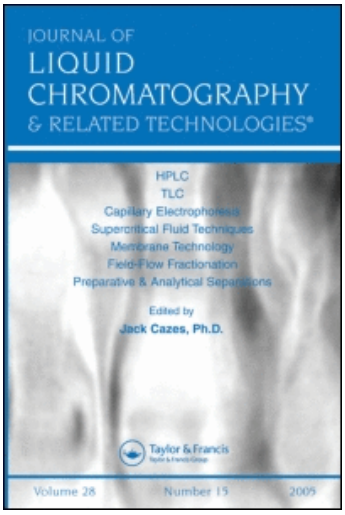

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Fast and Sensitive New High Performance Liquid Chromatography Laser Induced Fluorescence (HPLC-LIF) Method for Quinine. Comparative Study in Soft Drinks

X. Feás[a]; C. A. Fente[a]; A. Cepeda[a]
[a] Laboratorio de Hixiene, Inspección e Control Alimentario. Departamento de Química Analítica, Nutrición e Bromatoloxía. Facultade de Veterinaria. Campus de Lugo., Universidade de Santiago de Compostela, Lugo, Galiza, (Spain).

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Fast and Sensitive New High Performance Liquid Chromatography Laser Induced Fluorescence (HPLC-LIF) Method for Quinine. Comparative Study in Soft Drinks

X. Feás, C. A. Fente, and A. Cepeda

Laboratorio de Hixiene, Inspección e Control Alimentario. Departamento de Química Analítica, Nutrición e Bromatoloxía. Facultade de Veterinaria. Campus de Lugo. Universidade de Santiago de Compostela, Lugo, Galiza, (Spain).

Abstract: A simple, quick, and accurate new method for the determination of quinine (6′-methoxycinchonan-9-ol) in soft drinks is presented. The analysis is carried out using high performance liquid chromatography (HPLC), coupled laser induced fluorescence (LIF) that consisted of a 325 nm He-Cd laser and a ZETALIF detector. The chromatographic separation was performed on a Phenomenex Synergi Fusion-Reversed Phase (RP) column and allows good peak shape and symmetry in less than 1.5 min. A calibration curve ranging from 1 to 100 ng/mL was shown to be linear with a correlation coefficient (R) of 0.9999. The limit of detection of quinine was 3.2 pg on the column. The method was applied to the analysis of several beverages (n = 43) containing quinine, whose analysis required minimum pretreatment before direct injection, and can therefore be used for quality control in comparison to the classical methods. Data obtained from different commercial beverages containing quinine show no homogeneous concentration of this compound. This article describes, for the first time, the successful application of HPLC coupled LIF detection for quinine determination in common beverages.

Keywords: High performance liquid chromatography (HPLC), Laser-induced fluorescence (LIF), Quinine, Soft drinks

Correspondence: Xesus Feás, Laboratorio de Hixiene, Inspección e Control Alimentario. Departamento de Química Analítica, Nutrición e Bromatoloxía. Facultade de Veterinaria. Campus de Lugo. Universidade de Santiago de Compostela, E-27002 Lugo, Galiza, (Spain). E-mail: xesusfeas@gmail.com

INTRODUCTION

From the bark of *Cinchona* and *Remijia* species, trees of *Rubiaceou* genera, a number of various alkaloids can be isolated. Quinoline compounds are the principal Cinchona alkaloids, including cinchonine, cinchonidine, quinidine, and quinine, of which, this last represents the 70–90% of the total bark alkaloids.[1]

Quinine (Figure 1) has a long history of use and has been touted as an antimalarial agent, in the treatment of painful cramps and in influenza infections. Also having antipiretic, antiseptic, analgesic, and antalgic properties, it is a component in several pharmaceutical formulations. Moreover it has been utilized by the cosmetic industries and used to cut street heroin and cocaine.

The most important and extensive use is as an ingredient of regularly absorbed drinks such as tonic water, indian tonic water, bitter lemon, and related soft drinks, due to its bitter taste it has been added as a flavouring agent and provides a refreshing gustatory stimulation.

However, the use of quinine therapeutically or consumed excessively may cause cinchona, with toxic manifestations including gastrointestinal, visual, auditory, thrombocytopenia, cardiovascular and neurological effects, such as headache, confusion, coma, blindness, and psychosis.[2]

Toxicologically, quinine is important but should be avoided by pregnant women and people with hepatic failure,[3] and remains potentially an extremely toxic agent in children.[4] Also, an allergies to quinine and anaphylactic shock after drinking a glass of tonic water has been described.[5,6] A special report on the potential health risks linked to the consumption of quinine containing beverages can be accessed on line on the Germany's BfR.[7]

As a result, in China quinine is not legally permitted to be added to drinks.[8] In the United States quinine must be under 83 mg/kg and the

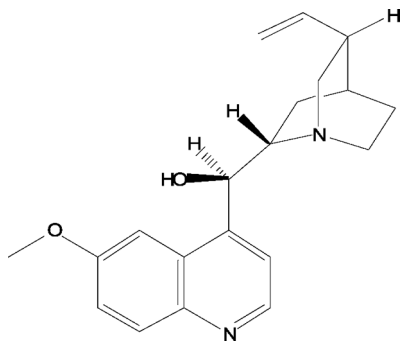

Figure 1. Chemical structure of quinine.

label shall bear a prominent declaration of the presence of quinine.[9] The European Commission has established new rules governing the labelling of quinine in drinks and food, making it necessary to clearly indicate to the consumer the presence of quinine in the list of ingredients, but current labelling rules do not require a warning message followed by an indication of the quinine content.[10] However, the commission directive recognised that consumption of quinine may be counter indicated for certain people for medical reasons, or because they are hypersensitive to the substance. The Member States shall prohibit trade in products which do not comply with this Directive as of 1 July 2004.

The determination of quinine, alone or their metabolites, has been of interest in different matrices such as hair,[11] biological fluids (plasma, urine, and whole blood),[12] bark extracts, pharmaceutical and cosmetic preparations.[13] Several methods were proposed, mostly for the concrete determination of quinine in soft drinks. These techniques include atomic absorption spectrometry (indirect determination) with reversed flow injection system,[14] flow through sensor with fluorimetric transduction,[15] isotachophoresis with UV and conductivity detection,[16] 4th-order derivative spectrophotometric,[17] spectrophotometric,[18] fluorimetry,[19,20] plasticized poly(vinyl chloride) membrane electrode with direct potentiometry,[21] optosensor with C_{18} silica gel as substrate in conjunction with a flow injection analysis system,[22] cyclic voltammetry,[23] and immunoassay.[24]

In this paper a simple, rapid, and sensitive method was developed for the routine determination of quinine based on high performance liquid chromatography (HPLC) with LIF detection. None of the previous methods described the determination of quinine in soft drinks using a laser as the excitation source, and the present technique offers advantages of improved sensitivity and selectivity.[25] Finally, our purpose was to determine the concentration of quinine in commercial beverages from different countries. The method has successfully been applied to the determination of quinine in soft drinks.

EXPERIMENTAL

Reagents and Chemicals

Quinine hemisulfate salt monohydrate, acesulfamate K, aspartame, saccharine, sodium benzoate, sorbic acid, glucose, and phosphoric acid were obtained from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Deionized double distilled water was purified with a Milly-Q system from Millipore (Bedford, MA, USA). All chemicals and reagents used in the preparation of standards and solutions were analytical or HPLC grade.

Standards

Stock standard solutions of quinine were prepared in distilled water at a concentration of 100 mg/mL. Standard working solutions were freshly prepared each day by dilution in deionized water, dissolving appropriate amounts of stock standard solutions by ultrasonic treatment.

Instrumentation

The liquid chromatographic system consisted of a pump model 980-PU from JASCO (Tokio, Japan). The injection device was a Rheodyne Model 7725 injector (Cotati, CA, USA).

The chromatographic separation was achieved on a Synergi Fusion-Reversed Phase (RP) column: silica-based dodecyl phase; 50 mm length x 2 mm inner diameter; particle diameter = 4 μm; nominal pore size = 80 Å (Phenomenex, Torrance, CA, USA). The column was kept at room temperature.

The mobile phase was a mixture of methanol-acetonitrile-ammonium acetate (45/15/40 v/v/v), subsequently filtrated and degassed (using nylon filter, 4 mm diameter, pore size 0.45 mm, about 20 min) under vacuum. The flow rate was maintained at 0.5 mL/min.

The HPLC was coupled to LIF system that consisted of a 325 nm He-Cd laser of 15 mW, a High Voltage Power Supply LC500–220RC (Melles Griot Laser Group, Carlsbad, CA, USA), and a ZETALIF detector (Picometrics, Ramonville, France). Flexible Fused Silica Capillary Tubing with standard polyimide coating (320 mm ID, 435 mm OD and 18 mm of coating thickness) from Galiza Analítica (Vigo, Galiza, Spain) was connected to the output of the HPLC column. It is made transparent by removing a portion of polyimide coating from the capillary at alture of the cell detection. A microscope objective is used to adjust the capillary and flow cell position. The capillary had a total length of 21 cm (10 cm to the detector). Every day prior to use, the capillary was washed with 0.1 M NaOH (2 min) and water (2 min).

Parameters adjusted in the ZETALIF detector are: photomultiplier high voltage (570 Volt), rise time (0 sec), maximum power (10 mW), and relative fluorescence units (2 RFU). Characteristics of the laser induced fluorescence system are: continuous wavelength output (325 nm), power (15 mV), excitation power (8 mV), and type (helium-cadmium).

External interfaces for the acquisition of dates Hercule-Lite was coupled and chromatographic data were recorded and processed with Jasco-Borwin software JMBS Developments (Le Fontanil, France).

Sample Preparation

The samples from different manufacturers, including tonic water (TW), indian tonic water (ITW), bitter lemon (BL), and grape tonic (GT) were purchased from a local market in Galiza, Spain (Gz), Portugal (Pt), France (Fr), Czech Republic (Cz), the Netherlands (Ne), Sweden (Sw), Denmark (Dn), Mozambique (Mz), Italy (It), United States of America (USA), Poland (Po) and Argentina (Arg). A total of 43 samples were used in this study, with the following distribution (n; country): 2 Dn, 6 Sw, 4 Po, 6 Cz, 2 Pt, 2 Arg, 1 Ne, 3 Fr, 3 It, 2 U.S.A., 1 Mz, and 11 from Galiza (Spain).

One mL of each soft drink in an eppendorf tube was degassed in an ultrasonic water bath to remove carbon dioxide for 5 min, and filtered through a 0.45 mm pore size nylon filter of 4 mm diameter (National Scientific Company, Duluth, GA, USA). Finally, 50 μL of each resulting sample was diluted accurately to 250 mL with water in a volumetric flask.

Interference and Stability Studies

In order to get an idea of possible interferences of common additives in soft drinks under the specified experimental conditions, synthetic tonic water was prepared. Known amounts of all the additives were investigated, thus achieving concentrations for each component lower than the corresponding legal limits set by the European Community.[26] In all cases, synthetic tonic water was prepared by dissolving quinine (10 ng/mL), saccharine (80 mg/mL), glucose (3%), phosphoric acid (150 mg/mL), acesulfamate K (350 mg/mL), aspartame (600 mg/mL) and sodium benzoate (30 mg/mL) in water.

The stability of quinine was studied at room temperature (20°C) in standard solutions, synthetic tonic water, and real soft drinks containing quinine (n = 6). Samples were analyzed over a period of 1 month every seven days as described previously in the sample preparation section.

Calibration

The linear relation between quinine concentration and peak area was estimated over the range of 1 ng/mL to 100 ng/mL to validate the procedure.

The detection limit (LOD) was calculated following the rules of the Food and Drug Administration, where LOD is defined as the signal corresponding to three times the noise standard deviation.[27] For LOQ (limit of quantification) we consider the minor point of calibration. Precision of the method was evaluated in terms of inter-day and intra-day

repeatability, injecting standard solutions at the same concentration six times over 3 different days, and nine times in the same day, respectively. Each assay was carried out at three different quinine concentrations: high (100 ng/mL), medium (10 ng/mL), and low (1 ng/mL). The relative standard deviations (RSD) of the data obtained were calculated and inter-day precision was defined as the highest R.S.D. recorded for the 3 days, and the repeatability (intra-day) as the highest R.S.D. obtained for the 3 levels injected at the same day.

To evaluate the accuracy of the method, three synthetic tonic waters, free from quinine were prepared (see previous interference and stability studies). Each sample was then spiked with appropiate amounts of quinine standard solutions to finally work within the stipulated calibration range at three different concentrations: 100 ng/mL, 10 ng/mL and 1 ng/mL. These spiked samples were taken through the entire analytical procedure described above. Results were derived from the corresponding calibration curve.

RESULTS AND DISCUSSION

The available methods of analysis for quinine in beverages have been compared in terms of linear range, detection limits, sample preparation, and detection methodologies in Table 1.

In this work, sonication to expel CO_2, filtration and dilution in water were involved in sample preparation. Neither a preliminary liquid-liquid extraction[18] with a crystallization of quinine from soft drinks,[22] nor additional special solutions (H_2SO_4 or Na_2PO_4) added to the dilution of sample are necessary[15,21] in this first step before analysis. The present method use 1 mL of soft drink for the sample preparation. The volume used for other authors ranges from 0.4 μL[16] to 100 mL.[15] In the present method it is not necessary to heat the sample[15] or to adjust the pH of sample to posterior analysis,[21] resulting in minor time consumption in sample preparation. The method proposed by García et al. is only valid for soft drinks without colouring matter and with low levels of benzoic acid.[15] Our method permits the analysis of all beverages even with coulorings and other compounds, as we demostrated in the study of interferences.

Since the concentrations of quinine were not stated on the label of soft drinks, in all but two cases, sample dilution was performed to reach the working range with the previous information and data obtained in literature. The pre-treatment consisted of a simple degassing step by sonication followed by filtration and dilution.

Quinine is a basic compound with complex multi-ring structure, and is a diprotic weak base, ionizable with two basic nitrogen atoms, with pKa_1 and pKa_2 values around 4.3 and 8.5, respectively.

Table 1. Figures of merit for comparable methods of quinine determination in beverages

Method	Analytical range	Detection limit	Sample preparation	Ref.
HPLC-LIF	1–100 ng/mL	0.64 ng/mL	1 mL SD, So (5 min), F, 50 μL, D (250 mL H_2O)	this work
$_r$FIA-AAS$_{id}$	5–110 μg/mL	2 μg/mL	So, D (1:1 H_2O)	[14]
FS-Fl	40–800 (iv: 40 μL)	2.2 μg/L	100 mL SD, H (<40°C), So, D (H_2SO_4 0.1 M)	[15]
ITP-UV-CD	1–10 μg	5 mg/L	So, 4 μL iv	[16]
^{4th}SP	1–6 μg/mL	nr	5 mL SD, D with 50 mL H_2SO_4 0.05 M and 5 mL D with 50 mL H_2O	[17]
HPLC-Fl	0.01–0.7 ng/μL	0.3 ng	So (10 min), D and F	[19]
IP-RP-HPLC	0.1–20 mg/mL	0.004 μg/mL (UV) 0.02 μg/mL (Fl)	2.5 g SD So (5 min), D with H_2O and F	[20]
PVC$_m$-DP	0.01–10 mM	6.3 μM	50 mL SD + 5 mL Na_2PO_4 bf	[21]
FIA-OP C_{18}	5–20 ng/mL	2.3 ng/mL	5 mL SD + 5 ml 0.1 M NaOH, LL 5 ml CH_3Cl (1.5 min), C and D with 0.1 M H_2SO_4.	[22]

Acronyms for sample preparation: So, sonication; SD, soft drinks; bf, buffer; C, crystalization; iv, injection volume; H, heated; D, dilution; LL, liquid-liquid extraction; nr, not reported.
Acronyms for methods: FIA-AAS$_{id}$, reversed flow-injection with atomic absorption spectrometry (indirect determination); FIA-OP C_{18}, flow-injection analysis with silica C_{18} optosensor; PVC$_m$-DP, plasticized poly(vinyl chloride) membrane electrode with direct potentiometry; SP, spectrophotometric; Fl, fluorimetric; ITP-UV-CD isotachophoresis with ultraviolet and conductivity detection; FS-Fl, flow-through sensor with fluorimetric transduction; HPLC-LIF, high-performance liquid chromatography with laser-induced fluorescence detection; HPLC-Fl, high-performance liquid chromatography-fluorimetric detection; ^{4th}SP, 4th-order derivative spectrophotometric; IP-RP-HPLC, ion-pair reversed-phase high performance liquid chromatography.

Stationary phase may be altered, with the compound's chargue and its pK_a relative to the pH of the mobile phase, and the residual unbonded silanols. Free ''residual silanols'' (non-bonded surfaces silanol

sites: Si−OH) on the support material are able to establish hydrogen bonding and ion-exchange interactions with samples and eluents. These interactions, however, can change the retention of polar analytes and lead to tailing peaks, especially with basic compounds and aqueous eluents.

Depending on the pH of the mobile phase, the ionisation of silanols varies. At neutral pH, silanols are able to create ionic interactions with protonated basic compounds, and it is accepted that isolated silanols mainly are responsible for these interactions with polar solutes.[28] For these reasons, the pH values controlled the ionization of silanols and the charge of quinine. Most workers have used acid mobile phase conditions[29] and the addition of an ion-pair reagent in mobile phase was employed.[20] Although some manufactures have introduced silica based technology that is more resistant to high pH, it is important to take note that silica dissolves at high pH and, if possible, it is not recommended to use solvents that exceed pH 7 (it can cause problems with shorten column lifetimes).

Both the use of high purity silica (99% metal free) and a full hydroxylation of silica (the C_{18} ligand allows good hydrophobic retention and selectivity, while the polar embedded groups provide increased polar retention) ensures minimal surface metal sites available for chelation and reduces silanol acidity, under neutral mobile phase conditions, providing an improvement in the peak symmetry of basic compounds.

In this paper with the silica based dodecyl phase Synergi Fusion-RP column it is possible obtain good peaks with fast run times (retention time: 1.5 min) under pH = 7.1 mobile phase, for quinine compared with traditional C_{18} columns (Figure 2).

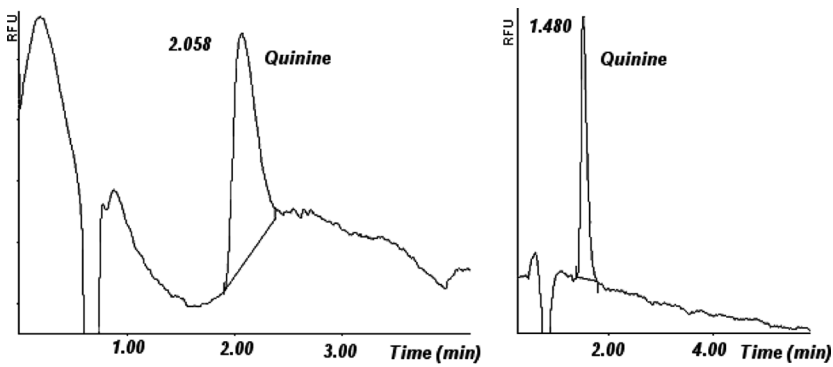

Figure 2. Chromatograms obtained with a Zorbax (a) and Synergi Fusion-RP column (b), mobile phase: methanol-acetonitrile-ammonium acetate (45/15/40 v/v/v) at a flow rate of 0.5 mL/min. The new column allows a good peak shape and symmetry.

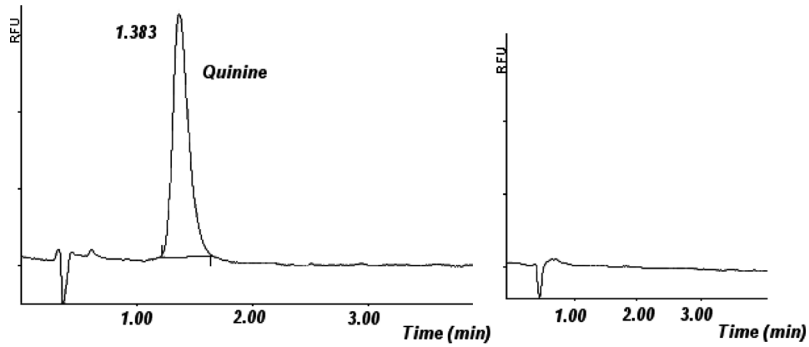

Figure 3. Chromatograms of a synthetic tonic water (a) and a blank soft drink (b). The proposed method is free from interferences of potential substances in large amounts of all species tested present normally in commercial soft drinks.

A study of interferences was performed by analyzing synthetic sample solutions. No interferences were noticed from other food additives and no interfering peaks were visible from the synthetic tonic beverage without quinine at the retention time of the analyte. Figure 3 shows the chromatogram of synthetic tonic water and a blank beverage, under the experimental conditions, the common additives present in soft drinks did not interfere with the quinine detection in real samples.

The stability of quinine was also studied. Samples were analyzed every seven days, and no decrease in concentration was seen after one month in standard solutions, commercial soft drinks, and synthetic tonic waters (data not shown).

The linearity of the method was determined over 3 days by the injection of 0.005, 0.025, 0.05, 0.2, and 0.5 ng of quinine. A calibration curve was constructed over the concentration range of 1 to 100 ng mL. Each injection assay was repeated three times. The peak area versus concentration plot showed a good linearity ($y = 1.1074x + 0.5489$) with a correlation coefficient (r) of 0.9999.

The theoretical detection limit was estimated to be 3.2 pg of injected material corresponding to a 0.64 ng/mL solution. Figure 4 shows the chromatogram of a standard solution 1 ng/mL corresponding to the minor point of the working curve and, in fact, we consider the minor point of calibration curve as the LOQ.

The repeatability inter-day of the method, defined as the highest relative standard deviation for all levels recorded for the 3 days, was 2.1%. Values ranged from 0.8% (100 ng/mL), 1.1% (10 ng/mL) to 2.1% (1 ng/mL). For repeatability intra-day, the R.S.D. did not exceed 1.8% (values ranged from 0.6% (100 ng/mL), 0.9% (10 ng/mL) to 1.8% (1 ng/mL).

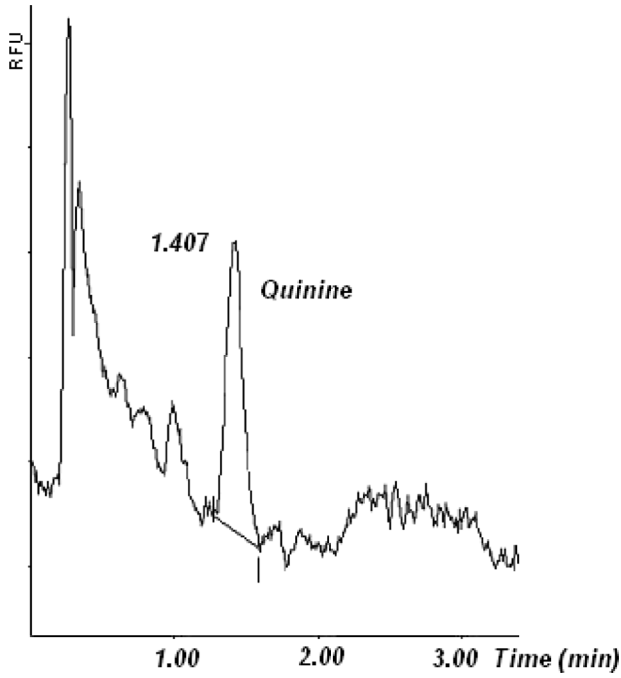

Figure 4. Chromatogram of standard solution 1 ng/mL corresponding to the minor point of the working curve.

The results for accuracy comparing spiking concentrations and measured concentrations at three levels (100 ng/mL, 10 ng/mL and 1 ng/mL) expressed as mean (%) ± S.D, were: 99.8 ± 0.1, 10.3 ± 0.2, and 0.98. ± 1.1, respectively.

Results for soft drink samples are summarized in Table 2. We wish to emphasize that information about quinine concentration in soft drinks was requested from various beverage enterprises, but unfortunately this information was not supplied. Directive 2000/13/EC[30] does not provide for a compulsory and specific mention of quinine in the list of ingredients, because it is used as a flavouring agent. However in the last directive,[10] quinine used in the production or preparation of a foodstuff, must be mentioned by name in the list of ingredients, immediately after the term "flavouring". Only this directive is clear for caffeine in a proportion in excess of 150 mg/L, the following message must appear on the label in the same field of vision as the name under which the product is sold. Soft drinks from different manufactures do not always comply with this directive.

Data obtained from soft drink analysis shows significant differences between countries and the kind soft drinks containing quinine. It could be necessary that the label shall bear a prominent declaration of the

Table 2. Results found on real samples by the procedure proposed. Concentrations expresed as mg/L ± RSD (n = 9).[+] nd (lower than limit of detection)

Soft drink sample	Country	Contains quinine
Indian tonic water	Denmark	83.97 ± 0.88
Bitter lemon		31.01 ± 0.44
Bitter lemon	Sweden	33.99 ± 0.41
Indian tonic water		24.02 ± 0.27
Tonic water		77.31 ± 0.71
Bitter lemon		37.93 ± 0.35
Grape tonic		7.20 ± 0.03
Grape tonic		8.43 ± 0.04
Indian tonic water	Poland	72.00 ± 1.05
Tonic water		37.86 ± 0.38
Tonic water		26.44 ± 0.35
Tonic water		nd[+]
Tonic water	Czech Republic	45.29 ± 0.60
Indian tonic water		77.58 ± 1.06
Lemon tonic		22.92 ± 0.25
Tonic water		26.40 ± 0.32
Tonic water		61.97 ± 0.62
Indian tonic water		41.28 ± 0.51
Tonic water	Portugal	45.30 ± 0.61
Tonic water		61.86 ± 0.83
Tonic water	Argentine	24.62 ± 0.34
Tonic water		17.68 ± 0.18
Tonic water	The Netherlands	50.65 ± 0.48
Tonic water	France	74.42 ± 1.00
Indian tonic water light		40.84 ± 0.37
Indian tonic water		77.76 ± 1.21
Tonic water	Italy	49.59 ± 0.59
Tonic water		55.80 ± 0.55
Tonic water		75.64 ± 0.69
Tonic water	United States of America	nd[+]
Tonic water		45.05 ± 0.65
Tonic water	Mozambique	92.98 ± 0.94
Tonic water	Galiza	54.02 ± 0.50
Tonic water		58.99 ± 0.62
Tonic water		98.74 ± 1.32
Tonic water		58.60 ± 0.72
Tonic water		79.75 ± 1.15
Tonic water		63.19 ± 0.94
Tonic water		65.95 ± 0.84
Tonic water		70.14 ± 0.72
Tonic water		95.88 ± 1.28
Lemon tonic water		72.22 ± 0.81
Tonic water light		76.78 ± 1.14

presence of quinine, since people who normally consume soft drinks containing quinine should be warned of the health risks. It is already known that quinine should be avoided by persons with certain metabolic disorders, or with a hypersensitivity to the substance.

There is a need to mandate that foodstuffs containing quinine include indication of the amount of quinine and information of possible effects, since it is common to use beverages containing quinine as homemade remedies to treat certain ailments.

In two samples, a tonic water from Poland and another from the United States of America, quinine was not detected after sample preparation. In this case, these samples without previous dilution were injected directly into the HPLC-LIF system, with negative results about the presence of quinine in these soft drinks. Information related by the labels of these products show that they should contain quinine. In only two of the samples was the quinine concentration declared on the labels, specifically samples from Mozambique and the Netherlands. The labels indicated 67 mg/L and 52 mg/L, respectively, while data analysis shows a result of 92.98 ± 0.94 mg/L and 50.65 ± 0.48 mg/L. Also the other tonic water from USA shows 45.05 ± 0.65 mg/L of quinine, according with USA legislation[9] that must be under 83 mg/kg.

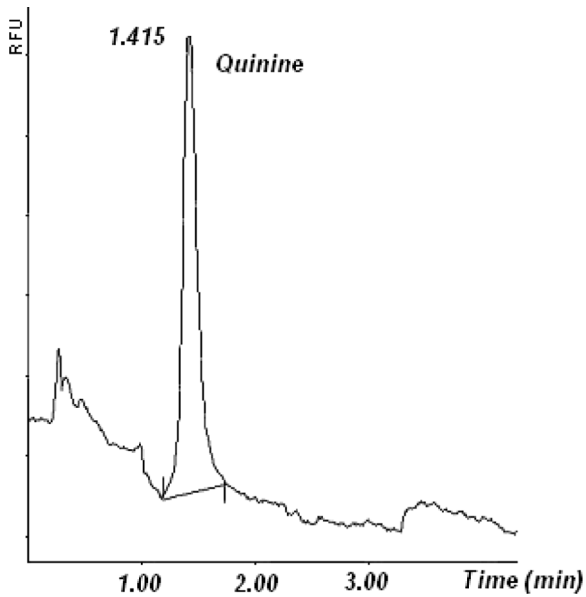

Figure 5. HPLC chromatogram of Indian tonic water sample from Denmark, under optimized conditions. Concentration of quinine in drink sample was observed 31.01 ± 0.44 mg/L.

In Sweden, there are grape tonic drinks in which the content of quinine is low (7.20 ± 0.03 mg/L) (8.43 ± 0.04 mg/L) in comparison with other soft drinks. Figure 5 shows a chromatogram obtained from an Indian tonic water from Denmark.

However, in Spain, it was possible to buy two tonic water drinks similar in composition with a final concentration of quinine that is almost doubled in one when compared to the other (54.02 ± 0.50 mg/L in comparison with 98.74 ± 1.32 mg/L).

In any case, data show that quinine concentrations in the analyzed beverages is variable. It could be because there is no specific regulatory legislation in the European Union and it is clear that people who consume tonic water like the bitter taste, and a higher concentration of quinine provides greater gustatory stimulation. We agree that new regulation is neccesary, and labels provided must not only declare quinine presence but also its concentration and potential health risks.

CONCLUSIONS

The new method presented here for quinine assay in beverages, which is based on laser induced fluorescence detection, excels in its precision, speed, and sensitivity. Samples must only be degassed, however, this step can be automated very easily. The analysis takes only 1.5 min, with a new Synergi Fusion-RP column it is possible to obtain good peaks with fast run times under neutral pH mobile phase, while peak tailing and broadening were eliminated. The food additives and ingredients normally present in soft drinks were found not to interfere in quinine analysis in the method described. This ultra sensitive methodology will be used in the future for metabolism studies of quinine and their metabolites, such as other principal cinchona alkaloids (quinidine, cinchonine, and cinchonidine), which exhibit native fluorescence. We believe there is a need for more extensive information for risk groups and to raise awareness among consumers about possible adverse reactions to this popular beverage flavouring.

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