Journal of Food Composition and Analysis xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

Preconcentration and indirect quantification of trace nitrite, nitrate and total nitrite in selected beverage and milk samples using ion-pairing cloud-point extraction with acridine orange

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ARTICLE INFO

Article history:
Received 12 December 2014
Received in revised form 4 April 2015
Accepted 6 April 2015
Available online xxx

Keywords:
Acridine orange
Food safety
Food analysis
Nitrite
Beverage and milk samples
Food composition
Triton X-114
Cloud-point extraction
Spectrophotometry

ABSTRACT

A new indirect preconcentration method was developed for micellar-sensitive detection of trace nitrite as analyte by spectrophotometry. It is based on selective ion-pairing complex formation of triiodide ion, I_3^- with acridine orange, AOH^+ (3,6-bis(dimethylamino) acridine) at pH 4.0 and its cloud-point extraction (CPE) with Triton X-114 as extracting agent. The extracted surfactant-rich phase is diluted with acetonitrile, and its absorbance is measured against analyte blank at 450 nm. The variables affecting CPE efficiency were investigated, and a set of optimized conditions was obtained. The calibration graph was rectilinear in the range of 1.5–150 μg L^{-1}. The detection limit was 0.42 μg L^{-1} with a precision lower than 4.1%. The validation was statistically confirmed by analysis of a certified reference material. The method was successfully applied to determination of nitrite, nitrate and total nitrite in selected beverage and milk samples before and after reduction with two different reducing agents.

1. Introduction

In recent years, an increasing interest concerning the determination of nitrate and nitrite levels in food products has been observed (Simion et al., 2008). The use of nitric acid as a sanitizer within dairy plants represents a further risk of nitrate contamination. Although generally associated with increased risk of several pathologies, it is notable that recent studies indicate that many health benefits such as important beneficial effects on cardiovascular function may be derived from consumption of dietary NO_3^- and NO_2^- based on nitrate-rich foods and beverages, through maintenance of systemic nitric oxide, NO_x homoeostasis (Hord et al., 2009). Nitrate ion is not directly toxic but it can readily be converted to harmful nitrite ion by microbial reduction. Nitrite can interact with hemoglobin to form methemoglobin by oxidation of ferrous iron, Fe(II) to the ferric state, Fe(III), a condition described as methemoglobinemia that is dangerous, especially in infants, and it is called as blue-baby syndrome. Nitrate is naturally present in leafy vegetables and nitrite is often added to meat and milk products as a preservative. Nitrite has several adverse effects upon human health (Cammack et al., 1999). Nitrite can interact with hemoglobin to form methemoglobin in infants and young children above 50 mg kg^{-1} of baby foods. An acceptable daily intake (ADI) of 0–3.7 mg (kg body weight)$^{-1}$ day^{-1} for nitrate was established by the Joint FAO/WHO Expert Committee on Food additives (JEFCA) in 2002 (FAO/WHO, 2003). The acceptable daily intake (ADI) for nitrite was set at 0.06 mg (kg body weight)$^{-1}$ day^{-1}. The maximum legally allowable limit for nitrate contents is 200 mg kg^{-1} for fruit- and vegetable-based foods. There was no legislated value for nitrite contents (FAO/WHO, 2003). For methemoglobinemia in infants, it was also confirmed that the existing guideline value for nitrate ion in drinking water of 50 mg L^{-1} is protective. For nitrite, human data reviewed by (JECFA) support the current provisional guideline value of 3 mg L^{-1} (Van Leeuwen, 2000). Due to their toxic effects onto food safety and human health, it is important to develop simple, low-cost, selective, accurate and reliable methods for their determination in beverage and foods.

Many reports previously published in literature have been published concerning the direct and indirect determination of

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http://dx.doi.org/10.1016/j.jfca.2015.04.009
0889-1575/

nitrite such in real matrices like beverages, foods and waters by means of flow analysis-vapor phase generation-Fourier transform infrared (FA-VPG-FTIR) (Gallignani et al., 2004), anodic voltammetry (Santos et al., 2009), amperometric detection (Salimi et al., 2007), diffuse UV–visible reflectance method (Khan et al., 2012; Luiz et al., 2012), optical and electrochemical sensors (Zhang et al., 2012; Ensafi and Amini, 2010, 2012; Chen et al., 2008; Mesároš et al., 1998), catalytic-spectrophotometric method (Sobhanardakani et al., 2013), spectrophotometric detection coupled with cloud-point extraction (CPE) (Afkhami et al., 2005; Pourreza et al., 2012; Filik et al., 2011), voltammetric techniques such as differential pulse voltammetry (DPV) and anodic stripping voltammetry (ASV) (Guanghan et al., 1997; Yilmaz and Somer, 2008; Thomas et al., 2012), solid-phase spectrophotofluorimetry (Li and Jiang, 2007), solid-phase fluorescent quenching method (Li et al., 2003), amperometric sensor based on ionic liquid (Zhou et al., 2013), microchip electrophoresis (Shiddiky et al., 2009), HPLC and ion-pair chromatography with chemiluminescence detection (Kodamatani et al., 2009, 2011), which suffer from limited linear dynamic range, low detection limit, poor precision, and low sensitivity/accuracy.

Among all the above-mentioned analytical methods, spectrophotometry is an important tool, which is most widely used in developing countries due to its low cost, easy operation, high accuracy/precision and good selectivity when a selective chromogenic or fluorogenic reagent for analyte is especially used. However, sometimes, there are some difficulties for the direct determination of nitrite by spectrophotometry since its accuracy and selectivity as well as sensitivity is not sufficient for the beverage samples due to matrix effect. For this reason, an extraction and preconcentration step is often required before spectrophotometric detection of the analyte at a suitable wavelength. Its sensitivity and detection power can be improved with the use of CPE procedure for preconcentration. In this way, CPE techniques exploit a peculiar property of most non-ionic surfactants that form micelles in aqueous solution: they become turbid when heated to the appropriate cloud-point temperature. Above the cloud-point temperature, the micellar solution separates into a small, surfactant-rich phase and a larger diluted aqueous phase. In the aqueous phase, the surfactant concentration remains near the critical micelle concentration (CMC). Any analyte solubilized in the hydrophobic core of the micelle in the unheated solution will be concentrated in the surfactant-rich phase following the CPE (Bezerra et al., 2005; Ojeda and Rojas, 2009).

When CPE technique is used for the extraction of a stable hydrophobic ion-pairing complex, spectrophotometry may efficiently be used as a detection tool. Though spectrophotometry has poor sensitivity, it is a very simple, rapid and low cost analytical tool, which can be found in almost every analytical research laboratory. CPE is also very simple, rapid, eco-friendly separation and preconcentration procedure with high preconcentration factor. Combination of CPE and spectrophotometry leads to a very simple, rapid and low cost analytical method with adequate sensitivity and selectivity.

In this context, in order to provide a way to monitor the levels of nitrite in selected beverage and milk samples, our main aim was to evaluate the feasibility of combining CPE preconcentration with spectrophotometry at 450 nm. In this procedure, acridine orange, AOH^+ (3,6-bis(dimethylamino) acridine) was used as the ion-pairing reagent, and Triton X-114 as the extracting agent in the presence of excess iodide at pH 4.0. The variables affecting CPE efficiency were extensively investigated. The method developed was successfully applied to the quantification of trace nitrite, nitrate and total nitrite in selected beverage and milk samples before and after reduction of nitrate to nitrite with two different reducing procedures.

2. Materials and methods

2.1. Instrumentation

Absorbance measurements at the selected wavelengths before and after CPE respectively were conducted on a double beam UV–Visible Spectrophotometer (Shimadzu UV-1800 PC, Kyoto, Japan) equipped with the 1.0-cm quartz cells. A centrifuge (Universal-320, Hettich Centrifuges, DJB Labcare Ltd., Newport Pagnell, UK) was used to accelerate the phase separation process at 1160 × *g*. A thermostatic water bath (EPC 4420, Thermal, and Istanbul, Turkey) was used to maintain the temperature in CPE experiments. Also, an ultrasonic power (UCS-10 model, Jeio Tech, Co., Ltd., Seoul, Korea) was used to degas and digest the beverages and milk samples with ultrasonic irradiation of 40 Hz at 300 watt, also used to maintain the temperature in CPE experiments. The pH measurements were carried out with a pH meter (pH-2005, JP Selecta, Barcelona, Spain). Eppendorf vary-pipettes (10–100 and 200–1000 μL) were used to deliver accurate volumes.

2.2. Standards and reagents

All chemicals and reagents used in were of analytical-reagent grade or higher purity. All solutions were prepared by using ultrapure water from Millipore Milli-Q System (with a resistivity equal to 18.2 MΩ at 25 °C). Sodium nitrite was dried at 110 °C for 4 h. A 1000 μg mL^{-1} stock nitrite solution was prepared by dissolving 0.375 g of $NaNO_2$, supplied by Merck (Merck, Darmstadt, Germany) in water in a 250-mL standard flask. The 50–100 mg of solid NaOH were added to prevent its decomposition, and 0.1 mL of chloroform was also added to prevent bacterial growth. The stock solution was kept in a refrigerator and diluted as required. The standard working solutions used for establishment of calibration curve were prepared before use by stepwise dilution of the stock solution with water. The solutions of 5.0% (v/v) of Triton X-114, Triton X-100 and Triton X-45 (Sigma, St. Louis, MO, USA) were prepared by dissolving 5.0 mL of non-ionic surfactant with 100 mL of water. A 1.0×10^{-4} mol L^{-1} of AOH^+ solution was prepared by dissolving 0.0332 g of dye (Sigma, St. Louis, MO, USA) in water in a 1 L standard flask. A 0.1 mol L^{-1} phthalate buffer at pH 4.0 was prepared by mixing 100 mL of 0.1 mol L^{-1} potassium hydrogen phthalate and 0.2 mL of 0.1 mol L^{-1} HCl, and adjusting pH to 4.0 by using a pH meter when necessary. The vessels and pipettes used for trace analysis were kept in 10.0% (w/v) HNO_3 for at least 24 h and subsequently washed five times with water.

2.3. The CPE procedure

An aliquot of standard or sample solution containing nitrite in the range of 1.5–150 μg L^{-1} was transferred to a 50 mL centrifuge tube, 0.3 mL of 0.02 mol L^{-1} KI, 0.8 mL of 1.0×10^{-4} mol L^{-1} AOH^+, 0.8 mL of 20% (w/v) NH_4Cl, 1.0 mL of 5.0% (v/v) Triton X-114, and 0.6 mL of pH 4.0 phthalate buffer solution were added. Then, the solution was made up to the mark with triply distilled water and allowed to stand for 12 min at 50 °C. Separation of the aqueous and surfactant-rich phase was accomplished by centrifugation for 10 min at 1160 × *g*. In order to increase the viscosity of the surfactant-rich phase and facilitate removal of the aqueous phase the solution was cooled in an ice-bath for 10 min. Then, the aqueous phase can be rapidly and easily separated by inverting the tube. The surfactant-rich phase of this procedure was dissolved and diluted to a volume of 0.75 mL with acetonitrile and transferred into a quartz cell. The absorbance of the solution was measured against reagent blank at 450 nm. Finally, nitrite contents were determined by making either direct calibration

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curve or standard additions calibration curve established by UV–Vis spectrophotometry at 450 nm.

2.4. Preparation of samples for analysis

All of the beverage and milk samples selected for analysis were supplied from local markets in Sivas, Turkey. Initially, all of the glassware and other mineralization containers used were acid-washed to avoid contamination. A 0.1% (v/v) of 2-octanol solution was added to the beer samples to prevent foaming, and the samples were degassed for 15 min using an ultrasonic bath. Wine samples were de-alcoholized at 80 °C using a reduced pressure evaporator until its total volume was approximately a quarter of its initial volume (100 mL). The beverages without alcohol were filtered using a membrane filter (0.45-μm pore size) to remove suspended solids before analysis. The applicability of the method was demonstrated by the analysis of a certified reference material (MOOS-1), different milk and beverage samples. The samples were prepared to analysis with five successive analytical steps like homogenization, dissolution, extraction, deproteinization and reduction as described below.

Initially, to a suitable volume of beverages and milk samples (in range of 5–15 mL) after homogenization by stirring in a vortex for 2 min, depending on their nitrite, nitrate and total nitrite contents, was added 25 mL of hot ultrapure water (60 °C) and 5 mL of saturated sodium tetraborate and heated on thermostatic water bath (in range of 50–55 °C) for 15 min to achieve a complete dissolution and extraction. After that the aqueous extracts were deproteinized and thoroughly clarified with potassium hexacyanoferrate (2 mL of 0.25 mol L^{-1}) and zinc acetate (2 mL of 0.25 mol L^{-1}), which are also known as Carrez-I and -II reagents, slurries were transferred to a volumetric flask of 250 mL and diluted by water, then filtrated by using a membrane filter after centrifugation at 1160 × g for 15 min (Topcu et al., 2006). After developing of colored ion-pairing complex due to addition of pH 4.0 phthalate buffer, iodide, AOH^+, NH_4Cl as salting out agent and Triton X-114 as extracting agent, diluted to volume of 50 mL and measured their absorptions in 450 nm against reagent blank before and after pre-reduction of a suitable aliquot of filtrate (in range of 2–10 mL) with (i) 0.2 mL of hydrazine solution (0.05 mol L^{-1}) containing only 3 mg L^{-1} Cu(II) as catalyst in 0.2 mL of 0.1 mol L^{-1} NaOH solution media under ultrasonic power (300 W, 40 Hz) at room temperature for 10 min and (ii) 0.3 mL of hydrazine solution at 0.07 mol L^{-1} containing 1.5 mg L^{-1} Cu(II) as catalyst and 100 mg L^{-1} Zn(II) in 0.2 mL of 0.1 mol L^{-1} NaOH solution media at 40 °C for 15 min as to fall in linear calibration range of the method. The nitrate contents of samples were calculated from the analytical difference between free nitrite and total nitrite concentrations after reduction. In order to control a systematic error originating from matrix effect, three-point standard addition approach was adopted for analysis of the digested and diluted beverage and milk samples where necessary. Then, the accuracy of results was verified by evaluating whether or not the percentage recoveries are quantitative. Two reduction procedures with and without sonication for 2–3 mL of the diluted samples were repeated five times in order to reduce nitrate contents of the samples to nitrite, and were subjected to CPE prior to detection. A five replicate blank analysis were also carried out in order to correct for any analyte contaminants in the reagents used for sample preparation. A certified sample, MOOS-1 in terms of free nitrite and total nitrite was also dissolved and prepared to the analysis with CPE in a similar way prior to spectrophotometric detection. Each point in optimization step and calibration curves with and without preconcentration with CPE was run in triplicate, and the results were indicated with error bars. The one- and two-paired ANOVA tests in optimization step and analysis step of samples were conducted for statistical comparisons.

3. Results and discussion

In the present study, we have for the first time used the ion-pairing interaction between triiodide, I_3^- and AOH^+ being a cationic fluorescent dye for indirect spectrophotometric determination of trace nitrite in beverages after separation and preconcentration with CPE. Acridine orange, AOH^+ is a cationic dye with planar structure (pK_a: 10.4), and it is protonated at the pH lower than 10. It has been observed that the diluted solution of AOH^+ in range of 1.0×10^{-6}–1.0×10^{-4} mol L^{-1} gives a second maximum peak at 465 nm as a result of dimerization in which its acid- and base-forms show a maximum absorption at 492 nm and 435 nm, respectively. Also, below $(4–5) \times 10^{-5}$ mol L^{-1}, an isobestic point is observed at 471.5 nm. However, at higher concentrations, it has been observed that a peak at 465 nm is changed to 450 nm with a blue shift of 15 nm, and the isobestic point disappears (Costantino et al., 1984). This may be due to charge transfer based on acid–base interactions between I_3^- and AOH^+ or $(AOH)_2^{2+}$ as well as dimerization of dye, as it is also observed in the existing study. It is well-known that cationic AOH^+ monomer forms aggregates with negatively charged hydrophobic surfactants such as sodium dodecyl benzene sulfonate (SDBS) and sodium-1,4-bis(2-ethylhexyl)sulfosuccinate (AOT), which alter the AOH^+ monomer-dimer equilibrium and hence, the fluorescence pattern of AOH^+ in literature (Wang et al., 2006; Falcone et al., 2002). Also, it is implied that its spectral properties of AOH^+ as a major limitation in quantitative measurement of pH are significantly affected by temperature and the presence of anions like NO_3^- ion; so that NO_3^- anion can induce aggregation of AOH^+ (Millot et al., 1997). Additionally, it has been observed in literature (Hayakawa et al., 1979) that an iodine molecule can interact strongly with a cationic surfactant, DTAC ion to give rise to a complex, which is sparingly soluble in water but is easily solubilized in DTAC micelle. At higher DTAC concentrations than CMC, it has been described that in micellar solution both iodine molecule and triiodide ion are efficiently solubilized in the surface region of the micelle with remarkable increase in the formation constant of complex, I_3^- at 360 nm. In other study (Khayathian et al., 2006), it has been observed that a charge transfer complex of iodine with cryptand 222 as an electroactive ionophore forms in a wide pH range. In the mentioned study, it is also implied that triiodide ion selective electrode exhibits very high selectivity for I_3^- ion over other ions due to formation of stable ion-pairing complex, $[C222.I^+]\,I_3^-$ with a new absorption band appearing at 363 nm, and successfully is used as an indicator electrode in potentiometric titration of copper. In lights of all these information's, it can be concluded that the developed method is based on micellar sensitized spectrophotometric monitoring of the basic form of dye produced by means of the proton transfer from ion-pairing reagent, AOH^+ or $(AOH)_2^{2+}$ to triiodide, I_3^-, which is proportional to nitrite concentration in a wide linear range at 450 nm. These interesting properties of AOH^+ and triiodide prompted us to study AOH^+–I_3^- interactions with the objective of developing an indirect nitrite detection method that could be useful in practical application by means of a mechanism, which may be postulated as follows:

$$2NO_2^- + 4H^+ + 3I^- \rightarrow I_3^- + 2NO + H_2O \text{ at low nitrite concentrations} \quad (1)$$

$$AOH^+ (\text{at } 492\,\text{nm}) \leftrightarrow AO\ (\text{at } 435\,\text{nm}) + H^+, \text{with a } pK_a \text{ value of } 10.4 \quad (2)$$

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$$AOH^+ + H^+ \leftrightarrow AOH_2{}^{2+}, \text{protonation at lower pHs than 6.0} \quad (3)$$

$$2AOH_2{}^{2+} \leftrightarrow (AOH_2)_2{}^{4+}(\text{Dimer, 465 nm}),$$
dimerization with a K_D of $(1.7 \pm 0.08) \times 10^4\ M^{-1}$ (4)

$$(AOH_2)_2{}^{4+} + H_2O \leftrightarrow AOH_2{}^{+}{}_{(\text{reducedform})} + AO(OH)_{(\text{oxidizedform})} + 3H^+,$$
disproportionation (5)

$$I_3{}^- + 2AOH^+ + H_2O \rightarrow I_2 + IO^- + 2AOH_2{}^+, \text{charge transfer at pH 4.0} \quad (6)$$

$$IO^- + 2AOH_2{}^+ \rightarrow 2AOH^+ + I^- + H_2O \quad (6a)$$

$$IO^- + 2Cl^- + 2H^+ \rightarrow ICl_2{}^- + H_2O,$$
in presence of NH_4Cl as salting out agent (6b)

$$ICl_2{}^- + 2AOH_2{}^+ \rightarrow 2AOH^+ + I^- + 2H^+ + 2Cl^- \quad (6c)$$

$$I_2 + I^- \rightarrow I_3{}^- \quad (7)$$

$$I_3{}^- + AOH^+ \rightarrow [AOH^+ \cdots I_3{}^-] \leftrightarrow [AOH_2{}^+ \cdots IO^-]I_2, \text{charge transfer complex at 450 nm} \quad (8)$$

At high nitrite concentrations above 150 μg L^{-1} in aqueous micellar solution, linear relation is disturbed due to following reactions:

$$2NO_2{}^- + 2H^+ \rightarrow {:}NO^+ + NO_2{}^- + H_2O \quad (9)$$

$${:}NO^+ + AOH^+ + H_2O \rightarrow \text{Protonated nitrosyl adducts, } P{-}N{-}N{=}OH^+ + H_3O^+ \quad (10)$$

From prior studies, the micellar improvement in absorbance of charge transfer complex at 450 nm with a blue shift of 15 nm in micellar media after preconcentration with CPE at pH 4.0 phthalate buffer has selectively been used for indirect spectrophotometric determination of nitrite, nitrate and total nitrite in an aqueous micellar medium. It has been observed that this absorption peak is linearly very sensitive to increasing nitrite concentration at levels of 3, 9 and 30 μg L^{-1} (Fig. 1).

Therefore, for further studies the different variables affecting CPE efficiency was optimized in order to achieve the maximum sensitivity. Finally, the usefulness of the method has successfully been demonstrated by measurement of the nitrite, nitrate and total nitrite contents in selected beverage and foods. The accuracy was also assessed by analyzing MOOS-1 nutrients in seawater due to lack of a CRM related to food and beverage samples and recovery studies after pre-treatment of samples with two different reduction approaches.

3.1. Effect of pH and buffer concentration

The pH plays a unique role on ion-pairing complex formation and subsequent extraction procedures in CPE. Separation and preconcentration of nitrite with CPE involve previous formation of a stable complex, which needs to present sufficient hydrophobicity to be extracted into the small volume of the surfactant-rich phase. The pH is a critical factor affecting both the redox reaction between nitrite and excess iodide, and produced triiodide ion, $I_3{}^-$ and ion-pairing ligand, AOH^+ and the extractability of ion-pairing complex into the surfactant-rich phase. Thus, the effect of different buffers such as phthalate, formate and universal Britton–Robinson (B–R) buffers were extensively studied for the extraction and determination of nitrite in the range pH 2.0–5.5 in Fig. 2(a). From the results, maximum absorbance was obtained with phthalate buffer system at pH 4.0. The small analytical signals at lower pH than 4.0 may be attributed to the instability of nitrite ions in acidic conditions due to its decomposition reaction of the conversion of $NO_2{}^-$ to NO and $NO_3{}^-$ as shown below:

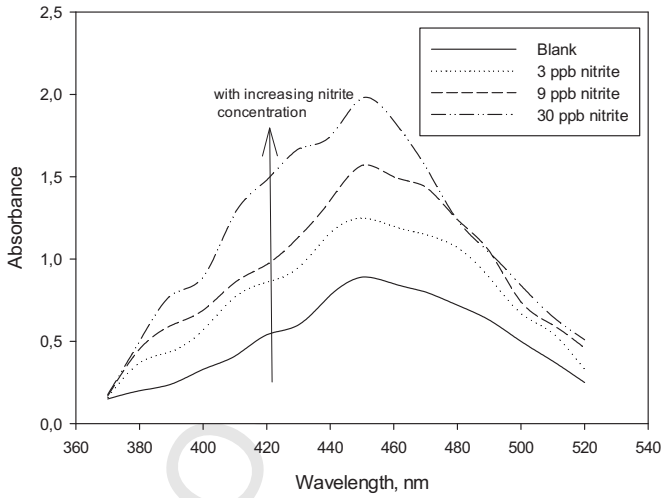

Fig. 1. The absorption spectra of micellar system in UV–visible region in absence and presence of trace nitrite ion as a function of increasing nitrite concentration at levels 3, 9 and 30 μg L^{-1}. Conditions: 0.3 mL of 0.02 mol L^{-1} KI, 0.8 mL of 1.0×10^{-4} mol L^{-1} AOH^+, 0.8 mL of 20% (w/v) NH_4Cl, 1.0 mL of 5.0% (v/v) Triton X-114, and 0.6 mL of pH 4.0 phthalate buffer at 50 °C.

$$2H^+ + 3NO_2{}^- \leftrightarrow 2NO + NO_3{}^- + H_2O$$

Also, the effect of buffer concentration on the analytical signal was studied in the range of $(0.4–6) \times 10^{-3}$ mol L^{-1} phthalate concentration, and the maximum absorbance was obtained with using 1.2×10^{-3} mol L^{-1} of buffer solution in Fig. 2(b). Therefore, 1.2×10^{-3} mol L^{-1} of phthalate buffer at pH 4.0 was used as optimal value for further studies.

3.2. Effect of reagent concentrations

The variation of the analytical signal as a function of the concentration of iodide as primary ligand in the range of $(0.02–0.5) \times 10^{-3}$ mol L^{-1} in the presence of 50 μg L^{-1} $NO_2{}^-$ was studied, and the results in Fig. 3(a) indicated that the signal intensity of the analyte linearly increased by iodide concentration up to 0.12×10^{-3} mol L^{-1}. The maximum signal intensity linearly decreased with increasing slope at the higher concentrations. So, an iodide concentration of 0.12×10^{-3} mol L^{-1} was selected as optimal value for further studies.

Different concentrations of AOH^+ as ion-pairing reagent in the range of $(0.4–6) \times 10^{-6}$ mol L^{-1} were studied for the influence of its concentration on analytical response for determination of nitrite at fixed concentration of 50 μg L^{-1}. The absorbance changes as a function of the concentration of AOH^+ were shown in Fig. 2(b). It can be seen that the signal intensity sharply depends on the concentration of AOH^+ at lower concentrations than 2.0×10^{-6} mol L^{-1} in CPE system. As shown in Fig. 3(b), the highest signal was obtained in concentration of 1.6×10^{-6} mol L^{-1}. Thus, a concentration of 1.6×10^{-6} mol L^{-1} was selected as optimal value, and this value was adopted and used for further studies.

3.3. Effect of non-ionic surfactant concentration

In CPE choosing an appropriate surfactant is important, since the temperature corresponding to cloud point is correlated with

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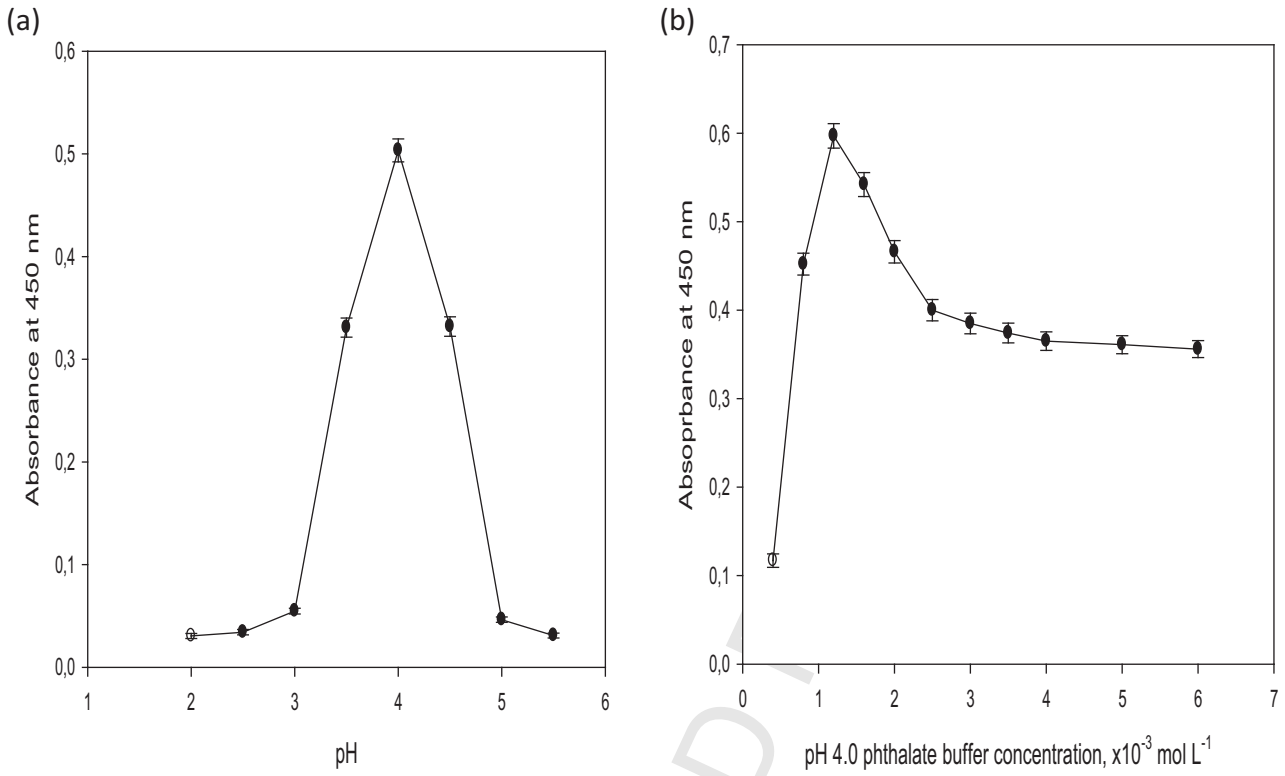

Fig. 2. Effect of (a) pH and (b) phthalate buffer concentration on CPE efficiency. Other experimental conditions are described in Section 2.

the hydrophilic property of a surfactant. A successful CPE should maximize the extraction efficiency by minimizing the phase volume, thus increasing its concentrating capability. In this work, three non-ionic surfactants such as Triton X-45, Triton X-100 and Triton X-114 were investigated in range of 0.02–0.2% (v/v) in Fig. 4, and the results showed that TritonX-114 gave better performance due to its higher density of the surfactant-rich phase, which facilitated phase separation. From the results, it was observed that the absorbance, and therefore, CPE efficiency increased up to 0.1% (v/v) Triton X-114 and decreased slightly and gradually above that concentration. Further increasing the concentration of Triton X-114 in range of 0.1–0.2% (v/v) would increase the amount of the

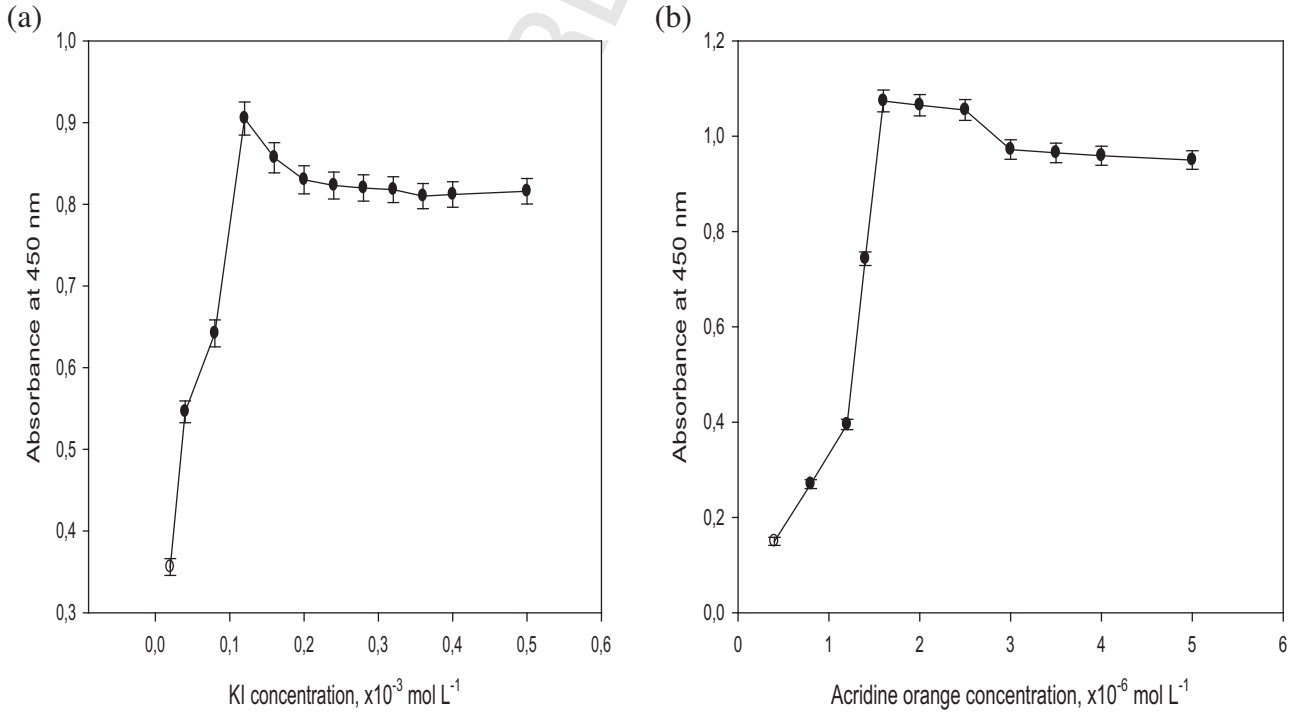

Fig. 3. Effect of (a) KI and (b) AOH^+ concentration on CPE efficiency. Other experimental conditions are described in Section 2.

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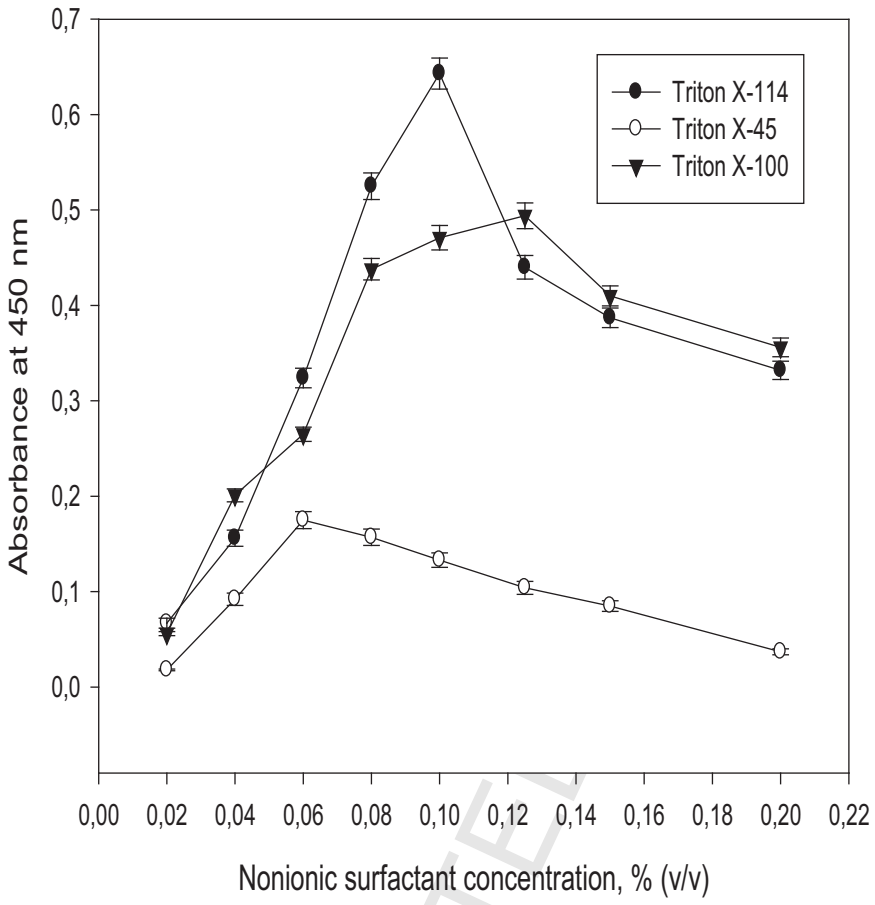

Fig. 4. Effect of nonionic surfactant concentrations on CPE efficiency. Other experimental conditions are described in Section 2.

surfactant-rich phase volume and thus decreasing the phase volume ratio. Thus, Triton X-114 concentration of 0.1% (v/v) was used in further studies in order to achieve the optimal analytical signal in conjunction with the highest possible extraction efficiency.

3.4. Effect of equilibrium temperature/time and centrifugation rate/time

The effects of the equilibrium temperature and the incubation time were examined due to their importance for the reaction completion and efficient separation of the phases, which reflect certainly the magnitude of preconcentration factor of an analyte. Consequently, a study was carried out to choose the range of temperature that enhances higher absorbance signals for nitrite ions. The temperature was varied from 20 °C to 70 °C in a search of optimal value. From the results, the highest absorbance signals were achieved when the temperature at 50 °C.

It was also observed that the incubation time of 12 min is sufficient for the maximum absorbance of ion-pairing complex. Thus, the temperature of 50 °C for 12 min was selected to fulfill efficient separation conditions. The effect of centrifugation rate and time also was investigated on extraction efficiency. A centrifuge time of 10 min at 1160 × *g* was selected for the entire procedure as being optimal and beyond this time no confirmation was observed for improving extraction efficiency.

3.5. Effect of electrolyte concentration

Studies on the effects of some additives, such as anionic and non-ionic surfactants and electrolytes, as NaCl, KCl and NH_4Cl, on the cloud-point behavior of non-ionic surfactants have been reported (Gu and Galera-Gomez, 1995; Nascentes and Arruda, 2003; Komaromy-Hiller et al., 1996). It was observed that the presence of electrolytes decreases the cloud point (salting-out effect), resulting in a more efficient extraction. The lower cloud point is attributed to electrolytes promoting dehydration of the poly (oxyethylene) chains (Armstrong et al., 1998). According to Komaromy-Hiller et al., the salting-out phenomenon is directly related to desorption of ions to the hydrophilic parts of the micelles, increasing inter-attraction between micelles and consequently leading to the precipitation of surfactant molecules. Based on this discussion, NaCl, KCl and NH_4Cl were investigated as electrolytes in the concentrations ranging from 0.08% to 0.80% (w/v) and the highest CPE efficiency was obtained at 0.32% (w/v) NH_4Cl concentration. The signal decreased considerably for increasing NH_4Cl concentrations in range of 0.32–0.80% (w/v). This effect might be explained by the additional surface charge when the NH_4Cl concentration is very high, thus changing the molecular architecture of the surfactant and consequently the micelle formation process. It is necessary to emphasize that different blank solutions were also evaluated and no significant signal was obtained. In this way, 0.32% (w/v) NH_4Cl concentration was used in all further experiments.

3.6. Selection of diluting agent

The volume of the surfactant-rich phase acquired after separation and preconcentration with CPE is small for detection with UV–Vis spectrophotometry. It is very important to choose the appropriate solvent in order to obtain the maximum signal after CPE. The effect of various solvents such as methanol, acetonitrile,

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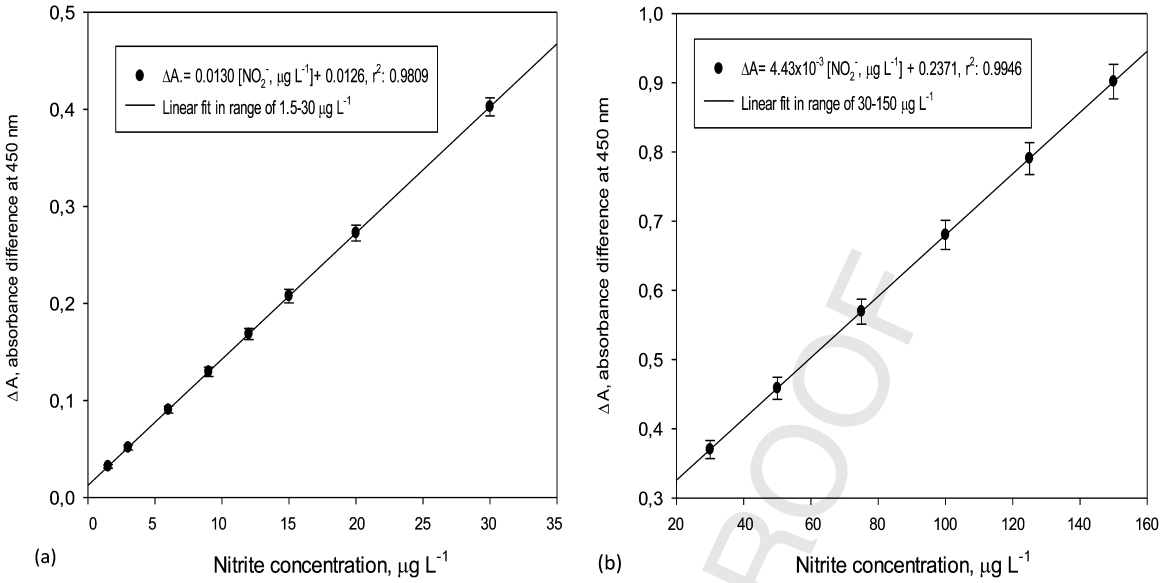

Fig. 5. The calibration curves obtained for a serial standard nitrite concentrations in range of (a) 1.5–30 and (b) 30–150 μg L^{-1} with changing sensitivity.

ethanol, acidic methanol, acidic ethanol, acetone and THF at volume range of 0.5–1.5 mL was separately studied in order to dilute surfactant-rich phase after phase separation. The best correlation, r^2 and analytical sensitivity, m/S_m, from slopes and standard deviation values of slopes of calibration curves obtained for three different nitrite concentration levels, 5, 15 and 25 μg L^{-1} (n: 3) was obtained after dilution of surfactant-rich phase to a volume of 0.75 mL with acetonitrile. Therefore, 0.75 mL of acetonitrile was selected for further studies.

3.7. Analytical figures of merit

The calibration graphs were constructed by measuring the difference between signals of sample and blank as a function of a serial standard nitrite concentrations. The analytical characteristics of the optimized method with and without preconcentration are as follows: Without preconcentration at 490 nm, linear regression equation for nitrite in linear range of 60–1800 μg L^{-1} with detection and quantification limits of 17.9 and 59.6 μg L^{-1} is $\Delta A: 1.76 \times 10^{-4} C_{NO_2^-}$ (μg L^{-1}) + 0.135, r^2: 0.9963. With preconcentration with CPE at 450 nm, linear regression equations for nitrite in the range of 1.5–30 μg L^{-1} and 30–150 μg L^{-1} is $\Delta A: 0.0130 C_{NO_2^-}$ (μg L^{-1}) + 0.0126, r^2: 0.9809; and $\Delta A: 4.43 \times 10^{-3} C_{NO_2^-}$ (μg L^{-1}) + 0.237, r^2: 0.9946 respectively. The calibration curves obtained for a serial standard nitrite concentrations in range of 1.5–150 μg L^{-1} with changing sensitivity are represented in Fig. 5. The limits of detection, LOD and quantification, LOQ defined as $3\sigma_{blank}/m$ and $10\sigma_{blank}/m$ (n: 12) (where σ_{blank} is the standard deviation of twelve replicate measurements of the analyte blank and m is the slope of the calibration graph) were found to be 0.42 and 1.40 μg L^{-1}, respectively. The precision of the method was controlled by the relative standard deviation (RSD) of five independent measurements taken in solutions containing all ions. The percentage recoveries and RSDs were in the range of 97.5–103.0% and 3.5–2.1% for three different concentration levels of 25, 75 and 100 μg L^{-1}, respectively. Because the amount of nitrite in 50 mL of sample solution is measured after preconcentration by CPE in a final volume of 0.75 mL acetonitrile, the preconcentration factor is 67. Because enhancement factor is calculated as the ratio of slope of preconcentrated samples to that obtained without preconcentration, the enhancement factor is 74.

3.8. Matrix effect

In order to evaluate the selectivity of the proposed method, a large number of anions and cations examined in Table 1 have no considerable effect on the preconcentration and determination of nitrite of 25 μg L^{-1} in tolerance limit ranging from 35 to 1000. The tolerance limit was identified as the concentration of added ion that caused greater relative error than ±5.0%. With a tolerance limit of 35, the possible weak interference of Fe^{3+} ions was improved up to 500-fold using 1.0 mL of 1.0×10^{-3} mol L^{-1} NaF. Due to give stable anionic complexes such as HgI_4^{2-} and BiI_4^-, Bi^{3+} and Hg^{2+} ions seldom found in beverage and food samples, interfered significantly up to a tolerance limit of 15- and 25-fold respectively, but their interferences were efficiently suppressed up to 150- and 250-fold after pretreatment with 50 μL of 1.0×10^{-3} mol L^{-1} thiourea solution. Due to their reducing natures, the SO_3^{2-} and $S_2O_3^{2-}$ ions do a serious interference. However, the higher concentrations of sulfite and thiosulfate (from tolerance limit of 30) can be tolerated up to 500-fold by the addition of 25 μL of 0.025% (w/v) formaldehyde to the sample solution prior to preconcentration and determination of nitrite. The interference of SiO_3^{2-} (with higher tolerance limit than 10) was overcome and improved up to 350-fold after pretreatment ant heating with 0.1 mL

Table 1
The interfering effect of matrix components on determination of 25 μg L^{-1} NO_2^-.

Interfering species	Tolerance limits
Na^+, K^+, NH_4^+, NO_3^-, HCO_3^-, thiourea, HPO_4^{2-}, Sr^{2+} and Mg^{2+}	□1000
Cl^-, Br^-, Ca^{2+}, Cr^{3+}, Zn^{2+}, hydrazine, formaldehyde, oxalate, Mn^{2+}, Cd^{2+} and Co^{2+}	400–800
Fe^{2+}, Al^{3+}, Br^-, SCN^-, citrate, tartrate, Ag^+, Pb^{2+}, Sn^{4+}, V^{5+}, Sb^{5+}, As^{5+} and Se^{4+}	250–350
Ni^{2+}, Mo^{6+}, V^{4+}, As^{3+} and Sb^{3+}	75–200
S^{2-}	50
CN^- and Fe^{3+}	35
Bi^{3+}[a], ($S_2O_3^{2-}$ and SO_3^{2-})[b]	20–30 (150[a], 500[b])
Hg^{2+}[a] and SiO_3^{2-}[d]	15, 10 (250[a], 350[d])
IO_3^-[c]	5 (150[c])

[a] After pretreatment with 50 μL of 1.0×10^{-3} mol L^{-1} thiourea solution.
[b] After pretreatment with 25 μL of 0.025% (w/v) formaldehyde solution.
[c] After pretreatment with 25 μL of 0.025% (w/v) hydrazine hydrochloride solution.
[d] After pretreatment ant heating with 0.1 mL of 1.0×10^{-3} mol L^{-1} NaF at 80 °C.

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Table 2
The speciative determination of dissolved inorganic NO_2^- and NO_3^- in artificially prepared binary mixtures after reduction with a mixture of hydrazine/Cu(II) as a catalyst in alkaline media under ultrasonic power (n: 3).

Added, μg L^{-1}		Found, μg L^{-1}			Recovery (%)*			RE (%)[c]		RSD (%)[c]	
NO_2^-	NO_3^-	NO_3^-	NO_2^-[a]	Total NO_3^- plus NO_2^-[a,b]	NO_3^-	NO_2^-	Total NO_3^- plus NO_2^-	NO_2^-	Total NO_2^-	NO_2^-	Total NO_3^- plus NO_2^-
20	5	4.7	19.7 ± 0.8	24.6 ± 1.1	98.0	98.5	98.4	−1.5	−1.6	4.1	4.5
15	10	10.0	14.7 ± 0.7	24.7 ± 1.0	100.0	98.0	98.8	−2.0	−1.2	4.8	4.0
10	15	15.1	9.7 ± 0.4	24.8 ± 0.9	101.0	97.0	99.2	−3.0	−0.8	4.1	3.6
5	20	19.8	4.9 ± 0.2	24.6 ± 0.9	99.0	98.0	98.8	−2.0	−1.6	4.1	3.7
0	25	24.5	–	24.5 ± 1.0	98.0	–	98.4	–	−2.0	–	4.1

[a] The average and standard deviation of three replicate measurements.
[b] The value found as total nitrite equivalent to nitrite after reduction with a mixture of hydrazine/Cu(II) as a catalyst in alkaline media under ultrasonic power by using calibration curve.
[c] The relative error and relative standard deviation of three replicate measurements.

of 1.0×10^{-3} mol L^{-1} NaF at 80 °C. Also, the serious interference of IO_3^- ions (with a tolerance limit of 5-fold) was removed and improved up to 150-fold after pretreatment with 25 μL of 0.025% (w/v) hydrazine hydrochloride solution. The results clearly point out the analytical performance of the proposed CPE method for complex sample applications such as beverages and milk samples.

3.9. Analytical applications

To ensure from complete reduction of nitrate to nitrite under ultrasonic power (300 W, 40 Hz) at room temperature for 10 min in alkaline media, a mixture containing 0.05 mol L^{-1} of hydrazine solution and 3 mg L^{-1} Cu(II) as catalyst at alkaline media (in optimal 0.2 mL of 0.1 mol L^{-1} NaOH) in a flask of 50 mL was preferentially used in range of 0.02–2 mL, and a reductant volume of 0.2 mL was chosen as optimum due to give maximum reduction efficiency. Reducing agent was adopted and used as a convenient and reliable reductant, which allowed rapid and complete reduction of nitrate (for levels of 20, 40 and 80 μg L^{-1}) to nitrite at room temperature for 10 min without any interfering of its excess amount in total nitrite determination step. Even at reducing volumes up to 1.0 mL for 100 μg L^{-1} NO_3^- an efficient and stable reduction signal could be obtained without disturbing effect. The percentage reduction with relative error of ±2.5% (n: 3) was verified to be quantitative by comparing the results obtained for measurement of nitrate after reduction with those obtained for measurement of directly free nitrite at same amounts by spectrophotometric detection of samples, which are submitted to CPE procedure at 450 nm. So, it has been concluded that a reducing volume of 0.2 mL is enough for complete reduction of nitrate to nitrite for lower concentrations of 80 μg L^{-1}.

The developed method was also used for speciative determination of nitrite, nitrate and total nitrite after reduction. For this purpose, the binary mixtures changing in 0–25 μg L^{-1} were prepared at known ratios. After reduction with a mixture of hydrazine/Cu(II) in alkaline media under ultrasonic power, total nitrite analysis was made by using spectrophotometry after preconcentration with CPE under optimized conditions. The concentration of nitrate was calculated from the analytical difference between total nitrite and free nitrite. The results are clearly represented in Table 2. As can be seen in Table 2, the REs and RSDs for measurement of free nitrite (5, 10, 15 and 20 μg L^{-1}; n: 3) in the presence of nitrate are between −(1.5–3.0)% and 4.1–4.8%, respectively while these values for total nitrite are between −(0.8–2.0)% and 3.6–4.5%. It is clear that the percentage recoveries obtained, are reasonable for trace analysis, in range of 97–98.5% and 98.4–99.5% for nitrite and total nitrite, respectively.

For analysis of milk/beverage samples and CRM, the standard calibration curve was employed. In order to establish the validity of the proposed procedure, the proposed method has been applied to the determination of nitrite, nitrate and total nitrite in the certified sample, MOOS-1. The results showed a good agreement between measured values (13.8 ± 0.7 and 109.0 ± 3.8 μg L^{-1}) obtained before and after fast and complete reduction of dissolved nitrate to nitrite with a mixture of Hydrazine/Cu(II) in alkaline media under ultrasonic power and the certified values (14.1 ± 0.6 and 109.0 ± 4.1 μg L^{-1}), respectively in Table 3. The tabulated student's t-values at a significance level of 0.05 are 2.78 for certified sample and the experimental t-values are 0.89 and 0.84, respectively. For certified sample, the experimental values obtained are also smaller than the tabulated values so it may be concluded that the values obtained in terms of free nitrite and total nitrite are significantly equal to the certified values.

The method was also applied to the determination of trace nitrite, nitrate and total nitrite in selected milk and beverages with and without alcohol. The results and the recoveries for the spiked samples at concentrations ranging from 20 to 50 μg L^{-1} after dilution were given in Table 4a(a). It can be seen that the percentage recoveries for the spiked samples is in the range of 97–99.8% with relative standard deviation of 2.8–4.6% (n: 5) for nitrite and total nitrite contents obtained by using both pre-treatment approaches. As can be seen from Table 3, the student's t-test for

Table 3
The nitrite and total nitrite (as NO_2^- plus NO_3^-) contents of CRM obtained by using the proposed CPE-spectrophotometric method (n: 5).

CRMs	Dilution ratio	Certified value (μg L^{-1})	Added (μg L^{-1} NO_2^- or NO_3^-)	Found[a] (μg L^{-1} NO_2^-)	Found[c] (μg L^{-1} Total NO_2^-/NO_3^-)	Recovery %	RSD %	Experimental t-value[b]
MOOS-1 nutrients in seawater	1:10	14.1 ± 0.6, NO_2^-	–	13.8 ± 0.7	–	98.0	5.10	0.89
			25	38.5 ± 1.5	–	98.8	3.90	–
		109.0 ± 4.1, NO_2^- plus NO_3^-	–	–	109.0 ± 3.8	99.5	3.50	0.84
			25	–	133.0 ± 4.2	98.0	3.16	–

[a] The average and its standard deviation of five replicate measurements at confidence interval of 95%.
[b] The experimental t-values calculated by using $\mu = x_{average} \pm tS/N^{1/2}$ for five replicate measurements at confidence interval of 95% in which the critical t-value is 2.78 for 4 degrees of freedom at confidence interval of 95%.
[c] The average and its standard deviation of five replicate measurements at confidence interval of 95% after reduction with a mixture of hydrazine/Cu(II) as a catalyst in alkaline media under ultrasonic power.

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Table 4a
Determination of nitrite, nitrate and total nitrite contents of the selected alcoholic and non-alcoholic beverages and recovery studies of spiked samples.

Samples	Sample volume, mL/Dilution ratio	After reduction with a mixture of hydrazine/Cu(II) in alkaline media at room temperature under ultrasonic power (*n*: 5)						After reduction with a mixture of Zn^{2+} and Cu^{2+}, and hydrazine in alkaline media at 40 °C (*n*: 5)						The calculated *t*- and *F*-values[c]
		Added, μg NO_2^- L^{-1}	Found[a], μg Nitrite L^{-1}	RSD%	Recovery%	Found[b], μg Nitrate L^{-1}	Found[a], total μg Nitrite L^{-1}	Added, μg NO_2^- L^{-1}	Found[a], μg Nitrite L^{-1}	RSD%	Recovery%	Found[b], μg Nitrate L^{-1}	Found[a], Total μg Nitrite L^{-1}	
Beverages without alcohol														
Plain iced tea	2/1:25	–	2.50 ± 0.1	4.0	–	142.0	145.0 ± 3.0	–	2.60 ± 0.1	3.8	–	142.0	145.0 ± 3.1	1.58, 1.00
		25	27.2 ± 1.0	3.7	98.8	–	–	25	27.4 ± 1.1	3.5	98.4	–	–	–
Peach-flavored iced tea	2/1:25	–	4.50 ± 0.2	4.4	–	136.0	140.0 ± 4.0	–	4.30 ± 0.2	4.6	–	136.0	140.0 ± 4.2	1.58, 1.00
		25	29.1 ± 1.1	3.8	98.4	–	–	25	28.8 ± 1.0	3.8	98.0	–	–	–
Green tea	2/1:25	–	2.30 ± 0.1	4.3	–	118.0	120.0 ± 3.6	–	2.35 ± 0.1	4.2	–	117.8	120.0 ± 3.8	0.79, 1.00
		25	27.0 ± 1.0	3.7	98.8	–	–	25	27.2 ± 1.1	4.0	98.8	–	–	–
Camomile tea	2/1:10	–	5.10 ± 0.2	3.9	–	85.0	90.0 ± 4.4	–	5.20 ± 0.2	3.8	–	85.3	90.5 ± 4.6	0.79, 1.0.0
		20	24.7 ± 0.9	3.6	98.0	–	–	20	24.8 ± 0.8	3.2	98.0	–	–	–
Grape vinegar	2/1:25	–	10.5 ± 0.4	3.8	–	120.0	131.0 ± 4.1	–	10.3 ± 0.4	3.9	–	120.0	131.0 ± 4.0	0.79, 1.00
		20	30.2 ± 0.9	3.0	98.5	–	–	20	29.8 ± 0.9	3.0	97.5	–	–	–
Apple vinegar	2/1:25	–	16.1 ± 0.3	4.3	–	126.0	142.0 ± 3.8	–	16.0 ± 0.3	4.2		127.0	143.0 ± 3.9	0.53, 1.00
		20	35.8 ± 1.2	3.4	98.5	–	–	20	35.4 ± 1.1	3.1	97.0	–	–	–
Beverages with alcohol														
Red wine	2/1:30	–	11.5 ± 0.4	3.5	–	106.0	118.0 ± 3.5	–	11.8 ± 0.5	4.2	–	106.0	118.0 ± 3.4	1.05, 1.56
		25	36.1 ± 1.2	3.3	98.4	–	–	25	36.3 ± 1.3	3.6	98.0	–	–	–
White wine	2/1:30	–	13.7 ± 0.6	4.4	–	124.0	137.5 ± 4.0	–	14.0 ± 0.5	3.6	–	124.0	138.0 ± 3.8	0.86, 1.44
		25	38.4 ± 1.2	3.1	98.8	–	–	25	38.6 ± 1.3	3.4	98.4	–	–	–
Efes malt beer	3/1:50	–	3.50 ± 0.12	3.4	–	109.0	112.0 ± 4.0	–	3.55 ± 0.13	3.7	–	109.0	113.0 ± 3.8	0.63, 1.17
		25	28.2 ± 0.8	2.8	98.8	–	–	25	28.5 ± 0.9	3.2	99.8	–	–	–
Tuborg malt beer	3/1:50	–	5.10 ± 0.2	3.8	–	143.0	148.0 ± 4.0	–	4.95 ± 0.18	3.7	–	144.0	149.0 ± 3.8	1.25, 1.44
		25	29.8 ± 1.0	3.4	98.8	–	–	25	29.6 ± 0.9	3.0	98.6	–	–	–

[a] The average plus standard deviation of five replicate measurements for nitrite and total nitrite after pre-treatment with two different reduction approaches.
[b] The nitrate value calculated from the difference between initial nitrite and total nitrite after pre-treatment with two different reduction approaches.
[c] In order to compare the mean values and their standard deviations for independent two samples *t*- and *F*-tests with equal sample size the statistical *t*- and *F*-critical values at 95% confidence level and 8 degrees of freedom are 2.31 and 6.39, respectively.

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Table 4b
Determination of nitrite, nitrate and total nitrite contents of milk samples after pre-treatment with two reduction approaches.

Samples	Sample volume, mL/Dilution ratio	After reduction with a mixture hydrazine/Cu(II) in alkaline media at room temperature under ultrasonic power (n: 5)						After reduction with a mixture of Zn^{2+} and Cu^{2+}, and hydrazine in alkaline media at 40 °C (n: 5)					
		Added[a], μg Nitrite L^{-1}	Found[a], μg Nitrite L^{-1}	RSD, %	Recovery %	Found[b], μg Nitrate L^{-1}	Found[a], Total μg Nitrite L^{-1}	Added[a], μg Nitrite L^{-1}	Found[a], μg Nitrite L^{-1}	RSD,%	Recovery %	Found[b], μg Nitrate L^{-1}	Found[a], Total μg Nitrite L^{-1}	The calculated t- and F-values[c]
Cow's milk	3/1:250	–	5.45 ± 0.3	5.5	–	48.6	54.0 ± 3.2	–	5.60 ± 0.3	5.3	–	49.0	54.5 ± 3.1	0.43, 1.00
		15	20.2 ± 0.7	3.5	98.0	–	–	15	20.2 ± 0.7	3.5	97.3	–	–	–
Sheep's milk	3/1:250	–	8.40 ± 0.4	4.8	–	85.6	94.0 ± 3.6	–	8.35 ± 0.4	4.8	–	86.2	94.5 ± 3.7	0.14, 1.00
		15	22.8 ± 1.0	4.4	96.0	–	–	15	22.7 ± 1.0	4.4	95.7	–	–	–
Goat's milk	2/1:250	–	10.3 ± 0.4	3.9	–	92.5	103.0 ± 3.8	–	10.5 ± 0.4	3.8	–	92.9	103.0 ± 4.0	0.58, 1.00
		15	24.7 ± 1.0	4.0	96.0	–	–	15	24.6 ± 1.0	4.1	94.0	–	−0	–
Banana flavored milk	2/1:250	–	12.5 ± 0.5	4.0	–	58.3	70.8 ± 3.6	–	12.1 ± 0.5	4.1	–	59.3	71.4 ± 3.7	1.58, 1.00
		15	26.6 ± 1.1	4.1	94.0	–	–	15	26.5 ± 1.1	4.5	96.0	–	–	–
Strawberry flavored milk	2/1:250	–	13.6 ± 0.6	4.4	–	102.2	116.0 ± 4.1	–	13.2 ± 0.6	4.5	–	103.0	116.0 ± 4.2	1.27, 1.00
		15	27.8 ± 1.2	4.3	94.7	–	–	15	27.5 ± 1.1	4.0	95.3	–	–	–

[a] The average plus standard deviation of five replicate measurements for nitrite and total nitrite after pre-treatment with two different reduction approaches.
[b] The nitrate value calculated from the difference between initial nitrite and total nitrite after pre-treatment with two different reduction approaches.
[c] In order to compare the mean values and their standard deviations for nitrite contents of independent two samples t- and F-tests with equal sample size the statistical t- and F-critical values at 95% confidence level and 8 degrees of freedom are 2.31 and 6.39, respectively.

comparison of the mean values and their RSDs demonstrated that there was no significant difference between the mean values obtained by two reduction procedures at the significance level of 0.05 (Miller and Miller, 2005). Because the experimental t-values ranging from 0.53 to 1.58 are lower than the tabulated t-value of 2.31, it can be concluded that the mean values obtained by two reduction approaches does not contain a significant difference for 8 degree of freedom at 95% confidence level. It is clear that the proposed method for beverage samples has a good reproducibility as a measure of precision by variance analysis based on pooled standard deviation with experimental $F_{(4,4)}$-value ranging from 1.00 to 1.56.

Also, the method was applied to the determination of trace nitrite, nitrate and total nitrite in selected milk samples. From the results obtained in Table 4b, it can be seen that the nitrite contents obtained by using both reduction approaches is highly quantitative with a lower RSD than 5.5% (n: 5). As can be seen from Table 4b, the student's t and variance ratio-tests based on two-paired ANOVA test statistically demonstrated that there was no significant difference between the mean values obtained by two reduction procedures at the significance level of 0.05. Because the calculated t-values ranging from 0.14 to 1.58 are lower than the tabulated t-value of 2.31, it can be concluded that the mean values do not contain a significant difference for 8 degree of freedom at 95% confidence level. It is clear that the proposed method for food samples has a good reproducibility as a measure of precision by variance analysis based on pooled standard deviation with experimental $F_{(4,4)}$-value ranging from 1.00 to 1.56. As a result, from the results obtained by the proposed CPE method after dilution of the digested samples at 10–50-fold for beverages with and without alcohol, 250-fold for milk samples, it is clear that the levels of nitrite found in beverages without alcohol (0.051–0.403 mg L^{-1}), beverages with alcohol (0.175–0.411 mg L^{-1}), milk and milk products (1.36–3.40 mg L^{-1}) are lower than the maximum permitted level of 50 mg kg^{-1} or mg L^{-1} while the levels of nitrate found in beverages without alcohol (0.85–3.16 mg L^{-1}), beverages with alcohol (3.17–7.15 mg L^{-1}), milk and milk products (12.1–25.6 mg L^{-1}) are lower than the maximum permitted level of 200 mg L^{-1}. Shortly, the results show that the proposed method is reliable and suitable for its application in a range of scenarios for monitoring of beverage and milk samples with high sensitivity.

In light of all these results, it can be concluded that our method shows a LOD that is better than those given by similar CPE methods including solid phase spectrophotofluorimetry, solid-phase fluorescent quenching and amperometric sensor based on IL-SWCNT reported in literature (Afkhami et al., 2005; Pourreza et al., 2012; Filik et al., 2011; Li and Jiang, 2007; Li et al., 2003; Zhou et al., 2013). The preconcentration methods based on detection with spectrophotometry were generally applied to water samples with low organic matrix. The other techniques with higher detection limits like FA-VPG-FT-IR, diffuse UV–visible reflectance method, microchip elecetrophoresis, optical nitrite sensor, catalytic spectrophotometry, electropolymerized Nile blue sensing film-based nitrite sensor, voltammetric techniques such as ASV and DPV, HPLC and IPC with chemiluminescence detection (Gallignani et al., 2004; Khan et al., 2012; Luiz et al., 2012; Shiddiky et al., 2009; Ensafi and Amini, 2012; Sobhanardakani et al., 2013; Chen et al., 2008; Guanghan et al., 1997; Santos et al., 2009; Mesaros et al., 1997; Kodamatani et al., 2009, 2011) are generally time-consuming, Q2 excess solvent consumption, expensive, selective, simple and/or complicated instruments, which needs an experienced user in her/his area according to spectrophotometer, which is available in almost every research laboratory. Moreover, some of these techniques might not be always available in all routine analytical laboratories. In the sense of robustness of the developed method in especially low concentrations, it is clear that the precise with a

lower RSD than 5.5% (n: 5) is within acceptable limits with those of other methods in literature. It is important to emphasize that 0.75 mL surfactant-rich phase in acetonitrile is sufficient to obtain the sensitivity enhancement and preconcentration factors of 76 and 67, respectively and the whole analytical procedure including sampling and detection steps can be done just in a centrifuge tube of 50 mL in an analysis time of 45 min.

4. Conclusions

The results indicate the usefulness of the proposed CPE/spectrophotometric method to quantitative extraction of trace nitrite, nitrate and total nitrite present in selected beverage and milk samples. The proposed preconcentration method reliably allowed the quantification of nitrite at 0.42 $\mu g\,L^{-1}$ levels in the linear range of 1.5–150 $\mu g\,L^{-1}$ at 450 nm with CPE after fast, accurate and reliable dissolution under ultrasonic power, and thus represents a promising approach in the monitoring of nitrite in different beverage and foods with low cost, simplicity, efficiency, versatility and a non-polluting process. The proposed CPE method gives very low LOD, high preconcentration and enhancement factors and good precision and extraction of indirect nitrite as ion-pairing complex with non-ionic surfactant, Triton X-114, from its initial matrix after pre-treatment with two reduction procedures. Because it is versatile, it can also be applied to the simultaneous monitoring of nitrite and nitrate in various samples in environmental, toxicological and medical analyses, including beverage and milk samples. The method can be considered as an alternative tool to selective, expensive, time-consuming, excess-solvent-consuming and experienced user-requiring analytical techniques such as FA-VPG-FT-IR, diffuse UV–visible reflectance method, HPLC and ion-pairing chromatography with UV- and fluorescence-detection, voltammetric techniques (ASV and DPV), optical and electroanalytical sensors based on catalytic effect.

Acknowledgments

Q3 Financial assistance from CUBAP, Sivas in Turkey, is sincerely thanked. Thanks are also due to Prof. Dr. Mehmet Akçay for his dedicated assistance and contribution in critical evaluation and publication steps of this research article. The present study was also presented in the 6th Black Sea Basin Conference on Analytical Chemistry with international participation, organized by Department of Chemistry, Faculty of Science, Karadeniz Technical University in 10–14 September 2013, Trabzon, Turkey.

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Please cite this article in press as: Gürkan, R., Altunay, N., Preconcentration and indirect quantification of trace nitrite, nitrate and total nitrite in selected beverage and milk samples using ion-pairing cloud-point extraction with acridine orange. J. Food Compos. Anal. (2015), http://dx.doi.org/10.1016/j.jfca.2015.04.009