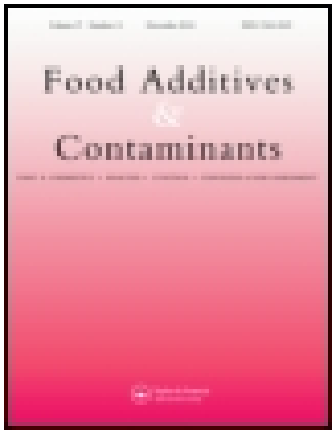

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Quantification of caffeine in dietary supplements and energy drinks by solid-surface fluorescence using a pre-concentration step on multi-walled carbon nanotubes and Rhodamine B

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Quantification of caffeine in dietary supplements and energy drinks by solid-surface fluorescence using a pre-concentration step on multi-walled carbon nanotubes and Rhodamine B

María Carolina Talio[b], María Gimena Acosta[b], Magdalena Alesso[a], Marta O. Luconi[a] and Liliana P. Fernández[a,b]*

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A new method for the determination of caffeine, a non-fluorescent analyte, based on the enhancement of the fluorescence of Rhodamine B dye on a membrane filter modified with multi-walled carbon nanotubes is proposed. The method comprises pre-concentration of caffeine on a solid support by chemofiltration in buffered solution onto multi-walled carbon nanotubes previously oxidised and dispersed in cationic surfactant admicelles. The effect of experimental parameters, including the nature of the buffer and pH, the nature of the solid support, filtration flow rate, dye and carbon nanotube concentration, and the nature of the surfactant and concentration were investigated by means univariation assays. Under optimum experimental conditions, the pre-concentration system gave detection and quantification limits of 0.3 and 1.1 μg l^{-1}, respectively. A wide linear range was achieved varying from concentrations of 1.1 to 9.7 × 103 μg l^{-1} (r^2 = 0.999). Satisfactory recovery values were obtained using the method of standard addition, confirming the feasibility of this method for caffeine determination in energising dietary supplements and energy drinks.

Keywords: caffeine monitoring; Rhodamine B; nylon membranes; multi-walled carbon nanotubes; solid-surface fluorescence

Introduction

Caffeine (1,3,7-trimethylxanthine; CF), is a substance found naturally in the leaves, beans and fruits of a variety of plants (Sawynok 2011). Although it is regularly consumed by a large percentage of adults, in Argentina recently the young have been added to this group. This compound has won popularity with adolescents due to the promotion of CF products found in the health benefits as follows: the stimulation of wakefulness, an increase in concentration and a decrease in the sensation of fatigue (Heckman et al. 2010). High levels of CF consumption may have adverse effects on fertility and have been associated with an increased risk of restriction to foetal growth. The recommendation for women trying to become pregnant is a CF limit < 300 mg day^{-1} (Nawrot et al. 2003; Kuczkowski 2009). In addition, pregnant women are advised to drink no more than two cups of coffee or four cups of tea per day (Heckman et al. 2010). Attending to the health risks, many countries have started action to establish the regulatory boundaries around CF.

Today in Argentina more than 10 brands of energy drinks are marketed, between imported and domestic processing those which are authorised as 'dietary supplements' because they provide nutrients such as sucrose, vitamins, amino acids, etc. Energy drinks are covered by the Argentine Food Code and are considered as foods if they can be acquired by free sale (see http://www.anmat.gov.ar/webanmat/Comunicados/Prensa/2005/Bebidas_Energizantes_17-10.asp).

Furthermore, dietary supplements containing botanical forms of CF and ephedra alkaloids have been widely promoted for both weight loss and the enhancement of athletic performance, despite the lack of adequate research on the pharmacology of these stimulants.

Foods are complex matrices. In order to determine CF at low levels in these samples, analytical methodologies with high sensitivity and adequate selectivity are necessary (Jacob et al. 2004). Diverse analytical methods have been proposed for CF determination and for the quality control of its associated products: these include spectrophotometry, fluorimetry, polarography, voltammetry, GC and HPLC (Fernández et al. 2000; Tsuda et al. 2000; Dinç et al. 2001; Alpdogan et al. 2002; Guo et al. 2011; Talio et al. 2013; Sereshti & Samadi 2014). The spectrophotometric methods lack adequate sensitivity for the determination of analytes at trace levels. Although fluorimetric methods have higher sensitivity than spectrophotometry, the poor selectivity represents a limitation; in addition their application is restricted to compounds that meet certain structural requirements. In the case of electrochemical methods, the main disadvantage is related to low reproducibility. On the other hand, chromatographic methods generally need sample pretreatment which is time-consuming and employs expensive

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instrumentation (Peri-Okonny et al. 2005; Shrivas & Wu 2007; Ayala et al. 2009).

Recently, a novel SPE method using multi-walled carbon nanotubes (MWCNTs) as the adsorbent was developed (Barbosa et al. 2007; Talio et al. 2013). The functionalisation of MWCNTs can introduce on their surface site oxygen-containing negatively charged functional groups. If a cationic surfactant is added to the functionalised MWCNTs, then interactions, such as hydrophobic and ionic, may lead to the formation of admicelle aggregates on the MWCNTs. A new kind of adsorbent, namely the admicelle capped, is obtained (Li et al. 2009).

In this work, oxidised MWCNTs were capped with hexadecyl-trimethylammonium bromide (HTAB) admicelles. As CF is a non-fluorescent analyte, the RhB dye introduces a fluorophore. The enhancement of the solid-surface fluorescence (SSF) of the dye on a membrane filter modified with the capped HTAB-MWCNTs was evaluated for chemofiltration and the determination of CF. The main aim of this work was provide a new tool for CF determination using low-cost instrumentation available in routine quality control laboratories.

Materials and methods

Reagents and materials

A stock of caffeine (Merck, Darmstadt, Germany; http://www.merck.com.ar) 1×10^{-4} mol l^{-1} and RhB 1×10^{-6} mol l^{-1} solution (Fluka AG, Chemische Fabrik, Buchs SG, Switzerland; http://www.sigmaaldrich.com/analytical-chromatography/fluka-analytical/about-fluka-and-riedel.html) were prepared weekly by dissolution of the appropriate amount of each reagent in ultrapure water.

Acetic/acetate (Mallinckrodt Chemical Works, New York, NY, USA) buffer solution 1×10^{-2} mol l^{-1} was prepared by dissolution of the appropriate amount of each reagent in ultrapure water. The pH was adjusted to the desired value by adding NaOH (Mallinckrodt Chemical Works; http://www.mallinckrodt.com/) solutions using a pH meter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) Model EA 940. Sodium dodecylsulfate (SDS), Triton® X-100 and hexadecyl trimethylammonium bromide (HTAB) were purchased from Tokyo Kasei Industries (Chuo-Ku, Tokyo, Japan; http://www.tcichemicals.com/es/ar/index.html). MWCNTs (CAS: 308068-56-6; diameter = 110–170 nm; length = 5–9 μm) were purchased from Merck (Darmstadt, Germany).

A solution of HTAB (Tokyo Kasei Industries) 1×10^{-3} mol l^{-1} was prepared by dissolution of the appropriate amount in ultrapure water.

Nylon membranes (Millipore, Sao Paulo, Brazil; http://www.millipore.com) with 0.45 μm pore size and 47 mm in diameter, cellulose acetate (Whatman, Maidstone, UK; http://www.whatman.com) of 0.45 μm pore size and 47 mm in diameter, mixed esters (Schleicher & Schuell, Dassel, Germany; http://www.ictsl.net/plaintext/productos/021b0796c20bcc817/schleicherschuell/default.html) of 0.45 μm pore size and 47 mm in diameter, Immobilon (+) (Millipore, Sao Paulo, Brazil) were used in sorption studies. All used reagents were of analytical grade.

Apparatus

Spectrofluorimetric measurements were made using a Shimadzu RF-5301 PC spectrofluorometer equipped with a 150 W xenon lamp and 1.00 cm quartz cells. A combined glass electrode and a pH meter (Orion Expandable Ion Analyzer) Model EA 940 were used for pH adjustments. A Gilson Minipuls 3 peristaltic pump with PVC pumping tubes coupled to an in-line filter holder 47 mm (Millipore) was used for filtrating samples/standard solutions. All glass materials used were previously washed with a 10% v/v HNO_3 water solution and then with ultrapure water.

Commercial samples – procurement and sample preparation

Dietary supplements and energy drinks were acquired in local shops. In order to guarantee representative samples, a randomised sampling strategy was applied. A total of three items of the same brand for each product were acquired. The whole of the contents of each product were homogenised and reserved for sample preparation. An adequate volume or weight of each sample containing 1.1–9.7 × 103 μg l^{-1} were dissolved/dispersed in ultrapure water, diluted to 100 ml in volumetric flasks and reserved for CF determination by applying the general procedure.

Activation and dispersion of MWCNTs

Several small portions of MWCNTs were put into Erlenmeyer flasks and activated with different solutions: H_2SO_4, HCl, HNO_3 and NaOH, 2 meq ml^{-1} in all cases. Moreover, a mix of H_2SO_4:HNO_3 (50:50) was also assayed in the activation step. MWCNTs were then rinsed with ultrapure water to remove the excess of reagent used and were then filtered using filter paper (S&S blue band) and dried at RT.

Portions of 5 mg of activated MWCNTs were weighed and suspended in solutions 1×10^{-3} mol l^{-1} of HTAB and SDS, respectively. Subsequently, they were allowed to stand for 24 h at RT; supernatant of the HTAB/MWCNTs sub-micellar solution was employed in membrane impregnation.

Membrane impregnation with MWCNTs

A total of 250 µl RhB 1×10^{-6} mol l^{-1} solution, 100 µl HTAB/MWCNTs sub-micellar solution and ultrapure water to 5 ml were mixed. Nylon membranes were submerged for 5 min in the previously prepared solution; they were then retired and dried at RT and stored in a dry environment (20–25°C) up to their use in the general procedure.

General procedure

A volume of 25–100 µl CF standard/sample containing 1.1–9.7 × 103 µg l^{-1} of analyte and 100 µl buffer acetic acid/acetate buffer solution 1×10^{-3} mol l^{-1} (pH 5.0) were placed in a 10 ml volumetric flask. The whole mixture was made to 10 ml with ultrapure water. Systems were filtered across the impregnated membranes using a peristaltic pump at 0.10 ml min^{-1} and dried at RT. SSF was determined at λ_{em} = 566 nm (λ_{exc} = 530 nm) using a solid sample holder.

Accuracy study

Adequate volumes of samples were spiked with increasing CF amounts 0.00, 1.94 and 3.88 µg l^{-1}. CF contents were determined by the proposed method.

Precision study

The repeatability (within-day precision) of the method was tested for replication of samples (n = 3) spiked with 1.95 µg l^{-1} CF and the contents were determined by this method.

Results and discussion

The CF molecule does not show native fluorescence. However, chemical associations of fluorescent dyes with this alkaloid have been reported (Zdunek et al. 2000; Bedner et al. 2001; Talio et al. 2013). This fact can be used as a starting point for the development of an analytical methodology in order to achieve CF determination by molecular fluorescence. Of all dyes, RhB and its derivatives have been extensively used with bioanalytical goals (Hojo et al. 2002; Alesso et al. 2012).

Experimental assays showed that in an aqueous solution the RhB fluorescence is kept unchanged by the addition of CF. However, when the system was filtered through a nylon membrane as a solid support, an increase in the fluorescent signal of RhB retained on the solid support (SSF) was observed. Additionally, in the presence of CNTs, the RhB SSF was more increased (Talio et al. 2013). This fact reinforces the theory of the existence of an association between RhB and CF, with an extra advantage in relation to the sensitivity due to the elevated superficial area of nanomaterials when SSF is used (Figure 1). As can be seen in Figure 1, the addition of CF produces an increase in the fluorescent signal of RhB (lines c and d). The increase was more noticeable when the solid support was modified with HTAB-MWCNTs (lines e and f). The enhancement factor (EF) was calculated with the experimental data of Figure 1 as follows:

$$EF = \Delta I2/\Delta I1 = (ICF\ 2 - I2)/(ICF1 - I1)$$

where ΔI is the difference in the relative fluorescence of the RhB dye when systems were filtered on solid supports in the absence (ICF1 and I1) and presence (ICF2 and I2) of MWCNTs, with (ICF1 and ICF2) and without (I1 and I2) CF. In this experimental condition, the obtained EF was 20, providing evidence about the improvement in the sensitivity of the proposed methodology for the MWCNTs.

Nanomaterial activation was realised by oxidation (Osorio et al. 2008; Tang & Xu 2011; Silva et al. 2012) using different chemical substances. The chemical functionalisation by mean HNO_3 (2 mequiv. ml^{-1}) resulted in the best means to increase the RhB and CF-RhB SSF signals. Therefore, this activation procedure was chosen for the following assays.

The proposed mechanism of reaction is based in the formation of HTAB admicelle aggregates on the MWCNT surface through electric interactions between oxygen-containing negatively functional groups on the surface of MWCNTs and cationic HTAB surfactant and then they were used as an adsorbent to pre-concentrate CF (Figure 2).

Among the experimental parameters, the volume of the HTAB-MWCNTs solution was optimised, varying from 0 to 0.250 ml. The best result for CF retention was

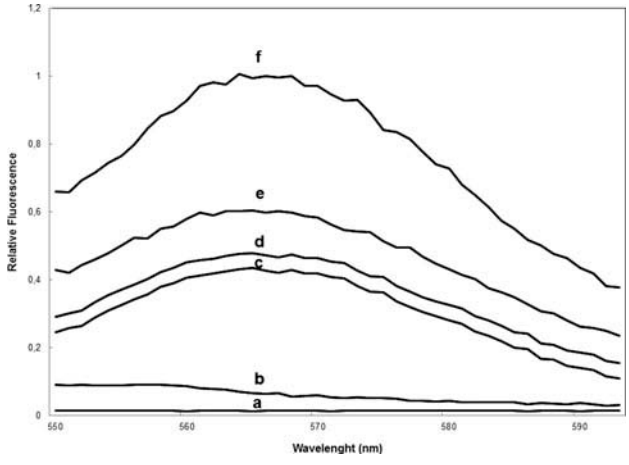

Figure 1. Solid-surface fluorescence emission of the RhB/MWCNTs/caffeine system (λ_{em} = 566 nm and λ_{exc} = 530 nm).

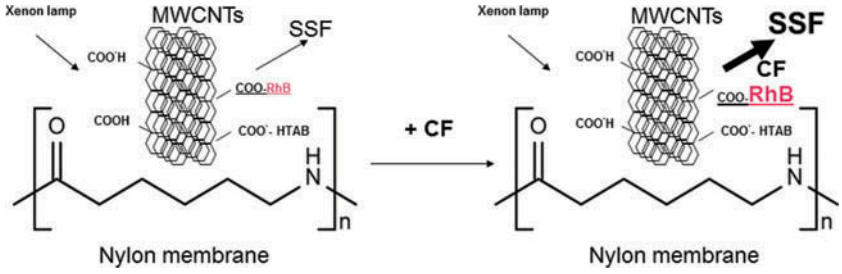

Figure 2. (colour online) Schematic representation of the proposed mechanism for the interaction of reagents in the developed methodology.

obtained by employing 0.10 ml of an HTAB-MWCNTs solution. The effect of the surfactant amount on the recovery of CF was studied. As can been seen from Figure 3, the analyte was better retained with the initial increasing concentration of HTAB. The results also showed that the CF recovery increased with surfactant concentration from 1×10^{-4} to 1×10^{-2} mol l^{-1}, above which the recovery decreased. Thus, 0.10 ml of 1×10^{-3} mol l^{-1} HTAB was selected for further study.

The other evaluated parameter was RhB concentration. In order to ensure the same contribution of the fluorescence signal, this parameter must be maintained constant in all samples/standards and the RhB concentration must be sufficiently high to guarantee the CF-RhB association and quantitative retention. A concentration of 5×10^{-8} mol l^{-1} was selected as optimal (Figure 4).

Nylon membranes were prepared by submerging them for different periods of time in an RhB/HTAB-MWCNTs solution. The minimal time that enabled quantitative RhB retention checked by the fluorescence signal was a time of 5 min, which was chosen as the optimal contact time (Figure 5).

The effect of the filtration flow rate was investigated by using a peristaltic pump over the range from 0.05 to 0.25 ml min^{-1}. The experimental results demonstrated that when the filtration flow rate was below 0.1 ml min^{-1}, the CF recoveries remain stable at over 99%. For a flow rate higher than 0.1 ml min^{-1}, a decrease in recovery was observed.

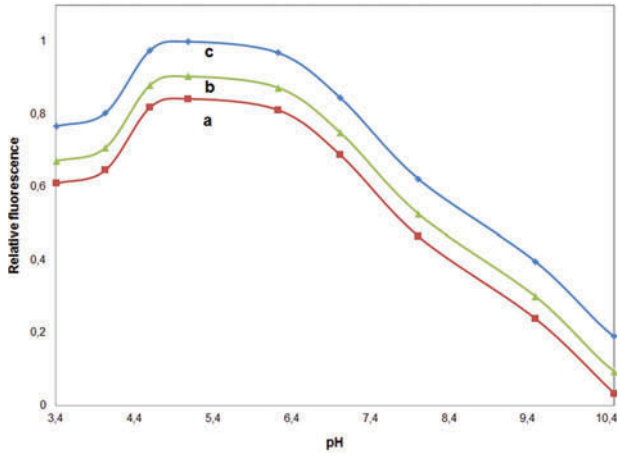

Figure 4. (colour online) Influence of RhB concentration and pH in caffeine retention.

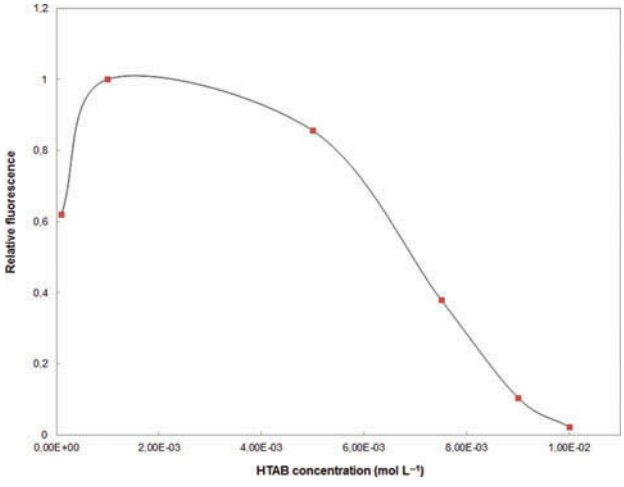

Figure 3. Influence of HTAB concentration on solid-surface fluorescence emission of the RhB/MWCNTs/caffeine system (λ_{em} = 566 nm and λ_{exc} = 530 nm).

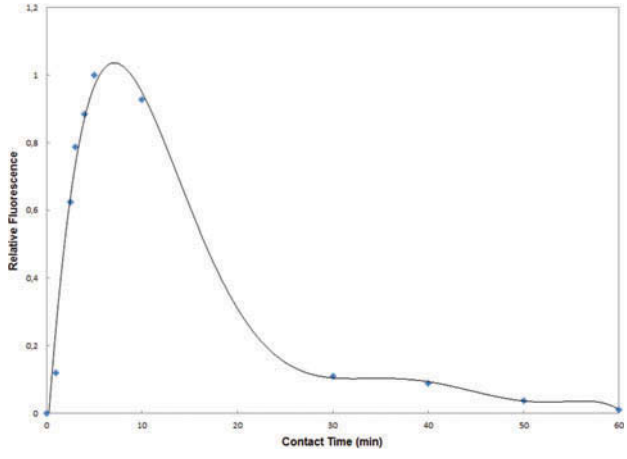

Figure 5. Influence of contact time on solid-surface fluorescence emission of the RhB/MWCNTs/caffeine system (λ_{em} = 566 nm and λ_{exc} = 530 nm).

Filtrate solutions were checked by molecular fluorescence in order to explore the possible presence of RhB that could cause a loss of signal in the following CF determinative step. In all cases, the fluorescence signal was null, indicating the quantitative retention of dye on the nylon membranes.

The effect of sample pH on the recoveries of CF was examined in a range of 3.0–11 by the addition of a diluted solution of HCl and/or NaOH. The results, shown in Figure 4, indicate that the retention amounts of CF by the admicelle-capped MWCNTs were greatly dependent on initial pH values. For maximum CF retention, the initial pH of the solution was adjusted to 5.0, and was selected as the optimum in this study. Filtrate solutions were checked by molecular fluorescence as previously indicated. Buffer concentration and nature were tested in order to obtain the maximal fluorescent signal. An acetic acid/acetate buffer solution was chosen as optimal at a concentration of 2×10^{-4} mol l^{-1}.

Analytical parameters

Calibration curve and tolerance

The calibration curve using zero-order regression for different concentration levels of CF from 1.1 to 9.7×10^{3} µg l^{-1} was attempted by applying the developed method. Table 1 summarises the main characteristics of the calibration plot and the optimised experimental conditions, which sustain the proposed procedure for CF quantification.

LOD and LOQ were calculated in accordance with the formulas given by the official compendia methods, using the relation k(SD)/m, where $k = 3$ for LOD and 10 for LOQ. SD represents the standard deviation from 15 replicate blank responses; and m is the slope of the calibration curve.

Table 2 presents the main analytical parameters of conventional methodologies by CF determination in different food and beverages matrixes. As can be seen, the new method showed adequate selectivity and sensitivity, comparable with other informed methodologies with the advantage of using a low-cost instrument (Shrivas & Wu 2007; Cianchino et al. 2008; Chen et al. 2010; Al-Othman et al. 2012; Hadad et al. 2012).

Validation and applications

In order to evaluate the versatility, the developed methodology was applied to commercial products with CF declared in the labels. Particular attention was paid to energy drinks and dietary supplements, since these products are usually consumed by the young and by athletes without professional surveillance.

In the experimental procedure, all commercial samples were dissolved in ultrapure water. In order to ensure quantitative CF retention, pH samples were conditioned by adding buffer acetic acid/acetate solution.

The validation and accuracy of this method were performed by using the standard addition method. In order to determine the repeatability (within-day precision) of the method, dietary supplement and energy drinks, replicate samples ($n = 3$) were analysed by the proposed methodology in a total of three levels of spiked CF for each sample. Precision was better than 0.09 CV for CF contents. Reproducibility (between-day precision) was also evaluated over 3 days by performing three determinations each day with a CV of 0.011. Table 3 show the recovery results achieved for each sample. The obtained results indicate that the proposed method is suitable for the determination of analytes in dietary supplement and energy drinks. As can be seen, an adequate concordance was obtained between CF nominal contents and the values found, with a relative error lower than 6% in all cases.

Quantitative retention of CF was checked by a double-filtration procedure, repeating the general procedure for the filtrate on a newly conditioned nylon membrane, and determining the SSF once more. In all cases, signals were not different from the blank signal, revealing quantitative retention of the analyte.

The results obtained showed that the proposed methodology result was appropriate for CF determination in such samples for all the range of studied concentrations.

Table 1. Experimental conditions and analytical parameters for the determination of caffeine.

Parameters	Studied range	Optimal conditions
pH	3.5–10.5	5.0
Buffer acetic/acetate	1×10^{-5}–1×10^{-2} mol l^{-1}	2×10^{-4} mol l^{-1}
MWCNTs volume	0–0.25 ml	0.1 ml
RhB concentration	1×10^{-8}–2×10^{-7} mol l^{-1}	5×10^{-8} mol l^{-1}
Contact time	0–700 min	5 min
Filtration flow rate	0.05–0.25 ml min^{-1}	0.1 ml min^{-1}
LOD	–	0.3 µg l^{-1}
LOQ	–	1.1 µg l^{-1}
LOL	–	1.1–9.7×10^{3} µg l^{-1}
r^2	–	0.999

Table 2. Analytical parameters of methodologies for CF determination in foods, beverages, dietary supplements and energy drinks.

Method	Comments	Reference
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. (2012)
GC-MS	LOL: 0.05–5.0 μg ml^{-1} Correlation coefficient = 0.980 RSD = 4.4% LOD = 4.0 ng ml^{-1} Applied to beverages and foods	Shrivas and Wu (2007)
CE	LOD = 0.42 μg ml^{-1} LOQ = 1.4 μg ml^{-1} Optimised experimental conditions were: background electrolite, sodium tetraborate buffer 20 mM, pH 9.2, voltage applied 30 kV, capillary temperature 25°C, injection sample at 0.5 Psi during 5 s RSD 0.24% Applied to herbal medicines and dietary supplements	Cianchino et al. (2008)
HPLC-DAD	LOD = 2.51×10^{-2} μg ml^{-1} LOQ = 8.36×10^{-2} μg ml^{-1} $R^2 = 0.9997$ Applied to commercial teas and dietary supplements	Hadad et al. (2012)
CEC	LOD = 0.48 μg ml^{-1} Reproducibility was tested by evaluating the intra- and inter-day precisions, and RSD < 8.4% Recovery (%) = 87.2–105.2% Applied to in beverages	Chen et al. (2010)
Voltammetry	LOD = 0.02 μg ml^{-1} LOL = 0.06–19 μg ml^{-1} LOQ = 0.06 μg ml^{-1} Applied to commercial teas samples	Guo et al. (2011)
GC	LOD = 0.02 μg ml^{-1} LOL = 0.05–500 μg ml^{-1} LOQ = 0.05 μg ml^{-1} $r^2 = 0.9990$ RSD = 3.2% Applied to commercial teas, coffees and beverages samples	Sereshti and Samadi (2014)
This method	LOL = 1.1 – 9.7×10^3 μg l^{-1} LOD = 0.3 μg l^{-1} LOQ = 1.1 μg l^{-1} $R^2 = 0.9989$ Recovery (%) = 97.40–105.25 Applied to dietary supplements and energy drinks	–

Conclusions

Oxidised MWCNTs capped with HTAB admicelles were successfully used as nylon membrane modifiers and a new luminescent methodology for CF determination was developed using RhB dye as a fluorophore. The presence of CF was evidenced by the increase of RhB SSF and performed by HTAB-MWCNTs. At the optimal experimental condition a quantitative retention of CF was obtained, representing an alternative to conventional methods for CF monitoring, with good reproducibility, low operational cost, generating non-polluting scarce waste, and also taking care of the analyst and the environment. In this sense, it represents a valuable contribution to green chemistry. The methodology was validated using the standard addition method and applied to commercial samples with good concordance with the declared CF contents. Considering the obtained results, health agents should pay attention to the indiscriminate use of CF products, including cosmetics, in order to prevent damage to health due to CF levels higher than the recommended maximal intake.

Additionally, taking into account the potential health risks, health authorities must start action to establish the regulatory boundaries around CF consumption.

Table 3. Caffeine determination in commercial samples: recovery study.

Sample	CF added (μg l^{-1})[a]	CF found ± CV (μg l^{-1})	Recovery (%, n = 3)	CF nominal content	CF found	RE%[b]
Dietary supplement I	–	2.43 ± 0.07	–	12 mg	12.15 mg	1.25
	1.94	4.35 ± 0.01	98.97			
	3.88	6.30 ± 0.02	99.74			
	9.70	12.15 ± 0.03	100.82			
Dietary supplement II	–	2.54 ± 0.08	–	12 mg	12.70 mg	5.8
	1.94	4.45 ± 0.02	98.45			
	3.88	6.44 ± 0.01	100.51			
	9.70	12.28 ± 0.07	101.60			
Dietary supplement III	–	1.65 ± 0.08	–	n.d.	0.0165%	–
	1.94	3.62 ± 0.03	101.55			
	3.88	5.50 ± 0.04	99.22			
	9.70	11.30 ± 0.09	96.70			
Energy drink I	–	1.97 ± 0.02	–	0.02%	0.0197%	1.5
	1.94	3.93 ± 0.04	101.00			
	3.88	5.84 ± 0.01	99.75			
	9.70	11.70 ± 0.06	101.50			
Energy drink II	–	3.02 ± 0.07	–	0.03%	0.0306%	2.0
	1.94	4.99 ± 0.04	101.55			
	3.88	6.89 ± 0.03	99.75			
	9.70	12.75 ± 0.05	101.00			
Tea I	–	2.77 ± 0.07	–	10 mg	10.20 mg	1.6
	1.94	4.79 ± 0.05	102.90			
	3.88	6.60 ± 0.06	98.20			
	9.70	12.38 ± 0.07	96.75			
Tea II	–	2.85 ± 0.04	–	10 mg	10.75 mg	1.8
	1.94	4.85 ± 0.01	102.10			
	3.88	7.00 ± 0.08	109.40			
	9.70	12.45 ± 0.09	96.50			

Notes: [a]Caffeine. [b]Relative error (%).

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