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Caffeine determination by flow injection analysis employing Bovine Serum Albumin as a fluorophore

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ABSTRACT

A new methodology for caffeine determination was developed based on the quenching effect on fluorescent emission of the molecule of Bovine Serum Albumin at $\lambda_{em} = 338$ nm ($\lambda_{ex} = 280$). A flow injection disposition was designed improving significantly the sampling rate to 60 samples/h using potassium dihydrophosphate $5 \cdot 10^{-3}$ mol L^{-1} buffer (pH 6.8) as carrier and flow rate of 1.5 mL min^{-1}. The experimental and instrumental conditions that influence on analytical quality parameters were systematically investigated, as consider: buffer nature and concentration, fluorophore nature and concentration, and carrier flow rate. The proposal is simple, fast, inexpensive and precise, with a linear range from $6.68 \cdot 10^{-6}$ to $4.0 \cdot 10^{-3}$ mol L^{-1} and SD of 0.0668, under optimized conditions. Methodology sensibility and selectivity allowed a variety of sample analyses. It was successfully applied to caffeine quantification in energy drinks, dietary supplements and sliming infusion samples without previous treatment.

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1. Introduction

Caffeine (CF) is an alkaloid of methylxanthines family, the most frequently ingested substance with pharmacological activity in the world [1]. Among its numerous physiological effects, it can be mentioned the stimulation of central nervous system, cardiac muscle, respiratory system and diuresis [2–5].

In the wild, it is found in more than 60 plants such as tea leaves, coffee beans, kola nuts and cocoa pods; CF has been part of many cultures for centuries and actually there are many new energy products, such as waffles, sunflower seeds, jelly beans, syrup, and bottled water. It also occurs in several other foodstuffs such as prescription medications, diuretics, and pain relievers. Also, more recently, CF has been added to some alcoholic beverages introduced to the marketplace.

CF is use in sports world as stimulant, enhancing the physical performance as well as mental aptitude. This substance can improve athlete's endurance in sports where long-term stamina is needed [6,7]; sports include cycling, running, and even soccer. CF can decrease fatigue in athletes and has been forbidden in sport by Word Anti-Doping Agency's (WADA) Monitoring Program [8].

With high doses unpleasant short-term side effects are exhibit, including palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure and insomnia [9–12]. In spite of numerous publications on the long-term consequences of CF consumption on human health, no clear picture has emerged, with reports of both protective and deleterious effects. However, there are severe concerns about unfavorable influences of CF on young children and pregnant women, including the risk of fetal death and miscarriage [13,14].

Because numerous adverse effects occurred, it results essential having CF monitoring methodologies. Several analytical methods had been proposed for the determination of CF in different samples and quality control of products including titrimetric spectrophotometry, polarography, GC and HPLC [15–18].

In other order of things, Bovine Serum Albumin (BSA) is one of the longest known and, probably, the most studied protein. It's conformed by 583 amino acids with 66 493 Da molecular weight. BSA has numerous applications, both in clinical medicine and basic research [19]. Because of the presence of Trp, Tyr and Phe residues, the BSA has a characteristic fluorescence spectrum. Fluorescence spectroscopy is a sensitive methodology chosen for studies of protein stability, hydrodynamics, kinetics, or ligand binding [20,21]. Many of these applications employed quenching phenomena. BSA binding property with several compounds enables the indirect study of many pharmacological drugs like CF [22,23].

Flow injection analysis (FIA) is well-established sample handling methodology, based on the injection of samples or standards in a continuous carrier stream, which provides the mechanization of different steps in the quantitative analysis [24]. Coupling the fluorescence determination with a flow injection analysis, multiple advantages are gained. Sampling times are usually just few minutes, furthermore, the small internal diameter of the PVC pumping tubes minimizes the amount of reagents consumed and the volume of waste generated. FIA is robust in

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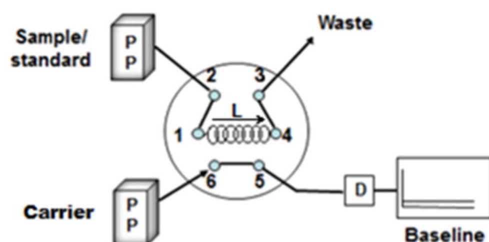

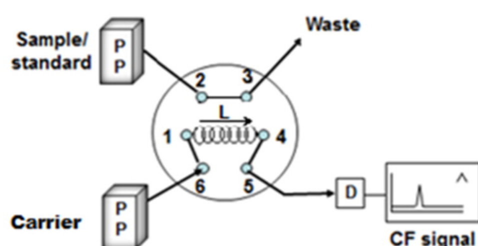

Fig. 1. Schematic representation of FIA system designed for caffeine determination by the developed methodology.

wide range of operating parameters which could be optimized in order to achieve successful analyses.

On this work, a simple, fast and inexpensive on line methodology for CF determination with fluorescence detection is proposed, employing BSA as a fluorophore. Experimental parameters that impact quality of analytical results have been optimized in order to apply the new methodology to the analyte determination in different energy supplement samples and slim tea.

2. Experimental

2.1. Reagents and materials

CF stock solution (Sigma-Aldrich, St. Louis, USA) $1 \cdot 10^{-2}$ mol L^{-1} was prepared by dissolution of the appropriate amount in methanol. Further dissolutions were weekly prepared in ultrapure water.

Stock of $1 \cdot 10^{-2}$ mol L^{-1} solution BSA (Fedesa, San Luis, Argentina) was weekly prepared in ultrapure water and storage al 278–283 K.

Potassium dihydrophosphate (Biopack, Buenos Aires, Argentina) buffer solution $1 \cdot 10^{-2}$ mol L^{-1} and sodium tetraborate (Biopack, Buenos Aires, Argentina) $1 \cdot 10^{-2}$ mol L^{-1} solutions were prepared dissolving the appropriate amount in ultrapure water. Acetic acid (Mallinckrodt Chemical Works, St. Louis, USA) $1 \cdot 10^{-2}$ mol L^{-1} buffer solution was prepared diluting in ultrapure water the appropriate volume.

The pH was adjusted to the desired value, by adding NaOH solution (Merck, Buenos Aires, Argentina) using a pH meter.

All used reagent were analytical grade.

2.2. Apparatus

Spectrofluorimetric measurements were made using a spectrofluorometer (Shimadzu RF-5301 PC) equipped with a 150 W Xenon lamp. In batch studies 1.00 cm quartz cells were used and 120 μL flow cell unit (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan) for the flow measurements.

The propulsion system consisted in two peristaltic pumps (Gilson Minipuls 3) with PVC pumping tubes. A Rheodyne model 5041 six-port two-way rotary valve (Rohnert Park, CA) was employed.

A combined glass electrode and a pH meter Orion Expandable Ion Analyzer (Orion Research, Cambridge, MA, USA, Model EA940) was used for pH adjustments.

2.3. Studied samples

A total of three recipients of the same brand for each CF containing products were acquired as a strategy of randomized sampling. The whole of the contents of each product was homogenized and reserved for sample preparation.

Adequate volume or weight of each sample, containing from 1.1 to $9.7 \cdot 10^{-3}$ g L^{-1} was dissolved in ultrapure water and diluted to 25 mL in a volumetric flask.

In the slimming infusion case, 200 mL of boiling ultrapure water was poured over a tea bag and let it sits for 5 min. A dilution 1:2 was carried out.

All the solutions were reserved for CF determination applying general procedure.

2.4. General procedure

In order to perform the calibration curve, an estipulate volume of CF standard solution containing 1.1 to $9.7 \cdot 10^{-3}$ g L^{-1} was placed in a volumetric flask and 200 μL of BSA $1 \cdot 10^{-5}$ mol L^{-1} was added. The whole mixture was made up to 10 mL with ultrapure water.

Standard/samples were injected into a flowing buffer stream conformed by phosphate $5 \cdot 10^{-3}$ mol L^{-1} pH 6.8. The continuous flow diagram is shown in Fig 1. In the “loading” position, the carrier was impulse by the first peristaltic pump (PP) to the fluorescence detector generating the baseline at $\lambda_{em} = 338$ nm ($\lambda_{ex} = 280$); while the second peristaltic pump aspirate the sample charging the sample loop (L) in the injection valve located among 1 and 4 positions. The loading was carried out for 30 s, enough time to fill L with sample/standard. In the “injection position” carrier flowed through the L and carried the analyte to the detector. The second PP is turned off.

3. Results and discussion

In order to use the molecular fluorescence to monitor CF, a variety of different fluorophores such as 8-hydroxyquinoline, dithizone, chromazurol, rhodamine B and BSA were assayed. No variation on native fluorescent response of mentioned fluorophores was observed on studied experimental conditions in the presence of analyte CF, with the exception of BSA that it showed quenching effect on fluorophore emission. So, this fluorophore was selected to follow the optimization studies.

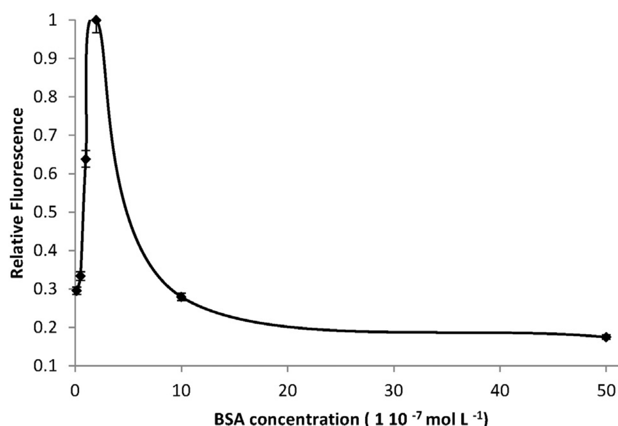

Fig. 2. Study of optimal fluorophore concentration.

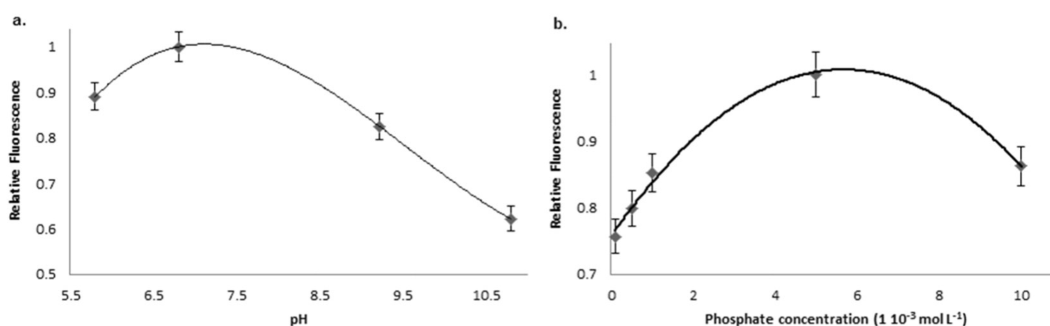

Fig. 3. a. Optimization of pH by developed methodology. b. Optimization of buffer concentration by developed methodology.

BSA contains two tryptophan residues with intrinsic fluorescence located in separate domains, the first one in position 134 (on the surface of the molecule) and the second in position 212 (located within a hydrophobic pocket of the protein) [25,26]. Fluorescence is due to indole group of tryptophan which absorbs near 280 nm and emits near 340 nm. Changes in the emission spectra of tryptophan are common in response to conformational transitions, subunit association, substrate binding, or denaturation [27]. The changes of the environment of tryptophan residues depend on the quencher structure. Caffeine interacts simultaneously with the interior hydrophobic pocket of BSA and with the hydrophilic surface of the protein [25,28]. Both caffeine rings are required in order for significant binding to occur [29].

In order to establish the optimal instrumental parameters, systems containing BSA were prepared and explored by molecular fluorescence showing the maximum emission at $\lambda_{em} = 338$ nm ($\lambda_{ex} = 280$ nm). Parameters that affect the fluorescence of the CF-BSA complex were analyzed.

To achieve maximum sensitivity in the new method, fluorophore concentration is other parameter that must be optimized. So that, systems containing BSA concentrations of $1.5 \cdot 10^{-8}$ to $5 \cdot 10^{-6}$ mol L^{-1} and constant concentration of CF were prepared and analyzed. As shown in Fig 2, maximum signal at $\lambda_{em} = 338$ nm ($\lambda_{ex} = 280$) corresponds to BSA optimal concentration of $2 \cdot 10^{-7}$ mol L^{-1}.

It was reported that BSA has different pH-dependent conformations, and the quenchers would preferentially interact with apolar regions of albumin [30]. So that, the solution pH has a direct implication in BSA molecule and the capability to complex formation with CF. Variation in pH generates conformational changes adopted by the molecule [31]. The albumin macromolecule in the N-form (pH 4.5 to 7.0) is globular. When media acidity rises, the surface charge of protein is altered, eliminating electrostatic interactions that stabilize tertiary structure. BSA domains are separated and molecular structure opens. Between pH 8.0 and 9.0 BSA loses some of its rigidity, affecting the protein amino-terminal region, and the macromolecule has a small increase in its radius [32] so the fluorescence decreases.

The optimum pH value, nature and concentration were evaluated employing different buffer solutions, to say: acetic acid/acetate pH 5.8, phosphate pH 6.8, sodium tetraborate pH 9 and 10.8. All were prepared in concentration $1 \cdot 10^{-2}$ mol L^{-1}. The best signal was achieved with phosphate at pH 6.8 as shown in Fig 3(a), the BSA molecule has the normal configuration, a globular form approachable to conform the CF-BSA complex, whit tryptophan residues in the suitable fluorescence way. In order to optimize buffer concentration, systems containing phosphate solution from $1 \cdot 10^{-4}$–$1 \cdot 10^{-2}$ mol L^{-1} (pH 6.8) were prepared. The maximum emission signal was obtained with $5 \cdot 10^{-3}$ mol L^{-1} as shown in Fig 3(b).

The methodology was adapted to on-line analysis using two peristaltic pumps: the first one, create a continuous carrier stream propelling the buffer solution, and the second one is turn on to impulse the sample only in "loading position" so that, two peristaltic pumps were needed. The injection valve controlled the sample input throw the system.

In order to generate an appropriated quenching effect, the interaction among CF and BSA must take place before the injection in FIA system. Other alternative modes to introduce the fluorophore and the analyte to FIA system were proved: in the case of BSA was part of the carrier, the resulted baseline generated was higher than the complex CF-BSA, so that, the peaks after CF injection were negative. So, systems containing BSA and CF were prepared "in batch"; as an additional advantage can be mentioned that the proposed configuration reduced the consumption of fluorophore.

On-line studies were conducted in order to verify the validity of experimental parameters that had been previously "in batch" optimized (pH and buffer concentration). It was shown that they were consistent with the improvement in FIA systems, with the added advantages of consuming less reactive and generating fewer amounts of waste.

The carrier effect on the analytical response was studied at flow rates between 1.0 and 3.0 mL min^{-1}. It was observed that the analytical signal increased with the carrier flow rate. However, despite the benefit of greater speed of analysis, flow rates greater than 1.5 mL min^{-1} generated undesirable pressures due to the high flow velocity, causing decoupling in the FIA system. Therefore, carrier stream of 1.5 mL min^{-1} was selected as optimal.

4. Analytical performance

Following the above mentioned conditions, a calibration plot was carried out from the injection of increasing concentrations of CF in FIA system (Fig 4). Main figures of merit of CF determination by FIA have been established.

The calibration of developed methodology was linear over CF concentration range from $6.68 \cdot 10^{-6}$ to $4 \cdot 10^{-3}$ mol L^{-1} ($r^2 = 0.9954$)

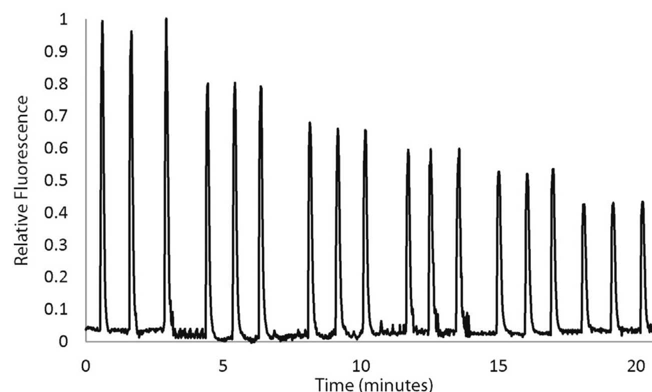

Fig. 4. Flow injection analysis graphic for CF calibration at optimal experimental conditions.

Table 1
Analytical methodologies applied to CF determination foods, dietary supplements and energy drinks.

Method	Comments	Reference
SPE/HPLC	SD: 2.5 LOD: $1 \cdot 10^{-6}$ mol L^{-1} Linear range: $5 \cdot 10^{-6}$–1.28 mol L^{-1} Sensibility: 48,177	Rodrigues et al. [33]
HPLC–UV	LOL: 0.16–250 μg ml^{-1} ($r > 0.995$, $n = 5$) RSD < 4.0% LOD: 0.05 μg ml^{-1} LOQ: 0.16 μg ml^{-1} Applied to food samples.	Al-Othman et al. [34]
GC–MS	LOL: 0.05–5.0 μg ml^{-1} RSD: 4.4% LOD: 4.0 ng ml^{-1} Correlation coefficient: 0.98 Applied to beverages and foods.	Shrivas and Wu [35]
CE	RSD: 0.24% LOD: 0.42 μg ml^{-1} LOQ: 1.4 μg ml^{-1} Applied to herbal medicines and dietary supplements.	Cianchino et al. [36]
Voltammetry	LOL: 0.06–19 μg ml^{-1} LOD: 0.02 μg ml^{-1} LOQ: 0.06 μg ml^{-1} Applied to commercial teas samples.	Sereshti and Samadi [37]
SP fluorescence	LOL: $1.1–9.7 \cdot 10^3$ μg L^{-1} LOD: 0.3 μg L^{-1} LOQ: 1.1 μg L^{-1} R^2: 0.9989 Applied to dietary supplements and energy drinks.	Talio et al. [38]
This methodology	LOD: $2.00 \cdot 10^{-6}$ mol L^{-1} (0.38 μg ml^{-1}) LOQ: $6.68 \cdot 10^{-6}$ mol L^{-1} (1.29 μg ml^{-1}) Lineal range: $6.68 \cdot 10^{-6}$–$4 \cdot 10^{-3}$ mol L^{-1} (1.29–766 μg ml^{-1}) Sensitivity: 9383 L mol^{-1} Standard deviation: 0.0668 Applied to dietary supplements, sliming teas and energy drinks.	–

SD: standard deviation; LOD: limit of detection; LOQ: limit of quantification; LOL: limit of linearity; RSD: relative standard deviation; R^2: coefficient linear determination; SPE/HPLC: solid phase extraction, high performance liquid chromatography; HPLC/UV: high performance liquid chromatography with ultraviolet detection; GC/MS: gas chromatography with mass spectrometry detector; CE: capillary electrophoresis.

with a standard deviation SD = 0.0668 calculated by 15 independent measurements of blank solution. The detection limit (LOD) is $2 \cdot 10^{-6}$ mol L^{-1} and was determined by a confidence of 99.6% ($k = 3$).The quantification limit (LOQ) was calculated by 10 standard derivation of a blank solution. The sensitivity is 9383 L mol^{-1} obtained from the slope of the calibration graph.

A comparison with other published results is shown in Table 1. Analytical parameters like LOD, LOQ, and linear range are similar. The proposed methodology presents the advantage of a significant lowest standard deviation, which implies a minor error in the measurements, improving accuracy. Other visible benefits of the method are the simplicity, low cost of equipment, versatility in sample application, as well as the low consumption of reagents and shorter time required for sample processing.

5. Application

The usefulness of the developed methodology was evaluated for CF quantification studies in a variety of energy samples and supplements employed in sports world (Table 2). The validation and accuracy of the methodology were performed using the standard addition method [39]. In this way, matrix interferences like the presence of other fluorophore or any compound which could form a complex with BSA, were solved. Adequate sample aliquots were spiked with increasing amounts of CF. Obtained results showed satisfactory agreement with the nominal concentrations of commercial studied samples and acceptable precision. The replicability of the method was evaluated repeating the proposed approach, 4 injections for each addition in a total of three levels of spiked CF for each sample. Table 2 shows the recovery results achieved for each sample, in almost all cases, it was near 100%. It shows that almost all analytes were determined, validating the methodology.

Table 2
Quantification studies of CF in a variety of samples employing the proposed methodology.

Sample	CF added (10^{-4} mol L^{-1})	CF found ± CV (10^{-4} mol L^{-1})	% recovery	CF nominal content (mol L^{-1})	% RE
1	0	5.28 ± 0.029	88.44	$5.97 \cdot 10^{-3}$	11.5
	2.5	7.26 ± 0.052			
	5	10.54 ± 0.038			
2	0	1.08 ± 0.031	104.85	$1.03 \cdot 10^{-3}$	4.8
	2.5	3.7 ± 0.021			
	5	5.98 ± 0.150			
3	0	1.04 ± 0.003	100.97	$1.03 \cdot 10^{-3}$	0.97
	2.5	3.47 ± 0.004			
	5	6.25 ± 0.012			
4	0	1.8 ± 0.001	109.09	$1.65 \cdot 10^{-3}$	9.09
	2.5	4.38 ± 0.006			
	5	6.76 ± 0.008			
5	0	0.723 ± 0.006	93.65	$0.772 \cdot 10^{-3}$	6.34
	2.5	2.985 ± 0.150			
	5	5.658 ± 0.074			

1 and 2: energetic gels; 3 and 4: energy drinks; 5: slimming infusion; % RE: porcentual relative error.

In proposed samples, usually there are no significant amounts of other methylxanthines as theophylline and/or theobromine which could be able to act in similar way as CF. Recovery studies with values near 100% demonstrated that the methodology allows quantitative study of CF content in different nature samples without interference of other species.

6. Conclusions

CF is widely consumed as a result of the benefits of the intake. The Nervous System stimulation and the energy increased is one of the most desired effects especially in the sports world. Nevertheless, the excess could cause irreparable damage leading to death, it's the reason why the monitoring is important. A novel methodology using BSA as a complexing fluorophore with fluorimetric detection for determination of CF in energy drinks, dietary supplements and sliming infusions has been developed with a linear range between $6.68 \cdot 10^{-6}$ mol L^{-1}–$4 \cdot 10^{-3}$ mol L^{-1} (SD = 0.0668). The proposed methodology offers a viable alternative to the conventional methods for CF determination, with the added advantage of rapid sample analysis due to the adaptation to flow injection analysis, which allows the processing of 60 samples per hour. With standard addition method, matrix interferences are remedied. The method is safe, rapid and inexpensive as well as environmentally friendly. The same general methodology could be suitable for the analysis of CF content in a variety of other products and it could be adopted for routine quality control analysis.

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