Online Supplementary Material 1

Development of Basic Ion-Selective Electrode-Based Methods for Fluoride Analysis

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The supplemental material presented in this manuscript describes part of the results of a study designed to develop standardized methods for analyzing fluoride in different types of samples used for dental research. The current supplement presents the data leading to the establishment of standard protocols. Detailed information describing the methods, their subsequent testing and a discussion of those results has been published elsewhere (*add main publication citation*).

Materials and Methods

In order to establish a preliminary measure of agreement, a group of nine laboratories analyzed a set of standardized samples for fluoride concentration using their own methods. All laboratories analyzed a standardized set of biological and non-biological samples. This initial sample set included:

Standard F solutions (0.0132, 0.02631, 0.0526, 0.2631 and 0.5263 μmol F/ml) prepared through the dilution of a commercially available Standard Fluoride Solution (0.1 mol/l NaF, Orion Fisher Scientific Co. Itasca, IL, USA).

Beverages, including two samples of orange juice, one spiked 0.02631 μmol F/ml; one diet cola-based soda (decarbonated); one 2% chocolate milk drink; and, one powder-beverage mix reconstituted with water (0.2631 μmol F/ml).

Food (homogenized for 1 and 10 min using a tissue homogenizer, single item (peas and carrots) and pooled meal-based samples (chicken and meat dinners).

Saliva (human, pooled and from individual donors).

Plasma (rabbit and human).

Urine (from healthy and systemically compromised donors).

The group then reviewed existing analytical techniques for fluoride analysis, identified inconsistencies in the use of these techniques and conducted testing to resolve differences. The material presented in this manuscript details the results of those tests.

All of the laboratories used different modifications of two techniques for F determination: 1) direct analysis using a F ion-selective electrode (Orion #96-09 or 94-09; Fisher Scientific Co., Itasca, IL, USA) and a pH/ion meter (Orion #420A, 720A or EA940); 2) modifications of the hexamethyldisiloxane (HMDS: Sigma Chemical Co., St. Louis, MO, USA) micro-diffusion method of either Taves [1968] or Venkateswarlu [1977].

Upon review of existing techniques, different comparative tests were performed for direct methods; these tests involved using different of types of TISAB solutions and the use of stirring

while analyzing the samples. Three different TISAB solutions were tested by analyzing a set of 24 standard F solutions with concentrations ranging from 0.0132 to 0.5263 μmol F/ml , prepared through the dilution of a commercially available Standard Fluoride Solution (0.1 mol/l NaF, Orion, Fisher Scientific Co. Itasca, IL, USA). According to the manufacturer, when using TISAB I, II or III, no differences should be found in analyzing samples above 0.0021 μmol F/ml that do not contain large amounts of aluminum. Finally, the effect of stirring vs. not stirring the sample during analysis was also tested, using TISAB II and analyzing the same set of standard F solutions with an additional set of beverages.

Comparative tests were also performed for the micro-diffusion analysis [Taves, 1968 and Venkateswarlu, 1977]; these differences centered on the use of different combinations of reagents. Several experiments were conducted to assess the analytical precision of different combinations of using a National Institute of Standards and Technology (NIST) certified reference material (RM). Eleven different combinations were selected and tested, using different types of acids, different volumes and concentrations for the F diffusion trap and different acid buffers and concentrations of these buffers. All acids used by collaborating laboratories were included in the comparison. Fifteen replicates of a RM with a concentration of 0.0348 ± 0.0005 μmol F/ml (as NaF) were analyzed following the International Organization for Standardization (ISO) Guide 33 [2000].

The basic technique used for testing of reagent combinations consisted of using 1 ml of sample with 2 ml of deionized water. A trapping solution of 50 μl of either 0.05, 0.50 or 1.65 mol/l sodium hydroxide (NaOH, A.R: Sigma Chemical Co., USA), was placed on the inside of a non-wettable petri dish lid previously ringed with Vaseline (Falcon 1007, Fisher Scientific Co.), and, after the addition of 1 ml of either 1.5 mol/l sulfuric acid (H_2SO_4: Sigma Chemical Co. USA), 5 mol/l perchloric acid ($HClO_4$: Sigma Chemical Co. USA) or 6 mol/l hydrochloric acid (HCl: Sigma Chemical Co. USA) saturated with HMDS, through a hole (2-3 mm diameter) previously burned into the lid with a soldering iron. The hole was then immediately sealed with petroleum jelly (Vaseline®, Unilever, USA). The HMDS acid was prepared by adding approximately 20-25 ml of HMDS to 1000 ml of acid in a separatory funnel and shaking it vigorously for 2-3 minutes until multiple HMDS droplets were observed dispersed throughout the acid. After shaking, the system was left to settle and the aqueous layer was used in the diffusion analysis. During overnight diffusion, F was collected in the trap. The trap sample was then

recovered and its pH was adjusted to 5.2 with either 25 µl of 0.1 mol/l or 0.2 mol/l acetic acid (CH_3CO_2H: Sigma Chemical Co., USA), plus 25 µl of TISAB II or 25 µl of 0.1 mol/l perchloric acid plus 25 µl of TISAB II. The recovered solution was adjusted in certain laboratories with deionized water to a final volume of 100 µl. Some modifications to this basic technique were additionally tested, such as drying vs. non-drying of the trap and sealing the system with petroleum jelly vs. parafilm M barrier film (Alcan, Fisher Scientific Co.). The same RM was used to study F recovery and calculate precision according to the ISO Guide 33 [2000].

A detailed review of each laboratory's protocol showed that there were a number of different approaches used to perform the mathematical calculations needed to obtain concentration values from raw analytical data. Different approaches were utilized for samples with values above and below 0.0105 µmol F/ml, a concentration that approaches the limit of detection of the electrode, to the point where the relationship between the logarithm of a sample's concentration and potential readings deviates from linearity. The approaches utilized included the use of the concentration function of the pH/ion meter, using the millivolt function of the pH/ion meter to externally calculate a linearised plot of potential against log concentration and a polynomial regression or a combination of both linear and polynomial regressions. In order to test which method of calculation rendered more accurate results, a set of 24 standard F solutions was tested. These solutions were used to construct curves using linear regressions, polynomial regressions with multiple terms, or a combination of both, for values above and below 0.0105 µmol F/ml. Results obtained through these calculations were compared to the results that the pH/ion meter automatically calculated using its concentration function. As indicators of accuracy, the slope of the line plotted between values and the coefficient of determination (r^2) were calculated. A separate set of experiments was conducted using a linear regression equation and a blank correction method, where the value of the F concentration in the blank was calculated as previously described by Villa [1988]. Based on the results of the testing undertaken to define the best approaches for analysis, the group developed recommendations for direct and micro-diffusion methods using the fluoride ion-selective electrode. Detailed information regarding the methods has been published elsewhere (*add main publication citation*). These basic methods were then initially tested using a certified RM.

Results

Results of the tests conducted for the differences found for direct methods are presented in Table 1. No TISAB solution consistently rendered higher or lower values for fluoride concentration when compared to the others. Results obtained while comparing stirring vs. non-

stirring during analysis showed no statistically significant differences (paired t-test, p = 0.94) for standard solutions. For the beverage set, a statistically significant difference was observed between the samples that were stirred vs. the samples that were not stirred (paired t-test, p = 0.04).

For the tests conducted with micro-diffusion techniques, results with the different combinations of reagents used to analyze selected samples showed different percentages of F recovery and some of them were unacceptable for precision testing (Table 2). Based on the tests recommended by ISO, the precision was acceptable for the trap concentration of 0.05 mol/l NaOH, while precision for the concentrations of 1.65 or 0.5 mol/l NaOH was not acceptable. When comparing acetic acid at two different concentrations (0.1 mol/l and 0.2 mol/l) and 0.1mol/l perchloric acid , 0.1 mol/l acetic acid's precision was acceptable. Results of the comparison of acids that were tested for saturation of HMDS (1.5 mol/l sulfuric, 5 mol/l perchloric and 6 mol/l hydrochloric) , showed that sulfuric acid produced better recoveries.

When comparing different formulas used to obtain values from raw data for both direct and micro-diffusion techniques, the values produced by linear calibration and the pH/ion meter were similar. As expected, there were no statistically significant differences between the values calculated using a linear regression model and those obtained using a combination of linear and polynomial regressions for values above 0.0105 μmol F/ml in the original sample. There were also no significant differences between the values obtained from the pH/ion meter and those obtained using a linear regression; or, for the pH/ion meter values and those obtained using a combination of both linear and polynomial regressions. Only when the polynomial regression values and the meter values were compared were significant differences found. The correlation values between the measured concentrations obtained using the standard curves and the target concentrations were similar for all regressions. A combination of a two-term polynomial regression for values below 0.0105 μmol F/ml, a concentration that approaches the limit of detection of the electrode, and linear regression for values above that concentration, produced the results closest to the theoretical F concentration in the samples. Results obtained incorporating a blank correction using a linear calculation demonstrated its usefulness for samples between 0.00105 and 0.0105 μmol F/ml.

Using the agreed protocol for direct F analysis for the reference material, the minimum percentage fluoride recovery ranged from 68.33 to 109.52% (Table 3). None of the laboratories exceeded the set variance level of 0.0083 μmol F/ml; therefore, no laboratory failed the precision test or the trueness test using the agreed protocol for the direct method. For the diffusion analysis, the minimum percentage fluoride recovery ranged from 75.38 to 98.48%

(Table 4). Using a goal of required within-laboratory precision of 5%, the estimated standard deviation would need to exceed 0.00847 μmol F/ml to conclude that the measurement was not as precise as required. Based on the analyses that are recommended by ISO, the precision was acceptable overall and for each laboratory. The overall trueness was also acceptable.

Legends

Table Headings

Table 1. Mean F concentration (± SD) and minimum % F recovery using different TISAB solutions and stirring technique

Table 2. Mean F concentration (± SD) for testing of samples using different reagents

Table 3. Mean F concentration (± SD) for analysis of reference material with the direct method using the agreed protocol

Table 4. Mean F concentration (± SD) for analysis of reference material using the agreed protocol for micro-diffusion method

Table 1. Mean F concentration (± SD) and minimum % F recovery using different TISAB solutions and stirring technique

TISAB/stirring	Mean F concentration ± SD (μmol F/ml)	Minimum percentage fluoride recovery (%)
I/stirring	0.034 ± 0.0015	98
II/stirring	0.035 ± 0.0015	98
II/no stirring	0.035 ± 0.0015	97
III/stirring	0.034 ± 0.0021	97

N=24 per laboratory. True value of reference material = 0.0348 ± 0.0005 μmol F/ml. No statistically significant differences were observed

Table 2. Mean F concentration (± SD) for testing of samples using different reagents

1 ml Acid + HMDS	Trap NaOH (mol/l; 50 µl)	Trap Buffer (25 µl)	Mean F concentration ± SD (µmol F/ml)
H_2SO_4(1.5 mol/l)	0.05	CH_3CO_2H (0.1 mol/l)	0.0337 ± 0.0015
HCl (6 mol/l)	0.05	CH_3CO_2H (0.1 mol/l)	0.0363 ± 0.0010
H_2SO_4(1.5 mol/l)	0.05	CH_3CO_2H (0.2 mol/l)	0.0332 ± 0.0021
HCl (6 mol/l)	0.05	$HClO_4$ (0.1 mol/l)	0.0332 ± 0.0010
H_2SO_4(1.5 mol/l)	0.05	$HClO_4$ (0.1 mol/l)	0.0326 ± 0.0021
HCl (6 mol/l)	1.65	CH_3CO_2H (0.66 mol/l - 40 µl)	0.0326 ± 0.0026
$HClO_4$ (5 mol/l)	0.50	CH_3CO_2Na (0.5 mol/l - 90 µl) CH_3CO_2H (2.5 mol/l -10 µl)	0.0326 ± 0.0026
$HClO_4$ (5 mol/l)	0.05	CH_3CO_2H (0.2 mol/l)	0.0321 ± 0.0026
$HClO_4$ (5 mol/l)	0.05	CH_3CO_2H (0.1 mol/l)	0.0205 ± 0.0015
HCl (6 mol/l)	0.50	$HClO_4$ (0.1 mol/l)	0.0947 ± 0.0142
H_2SO_4 (1.5 mol/l)	0.50	$HClO_4$ (0.1 mol/l)	2.6634 ± 0.0926

N=15 analyzed by three laboratories True value of reference material = 0.0348 ± 0.0005 µmol F/ml

Table 3. Mean F concentration ± SD and Minimum % F recovery for analysis of reference material with the direct method using the agreed protocol

Laboratory	Mean F concentration (μmol F/ml)	SD (μmol F/ml)	Minimum percentage fluoride recovery (%)
1	0.029	0.0005	89.00
2	0.032	0.0016	86.66
3	0.033	0.0005	98.41
4	0.0326	0.0021	79.03
5	0.035	0.0010	99.99
6	0.031	0.0037	73.33
7	0.029	0.0068	68.33
8	0.038	0.0016	109.52
9	0.0316	0.0010	95.22

N=15 per laboratory. True value = 0.0332 ± 0.0016 μmol F/ml

Table 4. Mean F concentration ± SD and minimum % F recovery for analysis of reference material using the agreed protocol for micro-diffusion method

Laboratory	Mean F concentration (μmol F/ml)	SD (μmol F/ml)	Minimum percentage fluoride recovery (%)
1	0.029	0.005	84.61
2	0.026	0.005	75.38
3	0.029	0.005	86.18
4	0.035	0.005	92.30
5	0.034	0.0005	98.48
6	0.031	0.002	93.22
7	0.038	0.005	89.23
8	0.033	0.0005	95.38
9	0.030	0.0016	87.68

N=15 per laboratory. True value = 0.0348 ± 0.0005 μmol F/ml

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