extent to its production. Therefore, oral fluid is generally accepted as a more accurate definition of this mixture of fluids [9].

Oral fluid contains the same electrolytes that are present in other body fluids, including water (99%) and mineral salts. Proteins (mucins) and some enzymes for digestion are also present, but at very low levels. This sample is hypotonic compared with serum, although its ionic concentrations are not constant due to the circadian rhythm.

The pH of oral fluid is approximately 6.8, but it can be higher in the case of an increase in the salivary flow. In this situation, its pH can even be greater than that of plasma [10].

An adult can produce as much as 1000 ml oral fluid per day, and typical flows are approximately 0.05 ml/min while sleeping, 0.5 ml/min while spitting and up to 3 ml/min while chewing gum [11]. This flow rate may be influenced by several factors, including the individual's emotional state, pregnancy or menopausal-related hormonal changes, or by the use of drugs [9].

One common feature when collecting oral fluid samples is the so-called 'dry mouth syndrome'. This can be due not only to the anxiety provoked by the collection procedure, but also to a lack of proper hydration by the examinee [12]. Furthermore, the use of anti-adrenergic and anticholinergic drugs, or even of illicit drugs such as opiates, amphetamines and cannabis [9], may also contribute to this syndrome.

Different mechanisms for drug incorporation are thought to occur: passive diffusion through the membrane, active processes against a concentration gradient, filtration through pores in the membrane and pinocytosis [3,13]. Nevertheless, most drugs appear to be incorporated by a simple passive diffusion process that depends on their physicochemical properties (e.g., pKa and molecular weight), the degree of binding to plasma proteins and the pH of both blood and oral fluid [9,14].

Therefore, nonionized lipophilic drugs can easily cross the barrier between plasma and saliva, and their concentration in the latter is dependent on the nonprotein-bound plasma concentration. The low concentrations of diazepam and other benzodiazepines usually

seen in this specimen represent a valid example of this dependence, since these compounds are highly protein bound [15,16].

Drugs that are basic in nature, such as amphetamines, cocaine and opiates, generally have higher concentrations in oral fluid than in blood, whereas acidic drugs have much lower concentrations. The concentrations of Δ^9 -tetrahydrocannabinol (THC) in this specimen are more variable in oral fluid than in blood [4], possibly due to the deposition of THC in the oral cavity. Furthermore, current belief is that the majority of THC found in oral fluid originates from this depot of active THC [6], since little THC is secreted into saliva [17]. However, this effect is also seen for other drugs, such as nicotine after tobacco smoking and buprenorphine after sublingual administration [12].

The pH of oral fluid plays an important role in the case of weakly basic drugs, and can greatly affect their salivary concentrations. Indeed, those drugs whose pKa is close to the saliva's pH will have their degree of ionization dramatically altered with pH changes, and this will be reflected in their saliva-to-plasma ratio [10]. This influence of the salivary pH on the saliva-to-plasma ratio of several drugs is perhaps the reason why ratios determined experimentally differ from theoretical values calculated from the Henderson–Hasselbach equation [3,18].

On the other hand, with an increase in the flow rate, the composition of the specimen will change, including its pH, and therefore the circumstances of the collection can affect the production of oral fluid, which in turn will also change the drug content [8]. For this reason, the protocol for saliva collection can differ depending on the study (with or without stimulation) and may be highly important for the determination of saliva-to-plasma ratios [10].

However, care should be taken in the establishment of these ratios, since significant intra- and inter-individual differences exist, which have been discussed elsewhere [19–25]. Nevertheless, these differences are not so large in the case of alcohol and antiepileptic drugs [22,25].

The research concerning oral fluid analysis in the case of alcohol consumption has been reviewed by Drummer [12], and the oral fluid-to-plasma concentration of this drug is similar to that predicted based on the water content of the two fluids, and has been used to assess alcohol exposure.

One important feature regarding oral fluid analysis is that, contrary to what happens when other biological specimens such as

blood or urine are analyzed, the parent drug is the main compound detected, rather than its metabolites.

It should be noted, however, that there is the possibility of passive contamination in this type of analysis. This can occur mainly at two levels: by exposure to an environment where drugs are being consumed by others (e.g., exposure to cannabis smoke) [26] and by contamination from drugs taken orally, meaning that the detected drugs may not proceed from the bloodstream. Therefore, this possibility should be taken into account in the interpretation of the obtained results.

Collection procedures & devices

There are two major advantages of oral fluid testing. First, as discussed above, drug concentrations in this specimen can be related to plasma free drug concentrations and, consequently, to the pharmacological effects of drugs. Second, saliva collection is made in a noninvasive and simple manner (under close observation, if necessary), without infringing on the examinee's privacy. This latter characteristic makes on-site specimen collection easy to perform, which is advantageous in the assessment of drug-impaired driving situations. Furthermore, the collection of this specimen is less liable to adulteration or substitution in comparison with urine sampling [27–29].

A variety of methods are available for oral fluid collection, with or without stimulation, and these include spitting, draining, suction and collection on various types of absorbent material. However, care should be taken since the concentration of the drugs can be affected by the collection procedure used.

Several techniques may be used to collect stimulated saliva, the simplest of which involves movements of the tongue, cheek or lip, without any external stimulus [30,31]. On the other hand, this stimulation can be made mechanically by chewing a variety of materials, such as paraffin wax, Parafilm®, teflon, rubber bands or gum base [13,32]. Likewise, a lemon juice drop or citric acid can be placed in the mouth to provide a gustatory stimulus for saliva production [11,30,32,33]. Following this stimulation, saliva can then be spat, suctioned, absorbed or swabbed for collection [33].

This production stimulation may present several problems capable of compromising the accuracy of the test, however. For instance, there are some drugs and/or metabolites that can be absorbed by Parafilm, and paraffin contains compounds that may affect chromatographic

analysis [34]. Furthermore, it is possible that the salivary composition is changed by the stimulation process, thereby potentially affecting drug concentrations in oral fluid [13,35]. In addition, citric acid changes saliva pH, which may alter drug concentrations in this specimen, and has also been shown to alter immunoassay drug test results [30,32]. In fact, acidic stimulation has been shown to lower codeine [29,36], methamphetamine [37] and cocaine [38] concentrations in oral fluid.

Nonstimulated saliva can be collected by the draining method, which is performed by allowing saliva to drip from the mouth into a collection container [33], or can be easily obtained by spitting, providing a neat sample. However, this is relatively viscous and less easily pipetted than, for instance, urine, and its collection can pose potential occupational health and safety problems. In addition, and because it may also be contaminated with food, it may not provide a fluid of uniform concentration.

Therefore, the sample is typically collected using an absorbent pad/foam, which is subsequently squeezed or mixed into a diluent (usually buffer) to extract the oral fluid and provide a less-viscous and easier-to-analyze specimen. Some devices have a volume indicator, showing if sufficient oral fluid has already been collected [29,36].

However, the collected sample volume will often be less than 1 ml and, therefore, sophisticated and sensitive detection techniques are mandatory to achieve the desired sensitivity, as will be further discussed.

The advantages and drawbacks of several collection devices have been extensively reviewed [39,40].

One of the problems that used to be associated with these collection devices was that the actual volume of sample was not accurately known. This has been overcome by the use of a volume adequacy indicator in the collection pad. This makes the collected volume well known, improving the accuracy of the measurements in this specimen. Indeed, for example, the Quantisal™ device's indicator turns blue when a sample volume of 1 ml ($\pm 10\%$) is collected [201].

Variable collection and recovery volumes between different devices may cause quantitative differences if it is assumed that:

- A consistent volume is collected
- A consistent volume is recovered from the device
- A consistent amount of drug is recovered from the device

This latter issue is important, since it is well known that analyte recovery from the collection device is concentration dependent and this assumes particular relevance at low concentrations. For this reason, the evaluation of each device's performance in terms of analyte recovery should be targeted at a screening assay cut-off or a relevant physiological concentration.

Another approach to overcome the variability of collected volumes may be the weighing of the collection devices before and after sample collection [41].

A variety of devices for oral fluid testing are commercially available, such as Omni-Sal[®] (Cozart Biosciences Ltd, Abingdon, UK), Salivette[®] (Sarstedt AG, Rommelsdorf, Germany), Intercept[®] (OraSure Technologies, Bethlehem, PA, USA) and Quantisal[™] (Immunalysis Corporation, Pomona, CA, USA). These devices usually consist of a sorbent material that becomes saturated in the mouth of the donor, and the oral fluid is recovered by centrifugation or by applying pressure [42,43]. The device is often placed in a container that contains a stabilizing buffer solution [44]. These buffer solutions are not the same for all the devices that use this type of approach, and this is why these devices show differences in their performance, for example concerning drug recovery. The acceptability of sample collection devices is determined both by the speed of collection and ease of handling, and the referred devices all have their advantages and drawbacks. In addition, attention should be paid to the devices' performance, as some collectors have been shown to absorb drug(s) irreversibly, meaning that the drugs are not able to leach out into the postcollection buffer, impairing the accuracy of quantitative analysis. Several studies have been made on this issue, and it appears that drugs are not affected to the same extent. Some studies have even obtained contradictory results.

Indeed, in one study, a recovery of codeine from the Salivette device of more than 80% was reported [36,45]; while, in another study, lower recoveries (39–42%) were found [29,39]. On the other hand, only approximately 38% of THC is recovered from the Intercept device, whereas recoveries of 3,4-methylenedioxymethamphetamine (MDMA), cocaine, morphine, codeine, diazepam and alprazolam from the same device are much higher [39]. Moreover, the Cozart[®] collector shows good recovery for THC [36,46,47] and methamphetamine [37,48].

Significant differences in drug recoveries from the sampling material are reported in the literature, and these will also lead to variations in the measured concentrations in oral fluid.

Several solutions are proposed to overcome this problem. For instance, a modification of the sampling procedure for the Intercept collector, which consisted of the addition of methanol to the elution buffer, resulted in complete recovery of THC over a large concentration range [41]. Other studies were conducted on the recovery of other drugs from the same device [49,50], and significant losses for 7-aminoclonazepam and lysergic acid diethylamide (LSD) were found. Another study, by Quintela *et al.* [48], evaluated the *in vitro* performance of the Quantisal device for amphetamine, methamphetamine, morphine, codeine, cocaine, benzoylecgonine (BE), methadone, oxazepam and THC. In general, recoveries were higher than 90%, but lower for BE (82%).

For THC, however, high recovery was obtained (81.3–91.4%). Other previous studies, using Salivette, showed that significant amounts of the drugs remained entrapped on the device [51–53].

Another important feature that should be taken into account in oral fluid analysis is drug stability or, more accurately, drug instability in the matrix, which can affect the utility of the collection process [40]. The inherent chemical instability of drugs may be exacerbated in oral fluid by the collection device and/or buffer. This issue is of utmost importance in oral fluid analysis, together with analyte recovery from the collection device, because drug concentrations are generally low in this specimen and, therefore, the detectability of the drug may be limited [8]. Stability of several analytes, such as THC, morphine, 6-acetylmorphine (MAM), BE and designer amphetamines, was evaluated in oral fluid samples after collection and was extensively discussed [29,48,54–57].

The stability of analytes can be affected by a number of conditions, including the use of preservatives in the case of flunitrazepam [58] and storage temperature and duration in the case of MAM [59] or THC [29].

In conclusion, no type of collection device is clearly superior based on design or ease of use. On the other hand, the recovery studies conducted on some devices do suggest that drug desorption may limit the usability of some collection materials. Therefore, more information is required for all drugs likely to be measured in oral fluid, and for each collection device.

Applicability of oral fluid analysis

Oral fluid can be used to assess an individual's exposure to virtually every class of compounds, but **drugs of abuse** are by far the most detected substances. This is mainly due to the deleterious consequences of these substances' misuse, both in workplace medicine and motor vehicle driving. For this reason, scientific literature is prolific in analytical techniques aimed at detecting and quantitating a variety of classes of abused drugs, namely opiates [52,60–65], cannabinoids [54,66–71], amphetamines [21,55,72–77], cocaine [78,79] and benzodiazepines [58,80–87]. Other substances such as ketamine, γ -hydroxybutyrate (GHB) [88,89], antibiotics [90], analgesics [91,92], antitussives [93], cyanides and other tobacco compounds [94–96], and sildenafil [97], have also been analyzed. Oral fluid has additional relevance in the case of opiate testing, since high amounts of 6-monoacetylmorphine can be detected in oral fluid in the case of heroin consumption, as well as detectable amounts of heroin itself, and therefore heroin abuse can be easily detected [12].

Oral fluid presents a wide field of analytical applications that take advantage of the aforementioned advantages [2] concerning, for example, therapeutic drug monitoring [1,25,45,80,98,99], pharmacokinetic studies [4,5,19,37,100], workplace medicine [27,101,102] or even detection of illicit drugs in driving situations [47,103–112].

The application of oral fluid analysis to assess drug prevalence is definitely desired, and a positive correlation to impairment has already been shown [106,113]. However, it should be pointed out that, for most substances, a measured oral fluid concentration cannot be used to predict the corresponding plasma concentration, due to the intra- and inter-individual differences discussed earlier.

Nevertheless, the assessment of drug-impaired driving is one of the most impacting applications of oral fluid testing, which has contributed to the development of several collection materials for laboratorial analysis and collection devices designated for on-site applications.

Analytical technologies

Due to developments in technology, it is possible to detect and quantitate drugs in biological specimens at levels that were unreachable a few years ago. As discussed earlier, one of the main disadvantages of oral fluid is the fact that one must be able to detect very small amounts of drugs, due both to their low concentration in this specimen and to the fact that usually there is little sample

available for analysis (often less than 1 ml). This latter drawback may be manageable by the use of multianalyte methods, providing the optimization of sample volume and eventually of confirmation analysis, if necessary. Therefore, the development of this type of method is highly desirable for oral fluid drug testing. For instance, Gunnar *et al.* have published a method by gas chromatography (GC)–mass spectroscopy (MS) that is capable of determining 30 derivatized drugs in 250 μ l of oral fluid [49]. A few LC–MS/MS multianalyte methods have also been published, allowing the simultaneous determination of several abused drugs [50,114,115], benzodiazepines and hypnotics [50,115]. A method for the simultaneous determination of 49 substances, including some antipsychotics and antidepressants, has also been published [116]. Therefore, sensitive and selective techniques are mandatory to achieve the desired low levels. The main analytical techniques usable for drug detection, including their advantages and drawbacks and the main parameters that can affect the analysis, will now be discussed, in light of existing literature on the matter.

■ On-site collection devices

The increased use of illegal drugs gave rise to the need for quick and reliable methods, which have been developed and improved since the 1990s [103], for the fast screening of drugged drivers.

Portable devices have been developed for drug detection in several biological fluids, namely urine, oral fluid and sweat. The performance of various urine on-site screening tests has been evaluated [117,118]. However, as stated previously, oral fluid concentrations of drugs can be related to their plasma concentrations and, therefore, to the pharmacological state of the individual [119,120], while drug detection in urine does not necessarily mean that the individual was influenced at that specific time [121]. Therefore, and due to the ease of its collection, oral fluid seems to be a better sample for this purpose compared with urine.

One advantage of these portable devices is that they allow the initial testing for drugs to be easily carried out in the field. A preliminary drug test result is usually provided within a few minutes, without the need for sophisticated and/or expensive laboratory equipment. Indeed, easy-to-use instruments are available that provide an electronic readout (e.g., Dräger DrugTest® and Orasure Uplink®, Cozart Rapiscan® and Drugread® hand photometer), while others

DRUGS OF ABUSE

Most publications concerning oral fluid analysis aim at detecting these substances

require visual identification (e.g., DrugWipe[®], iScreen OFD[™], OralScreen[®], Oratect[®] and SalivaScreen[™]) [2]. On-site collection kits may also provide an indirect aid to traffic control, since the tested individual is more likely to admit drug use after a positive test result. On the other hand, individuals under the influence of drugs may be deterred from driving, since people are starting to become aware that traffic control police are able to perform screening tests for drug use [103].

However, the assessment of these devices' performance is not objective, because, for most, the specifications are not consistent [2] and details concerning the detectability conditions are often missing. On the other hand, the ease of use of the devices should also be evaluated, taking into consideration the opinion of traffic regulation officers, since they are the ones who will be using them in the field.

The possibility of supervision of the sampling process is especially important in terms of law enforcement, since sample adulteration is avoided and the possibility of substitution or contamination appears to be minimal. For these reasons, the significance of oral fluid as a primary matrix in roadside testing can hardly be questioned. Indeed, oral fluid testing has revealed its usefulness in detecting drugs of abuse in various studies using several devices [46,106,122–130], and it can be seen as a good substitute for whole-blood samples [128], unless a quantitative analysis of blood levels is desired.

The advantages of on-site testing are rapid turnaround times, reduced costs and the fact that the test can be carried out virtually anywhere [129]. However, this type of technology presents some problems relating to THC detection, since the removal of the drug from the collection pad is difficult [67,130].

The scourge of driving under the influence of drugs or alcohol is responsible for thousands of accidents every year, and up to 25% of them involve drivers who tested positive for drugs [131]. For instance, in a roadside study by Samyn *et al.*, blood analysis of drivers suspected of impairment revealed the presence of MDMA in 35% of cases; in addition, amphetamine, cocaine and cannabis were detected in many of these [104].

The deleterious consequences of drug-impaired driving on road safety led the European Commission to promote scientific studies based on oral fluid analysis.

The first of these studies was the Roadside Testing Assessment (ROSITA), aimed at assessing the value of on-site tests at the roadside, and

also to give recommendations for the use of roadside testing equipment in European countries. Police officers from 16 countries were surveyed, and saliva was shown to be the preferred matrix for drug testing. The reasons for such a choice are quite obvious, namely its ready availability, low invasiveness and good correlation with impairment. Test configuration was also evaluated in this survey, and the ideal would be a single-use multianalyte test able to provide a clear and unambiguous test result within a 5-min interval [103,202]. A collaborative study between the USA and the European Union (ROSITA-2) has been conducted both to assess illegal drug use among motor vehicle operators and to evaluate the effectiveness of on-site oral fluid drug-detection technologies. Within the scope of this study, which was completed in 2005, Crouch *et al.* have tested the laboratorial performance of ten different devices, namely concerning their ability to meet the manufacturers' claimed cut-off concentrations for several drug classes, including amphetamines, cocaine and metabolites, opiates and cannabinoids [132]. Two devices were also evaluated for benzodiazepines. The devices' results were compared with laboratory-based immunoassay and MS results, both in terms of sensitivity and specificity.

Another study is ongoing in Europe, the Integrated Project Driving under the Influence of Drugs, Alcohol and Medicines (DRUID), which involves more than 20 European countries. The objective of this study is to gain new insights to the real degree of impairment caused by psychoactive drugs and their actual impact on road safety, giving scientific support to the EU transport policy to establish guidelines and measures to combat impaired driving [203].

A study has been conducted in the US (Pilot Test of New Roadside Survey Methodology for Impaired Driving), aimed at developing and testing new procedures for the assessment of impaired driving. In this study, oral fluid samples of over 600 randomly selected drivers were collected and analyzed, and blood was also collected in approximately half of those subjects. Several drug classes were included in this study, including both abused and prescription drugs [204].

■ Immunoassays

In recent years, extraordinary advances in analytical techniques have enabled the detection of drugs and metabolites in alternative specimens. The strategy for drug testing in biological fluids (e.g., urine, blood and oral fluid) usually

begins with screening procedures, which should be able to detect or exclude a drug class and elude false-negative results [3]. This is extremely useful, especially for those laboratories that are required to analyze a large number of samples routinely, saving time and money that would be wasted in more expensive confirmatory methods (usually MS-based techniques), while most of the samples would be negative.

Four interpretations are possible following a drug test:

- True-positive, when the test correctly detects a drug in the analyzed specimen
- False-positive, when the test incorrectly detects the presence of a drug where no drug is present
- True-negative, if the test correctly confirms the absence of a drug
- False-negative, when the test fails to detect a drug that is actually present in the sample

A test's sensitivity is determined by its ability to detect the presence of a drug at or above a cut-off level [133].

For obvious reasons, to choose this cut-off level one must optimize the number of true-positive and true-negative samples, and the number of false-positives and false-negatives should be kept to a minimum. Indeed, if the cut-off is set too low, the number of false-positive samples will be too high and there will be an unnecessary high number of confirmation analyses, as stated previously. On the other hand, a cut-off value that is too high will lead to a large number of false-negative samples, impairing the test's usefulness in its applications, namely in the clinical and forensic fields [44]. However, this is not an easy task, since these tests are designed for maximum sensitivity, in order to minimize the possibility of a false-negative sample, which increases the probability of obtaining a false-positive result.

Another common characteristic of immunoassays is their specificity or, more accurately, their lack of specificity. This means that these tests are, in general, not able to discriminate between different drugs or metabolites from the same class. On the other hand, there is also the possibility of a false-positive result deriving from the consumption of some over-the-counter medications, which are known to interfere with amphetamine screenings, originating a positive result. Perhaps the term 'presumptive positive' would be more accurate to

define this situation, since that sample would be positive, although not for drugs in the confirmation profile. This phenomenon is known as cross-reactivity, and it also occurs when the test cannot distinguish between chemically similar substances [81,133].

Despite these drawbacks, immunoassay techniques are the most commonly used methods for the screening of illicit drugs in biological specimens, including urine, blood and other matrices such as oral fluid and hair. This is due to the advantages that they present over other techniques, namely that the sample volume needed to perform the test is small (usually approximately 25 µl), which is extremely important in oral fluid analysis, since the amount of sample available is usually limited, and there is no need for sample pretreatment [44].

A few studies have been published on the use of immunoassays for drug screening in oral fluid, and these concerned the detection of opiates [40,54,60,134], including methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) [135], cocaine and metabolites [57,136–139], cannabinoids [40] and amphetamines [40,140,141]. Benzodiazepines were also screened using this specimen [40,81] and, more recently, dextromethorphan [95] was also studied.

Special attention should be paid to the analytical challenge that the screening of highly potent benzodiazepines, such as flunitrazepam, represents in oral fluid analysis. Indeed, these compounds are usually present in the low nanogram range [58,86] and, therefore, often remain undetected by traditional enzymatic assays. This lack of sensitivity may be overcome by the use of specific immunoassays for these substances or their metabolites. Indeed, studies have been performed on this type of assay [142], although their use in oral fluid analysis is not documented.

Immunoassay-based techniques represent highly sensitive and cost-effective technology; however, their lack of specificity makes the confirmation of presumptive-positive samples mandatory. This confirmation analysis is usually performed by highly specific MS-based methods, which are designed to identify unequivocally the present drug(s) and elude false-positive results.

The reader is referred to the review by Townsend *et al.* for a more comprehensive insight on immunoassay techniques, including biosensor technology [143].

■ Gas chromatography–mass spectrometry

Both qualitative and quantitative toxicological analyses are required to be reliable, and this applies to all fields of analytical toxicology, including clinical and forensics. The hyphenation of chromatographic techniques to mass spectrometers has brought a significant improvement in drug testing in biological specimens. This is of particular importance in the fields of clinical and forensic toxicology, for which the needed specificity and sensitivity could be met [144].

Gas chromatography–mass spectrometry was the most utilized technique for the confirmation of a number of organic compounds back in the 1990s, mainly due to the availability of spectral libraries for drugs and metabolites [145].

In addition, GC–MS is a very reproducible technique, and consistent mass spectra can be obtained in different laboratories, providing that the ionization conditions are the same (usually electron ionization at 70 eV). This is the reason why it is still the gold standard in mass detection specificity [146].

Gas chromatography–mass spectrometry is currently available in most laboratories, and this is still the main technique for confirmation and quantitation of drugs and their metabolites in biological specimens, including the so-called ‘alternative’ specimens such as oral fluid, hair and sweat.

Analysis by GC–MS generally requires the extraction of the analytes from the matrix and, in most cases, their chemical derivatization in order to improve peak shape and/or allow chromatographic analysis. Typical limits of quantitation are in the range of approximately 1–10 ng/ml, and the ability to achieve them obviously depends on the sample volume. This presents a limitation in terms of oral fluid, not only because of the low amounts at which drugs are present in this specimen, but also due to sample availability for analysis. For example, typical concentrations of 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid in oral fluid are in the low picograms per milliliter range [66]. Analysis of these low amounts of drugs in biological matrices are, in general, impaired by the presence of co-extracted endogenous compounds, which usually lead to an increase in the background noise, decreasing the analyte’s signal-to-noise ratio (S/N). Therefore, and to perform a valid quantification, the S/N must be increased, which can be performed using 2D chromatography. This method is able to

augment S/N by the selective transfer of a small segment of GC eluent that contains the analytes of interest to a second column coupled to a mass spectrometer, reducing or eliminating the interference of endogenous substances [67,147].

The S/N may also be increased using MS/MS, which allow LODs of approximately 0.01 ng/ml [66]. This type of technology was developed for the measurement of low-concentration analytes in biological samples, including blood and urine. However, it is also extremely useful in those situations where sample amount is critical, as frequently occurs when abused drugs and their metabolites are analyzed in alternative matrices. Of particular interest is its application to drugs that represent a more challenging analytical assay (e.g., cannabinoids), therefore requiring a MS/MS approach to achieve the required limits of detection.

Another type of GC–MS/MS technology applicable in oral fluid analysis is the ion-trap MS. These mass spectrometers create a magnetic field that holds the formed ions until they are sequentially released to the detector. Many of the advantages of an ion-trap derive from being able to monitor ions on demand, which means that they can be accumulated in the trap to improve sensitivity.

When the number of analytes is too big, an approach is to use fast GC methods, which allows a reduction in the time of analysis, maintaining acceptable resolution. Fast GC was developed following modifications on the quadrupole mass spectrometers, including reduced column bore size and more efficient capillary columns, rapid heating-rate ovens and high-pressure carrier gas control [147]. Applications include the ability to analyze 30 different drugs in oral fluid [49].

Several papers have been published on the use of GC–MS/MS for oral fluid analysis, including the detection and quantitation of several drug classes in this specimen. For a better understanding, most of the papers published over the last decade are summarized in **TABLE I** [148–156].

■ Liquid chromatography–mass spectrometry

Recent years have seen the development of powerful technologies that have provided forensic scientists with new analytical capabilities that were unthinkable only a few years ago [157]. The most spectacular analytical improvement was the advent of LC–MS, for which there has been an explosion in the range of new products available for solving many analytical problems,

LC–MS

This is a highly sensitive analytical technique, allowing the detection of extremely low amounts of drugs in biological specimens

Table 1. Current gas chromatography–tandem mass spectrometry procedures for oral fluid analysis.

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Derivatization	Stationary phase	Detection mode	LOD; LOQ	Ref.
COD, MOR, MAM, dihydrocodeine and metabolites	1 ml	Clin Rep® device	SPE (Chromabond drug® columns)	N-methyl-N-(trimethylsilyl) trifluoroacetamide	DB-5HT	GC-EI-MS	10 ng/ml	[62]
COC, BE, EME, AEME, MOR, MAM, COD, AP, MDA, MDMA, MDEA, MBDB, EPH, THC, CBN, CBD, OH-THC and THC-COOH	1–2 ml	Spitting	SPE (Bond Elut Certify®)	PFPA and pentafluoropropanol; BSTFA + 1% TMCS		GC-EI-MS		[63]
COD, MOR, MAM, hydrocodone, hydromorphone and oxycodone	0.25–1 ml		SPE (ZSDAU020)	BSTFA + 1% TMCS	DB-5MS	GC-EI-MS	2 ng/ml; 2 ng/ml for MOR and COD; 2 ng/ml; 3 ng/ml for MAM, hydromorphone and oxycodone; 3 ng/ml; 10 ng/ml for hydrocodone	[64]
COC and BE		Stimulation citric acid				GC-EI-MS	1 ng/ml; 8 ng/ml	[78]
COD, norcodeine, MOR and normorphine		Spitting and Salivette®	SPE (Clean Screen®)	MTBSTFA + 1% TBDMCS, BSTFA + 1% TMCS	HP-1 or phenomenex ZB1	GC-EI-MS	2.5 ng/ml; 2.5 ng/ml for all analytes	[20]
MOR, COD, dihydrocodeine, diacetylmorphine and MAM	0.025–0.5 ml	Cozart® Rapiscan	SPE (Bond Elut Certify)	BSTFA + 1% TMCS	DB-5MS	EIA, GC-EI-MS	5 ng/ml; 5 ng/ml for all compounds	[60]
COC, AEME, EME and COET	0.5 ml	Salivette	Automated SPE (HCX Isolute columns)		DB-5MS	GC-PCI-MS/MS	0.1 ng/ml for COC and 0.5 ng/ml for AEME, EME and COET; 2 ng/ml for COET and COC, and at 5 ng/ml for EME	[148]
COD, MOR and MAM	1 ml	Salivette	LLE (Tox tubes A®)	BSTFA + 1% TMCS	5% phenylmethylsiloxane	GC-PCI-MS	0.7 ng/ml; 2.3 ng/ml for COD, 2.0 ng/ml; 6.7 ng/ml for MOR, 0.6 ng/ml; 2 ng/ml for MAM	[52]

6-AC: 6-acetylcodeine; ACE: Acetone; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; BE: Benzoylcegonine; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; CBD: Cannabidiol; CBN: Cannabinol; COC: Cocaine; COD: Codeine; COET: Cocaehtylene; DMF: N,N-dimethyl formamide; DMSO: Dimethyl sulfoxide; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EI: Electron impact; EIA: Enzyme immunoassay; ELISA: Enzyme-linked immunosorbent assay; EME: Ecgonine methyl ester; EMIT: Enzyme multiplied immunoassay; EPH: Ephedrine; GC: Gas chromatography; GHB: γ -hydroxybutyrate; HFBA: Heptafluorobutyric acid anhydride; HFP: 1,1,1,3,3,3-hexafluoro-2-propanol; HMA: 3-hydroxy-4-methoxyamphetamine; HMMA: 3-hydroxy-4-methoxy-methamphetamine; IPA: Isopropyl alcohol; LLE: Liquid-liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Metamphetamine; MAM: 6-acetylmorphine; MBDB: N-methyl-1-(β ,4-methylenedioxyphenyl)-2-butanamide; MBTFA: N-methyl-bis-(trifluoroacetamide); MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyethamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MEK: Methyl ethyl ketone; MOR: Morphine; MS: Mass spectrometry; MSTFA: N-methyl-N-trimethylsilyl-trifluoroacetamide; MTBSTFA: N-methyl-N-tert-butyltrimethylsilyl-trifluoroacetamide; NEPH: Norephedrine; NMF: N-methyl formamide; OH-THC: 11-hydroxy- Δ^8 -tetrahydrocannabinol; PCI: Positive ion chemical ionization; PFPA: Pentafluoropropanoic anhydride; PFPA: Pentafluoropropanoic anhydride; PSEPH: Pseudoephedrine; S-HFBPCI: S-heptafluorobutylpropyl chloride; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; TBDMCS: Tert-butyl dimethylchlorosilane; THC: Δ^9 -tetrahydrocannabinol; THC-COOH: 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid; TMAH: Tetrabutylammonium hydroxide; TMCS: Trimethylchlorosilane.

Table 1. Current gas chromatography–tandem mass spectrometry procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Derivatization	Stationary phase	Detection mode	LOD; LOQ	Ref.
Methadone and EDDP		Spitting	SPME vs LLE		HP-5	GC-EI-MS	4 ng/ml for methadone and 8 ng/ml for EDDP; 45 ng/ml for methadone and 18 ng/ml for EDDP with SPME	[149]
THC	1 ml	Intercept™ DOA Oral Specimen Collection Device	SPE (Bakerbond C18)	MSTFA	FactorFour VF-1MS	GC-EI-MS	0.5 ng/ml; 2.4 ng/ml	[41]
THC	0.025–0.2 ml	Intercept DOA	LLE (hexane/ethyl acetate; 9:1, v/v)	BSTFA + 1% TMCS	5% phenyl methyl silicone	Intercept MICRO-PLATE enzyme immunoassay and GC-EI-MS/MS	0.37 ng/ml; with immunoassay, 0.2 ng/ml with GC-EI-MS/MS	[54]
THC	0.2 ml	Spitting	Polymer monolith microextraction		HP-5MS	GC-EI-MS	0.68 and 2.26 ng/ml	[150]
THC-COOH and THC	1 ml	Quantisal™	LLE (hexane/ethyl acetate/ acetic acid, 90:10:3, v/v)	BSTFA + 1% TMCS		ELISA and GC-EI-MS	1 ng/ml; 1 ng/ml for THC; 2 pg/ml for THC-COOH	[67]
MEK, IPA, DMF, ACE and NMF	1 ml	Spitting	HS-SPME		DB-WAX	GC-EI-MS	4, 3, 6, 50 and 100 ng/ml for ACE, MEK, IPA, DMF and NMF, respectively; 160 ng/ml for ACE, MEK and IPA and 300 ng/ml for DMF and NMF	[151]
11-nor-9-carboxytetrahydrocannabinol	0.1 ml	Intercept	SPE (CEREX® Polychrome)	HPIP and PFAA	DB-5	GC-NCI-MS/MS	0.01 ng/ml; 0.01 ng/ml	[66]
2-carboxy-tetrahydrocannabinol, THC, CBN and CBD	1 ml	Quantisal	SPE (Trace-N 315)	BSTFA + 1% TMCS	DB-5	GC-EI-MS	0.5 ng/ml for THC and CBN; 1 ng/ml for CBD and 2-carboxy-tetrahydrocannabinol	[68]

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Table 1. Current gas chromatography–tandem mass spectrometry procedures for oral fluid analysis (cont.).								
Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Derivatization	Stationary phase	Detection mode	LOD; LOQ	Ref.
THC	0.25–1 ml	OralLine® IV + Intercept devices	LLE (hexane/ethyl acetate; 90:10, v/v)	TMAH/DMSO and iodomethane	Optima 5-MS	GC–EI–MS	1 ng/ml	[152]
THC	0.25–0.5 ml	Intercept	LLE (hexane/ethyl acetate; 90:10, v/v)	TMAH/DMSO and iodomethane	HP-5-MS	GC–EI–MS	1 ng/ml	[105]
MDMA, MDA, MDEA, AP, MA, HMMA and HMA	0.4 ml	Spitting	SPE (SPEC C ₁₈ AR/MP1)	Triethylamine in heptane and HFBA	HP-5	GC–EI–MS	2.5 ng/ml; 5 ng/ml for AP, MDA, MDMA and MDEA; 1 ng/ml; 5 ng/ml for MA; 5 ng/ml; 25 ng/ml for HMA; 2.5 ng/ml; 25 ng/ml HMMA	[72]
MA and AP		Stimulation citric acid and Salivette				GC–EI–MS	50 ng/ml; 2.5 ng/ml for MA; 2.5 ng/ml for AP	[21]
MDMA, MDA, MDEA, AP and MA	0.05 ml	Spitting	LLE (cyclohexane)	S–HFBPCL	5% phenylmethyl siloxane	GC–NLI–MS	5 ng/ml for MDA; 25 ng/ml for the other compounds	[73]
AP, MA, MDMA, MDA, MDEA and MBDB	0.5–1 ml	Cozart RapiScan System, drug test cartridges	Buffer solution and SPE (Bond Elut Certify®)	PFPA	Rtx-5Sil	Cozart® RapiScan System and GC–EI–MS	2 ng/ml; 5 ng/ml for AP; 1 ng/ml; 2 ng/ml for MA; 5 ng/ml; 5 ng/ml for MDA; 1 ng/ml; 5 ng/ml for MDMA; 2 ng/ml; 5 ng/ml for MDEA; and 1 ng/ml; 5 ng/ml for MBDB	[74]

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Table 1. Current gas chromatography–tandem mass spectrometry procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Derivatization	Stationary phase	Detection mode	LOD; LOQ	Ref.
AP, MA, MDMA, MDA, MDEA, phentermine, BE, COET, EME, THC, THC-COOH, OH-TCH, CBN, CBD, MAM, MOR, COD, flurazepam, flunitrazepam, chlorazepate, alprazolam, diazepam, oxazepam, amitriptyline, paroxetine and sertraline, haloperidol, chlorpromazine, fluphenazine, chlormethiazole, loratidine, hydroxyzine, diphenhydramine, valproic acid and gabapentin	1 ml	Salivette	SPE (Bond Elut Certify)	MSTFA	Ultra 1	GC-EI-MS	0.3–6.9 ng/ml; 0.9–20.9 ng/ml	[153]
15 psychoactive amines	0.1 ml	Spitting	HFBA		DB-5MS	GC-EI-MS	5 ng/ml for all compounds except 10 ng/ml for PSEPH, NEPH and EPH; 20 ng/ml for all compounds	[154]
AP and AM	1 ml	Spitting and salivette	SPE (CSDAU Clean Screen Extraction columns)	MTBSTFA + 1% TBDMCS	HP-1 or phenomenex ZB1	GC-EI-MS	10 ng/ml; 2.5 ng/ml	[37]
MDMA, MDA and HMMA	1 ml	Spitting	SPE (Bond Elut Certify)	MBTFA	HP Ultra-2	GC-EI-MS	5.7 ng/ml for MDMA, 1 ng/ml for MDA and 2.9 ng/ml for HMMA	[35]

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Table 1. Current gas chromatography–tandem mass spectrometry procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Derivatization	Stationary phase	Detection mode	LOD; LOQ	Ref.
Amphetamines: AP, AM, MDA, MDEA, MDMA and MBDB; benzodiazepines: clordiazepoxide, diazepam, lorazepam, nordiazepam, oxazepam and temazepam; buprenorphine: buprenorphine and norbuprenorphine; cannabinoids: CBN, CBD, THC, HO-THC and HCCOOH; cocaine: AEME, BE, COET, and COC; methadone: methadone and EDDP; opiates: 6-AC, MAM, COD, dihydrocodeine, heroin and MOR	0.3 ml	Intercept	SPE (HCX solid-phase extraction)	BSTFA + 1% TMCS		MICRO-PLATE Enzyme immunoassays, SINGLESTEP Buprenorphine ELISA®, GC-EI-MS or GC-NCI-MS or GC-EI-MS/MS	LOQ between 0.03 and 9 ng/ml	[155]
AP	0.25 ml	Intercept device	Buffer solution and SPE			Immunoassay; GC-MS	25 ng/ml with immunoassay	[75]
Flunitrazepam and 7-aminoflunitrazepam	1 ml	Spitting	SPE (Bond Elut Certify)	HFBA	HP-1	GC-EI-MS; GC-NCI-MS	0.05 ng/ml; 0.10 ng/ml for flunitrazepam; 0.1 ng/ml; 0.15 ng/ml for 7-aminoflunitrazepam	[58]
GHB	1 ml	Salivette	LLE (chloroform)	Trifluoroacetic acid	DB-5MS	GC-EI-MS	100 ng/ml; 100 ng/ml	[88]
Propoxyphene	1 ml	Quantisal	SPE (Clin II)	No derivatization	DB-5MS	ELISA; GC-EI-MS	40 ng/ml with ELISA; 2 ng/ml; 5 ng/ml with GC-EI-MS	[91]

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Table 1. Current gas chromatography–tandem mass spectrometry procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Derivatization	Stationary phase	Detection mode	LOD; LOQ	Ref.
Meperidine, tramadol and oxycodone	1 ml	Quantisal	SPE (Clin II)	BSTFA + 1% TMCS; MSTFA + 1% TMCS	DB-5MS	ELISA; GC–EI–MS	25 ng/ml for oxycodone and 50 ng/ml for meperidine and tramadol with ELISA; 10 ng/ml for all compounds with GC–EI–MS	[92]
Dextromethorphan, dextrorphan	1 ml	Quantisal	SPE (Clin II)	BSTFA + 1% TMCS	DB-5MS	ELISA; GC–EI–MS	1 ng/ml; 10 ng/ml	[93]
30 drugs of abuse	0.25 ml	Spitting	SPE (HCX)	MSTFA; MTBSTFA; HFBA	DB-35MS	Fast GC/EI–MS		[49]
Nicotine and cotinine	2 ml	Monovette® and Salivette	LLE (dichloromethane)	No derivatization	DB-1MS	GC–EI–MS	0.60 ng/ml; 0.011 µg/ml for nicotine and cotinine	[94]
Nicotine, cotinine, norcotinine and trans-3-hydroxycotinine	0.5 ml	Salivette	SPE (Clean Screen)	BSTFA + 1% TMCS	HP-5	GC–EI–MS	5 ng/ml; 5 ng/ml	[95]
Cyanide and thiocyanate	0.5 ml	Spitting	LLE (ethyl acetate)	Pentafluorobenzyl bromide	5% phenyl polysiloxane	GC–EI–MS	26 ng/ml for cyanide; 290 ng/ml for thiocyanate	[96]
Modafinil, selegiline, croretamide, cropropamide, pentetrazol, EPH, NEPH, sibutramine, COC, BE, EME, AP, MA, COD and dhydrocodeine	1 ml		LLE (t-butylmethyl-ether) LLE (chloroform/isopropanol) for COC and metabolites	PFAA + pentafluoropropanol for derivatization of COC and metabolites	5% phenyl methylsilicone	GC–EI–MS	10 ng/ml for AP, 2 ng/ml for croretamide, cropropamide, COD and modafinil, 5 ng/ml for the other compounds; 25 ng/ml for AP, 5 ng/ml for croretamide, cropropamide, COD and modafinil 10 ng/ml for the other compounds	[156]
49 illicit drugs	1 ml	Omni-Sal®	SPE (Bond Elut Certify)	No derivatization	HP-1	GC–EI–MS	0.3–2.5 ng/ml; 0.8–11.4 ng/ml	[116]
Levetiracetam	1 ml	Omni-Sal	SPE (Bond Elut Certify)	MSTFA + 2% TMCS	HP-5MS	GC–EI–MS	10 ng/ml; 2200 ng/ml	[22]

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especially for those applications aimed at analyzing nonvolatile, labile and/or high-molecular-weight compounds. In the last 10 years, an important step was made toward LC–MS-based scientific investigation. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have become the most widespread ionization techniques [158].

However, assessing which of these ionization techniques is more suitable for developing a new procedure must be made on a case-by-case basis. For example, APCI is more appropriate for unionized analytes, and the sensitivity depends on the analyte structure and apparatus [144]. On the other hand, ESI interfaces permit MS analyses of molecules in the molecular weight range of drugs of abuse (50–600 Da) or larger molecules, including proteins as large as 232 kDa [147]; this is the most used ionization technique.

Related to LC–MS, tandem mass spectrometry (LC–MS/MS) offers superior sensitivity and specificity, especially if compared with the use of a single quadrupole. These instruments, when operated in the multiple reaction monitoring mode (MRM), allow the detection of extremely low levels of analytes in complex biological samples, reducing sample pretreatment and analysis time.

One limitation of LC–MS/MS, mainly in ESI mode, is its susceptibility to matrix effects, causing unwanted ion suppression or enhancement [159–162]. For example, Dams *et al.* found that ESI and APCI showed matrix effects, with ESI being much more susceptible than APCI [161]. Indeed, the common and early perception that utilization of LC–MS/MS practically guarantees selectivity is being challenged by a number of reported examples of lack of selectivity due to ion suppression or enhancement phenomena caused by the sample matrix and interferences from metabolites, as well as ‘cross-talk’ effects.

These effects are capable of affecting ion ratios in the mass spectrum, potentially impairing the assay’s accuracy. Several strategies can be used to evaluate and overcome this problem, and these have been reviewed by Matuszewski *et al.* [163].

Ion-suppression effects may occur to a greater extent with increased solvent amount in the chamber. This problem can be addressed via the reduction of the amount of mobile phase exiting the column by means of nano-LC systems, which will be further discussed below. Another possible approach is to reduce matrix constituents by reducing sample volume or adjusting

mobile-phase composition in order to reduce co-elution of matrix components with target analytes. In addition, matrix-matched calibrators and deuterated internal standards should also be used.

Another problem that LC–MS and LC–MS/MS methods may present is the formation of adducts by combination with Na⁺, K⁺ or NH₄⁺ introduced by the solvent in the chamber. These adducts produce ions of mass-to-charge ratios higher than expected by factors of 23, 40 or 18 Da. In addition, these adducts do contain multiple salt ions and are capable of forming bridges between ions of differing masses, which will complicate mass spectra interpretation [164,165].

Unlike GC–MS, the development of searchable libraries for LC–MS and LC–MS/MS is still a problem, since fragmentation and spectra differ between instruments. However, despite interinstrument reproducibility, these spectra do have many similar features [166,167].

Recently, two research groups have been creating homemade libraries, allowing the identification of therapeutic agents and drugs of abuse, by means of the collision-induced-dissociation (CID) approach [168–172]. Briefly, the ions are accelerated and a high number of collisions are produced with gaseous solvent molecules, leading to analyte dissociation. These fragments allow the confirmation of the compound’s identity, and it is possible to design a library for each instrument.

Liquid chromatography–MS presents a number of advantages over GC–MS, especially for the quantitation of more polar, thermolabile or low-dosed drugs [144], avoiding the complicated and laborious derivatization procedures usually necessary in GC analysis [173,174]. Furthermore, these needed derivatization steps may differ for different compound classes according to their chemical structure. Indeed, for example, amphetamines are often derivatized by acetylation, while opiates originate better signals using silylating agents. This will pose several difficulties in the development of multi-analyte methods, which is desirable in oral fluid analysis, as stated earlier.

As discussed previously, nano-LC systems represent a valid approach to overcome ion-suppression phenomena, since the amount of solvent present in the chamber is reduced, achieving lower limits of detection. In fact, Tomkins *et al.* reported a chip-based nanoelectrospray MS/MS method that could detect 0.49 ng of cotinine (metabolite of nicotine) in 1 ml of oral fluid [175]. The amount of specimen extract on the chip was 10 µl, providing a detection limit of 4.9 pg on the column. Accuracy and precision results were not

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis.

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
AP, MA, MDA, MDMA, MDEA, MOR, COD, COC and BE	0.2 ml	Spitting	SPE (Bond Elut Certify®)	Hypersil BDS phenyl	Methanol and ammonium formate (10 mM, pH 5)	LC–ESI–MS–TOF	1.07 ng/ml; 2 ng/ml for MDA; 0.71 ng/ml; 2 ng/ml for MDMA; 0.22–0.37 ng/ml; 2 ng/ml for other compounds	[61]
MOR, COD, MAM, acetylcodeine and heroin	0.5 ml	Intercept®	Methanol and ammonium acetate	EXSIL BDS C8	Mobile phase A: 5% methanol in ammonium acetate 4 mM; mobile phase B: propan-2-ol 1% and formic acid 0.05% in methanol	LC–ESI–MS–MS	1 ng/ml; 2 ng/ml for MAM, COD and acetylcodeine; 2 ng/ml; 6 ng/ml for MOR; 6 ng/ml; 10 ng/ml for heroin	[65]
THC	0.2 ml	Spitting	LLE (hexane)	XTerra® C18 MS	Formic acid 0.1%/acetonitrile (15:85, v/v)	LC–ESI–MS	2 ng/ml; 2 ng/ml	[70]
THC	0.1–0.5 ml	Intercept	LLE (hexane)	XTerra C18 MS	Ammonium formate–methanol 1 mM (10:90, v/v)	LC–ESI–MS/MS	0.5–0.1 ng/ml	[71]
THC and THC-COOH	0.5 ml	Artificial saliva, spitting, Intercept, Quantisal™	LLE (hexane/ethyl acetate; 9:1, v/v)	XTerra C18 MS	Mobile phase A: aqueous ammonium formate 10 mM (pH 3.5); mobile phase B: methanol	LC–ESI–MS–TOF	0.05 ng/ml; 0.1 ng/ml for THC; 0.2 ng/ml; 0.5 ng/ml for THC-COOH	[69]
49 illicit drugs	1 ml	Orni-Sal®	SPE (Bond Elut Certify)	Luna C18(2)	Mobile phase A: ammonium formate 3 mM and formic acid 0.001% in water; mobile phase B: acetonitrile	LC–MS/MS (ion trap)	0.3–2.5 ng/ml; 0.8–11.4 ng/ml	[116]
AP, MA, MDA, MDMA, COC, BE, MOR, MAM and COD	0.25 ml	Intercept	SPE (Oasis MCX®)	XTerra C18 MS	Mobile phase A: ammonium bicarbonate 10 mM, pH 10; mobile phase B: methanol	LC–ESI–MS/MS	0.2–0.5 ng/ml; 2 ng/ml for all compounds	[181]
MDMA, MDEA and AP	0.05 ml	Spitting	Methanol	Hypersil BDS C18	Mobile phase A: ammonium acetate 10 mM; mobile phase B: acetonitrile 95%, 5% ammonium acetate 10 mM (85/15 v/v)	LC–ESI–MS/MS	1–5 ng/ml	[76]

6-AC: 6-acetylcodeine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylcegonine; COC: Cocaine; COD: Codeine; COET: Coaethylene; EDDP: 2-ethylidene-1,5-dimethyl-3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMIT: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid–liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Metamphetamine; MAM: 6-acetylmorphine; MBD: N-methyl-1-β,4-methylenedioxyphenyl-2-butanamide; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy-Δ⁸-tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC: Tetrahydrocannabinol; THC-COOH: 11-nor-Δ⁸-tetrahydrocannabinol-9-carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis (cont.).								
Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
MDMA, MDA, MDEA, AP, MA and EPH	0.05 ml	Spitting	Methanol	Hypersil BDS C18	Ammonium acetate and acetonitrile (75:25 v/v)	LC–ESI–MS/MS	0.15 ng/ml for MDEA, 0.20 ng/ml for MDMA, 0.5 ng/ml for MDA and EPH, 0.2 ng/ml; 0.5 ng/ml for MA, 0.5 ng/ml; 0.5 ng/ml for AP	[77]
MOR, MAM, COD, buprenorphine, methadone, AP, MA, MDA, MDMA, MDEA, COC, BE, OH-THC, lysergic acid diethylamide, alprazolam, bromazepam, clonazepam, 7-aminiclonazepam, diazepam, <i>N</i> -desmethyldiazepam, 3-OH-diazepam, fenazepam, flunitrazepam, 7-aminoflunitrazepam, lorazepam, nitrazepam, 7-aminonitrazepam, oxazepam, zopiclone, zolpidem, carisoprodol and meprobamat	0.5 ml	Intercept	LLE (ethylcetate/heptane, 4:1)	Atlantis dC18	Mobile phase A: acetonitrile; mobile phase B: ammonium acetate 5 mM (pH 5) in water	LC–ESI–MS/MS	0.15–31.8 ng/ml	[50]
MDMA, MDA, MDEA and MBDB	1 ml		LLE (Tox tube A [®])	Spherisorb1 S5	Potassium dihydrogen phosphate (KH ₂ PO ₄) 0.03 M (pH 5) and acetonitrile (75:25 v/v)	HPLC with fluorescence detection	2 ng/ml; 10 ng/ml for all compounds	[55]
COC, BE and COET	0.1 ml	Salivette [®]	SPE (ISTM [™] confirm HCX)	Hypersil BDS C18	Mobile phase A: 0.05 M solution of ammonium acetate (82.5%), methanol (8.75%) and acetonitrile (41.2%); mobile phase B: methanol (41.2%) acetonitrile (41.2%), and water (17.6%)	LC–ESI–MS–TOF	1 ng/ml; 10 ng/ml for all compounds	[79]
R- and S-methadone	0.2 ml		LLE (n-hexane, 2-propanol)	Luna C18	Acetonitrile: ammonium acetate 10mM buffer (pH 5.5)	LC–APCI–MS	0.08 ng/ml	[182]

6-AC: 6-acetylcodeine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylcegonine; COC: Cocaine; COD: Codeine; COET: Cocaehtylene; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMIT: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid–liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Methamphetamine; MAM: 6-acetylmorphine; MBDB: *N*-methyl-1-(β -4-methylenedioxyphenyl)-2-butanamide; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy- Δ^8 -tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC-COOH: 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
R- and S-methadone, R- and S-EDDP	0.5 ml	Salivette	No extraction	Chiral 1-acid glycoprotein (chiral-AGP)	Acetonitrile: ammonium acetate 10 mM buffer (pH 7)	LC-ESI-MS	0.10 ng/ml; 5 ng/ml for R- and S-methadone; 0.25 ng/ml; 0.5 ng/ml for R- and S-EDDP	[183]
AP, MA, MOR, MAM, MDA, MDEA, MDMA, COC, BE, THC, THC-COOH, ketamine and phencyclidine	0.15 ml	Spitting	No extraction (sample precipitation with methanol)	Altima C18	Mobile phase A: acetonitrile; mobile phase B: formic acid 5 mM in water	LC-MS/MS	0.2–2.8 ng/ml; 0.5–5.0 ng/ml	[114]
AP, MA, MDA, MDMA, phencyclidine, COD, COC, BE, MOR, hydrocodone, hydromorphone, oxycodone and oxycodone	0.5 ml	Quantisal	LLE for amphetamines and phencyclidine (hexane/ethyl acetate [50:50] SPE for opiates and cocaine group [UCT Zsdau005])	Pinnacle II C18 for amphetamines and Allure; PFP propyl column for all compounds	Ammonium acetate 5 mM and methanol containing 0.1% formic acid for amphetamines, 0.1% formic acid in water and 80:20 methanol/acetone containing 0.1% formic acid for opiates, 0.1% formic acid in water and 80:20 methanol/acetone containing 0.1% formic acid for cocaine and metabolites, 0.1% formic acid in water and 80:20 methanol/acetone containing 0.1% formic acid for phencyclidine	LC-MS/MS (Qtrap)	2–10 ng/ml; 2–10 ng/ml	[184]
THC, amphetamines (including MDMA, MA and MDA), opiates, cocaine and its metabolites, and benzodiazepines	Cozart	Cozart	LLE for amphetamines, THC and benzodiazepines; SPE for opiates and cocaine groups			Cozart drugs of abuse microplate EIA, GC-MS, LC-MS/MS		[185]

6-AC: 6-acetylcocaine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCL: Atmospheric pressure chemical ionization; BE: Benzoyllecgonine; COC: Cocaine; COD: Codeine; COET: Cocaehtylene; EDDP: 2-ethylidene-1,5-dimethyl-3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMIT: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid-liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Methylamphetamine; MAM: 6-acetylmorphine; MBDB: N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamide; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MSI/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy- Δ^8 -tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC: Tetrahydrocannabinol; THC-COOH: 11-nor- Δ^8 -tetrahydrocannabinol-9-carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
Cocaine: COC, BE, COET, EME, AEME, ecgonine ethyl ester, ecgonine, p- and m-hydroxybenzoyllecgonine, p- and m-hydroxycocaine, benzoylnorecgonine, norcocaine and norcocaehtylene; methadone: methadone and EDDP; opiates: 6-AC, MAM, COD, norcodeine, normorphine, heroin, propoxyphene, noscapine, papaverine, methadol and MOR	0.2 ml		No extraction (sample precipitation with acetonitrile)	Synergi Polar RP	Mobile phase A: ammonium formate 10 mM in water, formic acid 0.001% (pH 4.5); mobile phase B: acetonitrile	LC–APCI–MS/MS	0.25–5 ng/ml; 5–10 ng/ml	[186]
Cocaine: COC, BE, COET, EME, ecgonine ethyl ester, ecgonine, p- and m-hydroxybenzoyllecgonine, p- and m-hydroxycocaine, benzoylnorecgonine, norcocaine and norcocaehtylene; methadone: methadone and EDDP; opiates: 6-AC, MAM, COD, norcodeine, normorphine, heroin, propoxyphene, noscapine, papaverine, methadol and MOR	1 ml	Salivette	No extraction (sample precipitation with acetonitrile)	Synergi Polar RP	Mobile phase A: ammonium formate 10 mM in water, formic acid 0.001% (pH 4.5); mobile phase B: acetonitrile	LC–APCI–MS/MS	0.25–5 ng/ml; 5–10 ng/ml	[45]
Phencyclidine	1 ml	Quantisal	SPE (Clin II)	Zorbax Eclipse XDB C18	Mobile phase A: ammonium formate 20 mM (pH 6.4); mobile phase B: methanol	LC–APCI–MS/MS	5 ng/ml	[187]
Lorazepam	0.5 ml	Spitting	LLE (dichloromethane/diethyl ether; 80:20, v/v)	XTerra® C18	Acetonitrile 0.5% and NH ₄ COOH 95% 2 mM (pH 3.6)	LC–ESI–MS/MS	0.05 ng/ml; 1 ng/ml	[82]

6-AC: 6-acetylcodeine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoyllecgonine; COC: Cocaine; COD: Codeine; COET: Cocaehtylene; EDDP: 2-ethylidene-1,5-dimethyl-3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMI: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid–liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Metamphetamine; MAM: 6-acetylmorphine; MBDB: N-methyl-1-β-4-methylenedioxyphenyl)-2-butanamide; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy-Δ⁸-tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC-COOH: 11-nor-Δ⁹-tetrahydrocannabinol-9 carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
Alprazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, bromazepam, clobazam, diazepam, lorazepam, lormetazepam, midazolam, nordiazepam, oxazepam, temazepam, tetrazepam, triazolam, zaleplon, zopiclone and zolpidem	0.5 ml	Intercept	LLE (methylene chloride/diethylether; 50:50, v/v)	XTerra C18	Acetonitrile and formic acid 0.1%	LC-ESI-MS/MS	0.1–0.2 ng/ml	[83]
Midazolam, bromazepam, tetrazepam, alprazolam, lorazepam, triazolam, flunitrazepam, diazepam and lormetazepam	0.5 ml	Salivette	LLE (diethyl ether)	XTerra C18	Acetonitrile in formic acid 0.1%	LC-ESI-MS	0.2 ng/ml; 0.5 ng/ml for alprazolam, lorazepam and bromazepam; 0.1 ng/ml; 0.2 ng/ml for diazepam, flunitrazepam, lormetazepam, midazolam, tetrazepam and triazolam	[80]

6-AC: 6-acetylcodeine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylcegonine; COC: Cocaine; COD: Codeine; COF: Cocaethylene; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMIT: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid–liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Metamphetamine; MBD: 6-acetylmorphine; MBDB: N-methyl-1-β,4-methylenedioxyphenyl-2-butanamide; MDMA: 3,4-methylenedioxymethamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy-Δ⁸-tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC-COOH: 11-nor-Δ⁸-tetrahydrocannabinol-9-carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis (cont.).								
Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
Oxazepam, temazepam, nordazepam, midazolam, OH-midazolam, alprazolam, OH-alprazolam, OH-Ethylflurazepam, lorazepam, lormetazepam, diazepam, zolpidem, zopiclone and nitrazepam	0.5 ml	Salivette	LLE (ChemElut)	XTerra RP18	Methanol/formic acid (0.006 M, pH 3, 30–60%, v/v)	EMIT and LC-APCI-MS/MS	10 ng/ml for EMIT; 3.9 ng/ml; 13.1 ng/ml for oxazepam, 2.4 ng/ml; 8.1 ng/ml for temazepam, 2.3 ng/ml; 7.6 ng/ml for nordazepam, 0.7 ng/ml; 2.3 ng/ml for midazolam, 0.3 ng/ml; 1.1 ng/ml for OH-midazolam, 0.3 ng/ml; 1.2 ng/ml for alprazolam, 0.2 ng/ml; 0.5 ng/ml for OH-alprazolam, 0.8 ng/ml; 2.7 ng/ml for OH-ethylflurazepam, 0.3 ng/ml; 1.2 ng/ml for lorazepam, 0.4 ng/ml; 1.3 ng/ml for lorazepam, 2.1 ng/ml; 6.9 ng/ml for diazepam, 3.0 ng/ml; 10.0 ng/ml for zolpidem, 3.9 ng/ml; 13.0 ng/ml for zopiclone, 0.0 ng/ml; 0.1 ng/ml for nitrazepam	[81]
Midazolam, 1-hydroxymidazolam and 4-hydroxymidazolam	1 ml	Spitting	LLE hexane/dichloromethane (73:27, v/v)	Luna C18	Mobile phase A: acetic acid 0.1% in water/acetonitrile (90:10, v/v); mobile phase B: acetic acid 0.1% in acetonitrile	LC-ESI-MS/MS (ion trap)	0.025 ng/ml; 0.05 ng/ml	[84]
Temazepam, oxazepam, nordiazepam, tetrazepam and diazepam	0.5 ml	Intercept	LLE (1-chlorobutane)	Gemini C18	Formic acid 0.1% and methanol	LC-ESI-MS/MS	0.20 ng/ml for temazepam and tetrazepam, 0.05 ng/ml for oxazepam, nordiazepam and diazepam	[85]

6-AC: 6-acetylcodeine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoyllecgonine; COC: Cocaine; COD: Codeine; COE: Cocaehtylene; EDDP: 2-ethylidene-1,5-dimethyl-3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMIT: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid-liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Metamphetamine; MAM: 6-acetylmorphine; MBDB: N-methyl-1-β-4-methylenedioxyphenyl-2-butanamide; MDA: 3,4-methylenedioxymphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy-Δ⁸-tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC-COOH: 11-nor-Δ⁸-tetrahydrocannabinol-9-carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis (cont.).

Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
Diazepam, oxazepam, temazepam, nordiazepam, lorazepam, chlordiasepoxide, alprazolam, OH-hydroxyalprazolam, desalkylflurazepam, hydroxyethylflurazepam, clonazepam, 7-aminoclonazepam, flunitrazepam and 7-aminoflunitrazepam	0.4 ml	Intercept	SPE (Bond Elut Certify)	Zorbax Bonus-RP	Methanol in formic acid 0.1%	LC–APCI–MS/MS	0.02 ng/ml; 0.1 ng/ml for flunitrazepam and alprazolam 0.05 ng/ml; 0.1 ng/ml for oxazepam, diazepam, lorazepam, desalkylflurazepam, clonazepam, temazepam, hydroxyethylflurazepam and nordiazepam 0.5 ng/ml; 1 ng/ml for 7-aminoclonazepam and flunitrazepam 0.2 ng/ml; chlordiasepoxide 0.5 ng/ml for OH-alprazolam 0.1 ng/ml; 0.5 ng/ml for 7-aminoflunitrazepam	[86]
Bromazepam, alprazolam, clonazepam, lorazepam, oxazepam, diazepam, midazolam, flurazepam, flunitrazepam, nordiazepam, triazolam, temazepam, nitrazepam and chlordiasepoxide	1 ml	Quantisal	SPE (CSDAU020)	Zorbax, Eclipse XDB C18	Mobile phase A: ammonium formate 20 mM (pH 8.6); mobile phase B: acetonitrile	LC–ESI–MS/MS	0.5–5 ng/ml	[188]
MOR, COD, MAM, methadone, AM, MDA, MDMA, MDEA, MA, BE, COC, THC, zolpidem, zopiclone, alprazolam, clonazepam, oxazepam, nordiazepam, lorazepam, flunitrazepam, diazepam, diphenhydramine and amitriptyline	0.5–1 ml	StatSure Saliva Sampler™	SPE (OASIS HLB)	Atlantis C18	Acetonitrile and formic acid 0.1%	LC–ESI–MS/MS	500 ng/ml; 1000 ng/ml for all compounds	[115]
Zopiclone, zolpidem, flunitrazepam, zopiclone, N-desmethyl, 7-aminoflunitrazepam and 3-OH- flunitrazepma, N-desmethyl-flunitrazepam	0.2 ml	No extraction (sample precipitation with acetonitrile)	C18	Mobile phase A: methanol/water/formic acid (20:80:0.1, v/v/v); mobile phase B: methanol/water/formic acid (80:20:0.1, v/v/v)	LC–ESI–QTOF–MS; LC–ESI–MS (ion trap)	2–100 ng/ml	[189]	

6-AC: 6-acetylcodeine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylcegonine; COC: Cocaine; COD: Codeine; COET: Cocethylene; EDDP: 2-ethylidene-1,5-dimethyl-3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMIT: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid–liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Metamphetamine; MAM: 6-acetylmorphine; MBDB: N-methyl-1-β-4-methylenedioxyphenyl)-2-butanamide; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy-Δ⁸-tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC-COOH: 11-nor-Δ⁸-tetrahydrocannabinol-9 carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

Table 2. Current liquid chromatography–tandem mass spectroscopy procedures for oral fluid analysis (cont.).								
Drug class/compound(s)	Volume of sample	Sample collection	Preparation	Stationary phase	Mobile phase	Detection mode	LOD; LOQ	Ref.
Methylphenidate and ritalinic acid	0.1 ml		No extraction (sample precipitation with acetonitrile)	Thermo Electron-Hipersil™ gold	Mobile phase A: ammonium formate 10 mM; mobile phase B was acetonitrile	LC-ESI-MS	0.15 ng/ml for methylphenidate and 0.33 ng/ml for ritalinic acid; 0.5 and 1.0 ng/ml for methylphenidate and ritalinic, respectively	[23]
Ketamine	1 ml	Cozart RapiScan	SPE (OASIS MCX)	Alltech Platinum EPS C18	Acetonitrile: ammonium formate 0.1 M (pH 3; 50:50, v/v)	LC-ESI-MS/MS	20 ng/ml; 45 ng/ml	[190]
Metronidazole and spiramycin I	0.5 ml		LLE (ethyl acetate)	Kromasil C18	Mobile phase A: acetonitrile/water/formic acid (15:85:0.1, v/v/v); mobile phase B: acetonitrile/water/formic acid (50:50:0.1, v/v/v)	LC-ESI-MS/MS	50 ng/ml for metronidazole; 15 ng/ml for spiramycin	[90]
Docetaxel, paclitaxel, 6-OH-paclitaxel and p-3'-OH-paclitaxel	0.25 ml	Salivette	LLE (methyl-t-butyl ether)	Merck Purospher Star RP-18	Mobile phase A: acetic acid 2 mM/ammonium acetate 0.2 mM in water; mobile phase B: acetic acid 2 mM/ammonium acetate 0.2 mM in methanol	LC-ESI-MS/MS	4 ng/ml for docetaxel and paclitaxel 2 ng/ml for 6-OH-paclitaxel and p-3'-OH-paclitaxel	[191]
Sildenafil	3 ml		LLE (chloroform/2-propanol/n-heptane; 25:10:65, v/v)	NovaPak C18	Mobile phase A: acetonitrile and TMA 10 µg/ml; mobile phase B: NH ₄ COOH 2 mM (pH 3.0 buffer)	LC-ESI-MS	0.2 ng/ml; 0.5 ng/ml	[97]
Amitriptyline, imipramine, clomipramine, fluoxetine, paroxetine, sertraline, fluvoxamine, citalopram, venlafaxine, nortriptyline, desipramine, norclopimpramine and norfluoxetine	0.2 ml	Spitting	SPE (OASIS MCX)	Sunfire C18	Acetonitrile and ammonium formate (pH 3; 2 mM)	LC-ESI-MS/MS	2–10 ng/ml	[121]
Nicotine, cotinine, nornicotine and anabasine	0.1–0.2 ml	Salisoft®	In-tube SPME	Synergi 4u POLAR-RP 80A	Ammonium formate 5 mM/ methanol (55/45, v/v)	LC-ESI-MS	0.015–0.040 ng/ml; 0.5 ng/ml	[192]

6-AC: 6-acetylcocaine; AEME: Anhydroecgonine methyl ester; AP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylcegonine; COC: Cocaine; COD: Codeine; COE: Coccaethylene; EDDP: 2-ethylidene-1,5-dimethyl-3-diphenylpyrrolidine; EIA: Enzyme immunoassay; EME: Ecgonine methyl ester; EMT: Enzyme multiplied immunoassay; EPH: Ephedrine; ESI: Electrospray ionization; LC: Liquid chromatography; LLE: Liquid-liquid extraction; LOD: Limit of detection; LOQ: Limit of quantitation; MA: Metamphetamine; MAM: 6-acetyl morphine; MBDB: N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamide; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; MOR: Morphine; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OH-THC: 11-hydroxy- Δ^8 -tetrahydrocannabinol; SPE: Solid-phase extraction; SPME: Solid-phase microextraction; THC-COOH: 11-nor- Δ^8 -tetrahydrocannabinol-9-carboxylic acid; TMA: Trimethylamine; TOF: Time of flight.

reported and, despite the small injection volume, occasional specimens had reduced signal, which was possibly due to ion suppression from oral fluid interferences.

An approach to improve laboratory throughput and analyze a large number of analytes in a single run is to use ultraperformance liquid chromatography (UPLC) systems, which employ particle diameters as small as 1.5 μm and operating pressures higher than 5000 psi. However, despite having several applications in the field of toxicology [164,176–179], this technology has scarce application in oral fluid analysis [180].

However, identification of unknowns is a hard task for LC–MS/MS instruments. This can be overcome by the use of time-of-flight (TOF) mass spectrometers, which allow accurate mass measurement. This determination is performed by measuring flight time after acceleration in a vacuum tube by high voltage, and ion paths in the order of 2 m with flight times of 5–100 μs allow rapid and accurate time measurements. This results in short analysis times, accurate mass measurements and large dynamic ranges.

The elemental formula can be determined from molecular mass, and interpretation is usually unambiguous. Indeed, there are reports on the reduction of choices to two or three unknown substances using a library of 7640 compounds without considering LC retention times [147]. The application of TOF technology to oral fluid specimens is reported in studies such as those by Clauwaert *et al.* [79] for the determination of cocaine and metabolites in oral fluid, by Mortier *et al.* [61] for amphetamines, opiates and cocaine, and by Quintela *et al.* [69] for cannabinoids.

Liquid chromatography–MS/MS-based methods are the state-of-the-art in analyzing oral fluid samples, due to the high sensitivity and specificity provided, and there has been a considerable number of published papers on the topic, which are summarized in **TABLE 2**.

Conclusion & future perspective

Oral fluid testing is becoming more and more important in analytical toxicology, namely in the fields of clinical and forensic toxicology. Indeed, sample collection is performed easily in a noninvasive manner and, if necessary, under close supervision. This brings several advantages, including the cooperation of the person being analyzed and the difficulty in sample substitution or adulteration. One of the most prominent issues concerning the use of this biological specimen is that it can provide the ability to assess situations of

driving under the influence of drugs, to which the development of easy-to-use on-site collection devices has contributed. Indeed, this type of instrument can provide a result within minutes of sample collection, which should be confirmed afterwards in laboratorial analysis.

This laboratorial analysis is only possible due to the huge improvement in analytical technologies seen over the last two decades, including both screening and confirmatory techniques. None of these techniques should be regarded as the ‘unique solution to all the problems’. On the contrary, these techniques complement each other and, currently, their use is common in most laboratories.

Still, analytical instruments are becoming more sensitive and specific, which enables both drug detection and quantitation in very low amounts, and analyses where concentrations are expected to be low, as occurs in oral fluid. While the main analytical problems are adequately dealt with, more investigation is needed, namely on the establishment of saliva-to-plasma ratios of several drugs, aiding result interpretation.

In general, biological matrices are complex, as is oral fluid. Therefore, despite the high selectivity presented by analytical instruments, it is generally mandatory that the samples are thoroughly cleaned-up before chromatographic analysis can be performed. In addition, ion-suppression/enhancement effects should be comprehensively studied, since the precision and accuracy of the method may be compromised. This may be a problem in terms of detection limits, which represent a very important issue when analyzing oral fluid, since, in most situations, the amount of available sample is small.

No one knows what the future holds for oral fluid analysis, but it is expected that analytical equipment will become more sensitive and miniaturization will be a reality. In fact, the concept of a system with small specimen size, low detection limits, multiple drug testing platforms and high throughput is promising.

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Executive summary**Physiology & mechanisms of drug incorporation**

- Drug incorporation can occur by passive diffusion through the membrane, active processes against a concentration gradient, filtration through pores in the membrane and pinocytosis.
- Drug incorporation is pH dependent.
- Little Δ^9 -tetrahydrocannabinol (THC; main constituent of cannabis) is secreted into saliva and, therefore, the majority of THC found in oral fluid originates from a deposit after smoking.
- The parent drug is the main compound detected in oral fluid, rather than its metabolites.
- Passive contamination is possible by exposure to an environment where drugs are being consumed by others.

Collection procedures & devices

- The collection of saliva after stimulating its production can alter drug concentrations in this specimen.
- Saliva can be easily obtained without stimulation by spitting or the draining method.
- The recovery of drugs from the collection devices is concentration dependent and varies according to the analyte and collection device.
- Drug stability in the matrix should be studied, since it can affect the utility of the collection process.

Applicability of oral fluid analysis

- Oral fluid can be used to assess an individual's exposure to virtually every class of compounds, but drugs of abuse are by far the most detected substances.
- Analytical applications of oral fluid include therapeutic drug monitoring, pharmacokinetic studies, workplace medicine and detection of illicit drugs in driving situations.

Analytical technologies

- On-site collection devices
 - On-site portable devices can be used for the assessment of drug-impaired driving situations. The advantages of on-site testing are rapid turnaround times, reduced costs and the fact that the test can be carried out virtually anywhere.
 - Several studies have been conducted in the EU and USA to assess the utility of oral fluid analysis in driving situations.
- Immunoassays
 - Immunoassays are extremely useful, since they allow the saving of time and money, which would be wasted in more expensive confirmatory methods (usually mass spectrometric-based techniques), while most of the samples would be negative.
 - Immunoassays are in general aimed at the parent compound.
 - The presumptive positive samples must be confirmed by more specific techniques, namely mass spectrometric methods.
 - Their poor sensitivity for low-dose compounds, such as flunitrazepam, might still be a problem.
- Gas chromatography–mass spectrometry (GC–MS)
 - GC–MS has been the most utilized technique for the confirmation of a number of organic compounds, mainly due to the availability of spectral libraries for drugs and metabolites.
 - GC–MS is currently available in most laboratories, and this is still the main technique for confirmation and quantitation of drugs and their metabolites in biological specimens, including the so-called 'alternative' specimens, such as oral fluid, hair and sweat.
 - The limits obtained when GC–MS-based techniques are used present a limitation in terms of oral fluid, mainly due to little sample availability for analysis.
 - One possibility to overcome this problem is the use of tandem mass spectrometry.
- Liquid chromatography–mass spectrometry (LC–MS)
 - LC–MS permits the analysis of nonvolatile, labile and/or high-molecular-weight compounds.
 - Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have become the most widespread ionization techniques.
 - LC–MS is highly susceptible to matrix effects (ion suppression or enhancement), and these must be assessed during method development and validation.
 - Tandem mass spectrometry improves the detection limits.
 - The use of time-of-flight mass spectrometers, which allow accurate mass measurement, allows the identification of unknowns.

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