

Short Communication

Evaluation of a novel standardized system for collection and quantification of oral fluid

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Abstract

Background: Standardization in collection and testing of oral fluid is lacking.

Methods: A novel standardized collection and quantification system for oral fluid testing was evaluated. Sample collection volumes were determined. For determination of the saliva content in oral fluid samples, tartrazine, which is an integral part of the saliva extraction solution serving as an internal standard, was used. Results were compared with those obtained by employing glucose as a 'supplemental internal standard' for determination. Analytical performance of the new system for collection and quantification of oral fluid was evaluated. Calcium and magnesium analyte concentrations in oral fluid samples were measured with a routine laboratory method and compared to a reference method.

Results: Volumes of oral fluid samples collected ranged from 4.9 to 10.5 mL. The mean saliva content in oral fluid samples was found to be 65.5 percent by volume (vol.-%) when determined through the tartrazine concentration and 65.0 vol.-% when determined through the glucose concentration. Evaluation of analytical performance revealed interassay imprecision coefficients of variation (CVs) ranging from 1.5% to 3.2% and intraassay imprecision CVs from 1.1% to 2.5%. When linearity was tested, a quasi-linear curve was observed ($R^2=0.99$). Comparison of two different

methods for determination of calcium and magnesium concentrations showed correlations of $R^2=0.96$ for calcium and $R^2=0.97$ for magnesium.

Conclusions: The new system for collection and quantification of oral fluid helps to improve standardization of pre-analytics in oral fluid testing and to provide reliable and accurate quantification of analytes in oral fluid samples.

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Several fluids combine to constitute what is commonly referred to as 'saliva'. These fluids are excreted by major salivary glands, minor salivary glands and gingival crevices. A mixture of fluids from these sources including other constituents of non-salivary origin that may be present in the mouth, such as bronchial and nasal secretions, serum and blood derivatives from oral wounds, pathogens, desquamated epithelial cells, other cellular components and food debris is referred to as 'oral fluid' or 'whole saliva' (1–4). Saliva testing has advantages over more conventional biological matrices, such as blood and urine. Sample collection is rapid, non-invasive, less objectionable to patients and easier in those with poor venous access. Furthermore, it is less stressful and has fewer risks for the patient. Saliva can be collected in remote sites by unskilled personnel and, with certain collection devices, is stable at room temperature for extended periods (5). Saliva can be collected in a variety of ways including the spitting method with the saliva running freely from the mouth into a collection container or through stimulation by chewing, by citric acid or other means (6, 7). For sample collection, a wide variety of devices are commercially available (1). However, devices show several disadvantages including great variations in the sample volume recovered from the collection matrix. This results in limited or unknown saliva volume and thus imprecise quantification of the analyte (2, 8). Because a standardized system for collection and quantification of oral fluid is lacking, oral fluid testing is rarely implemented in the routine clinical chemistry laboratory.

In this study, the novel standardized CE-labeled Saliva Collection System (SCS) and Saliva Quantification Kit (SQK) (Greiner Bio-One GmbH, Kremsmuenster, Austria), which contain an internal standard for accurate determination of the saliva content (reported in vol.-%) in oral fluid samples, were evaluated (Figure 1). Oral fluid was collected with the new oral fluid collection device according to the

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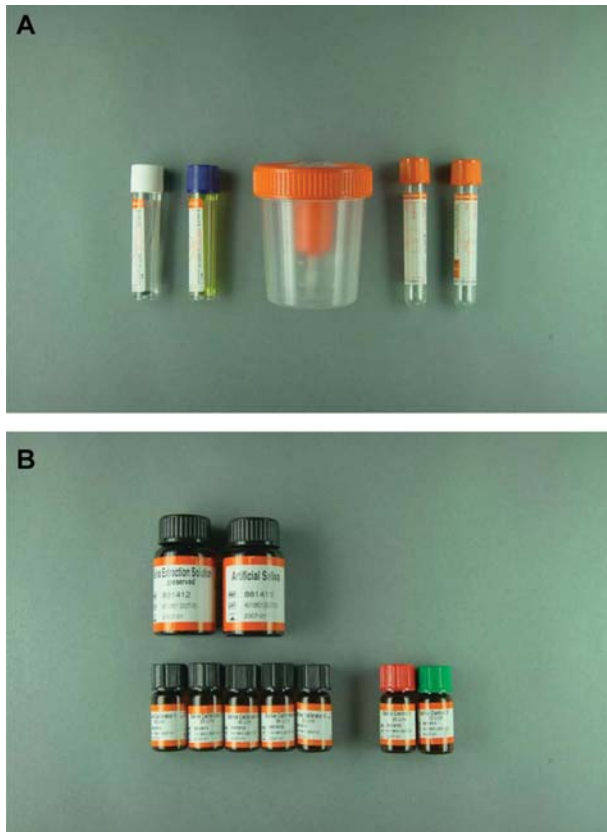


Figure 1 The new system for oral fluid collection and determination of its saliva content consists of the Saliva Collection System (SCS) and the Saliva Quantification Kit (SQK). The SCS (A; from left to right) includes one tube containing 6 mL of rinsing solution (27 mmol/L citric buffer, pH 7.0), one tube containing 4 mL of saliva extraction solution (39 mmol/L citric buffer, pH 4.2, and tartrazine, a synthetic, water-soluble yellow food dye), one collection beaker and two 3.5-mL scaled vacuum sample-transfer tubes containing sodium azide in crystalline form as preservative. The SQK (B; upper row, from left to right) includes one vial containing preserved saliva extraction solution and one vial containing preserved artificial saliva; lower row, from left to right, five vials containing calibrators and two vials containing controls.

manufacturer's instructions (<http://www.vacurette.com/en/company/3698.php>). Intake of food or liquids and smoking was not permitted within 10 min prior to sample collection. Immediately before sample collection, the oral cavity was cleaned with the rinsing solution. After emptying the oral cavity, it was rinsed with 4 mL saliva extraction solution (SES) for 2 min and the entire content of the oral cavity was spit into the beaker. The beaker content referred to as 'oral fluid sample' (SES plus oral fluid) was then transferred into the scaled vacuum sample-transfer tubes. The total collection volumes were recorded by reading off the scales. Sample-transfer tubes containing the oral fluid sample were centrifuged at $2200 \times g$ for 10 min. Samples were stored at room temperature when analyzed within 6 h, otherwise frozen at -20°C .

Oral fluid samples were produced by healthy volunteers. To evaluate sample collection volumes, 25 oral fluid samples were collected. For determination

of saliva contents in oral fluid samples, tartrazine, which is an integral part of the saliva extraction solution serving as an internal standard, was used. Results were compared with those obtained by employing glucose as a 'supplemental internal standard' for determination of saliva contents. Concentrations of tartrazine and glucose were determined photometrically and values were used for calculation of the saliva content in oral fluid samples. Saliva contents obtained through either tartrazine or glucose concentrations were compared. The analytical performance of the new system for oral fluid collection and determination of its saliva content was evaluated by testing inter- and intraassay imprecisions and linearity. Calcium and magnesium analyte concentrations in 12 oral fluid samples were determined using a routine laboratory analyzer. Results were compared to those obtained by an inductively coupled plasma mass spectrometer (ICP-MS; Agilent, Stuttgart, Germany) serving as reference method.

With the new system for oral fluid collection and determination of its saliva content, sample collection volumes of 25 oral fluid samples produced by healthy volunteers ranged from 4.9 to 10.5 mL (mean 7.0 mL). When contents of saliva in 12 oral fluid samples were determined by using either tartrazine or glucose as internal standards, contents of saliva calculated through tartrazine concentrations were found to be between 48.5 and 71.0 percent by volume (vol.-%) (mean 65.5 vol.-%) and contents of saliva calculated through glucose concentrations were found to be between 50.5 and 72.0 vol.-% (mean 65.0 vol.-%; Figure 2). For testing interassay imprecision, four oral fluid samples containing four different concentrations of glucose were analyzed twice per day on 20 different days. Coefficients of variation (CVs) were found to be between 1.5% and 3.2% (Table 1). For testing intraassay imprecision, another four oral fluid samples containing four different concentrations of glucose were analyzed 40 times in one run. CVs were found to be between 1.1% and 2.5% (Table 2). When linearity was tested with a dilution series of an oral fluid sample containing a known high concentration of glucose, a quasi-linear curve was observed ($R^2=0.99$; Figure 3). Calcium and magnesium concentrations were determined in 12 individually produced oral fluid samples collected with the SCS. Samples were tested photometrically using a Hitachi 912 analyzer (Roche Diagnostics, Mannheim, Germany) and by ICP-MS. Calcium and magnesium values obtained by different methods showed correlations of $R^2=0.96$ for calcium and $R^2=0.97$ for magnesium (Figure 4).

A standardized procedure for oral fluid collection and determination of its saliva content is lacking. In this study, a novel standardized collection and quantification system for oral fluid testing was evaluated. Oral fluid can be collected with a wide variety of devices. One of the major disadvantages of oral fluid collection devices currently available is the poor sample recovery volume, allowing only a limited number of analyses (2, 8). The new Greiner Bio-One SCS is based on liquid phase-based specimen collection.

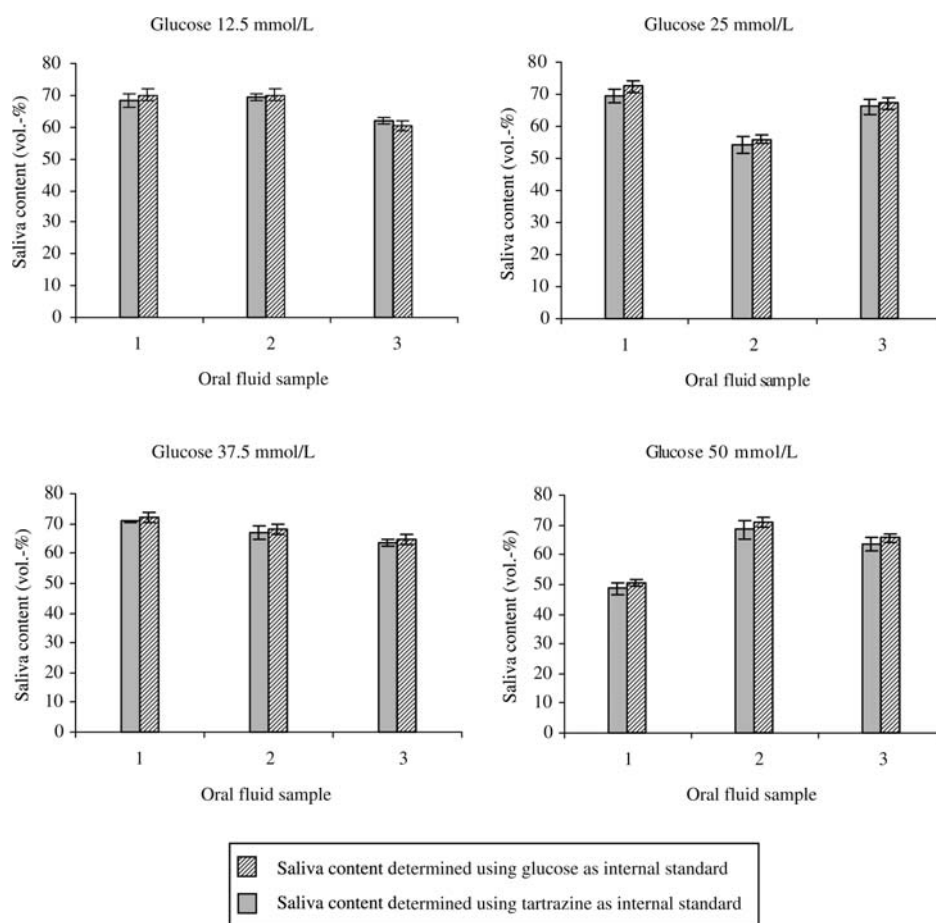


Figure 2 Determination and comparison of saliva contents in 12 oral fluid samples by using either tartrazine or glucose as internal standards.

Tartrazine: prior to measurement of saliva contents in oral fluid samples, a calibration curve was generated with a Hitachi 912 analyzer (Roche Diagnostics) according to the manufacturer's instructions (<http://www.vacurette.com/en/company/3698.php>). The two controls containing saliva contents of 30 and 70 vol.-% were included in each run. **Glucose:** prior to measurement of saliva contents in oral fluid samples, a calibration curve was generated with six self-prepared calibrators on a Hitachi 912 analyzer employing Glucose Roche/Hitachi 912 reagent (Roche Diagnostics). Calibrators were prepared by mixing preserved artificial saliva and preserved SES (1:1) provided in the SQK. This mixture was then spiked with different concentrations (2.6, 14.8, 28.8, 39.7 and 51.6 mmol/L) of glucose monohydrate p.a. (Merck Pharmaceuticals, Munich, Germany). Controls were prepared by using oral fluid samples obtained by the SCS and spiking them with a low (12.9 mmol/L) and a high (34.2 mmol/L) concentration of glucose monohydrate p.a.. Glucose concentrations of both the calibrators and the controls were determined with the reference method according to the manufacturer's instructions (D-Glucose UV-method, Roche Diagnostics). Both of the controls were included in each run. A total of 12 SES were spiked with four different concentrations (11.9, 24.4, 34.6 and 44.0 mmol/L; three each) of glucose monohydrate p.a. prior to sample collection. After collection of 12 authentic oral fluid samples, 200 μ L of each sample were pipetted into Hitachi cups (system water as cuvette blank value) and the glucose concentration was determined on a Hitachi 912 analyzer. Calculation of the saliva content in oral fluid samples was based on the concentration of glucose in SES and in oral fluid samples. The change of glucose concentration in oral fluid samples is inversely proportional to the dilution and thus can be used to calculate the saliva content in oral fluid samples. Samples were tested three times; values are expressed as means.

Table 1 Results of interassay imprecision testing^a.

Glucose concentration, mmol/L	\pm SD	CV, %
4.3	0.1	3.2
12.5	0.4	3.1
14.4	0.3	1.8
33.9	0.5	1.5

^aFor determination of interassay imprecision, four oral fluid samples containing the four different concentrations of glucose were taken. Samples were tested twice per day on 20 different days; values of glucose concentrations are expressed as means.

Table 2 Results of intraassay imprecision testing^a.

Glucose concentration, mmol/L	\pm SD	CV, %
4.8	0.1	2.5
12.8	0.3	2.2
14.4	0.2	1.1
33.4	0.7	2.1

^aFor determination of intraassay imprecision, four oral fluid samples containing four different concentrations of glucose were taken. Samples were tested 40 times in one run; values of glucose concentrations are expressed as means.

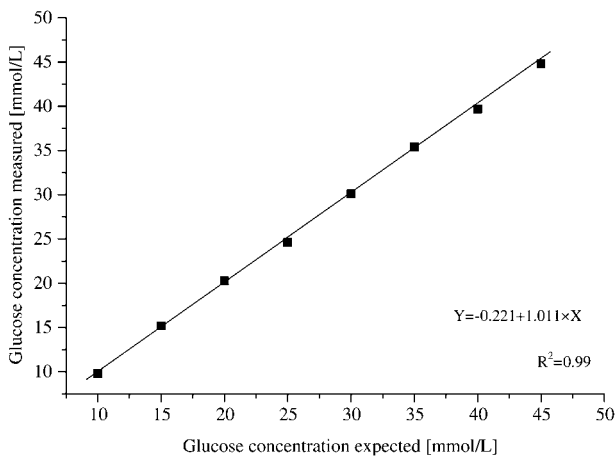


Figure 3 Linearity testing.

For determination of linearity, an oral fluid sample with a known high concentration of glucose was used. A dilution series was prepared by using preserved artificial saliva. Each dilution was analyzed three times and the mean concentration of each sample was determined; values of glucose concentrations are expressed as means.

This oral fluid collection system allows rinsing the oral cavity with SES, a citric buffer including tartrazine. This non-toxic dye serves as internal standard and its optical density can easily be determined photometrically. With the corresponding Greiner Bio-One SQK, the saliva content in oral fluid samples can be calculated accurately, as demonstrated in this study. Exact recalculation of analyte concentrations in oral fluid samples is thus provided. Moreover, by using an internal standard for calculation of the saliva content in an oral fluid sample, the measurement of analyte concentrations is independent from a change in sample volume. With this new collection and quantification system, oral fluid sample collection volumes were found to be sufficient, despite a minor loss of oral fluid sample because of a remnant in the collection beaker and possibly an amount swallowed while rinsing the oral cavity. The system thus allows multiple and, if necessary, repeat determination of analytes. To investigate whether tartrazine is suitable for serving as internal standard added by the manufacturer, a 'supplemental internal standard' for the calculation of the saliva content in oral fluid samples was introduced and compared to tartrazine. For that reason, a total of 12 SESs used for sample collection were spiked with four different concentrations (three each) of glucose monohydrate p.a. (Merck Pharmaceuticals, Munich, Germany), serving as 'supplemental internal standard'. Glucose was chosen as a secondary test to evaluate the accuracy of tartrazine standardization, because glucose concentrations in oral fluid obtained by healthy, non-diabetic volunteers are found below 0.04 mmol/L and therefore negligible (9). In addition, calibrators and controls for glucose testing were prepared and concentrations were determined with a reference method with no differences observed when concentrations measured and nominal concentrations were compared. Controls were included in every run ensuring accurate measure-

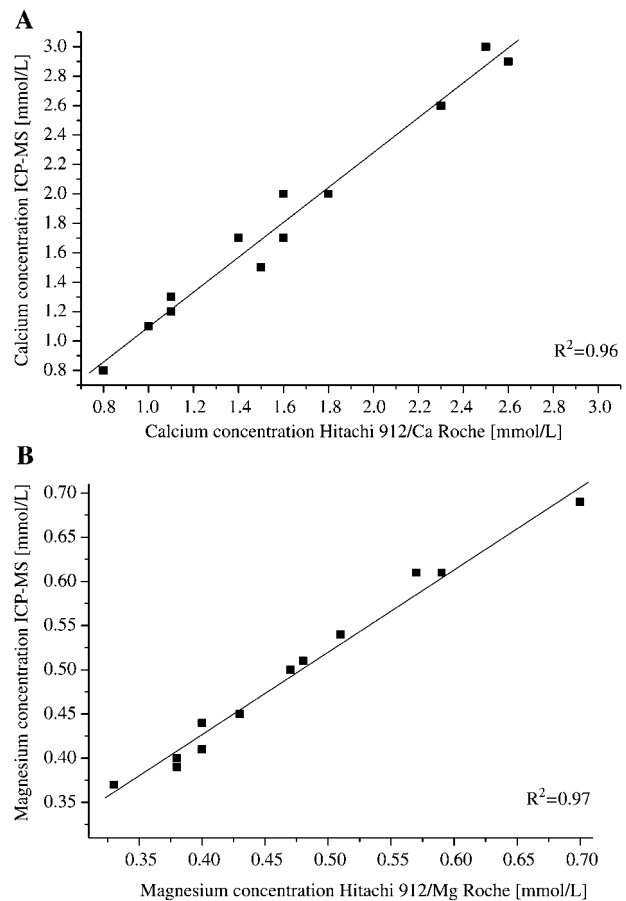


Figure 4 Determination of calcium and magnesium concentrations.

Calcium (A) and magnesium (B) concentrations were determined on a Hitachi 912 analyzer using Ca and Mg Roche/Hitachi 912 reagents according to the manufacturer's specifications (Roche Diagnostics). For determination of calcium and magnesium concentrations by ICP-MS, an inductively coupled plasma mass spectrometer (Agilent) equipped with a PFA 50 microflow nebulizer was used. The spray chamber temperature was set to 2°C. The performance of the instrument was within the manufacturer's specifications. To minimize possible polyatomic interferences, helium was used as collision gas. Prior to the measurements, samples were diluted (1+49) with 1% nitric acid. The following isotopes were used for the measurements: calcium (m/z 43) and magnesium (m/z 24 and m/z 25). Calibration standards (CPI International) were prepared using 1% nitric acid in the following calibration range: 0.50/0.050, 1.0/0.10, 2.0/0.20 and 5.0/0.50 mg Ca/Mg. Indium (m/z 115) was used as internal standard throughout all measurements.

ments. Similar results were obtained when saliva contents of oral fluid samples were calculated by either using tartrazine or glucose concentration as internal standard proving the validity of the whole system. The analytical performance of the new system for collection and quantification of oral fluid was evaluated. Excellent results were found with both the interassay and intraassay imprecision testing. Linearity was determined by analysis of a dilution series of one oral fluid sample that contained a high concentration of glucose. The new collection and quantification system revealed sufficient linearity. Calcium and magnesium appeared as proper parameters, because calcium and

magnesium ions are actively transferred from blood to saliva (10). While the transport of alkaline and acidic substances depends on external factors, such as the pH value, the transport of calcium and magnesium ions is largely independent from the pH value and other external factors (10). Furthermore, those parameters appear to be potential targets for routine determination in oral fluid samples since reference ranges have been established and these results were found to be within these reference ranges (11). To exclude a possible matrix-induced effect by the established photometrical method for determination of calcium and magnesium, results were compared to ICP-MS as reference method. No significant differences between the methods were observed, indicating a negligible matrix-induced effect for the photometrical method, if any. However, a matrix-induced effect caused by interference with the buffer system (e.g., by complex forming of bivalent ions) might occur if alternative methods are used. Future applications for this system may include testing for drugs of abuse, determination of steroid levels and detection of nucleic acids. The most important problem with currently available saliva collection systems is adsorption of drugs and drug metabolites to the collection device resulting in inaccurate determination of the concentration of a drug or drug metabolite in oral fluid. This is avoided through the major advantage of the new system, the liquid phase-based specimen collection. Limitations of this system include the number of steps and reagents that need to be used for volume determination, which makes it too complex for on the roadside analyses, the SES volume which may reduce the concentration of drugs and metabolites or other analytes, and the extended waiting period prior to specimen collection. For several approaches considering oral fluid as a matrix, such as detection of drugged driving and workplace drug testing, it would be preferable to keep the waiting period prior to specimen collection as short as possible.

In conclusion, the novel system for collection and quantification of oral fluid proved to be a useful tool for collection of oral fluid samples and determination of its saliva content. This system allows for an easy sample collection with no major complaints by the users (healthy volunteers); clinical samples may thus be obtained not only at healthcare units but also at home. The format of saliva sample-transfer tubes per-

mits large-scale testing on routine laboratory analyzers acting as a closed system and thus avoiding additional pipetting steps. The evaluated system is able to generate valid results with the established routine laboratory method used in this study. The novel system helps to improve pre-analytics in oral fluid testing significantly and provides both reliable detection and accurate quantitation of calcium and magnesium in oral fluid samples.

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