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www.elsevier.com/locate/talanta

PII: S0039-9140(18)30892-0
DOI: https://doi.org/10.1016/j.talanta.2018.08.081
Reference: TAL19002

To appear in: *Talanta*

Received date: 30 July 2018
Revised date: 27 August 2018
Accepted date: 28 August 2018

Cite this article as: Diogo La Rosa Novo, Julia Eisenhardt Mello, Filipe Soares Rondan, Alessandra Schneider Henn, Paola Azevedo Mello and Marcia Foster Mesko, Bromine and iodine determination in human saliva: challenges in the development of an accurate method, *Talanta,* https://doi.org/10.1016/j.talanta.2018.08.081

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Bromine and iodine determination in human saliva: challenges in the development of an accurate method

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ABSTRACT

In this work, an analytical method for bromine and iodine determination in human saliva was proposed. A simple protocol based on centrifugation and direct analysis of supernatant by inductively coupled plasma mass spectrometry (ICP-MS) was investigated. Although this method was feasible for bromine determination, iodine was partially present in the supernatant and an agreement about 54% with reference value was obtained. In addition, microwave-induced combustion (MIC) and microwave-assisted dissolution were also evaluated. Using MIC, 0.2 mL of saliva added on 300 mg of microcrystalline cellulose were efficiently digested. A diluted solution (50 mmol L^{-1} NH_4OH) was used for analytes absorption and a reflux step of 5 min was applied to ensure quantitative recoveries of Br and I. Accuracy was evaluated by analyte recovery experiments, and recoveries between 94% and 98% were obtained. Microwave-assisted

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dissolution was also evaluated for 2.0 mL of saliva using a diluted alkaline solution (25 mmol L^{-1} NH_4OH) and a microwave irradiation program of 35 min (including the cooling step). Results for this method agreed with those obtained using MIC method. Although MIC has also been appropriated for further determination of Br and I in saliva, microwave-assisted dissolution can be considered a simple sample preparation method and it was effective for high amount of sample (up to 2.0 mL). Moreover, final solutions were compatible with ICP-MS analysis, allowing the quantification of Br and I in human saliva at ultra-trace concentrations (limits of quantification were 0.052 μg mL^{-1} for Br and 0.022 μg mL^{-1} for I).

Keywords: Human saliva analysis; Biological samples analysis; Trace elements; Microwave-assisted dissolution; Microwave-induced combustion; Inductively coupled plasma mass spectrometry.

1. Introduction

Analysis of human saliva has been used to evaluate the presence of essential and potentially toxic elements at trace level in the body [1-6]. Saliva reflects the physiological conditions of the organism, once the salivary glands have a high blood flow. Saliva is composed of water, specific enzymes and a variety of inorganic and organic compounds, becoming an interesting tool for the diagnosis and prognosis of several diseases [1-6]. Currently, there is an increasing interest on bromine and iodine determination in biological samples, once they are responsible for important physiological functions. Bromine acts as a sedative-hypnotic and it has important functions in the formation of collagen IV and in the activation of α-amylase in saliva. Iodine is primarily involved in the synthesis of thyroid hormones [7-15]. However, depending on the concentration, they can be associated with several adverse effects. In

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this sense, bromine is associated with hematologic and thyroid diseases, growth retardation and insomnia, while I is associated with dysfunction and pathologies in the human body related to the thyroid gland [7-15]. Moreover, studies have suggested that Br and I determination in saliva is important because the salivary glands are strongly affected by these elements [10,16,17].

Bromine and I determination at low concentration is a challenge and just a few techniques may provide accurate results [18-22]. Inductively coupled plasma mass spectrometry (ICP-MS) is one of the most sensitive technique for ultra-trace elemental determination [19,20,22,23]. It has been applied for metals determination in saliva [3,24], but it has not been applied for Br and I determination in saliva. For ICP-MS analysis, sample preparation is crucial to avoid incorrect results [19,20,22,23]. Matrix effects are the main source of errors, highlighting interferences by isobaric ions, excitation, ionization, and among others that are closely related to the carbon content in solution and high acid concentration [19,20,22,23]. The presence of high amounts of carbon in solution is strongly related to interference for elements with high ionization potential, such as Br and I [19]. Moreover, accumulation of carbon in the equipment interface can also occur, impairing the determination and requiring additional maintenance [19,20]. Although some approaches have been used for interference suppression, the use of an efficient sample preparation method can be considered as a useful approach.

Typically, saliva is simply centrifuged or filtered prior to the analysis [10,15,25]. However, representativeness of Br and I concentrations in the supernatant or filtrate compared to whole saliva was not carefully evaluated. Moreover, a high dilution factor after centrifugation or filtration procedure is necessary to minimize the interferences in view of the saliva viscosity, increasing the limits of detection (LODs) that can be

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unsuitable for the analytical concern [10,15,25]. Digestion using concentrated acids, which is commonly used to convert complex solid or liquid samples in an aqueous solution free of interferences, is also not suitable for Br and I determination in saliva. The use of concentrated acids results in non-quantitative recoveries for Br and I due to halogens losses as volatile compounds, such as HX or X_2 (X = Br and I) [19,20,22,23]. Thus, trends in sample preparation methods such as microwave-induced combustion (MIC) and microwave-assisted alkaline extraction or dissolution may be excellent alternatives. These methods have been reported for subsequent Br and I determination in several matrices in view of their advantages [19-21, 26-29]. The use of alkaline solutions and closed vessels during sample preparation step minimizes the risk of element losses and increases the process efficiency [19-21, 26-29].

Thus, the aim of this research is to propose a suitable analytical method for the determination of Br and I in human saliva. Microwave-induced combustion and microwave-assisted alkaline dissolution were evaluated as sample preparation method and the analytes were determined by ICP-MS. Several parameters were optimized such as the volume of saliva, the microwave irradiation program and the most suitable solution to absorb the analytes or to dissolve the sample allowing adequate analytes recoveries. Supernatant of saliva was analyzed after centrifugation to compare the results. Accuracy was evaluated by recovery tests and by comparison of the results among all methods. Finally, the proposed method was applied for the determination of Br and I in saliva of some volunteers to demonstrate its applicability. It is important to mention that this study does not focus on the evaluation of the clinical status of the volunteers and it is simply devoted to the development of the analytical method.

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2. Experimental

2.1. Instrumentation

Microwave-induced combustion and microwave-assisted alkaline dissolution were performed in a microwave oven (Multiwave 3000®, Microwave Sample Preparation System, Anton Paar, Austria). For MIC, the system was equipped with eight high-pressure quartz vessels, with an internal volume of 80 mL, and homemade quartz sample holders that were used to support the sample inside the quartz vessels [26,27]. The temperature and pressure of this process were limited to 280 ºC and 80 bar, respectively, as recommended by the manufacturer [30]. For microwave-assisted alkaline dissolution, eight chemically modified polytetrafluoroethylene (PTFE-TFM) vessels with an internal volume of 100 mL were used. The temperature and pressure of this process were limited to 260 ºC and 60 bar, respectively, as recommended by the manufacturer [30]. The maximum operational temperature and pressure were real-time monitored during the procedures using sensors available in the microwave oven. A pHmeter (MPA-210, Tecnopon, Brazil) equipped with a glass combined electrode was used to determine the pH of the digests. A centrifuge (80-2b centrifuge, Daiki, Seoul) was used to perform the centrifugation process.

Bromine and I determination was carried out using an inductively coupled plasma mass spectrometer (NexION 300X, Perkin-Elmer, Canada), equipped with a concentric nebulizer (Meinhard Associates, USA), a cyclonic spray chamber (Glass Expansion Inc., Australia), and a quartz torch with a quartz injector tube (2 mm i.d.). Carbon content in solution was determined using an inductively coupled plasma optical emission spectrometer (Spectro Ciros CCD, Spectro Analytical Instruments, Germany) with axial view configuration. A crossflow nebulizer coupled to a Scott double pass type nebulization chamber was used. In order to remove the volatile carbon compounds

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before carbon determination, samples aliquots were bubbled with an inert gas (Ar, 0.1 L min^{-1}) for 2 min [31,32]. The parameters for the determination of Br and I by ICP-MS and C by ICP OES are shown in Table 1.

2.2. Reagents and solutions

All solutions and sample dilutions were prepared using ultrapure water (resistivity of 18.3 MΩ cm) obtained from a purification system (Mega UP, MegaPurity, South Korea) and all chemicals used in this study were of analytical grade. The cleaning of vessels and holders was carried out with 6 mL of 14.4 mol L^{-1} HNO_3 (Vetec, Brazil) using a microwave heating program set at 1000 W for 10 min and 0 W for 20 min (cooling step). After cleaning with HNO_3, the procedure was repeated using 6 mL of ultrapure water to decrease the blank values and eliminate the traces of acid.

Ammonium hydroxide and tetramethylammonium hydroxide (TMAH) solutions used as absorbing solution for MIC and for microwave-assisted alkaline dissolution, were prepared from 25% NH_4OH (Merck, Germany) and 25% TMAH in water (Sigma-Aldrich, USA). Ammonium nitrate solution (6 mol L^{-1}) was used as an igniter for MIC and it was prepared by dissolution of solid reagent (Merck) in water. Oxygen with a purity of 99.5% (Linde, Brazil) was used for the pressurization of quartz vessels in the MIC method. Small discs of filter paper (15 mm in diameter, 12 mg) with low ash content (Black Ribbon Ashless, Schleicher and Schuell GmbH, Germany) were used as combustion aid for MIC. Paper discs were previously cleaned with 20% (v/v) ethanol solution for 20 min in an ultrasonic bath (USC-1800 A, 40 kHz, 155 W, Unique, Brazil), subsequently rinsed with ultrapure water, and dried in a class 100 laminar bench (CSLH-12, Veco, Brazil). This same procedure was applied to the polyethylene films (Congelito, Brazil) used to wrap the samples for digestion by MIC.

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Stock solutions (100 mg L^{-1}) for Br and I were obtained by dissolution of KBr and KI salts (Merck) in water and they were used to prepare the calibration curves for ICP-MS analysis. These same solutions were also used in the recovery experiments for the evaluation of the absorbing solutions in the MIC method and for the accuracy evaluation of microwave-assisted alkaline dissolution method. For Br and I determination by ICP-MS, a calibration curve containing five standard solutions was prepared in 10 mmol L^{-1} NH_4OH, and the concentrations ranged from 1.0 to 10 μg L^{-1} for Br and from 0.1 to 1.0 μg L^{-1} for I. For the determination of carbon content in solution by inductively coupled plasma optical emission spectrometry (ICP OES), the external standard calibration curve (25 to 100 mg L^{-1} of C) was prepared from the dissolution of citric acid (Merck) in 5% v/v HNO_3 (Merck). In addition, an yttrium solution (1 mg L^{-1}, SpexCertPrep, USA) was used as internal standard for carbon determination [31,32]. Argon with a purity of 99.998% (White Martins, Brazil) was used for plasma generation and nebulization.

2.3. Sample collection

Saliva of five volunteers was collected, mixed and homogenized to obtain a representative sample for method optimization (total volume of 200 mL). For applicability of the proposed method, saliva of six women volunteers from Rio Grande do Sul, Brazil, were analyzed. Women were chosen because the occurrence of thyroid dysfunctions is more frequent than in men [33]. Samples named as A and B are from two hypothyroidism patients in treatment: age, 26 ± 2 years; weight, 60 ± 6 kg; height, 165 ± 3 cm; whereas samples named as C and D are from two type one diabetic patients in treatment: age, 26 ± 2 years; weight, 65 ± 5 kg; height, 170 ± 4 cm, and samples named as E and F are from two volunteers considered healthy: age, 28 ± 4 years;

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weight, 65 ± 3 kg; height, 167 ± 2 cm. Samples were collected early morning and the volunteers are in fasting and washed the oral cavity three times with ultrapure water. The samples were stored under refrigeration (-6 ºC) prior to the analysis. All experiments were conducted as approved by Research Ethics Committee of the Federal University of Pelotas (opinion number: 2.251.932).

2.4. Microwave-induced combustion

Some parameters such as the volume of saliva, the mass of combustion aid (microcrystalline cellulose) and the type and concentration of absorbing solution were optimized. Saliva has a high content of water, which limits its combustion. Thus, microcrystalline cellulose was used as combustion aid and it was selected based on studies reported in the literature [34,35]. Saliva samples (0.05 to 0.3 mL) were added to microcrystalline cellulose (100 to 500 mg) and wrapped with a polyethylene (PE) film (8×8 cm) as small packets sealed by heating, and the excess of the PE film was removed. The packets containing sample and microcrystalline cellulose were placed on a small disc of filter paper on the quartz holder. Ammonium nitrate solution (6 mol L^{-1}, 50 µL) was immediately added to the paper. Then, holders with the samples were placed into the quartz vessels, containing 6 mL of absorbing solution. The vessels were closed, fixed on the rotor, and pressurized with 20 bar of oxygen. The rotor with the vessels was placed inside of the microwave oven, and the irradiation program was started. The microwave irradiation program was: *i*) 5 min at 1400 W (ignition and reflux steps) and *ii*) 20 min at 0 W (cooling step). Water and 50 or 100 mmol L^{-1} NH_4OH were evaluated as absorbing solution. Microwave irradiation program, absorbing solutions, volume of NH_4NO_3 solution, and oxygen pressure were selected based on studies reported in the literature [19,20,26,35]. After combustion, the pressure of each vessel

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was released and resultant solutions were transferred to a volumetric flask and diluted with water up to 20 mL for further Br and I determination by ICP-MS. For evaluation of the digestion effiency carbon content in solution was determined by ICP OES.

Accuracy of the MIC method was evaluated by recovery tests by adding 20 or 30 μL of a standard solution containing 15 μg mL^{-1} of Br and 1.0 μg mL^{-1} of I on the sample (saliva and microcrystalline cellulose) before closing the PE film. Moreover, the results obtained after MIC were compared with those obtained after microwave-assisted alkaline dissolution and after a centrifugation procedure for determination of Br and I in saliva by ICP-MS.

2.5. Microwave-assisted alkaline dissolution

Saliva samples (0.5 to 2.0 mL) were transferred to PTFE-TFM vessels, and 6 mL of alkaline solution (25, 50 or 100 mmol L^{-1} NH_4OH or 110 mmol L^{-1} TMAH) were added. Water was also evaluated for comparison. These solutions were selected based on works reported in the literature for determination of Br and I in several matrices [28,29]. Microwave irradiation program was as follows: *i*) 1000 W for 5 or 10 min (ramp), *ii*) 1000 W for 3, 5, 10, 20 or 50 min, and *iii*) 0 W for 20 min (cooling step). When TMAH was used, the maximum temperature was limited at 90 °C to avoid its degradation [29]. This procedure was performed according to studies reported in the literature using a high-pressure microwave-assisted alkaline extraction or dissolution methods [28,29]. The final solutions were then transferred to volumetric flasks and diluted with ultrapure water up to 20 mL. The determination of Br and I was performed by ICP-MS and the carbon content in solution was determined by ICP OES. Accuracy was evaluated by recovery tests by adding 200 or 300 μL of a standard solution

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containing 15 μg mL^{-1} of Br and 1.0 μg mL^{-1} of I on the saliva before sample preparation, and by comparison of results with those obtained after MIC method.

2.6. Analysis of saliva after simple centrifugation and dilution

The centrifugation of saliva prior to the determination step was performed as reported in the literature [10, 15, 25]. Initially, 5 mL of saliva were centrifuged at 3500 rpm for 5 min in polyethylene flasks. Then, 1 mL of the supernatant was collected for analysis and the dilution factor necessary to obtain a more compatible solution with ICP-MS analysis was evaluated. Carbon content in solution was determined by ICP OES and accuracy was evaluated by comparison of the results with those obtained after MIC and microwave-assisted alkaline dissolution.

All the results were statistically evaluated by Student's *t*-test (confidence level of 95%, $P < 0.05$) using the GraphPad InStat version 3.00 computer software package (GraphPad, USA). Limits of detection and quantification (LOQ) were calculated from the mean of the blank values plus three times (for LOD) or ten times (for LOQ) the standard deviation for ten replicates. The sample volume, final volume and dilution factor were also taken into account.

3. Results and discussion

3.1. Microwave-induced combustion for sample preparation of saliva and determination of bromine and iodine by ICP-MS

High water content in saliva limits the combustion process and a combustion aid was necessary for saliva digestion by MIC. Microcrystalline cellulose was used as combustion aid in order to digest a high volume of saliva and improve the LODs. Initially, the maximum volume of saliva that could be digested by MIC, using 100 mg

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of microcrystalline cellulose, was evaluated. As result, only 0.05 mL of saliva was efficiently digested. It occurred because the higher volumes of saliva made the microcrystalline cellulose wet and not feasible for combustion. Aiming an efficient digestion of a higher volume of saliva, a higher mass of combustion aid was evaluated. In this evaluation, volumes of saliva (0.1 to 0.3 mL) were added on different masses of microcrystalline cellulose (200 to 500 mg). The volume of 0.1 mL of saliva was efficiently digested added on 200 mg of microcrystalline cellulose, while 0.2 mL of saliva was efficiently digested only if added on 300 mg of microcrystalline cellulose. The combustion of 0.3 mL of saliva added on 300 mg of microcrystalline cellulose was not efficient.

An additional study was performed aiming to digest 0.3 mL of saliva added on 400 or 500 mg of microcrystalline cellulose. However, an inefficient combustion with the presence of soot in the absorbing solution was observed in all evaluations. A drying step of saliva mixed with microcrystalline cellulose prior to the combustion was not evaluated, once it would increase sample preparation time and contamination, and it may cause analyte losses by volatilization. Thus, 0.2 mL of saliva added on 300 mg of microcrystalline cellulose was the condition chosen for further optimization. It is important to mention that this is the first study that evaluate the use of the MIC for digestion of a sample with a high water content, such as saliva.

After the volume of saliva and the mass of combustion aid have been chosen, ultrapure water and 50 or 100 mmol L^{-1} NH_4OH solutions were evaluated for Br and I absorption. Recovery tests were performed for the all absorbing solutions by adding 1.5 µg of Br and of 0.10 µg of I per mL of saliva, prior to digestion by MIC. These concentrations corresponded to about 50% of the concentration of Br and I present in the initially studied sample (2.94 ± 0.12 µg mL^{-1} for Br and 0.178 ± 0.008 µg mL^{-1}

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for I). Figure 1 shows the results for Br and I obtained when the recovery test was performed.

As shown in Fig. 1, the recoveries for Br were between 94% and 97% for all evaluated solutions and no significant difference ($P < 0.05$) was observed between the results. Relative standard deviations (RSDs) ranged from 3% to 6%. However, for I, significant difference ($P < 0.05$) was observed between the results obtained using ultrapure water and those obtained using 50 or 100 mmol L^{-1} NH_4OH solutions. When ultrapure water was used, the recovery for I was about 76% and this lower recovery can be related to the pH of the final digests, which ranged from 2 to 3. These pH values may have caused analyte losses by volatilization [19-21,26]. On the other hand, when 50 or 100 mmol L^{-1} NH_4OH were used for analytes absorption, the recovery for I was around 97%. Using those solution, the RSDs for I measurements were lower 3%, and the pH value after digestion was 7.

Additionally, a recovery test containing a higher concentration of the analytes (corresponding to 75% of the analytes concentration in the sample) was carried out in the selected conditions. This recovery experiment was performed by adding of 2.3 µg of Br and of 0.15 µg of I per mL of saliva on the sample, prior to digestion by MIC. Recoveries for both analytes ranged from 96% to 98%, and the RSDs were lower than 4%. Thus, 50 mmol L^{-1} NH_4OH was considered suitable to absorb Br and I after combustion of saliva. It is considered a diluted solution, which minimizes reagent consumption and waste generation, provides more security for the analyst and presents a great compatibility with different determination techniques, including ICP-MS. Carbon content in solution obtained by MIC was lower than 5 mg L^{-1} and interferences related to matrix compounds during the determination of Br and I by ICP-MS were not

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observed. Thus, the MIC method was considered suitable for further Br and I determination in saliva by ICP-MS.

3.2. Microwave-assisted alkaline dissolution for sample preparation of saliva and determination of bromine and iodine by ICP-MS

Solutions of 110 mmol L^{-1} TMAH or 25, 50 and 100 mmol L^{-1} of NH_4OH or ultrapure water were evaluated for dissolution of saliva and subsequent determination of Br and I by ICP-MS. During this evaluation, 0.5 mL of saliva and a microwave irradiation program of *i*) 1000 W for 10 min (ramp), *ii*) 1000 W for 50 min, and *iii*) 0 W for 20 min were used based on works reported in the literature [28,29]. Bromine and I concentrations in saliva obtained using different solutions were compared with those results obtained after MIC method. Results are shown in Table 2.

As shown in Table 2, no significant difference ($P < 0.05$) was observed between Br and I concentrations in saliva using microwave-assisted alkaline dissolution with different solutions. All results agreed with values obtained using MIC. Ultrapure water was also evaluated for comparison, however, the RSDs were higher (≤ 12%) than those using alkaline solutions (≤ 6%). Thus, 25 mmol L^{-1} NH_4OH solution was chosen for further evaluations once it was the most diluted alkaline solution and presented accurate results and with lower RSDs (≤ 6%). Furthermore, the pH of the final solution using 25 mmol L^{-1} NH_4OH was about 7, which ensures the stability of the analytes. The maximum volume of saliva (0.5, 1.0 or 2.0 mL) that could be used in the microwave-assisted alkaline dissolution method was also evaluated aiming to improve the LODs. As result, no significant difference ($P < 0.05$) was observed between Br and I concentrations by using different volumes of saliva in comparison with results obtained after MIC. Thus, 2 mL of saliva were chosen for further optimization. Higher volumes

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of saliva were not evaluated once the collection of a high amount of saliva is difficult for volunteers.

An additional study, in order to reduce the sample preparation time, modifying the microwave irradiation program was performed. In the initial optimizations, a ramp time of 10 min to 1000 W was used, to avoid any sudden increase in the pressure. However, no sudden increase in the pressure was observed when preparing saliva, and the ramp time was reduced to 5 min. In addition, the hold time at 1000 W was also evaluated (3, 5, 10, 20 or 50 min). The aspect of solutions after different microwave irradiation times and the aspect of the solution obtained after MIC method are shown in Fig. 2.

As can be observed in Fig. 2, the solution obtained after microwave-assisted alkaline dissolution using 3 and 5 min of hold time at 1000 W presented similar aspect, with high viscosity and foam, as represented in Fig. 2 A. On the other hand, clean solutions with low viscosity and without foam were obtained using 10, 20 or 50 min of hold time at 1000 W, and the aspect observed for all solutions were similar, as presented in Fig. 2 B. Moreover, they present similar aspect to that observed after MIC (Fig. 2 C). The aspects of solutions using different microwave irradiation program are related to the temperature reached during the dissolution. The maximum temperature reached using 3 and 5 min of hold time at 1000 W was 140 ºC, while the maximum temperature using 10, 20 or 50 min of hold time at 1000 W was 230 ºC. At these higher temperature, probably, degradation of some compounds of saliva matrix occurred. Bromine and I concentrations in the solutions obtained using 10, 20 or 50 min of hold time at 1000 W were determined, and no significant difference ($P < 0.05$) was observed between these results and those obtained after MIC. Relative standard deviations were always lower than 4%. Thus, the microwave-irradiation program selected was *i*) 1000 W for 5 min

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(ramp), *ii*) 1000 W for 10 min, and *iii*) 0 W for 20 min (cooling step). The pH of the final solutions was around 7 and the carbon content in solution was about 3000 mg L^{-1}. A dilution factor of around ten times was always performed to eliminate carbon interferences during the determination of Br and I by ICP-MS [36].

Accuracy of microwave-assisted alkaline dissolution method was also evaluated by analyte recovery experiments adding 1.5 and 2.3 µg of Br and of 0.10 and 0.15 µg of I per mL of saliva, prior to dissolution using the selected conditions. These additions correspond to about 50% and 75% of the concentrations of Br and I in the sample, which were previously determined by ICP-MS using this method and the optimized conditions. Recoveries for both analytes ranged from 97% to 100%, and the RSDs were lower than 5%. Thus, the microwave-assisted alkaline dissolution method, as well as the MIC method, were considered suitable for further Br and I determination in saliva by ICP-MS.

3.3. Determination of bromine and iodine in saliva by ICP-MS after centrifugation and dilution

A simple centrifugation and dilution procedure was evaluated for the determination of Br and I in saliva by ICP-MS. This procedure has been performed to remove cellular debris, mucin, and other large molecules and particles of saliva, which may interfere during the analysis by ICP-MS [1,19]. However, after centrifugation, a solution with high viscosity and with significant carbon content (1000 mg L^{-1}) was obtained. In order to minimize the occurrence of interferences due to sample transport to the ICP-MS equipment, a dilution factor of at least thirty times was necessary. The saliva supernatant was diluted and then analyzed by ICP-MS. The concentration of Br (3.03 ± 0.13 µg mL^{-1}) in saliva supernatant agreed ($P < 0.05$) with the value obtained after the

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MIC method (Br: 2.94 ± 0.12 μg mL^{-1}). However, the concentration of I (0.097 ± 0.005 μg mL^{-1}) in the saliva supernatant was not in agreement ($P > 0.05$) with the value obtained after MIC (I: 0.178 ± 0.008 μg mL^{-1}). Iodine may have remained bound to some enzyme or/and protein in the precipitate. These results indicate that the concentration of I in supernatant of saliva was 45% lower than in whole saliva. Thus, considering this results, it is possible to obtain underestimated results and make wrong interpretations about the total concentration of I in saliva using this sample preparation method.

Reports in the literature emphasize that the concentration of some elements such as Si, Ti, Mn Ag, Al, Cu, Fe, Mg, and Zn in the supernatant of saliva are lower when compared to those in the whole saliva [1]. The authors mention that in some cases, saliva should be mineralized for determination of some chemical elements because the results in the supernatant of saliva are not representative for whole sample [1]. Thus, in the present study is demonstrated, for the first time, that the concentration of I in supernatant of saliva is lower than in whole saliva. Only a centrifugation procedure was not considered as suitable for further simultaneous quantitative determination of Br and I in saliva.

3.4. Comparison of sample preparation methods for determination of bromine and iodine in saliva by ICP-MS

Microwave-induced combustion and microwave-assisted alkaline dissolution were considered suitable for determination of Br and I in saliva by ICP-MS. The blank values using MIC (1.5 μg L^{-1} for Br and 0.38 μg L^{-1} for I) were higher than those using microwave-assisted alkaline dissolution (0.20 μg L^{-1} for Br and 0.07 μg L^{-1} for I) and this is probably due to microcrystalline cellulose used as combustion aid in the MIC

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method. On the other hand, the carbon content in solution after MIC (5 mg L^{-1}) were lower than those obtained after microwave-assisted alkaline dissolution (around 3000 mg L^{-1}). In the MIC method, the organic matter of sample is completely digested and interferences related to matrix are minimized. A systematic study was carried out aiming to evaluate the lowest carbon concentration for Br and I determination by ICP-MS free of interferences. In the presence of a carbon concentration up to 400 mg L^{-1}, no matrix effects were observed for $^{79}Br^{+}$ and $^{127}I^{+}$. Thus, a dilution factor of around ten times after microwave-assisted alkaline dissolution was necessary to avoid interferences during Br and I determination by ICP-MS, while after MIC an additional dilution factor was not necessary. Using both methods, clear solutions with low viscosity and without foam were always obtained and interferences due to sample transport to the ICP-MS equipment was not observed.

Although both sample preparation methods are appropriate for the determination of Br and I in saliva, using microwave-assisted alkaline dissolution it was possible to use higher amount of sample (2.0 mL) in comparison to MIC (0.2 mL). Thus, the LODs using microwave-assisted alkaline dissolution (0.03 μg mL^{-1} for Br and 0.01 μg mL^{-1} for I) were lower than those using MIC (0.233 μg mL^{-1} for Br and 0.053 μg mL^{-1} for I). The LOQs using microwave-assisted alkaline dissolution were 0.052 μg mL^{-1} for Br and 0.022 μg mL^{-1} for I, while using MIC they were 0.409 μg mL^{-1} for Br and 0.090 μg mL^{-1} for I. Thus, the LODs obtained by microwave-assisted alkaline dissolution were about eight and five times better for Br and I, respectively, when compared with the LODs obtained using the MIC.

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3.5. Determination of bromine and iodine in saliva by ICP-MS after microwave-induced combustion and microwave-assisted dissolution as sample preparation methods

Based on the results previously discussed, samples of six volunteers were prepared by both methods for further Br and I determination by ICP-MS and the results are shown in Table 3.

As can be observed in Table 3, no significant difference ($P < 0.05$) was observed between the results for Br and I in saliva using MIC and microwave-assisted alkaline dissolution. Although the RSDs during the optimization of MIC method were lower than 6%, in the applicability of MIC method for different saliva the RSD were up to 14%. This is probably associated with the lack of homogeneity of some sample. However, the RSDs for both analytes using microwave-assisted alkaline dissolution were always lower than 6%. This is probably because in the microwave-assisted alkaline dissolution, it is possible to prepare higher volume of saliva when compared to MIC (2.0 mL of sample in microwave-assisted dissolution and 0.2 mL of sample in MIC).

The concentration of Br and I in saliva samples presented a wide range (1.20 to 5.14 μg mL^{-1} and 0.074 to 0.250 μg mL^{-1} for Br and I, respectively). This may be associated with different habits or environmental exposure. Bromine concentration was always higher than I concentration in all saliva samples analyzed in this work. This should be highlighted because Br can reduce I absorption in the thyroid gland [13]. It is important to mention that the evaluation of the clinical status of the volunteers was not the focus of this study, but the high concentration range for Br and I in human saliva deserves attention. Thus, these results only indicate that Br and I concentration in saliva should be carefully investigated, and that the proposed method is an excellent

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alternative for this purpose, considering that it was possible to determine the analytes in a wide range.

4. Conclusion

Microwave-induced combustion and microwave-assisted alkaline dissolution methods were suitable for sample preparation of saliva and subsequent Br and I determination by ICP-MS. Quantitative recoveries for Br and I were obtained using a diluted solution for analytes absorption, in MIC, or by dissolution of sample. Only a simple centrifugation and dilution procedure was not suitable for quantitative simultaneous determination of Br and I by ICP-MS, because the concentration of I in supernatant of saliva was around 45% lower than in whole saliva. In addition, microwave-assisted alkaline dissolution showed to be efficient for sample preparation of a relatively high volume of saliva (up to 2.0 mL) under safe conditions, allowing the quantification of low Br and I content in saliva samples (LOQs were 0.052 μg mL^{-1} for Br and 0.022 μg mL^{-1} for I), useful for routine analysis. The proposed analytical methods are powerful tools to determine Br and I at ultra-trace concentration in human saliva. They could assist different research fields to better understand several diseases, which involve variations in Br and I concentration in human organism, such as hyperthyroidism and hypothyroidism.

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Acknowledgements

The authors are grateful to CAPES, CNPq and FAPERGS from Brazil for supporting this study.

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Fig. 1. Bromine and I recoveries in human saliva using ultrapure water and 50 or 100 mmol L^{-1} NH_4OH solutions after digestion by MIC and determination by ICP-MS (n = 3).

Fig. 2. Aspect of solutions obtained after microwave-assisted alkaline dissolution of 2 mL of saliva in 25 mmol L^{-1} NH_4OH. Microwave irradiation programs using A) 3 or 5 min, B) 10, 20 or 50 min of hold time at 1000 W (ramp at 1000 W for 5 min) and C) solution obtained after MIC method using 0.2 mL of saliva added on 300 mg of microcrystalline cellulose and 50 mmol L^{-1} NH_4OH as absorbing solution.

Table 1. Operational parameters for Br and I determination by ICP-MS and C by ICP OES.

Parameter	ICP-MS	ICP OES
RF power (W)	1300	1400
Plasma gas flow rate (L min^{-1})	18.0	14.0
Auxiliary gas flow rate (L min^{-1})	1.20	1.00
Nebulizer gas flow rate (L min^{-1})	0.95	1.00
Spray chamber	Cyclonic	Scott type, double path

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Nebulizer	Concentric	Crossflow
Sampler and skimmer cones	Pt	-
Ion lens	Auto lens "on"	-
Observation view	-	Axial
Isotope (m/z)	^{79}Br and ^{127}I	-
Emission line (nm)	-	C (193.091)

Table 2. Bromine and I concentration in saliva by ICP-MS after microwave-assisted alkaline dissolution using different solutions (mean ± standard deviation, n = 3).

Solution	Concentration (μg mL^{-1})	
	Br	I
110 mmol L^{-1} TMAH	3.24 ± 0.14	0.188 ± 0.010
25 mmol L^{-1} NH_4OH	3.19 ± 0.18	0.185 ± 0.008
50 mmol L^{-1} NH_4OH	3.19 ± 0.12	0.187 ± 0.008
100 mmol L^{-1} NH_4OH	3.04 ± 0.14	0.178 ± 0.011
Ultrapure water	2.90 ± 0.34	0.170 ± 0.025
Results using MIC *	2.94 ± 0.12	0.178 ± 0.008

Table 3. Results for Br and I determination in saliva by ICP-MS after microwave-assisted alkaline dissolution and MIC (mean ± standard deviation, μg mL^{-1}, n =3).

Sample	Microwave-assisted alkaline dissolution		MIC	
	Br	I	Br	I
A	2.60 ± 0.07	0.120 ± 0.003	2.51 ± 0.15	0.132 ± 0.011
B	1.20 ± 0.04	0.074 ± 0.002	1.09 ± 0.09	0.069 ± 0.006
C	2.17 ± 0.02	0.180 ± 0.004	2.27 ± 0.08	0.202 ± 0.020

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D	5.14 ± 0.22	0.250 ± 0.010	4.81 ± 0.32	0.272 ± 0.016
E	4.21 ± 0.19	0.121 ± 0.007	3.60 ± 0.50	0.105 ± 0.014
F	1.82 ± 0.10	0.182 ± 0.004	1.63 ± 0.20	0.192 ± 0.008

Highlights:

- Bromine and iodine can be determined at ultra-trace concentration in human saliva.
- Proposed analytical methods are accurate for Br and I determination in human saliva.
- Drawbacks of analytical methods reported in the literature were overcome.
- Proposed analytical methods can be used in different research fields.

Fig. 1.

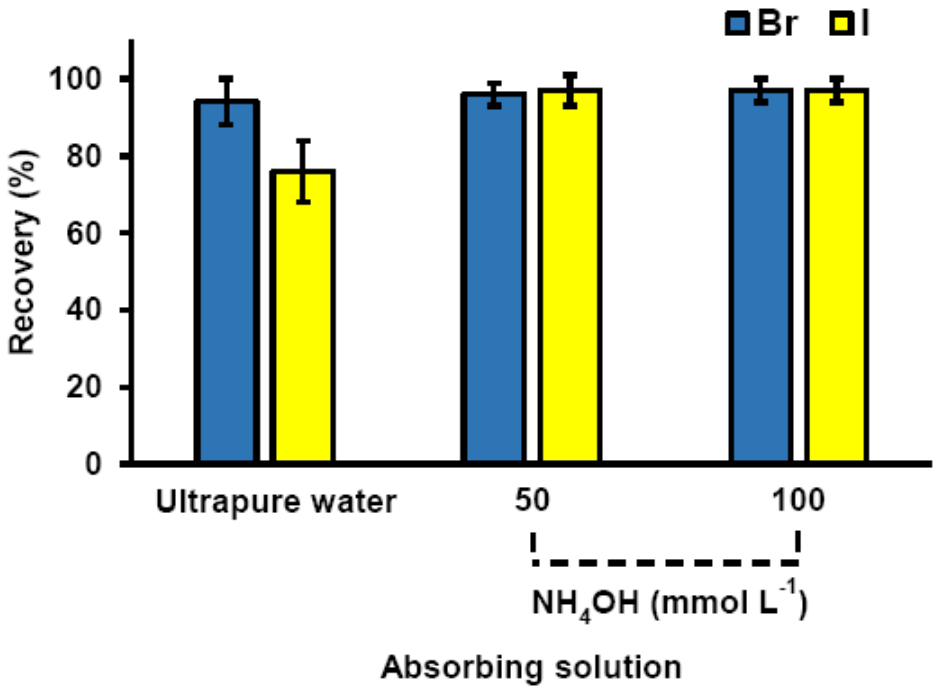
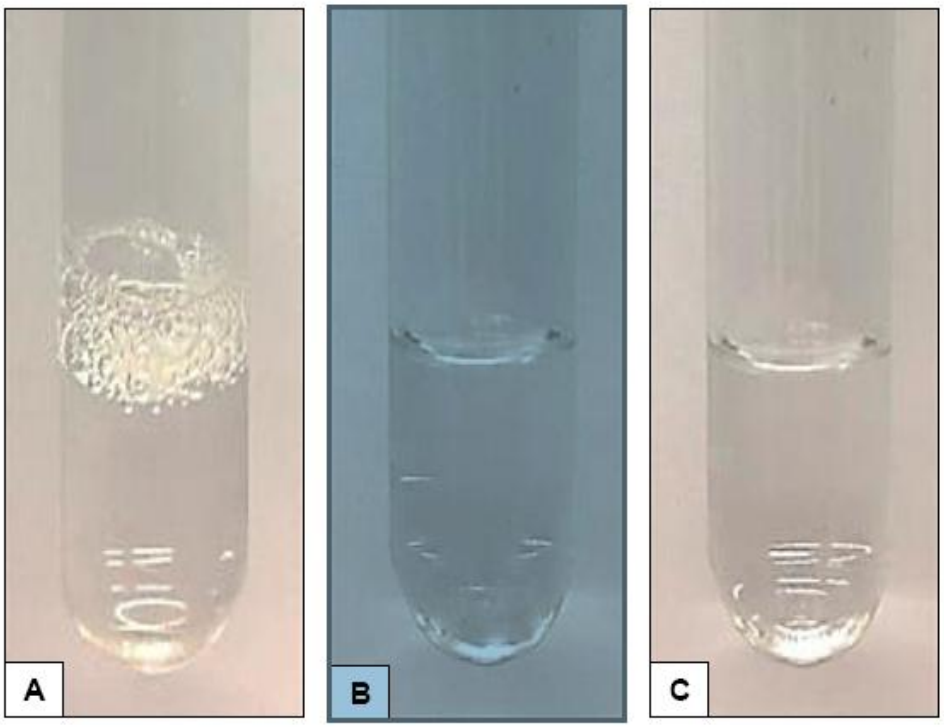
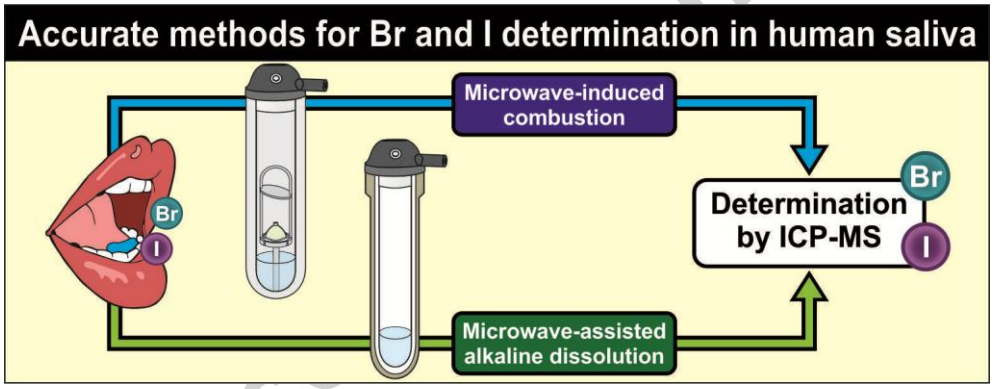


Fig. 2.





Graphical abstract