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REVIEW ARTICLE

Saliva as an analytical matrix: state of the art and application for biomonitoring

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Abstract
Analytical tests to measure chemicals in saliva can be employed for numerous analytes, endogenous compounds or xenobiotics. The objective was to determine which chemicals can be analysed with this matrix, which analytical methods are applicable, and what application is possible for biomonitoring. We reviewed the literature using three databases, MEDLINE, PubMed and Scopus, collecting articles on different kinds of analysis in saliva. Studies were principally about molecules of clinical interest, xenobiotics, especially drugs of abuse, and chemicals used at workplaces; some substances show no relevant correlation with exposure data while others seems to be of particular interest for systematic use for biomonitoring. Currently, saliva is used far less than other biological fluids but its use for biomonitoring of exposure to chemicals might open up new areas for research and would certainly simplify the collection of biological samples.

Keywords: *Saliva; biological monitoring; analytical chemistry*

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Introduction

Saliva is a biological fluid that is produced by the salivary glands at a relatively constant rate, from 0.5–1.5 l daily. It is 97–99% water, containing plasma electrolytes such as Na^{+}, K^{+}, Ca^{2+}, Cl^{-}, HCO^{3-}, whose concentrations vary depending on the salivary flow (Blomfield et al. 1976). Ninety-nine per cent of the lipids in saliva are cholesterol and its esters, various forms of glycerides (Larsson et al. 1996) and some fatty acids, which are believed to come solely by passage from plasma (Actis et al. 2005). The proteins present in saliva are albumin from plasma, in low concentrations (Oppenheim 1970) and glycoproteins which are responsible for the viscosity, and are the most abundant and protect the buccal epithelium (Ramachandran et al. 2006). Then there are proteic enzymes such as lysozymes, amylase and antiproteases (Rantonen et al. 2000). Saliva also contains buccal epithelial cells, lymphocytes and food residues, analytes of diagnostic interest such as steroids and non-steroidal hormones (Vining et al. 1987), some polyamines (Venza et al. 2001), traces of uric acid (Inoue et al. 2003), residues of medicinal substances and drugs of abuse, antibiotics and numerous other substances which, through various processes, can pass into saliva from the plasma (Chiappin et al. 2007).

To characterize the human saliva metabolome, some authors (Bertram et al. 2009, Takeda et al. 2009) have measured salivary metabolite concentrations under a variety of conditions in a healthy population using ^{1}H nuclear magnetic resonance (NMR) spectroscopy; metabolite concentrations were measured in resting (basal) and stimulated saliva from the same subject and they revealed citrate, lactate, pyruvate, sucrose, acetate, formate, glycine, lactate, methanol, propionate, propylene glycol, succinate and taurine.

The international scientific literature in recent years attests to the utility of saliva tests, mainly for identifying primary glandular lesions, but also for detecting metabolic or immunological systemic disorders, and checking for the use of medicinal substances and drugs of abuse. Interesting results have been reported on antibodies to the human immunodeficiency virus (HIV) in saliva (Schramm et al. 1999, Chamnanput & Phanupphak 1993, King et al. 1995).

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Saliva has various functions in the body: digestion, when amylases in the mouth start breaking down carbohydrates (Valdez & Fox 1991); clearance of oral glucose (Sreebny et al. 1985); antibacterial activity, through a peroxidase system (Iwamoto et al. 1972); is one of the routes of excretion of iodine, cadmium, mercury, gallium and technetium, i.e. a portion of these elements is eliminated by the human body through the saliva (Smith & Borzelleca 1977, Garhammer et al. 2004); and protection for teeth and the buccal epithelium (Haeckel & Hanecke 1993).

Recent years have seen a growth in scientific and clinical interest in saliva as a matrix for analysis of a variety of chemicals, and, on the basis of the correlations between blood and salivary levels of different analytes, saliva tests have been developed for several classes of compounds, offering greater specificity and sensitivity than traditional methods (Cone et al. 2002, George 1997, Hodinka et al. 1998, Kefalides 1999, Malamud & Tabak 1993).

The last decade has seen a significant development in the understanding of the pharmacokinetics of chemicals in oral fluid; this has applied particularly to abused drugs (Drummer 2005), but also for diagnostic tool (Choo & Huestsis 2004) or for workplace applications (Caplan & Goldberger 2001). Factors that influence the passage of xenobiotics from blood to saliva include: the pH of both biological fluids, the pK_a, molecular weight, spatial configuration and lipid solubility of the analyte and degree of protein binding. As a general rule there is some similarity between oral fluid concentration and a blood/plasma concentration; the pH of saliva is usually less than that of plasma, so acidic chemicals may concentrate in plasma while basic ones are commonly higher in saliva. Compounds with pK_a values less than 5.5 or greater than 8.5 are not significantly influenced by small changes in salivary pH due to flow rate changes; saliva-to-plasma concentration ratios (S/P) tend to remain relatively constant for these analytes. For neutral, non-protein-bound drugs the S/P ratio is about 1. The pharmacokinetics of xenobiotics in saliva is more complex than that of blood; detection times in saliva will depend on a range of factors including dose, frequency of exposure to chemicals and detection limit of analytical assays.

The use of saliva has been found to offer significant promise as a non-invasive alternative to blood, overcoming any possibility of a health risk for the donor and for the researcher, but also as an alternative to urine when substitution or adulteration is suspected, even if each specimen has its own distinct advantages and disadvantages (Kintz & Samyn 2002). In particular, spitting provides neat oral fluid but this is relatively viscous and can be difficult to work with in the laboratory; it may also be contaminated with food or other debris from the mouth. More often the volume will be less than 1 ml, requiring the use of sensitive detection techniques. Furthermore when oral fluid is stimulated by the use of agents such as citric acid candy, chewing gum or other agents there will inevitably be a change of pH and a corresponding lower concentration of xenobiotic (Kato et al. 1993, O'Neil et al 2000).

This article is a systematic review of the literature on the use of saliva as a matrix for analysis for the determination of xenobiotics, including its application in the practice of biological monitoring of workers occupationally exposed to chemicals. The aim was to obtain an overview of the reliability of the results obtained with the application of different analytical methods. For this purpose a literature search was performed using three different search engines for scientific literature: MEDLINE, PubMed and Scopus. We have mainly collected the publications on analytical investigations into saliva: for molecules of clinical interest, xenobiotics and chemicals used in different workplaces.

The structure of this work involves a description of the transport mechanisms of various chemical compounds in saliva, then a review of studies evaluating the presence of molecules in saliva: those of purely clinical interest, the xenobiotics such as substances of abuse or drugs and finally the chemical compounds used in different cycles of production in which exposure of subjects is linked to the workplace (separately in metals, pesticides and compounds of industrial use).

Mechanisms of transport from bloodstream to saliva

Many of the substances found in saliva – water, proteins and immunoglobulins, for instance – are secreted by the salivary glands, and their concentrations depend on saliva flow; these have therefore to be normalized in relation to a substance that allows to define the flow, and rather the volume of saliva produced, like creatinine for urine analysis, unfortunately at the moment was not identified a compound useful for the purpose (Haeckel & Hanecke 1993).

For a molecule to be assayed in saliva, unless it is actually produced by the salivary glands, it must have certain chemical features that enable it to cross the biological barriers between the bloodstream and the salivary glands – the capillary wall, the basement membrane and the cell membrane of the glandular epithelium. Alternatively, interstitial extracellular secretory fluid can pass into the saliva through the secretory epithelial junctions. There are various mechanisms for this transport, involving different molecular structures:

(1) Ultrafiltration through the cell junctions of the secreting unit. This mechanism can only deal with very small molecules, i.e. with molecular weight less

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than 1900 Da, such as electrolytes and hormones (catecholamines and some steroids) dissolved in plasma or interstitial fluid. Molecules that pass by this route have a lower concentration in saliva than plasma (from 1/300 to 1/3000) (Lac 2001, Marini & Cabassi 2002).

(2) Ultrafiltration through the cell membrane pores; the molecular weight limit here is 400 Da, meaning water and electrolytes.

(3) Trans/exudation of plasma into the oral cavity. Transudation comes either from the gingival sulcus or directly from the oral mucous membrane and the transudate carries low-molecular-weight molecules and proteins, such as albumin, into the saliva. Exudation occurs when there is a disorder such as gingivitis, and carries into the saliva molecules with larger molecular weight, such as globulin and fibrinogen whose plasma concentrations are generally higher than in saliva (Lac et al. 1993, Vining et al. 1983b).

(4) Selective transport through cell membranes:

a. *Passive diffusion of lipophilic molecules*. Highly liposoluble molecules can cross the cell membrane, particularly the plasma membrane of secretory cells. Non-esterified and unbound steroid hormones enter saliva through this mechanism (Haeckel & Hanecke 1993, Vining et al 1983a). In this case the salivary and free plasma concentrations are kept in fairly constant equilibrium, the former being 10–100 times lower than the latter, because of the fast passage. Many other substances besides steroids (xenobiotics) enter the saliva by passive diffusion.

b. *Active transport by a membrane system (pumps) against a concentration gradient.* Water and small molecules can flow into the cell by this mechanism, which operates for many electrolytes and molecules such as IgA; it is also active for penicillin and tetracycline, and presumably also for other molecules too. In general active transport is believed to work for molecules with a S/P close to 1 (Marini & Cabassi 2002).

Saliva as an analytical matrix for molecules of clinical interest

Current saliva tests are used to investigate the physiological molecular pool and some exogenous compounds, for pharmacokinetic studies (Matin et al. 1974), or to detect molecules that have been used inappropriately or fraudulently in medicine (Marquet et al. 1996, Barbera et al. 2002, Grootveld et al. 2006, Rodopoulos & Norman 1996). This sort of test is particularly important to hospital pediatricians and neonatologists, as the non-invasive approach is accepted by their small patients (Lee et al. 1996).

The evaluation of collectors that could be used in oral fluid sampling is presented in the literature with particular attention to analysis of pathogens, antibodies, drugs and nucleic acids in saliva (Holm-Hansen et al. 2004); the results show that a collector such as the Salivette® is better for a complete transfer of whole fluid but for protein or nucleic acid analysis OraSure® or UpLink® seem to be more suitable; however, each analysis requires the use of the appropriate instrument for sampling and the evaluation of correct storage conditions to avoid blunders.

Seidel et al. (2001) provide an example in their study in which they assayed IgA and IgD in saliva of newborns, for studying local defence mechanisms against bacterial and viral infection in the first day of life, by radial immunodiffusion; the findings indicated that the immune status of the oral mucosa already had biological significance on the infant's very first day.

Immunoglobulins are secreted by the plasma cells of the mucosal lamina propria, mainly the minor salivary glands, and constitute a barrier preventing antigens

Table 1. Analytical methods applied to molecules of clinical interest in saliva.

Molecules	Analytical method	References
IgA, IgD	Radial immunodiffusion, RIA, EIA techniques	Siedel et al. 2001; Mackinnon et al. 1993; Nurkka et al. 2003
HIV-1, HIV-2 antibodies	Strip test	Schramm et al. 1999; Chamnanput et al. 1993; King et al. 1995
Melatonin	LC-RIA	Simonin et al. 1999; Harumi et al. 1996; Vitale et al. 1996
	GC-MS	
Cortisol	Immunoassay system,HPLC/MSHPLC/MS/MS	Vining et al. 1987; Chiu et al. 2003; Kirschbaum et al. 1994; Neu et al. 2007
Testosterone	Immunoassay system	Vining et al. 1987; Lac et al. 1993
Estradiol	Immunoassay system	Vining et al. 1987; Worthman et al. 1990
Progesterone	Chemiluminescence immunoassay	Vining et al. 1987; De Boever et al. 1986
Polyamines	HPLC/ fluorescent detector	Venza et al. 2001
Uric acid	HPLC/amperometric detector	Inoue et al. 2003

RIA, radioimmunoassay; EIA, enzyme immunoassay; HIV, human immunodeficiency virus; HP, high performance; LC liquid chromatography; GC, gas chromatography; MS mass spectrometry.

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entering the organism (Grimound et al. 1996). IgAs have been determined by radial immunodiffusion or radio- and immunoenzymatic techniques to examine immune responses in the oral cavity and upper respiratory tract, and to assess the immune status of competitive athletes (Mackinnon et al. 1993, Nurkka et al. 2003). The sampling method and storage of the sample are important in these assays, as differences can lead to over- or underestimates (Nurkka et al. 2003): four collection methods (collecting drool, pipette suction and two commercial kits, OraSure® and Oracol®) and three storage protocols (snap-frozen with glycerol in liquid nitrogen, stored for 4–8 h at 4°C with and without addition of protease enzyme inhibitors prior to storage at -70°C) were applied; results showed that IgA concentrations were not significantly different with different collector methods but snap-freezing the specimens in liquid nitrogen led to concentrations about 45% higher than other methods.

There is ample literature on the use of saliva assays to measure human immunodeficiency virus (HIV)-1 and HIV-2 antibodies (Chiappin et al. 2007, George 1997, Kefalides 1999), with the use of immunochemical methods; the sensitivity (compared with serum) for detection in saliva was between 90 and 98%, and rapid strip tests are considered reliable for use in the field when no laboratory is available for conventional analysis (Schramm et al 1999).

Melatonin plays an important role in the circadian regulation of various physiological and neuroendocrine processes; it is detectable in saliva, but usually at a very low concentration, and quantification calls for highly sensitive techniques. Liquid chromatography (LC)-radioimmunoassay (RIA) has given good results (mean CV% 8, accuracy 88–112%), coupling chromatographic separation with highly specific detection to reach a limit of quantification of 15 pg ml^{-1}. The authors nevertheless confirmed their findings by gas chromatography (GC)-mass spectrometry (MS) (mean CV% 16%, accuracy 84–116%), achieving a limit of quantification of 1 pg ml^{-1}, which makes this latter method more suitable for salivary assays of melatonin; the stability of melatonin in saliva was studied following storage for 7 days at 4°C than 20°C while for 6 months at -20°C, in every situation there was a maximum loss of 20% (Simonin et al. 1999, Harumi et al. 1996, Vitale et al. 1996).

Hormone assays in saliva are a wide field of analysis and clinical research. Among non-steroidal hormones the catecholamines are detectable in saliva at between 250 and 800 pg ml^{-1}. They arrive either by diffusion from serum or from stimulation of the glandular sympathetic nerve endings. As a consequence their salivary concentrations show little relation to the plasma content (Marini & Cabassi 2002).

The close correlation between salivary and serum concentrations of steroid hormones was first reported in the early 1980s (Vining et al. 1983a, Price et al. 1979, De Boever et al. 1986). This is related to the way these compounds enter saliva, and the ratio of salivary concentration to total plasma content – from 1:10 to 1:100 – depends on the strength of the bond between the hormone and its protein carrier, and the high permeability constant. As a result, therefore, the salivary levels of steroid hormones are representative of the free plasma fraction even in situations in which the concentrations in blood change rapidly (Lac 2001). The mean salivary concentration of cortisol in male subjects ranges from 15 nmol l^{-1} in the morning to 3 nmol l^{-1} in the evening (Lac 2001), and the S/P ratio is around 1:30 (Lac et al. 1993). Some of the cortisol is converted to cortisone by 11-β-hydroxysteroid dehydrogenase in the salivary glands (Vining & McGinley 1987). Salivary and serum cortisol levels are closely correlated in mammals (from 0.6 to 0.9) (Lac et al 1993, Vining & McGinley 1987).

Cortisol in saliva is a diagnostic test in current clinical use for the follow-up of Cushing syndrome (Chiu et al. 2003), for psychological disorders (Kirschbaum & Hellhammer 1994) and for assessing certain targeted stress situations. For each of these tests the saliva sampling methods (Neu et al. 2007) and the analytical methods (Kataoka et al 2007, Jönsson et al. 2003, Yehuda et al. 2003, Anfossi et al. 2002, Sgoifo et al. 2003) have been compared. The comparison between direct sampling of saliva in a conical tube and a paper filter wetted with saliva showed that this second collection method is the most useful; the analyte seems to be more stable even at room temperature for more than 6 weeks, while whole saliva needs storage at -20°C for a comparable stability. Analytical methods used were immunoenzymatic or chromatographic; the first showed interday and intraday coefficient of variability <6%, with a limit of detection (LOD) around 1.0 nm l^{-1} while using solid-phase microextraction (SPME)/LC/MS the performances obtained, were: a LOD at 5 pg ml^{-1} with recoveries above 95% and CV% between 4.6 and 8.9% were optimized also numerous parameters to assess possible analytical interference such the presence of bacteria in the oral cavity (Whembolua et al. 2006). The concentration in saliva is in fact widely believed to be more accurate than the plasma level (Lac 2001, Seidel et al. 2001) as it is independent of the patterns of cortisol-bound globulin (CBG) which can complicate the interpretation of blood test results. The test on saliva has therefore been proposed even for preterm babies, to assess their hypothalamic, pituitary and adrenocortical gland function (Neu et al. 2007).

The correlations between salivary and free serum concentrations and the S/P ratio have been defined for testosterone, estradiol, estrone, estriol and progesterone, and for adrenal cortical androgens (as indicated above

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the S/P ratio is around 1/10–1/100 owing to the unbound hormone). Serum testosterone is bound at 98.5% to a specific protein carrier (TeBG or SHBG). GC-MS measurements indicate that the salivary concentration is the same as the free serum fraction, i.e. 1.5% of total serum testosterone. However, radioimmunological assays set it between 1 and 5%. This difference is quite possibly due to cross-reactions between dihydrotestosterone and different pools of polyclonal antibodies. These could lead to overestimation because the salivary glands contain a hydroxylase that converts more than half the testosterone to dihydrotestosterone. Despite these conflicting findings, salivary testosterone assays are still widely used (Lac et al. 1993).

Female hormones are assayed in human saliva mainly as a clinical follow-up of ovarian pathologies, or for assisted reproduction. Findings are limited to date for estradiol and estrone, probably because of their low concentrations in saliva, which makes them hard to measure. There is, however, more information on salivary progesterone, which is frequently measured for menstrual problems and in pregnancy. Salivary estriol is sometimes tested during pregnancy to check fetal vitality (Worthman et al. 1990).

The concentrations of adrenal cortical androgens in saliva are high enough to allow radioimmunological tests without difficulty, in male and female subjects. In clinical practice these compounds, together with 17-hydroxyprogesterone, are assayed to diagnose congenital adrenocortical hyperplasia and hyperandrogenism (Lac 2001). Adrenal androgens are often assayed in saliva to check pubertal development in boys.

Other pathologies too can change the salivary concentrations of certain compounds, which can therefore be usefully assayed. These include polyamines (spermine, spermidine and putrescine) whose presence in the oral cavity is a pointer to various disorders; these compounds, after collection of saliva with a polyethylene pipette and centrifugation for 30 min to remove cellular elements, were analysed with high-performance LC (HPLC) and fluorescence detection: the detection limits were 0.04, 0.05, 0.06 nmol ml^{-1}, respectively, for spermine, spermidine and putrescine, with recovery over 90% and mean CV% 5 (Venza et al. 2001). Uric acid can be assayed to diagnose gout, hyperuricemia, or Lesch–Nyhan syndrome and helps detect alcohol abuse, obesity, diabetes, hypercholesterolemia, hypertension, and renal and cardiac problems. An HPLC/electrochemical detection (ED) method was carried out with LOD 3 nM for Ampero-ED and 6 nM for Coulo-ED, and recoveries above 95% with a relative standard deviation (RSD) of less than 15% (Inoue et al. 2003). Table 1 resumes the above.

Xenobiotics

Recent experimental studies have looked into the possibility of chemical–toxicological tests on biological samples other than blood. For substances of abuse, the difficulties of confirming acute intoxication due to drugs and the impossibility of obligatory blood sampling has led researchers to seek other matrices that could be sampled without affecting the subject's physical integrity (Schramm et al. 1992, Johan & Luc 2005, Drummer 2006).

Heroin (Drummer 2006, Goldberger et al. 1993) and cocaine (Jenkins et al. 1995, Cone et al. 1997) taken intranasally or intravenously, or by inhalation, gave salivary

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Table 2. Analytical methods applied to different xenobiotics.

Molecules	Analytical method	References
Heroin	GC/MS	Drummer 2006; Goldberger et al. 1993
Cocaine	GC/MS	Jenkins et al. 1995; Cone et al. 1997; Lo Murzio et al. 2005
	Immunochemical	De Giovanni et al. 2002
Cocaine metabolites	EIA microplatesGC/MS	Cooper et al. 2004; Niedbala et al. 2001
Amphetamines		Cooper et al. 2006
Opiates		Cooper et al. 2006
Methadone		Moore et al. 2001; Cooper et al 2005b
THC	GC/MS	Gross et al. 1985; Somers et al. 2003
Cotinine	LC-MS/MS	Lee et al. 2005
Cotinine-nicotine	GC/MS	Lee et al. 2005; Shin et al. 2002
Levitracem (antiepileptic)	HPLC-ESI-MS/MS	Guo et al. 2007
Spiramycin	LC-MS/MS	Sagan et al. 2005
Metronidazole		
Propranolol		Hold et al. 1995
Caffeine	TLC with UV detector	Fenske 2007
Ethanol	GC-HS	Wojciech et al. 2002

EIA, enzyme immunoassay; HP, high performance; LC, liquid chromatography; GC, gas chromatography; MS mass spectrometry; THC, Δ^9-tetrahydrocannabinol; ESI, electrospray ionization; TLC, thin-layer chromatography.

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concentrations detectable by GC/MS using a selected ion recording mode. Validation parameters for cocaine were: LOD of 20 ng ml^{-1} and a mean CV% of 4.5; for heroin LOD of 1 ng ml^{-1}, a mean CV% of 5; recovery above 95% with a solid-phase extraction (SPE); the mean accuracy of both analytes was 98% (Lo Murzio et al. 2005, Fucci et al. 2003). Also immunochemical methods, even if only for a qualitative detection, were proposed with a GC/MS confirmation of results for an identification of false positives; the sensitivity was 25 ng ml^{-1} while the GC method had a LOD of 10 ng ml^{-1} (De Giovanni et al. 2002).

One of the major disadvantages of salivary drug testing is the shorter detection times for drugs, about 1 day. The method of saliva collection may also impact on the analytical findings; if the collection is stimulated to obtain sufficient specimen volume for testing, it is known that the saliva flow rate is increased, leading to an increased pH and potentially a decreased concentration of drugs; this is particularly true for cocaine (Kato et al. 1993). It as been found that to determine heroin and cocaine excretion profiles by a sensitive method requires the collection of 5 ml of saliva in 30 s.

The validity of enzyme immunoassay (EIA) microplates has been assessed for detecting cocaine and its metabolites (sensitivity 96%, specificity 83%) (Cooper et al. 2004, Niedbala et al. 2001), amphetamines (sensitivity 91.7%, specificity 95.9%) (Cooper et al. 2006), opiates (sensitivity 99.1%, specificity 94.4%) (Cooper et al. 2005a) and methadone (sensitivity 91.3%, specificity 100%) (Moore et al. 2001, Cooper et al. 2005b) in saliva and the sensitivity and specificity were all good, as confirmed by GC-MS. That heroin and cocaine are found in saliva because of redistribution is confirmed by the presence of their metabolites (monoacetylmorphine, morphine and benzoylecgonine).

Cannabis derivatives are highly liposoluble, and salivary concentrations of Δ^9-tetrahydrocannabinol (THC) are indicative of the blood levels (Gross et al. 1985) although not strictly correlated. It even seems that in some cases THC is detectable in saliva for longer than in blood (Thompson et al. 1987b). Understanding the relationship of THC concentrations in oral fluid and plasma is important in the interpretation of oral fluid test results. Current evidence suggests that THC is deposited in the oral cavity during cannabis smoking. This 'depot' represents the primary or sole source of THC found when oral fluid is collected and analysed. Plasma and saliva specimens were analysed by GC-MS (LOD 0.5 ng ml^{-1}, mean CV% of 2, recovery from collection pad of 100 ± 10%); oral fluid was also analysed by RIA. THC ratio of oral fluid to plasma THC concentrations was 1.18 with a range of 0.5–2.2. Within 12 h, both oral fluid and plasma THC concentrations generally declined below 1 ng ml^{-1}. RIA analyses (LOD 10 ng ml^{-1}, mean CV% 6) of oral fluid specimens demonstrated the same pattern of initial high levels of contamination immediately after smoking, followed by rapid clearing, and a slower decline over 12 h. The decline in THC oral fluid concentration over this time suggests that there may be absorption of THC into blood (Huestis et al. 2004).

The findings confirm that saliva can be used as the starting matrix for investigating cannabinoid use (Barbera et al. 2002, Verstraete 2004, Cirimele et al. 2006, Moore et al. 2007, Somers et al. 2003).

The list of substances of abuse must also include cigarette smoke, and tests on saliva collected from adolescents have been used to assess exposure, analysing cotinine by LC-MS/MS (LOD 0.10 ng ml^{-1}); cotinine was assayed as the biomarker (Lee et al. 2005). Both cotinine and nicotine have been assayed in saliva and other biological fluids (urine, plasma), using GC/MS; analytes were extracted from alkalinized samples with ethyl ether and concentrated with a nitrogen stream; the detection limits for both were 1 ng ml^{-1}, intraday precision was $<5\%$ as RDS, with recoveries between 88 and 99%. Salivary cotinine was highly significantly correlated with plasma concentrations ($R = 0.96$, $p = 0.00$), indicating that this is a good biomarker for cigarette smoke exposure, while salivary nicotine showed no significant correlation with nicotine in plasma ($R = 0.090$, $p = 0.499$) (Lee et al. 2005, Shin et al. 2002).

Grootveld et al. (2006) used high-resolution ^{1}H NMR spectroscopy to investigate the detection and quantification of the illicit 'date-rape' drug γ-hydroxybutyrate (GHB) in human saliva. They demonstrated the advantages offered by this technique when applied to the analysis of illicit drugs in multicomponent sample matrices such as human biofluids, even though this technique does not have a sensitivity high enough to permit an analysis at the low concentrations that are often present in saliva without voluntary intoxication.

Instrumental methods for assaying medicinal drugs in saliva have also been developed; for example levetiracetam, an antiepileptic drug, was easily analysed in saliva. The pretreatment of the sample involved collection with a plastic centrifuge tube, then the sample was mixed with a solution of acetonitrile and ritonavir to precipitate proteins, and finally the tube was vortexed and centrifuged. The supernatant was analysed using an HPLC-electrospray ionization (ESI)-MS/MS method, in multireaction monitoring mode, which gave an excellent recovery (103–108%), precision (mean CV% 6.5) and detection limits (0.1 μg ml^{-1}) (Guo et al. 2007), showing a S/P ratio of 0.9. LC-MS/MS has been used to determine spiramycin and metronidazole in saliva. The extraction of analytes was carried out adding to the sample sodium hydroxide, a buffer solution of pH 9 and ethyl acetate, then after centrifugation the upper organic phase was transferred to a polypropylene tube, evaporated to dryness under nitrogen and the dried extract

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was reconstituted with acetonitrile, and finally it was analysed with the chromatograph. The validation data were: precision of CV% 12, accuracy of 85–115%, recovery above 76.3%, LOD 15 ng ml^{-1} for both analytes (Sagan et al. 2005).

Studies of the modes of transport of propranolol enantiomers after an oral dose of 10 mg showed that passage from the bloodstream to the salivary glands was not stereospecific. The salivary concentrations of both enantiomers were higher than in venous blood, and with time the S/P ratio declines from about 5 to 1, indicating that this assay measures the internal dose more accurately than blood tests (Hold et al. 1995), even if it is necessary to consider the method of sampling saliva. In fact the use of some kits for sampling commercially available like Parafilm® and Salivette®, owing to their constituent materials, give incorrect results for the possibility of partial absorption of the analyte.

The instrumental methods employed are extremely accurate, but are usually costly. However, thin-layer chromatography (TLC) with dosimetric ultraviolet (UV) absorption detection to assay caffeine in saliva and urine was judged an excellent method, inexpensive and just as effective as GC and immunoenzymatic techniques: diluted saliva (0.5 ml) was pipetted on the top of glass columns for TLC, the LOD was fixed at 20 ng, mean recovery data were 94% with a mean precision of RSD 2.2% (Fenske 2007).

This rapid overview of the types of molecules analysed in saliva ends with ethanol, which is easily assayed using a head space sampler coupled to the GC (Wojciech & Zuba 2002). Table 2 summarizes the analytical methods used for the different xenobiotics.

Saliva as an analytical matrix for biological monitoring of workers

Biological monitoring of occupational exposure to chemicals always requires the worker concerned to be willing to take part in the investigation so as to assess whether the conditions for exposure were sufficient to permit detection of the dose absorbed. When a blood sample is needed, however, workers are not always willing because the sampling is invasive. An analytical matrix that is easier to sample than blood would therefore be very useful – also in economic terms. However, salivary concentrations of molecules of interest are normally much lower than in plasma, so highly sensitive analytical equipment is essential to reach the low levels of detection, especially when workers are not exposed to high doses.

Exposure to inorganic elements

Many studies have looked into the possibility of using saliva assays to measure exposure to inorganic substances of toxicological interest, particularly metals. These studies have involved various different scenarios, but their findings are often similar. Extremely sensitive analytical methods are normally employed, such as inductively coupled plasma/mass spectrometry (ICP/MS) (Menegario et al. 2001) or atomic absorption (AA) (Poczatek et al. 2004), but even so problems can arise because the concentrations are often below the limit of detection, and quantitative analysis is not possible.

The question of mercury in dental amalgams triggered studies to determine this heavy metal in saliva, in order to assess contamination of the oral cavity (Zimmer et al. 2002, Ganss et al. 2000). The correlations

Table 3. Analytical methods applied for biomonitoring of workers exposed to chemicals.

Molecule	Analytical method	References
Inorganic molecules		
Mercury	ICP/MS	Zimmer et al. 2002; Ganss et al. 2000; Pesch et al. 2002
	AA	
Lead	ICP/MS	Wilhelm et al. 2002; Barbosa et al. 2006
	Zonal capillary electrophoresis with UV detection	Luconi et al. 2006
Cadmium	Electrothermal atomic absorption spectrophotometry	With et al. 1992
Exposure to pesticides		
Diazinon	ELISA	Lu et al. 2003
Atrazine	ELISA	Denovan et al. 2000; Koivunen et al. 2007
Paraquat	ELISA	Koivunen et al. 2007
Compounds used in industry		
Phthalates	LC-MS/MS	Silva et al. 2005
Isopropanol	HS-GC	Rose et al. 1999; Ernstgård et al. 2003a
Xylene	HS-GC/FID	Ernstgård et al. 2003b
Methanol	Enzymatic assay	Ernstgård et al. 2005
Toluene	GC/MS	Ferrari et al. 2008

ICP, inductively coupled plasma; MS, mass spectrometry; AA, atomic absorption; ELISA, enzyme-linked immunosorbent assay; LC, liquid chromatography; GC, gas chromatography; FID, flame ionization detector.

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with blood and urinary levels in the study subjects were poor, statistically not significant, and saliva tests were therefore judged inappropriate for investigating this type of exposure. Pesch et al. (2002) reached a similar conclusion in their study in children: salivary concentrations were undetectable in 70% of the sample, whereas the concentrations in urine and hair were complete and correlatable. A similar situation was reported by Wilhelm et al. (2002) who assayed lead (Pb) in saliva of 254 German children and the values were below the limit of detection (1.5 μg l^{-1}) in 89%.

There are more experimental investigations assessing the applicability of saliva tests for exposure to Pb. The Pb fraction in saliva is correlated with the portion not bound to plasma proteins, so it is normally very small – about 100 times lower than the dose detectable in other biological compartments (Barbosa et al. 2006). Such very low concentrations of the analyte limit application to situations where extremely highly sensitive techniques are available (Barbosa et al. 2005), unless one preconcentrates the analyte, so as to use methods such as capillary zone electrophoresis with indirect UV detection. In this work the detection limit value of Pb for the preconcentration of 8 ml of saliva was 11.4 μg l^{-1}, the reproducibility was 0.68–3.6%, but to obtain the necessary volume of saliva the collection was done using citric acid solution for salivation stimulation and the correlation with plasma levels was not studied (Luconi et al. 2006). Procedures like this have been refined and are considered effective for measuring Pb in saliva.

Studies to correlate blood and salivary Pb concentrations have not given the expected results as the correlations were not significant. Researchers therefore strongly advised against using this matrix for testing people exposed to Pb (Barbosa et al. 2005, Liu 1991, Thaweboon et al. 2005). Attempts to apply this analytical approach for biological monitoring of occupationally exposed workers has also given contradictory data, because with blood concentrations between 10 and 50 μg dl^{-1} no useful salivary levels were found (Kho et al. 2003).

The only discordant finding was reported by Nriagu et al. (2006) who found a weak but statistical significant correlation between the results of assays (with ICP/MS) in blood and saliva in an urban population, suggesting that salivary Pb tests might be useful for qualitative applications: the average plasma Pb concentration was 27 ± 0.1 μg dl^{-1} while average saliva concentration was 2.4 ± 0.1 μg dl^{-1}; the Pearson coefficient of correlation was found to be $r = 0.49$ for older participants (>49 years) vs $r < 0.15$ for younger participants; the correlation was stronger for male ($r = 0.33$) than female ($r = 0.15$) subjects.

The analysis of cadmium in saliva as a biological marker of occupational exposure to the metal proved fairly suitable for this purpose (LOD 0.6 nmol l^{-1}), salivary cadmium levels were significantly raised in groups of currently exposed individuals (median value 17 nmol l^{-1} and 70 nmol l^{-1} a second group) and in past workers with previous long-term exposure (median 2.5 nmol l^{-1}) when compared with unexposed population, with results indicating recent exposure. However, great care is needed in the sampling procedure because of the possible risks of contamination (Withe et al. 1992). Nevertheless, these researchers note that there is much interest in this matrix, particularly for biological monitoring, on account of the practical advantages it offers (Pesch et al. 2002).

Exposure to pesticides

The use of saliva for determining pesticides is attracting considerable interest, not only on the experimental level but also in practical biological monitoring.

In the literature information is present for pesticide administration to animals and salivary concentrations, while there are fewer investigations on workers, as is shown below. In this case it is important to emphasize that the concentrations of chemicals in workplaces are, of course, different from one plantation to another and that they are frequently much lower than experimental data on animals; the low concentrations of exposure call for analytical methods with high sensitivity to check the real dosage in blood and urine, and especially in saliva.

Laboratory tests on rats, investigating the potential application of saliva assays to check for occupational exposure, found a close correlation between salivary and plasma levels of diazinon (Lu et al. 2003). In saliva the proportion of the total intake of the compound was about ten times lower than in blood (the mean S/P concentration ratios of diazionon were 0.16 and 0.13 for 1 and 10 mg kg^{-1} of bolus doses, respectively), indicating incomplete transfer of the xenobiotic from blood to the salivary glands. Further study, however, showed that the ratio of the concentration of the unbound substance in plasma to the salivary concentration was practically 1:1, which clarified the reason for the partial transport.

In view of this close correlation salivary diazinon was proposed as a biological marker of occupational exposure and it was in fact used in Nicaragua on banana plantation workers. Saliva samples were centrifuged after collection with Salivette®, the cotton roll and inner plastic tube were then removed and discarded, leaving the ultrafiltrate in the centrifuge tube. Salivette® collects approximately 2 ml of saliva in 2 min. Saliva and plasma samples were analysed for diazinon using an enzyme-linked immunosorbent assay (ELISA). This ELISA kit is specific for diazinon with the limit of detection of 0.022 μg l^{-1}. There is approximately a 20% degradation of diazinon in saliva and plasma samples during the course of sample transportation and freezer storage. Recoveries were about of 80% and precision was

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very variable CV% 17.3–142.9. The results were confirmed as reliable and for the authors the procedure proved applicable for biological monitoring (Lu et al. 2006). A study to determine trichloropyridinol, a metabolite of the organophosphorus insecticide chlorpyrifos, in rats also found significant correlations, with a blood:saliva difference increased over a range tested (1–50 mg kg^{-1}) from 11 to 67. Initial results suggest that the response is non-linear over the dose levels evaluated and a computational model confirmed this situation also at relevant occupational and environmental exposure levels. So, even if the analyte is detectable in saliva and the kinetics observed is parallel to that of blood, more studies are needed to understand the real possibility of use of this biomarker. The authors proposed this method for biological monitoring of occupational exposure once there was a fully developed and validated immunosensor platform coupled with a pharmacokinetics/pharmacodynamic model to study for a real-time biomonitoring"(Timchalk et al. 2007).

Atrazine is one of the most widely used triazine herbicides and particular efforts have been made to establish non-invasive monitoring methods. Laboratory findings indicate that atrazine is found in saliva and that the S/P (0.66) ratio remains stable with time, independently of changes in saliva flow, so this assay could be useful in humans (Lu et al. 1997). Denovan et al. (2000) confirmed this in herbicide applicators, where they found that atrazine passed through the salivary gland cell membrane, and could be detected in saliva with high sensitivity using the ELISA method, with a limit of detection of 0.22 ng ml^{-1}; median salivary concentrations of atrazine on non-spray days were significantly lower than on spray days (Mann–Whitney *U*–Wilcoxon rank sum test $p < 0.01$). Koivunen et al. (2007) suggested using immunoenzymatic methods for this purpose, particularly for atrazine and paraquat in saliva.

Exposure to industrial compounds

To assess exposure to phthalates, chemicals used as plastifiers, 14 metabolites were examined by isotopic dilution and SPE, with LC/MS/MS analysis; seven were not identifiable because they fell below the limit of detection of the method, 1 ng ml^{-1}. In 40 occupationally exposed workers mean levels were significantly higher than in the controls. The concentration of each compound in saliva was always lower than in urine, but comparable with serum levels. The authors therefore suggested this matrix for measuring the internal dose absorbed, but the correlation was investigated using serum data available from literature and this methodological approach is not correct for the study of passage from blood to saliva of chemicals, furthermore the validation parameters of analytical methods are not presented in detail (Silva et al. 2005).

Methanol is widely employed in the workplace and to assess the risk of exposure to this organic solvent toxicokinetic and toxicodynamic studies have been done to establish which biological compartments are useful indicators of its presence in the body (Ernstgård et al. 2005). Immediately after exposure saliva had higher levels than blood, although the difference disappeared within a few minutes. Concentrations in blood, saliva and urine were comparable with a half-life, 1.4, 1.3 and 1.7 h, respectively, and were more or less equally reliable. A GC/flame ionization detector (FID) was used, obtaining a LOD of 0.1 μM, and a CV% <3%.

Efforts to find alternatives to blood sampling methods for studying occupational exposure to chemicals have lead to a method for determining acetone, a metabolite of isopropanol, in saliva using GC. In workers exposed to isopropanol, correlations between blood and salivary acetone were high ($R = 0.8568$). However, the researchers felt that work was still required to standardize the procedures and validate the method for biological monitoring in the workplace (Rose et al. 1999). Tests have also been done to check for sex differences in the toxicokinetics of isopropanol, using saliva, blood, urine and expired air analysed in a GC with a head space sampler (detection limit of 1 μM, a method error estimated <3–11%). Some differences were found, especially for salivary acetone in exposed women, although the authors of the study could not explain this (Ernstgård et al. 2003a).

A similar study was done for m-xylene, which was analysed by HS/GC/FID in blood, saliva, expired air and urine. Here the men had a higher volume of distribution and excretion of m-methylhippuric acid, but there were no significant differences involving genotype or phenotype. Again, analysis in saliva gave accurate results with a detection limit of 1 μM, and a method error estimated at <3–11% comparable to the other biological compartments. The postexposure time course of m-xylene in saliva was parallel to that in blood, and saliva levels were, on average, seven time lower than the blood levels (Ernstgård et al. 2003b).

A recent study describes a rapid and sensitive method for the determination of toluene in saliva through the use of GC/MS (LOD 0.10 μg l^{-1}, intraday CV% 1.9%, interday CV% 3.5). The investigation in question has provided environmental monitoring and analysis of salivary toluene and urinary hippuric acid, *o*-cresol and toluene itself in 36 workers exposed to toluene in the synthetic leather industry and five healthy volunteers. The results showed a correlation coefficient (r) between environmental and biological data in saliva of 0.77 against 0.93 in urine. The authors believe that the data obtained indicate that the determination of salivary toluene can be proposed as new biomarkers of toluene exposure (Ferrari et al. 2008). Table 3 summarizes the analytical methods for chemicals used in worplaces.

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Table 4. Advantages and drawbacks of use of saliva as an analytical matrix.

Advantages	Drawbacks
▪ Non-invasive sampling	▪ Small sample volume
▪ Simple sampling in patients with limitations and in children	▪ Possibility of interference
▪ Inexpensive	▪ Low concentrations of analytes of interest in saliva, making detection difficult
▪ Samples can be taken by non-qualified personnel	▪ Possibility of assaying analytes only after recent exposure
▪ Samples can be taken by the donors themselves	▪ Risk of rise in pH of the matrix after stimulation of saliva flow, with potential lower concentration of analytes of interest
▪ There is no health risk for the donor	
▪ Controlled sampling is possible, avoiding the risk of adulteration	

Discussion

Further research is undoubtedly needed on saliva as an analytical matrix to standardize and validate procedures. Reports so far indicate that for inorganic compounds this approach is not accurate or applicable quantitatively; however, for various organic compounds, in particular drugs and substances of abuse, assays clearly highlight recent exposure and give qualitative information. Quantitative analysis in saliva calls for highly sensitive instrumental techniques with the lowest possible detection limits. MS offers excellent detection, coupled with either LC or GC (Cooper et al. 2005b, Cirimele et al.2006, Ernstgård et al. 2003b, Ferrari et al. 2008, Shipper et al. 2007) and solid-phase microextraction (SPME) is another possible method, with preconcentration of the analyte (Fucci et al. 2003). Table 4 lists the pros and cons of using saliva.

For routine biological monitoring using saliva as the analyte would be a practical way of simplifying periodic screening of workers exposed to chemicals (Esteban & Castano 2009). Before it can be employed on a large scale further specific research is needed on the transport mechanisms and distribution in the body. We need to know exactly which compounds can be detected in saliva, in what conditions of exposure (low or very low doses definitely impose appreciable limits), and which analytical methods will give data that can really be used to assess the risk of exposure to chemicals.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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