



## Turbidimetric and photometric determination of total tannins in tea using a micro-flow-batch analyzer

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### ABSTRACT

Both turbidimetric and photometric determinations of total tannins in samples of green and black tea, using a micro-flow-batch analyzer ( $\mu$ FBA) were studied. The miniaturized system was formed using photocurable urethane-acrylate resin and ultraviolet lithography technique. The turbidimetric method was based on the precipitation reaction of Cu (II) with tannins in acetate medium at a pH of 4.5. The photometric method was based on the complexation reaction of tannins with ferrous tartrate. The turbidimetric  $\mu$ FBA was able to test 200 samples per hour. The photometric  $\mu$ FBA allowed 300 analyses per hour, generating 136  $\mu$ L of residue per analysis. The paired *t* test, at a 95% confidence level, showed no statistically significant differences between results obtained by both methods and the reference method. The urethane-acrylate  $\mu$ FBA maintained satisfactory physical and chemical properties, and represents an improvement over conventional flow-batch analyzer.

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### 1. Introduction

Tannins are a polyphenol group with high molecular weights. They are astringent and have the natural ability to complex with various macromolecules such as proteins, starch, cellulose, as well as minerals [1]. Tannins in general have antioxidant, anticarcinogenic, antimutagenic and antimicrobial activities and can assist in the prevention and treatment of diseases [2,3]. Tannins are found in common foods and drinks, but especially in green and black tea, which are consumed worldwide [4,5]. Green tea is derived from the infusion of twigs and dried leaves of *Camellia sinensis* [6], while black tea is obtained by oxidation of the same plant [7]. Quality control is of course important for the world's tea consumers, but the quantification of tannins has always been of particular interest to the industry, as well as governmental regulators [8,9].

Determination of tannins in tea samples is carried out regularly in food laboratories around the world [10]. The Folin–Ciocalteu spectrophotometric method is currently the official method for

the determination of tannins in samples from plant tissues by the AOAC (Association of Official Analytical Chemists) [11]. However, this method is a relatively slow process, both in reagent preparation, and in product formation with tannins, it is also susceptible to interference from any reductive species present in the sample [10,12].

The official method in Japan (Official Chemical Analysis of Tea) uses the ferrous tartrate method as the reference method for determination of tannins in tea samples [13]. This method is based on the formation, in a buffered medium (pH 6.8) of a complex between ferrous tartrate and tannins in the tea, which absorbs light at around 560 nm. The ferrous tartrate method is more selective than the Folin–Ciocalteu method, and is unaffected by the coexistence of reducing agents, such as ascorbic acid [14]. It is being used by researchers for the determination of tannins in tea [15–18].

Another alternative for the determination of tannins in tea is the turbidimetric method [10]. This method consists in the precipitation reaction of Cu (II) with tannins in acetate medium at a pH of 4.5 monitored at around 470 nm [19]. This precipitation is due to the presence of functional groups in tannins, such as ortho-dihydroxyphenyl and carboxyl groups, that act as mono- or bidentate ligands [20].

Methods automation for analysis of tannins allows: faster controlled analysis, greater precision, less analyst interference, and a

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reduction of expenditures on samples and reagents. Several studies have been reported in the literature using automated flow systems for analysis of tannins in tea samples using both the turbidimetric [10,19] and ferrous tartrate [14–18] methods. But it would be interesting to have an automatic system capable of allowing the analyst to choose, depending on the reagents available in the laboratory, determine tannins using the same manifold and selecting one of these methods via software. This can be achieved by developing an automatized flow-batch analyzer.

The flow-batch analyzers are automated systems that use an instantaneous stop chamber and integrate batch and flow methods by using programmed multi-commutation [21]. The main component is the mixing chamber where the whole analytical process including: fluids addition, sample pretreatment, homogenization, precipitation, extraction, preparation of calibration solutions, and detection, takes place under the total control of the software [22–24]. The sample is processed seamlessly with less manipulation, less consumption of reagents and samples, less chemical waste, and less chance for human error. Classical methods (discrete) can be performed with precision, accuracy and speed similar to other flow analysis methods [25].

The flow-batch systems flexible and versatile have been developed for different applications such as: titration [26], screening analysis [27], standard addition [28] chemiluminescence [21], nephelometric [29] and liquid–liquid extractions [30], for example.

Recently, the flow-batch analyzer was miniaturized ( $\mu$ FBA) and applied analysis of Fe (II) ions in medicinal samples [31]. The microfabrication used deep ultraviolet lithography and photopolymerizable urethane-acrylate photoresist. It contained an integrated photometer (LED)/phototransistor for detection, and a nylon line homogenizing system. The same fabrication technique was applied to develop a new  $\mu$ FBA for total tannin determinations in green and black tea samples using the photometric (ferrous tartrate) and turbidimetric (copper (II) in acetate medium) methods.

## 2. Experimental

### 2.1. Reagents and standards

All reagents were of analytical grade and freshly distilled and deionized water ( $18\text{ M}\Omega\text{ cm}^{-1}$ ) was used to prepare all solutions. A stock solution of  $1000\text{ mg L}^{-1}$  of tannic acid was prepared with  $0.1000\text{ g}$  of tannic acid (Labsynth) diluted to  $100.0\text{ mL}$  with water.

For the photometric analysis a ferrous tartrate reagent solution was obtained by mixing  $1.0000\text{ g}$  of heptahydrated ferrous sulfate (Vetec) with  $2.0000\text{ g}$  of potassium sodium tartrate (Vetec) and  $0.1000\text{ g}$  of sodium bisulfite (Reagen) diluted in water to  $100.0\text{ mL}$ . The phosphate buffer pH 6.8 was prepared by mixing heptahydrated sodium phosphate  $0.10\text{ mol L}^{-1}$  (Reagen) with  $0.10\text{ mol L}^{-1}$  sodium hydroxide (Vetec).

For the turbidimetric analysis we used a solution of copper (II) at  $0.1\text{ mol L}^{-1}$ , this solution was prepared by dissolving  $0.6354\text{ g}$  of metallic copper (Merck) in nitric acid 50% (v/v) and diluted to  $100.0\text{ mL}$  with a solution of 1% nitric acid, a solution of ammonium acetate  $1.5\text{ mol L}^{-1}$  (Vetec), and a nitric acid solution 10% (v/v).

The commercial urethane-acrylate photoresist used for fabrication of the  $\mu$ FBA was acquired by Carimbo Medeiros Ltda, Brazil (MacDermid, flex-light trademark M050).

### 2.2. Preparation of samples

Samples were obtained from six emulsions of green and black tea purchased from different manufacturers and lots. These tea samples were purchased in local shops in Bahía Blanca, Argentina. For sample preparation, an amount of  $0.5000\text{ g}$  of tea was heated

for 10 min at  $90^\circ\text{C}$  in about  $50\text{ mL}$  of deionized water, the mixture was filtered, washing the residues and completing to  $100.0\text{ mL}$  with deionized water.

### 2.3. Apparatus

To fabricate the micro-chamber ( $\mu$ CH) in urethane-acrylate resin, we used a commercial UV light source (Fotolight-MD2-A4, Carimbo Medeiros Ltda, Brazil), with two sets of mercury lamps (BLB-15W-T8, SCT black light).

For layout design of the  $\mu$ CH, the CorelDraw® X5 program was used. The layout printing was on polyester transparency films for laser printing using an HP LaserJet P2014. After UV exposure, channels on the substrate were revealed by removal of the non-exposed resin with an ultrasonic bath (model UltraCleaner800, Unique, Brazil).

A spectrophotometer model 8453 Hewlett-Packard (HP) diode array UV–vis, equipped with cuvette (with an inner volume of about  $4\text{ mL}$ , and an optical path of  $1.0\text{ cm}$ ) was used for absorbance measurements when employing the reference method.

### 2.4. Assembly of the $\mu$ FBA system

A peristaltic pump (model 78001-12, 8 channel, Ismatec) for fluids propulsion was used which operated at 10 rpm. Minisolenoid valves (model LHDA 0531415H, Lee Company) were used for controlled fluid additions to the micro-mixing chamber. A  $0.4\text{ mm}$  nylon wire was used for agitation within the micro-mixing chamber to ensure mix of added products. The nylon was adapted to a CD/DVD-ROM motor drive (model MDN3GT3CPAC, 2000 rpm, 5 Vdc). Teflon® tubes with  $0.5\text{ mm}$  internal diameter were used for fluids transport.

The detection system integrated into the micro-mixing chamber was composed of an LED (light emitting diode) as source radiation, and a phototransistor for detection [32]. For the photometric detection we used a green LED with a maximum emission of  $560\text{ nm}$ . For the turbidimetric detection we used a blue LED with a maximum emission of  $470\text{ nm}$ . These devices, both LED and phototransistor having  $5\text{ mm}$  of diameter, were mounted firmly outside the mixing chamber against the glass tube openings which have an internal diameter of  $2\text{ mm}$ . The  $2\text{ mm}$  diameter was chosen for the glass tubes to minimize the resulting chamber volume.

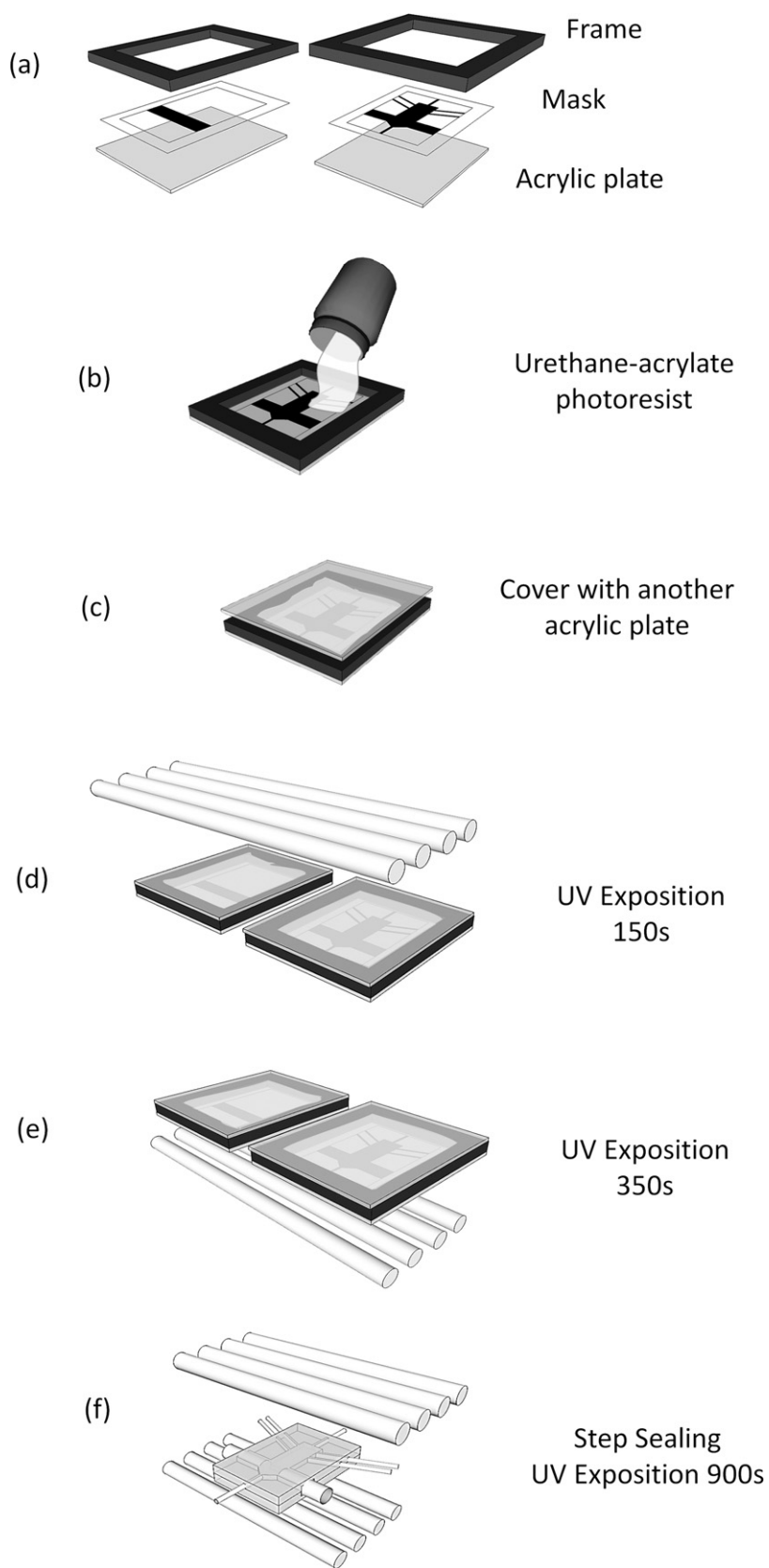
All tasks, such as data acquisition, valve, and drive motor activation, were done using a USB interface (USB6009, National Instruments®), which activated a lab made controller module. The software was developed in LabVIEW® 7.1 (National Instruments®).

### 2.5. Lithographic micro-chamber fabrication process

As outlined in Fig. 1, the  $\mu$ FBA was fabricated based on the methodology described by Monte-Filho et al. [31].

Initially the required layouts or templates were developed and printed. Each one was set between a  $2.0\text{ mm}$  acrylic plate, and a  $3.4\text{ mm}$  rubber frame forming the mold, as shown in Fig. 1a. The urethane-acrylate resin was then deposited on the mold and immobilized by another acrylic plate, as sketched in Fig. 1b and c, respectively. The thickness of the rubber framing (in this case  $3.4\text{ mm}$ ), allows you to define the volume of the system.

The layouts were engraved in the resin by exposure to UV radiation in two steps. First the top of both layers were cured (polymerized) when exposed for  $150\text{ s}$ , as shown in Fig. 1d. Next, the bottom was cured by exposure for  $350\text{ s}$ , as illustrated in Fig. 1e. The uncured resin layers were removed with the aid of an ultrasonic bath in detergent solution 10% (v/v) for  $15\text{ min}$ . These layers were then dried in a nitrogen flow.



**Fig. 1.** Schematic diagram illustrating the procedure for construction of the  $\mu$ FBA. (a) Mold mounting with the required layout, (b) depositing the urethane acrylic resin over the mold, (c) immobilizing the resin with the second acrylic plate, (d) radiation exposure UV for 150 s, (e) radiation exposure UV for 350 s, (f) layer sealing with inserted teflon and glass tubes, radiation exposure UV for 900 s.

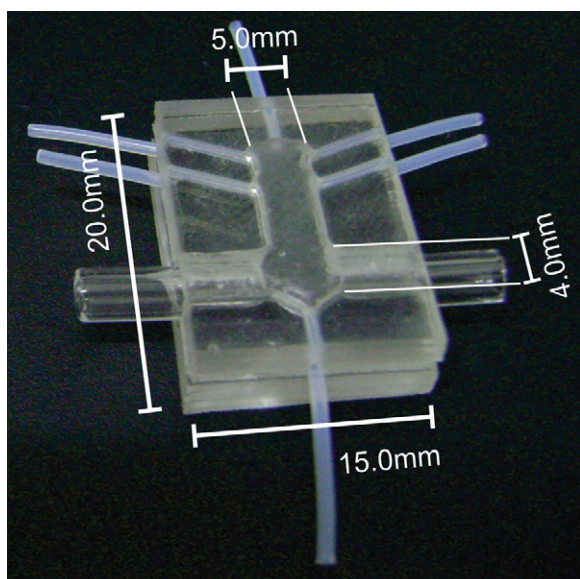


Fig. 2. Photograph of the microfabricated  $\mu$ FBA with its dimensions (mm).

In the sealing step, the layers were superimposed appropriately to form the required channels. Tubes of Teflon<sup>®</sup> and glass were carefully inserted into their respective channels. The system was again exposed to UV radiation in a single step of 900 s on both sides, as shown in Fig. 1f. This exposure time allows for an efficient and irreversible seal.

Teflon<sup>®</sup> tubes (0.8 mm diameter) fixed in the channels allow  $\mu$ CH communication with the external environment, making possible the insertions of fluids as well as the agitator shaft. The glass tubes used have one end inside the closed  $\mu$ CH, an external diameter of 4.0 mm and a 2.0 mm internal diameter. These tubes allow flexibly, the integrated detection in the  $\mu$ CH.

Fig. 2 shows a photo of the microsystem and its dimensions, as manufactured by the procedure described above. The detail of the attachment and sealing of the tubes can be seen in this photography. This microsystem is mounted onto a suitable support in a black (darkroom) box (10.0 cm  $\times$  8.0 cm  $\times$  4.0 cm), to preserve the system from the effects of spurious environmental radiation while in operation.

## 2.6. Analytical procedure

Before starting the analytical procedure, working solutions for each channel are pumped and re-circulated to their respective reservoirs (Fig. 3a). Then the mini-valves  $V_S$ ,  $V_{R1}$ ,  $V_{R2}$  and  $V_C$  are opened for 3.0 s and the working solutions (S, R1, R2, and C) are pumped to the micro-chamber to fill the channels between the valves and the chamber. Then immediately, the discard valve  $V_W$  is opened for 5.0 s and then any solution inside the  $\mu$ CH is emptied using the peristaltic pump for aspiration. This channels filling procedure is very important and must be carried out whenever there is a change of the reservoir liquids.

Fig. 3a represent the schematic diagram of the  $\mu$ FBA used for determination of tannins by the Cu (II) in acetate medium and the ferrous tartrate methods. They have in common every preparation step for mixing/homogenization in the  $\mu$ CH, measurement of absorbance, disposal and cleaning. Homogenization is performed by the drive motor (DM), the absorbance is measured and the  $\mu$ CH is emptied. Afterwards the  $\mu$ CH is cleaned by simultaneous activation of  $V_C$  and the drive motor, adding cleaning solution (C) while activated. Then,  $V_W$  is opened to discard the contents of the  $\mu$ CH. This

cleaning and disposal procedure must be done twice to effectively clean the  $\mu$ CH.

The timing diagrams that illustrate the analytical procedures for both the turbidimetric and photometric methods are described in Fig. 3b and c, respectively. The time intervals chosen for each in-line preparation are for the drive motor ( $T_{DM}$ ), valve switchings ( $T_S$ ,  $T_{R1}$ ,  $T_{R2}$ ,  $T_C$  and  $T_W$ ), and absorbance measurements ( $T_A$ ). All preparations used a peristaltic pump (PP) with a flow of  $34.0 \pm 0.3 \mu\text{L s}^{-1}$  ( $n = 10$ ) for all channels.

### 2.6.1. Procedure for Cu (II) in acetate medium

For in-line blank preparation, valves,  $V_{R1}$  and  $V_{R2}$  are simultaneously activated for 1.0 s, the mixing for 2.0 s and after that the absorbance signal is measured.

The in-line preparation of calibration solutions from 20 to  $100 \text{ mg L}^{-1}$  were performed using the working solution of  $200 \text{ mg L}^{-1}$  of tannic acid prepared from the appropriate dilution of stock solution of  $1000 \text{ mg L}^{-1}$ . In these preparations valves  $V_S$ ,  $V_{R1}$ , and  $V_{R2}$  are activated simultaneously. Working solution (S), acetate reagent (R1) and  $\text{Cu}^{2+}$  (R2) are added to the  $\mu$ CH, homogenized and the absorbance is monitored at 470 nm.  $V_{R2}$  is opened for 0.5 s and the on times for  $V_S$  and  $V_{R1}$  vary proportionately with the concentration of the standard solution being prepared.

The procedure for in-line preparation of the sample is similar to the preparation of calibration solutions. The prepared samples (see Section 2.2) were used without further dilution. Samples are used instead of the working standard solution. The time intervals used for this analysis are shown in Fig. 3b. This method used a 10% nitric acid solution for  $\mu$ CH cleaning.

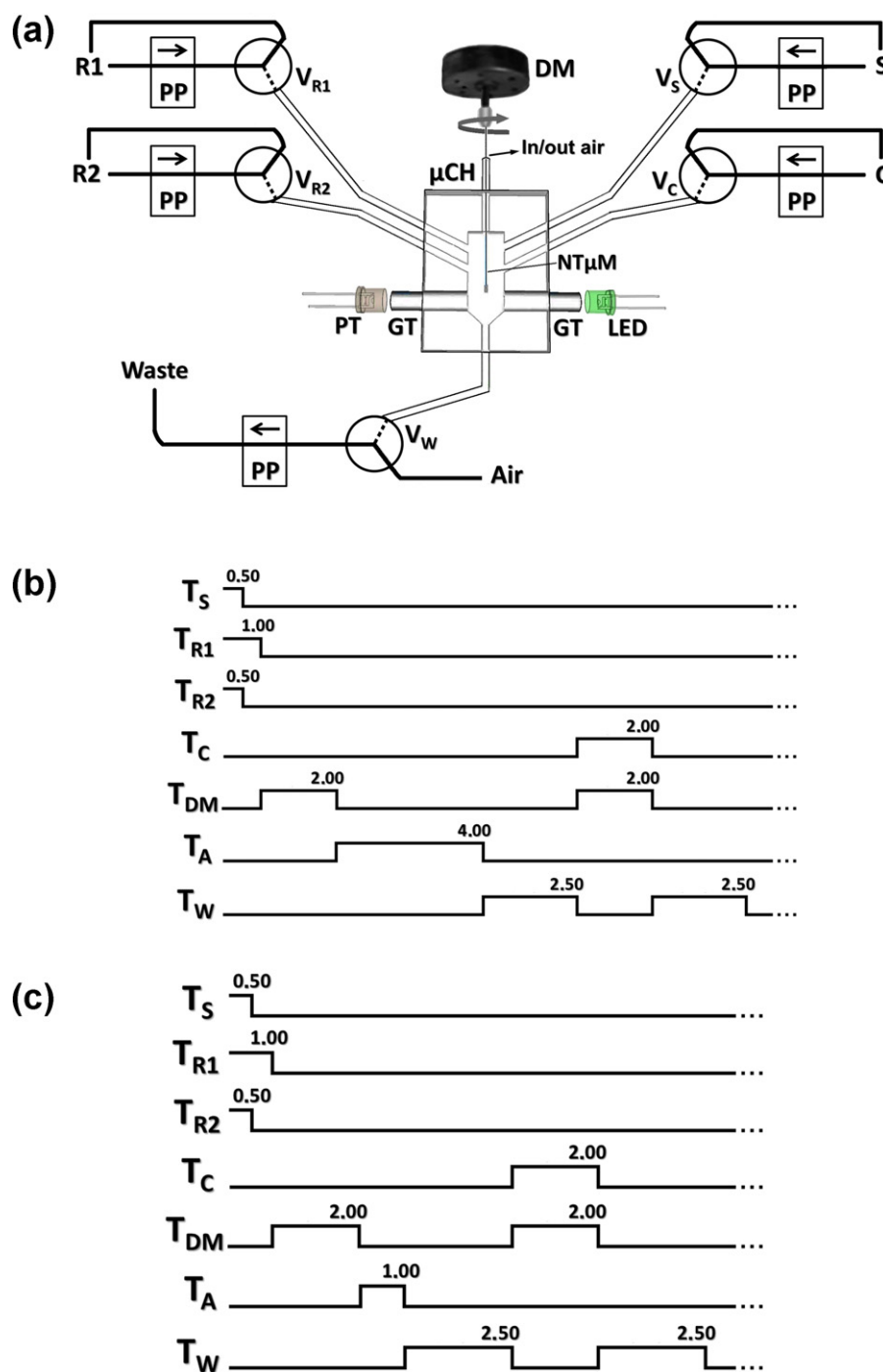
### 2.6.2. Procedure for ferrous tartrate

The procedure for blank preparation and the calibration solutions is similar to that described in Section 2.6.1. For the calibration solutions, the working solution (S), phosphate buffer pH 6.8 ( $R_1$ ) and ferrous tartrate reagent ( $R_2$ ) are added to the  $\mu$ CH, homogenized and the absorbance is measured at 560 nm.  $V_{R2}$  is opened for 0.5 s and the on times  $V_S$  and  $V_{R1}$  vary proportionately with the concentration of the standard solution being prepared.

For in-line preparation of the sample, the in-line procedure is similar to the preparation of calibration solutions. The prepared samples as described in Section 2.2 were used without further dilution. The difference here is also that the samples are used instead of the standard working solution. The time intervals used for this analysis are shown in Fig. 3c. This method used deionized water for cleaning the  $\mu$ CH.

## 2.7. Reference method

Results obtained with the proposed mini-system were compared with the official Japanese ferrous tartrate method [13,14]. Calibration solutions from 20 to  $100 \text{ mg L}^{-1}$  of tannic acid were prepared by adding appropriate volumes of  $1000 \text{ mg L}^{-1}$  stock solution, 10.0 mL of ferrous tartrate solution, 50.0 mL of phosphate buffer (pH 6.8) and water to complete to a final volume of 100.0 mL in a volumetric flask. For determination of tannins in the tea samples we added 10.0 mL aliquots of the sample, 10.0 mL of reagent and 50.0 mL of buffer using the same conditions as were used in determining the calibration solutions. After 10 min of reaction, the absorbance of all solutions, both calibration and sample were measured at 550 nm.



**Fig. 3.** (a) The  $\mu$ FBA diagram, (b) photometric and (c) turbidimetric time diagrams for samples. Micro-chamber ( $\mu$ CH), peristaltic pump (PP), drive motor (DM), nylon line mixer (NT $\mu$ M), glass tube (GT), light emitting diode (LED), phototransistor (PT), minivalves solenoids ( $V_S$ ,  $V_{R1}$ ,  $V_{R2}$ ,  $V_C$  and  $V_W$ ), working solution or sample (S), discharge (W). For turbidimetric determination: R1, ammonium acetate  $1.5 \text{ mol L}^{-1}$ ; R2, Cu (II)  $0.1 \text{ mol L}^{-1}$ ; C,  $\text{HNO}_3$  10% (v/v). For photometric determination: R1, phosphate buffer pH 6.8; R2, ferrous tartrate; C, water. The time intervals (in s)  $T_S$ ,  $T_{R1}$ ,  $T_{R2}$ ,  $T_C$  and  $T_W$ , correspond to the minivalves ( $V_S$ ,  $V_{R1}$ ,  $V_{R2}$ ,  $V_C$  and  $V_W$ );  $T_{DM}$ , drive motor;  $T_A$ , absorbance measurements.

### 3. Results and discussion

#### 3.1. Characterization of the microsystem

Lithographic microfabrication with ultraviolet urethane-acrylate resin is easily reproducible. The sealing of the two partially cured layers with UV exposure was performed with both the Teflon and glass tubes already properly positioned in their respective channels. Due to the risk of clogging, the technique

requires caution, and proper control of the exposure time. A time of 900 s is needed to ensure effective sealing of the layers. After curing the photoresist, a colorless and transparent material is obtained which does not absorb radiation in the visible region, allowing the use of optical detectors [33,34].

Monte-Filho et al. [31] describe the  $\mu$ FBA using detection devices permanently inserted into the resin and polymerized. For a more flexible system, glass tubes were attached to  $\mu$ CH. Thus, we employed the same system for both determinations,

measuring turbidity with a blue LED and for photometric absorbance, a green LED for. The use of glass tubes allows the micro flow-batch to be used with different LED/phototransistor couplings for various applications.

In conventional FBA systems the mixing chamber contains a magnetic strip that allows fluids agitation. In the  $\mu$ FBA system agitation is performed using nylon line with a shovel tip. Complete mixing of the solutions in the micro-chamber was obtained in less than 2 s, due to the high speed of (2000 rpm), CD/DVD-ROM motor.

### 3.2. $\mu$ FBA application

The copper (II) in acetate medium method obtained a satisfactory analytical curve for the determination of tannins in tea samples with the regression equation  $Y = -0.0025 + 0.0002C$ ; where  $Y$  is the absorbance and  $C$  is the analyte concentration in  $\text{mg L}^{-1}$  of tannic acid. The linear correlation coefficient,  $r^2$ , was 0.997 in the range between 20 and  $100 \text{ mg L}^{-1}$ . Its calibration curve was statistically validated by variance analysis showing no lack of fit at a 95% confidence level. The LOD and LOQ were  $6.89$  and  $10.06 \text{ mg L}^{-1}$  respectively.

For the reference method applied to the  $\mu$ FBA the regression equation was  $Y = -0.0043 + 0.0010C$ ; where  $Y$  is the absorbance and  $C$  is the analytic concentration in  $\text{mg L}^{-1}$  of tannic acid. The linear correlation coefficient,  $r^2$  was 0.9994 in the range between 20 and  $100 \text{ mg L}^{-1}$ . The calibration curve was statistically validated by variance analysis showing no significant lack of fit in the proposed model at a 95% confidence level. The LOD and LOQ were  $4.74$  and  $6.97 \text{ mg L}^{-1}$  respectively.

The LOD and LOQ for both methods were calculated based on the criteria established by IUPAC, the LOD was evaluated as three times the standard deviation of the blank measurement, and the LOQ was evaluated as being 10 times the standard deviation of the blank measure [35]. The LOD and LOQ values, although relatively high may be acceptable for the analyte under study, considering the high concentrations of tannin in green and black tea [10].

Table 1 presents the results obtained for the proposed microsystem for turbidimetric and photometric analysis, and those obtained for the reference spectrophotometric method. These results were statistically significant at a 95% confidence level when using the paired  $t$  test.

The  $\mu$ FBA presented an analytical frequency of 300 samples per hour for the ferrous tartrate method, with a waste generation of  $136 \mu\text{L}$  per analysis. The turbidimetric method, copper (II) in acetate medium displayed an analytical frequency of 200 samples per hour, with a waste generation of  $204 \mu\text{L}$  per analysis.

The turbidimetric analysis needed more signal monitoring (4.0 s), due to the need to maintain a reproducible precipitate formation. The turbidimetric method's analytical frequency was lower than that of the photometric method.

The analytical curves obtained using the  $\mu$ FBA system had less slope or sensitivity, because absorbance measurements were made

**Table 1**

Results for tannin determinations in samples of green and black tea ( $\text{mg L}^{-1}$ ) using both turbidimetric ( $\mu$ FBA-Turb.), photometric ( $\mu$ FBA-Phot.) methods and the spectrophotometric method with UV-vis as reference.

Samples	$\mu$ FBA-Turb.	$\mu$ FBA-Phot.	Reference
Green tea	$41.48 \pm 0.05$	$41.50 \pm 0.03$	$41.74 \pm 0.01$
Green tea	$49.21 \pm 0.03$	$48.01 \pm 0.05$	$47.92 \pm 0.02$
Green tea	$61.62 \pm 0.05$	$63.76 \pm 0.03$	$64.35 \pm 0.03$
Black tea	$46.47 \pm 0.04$	$47.21 \pm 0.01$	$47.40 \pm 0.03$
Black tea	$54.57 \pm 0.05$	$54.97 \pm 0.01$	$55.19 \pm 0.02$
Black tea	$44.14 \pm 0.06$	$45.01 \pm 0.03$	$44.87 \pm 0.00$
RSD% ( $n=5$ )	0.05	0.03	0.02

using an LED/phototransistor pair in a shorter optical path, about 0.5 cm. In the reference method, absorbance measurements were performed at 560 nm with a cuvette with 1.0 cm optical path.

## 4. Conclusion

The  $\mu$ FBA system proposed, successfully carried out the analysis of tannins in samples of green and black teas. The glass tubes attached to the  $\mu$ CH allowed changing the source radiations, a blue LED was used for turbidimetric monitoring and a green LED for the photometric determination. The urethane-acrylate resin used as substrate, proved chemically stable throughout the study and the fabrication methodology proved to be fast, and efficient while producing a robust or sturdy assemblage.

We obtained high speed microanalysis, thanks to optimizations in the injection times and volumes, in a system that allowed both homogenization (2 s) and discrete measurements. Essentially equal to a traditional flow-batch analyzer [22,24], the miniaturized or  $\mu$ FBA proposed can be used in many analytical applications; determinations for pharmaceuticals, foods, and clinical laboratory analysis of biological fluids, while guaranteeing significant reductions in the consumption of reagents and samples, and generating much less chemical waste.

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