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Flow injection on-line dilution for zinc determination in human saliva with electrothermal atomic absorption spectrometry detection

Margarita Burguera-Pascu[a,*], *Alberto Rodríguez-Archilla*[a], *José Luis Burguera*[b], *Marcela Burguera*[b], *Carlos Rondón*[b], *Pablo Carrero*[b]

[a] *Department of Oral Medicine, School of Dentistry, University of Granada, Granada, Spain*
[b] *Department of Chemistry, Faculty of Sciences, University of Los Andes, Mérida, Venezuela*

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ABSTRACT

An automated method is described for the determination of zinc in human saliva by electrothermal atomic absorption spectrometry (ET AAS) after on-line dilution of samples with a significant reduction of sample consumption per analysis (<0.4 mL including the dead volume of the system). In order to fulfill this aim without changing the sample transport conduits during the experiments, a flow injection (FI) dilution system was constructed. Its principal parts are: one propulsion device (peristaltic pump, PP) for either samples, standards or washing solution all located in an autosampler tray and for the surfactant solution (Triton X-100) used as diluent, and a two-position time based solenoid injector ($TBSI_1$) which allowed the introduction of 10 μL of either solution in the diluent stream. To avoid unnecessary waste of samples, the $TBSI_1$ also permitted the recirculation of the solutions to their respective autosampler cups. The downstream diluted solution fills a home made sampling arm assembly. The sequential deposition of 20 μL aliquots of samples or standards on the graphite tube platform was carried out by air displacement with a similar time based solenoid injector ($TBSI_2$). The dilution procedure and the injection of solutions into the atomizer are computer controlled and synchronized with the operation of the temperature program. Samples or standards solutions were submitted to two drying steps (at 90 and 130 °C), followed by pyrolysis and atomization at 700 and 1700 °C, respectively. The aqueous calibration was linear up to 120.0 μg L^{-1} for diluted standard solutions/samples and its slope was similar ($p > 0.05$) to the standard addition curve, indicating lack of matrix effect. The precision tested by repeated analysis of real saliva samples was less than 3% and the detection limit (3σ) was of 0.35 μg L^{-1}. To test the accuracy of the proposed procedure, recovery tests were performed, obtaining mean recovery of added zinc of 97.8 ± 1.3%. Furthermore, Zn values estimated by the procedure developed in this work were compared with those obtained by a standard addition flame-AAS method applied to 20 randomly selected saliva samples. No significant differences ($p > 0.05$) were obtained between the two methods. Zinc levels in saliva samples from 44 healthy volunteers, 15 male and 29 female, with ages between 20 and 51 years (mean 30.50 ± 9.14 years) were in the range 22–98 μg L^{-1} (mean of 55 ± 17 μg L^{-1}), similar to some and different from others reported in the literature. It was found that zinc values for male were statistically higher ($p = 0.006$) than for female.

* *Corresponding author.*
E-mail address: margaburpas@hotmail.com (M. Burguera-Pascu).
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1. Introduction

Whole saliva is a unique and complex secretion, containing a mixture of oral fluids and constituents of non-salivary origin, with numerous functions in the oral cavity [1]. Water represents 99% of its composition and the remaining 1% consist of organic molecules, electrolytes and oligoelements. It is well known that the composition of saliva depends on a number of factors related to physiological, pathological and environmental changes. The fluid composition may differ on account of varying contribution rate and quality from the individual glands, the presence or absence of a stimulatory source, the type of food ingested, the circadian cycles and the presence of infections [1,2]. Monitoring one or various salivary parameters, one can lead to the cause of any baseline levels impairment.

Zinc is an essential trace element which is present in small amounts in all tissues and body fluids, including saliva. Several diseases that affect the oral cavity have been shown to be related to changes in salivary zinc concentration: incidence of dental caries [3], periodontal diseases [4], abnormal test acuity [5], etc. Therefore, knowledge about concentrations of zinc in saliva has become increasingly important. Estimation of baseline salivary zinc concentrations is sensitively carried out by inductively coupled plasma-mass spectrometry (ICP-MS) [5,6] or atomic absorption spectrometry (AAS) with electrothermal atomization (ET AAS) [7,8], as conventional flame-AAS proves impractical: (1) because zinc values are below or near the detection limit of the instrument, which makes necessary the use of the standard addition method, thus increasing the time of analysis and sample handling, and (2) due to a high sample consumption (see below).

Given its high selectivity and sensitivity, its wide availability in analytical and clinical laboratories, as well as its relatively low cost and sample economy, ET AAS is the method of choice for the routine analysis of biological samples. However, there are two major problems for its application to the determination of zinc: (i) the achievement of a too high sensitivity (less than 0.3 pg of zinc for 1% absorption), and (ii) the great risk of contamination due to excessive sample handling and/or addition of reagents, as zinc is an over-abundant element in the environment.

There are different means of decreasing the sensitivity in ET AAS: the use of an alternative atomic line or the dilution of samples with water or any appropriate reagent. The alternative zinc line at 307.6 nm has about 1000-fold less sensitivity than its resonant line at 213.9 nm, which makes it too insensitive for the purpose of this work [7,8]. An adequate dilution scheme with water or any reagent looks suitable to bring samples concentrations within the instrumental working range and also to reduce matrix interferences which might occur with concentrated samples. The use of mineral acids and oxidizing agents to digest the samples, and also of chemical modifiers to correct the background should be avoided as it proved inappropriate for zinc analysis due to impaired blank signals which lead to inaccurate results [9]. The dilution procedure with pure reagents or water fulfills the requirements mentioned above and it is advantageously facilitated by coupling the detection system with a flow injection (FI) manifold which reduces: the analysis time, the number of preparatory steps and the sample volume needed for each analysis.

Up to date, only spectrometric techniques with continuous detecting systems like flame-AAS [10–18], ICP-MS [19–21], spectrophotometry [22–25] and fluorimetry [26] have been coupled with FI manifolds for on-line dilution. Generally, the purpose was to extend the analytical dynamic range [10,12,14,17,26], to increase sample throughput and precision and to reduce the sample pretreatment steps, thus minimizing the risk of contamination [11,13,16,18,19,22,23].

In such systems, the on-line dilution was achieved in manifolds based on different principles, like zone penetration [11], dispersion of micro-volumes [12,14,18] and zone sampling [26], implementation of the cascade method [16,22] or using different devises, like recirculating loops [10,19], dialysis units [24], dilution chambers [16,17,25], binary inlet static mixers [23], etc. Each of these approaches have their drawbacks: some manifolds are too complex to be implemented in other laboratories, the precision is impaired by the peristaltic pumps fluctuations at large flow rates and by the generation of air bubbles in the flow streams, the dialysis membranes or the manifold tubing are fragile for long-term usage, etc.

Coupling a continuous flowing system with the discontinuous ET AAS detection is guaranteed by the characteristic precise timing of FI and by the development of an adequate interface to perfectly synchronize the injection of the desired flowing segment into the atomizer [27–30]. Our experience in the development of such systems allows us to develop a FI on-line dilution manifold with ET AAS detection for the contamination-free determination of zinc in whole, unstimulated saliva samples. Such a system will allow us to achieve the main purposes of this study: (i) bring zinc concentrations of samples within the instrumental working range, (ii) there is a significant reduction of sample and reagent consumption, (iii) reduce sampling handling, (iv) avoid samples digestion, (v) avoid the use of chemical modification, and (vi) demonstrate the robustness of the experimental arrangement and make complete automation of the analysis possible.

2. Experimental

2.1. Reagents

All the chemicals used in this study were of analytical-reagent grade or higher. Only high-purity de-ionized and distilled water (DDW) with specific conductance <0.1 $\mu S\,cm^{-1}$, obtained in a Milli-Q system (Millipore, Bedford, Massachusetts, USA) and frequently tested to be zinc-free, was used in the experiments. A zinc stock solution of 1000 $mg\,L^{-1}$ (Titrisol, Merck) was used to prepare daily standards. The carrier (diluent) solution was prepared by diluting Triton X-100 (Merck) in water to obtain a concentration of 0.02% (v/v). At this surfactant concentration no traces of zinc were detected. All standards were stored in appropriate polystyrene screw-cup containers soaked overnight in 2% (w/v) EDTA and then thoroughly washed with several portions of zinc-free water to remove any traces of zinc. Argon gas of 99.99% purity served as purge gas for the graphite furnace. The only glassware employed in this

work was few volumetric flasks used to prepare the calibration solutions.

2.2. Subjects and samples

A group of 44 volunteers, 15 men and 29 women with ages between 20 and 51 years (mean age of 30.50 ± 9.24 years) were randomly recruited among students, teachers and technicians of the Department of Oral Medicine, School of Dentistry, University of Granada, Spain.

In order to get a representative sample for analysis, an accurate standardization of the sampling procedures was carried out. Therefore, before starting the sampling, each subject signed an informed consent form, was asked to complete a medical questionnaire and to undergo an examination of the oral cavity in order to fulfill the inclusion criteria: lack of metal restorations and/or amalgam fillings, healthy gums, caries-free and normal gustatory acuity. In order to minimize the effect of circadian variations and other disturbance factors on salivary composition, 0.5–1.0 mL of saliva samples were collected under fasting conditions between 8 and 9 a.m. without any mouth washing other than water. After rinsing the mouth thoroughly with several portions of pure water, each subject was instructed to remain sited, maintain the head in a sub-horizontal position and keep the saliva in the vestibule zone of the oral cavity for one minute. The fluid was afterwards transferred to small cupped polyethylene tubes and frozen until the sampling procedure ends and the analysis is ready to be performed. Just before analysis, the samples are allowed to defrost at room temperature and vigorously shaken in a Vortex for 30 s to re-homogenize it just before analysis. This step was included in the procedure because the samples could have been stratified on standing by vertical gravitational fractionation of the different organic fractions of saliva, to which zinc might be bound [9].

2.3. Apparatus

The detection unit used in the FI-ET AAS system was a Perkin-Elmer (PE) atomic absorption spectrometer, Model 4100 ZL with a transversally heated graphite atomizer and a Zeeman-effect background corrector. The instrument control and data processing was accomplished with an Epson personal computer (PC), Model EL 486UC through PE 4100PC software (Version 7.3) under Gem Desktop (Version Gem/3). A PE zinc hollow cathode lamp, pyrolytic graphite – coated graphite tubes and pyrolytic graphite – coated graphite platforms was used. Integrated absorbance values (peak area), peak profiles and statistical data were printed with an Epson LX-810 printer.

In order to obtain the desired samples dilution without changing the sample transport conduits, a FI dilution system was constructed and connected to ET AAS apparatus (Fig. 1). Its principal parts are:

- An autosampler (Perkin Elmer AS-71) containing vials with standards/samples was programmed to choose an appropriate solution from the carousel and to introduce it in a FI manifold as described in the procedure. The vertical and horizontal positions of the autosampler's capillary tubing with respect to the vials were controlled by the spectrometer computer. The washing solution (0.02% (v/v) Triton X-100), located on the reservoir bottle provided with the instrument, was pumped into the overflow container which was then slewed by a stepping motor to the required position during the washing step of the procedure. The used washing liquid runs to waste down the drain tube into a waste bottle after each cycle.
- One propulsion device Gilson Minipuls 3 (peristaltic pump, PP) for either standards/samples and diluent solution, which was also a solution of 0.02% (v/v) Triton X-100. A 75 cm long Tygon® tubing with an internal diameter of 0.8 mm (which houses a total volume of 375 μL) was used for the closed-flow circuit channel indicated by arrows in Fig. 1.
- Two, two-position time based, electronically controlled solenoid injectors from Cole Palmer: The solenoid valve $TBSI_1$ is configured to have its set of tubing "a" and "b" closed and open, respectively (stand-by position) to allow the recirculation of either solution in its flow circuit, as indicated by the arrows in Fig. 1, and also to introduce certain volume of either solution in the diluent stream, when the set of tubing "a" and "b" are open and closed, respectively. The other solenoid valve, $TBSI_2$, permits the sequential deposition of

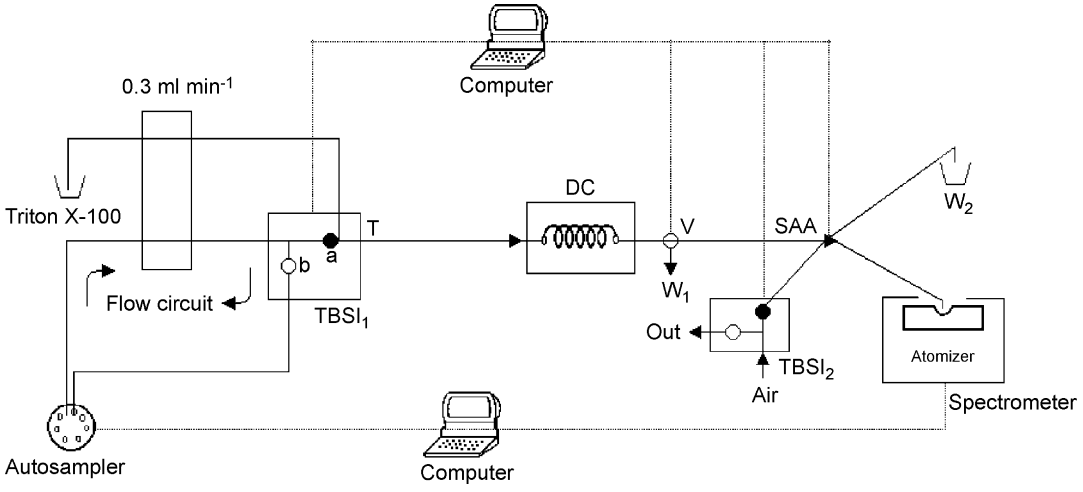

Fig. 1 – Schematic diagram of the FI-ET AAS dilution manifold.

20 μL aliquots of diluted samples/standards on the graphite tube platform by air displacement.
- One four-way Latek RMW rotary valve (V) which permitted the filling of a home made sample arm assembly (SAA) described elsewhere [31].
- A TDK model 286 computer controlled the functioning of $TBSI_1$, V, $TBSI_2$ and SAA. The sequences programmed by this computer were synchronized with those controlled by the spectrometer computer, which had been pre-programmed to control the instrument autosampler and the furnace temperature program. Either the FI or the ET AAS components were easily interfaced and the instrument computer was readily available for other purposes.

Neoprene pump tubes with small bore size (0.8 mm i.d.) and poly(tetrafluoroethylene) (PTFE) tubing (0.8 mm i.d.) were used for the PP and for the FI manifold, respectively.

A Heidolph Model Reax1 Vortex-type stirrer was used to homogenize saliva samples just before their introduction into the FI manifold.

2.4. *FI-ET AAS manifold procedure*

The operation of the FI dilution manifold as well as the ET AAS optimized conditions for the determination of zinc concentrations in the diluted saliva samples are summarized in Fig. 1 and Table 1, respectively. The peristaltic pump continuously propels either the washing or standard/sample and diluent solutions at a continuous flow rate of 0.3 mL min^{-1}. The principal steps of the dilution and determination processes are described below.

2.4.1. *Sequence 1: sample loading*

To avoid uncontrollable dilution of the next sample, the Triton X-100 solution left from the last sequence in the flow circuit, was flushed out with air through the autosampler flush port during 100 s. Then, the autosampler take-up capillary moves to the next sample vial filling the flow circuit with the next sample, which is kept flowing in a closed flow manner.

2.4.2. *Sequence 2: dilution*

$TBSI_1$ is activated for 4 s to allow 10 μL of the concentrated saliva sample to be introduced into the diluent stream at confluence "T" and to thoroughly mix in the 30 cm long dilution coil. In order to ensure that only the most homogeneous part of the diluted sample segment will be used for zinc analysis, about one third of this mixture is discarded, as valve V continues in W_1 position for 25 s. Thereafter, valve V is rotated during 25 s to fill the SAA which immediately moves to injecting position, ready to initiate again the ET AAS measurements, as described in sequence 3. Thus saliva samples are diluted about 20-fold, and their zinc concentrations are brought within the working range of the instrument. Standards are processed exactly as samples for calibration purposes. This sequence lasts 50 s.

2.4.3. *Sequence 3: analytical measurement*

In this sequence, there are two parallel-operated steps: (1) the diluent flows to waste through valve V in waste (W_1) position, while the set of tubing "a" and "b" of $TBSI_1$ are, respectively, closed and opened allowing the washing solution to flow within the flow circuit, and (2) the SAA moves to inject the previously diluted sample into the graphite tube as $TBSI_2$ is activated to allow the sequential deposition of 20 μL of solution on the graphite platform. The last step was timed to synchronize with the spectrometer computer, which had been pre-programmed to run the furnace temperature program. This operation was repeated, as the integrated absorbance was in all cases the average of three or more determinations. The duration of this sequence was of 200 s for three measurements.

2.4.4. *Sequence 4: washing*

Once the measurement is carried out, the SAA moves to its waste position (W_2) and V rotates to the alternate position so that the diluent flows to W_2. Concomitantly, $TBSI_1$ is activated for 60 s to open tubing "a" and close "b" to allow a large volume of washing solution (300 μL) to meet the diluent at point T. In this way, the entire system is washed-out with surfactant solution. The duration of the washing sequence is of 200 s.

Finally, the autosampler capillary tubing moves to the washing solution vial and the valves to the positions indicated in sequence 3 and so the system is ready to start again the described operation.

3. Results and discussion

3.1. *Optimization of experimental variables*

Preliminary experiments carried out in the FI-ET AAS system described above but without the dilution coil incorporated, showed that the introduction of saliva samples on the graphite platform produced analyte signals well above the upper limit

Table 1 – Conditions for the determination of zinc in diluted saliva samples by FI-ET AAS

Spectrophotometer parameter	HGA-700 Zeeman				
	Value	Step	T (°C)	Ramp (s)	Hold (s)
Wavelength	213.9 nm	Drying 1	90	10	5
Bandwidth	0.7 nm	Drying 2	130	15	5
Lamp current	20 A	Pyrolysis	700	10	5
Reading time	5 s	Atomization	1700	1	4
		Clean	2200	1	3

of the linear calibration graph. The use of mineral acids, like analytical-grade nitric acid to digest and consequently to dilute the samples was avoided as high blank readings were recorded. Consequently, Milli-Q water as well as Triton X-100, frequently checked to be zinc-free, was used to dilute standards and saliva samples. In order to enhance the sample transport process and to avoid the effect caused on the dilution pattern by the difference in the viscosity of saliva samples and standards, Triton X-100 was preferred for further experiments. Its concentration was kept as low as it was possible (0.02%, v/v) that any surfactant foam was not formed in the dilution coil due to the flow movement.

The dilution factor will be determined by the range of zinc concentrations in the samples and by the linear working range of the instrument. In-batch dilution of 20 randomly selected samples indicated that a 20-fold dilution would bring their zinc concentration, which varied from 20 to 100 $\mu g\,L^{-1}$, within the working range of the instrument (0.5–6.0 $\mu g\,L^{-1}$). Based on these results, the target dilution factor for our FI on-line operation was selected to be 20. However, this dilution factor can be easily changed either by varying the length of the dilution coil [12] or the time pulses sent to the solenoid valves (see below) to control the sample volume introduced in the diluent stream. Additionally, the precision, expressed as relative standard deviation (R.S.D.%) of three manual dilutions applied to each of the 20 samples was between 3 and 5%. It was expected that the on-line dilution will improve the precision, will better control all FI experimental variables and will avoid contamination. In such a system, the dilution process is directly influenced by the sample injected volumes, the length of the manifold tubing and the solutions flow rates.

The timed pulses sent to $TBSI_1$ and $TBSI_2$ could vary, without overheating the solenoids, between 0.5 s and 1 min which correspond to 2.5–300 μL at a sample flow rate of 0.3 mL min^{-1}. In order to achieve the targeted dilution factor and a sample throughput reasonable for routine analysis, a compromise might be made between the volume of sample taken for dilution, the solutions flow rates and the length of the dilution coil. The dilution of samples might be also possible by using the sequential injection (SI) approach. However, in our case, the use of injection valves may be impractical as their dead volume is high, and on the other hand, time-based solenoid valves are not flexible enough to allow dilution of samples in SI systems.

As the entire manifold was constructed with PTFE tubing of the same internal diameter (0.8 mm), only the tubing length had to be optimized. There are several portions of the manifold tubing which might or might not affect the dilution and consequently the analytical results. First of all, there is the sample closed-flow circuit which only recirculated the sample from its respective vial through $TBSI_1$ at the constant PP flow rate of 0.3 mL min^{-1}. The only limitation for the dimensions of this tubing is the volume of the collected saliva samples, which in some cases was of only 0.5 mL. To ensure that this circuit is full of sample and to avoid the insertion of air bubbles from an empty vial, its length was of 75 cm, which is able to recirculate a maximum volume of 375 μL. Here we must emphasize one of the main advantages of the proposed method: a significant reduction of sample consumption, which is a very important point to be taken into account when most clinical samples are analyzed. The elasticity of the Neoprene tubing minimized deformation and allowed its use for extended periods with acceptable reproducibility (<2%). Secondly, the length of the diluent channel which meets the sample bolus introduced by $TBSI_1$ at point "T" has no influence on the overall performance of the manifold. The "T" junction is located at the exit of the injection valve, while the tubing length from V to W_2 was only enough to perform repeated introduction of the diluted sample on the graphite platform (25 cm). Experiments carried out with such a system without the dilution coil incorporated and using standards in the working range, showed that for a volume of 10 μL inserted into the system, only an eight-fold dilution was accomplished. This dilution effect was due to contributions from the tubing connecting $TBSI_1$ with the "T" junction and with the SAA. As the dilution factor required is known to be 20, the optimum length of the dilution coil was of 30 cm. To improve mixing, this tubing was coiled with parallel windings on a rigid plastic support ring to produce a 4 cm diameter coil.

The use of peristaltic pumps as propelling devises poses reproducibility problems due to the action of the rollers [14]. Therefore, poor precision was expected given the peristaltic pump pulsations. However, this was not found to be the case in this work as the precision obtained was around 1.5% in all cases. The use of small internal diameter peristaltic pump tubing (0.8 mm i.d.), their lubrication with silicone oil [12,14] and the low flow rates (0.3 mL min^{-1}), limited the influence from pump pulsations, which appeared to be dumped to an extent that did not affected the precision. This good precision favored the applicability of this system configuration to the determination of zinc in saliva samples after their on-line dilution. Also, to avoid entrainment of a small air segment in the closed flow circuit, the PP was stopped during the movement of the take-up capillary from the standard/sample vial to the washing container.

3.2. *FI-ET AAS performance*

Following the procedure described above and under the optimized conditions, a linear calibration curve for aqueous zinc standards was obtained over the range 8.0–120.0 $\mu g\,Zn\,L^{-1}$ (0.4–6.0 $\mu g\,Zn\,L^{-1}$ in undiluted solutions) with a correlation coefficient (r) of 0.9980 and fall off at higher concentrations. The concentration of zinc in all analyzed samples (22.0–98.0 $\mu g\,Zn\,L^{-1}$) was within, and well above the lowest limit of this range. In order to estimate the effect of protein-bound zinc in saliva, standard addition curves were constructed. The slope of the calibration graph of zinc added to saliva samples was not statistically distinguishable from that of zinc in aqueous standards, therefore aqueous standards calibration was chosen to estimate zinc in saliva samples. The limit of detection defined as three times the standard deviation (95% confidence level) of 10 replicate blank measurements was of 0.35 $\mu g\,L^{-1}$ which corresponds to 3.5 pg in the 10 μL of sample injected. On the other hand, the sensitivity, defined as the amount of zinc necessary to give an instrumental response of 0.0044 absorbance units was determined to be 4.8 pg of zinc. Precision, expressed as relative standard deviation (R.S.D.%) was determined by making replicate measurements of 10 on-line diluted saliva samples randomly selected. The mea-

Table 2 – Basic levels of zinc in saliva

Sample type	Zinc concentration ($\mu g\,L^{-1}$)				
	Mean ± S.D.	Range	Method	*n*	Reference
Parotid saliva	51 ± 14	27–82	ET AAS	34	[32]
	112 ± 48	NR	ET AAS	24	[33]
Whole saliva	80 ± 43	NR	ICP-MS	35	[5]
	36 ± 17	28–70	ET AAS	6	[9]
	102 ± 56	46–170	ICP-MS	5	[6]
	55 ± 17	22–98	FI-ET AAS	44	This work

S.D.: standard deviation; *n*: number of subjects; NR: not reported.

surements yielded, in all cases, a R.S.D. <3%. If higher values were obtained, a clear sign of contamination of one sample or a set of samples during the sampling procedure was suspected. To test the validity of the proposed procedure, recovery tests over the entire linear range were performed in triplicate by adding known quantities of zinc standards to previously analyzed saliva samples. The mean recovery of added zinc was 97.8 ± 1.3% and was never less than 95%. Additionally, as saliva reference standards with certified zinc levels are not available, Zn values estimated by the procedure developed in this work were compared with those obtained by a standard addition flame-AAS method applied to four pools of saliva samples each obtained from five randomly selected subjects. No significant differences ($p > 0.05$) were obtained between the two methods, with a linear regression equation $Y = 1.005X + 0.080$ ($r = 0.9970$), were X and Y are zinc concentrations determined by the proposed ET AAS method and by standard addition flame-AAS, respectively, which give faith of the accuracy of zinc determinations. However, in should be pointed out that by using the here proposed method a considerable saving of sample is achieved. In this case, the sample consumption was <0.4 mL per analysis in contrast with ca. 3 mL consumed by the AAS method (minimum 1 mL per measurement). Besides, the FI-ET AAS system reduced sample handling and allowed a complete automated analysis possible.

In order to obtain reasonable sample throughput, the sample volume introduced into the FI system should be small (10 μL) but reproducibly injected, the dilution coil as short as the achievable dilution factor allows and the carrier flow slow enough to achieve through mixing. Based on Fang et al. dilution principle [12], the theoretical sampling throughput obtained under our conditions is of $45\,h^{-1}$. However, as described in the procedure section, our system needs to be thoroughly washed and the closed flow circuit emptied of the previous sample, before starting a new cycle. Additionally, the interface of the FI manifold with ET AAS detection delayed the analytical signal due to its intrinsic discontinuous nature, thus the experimental sample throughput obtained was of only nine samples per hour for three measurements, with a diluent consumption of ca. 3 mL for each measurement.

3.3. *Analysis of real samples*

The concentration of zinc found in unstimulated saliva from 44 volunteers was of $55 \pm 17\,\mu g\,L^{-1}$ within the range 22–98 $\mu g\,L^{-1}$. The coefficient of variation of 30.9% indicates the scatter of individual values. It was found that zinc values for male (66 $\mu g\,L^{-1}$) were statistically higher ($p = 0.006$) than for female (50 $\mu g\,L^{-1}$). Their age (20–51 years), did not appears to have any influence on salivary zinc concentrations. Only few studies have reported zinc baseline values in saliva. Comparison with literature data shown in Table 2, indicate that our values may be considered to be within "normal" zinc levels or are similar to those zinc baseline levels determined by ICP-MS [5,6] or ET AAS [9,32,33], either in parotid [32,33], or whole stimulated [5] or unstimulated [6,9] saliva. Some differences may be attributed to different sampling procedures used by these authors and/or to the kind of saliva samples.

4. Conclusions

This work provides a useful and convenient procedure to decrease the sensitivity of ET AAS for zinc determination by performing precise on-line dilution of small volumes of saliva samples without reconfiguration of the flow manifold. The developed methodology enabled the determination of zinc in a complex organic matrix with the minimum samples and reagents consumption, thereby reducing the errors related to manual large dilutions and the risk of contamination, respectively. At the same time, the data reported in this study contribute to the assessment of baseline zinc levels in whole, unstimulated saliva, as the current literature reports are rather conflicting. Perhaps, different sampling procedures for whole or specific fractions of saliva, as well as not detected contamination problems could account for the difference in the literature results. An accurate standardization of the sampling procedures, as the one described in this study, is highly recommended in order to get a representative sample for analysis. Also, similar FI-ET AAS systems to that presented here could be adaptable for the analysis with appropriate direct reader-ICP and ICP-MS equipment, which may benefit laboratories devoted to the determination of different species in clinical samples, which are available in limited volumes, especially when additional determinations are required.

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