

ORCA Workshop: Methodology for Determination of Potentially Available Fluoride in Toothpastes

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Declaration of Interest

The following authors EA Martinez-Mier, LM Tenuta, CM Carey, JA Cury, C van Loveren, K Ekstrand, C Ganss, A Schulte, P Bottenberg, MJ Buijs, JC Carvalho, C Gonzalez-Cabezas, C Holmgren, F Lippert, DJ Manton, S Martignon, A Jablonski Momeni, W Plett, C Rahiotis, F Sampaio, and DT Zero declare no potential conflicts of interest. A Baig is an employee of the Procter & Gamble Company, A Ceresa, R Ellwood, and M Knapp are employees of Colgate-Palmolive, A Joiner is an employee of Unilever Oral Care, and S Mason is an employee of GlaxoSmithKline Consumer Healthcare.

Abstract

Toothpastes are the most universally-accepted form of fluoride delivery for caries prevention. In order to provide anti-caries benefits, they must be able to release fluoride during the time of tooth brushing or post brushing into the oral cavity. However, there is no standard procedure accepted to measure how much fluoride in a toothpaste may be (bio) available for release. The European Organization for Caries Research proposed and supported a workshop with experts in fluoride analysis in toothpastes and representatives from industry. The objective of the meeting was to discuss issues surrounding fluoride analysis in toothpaste and reach consensus on terminology and best practices, wherever the available evidence allowed it. Participants received a background paper and heard presentations followed by structured discussion in order to define the problem. The group also reviewed the evidence on the validity, reliability and feasibility of each technique (namely chromatography and fluoride electroanalysis) and discussed their strengths and limitations. Participants were able to reach consensus on terminology and were also able to identify and summarize the advantages and disadvantages of each technique. However, they agreed that most currently available methods were developed for regulatory agencies several decades ago utilizing the best available data from clinical trials at the time, but are in need of updating. They also agreed that although significant advances to our understanding of the mechanism of action of fluoride in toothpaste have been achieved over the past four decades, this clearly is an extraordinarily complex subject and more work remains to be done.

Introduction

Fluorides have a pronounced caries preventive effect that is dose-dependent, with toothpastes being the simplest, consumer-friendly, and most universally-accepted form of fluoride delivery [Marinho et al., 2003; Twetman et al., 2003; Walsh et al., 2010; dos Santos et al., 2013]. However, as demonstrated by pioneer studies [Bibby, 1945; Muhler, 1957], not all fluoride toothpastes have similar efficacy. These studies point to the concept that toothpastes must be able to release fluoride to provide anti-caries benefits during the time of tooth brushing or post brushing. For that, it is believed that fluoride should be (bio) available for release from the toothpaste formulation into oral cavity [von der Fehr & Moller, 1978; Hattab, 1989].

Toothpastes are extraordinarily complex products with a plethora of possible formulation permutations. Generally, they contain abrasive mixtures suspended in an aqueous humectant phase, with embedded surfactants, active ingredients, flavor compounds, sweeteners, colorings, binding gums, preservatives and other excipients. The most common toothpastes available in the market today may contain anti-caries agents (fluorides and other miscellaneous remineralizing agents), anti-malodor agents, anti-tartar/anti-calculus, anti-plaque/anti-gingivitis agents, whitening agents, agents for the relief of dentin hypersensitivity, and erosion-prevention agents, [Lippert, 2013]. The main ingredients in currently available toothpastes are described in Table 1.

Fluoride salts added to toothpastes can interact with other ingredients, formulation excipients and minor impurities within the ingredients to form poorly soluble salts with little to no anti-caries efficacy. Therefore, an important distinction needs to be made between the total fluoride present in a formulation and the soluble fluoride (as ionic fluoride or monofluorophosphate [MFP]) that could become (bio) available during brushing to exert its cariostatic effect. Table 2 presents the definitions agreed upon by the work group for these terms.

As toothpastes of similar total fluoride concentration can differ in their ability to release fluoride into the oral environment [Issa and Toumba, 2004; Nagata et al., 2017], which is arguably a measure/predictor of their anti-caries effect, the determination of potentially (bio) available fluoride in toothpastes is vital to ensure that the public is protected from toothpastes

with little to no efficacy. Today, it can be assumed that most toothpastes in developed countries present adequate concentrations of potentially (bio) available fluoride to control caries. However, there have been reports of commercially available toothpastes containing less total fluoride than declared and/or low potentially available fluoride [Cury et al., 2004; van Loveren et al., 2005; Kikwilu et al., 2008; Cury et al., 2010; Benzian et al., 2012; Carrera et al., 2012; Giacaman et al., 2013; Marin et al., 2015], with some of these observations made in countries with a high burden of caries.

At this time, there is no standard procedure for the measurement of potentially (bio) available fluoride in toothpastes, leading to substantial inter-laboratory variation. Historically, fluoride analysis evolved from simple colorimetric analysis, which yielded crude results and suffered interference from other ions present in the solutions, to more complex methods of analysis [Martinez-Mier et al., 2010]. Among these more complex methods of analysis are: mass spectrometry, gas chromatography, electroanalysis, catalytic, enzymatic and radioanalytical methods.

Different toothpaste formulations require a range of approaches for the determination of their potentially available fluoride. The complex ingredients utilized in toothpastes can also interfere with the accuracy and precision of analytical methods. Therefore, methods used today vary widely [van Loveren et al., 2005; Cury et al., 2010]. The different approaches employed in the determination of fluoride levels may require the pretreatment of samples in order to achieve the separation of the fluoride ion and true representation of the concentration of fluoride [Venkateswarlu, 1990].

Of major importance for the determination of potentially available fluoride are the fluoride source (sodium fluoride [NaF], sodium MFP [Na_2FPO_3], stannous fluoride (SnF_2), and amine fluorides [AmF], such as $\text{C}_{27}\text{H}_{60}\text{F}_2\text{N}_2\text{O}_3$) and the abrasive system (calcium-free or calcium-based). Nevertheless, other formulation excipients can affect potentially available fluoride which in turn may influence anti-caries caries performance, and therefore require further testing (such as the 1 min fluoride release rate test described in the Guidelines of Fluoride-Containing Dentifrices of the American Dental Association) [ADA, 2005].

Among the different methodologies currently used to assess available fluoride in toothpastes, two can be highlighted: the fluoride-specific electrode and gas chromatography. Both have strengths and limitations and need specific sample preparation in order to provide reliable results. There are also differences in the sample preparation when MFP toothpastes are assessed by fluoride-specific electrode; MFP hydrolysis, necessary to release the fluoride ion, can be performed either chemically [Pearce, 1974; Cury et al., 2010] or enzymatically [van Loveren et al., 2005], again with strengths and limitations for each method. It should also be considered that many formulations have important ageing issues. In toothpastes containing MFP and a calcium-based abrasive, a decrease in available fluoride occurs over time [Cury et al., 2004], and methods to assess fluoride availability allowing the test of artificially aged samples (accelerated aging) are useful [Tabchoury and Cury, 1994].

In summary, there is/are no ideal method(s) for sample preparation and the measurement of available fluoride in different types of toothpaste. In addition to the methodological concerns discussed above, there has been debate about the clinical relevance of the currently available methods. Ideally, methods used to determine fluoride availability as a surrogate measure of effectiveness in toothpaste should mimic the clinical environment. Aiming to discuss the strengths and limitations of each method, reaching out for a consensus among those available, a workshop was proposed by the European Association for caries Research.

Workshop Process

The European Organization for Caries Research (ORCA) supported a two-day meeting (in February 2015) of a workgroup consisting of experts in the field of fluoride analysis in toothpastes. The workgroup also included representatives from industry. The objective of the meeting was to discuss in detail the issues surrounding fluoride analysis in toothpastes and reach consensus on terminology and best practices, wherever the available evidence allowed for it. The meeting was designed to foster the exchange of ideas and discussion with the assistance of a moderator.

One week prior to the workshop, participants received a background paper prepared by the organizing committee, describing the 'state of the art' and of the science on techniques to determine fluoride concentration in toothpastes. The workgroup was asked to consider that there is little consensus on how to measure potentially available fluoride; that there are reports in the literature of toothpastes having low levels of potentially available fluoride; and that there is little evidence on what level of potentially available fluoride constitutes a clinically relevant and effective concentration.

During the first day of the workshop, participants heard a series of presentations followed by structured discussion on the following topics:

- Definition of the problem
- Strengths and limitations of methods and standards for determining fluoride in toothpastes
 - Methods approved by regulatory agencies, such as the U.S. Food and Drug Administration (FDA), American Dental Association (ADA), and the International Organization for Standardization (ISO)
 - Using chromatography to determine potentially available fluoride in different toothpaste formulations
 - Using the fluoride electrode to determine potentially available fluoride in different toothpaste formulations

The workgroup was tasked with reviewing the evidence on the validity, reliability and feasibility of each technique to determine fluoride in toothpastes, and to:

- Reach consensus on the terminology to be used
- Identify and summarize the advantages and disadvantages of each technique
- Discuss strengths and limitations of different sample preparation methods
- Reach consensus on what available methods are appropriate for different types of toothpastes or if new methods need to be developed
- Identify gaps in knowledge (and make research recommendations) to optimize these techniques
- Identify any new approaches, methods or technologies that are still in initial development

Consensus was reached post-workshop, by affording participants the opportunity to be authors in this publication, participate in its development, and approve the final version.

Definition of the problem

State of the science/evidence (Presented by E.A. Martinez Mier [EAMM])

During her presentation, EAMM reviewed the history of fluoride analysis in toothpastes and posited that the issue of clinical relevance needs to be embedded in the discussions regarding methodological issues. When considering the task at hand, the work group was urged to always keep at the forefront of discussions if a technique measured what it intended to measure and if the results could be potentially extrapolated to the clinical situation. The problem at hand was then defined as the fact that the analytical techniques currently in use to determine fluoride in toothpastes are not standardized, and that clinically-relevant procedures for determination of available fluoride have not been established.

The consequences of drawing wrong conclusions based on the results of imprecise and non-valid techniques were discussed. Studies which proposed that there were homeostatic mechanisms maintaining fluoride levels in the body independent of the amount ingested [Singer and Armstrong, 1960] or the studies that supported the belief that the placenta acted as a partial barrier to the passage of fluoride [Gedalia, 1970] were discussed as examples of such types of erroneous conclusions. It was discussed how these conclusions were reached in part due to the inability of the available techniques to measure ionic fluoride instead of total fluoride at the time.

Finally, the results of a multi-laboratory study (reported elsewhere) which demonstrated that the development of standard protocols without direct inter-laboratory training increased fluoride recovery and resulted in very precise and true values, as measured by the analysis of certified reference material, were discussed [Martinez Mier et al., 2010]. The results of a multi-laboratory study that included participation in a training program, in addition to use of standardized protocols allowing laboratories to improve or maintain the accuracy of their analytical work by periodically comparing their results, were also discussed [Weber, 1988].

Discussion of the ideal method

After EAMM's presentation, workshop participants engaged in discussion to further define the problem and the issues surrounding the efforts to standardize available methods or develop new ones as needed. Participants questioned the feasibility of developing just one method to fit all types of toothpaste formulations or if there was a need for modification for each toothpaste formulation. There was concern that clinical testing is needed but it was recognized that attempting to fully mimic what happens in the mouth under laboratory conditions is difficult.

Participants also agreed that results of laboratory testing should not be used in isolation to draw conclusions regarding a toothpaste's clinical efficacy. However, there was consensus that developing methods to determine total and available fluoride is still important to comply with regulations. It was discussed that although clinical relevance was key, methods for quality control which measured simpler outcomes (such as total fluoride content) should also be developed.

For example, total fluoride may be used to assess the quality of the manufactured product and confirm it meets the country specific legal limits for maximum total fluoride concentrations (e.g. 1500 ppm USA, 1500 ppm Europe, 1000 ppm India). On the other hand, available fluoride may be used to assess toothpaste quality within the same formulation chassis e.g. NaF/silica. However, the addition of ingredients to provide functional benefits (e.g. stain removal, 'tartar control' ingredients) may require available fluoride assessments to confirm predicted efficacy. Available/soluble fluoride alone was deemed as insufficient to assess the quality of complex toothpaste formulations (e.g. where calcium phosphate compounds have been added or used as abrasives).

Participants also concluded that comparisons of available/soluble fluoride values alone across different fluoride sources and toothpaste formulations (NaF vs MFP vs SnF₂ vs AmF) should be treated with caution. Participants pointed to the need to recognize different formulations have different target fluoride concentrations to achieve efficacy. It was mentioned that an ideal method should not only consider solubilization of fluoride in a clinically relevant time, but also measure efficacy. Furthermore, it was proposed that the age of the sample being assessed must be considered prior to drawing conclusions.

After extensive discussion, participants agreed that the description of an ideal method should cover the following points:

- Standardization, which has to be simple
- Description of steps to ensure blindness
- Number of standards needed for calibration, including spot checks
- Determination of threshold for measurement
- Determination of the ideal and most clinically relevant dilution
- Description of financial aspects which may play a role in sample preparation, emphasizing a simple approach
- Training and calibration of technicians
- Recommendations for external validation

Definition of terms

Workshop participants engaged in an extensive discussion regarding terminology to be used for fluoride analysis. It was stated that an agreement on the terms and definitions to be used would be a positive first step towards harmonization. Table 2 presents the terms and definitions agreed upon by consensus through workshop discussions and subsequent manuscript development.

Discussion on terminology

Participants agreed that total measured fluoride is affected greatly by the technique of choice and this may need to be mentioned in the definition. They also agreed that the

determination of total fluoride is important to assess toxicity and for quality control (QC). Participants pointed out that the total fluoride present in sample may or may not be equivalent to the total fluoride measured by a particular method. In principle, participants agreed that total fluoride is the amount added to toothpaste as well as that already present in the raw materials.

According to the workshop participants, total fluoride is comprised of potentially (bio) available and unavailable fluoride. An important distinction was made between fluoride in toothpastes that can be measured by analytical means and fluoride in toothpastes that exerts anti-caries properties – the former was the focus of the present workshop whereas the latter can only be indirectly determined through caries clinical trials, as toothpaste excipients can also contribute to caries reduction. Analytically, investigators have tackled the issue of fluoride bioavailability by differentiating between the determinations of potentially (bio) available vs. total fluoride. Investigators have attempted to determine free fluoride in multiple reservoirs *in vivo*, including saliva, soft tissue and biofilm. Among these, free fluoride in the biofilm has been found to be the better indicator of the anti-caries effectiveness of toothpastes [Vogel, 2011].

It was agreed that potentially available fluoride can be defined as the amount of chemically soluble fluoride, while potentially bioavailable fluoride carries a biological dimension, as was described as fluoride that is chemically soluble and can be released into the oral cavity during and after tooth brushing. On the other hand, total fluoride is the measurable fluoride which may or may not be equivalent to available fluoride. Dilution, pH, sample preparation and time are factors that influence chemically soluble fluoride concentrations. It was also agreed that fluoride unbound in formulations may be considered available and that when using the term available fluoride one may be referring to readily available or potentially available for some formulations (e.g. formulations of lower water activity in which fluoride compounds are not solubilized but may solubilize during brushing).

Participants agreed that the determination of potentially (bio) available fluoride differs from that of available fluoride in that the latter should be done under a clinically relevant solubilization time, dilution, and pH. Also, for formulations requiring pretreatment (e.g. MFP), this step has to be biologically possible within a clinically relevant exposure time. Any determination has to be within the scope of clinical relevance. Finally, it was proposed that the amount of fluoride

remaining in oral reservoirs (biofilm or oral hard and soft tissues) after brushing may also be considered (bio) available fluoride.

Participants agreed that there are several methods that have attempted to determine bioavailability within a clinically relevant timeframe. One min has been proposed as the clinically relevant time for exposure [Carey et al., 2014]. Among the methods, the most common is acid diffusion; other investigators have attempted to pretreat samples to determine availability. The currently available methods may not be appropriate for new toothpaste formulations aimed at increasing fluoride retention by means of creating reservoirs of fluoride.

Strengths and limitations of methods and standards for determining fluoride in toothpastes approved by regulatory agencies (FDA, ADA, ISO)

State of the science/evidence for the available techniques (Presented by C.M. Carey [CMC])

This presentation reviewed the methods, discussed factors that reduce their accuracy, and presented data from international round robin studies that highlight the issues in the techniques. The presenter posited that there are three types of fluoride amounts that are of interest in toothpaste products: the total fluoride, the potentially available fluoride, and the amount of fluoride taken up by the tooth (enamel fluoride uptake – EFU). CMC proposed that total fluoride is the entire quantity of fluoride in a toothpaste, that potentially available fluoride could be defined as the amount of fluoride ion that becomes available in the oral cavity after tooth brushing with a fluoridated toothpaste, and that EFU is the amount of fluoride bound to the tooth as a result of exposure to fluoride-containing products. This definition was later taken into consideration when the group reached consensus regarding definition of terms. These include the following forms of fluoride: “ionic, precipitated, and pro-fluoride compounds”. Table 2 presents the definition of pro-fluoride compounds.

Fluoride salts in toothpastes can react with toothpaste excipients including abrasives, detergents, and other active ingredients to form insoluble fluoride salts that do not become available during use and therefore do not provide anti-caries benefits. Failure to release fluoride can be due to toothpaste matrix components that interfere with the solubilization of the fluoride

salts during brushing. These components regulate potential availability of fluoride, which can negatively affect clinical efficacy.

Total Fluoride Analysis

Currently, there are several methods for determining the total fluoride content in toothpastes which are accepted by the governing bodies who oversee the quality of these products in the marketplace. The American Dental Association (ADA) does not have regulatory authority; however, many manufacturers submit their products to the ADA to obtain the ADA's Seal of Approval. The Food and Drug Administration (FDA) has regulatory authority in the United States, and the International Standards Organization (ISO) standards are adopted by governing bodies in many other countries throughout the world. Table 3 presents a summary of analytical methods for total fluoride acceptable by governing bodies.

- The ADA seal program specifies one method based on Taves' use of an ion specific fluoride electrode (F-ISE)
- The FDA allows alternative methods, and requires comparison to reference standards for equivalency
- The ISO-11609 Dentifrices standard lists two methods and allows "Other validated methods of similar sensitivity and accuracy..."
 - 'ADA method' EDTA at a pH 8 digestion / HClO_4 diffusion to NaOH / F-ISE
 - 'Indian Standard 6356:2001' Extract into H_2O / fuse with Na_2CO_3 / F-ISE

The ADA method is based on the Taves method for the separation of fluoride from complex samples [Taves, 1968] and has the following advantages:

- Applicable to the greatest variety of products
- Digests the fluoride complexes that may be in the toothpaste, releasing HF
- Removes the fluoride from the sample matrix into a consistent sample
- Fluoride analysis by F-ISE is not hampered by complex matrix background
- Dilute samples may be concentrated through the diffusion step
- Reproducibility of the method is sufficient with a standard deviation of approximately 5%

The ADA method has the following disadvantages:

- Requires specialized diffusion dishes
- Diffusion efficiency is ~80% and may be inconsistent between analyses. Thus, internal standards must be included in each set of analyses
- The method requires the assumption that diffusion efficiency is the same for standards and samples. Spiked samples can reduce this uncertainty
- The diffusion step is time-consuming and therefore does not allow for rapid analysis

The Indian Standard method for determination of fluoride ion is incorporated into the Indian Standard for Toothpastes as Annex G [Bureau of Indian Standards, 2001 - IS6356:1993]. Sodium MFP or fluoride ions are extracted with water from toothpaste and the extract is fused with sodium carbonate to convert it into sodium fluoride. The fluoride content is then determined using a F-ISE. No publication could be retrieved that provides information about the accuracy and reproducibility of this method. The updated IS6365:2001 standard does not include this method, while the ISO 11609:2017 retains this method in section C.2.2 [ISO, 2017].

Potentially Available Fluoride Analysis

There are fewer methods for the analysis of potentially available fluoride in toothpastes. The analysis of fluoride that is available during tooth brushing requires that the method accounts for the need to solubilize the fluoride salt within the brushing time capturing the concentration of fluoride at that time as well as eliminating the possibility of fluoride reactions that could occur during the sample handling for analysis, for example, long centrifugation periods prior to analysis.

Common methods for quantification of potentially available fluoride in toothpaste have been to suspend the toothpaste into a slurry for 1 min, and then centrifuge the samples for 10 min followed by analysis of the supernatants for fluoride content using the same techniques as for total fluoride analyses [ADA, 2005]. These methods work well for the analysis of many NaF toothpastes where solubilized fluoride ions do not precipitate. Analyses of MFP-containing toothpastes require an additional hydrolysis step prior to fluoride ion analysis.

Recently, a new generation of toothpastes has been introduced that incorporates chemical agents resulting in the precipitation of fluoride reservoirs such as MFP, ACP or CaF₂-like deposits in dental plaque, and oral soft and hard tissues. Many of these newer-generation toothpastes produce fluoride reservoirs within the first minute of use. These potential fluoride reservoirs later release fluoride to the teeth over a longer period of time, which is claimed to contribute to the products' anti-caries efficacy. Measurements of fluoride that do not account for these phenomena underestimate the potentially available fluoride. This may be due to fluoride precipitation during the long centrifugation step resulting in lower fluoride concentrations in the supernatant.

At present, there are no methods for available fluoride accepted by the ISO. Therefore, the ISO dentifrice standard ISO-11609:2017 does not contain any requirement for available fluoride. The ADA offers older methods that have been shown not to be able to quantify available fluoride from products designed to precipitate fluoride reservoirs. Table 4 presents a summary of available fluoride determination methods. The FDA allows alternative methods, and requires comparison to reference standards for equivalency. Table 4 presents a summary of ADA tests.

The ADA Test 2a and 2b are based on a 1:100 dilution in H₂O, centrifugation for 10 min, filter and determine fluoride by F-ISE for NaF and SnF₂ salts or ion chromatography for MFP salts. The ADA methods have the following advantages:

- Applicable to the greatest variety of products
- Simple methodology
- Reproducibility of the method is sufficient with a standard deviation of approximately 5%

The ADA methods have the following disadvantages:

- Sample dilution not relevant for clinical evaluations (should be 1:3)
- May release more fluoride than would occur during use due to large dilution.
- 10 min centrifugation may remove fluoride complexes and fluoride precipitates that have clinical relevance. Pro-fluoride complexes may adsorb to the oral surfaces and can breakdown to release fluoride in the oral cavity over time. Examples include MFP and CaF₂

- Filtration (0.22 μm) also may remove pro-fluoride complexes and fluoride precipitates that have clinical relevance
- Soluble toothpaste matrix components may interfere with the F-ISE

One of Winston's methods [Winston, 2006] is based on a 1:100 dilution in H_2O , filtration through a 0.22 μm filter, and analysis via F-ISE methods for NaF-containing toothpastes.

The Winston method has the following advantage:

- Quick filtration avoids long centrifugation steps

The Winston method has the following disadvantages:

- Disadvantages: filters clog quickly and only a small amount of sample is gained
- Sample dilution not relevant for clinical evaluations (should be 1:3)
- May release more fluoride than would occur during use due to large dilution
- The small sample size gained may not reflect the bulk sample composition
- Filtration (0.22 μm) also may remove fluoride complexes and fluoride precipitates that may have clinical relevance
- Soluble toothpaste matrix components may interfere with the F-ISE

One min Potentially Available Fluoride Analysis

At present there are very few methods for 1 min potentially available fluoride analysis. These are based on the same methods as above but restrict the extraction of the sample to 1 min followed by a variety of separation methods to yield clear samples for analysis. The ADA seal program specifies one method for 1 min available fluoride, whereas the FDA and ISO do not have required methods at this time. Table 5 presents a summary of 1 min testing. The ADA Tests 3a and 3b are based on a 1:3 dilution in H_2O , centrifugation for 10 min, filtration and determination of fluoride by F-ISE for NaF and SnF_2 salts, or ion chromatography for MFP salts.

The ADA methods have the following advantages:

- Applicable to the greatest variety of products
- The dilution is for 1 min and is clinically relevant at 1:3
- Reproducibility of the method is sufficient with a standard deviation of approximately 5%

The ADA methods have the following disadvantages:

- 10 min centrifugation may remove pro-fluoride complexes and fluoride precipitates that have clinical relevance
- Soluble toothpaste matrix components may interfere with the F-ISE

A second Winston method [Winston, 2006] is based on a 1:3 dilution in H₂O for 1 min, filtration through a 0.22 µm filter, and analysis via F-ISE methods for NaF-containing toothpastes. The Winston method has the following advantages:

- The dilution is for 1 min and is clinically relevant at 1:3
- The quick filtration avoids long centrifugation steps

The Winston method has the following disadvantages:

- The filters clog quickly and only a small amount of sample is gained
- The small sample size gained may not reflect the bulk sample composition.
- Filtration (0.22 µm) also may remove fluoride complexes and fluoride precipitates that have clinical relevance
- Soluble toothpaste matrix components may interfere with the F-ISE

The Carey method [Carey et al., 2014] is based on a 1:3 dilution in H₂O for 1 min, 15 s collection of aqueous phase into a coil of filter paper, centrifugation of the sample-soaked filter paper to obtain fluid sample, 1 h digestion with HCl, KOH neutralization analysis via F-ISE methods for NaF, SnF₂, and MFP containing toothpastes. The Carey method has the following advantage:

- Applicable to the greatest variety of products
- The dilution is for 1 min and is clinically relevant at 1:3
- The absorption into filter paper of the fluid phase of the slurry quickly separates this phase
- Reproducibility of the method is sufficient with a reported standard deviation of 3%

The Carey method has the following disadvantages:

- The recovered samples are very small at ~100 µl
- Small sample is difficult to handle – introduces large variations from pipetting errors

- Dilution factor of 1200 multiplies the errors requiring careful analytical techniques to obtain small standard deviations
- Soluble toothpaste matrix components may interfere with the F-ISE

The ISO TC106/SC7/Working Group 4 – Dentifrices has conducted one pilot inter-laboratory study to evaluate the methods for the coiled filter paper method, and one large scale international inter-laboratory study to evaluate a modified the ADA 1 min potentially available fluoride based method. The method specified a shorter two-minute centrifugation step. The results of the pilot study indicated that the method was not feasible for most laboratories and there was very large inter-laboratory variation. The major issue was the very small sample size recovered from the coiled filter paper.

As part of an international inter-laboratory study, six different commercially available products and one non-commercial NaF sample specifically formulated for this study to have large amounts of bound fluoride were analyzed in the Carey laboratories. The samples contained NaF, NaF+Ca-PO₄, MFP, KF+Ca and AmF and were analyzed for total and potentially available fluoride. The potentially available fluoride method that was evaluated included a 1:3 dilution with 0.1 mol/l K₂HPO₄ (pH 7) with vigorous mixing of the slurry for 1 min immediately followed by a 2 min centrifugation at 12,000 g. A 100 µl aliquot of the supernatant was recovered and 200 µl 1 mol/l HCl was added and left to stand overnight. Then, 300 µl TISAB-II was added to the sample, which was analyzed using F-ISE methods. The slurry ratio of 1 g toothpaste mixed with 3 ml 0.1 mol/l K₂HPO₄ was chosen to mimic the toothpaste: saliva ratio used in the ADA Guidelines for Fluoride Containing Dentifrice [2005], and to mimic the buffering capacity of saliva [Lilienthal, 1955].

The results from the Carey laboratories are shown in Figures 1 and 2. It can be seen that the total fluoride concentrations reported were generally lower than the concentrations declared on the labels. The concentration of the potentially available fluoride was higher than the total fluoride for two of the products. The variability was somewhat larger than desired for some products. Three reasons that led to the high variations and lower concentrations were: the 1 min sample mixing was often incomplete resulting in lower amounts of toothpaste in the slurry; the use of K₂HPO₄ buffer instead of H₂O was unnecessary and may have caused precipitation during mixing and centrifugation. It was found that the soluble toothpaste matrix components

interfered with the F-ISE analysis, when the laboratory repeated the testing using the Taves diffusion methods to determine the fluoride concentrations. On the basis of these results and observations, it was decided to modify the methods accordingly and repeat this study in the ISO working group in the next year.

Discussion of the strengths and limitations of methods and standards for determining fluoride in toothpastes approved by regulatory agencies

After a thorough review of the methods requested by regulatory agencies, the discussion centered on the issues surrounding the determination of (bio) available fluoride. It was agreed that a feasible method still needs to be developed to determine the concentration of potentially (bio) available fluoride in toothpaste products, and that a consensus on how to interpret those concentrations will need to occur. At present, there are no data that support the minimal amount of potentially available fluoride needed for the prevention of caries. Other tests that have been used to determine the anti-caries efficacy of products include EFU, enamel solubility reduction (ESR), and animal (rat) caries studies. None of these tests have been related to clinical efficacy in humans. Therefore, we are left with the possibility that there are no *in vitro* or animal model quantities that are indicative of caries prevention efficacy.

Clinical trials indicate that fluoride-containing toothpastes at least 1000 ppm have better caries prevention efficacy than toothpastes containing <600 ppm fluoride [Santos et al., 2013]. Thus, fluoride concentration is a significant factor in the efficacy of toothpastes. There are a number of different fluoride salts that are used in toothpastes, and the amount of available fluoride from these salts varies considerably. Yet, there are clinical trials of toothpastes that use different fluoride salts as their active ingredient that significantly reduce the caries experience in children [Adair et al., 2001]. As an example, SnF₂-containing toothpastes typically exhibit low available fluoride contents (~50 % of total fluoride), yet toothpastes containing this salt are as effective as those containing NaF with 90% available fluoride. Thus, the discussion about how to interpret available fluoride will need to address these issues. What remains to be discovered is how the concentration of fluoride works to decrease caries experience and what effects come about from the specific fluoride salt or the presence of other potentially active ingredients in the toothpaste products.

At present, we are using a perspective developed for regulatory agencies several decades ago. When developed, the best available data from clinical trials was used and the FDA set requirements for toothpastes that established the concept of equivalency. That is, a new composition for toothpaste is required to demonstrate equivalency to products that have been shown to have anti-caries efficacy. Tests included EFU, ESR, and rat caries increment. The manufacturer is required to show equivalency for two of these tests, e.g., EFU or ESR and animal caries reduction, to be allowed to market their new compositions without providing efficacy derived from two caries clinical trials. Given the cost of clinical trials, it is not surprising that manufacturers have opted to follow the FDA requirements of equivalency rather than to conduct new clinical trials. This is also the reason that many new technologies have not made it to the marketplace [Pfarrer and Karlinsey, 2009]. It is therefore worrisome that none of the original products that the FDA based their requirements upon still exist in the marketplace.

Fluoride Analysis by Gas Chromatography - Potentially available fluoride analysis of MFP toothpastes after acidic phosphatase treatment

State of the science/evidence for the analysis of potentially available fluoride of MFP toothpastes after acidic phosphatase treatment (Introduced as part of C.M. Carey and presented by M.J. Buijs and C. van Loveren).

The van Loveren method for the potentially available fluoride analysis of NaF- and MFP-containing toothpastes requires the dilution of a 4 g sample with 8 ml artificial saliva, agitation for 2 min, centrifugation for 10 min with subsequent collection of the supernatant. For MFP toothpastes, an aliquot of the supernatant is treated with acidic phosphatase for 24 h before analysis via F-ISE methods [van Loveren et al., 2005]. After 1:75 dilution, the samples are treated with four units of acidic phosphatase (Sigma Chemical Co., St. Louis, MO, USA) for each 12.5 mg of toothpaste [Duckworth et al., 1987, 1991]. Acidic phosphatase is dissolved in a fresh mixture with final concentrations of 89 mM NaAc (Merck & Co., Kenilworth, NJ, USA) and 116 mM glacial acetic acid (Merck & Co., Kenilworth, NJ, USA) adjusted to pH 4.8 with KOH.

The van Loveren method has the following advantages:

- The artificial saliva does not contain calcium or magnesium, avoiding precipitation of fluoride salts with these ions

- Artificial saliva and phosphatase digestion are clinically relevant

The van Loveren method has the following disadvantages:

- Two-minute centrifugation may remove pro-fluoride complexes and fluoride precipitates that have clinical relevance
- Effect of long digestion allowing possible precipitation of fluoride complexes is unknown
- Soluble toothpaste matrix components may interfere with the F-ISE

Discussion of the strengths and limitations of the analysis of potentially available fluoride of MFP toothpastes after acidic phosphatase treatment

Workshop participants discussed the ability of the technique to determine available fluoride in a manner that could be clinically relevant. The discussion particularly touched upon the 20 h needed to enzymatically digest the samples. Participants agreed there is a distinction between soluble fluoride and clinically relevant (bio) available fluoride. It was mentioned that the fluoride made available after such a long period of enzymatic digestions (known as free ionizable fluoride) may not mimic what happens in the mouth. It was suggested that in order to have clinical relevance the preparation of the MFP sample should probably not be more than 3 to 8 h.

Participants agreed that it would be difficult to find an enzymatic method to reproduce what happens in the mouth and that the current method was developed for convenience. Participants also discussed that a method for MFP toothpaste is needed and at the moment, gas chromatography seems to be the most frequently used.

State of the science/evidence for the fluoride analysis by gas chromatography (Presented by M.J. Buijs and C. van Loveren)

The fluoride analysis by gas chromatography is an indirect method to analyze fluoride in the form of Trimethylfluorosilane (TMFS). The analysis is based on the acid promoted reaction between trimethylchlorosilane (TMCS) and fluoride ions. This reaction has to be carried out in a gas tight vessel in which TMCS, a strong acid, and the organic solvent toluene are heterogeneously mixed with the fluoride solution or compound. The reaction between fluoride

and TMCS happens immediately and the resulting apolar compound TMFS will be trapped in the toluene. After separating the phases through centrifugation, aliquots of the TMFS toluene solution are injected into a gas chromatograph in which the trimethylfluorosilane (TMFS) will be separated from the solvent.

Chromatographic separations are based on differences in molecular size, charge or polarity of components in a mixture. In gas chromatography, the mixture is vaporized and carried by gas (mobile phase) into a chromatographic column (stationary phase). This carrier gas is inert and has no interaction with the components. The components interact through affinity with the stationary phase coated on the wall of the column. Their passage through the column will be slowed down based on their relative interaction. The individual components will pass a detector and are registered on a chromatogram as peaks. A reliable system produces chromatograms with highly reproducible retention times for the analyte components as well as linear responses for peak surface area and height. Addition of an internal standard (isopentane) to the toluene will help to prevent errors due to variation in injection volumes and improve duplicate measurements.

The derivatization of the fluoride ion into a volatile compound (TMCS) is comparable to the one of HMDS with fluoride in the Taves method [Taves, 1968]. The difference between both methods is that in the Taves method the airborne TMFS releases the fluoride ion into a basic environment, the KOH droplet, which needs to be dried by evaporation. In the gas chromatographic method, the TMFS dissolves into the toluene solvent which can directly be injected for analysis. The gas chromatographic method is less labor intensive than the Taves method.

In the chromatographic method, the acid digestion of the fluoride compounds is done in one vessel with all chemicals present. The limiting factor for fluoride derivatization is in effect the effectiveness of the sample digestion. Usually sample and fluoride standard containing vessels are incubated overnight. Digestion in strong acid makes the method suitable for many materials such as toothpastes, dental plaque, saliva, cow and human milk, foods in general, surface water, glass ionomers, fingernails, salts etc. [Damen et al., 1996; Heijnsbroek et al., 2006; van Loveren et al., 2005; Benzian et al., 2012]. This method allows for determination of total fluoride

present in the sample. Determination of potentially available, soluble, and ionic fluoride requires pre-preparation of the sample from which these fractions are separated.

Discussion of the validity, reliability, feasibility, strengths and limitations of fluoride analysis by use of a gas chromatography

Workshop participants discussed the calibration curve for measuring fluoride in toothpaste, which is typically made between 0.5 and 50 ppm F. It was highlighted that the minimum detectable concentration of TMFS in toluene is 0.025 ppm. It was agreed that the calibration solutions should be measured by duplicate measurements, while toothpastes should be prepared as duplicate samples followed by duplicate measurements of each sample.

Participants also discussed that the repeatability of duplicate measurements is 0.4% from one sample and repeatability between duplicate samples, while the internal control toothpaste is 30 ppm F (2.2% for a 1450 ppm F toothpaste). The presenter shared that in his laboratory's experience, monitoring the internal control toothpaste (with different batches of toothpaste) for 10 experiments has a validity of 94% (1366 ± 56 ppm F) compared to the declared concentration of 1450 ppm. To the presenter's knowledge, no study on the reproducibility of this method, as defined in table 2, has been published.

Operating a gas chromatograph requires qualified personnel and a higher degree of laboratory infrastructure than what would be required for the F-ISE. The system needs pure nitrogen gas (5.0 purity), hydrogen gas and air. A filter system is needed to clean moisture and hydrocarbon impurities from the gases. Chemicals need to be of analytical grade or at least gas chromatographic quality. The volatile chemicals have to be handled in a fume hood and are hazardous for health. The chemicals need to be free from interfering compounds resulting in peaks at the TMFS's retention time. CaCO_3 -containing salts and dentifrices can create CO_2 build up in the vessel and therefore require special attention when adding the acid to prevent spillover.

The gas chromatographic method has the following advantages:

- There is a linear relationship between signal and fluoride levels

- It is possible to detect low concentrations of fluoride in small volumes; the minimum detectable concentration in toluene 0.025 ppm TMFS
- Automated injections allow for many samples to be run in a short period of time and overnight
- Addition of an internal standard strengthens the reliability of measurements
- The digestion in strong acid makes the method suitable for many types of samples: saliva, dental plaque, toothpastes etc.
- One vessel for all steps during sample preparation
- High repeatability
- Possible to concentrate dilute samples by changing volume ratio between water and toluene

The gas chromatographic method has the following disadvantages:

- Technically challenging method
- High initial investment and operational costs

Use of the fluoride electrode to determine potentially available fluoride in different toothpaste formulations

State of the science/evidence for the technique (Presented by J.A. Cury)

This presentation reviewed the use of the fluoride ion-specific electrode for determination of total and (bio) available fluoride in toothpastes. The fluoride electrode is by far the most commonly used and simple method for fluoride detection in different types of samples. Its use for the determination of fluoride in toothpastes is also simple, considering that few requirements need to be met.

The fluoride electrode method has the following advantages:

- The fluoride electrode detects only ionic fluoride. Therefore, in any toothpaste containing NaF, SnF₂ and AmF, fluoride determination using the electrode would be possible by a direct measurement of the toothpaste slurry, provided that it is adequately buffered with TISAB.

However, some limitations to the direct use of this technique exist:

- Fluoride is commonly added to toothpastes in an ionizable (not yet ionic) form, such as MFP. This requires prior hydrolysis for detection with the fluoride electrode.
- Many toothpaste formulations are based on calcium-containing abrasives; when fluoride is already ionic, or ionized from the formulation during preparation for analysis, such calcium can bind fluoride ions, compromising an accurate determination of the fluoride concentration.

To overcome both limitations, a standardized technique [Cury et al., 2010], adapted from Pearce [1974], has been used for almost 40 years in the Oral Biochemistry laboratory of the Piracicaba Dental School, Brazil [Cury et al., 2004], which has been able to demonstrate results on the availability and stability of fluoride in toothpastes from all over the world [Cury et al., 1981; Sarmiento et al., 1994; Conde et al., 2003; Hashizume et al., 2003; Cury et al., 2006; Cury et al., 2010; Carrera et al., 2012; Ricomini Filho et al., 2012; Giacaman et al., 2013; Soysa et al., 2015; Cury et al., 2015, Cury et al., 2016]. With this technique, it is possible to estimate total, soluble (ionic and ionizable, separately) and unavailable fluoride in toothpastes.

The method (figure 3) is based on the dilution of the toothpaste in water (0.1 g/10 mL), followed by centrifugation/acid hydrolysis steps in order to estimate all fluoride forms in the formulation using the fluoride electrode. The acid is used not only to hydrolyze ionizable fluoride forms (.e.g. MFP), but also to dissolve insoluble fluoride salts in the determination of total fluoride concentration in the formulation. The costs are similar to a direct fluoride analysis using the electrode, making the method highly feasible.

The reproducibility and validity of total fluoride and total soluble determinations in toothpastes with the fluoride electrode were presented, based on the results of four independent studies [Cury et al., 2006; Cury et al., 2010; Carrera et al., 2012; Giacaman et al., 2013]. A low variation was shown for the determination of total fluoride ($1.5\% \pm 0.9\%$) and total soluble fluoride ($1.4\% \pm 1.1\%$). The validity, assessed by the correlation between the expected and detected total fluoride concentrations, ranged from 0.992 to 1.000 for the NaF/silica-based formulations, and from 0.918 to 0.980 for the MFP/calcium carbonate-based formulations [Marín et al., 2016].

In fact, the validity of the fluoride electrode to determine total soluble fluoride in toothpastes, using the acid hydrolysis of MFP, has been previously shown to be high ($r = 0.997$) [Hattab, 1989].

Discussion on the validity, reliability, feasibility, strengths and limitations of the technique

Participants agreed that the F-ISE is the most used and simple method for fluoride detection and that as such there was a need for a protocol for its use that needs to discuss potential systematic error. It was agreed that there is a need to describe why there is large variation when using this technique with small concentrations of fluoride.

Participants also mentioned that despite the clear advantages of the F-ISE method, the fact that the different fluoride salts are not easily analyzed by one simple F-ISE method makes its adoption as the universal method problematic. This is complicated further by the wide variety of components within the matrix of the toothpaste products. This problem is being addressed by the use of fluoride diffusion technology first described by Taves [1968] with modifications that are making the analysis of fluoride in almost any matrix possible. However, because different fluoride salts may require differing amounts of potentially available fluoride to exert caries preventive amounts, a unified recommendation on the ideal amount of available fluoride in toothpaste is not likely, even if the analysis relies solely on F-ISE.

Discussion on the Need for Clinical Relevance when Developing Tests and Specific Formulations Issues

During the second day, participants spent some time defining the steps needed to provide clinical relevance to any accepted methods. The participants agreed to a stepwise approach that starts with the current *in vitro* analysis and moves to more complicated *in situ* models. The group recognized that the current analysis of potentially available fluoride does not include factors such as saliva components interacting with the toothpaste. This could include protein interactions with abrasive, detergent, or fluoride complexes. The current analytical method would represent the baseline for potentially available fluoride concentration without interferences from salivary components. The participants chose a stepwise approach starting with the development of a reliable analytical technique for toothpaste slurries in water followed with procedures to be integrated that bring the analyte closer to what is clinically observed.

There was also time allotted to the discussion of the specific needs for the analysis of specific formulations.

Participants agreed that what happens in the mouth, specifically dispersion *in vivo*, needs to be better understood and that there is a need to develop methods to simulate it *in vitro*. These methods would need to replicate the *in vivo* brushing experience in a laboratory (taking into account release kinetics). There was agreement that there is a need for data on pH cycling models capable of predicting the likely clinical outcome (in terms of caries prevention). Similarly, there is a need to revisit *in situ* models which replicate the *in vivo* brushing experience and are not limited by the fluoride source. In general, pH cycling methods will not work effectively with MFP, but *in situ* (intra-oral models) methods work with all species of fluoride because they take into account the digestion of MFP in the mouth. Likewise, there is a need to revisit models capable of predicting the intra-oral retention of fluoride. And finally, there is a need to understand the importance of intra-oral fluoride reservoirs and their contribution to caries prevention. A recommendation was made for the creation of a validation matrix to provide evidence to support the understanding of what the different data tell us.

The following points were raised during discussion regarding the analysis of specific fluoride formulations:

- MFP
 - Analysis has proven to be challenging
 - The current methods for MFP may not have clinical relevance
 - The digestion of MFP vs. its hydrolysis requires very different time periods
 - Ion chromatography is a suitable analytical methodology for MFP
 - Because the analysis of MFP-containing toothpaste typically yields lower fluoride concentrations than expected, the analytical technique may require additional steps or may require a different analytical technique. This in turn may introduce one or more sets of analytical parameters
 - Any proposed method to analyze MFP-containing toothpastes will need to be validated with chemically pure MFP

- To avoid analytical techniques that are not able to accurately quantify fluoride in the wide variety of toothpaste compositions, it is recommended that commercial samples of known stability be used as controls
- NaF
 - The available ADA methods seem to work since NaF is relatively simple to analyze
 - The use of a dilution of 1:3 and its clinical relevance needs to be revisited. It needs to be considered that the dilutions are time sensitive. A range of two to 6 min was suggested
- Amine fluoride
 - Any method used for NaF can be utilized for AmF while using the same TISAB
 - If pH is too low, AmF may be bound to silica; however, this bond is fully reversible when the pH is raised to 7
- SnF₂
 - The same methods that are used for NaF are appropriate; it is simple to analyze, but has to be done at low pH
 - For more concentrated samples (1:30); it is recommended to use TISAB IV
 - A dilution of 1:100 is needed if EDTA–TAM or CTAB are being used
 - Numbers seem to be lower, at 90% of what may be expected if stannous species are being formed

Participants agreed that fluoride-concentration in toothpastes may vary due to production. This is minimized by always weighing fluoride is twice in production and carefully monitored before release to public. For that reason, there is a need to take into account that different types of fluoride may require a different protocol in the preparation of samples and standard solutions.

- NaF sample preparation
 - No special requirements

- AmF sample preparation
 - These formulations have usually a low pH (approximately 4.0-5.0), so adequate solutions have to be added to increase pH of sample, (i.e. NaOH); it is advised to have a pH of 7 in the sample. F-ISE analysis requires use of TISAB to set the pH and disassociate fluoride-matrix complexes.

- SnF₂ sample preparation
 - These formulations have a low pH (approximately 5.0-5.5); the lower the pH the more the silica can adsorb fluoride. F-ISE analysis requires use of TISAB to set the pH and disassociate fluoride-matrix complexes.

- MFP sample preparation
 - There is a need to add enzymatic or chemical ingredients to disassociate the MFP

Identification of gaps in knowledge to be addressed by future research

Based on the meeting presentations and discussions, the work group drafted recommendations and identified areas in which additional evidence review was necessary.

Research gaps

- There is an urgent need to develop new methods to determine (bio) available fluoride grounded in clinical relevance. In order to achieve this, there is a need to:
 - Conduct studies to test if there is correlation between the concentration of chemically determined total soluble fluoride in a toothpaste and the concentration found in the oral cavity during and after tooth-brushing
 - Better understand fluoride dispersion in the oral environment and to develop methods to simulate it *in vitro*
 - Understand the importance of intra-oral fluoride reservoirs and their contribution to caries prevention and to develop methods that may simulate them *in vitro*

- There is an urgent need to refine existing methods based on new data to better understand their limitations and modify them if needed. In order to achieve this, there is a need to:
 - Generate data on pH cycling models to determine if they are capable of predicting caries prevention efficacy *in vivo*
 - Compare chemical hydrolysis vs. enzymatic hydrolysis of MFP toothpastes for its analysis using IC in order to evaluate the clinical relevance of both methods
 - Develop a F-ISE universal methodology and define its limitations
- There is a need to develop protocols for the accelerated ageing of toothpaste that replicate the effects on potentially (bio) available fluoride of storage at room temperature until the expiry date

New Methods

Capillary electrophoresis was discussed as a potential new method. Participants agreed that it is less reliable than ISE and IC. It was also mentioned that it requires a secondary step for internal availability control, which makes it less desirable.

Discussion on Public Health Implications

During the second day, participants spent some time discussing the public health implications of developing a method capable of determining fluoride (bio) availability. It was agreed that, although the efficacy of fluoride toothpastes can ultimately only be proven in well conducted randomized controlled clinical trials, the central role of fluoride toothpaste in the context of oral health worldwide makes it critical that standardized techniques for the analysis of their potentially (bio)available fluoride are defined. Such standards are, however, only useful if they are translated into regional and national guidelines which can be adopted by local governments. For this reason, the development of relevant, but simple and reproducible methods remains crucial.

Participants recognized that the issues and challenges discussed by the experts during the workshop are highly relevant to ensure anti-caries efficacy of fluoride toothpastes, but that

many additional areas need to be considered from broader public health and consumer protection perspectives. Toothpastes are the most important and most widely used vehicle for the delivery of fluoride for caries prevention. The central role of fluoride toothpastes in the context of oral health worldwide makes it critical that they have a minimum concentration of potentially (bio) available fluoride to have anti-caries potential during the expected shelf life. To ensure this, it is essential that international norms be defined for minimum potentially (bio) available fluoride along with standardized techniques for its analysis that are relevant, straightforward and reproducible.

With some notable exceptions, international and most regional and country norms only specify the maximum amount of total fluoride that a toothpaste should contain. Consequently, there is an urgent need for the relevant organizations to advance rapidly in terms of norms and analysis techniques for fluoride toothpaste to ensure anti-caries potential. From a public health and regulatory perspective, it would be crucial to strengthen quality control in a pragmatic and cost-effective way. In order to maximize the potential of fluoride toothpaste as an essential public health tool to address the high burden of tooth decay worldwide, comprehensive national, regional and global strategies are required to make effective fluoride toothpaste universally available. Workshop participants commended ORCA for taking the initiative and for providing a forum to advance the global agenda in this context; ORCA and all relevant international stakeholders were encouraged to maintain momentum and to intensify their collaborative efforts.

Workshop Conclusions

The workgroup was tasked with reviewing the evidence on the validity, reliability and feasibility of each technique to determine fluoride in toothpastes, and was able to reach consensus on the terminology to be used. Workgroup participants were also able to identify and summarize the advantages and disadvantages of each technique, discuss strengths and limitations of different sample preparation methods for different types of toothpastes.

Reaching consensus on what available methods are appropriate to assess potential (bio)availability proved a more difficult task, since participants agreed that most currently available methods were developed for regulatory agencies several decades ago utilizing the

best available data from clinical trials at the time. Participants agreed that interpretation of the results of current or newly developed methods needs to be carefully considered based on toothpaste formulation/excipients and the analytical methods chosen. Although significant advances to our understanding of the mechanism of action of fluoride in toothpaste has been achieved over the past four decades, this clearly is an extraordinarily complex subject and more work remains to be done.

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Legends

Figure 1: Results from the Carey's Laboratory on Total Fluoride versus Potentially Available Fluoride.

Figure 2: Results from the Carey's Laboratory on Percentage of Potentially Available Fluoride.

Figure 3: Determination of Total, Soluble, Ionic and Insoluble Fluoride by the Direct Method Using the Fluoride Electrode.

Table 1. Main Ingredients in Toothpastes.

Type of fluoride agent
Amine fluoride (AmF)
Sodium fluoride (NaF)
Sodium monofluorophosphate ($\text{Na}_2\text{PO}_3\text{F}$)
Stannous fluoride (SnF_2)
Abrasive system
Alumina
Calcium carbonate
Calcium pyrophosphate
Dicalcium phosphate
Silica
Sodium bicarbonate
Other ingredients
Binding agents
Coloring
Flavorings and sweeteners
Humectants
Preservatives
Surfactants
Delivery form
Foam
Gel
Liquid
Paste

Table 2. Terminology and Definitions.

Term	Definition
Total Fluoride	Total fluoride contained in the sample measurable by currently available methods.
Labeled/Declared Fluoride	Fluoride declared by the manufacturer on the toothpaste label
Potentially Available Fluoride in Toothpaste	Fraction of total fluoride in the formulation that is chemically soluble in water or acid.
Potentially Bioavailable Fluoride in Toothpaste	Chemically soluble fluoride present in a toothpaste that would be potentially available to be released into the oral cavity during and after tooth brushing for caries prevention and absorbed in gastro-intestinal tract.
Unavailable Fluoride	Fraction of total fluoride that is not chemically soluble in the formulation.
Pro-fluoride compounds	Fluoride complexes that can adsorb to the oral surfaces and breakdown to release fluoride in the oral cavity over time. Examples include MFP and CaF ₂ . These should be considered to be part of the potentially available fluoride concentration.
Soluble Fluoride	Fraction of total fluoride that is ionizable through dissolution in an aqueous media or enzymatic breakdown.
Ionic fluoride	Fraction of total fluoride that is readily ionic upon dissolution in an aqueous medium.
Validity	The extent to which an analytical procedure accurately measures what it intends to measure.
Accuracy or Trueness ¹	The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.
Precision ¹	The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.
Repeatability ¹	Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.
Reproducibility ¹	Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).
Intermediate precision	Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

¹ INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED TRIPARTITE GUIDELINE VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY Q2(R1) Current Step 4 version Parent Guideline dated 27 October 1994

Table 3: Analytical Methods for Total Fluoride Acceptable to Several Regulatory Agencies.

Method source	Fluoride source	Comments
ADA Test 1 ISO 11609 C.2.1	NaF / SnF ₂ / MFP /Amine F	Digestion in HClO ₄ with diffusion to NaOH for ≥ 6 h / F-ISE
Indian Standard ISO 11609 C.2.2	NaF / SnF ₂ / MFP /Amine F	Extract into H ₂ O 30 min / 10 min centrifuge / fuse with Na ₂ CO ₃ to convert all forms of F into NaF / F-ISE
van Loveren (CEP048)	NaF / MFP	HCl digestion 1h / extraction into toluene 12 h / Gas Chromatography
van Loveren (Taves CEP021)	NaF / SnF ₂ / MFP	Digestion in HClO ₄ / HCl-HDMS with diffusion to NaOH for 24 h / F-ISE
Cury JA et al., (2010)	NaF/MFP	1% Toothpaste suspension in H ₂ O, HCl 45°C/1h, NaOH neutralization, TISAB buffering, direct analysis with F-ISE

Table 4: Determination of Available Fluoride.

Method source	Fluoride source	Comments
ADA Test 2a	NaF & SnF ₂	H ₂ O extraction / 10 min centrifugation / F-ISE
ADA Test 2b	MFP	H ₂ O extraction / 10 min centrifuge; Ion chromatography for MFP
Winston	NaF	For Ca-PO ₄ containing toothpaste: H ₂ O extract / 0.22 µm filter / F-ISE
Cury JA et al. (2010)	NaF / MFP	1% Toothpaste suspension supernatant, HCl 45 °C/1h, NaOH neutralization, TISAB buffering, direct analysis with F-ISE
van Loveren (CEP044)	NaF / MFP	Dilute with artificial saliva / digest 24 h with acidic phosphatase / F-ISE

Table 5: Summary of 1 min Testing.

Method source	Fluoride source	Comments
ADA Test 3a	NaF and SnF ₂	H ₂ O extract for 60 s / 10 min centrifugation / 0.22 µm filter / F-ISE
Winston	NaF	For Ca-PO ₄ containing toothpaste: H ₂ O extract for 60 s / 0.22 µm filter / F-ISE
ADA Test 3b	MFP	H ₂ O extract for 60 s / 10 min centrifuge / Ion chromatography for MFP
Carey	NaF, MFP, SnF ₂	Coiled filter paper extract from slurry / digestion / F-ISE