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Sequential injection spectrophotometric determination of tetracycline antibiotics in pharmaceutical preparations and their residues in honey and milk samples using yttrium (III) and cationic surfactant

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ABSTRACT

A sequential injection analysis (SIA) spectrophotometric method for determining tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC) in different sample matrices were described. The method was based on the reaction between tetracyclines and yttrium (III) in weak basic micellar medium, yielding the light yellow complexes, which were monitored at 390, 392 and 395 nm, respectively. A cationic surfactant, cetyltrimethylammonium bromide (CTAB) was used to obtain the micellar system. The linear ranges of calibration graphs were between 1.0×10^{-5} and 4×10^{-4} mol L^{-1}, respectively. The molar absorptivities were 5.24×10^5, 4.98×10^4 and 4.78×10^4 L mol^{-1} cm^{-1}. The detection limits (3σ) were between 4.9×10^{-6} and 7.8×10^{-6} mol L^{-1} whereas the limit of quantitations (10σ) were between 1.63×10^{-5} and 2.60×10^{-5} mol L^{-1} the interday and intraday precisions within a weak revealed as the relative standard deviations (R.S.D., $n = 11$) were less than 4%. The method was rapid with a sampling rate of over 60 samples h^{-1} for the three drugs. The proposed method has been satisfactorily applied for the determination of tetracycline and its derivatives in pharmaceutical preparations together with their residues in milk and honey samples collected in Chiang Mai Province. The accuracy was found to be high as the Student's *t*-values were found to be less than the theoretical ones. The results were compared favorably with those obtained by the conventional spectrophotometric method.

1. Introduction

Tetracycline and its derivatives are extensively used in current therapy owing to their broad-spectrum antibacterial activity in human, animal nutrition as feed additives and veterinary medicines. They contain a 4-ring structure with a wide variety of functional groups that form complexes with various metal ions in aqueous solution. This ability is responsible for its antibacterial action [1]. This group of antibiotics can be contaminated in the environment via their broad-spectrum antibacterial activity as described above. Tetracycline residues can be found in various sample matrices such as water, soil, stream sediments agricultural products such as fruits, vegetables, milk and honey. TCs residues found in milk samples arise from the applications of TCs in animals for preventive treatment of bacterial inflections and increase growth rates. The rate of metabolism of TCs in dairy cow has been reported to be 25–75% [2] and a significant percentage of administrated TCs is excreted in bovine milk. If the TCs have been improperly administrated or If the withdrawal time for the treated cows has not been observed, TCs and their degradation products may be present in market milk which cause harmful effects on consumers, such as possible allergic reactions, liver damage, yellowing of teeth and gastro-intestinal disturbance due to the selective pressure of antibiotics on human gut micro flora. The maximum residue limit (MRLs) in milk of 0.1 mg L^{-1} for each TC derivative was recommended by WHO. However, no MRLs have restricted for use with bee products. Antibiotics such as TCs have had MRLs fixed for their

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utility in large animals but are illegal for use with bees. Honey is considered to be natural healthy food containing primarily sugar and water [3]. Therefore, the presence of TCs in honey is illegal at any level [4]. The Spanish plan for residue control and healthy food (Plan CRE HA) of 2002 has established maximum allowable limit for the concentration of residual substances [5]. The maximum acceptable limit for the tetracyclines (TC, OTC and CTC) has been set at 100 ng g^{-1} or 100 ng mL^{-1} after the three antibiotic have been analyzed in meat, milk, honey and eggs by HPLC with the detection limit of 20 ng g^{-1} or 20 ng mL^{-1}. These limits are similar to those recommended by the Belgian Agency for Safe Foods.

According to the harmful effects of this group of antibiotics and their residues contaminated in the environment therefore sensitive, cost effective, rapid and reliable analytical techniques based on green chemistry are increasingly demand.

Most spectrophotometric procedures for the determining tetracyclines are based on their complexation abilities to combine with various metal ions, such as iron (III) [6,7], copper (II) [8], magnesium (II) [9,10] and some lanthanide ions, especially those of europium (III) [11], and indium (III) ions [12].

Since the conventional or batch-wise spectrophotometric methods for tetracyclines determinations are tedious and time consuming together with rather large amounts of sample and reagent consumption and waste released. Flow injection analysis (FIA) methods with spectrophotometric detections, based on complex formation between the drugs and various metal ions, have been employed instead [13,14]. Although FIA is superior to the batch-wise methods in that they provide relatively high sample throughput, low reagents and sample consumptions, low waste production, low analysis time and cost effectiveness, some of which typically exhibits considerably high reagents consumptions and produce a relatively large amount of waste especially dealing with expensive chemicals, hazardous reagents, or online/remote site applications. Thus, the FIA technique is a relatively expensive method by comparison with the second generation termed sequential injection analysis (SIA) [15]. This technique has been used for pharmaceutical assays for a number of years. Several articles dealing with pharmaceutical analysis have been published on the recent review [16]. Therefore, SIA seems promising to be adopted as a basis for the development of greener analytical method for determination of tetracycline in several sample matrices.

Currently, the use of surfactant in analytical chemistry is growing [17–23] especially in the beneficial alteration of metal ion–ligand complex spectral properties via surfactant. The system used in development of several spectrophotometric methods for determining microgram amounts of metal ions to improve the sensitivity [24]. Usually, the metal-complexes formed in the micellar systems, are more stable than those formed in the absence of micelles because the complexes are stabilized by a pseudo-single-phase formed by surfactant micelles in an aqueous solution. Surfactants and micellar systems stabilized water-insoluble complexes, and the size of micelles provided the equilibrium to be attained quickly with a change in pH.

Synthesis and spectroscopic characterization of chelates formed by a number of rare-earth elements (lanthanides) with tetracycline antibiotics (TCs) together with their antibacterial activity have been reported. However no analytical aspects of such chelates have been revealed [25]. Lanthanides have been used as reagents (metal ions) for TCs determinations excluded yttrium (III). Very few other rareearth elements such as Eu^{3+} [11,26], In^{3+} [12] and Tb^{3+} [26] have been previously employed as reagents for spectrophotometric determination of tetracyclines. Yttrium is one of the rare earth elements (lanthanide) its chemistry should be very similar to those of other lanthanides. The structure of Y^{3+}–TCs complex should be corresponding to the general structure of lanthanide-TCs chelate (Fig. 3a) proposed by Karthikeyan et al. [25]. Therefore, the structure of Y^{3+}–TCs complex is shown in Fig. 3b. Among the rare earth elements employed for chelation with TCs for TCs determination Eu^{3+} is the most frequently used [11,26–29] for spectrofluorimetric and/or spectrophotometric determination of TCs in various sample matrices such as citrate in tablet and OCT in serum [27], methacycline and glucose in biological fluids [28] and TCs in blood plasma [29]. To our present knowledge SIA determination of TC, CTC and OTC in pharmaceuticals, milk and honey samples based on complexation of Y^{3+} with TCs in the presence of cationic surfactant (CTAB) using home-made software to control the home-made SIA manifold has not been previously reported.

The present works describes a cost effective, rapid, sensitive and reproducible SIA spectrophotometric procedure for determining TC, CTC and OTC in pharmaceutical honey and milk samples using a home-made SIA manifold coupled with the PC using the home-made software to control the whole SIA system. The method is based on the complexation of tetracyclines with yttrium (III) in Tris-(hydroxymethyl)aminomethane buffer (Tris-buffer) in the presence of cationic surfactant, CTAB as a micellar system in a weak basic medium (pH 7.5–8.0). The resulting light yellow complexes arising from these drugs with Y^{3+} were measured at different λ_{max} values. Comparative determinations of tetracyclines by conventional spectrophotometric method based on the complexation of iron (III) with tetracyclines in 0.001 mol L^{-1} sulphuric acid were also carried out. A brown solution was obtained, with each TC derivative giving characteristic wavelengths of maximum absorption (λ_{max}) 423 nm for tetracycline and 435 nm for the others [30].

2. Experimental

2.1. Instrumentation

2.1.1. Commercial SIA manifold

The commercial SIA manifold was use to validate the home-made one. The system consists of the following equipments: a FIA Lab®-3000 system (FIA Lab Instruments, USA) consisting of a syringe pump (syringes reservoir 2.5 mL) and a 6-port selection cheminert valve (Valco Instrument Co., Ltd., USA) which was connected to a 4-port RS-232 switching box. A Jenway 6400 Spectrophotometer (Jenway, Dunmow, Essex, UK) equipped with a 10 mm path-length flow through cell (80 μL Hellma, Germany) was used as detector. All manifolds were used PTFE tubings were used as flow lines including holding coil which was made from PTFE tubing by winding around small test tube.

2.1.2. Home-made SIA manifold

The home-made SIA manifold was developed using an ATMEGA-8 microcontroller to control the syringe pump (XCALIBIR-3000, CAVRO Co., Ltd., USA) and a 12-port selection valve (VICI Instruments, USA). The home-made detector was consisted of a Triband RGB LED controlled with PWM generated by ATMEGA-8 microcontroller to the designed wavelength and the LDR which was responsible for measuring the intensity of the light after passing through a 10 mm path-length flow through cell (80 μL Hellma, Germany) with the same manifold. All flow cell housing was made from a black acrylic engraved with LASER performing a cell holder abutting together with nuts and screws. The microcontroller has built in 8 port with the resolution of 10-bit analog to digital converter where the light intensity (in term of voltage) could be transferred digitally.

The differentiation between the commercial SIA and the home-made SIA is the switching box. FIA Lab®-3000 connected to the switching box at the main port while syringe pump and multi-position valve are connected through port A, B, C or D as configure in the same protocol and baudrate

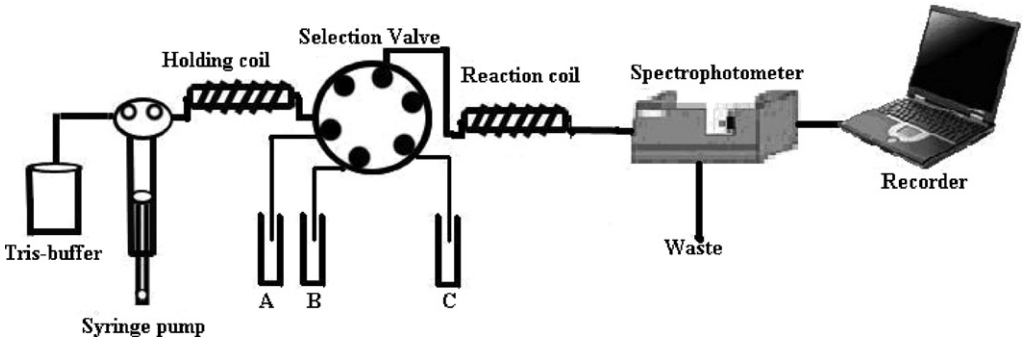

Fig. 1. SIA diagram for determination of tetracycline and its derivatives, A = standard/sample; B = CTAB solution and C = yttrium (III) solution.

while the microcontroller processes this protocol in one session. The home-made software was developed by Microsoft Visual Basic 6.0 coupled with Microsoft Communication Control ActiveX and the ATMEGA-8 firmware which was written in BASCOMAVR developing suite. Both commercial and home-made softwares were linked with the personal computer using RS-232 protocol.

2.2. Reagents and solutions

All reagents used were of analytical reagent grade and used without further purification. Deionized water was used for all solution preparation throughout the experiments.

Standard stock solutions of TC and CTC (1×10^{-3} mol L^{-1}) were prepared by accurately weighing and dissolving appropriate amounts of tetracycline hydrochloride (Fluka, Switzerland) (accurately weighed) in 1000 mL water in a 1000 mL volumetric flask and were kept in a refrigerator at a temperature below 4 °C. The working solutions were prepared daily by appropriate dilution of the stock solution. For OTC, appropriate amount of this drug (accurately weighed) was dissolved in a suitable volume of water (250 mL) in a beaker. The solution was warmed at 60 °C with constant stirring for 20 min. The solution was transferred into a 1000 mL volumetric flask and made up to the mark with water.

A stock solution of yttrium (III) (100 ppm) was prepared by dissolving about 4.3 g (accurately weighed) (0.4308 g) of yttrium (III) nitrate hexahydrate (Aldrich, USA) in 5.0×10^{-3} mol L^{-1} cetyltrimethylammonium bromide (CTAB). Further dilutions of the stock solution in 5.0×10^{-3} mol L^{-1} CTAB were made for appropriate concentrations.

A stock solution of CTAB solution (0.10 mol L^{-1}) was prepared by dissolving 36.45 g of cetyltrimethylammonium bromide (Fluka, Switzerland) in 1000 mL water. Further dilutions were made for appropriate concentrations.

A 0.01 mol L^{-1} Tris-buffer solution was prepared by dissolution of 1.21 g Tris (hydroxymethyl) aminomethane in 1000 mL of water and adjusting the pH to a desire values (7.5) with 1 mol L^{-1} hydrochloric acid (J.T. Baker Inc., USA).

2.3. Sample preparation

2.3.1. Pharmaceutical samples

2.3.1.1. Capsules samples. The drug powder content of ten capsules were transferred into a beaker then dissolved in deionized water, warmed at 60 °C for 20 min and transferred into a 1000 mL volumetric flask by filtering through a No. 41 filter-paper (Whatman), cooled to room temperature and diluted to the mark with the distilled deionized water. Afterward, appropriate volume of the sample solutions were taken and diluted into 100 mL volumetric flask with distilled deionized water to obtain the drug concentration of about 5.2208×10^{-5} mol L^{-1}.

2.3.1.2. Ointments. Ophthalmic ointment samples, about 0.5 g of sample was accurately weighed and dissolved in water in a 50 mL volumetric flask. The solution was incubated at 60 °C for 10 min in a water bath. Then a 20 mL aliquot of the solution was pipetted into a 50 mL volumetric flask and diluted to the mark with water.

2.3.2. Food samples

2.3.2.1. Milk samples. An aliquot 15 mL of each milk sample was taken (by pipette) and diluted to 25 mL with McIlvaine buffer-EDTA solution (pH 4) followed by centrifuging for 15 min. The supernatant was collected and passed through a Florisil syringe column. The TCs sorbed on the Florisil column were eluted with 5 mL of a mixture consisting of 0.2 mol L^{-1} oxalic acid and acetonitrile (4:1, v/v).

2.3.2.2. Honey samples. An appropriate amount of honey sample (50 g) was weighted (accurate to 0.1 g) and dissolved in 200 mL of 2% citric acid buffer solution (pH 4) the pH was adjusted to 4.5 with 2% citric acid solution and 60 mL of pH 4.5 phosphate buffer solution (0.1 mol L^{-1}) was added. The sample solution was analyzed by the SIA method.

2.4. Sequential injection method

A diagram of the SIA instrument used for tetracycline and its derivatives determination is shown in Fig. 1. The carrier steam (Tris-buffer) was provided by the syringe aspirating with the desired volumes. The sequence was started by aspirating the sample/standard solution into the holding coil (1.02 mm i.d., 150 cm long) directly, followed by CTAB solution and yttrium (III) solution, respectively. The syringe pump transported the complex through the reaction coil (1.02 mm i.d., 80 cm long) to a flow-through spectrophotometric detector. The maximum peak height was also detected at an appropriate wavelength and displayed. Table 1 lists the steps of the procedure entered to the home-made software to control syringe pump and selection valves. The software was also used for controlling, recording and analyzing the data from the procedure. In order to evaluate the performance of the home-made SIA device and software, the same procedure was repeated using the FIAlab® for windows software for controlling the syringe pump and selection valves. The home-made software was used to control readout and data analyzing device.

Table 1
Experimental procedure used in the home-made software and FIAlab® for Windows with the same protocol.

Loop Start (#) 4	
	'Send sample to detector
'Fill Syringe	Valve port 6
SyringePump Flowrate (μL s^{-1}) 100	SyringePump Valve Out
SyringePump Valve In	SyringePump Flowrate (μL s^{-1}) 35
SyringePump Delay Until Done	SyringePump Empty
	SyringePump Delay Until Done
SyringePump Aspirate (μL) 1500	
SyringePump Valve Out	**Clean detector**
SyringePump Delay Until Done	SyringePump Flowrate (μL s^{-1}) 100
	SyringePump Valve In
'standard to holding coil	SyringePump Delay Until Done
Valve port 1	
SyringePump Valve Out	SyringePump Aspirate (μL) 250
SyringePump Flowrate (μL s^{-1}) 25	SyringePump Valve Out
SyringePump Aspirate (μL) 50	SyringePump Delay Until Done
SyringePump Delay Until Done	
	Valve port 6
Valve port 2	SyringePump Valve Out
SyringePump Valve Out	SyringePump Empty
SyringePump Flowrate (μL s^{-1}) 25	SyringePump Delay Until Done
SyringePump Aspirate (μL) 50	
SyringePump Delay Until Done	**Loop End**
Valve port 3	Total time: 50 s
SyringePump Flowrate (μL s^{-1}) 25	
SyringePump Aspirate (μL) 30	
SyringePump Delay Until Done	

3. Results and discussion

3.1. Preliminary investigation

3.1.1. Choice of surfactants

Surfactants can stabilize the solution by preventing the sedimentation of the particles. Preliminary experiments pointed out that the addition of certain non ionic and anionic surfactant into the TCs–Y^{3+} system, the SIA signals were not observed. Among the different cationic surfactants tested, CTAB exhibited the greatest peak height which was selected because the attainable sensitivity was the highest with reasonable low back ground signals and high reproducibility.

The advantages to employ yttrium (III) as metal ions (as reagent) to complex with TCs for their determinations are as follows: (i) yttrium is one of the rare earth elements as well as other lanthanides. In contrast with Eu^{3+}, TCs are ideal ligand to chelate with Eu^{3+} leading to the development of sensitive method for these drug determinations Y^{3+} should behave similarly. (ii) Preliminary experiments revealed that Y^{3+} seemed promising to complex with TCs as well as Eu^{3+} which can be adopted as a basis for the development of a sensitive method for TCs determinations. (iii) Y^{3+} as oxide is one of the starting materials for the preparation of the Y^{3+}-system high critical temperature (T_c) superconductor and it is available in the author's laboratory. It is possible to develop a sensitive method for determining Y^{3+} in superconducting material samples containing Y^{3+} using TCs as chelating agent which may be published elsewhere in the near future.

3.1.2. Evaluation of the home-made SIA manifold and the home-made software

The performance of the home-made SIA manifold with the home-made software have been evaluated initially by comparative determination of tetracycline in standard tetracycline solution containing 3.0×10^{-5} mol L^{-1} tetracycline using the home-made SIA device and the commercially available one it was found that the % recoveries of both methods were $99.8 \pm 0.01\%$ and $99.8 \pm 0.02\%$ ($n = 5$), respectively, indicating that results obtained from both devices were in good agreement.

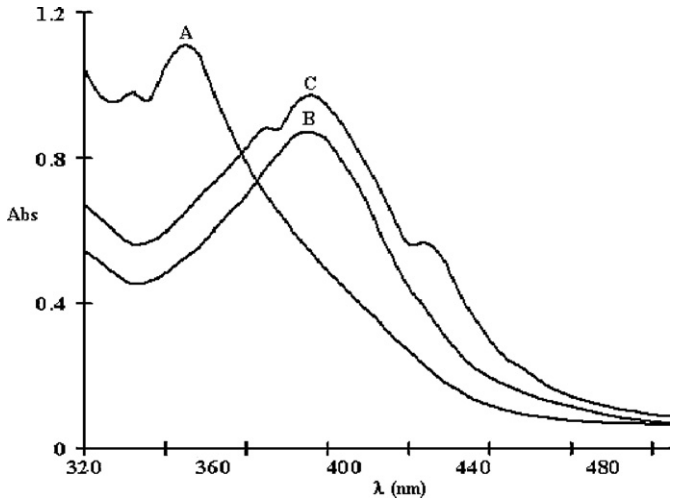

Fig. 2. Absorption spectra of (A) TC, (B) TC–yttrium(III) complex and (C) TC–yttrium(III) in the presence of CTAB.

3.2. Absorption spectra of tetracyclines and their yttrium (III) complexes

The absorption spectra of CTC and its yttrium (III) complex in the absence and presence of CTAB are shown in Fig. 2. In the presence of the cationic surfactant, the light yellow complex shows maximum absorbance of 0.967 (Fig. 2C), which is relatively grater than that in the absence of the surfactant, presenting a maximum absorbance of 0.864 (Fig. 2B) at the λ_{max} of 389 nm and 392 nm, respectively. A slight bathochromic shift was observed in the presence of CTAB which was responsible for the enhancement of sensitivity. The CTC exhibits its absorption maximum at 353 nm (Fig. 2A). For other TC derivatives (CTC and OTC), as soon as they reacted with yttrium (III) under the same conditions resulting in soluble, and stable, light yellow complexes with the absorption maxima at 392 and 395 nm for CTC and OTC, respectively. All absorption spectra studied in Tris-buffer pH 7.5 medium, 10 mg L^{-1} of yttrium (III) and 5×10^{-5} mol L^{-1} of all tetracyclines. The stoichiometry of the metal-to-ligand ratio of each complex was determined by the mole ratio and the continuous variation methods, and was found to be 1:1 which may be similar to that suggested for lanthanide–tetracycline complexes [25]. Tetracyclines provide a number of potential donor atoms in various positions which can bind to various metal ions, known as chelating ligand. Tetracyclines behave as a bivalent anion after losing an appropriate number of protons and forming stable crystalline complexes with trivalent lanthanide metal ions. In the binuclear systems, each lanthanide metal ion is bound to four oxygen of the ligand and three chloride ions, with two molecules of water of hydration in the lattice, representing a coordination number of seven. The most probable molecular shape of the complex is a pentagonal bipyramid, as shown in Fig. 3. The molar absorptivities at the optimum wavelengths for the complexes formed by TC, CTC and OTC with yttrium (III) were 5.24×10^5, 4.98×10^4 and 4.78×10^4 L mol^{-1} cm^{-1}, respectively in Tris-buffer pH 7.5 and the CTAB micellar medium.

3.3. Investigations of the experimental conditions

This investigation was aimed at development of a simple SIA spectrophotometric method for determination of TC, CTC and OTC in pharmaceutical preparations and their residues in real samples (milks and honey samples). The parameters including pH, yttrium concentration, CTAB concentration, aspiration volumes, flow rate and holding reaction coil length related to the signal response were studied.

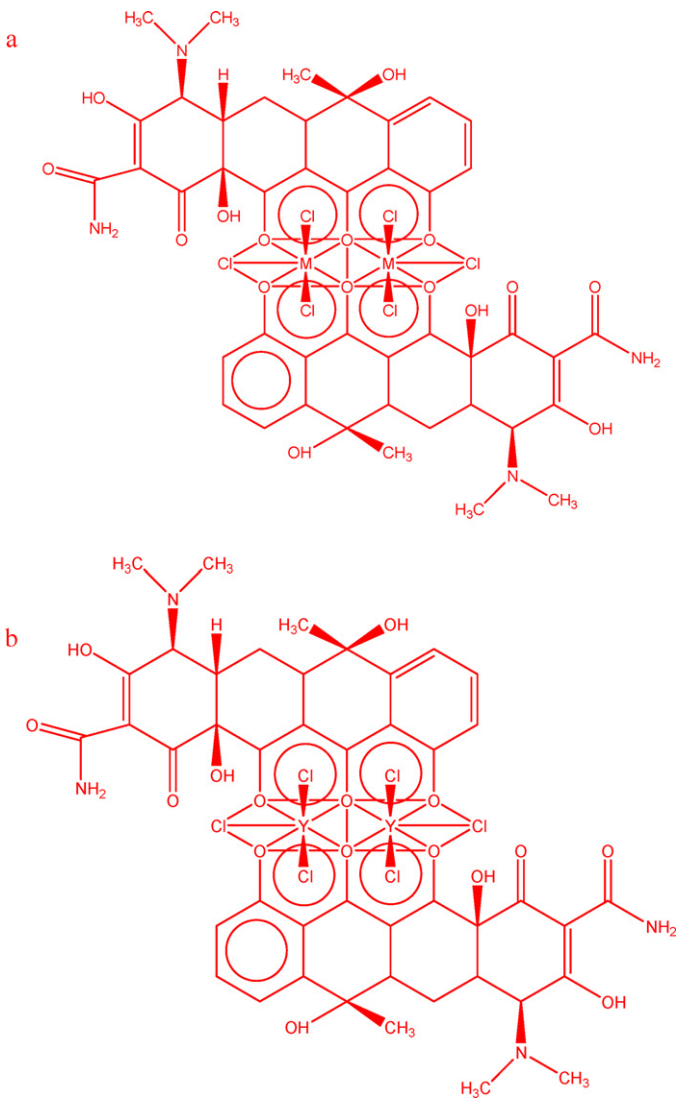

Fig. 3. Structure of lanthanide–tetracycline complex: (a) general structure and (b) structure of Yttrium-tetracycline complex.

3.3.1. *Effect of pH on the formation of TCs – yttrium (III)*

The complexations between three tetracyclines and yttrium (III) in Tris-buffer medium were studied over the pH range of 5.0–9.0. The peak heights (as absorbance in mV) of the complexes were measured in Tris-buffer solution with the addition of a sufficient volume of 1 mol L^{-1} HCl to give the required pH value for tetracycline or each tetracycline derivative. It was found that, the pH values were higher than 6 (Fig. 4), the peak height increased significantly when the pH exceeded 7.0 (for TC) and 7.5 (for CTC and OTC), the peak height decreased slightly and reached the minimum value at pH about 8 then the peak height remained constant. The pH values of 7.0, 7.5 and 7.5 were chosen for TC, CTC and OTC, respectively, as the absorbances at these pH values exhibited the greatest peak heights (Fig. 4) and the best reproducibility with reasonable low background signals for the above three antibiotics.

3.3.2. *Effect of yttrium (III) concentration*

The effect of yttrium (III) concentrations on the absorbance (as peak height) of the complex was investigated at various yttrium (III) concentrations from 0.25 to 20 mg L^{-1}. The peak height increased with increasing concentration of yttrium (III) and remained constant when the concentration was above 10 mg L^{-1} (Fig. 5). In order to obtain the greatest peak height and repeatability, the yttrium (III)

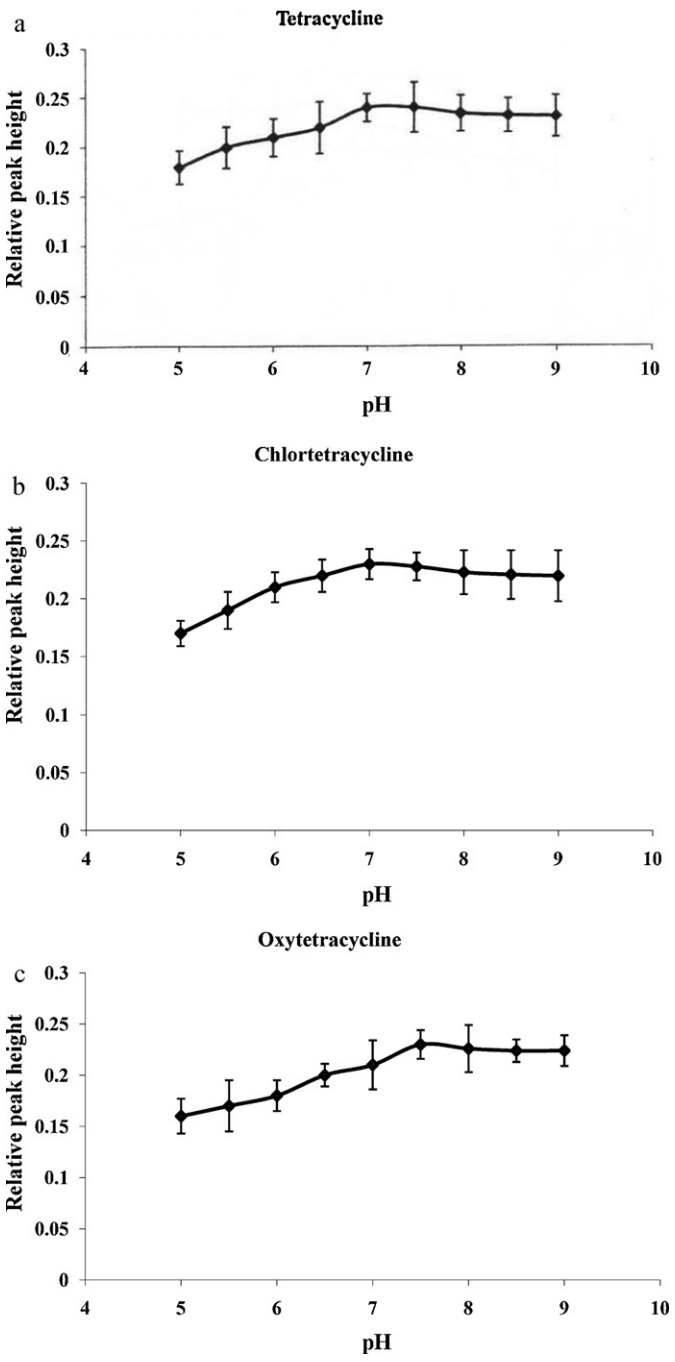

Fig. 4. The effect of pH on the peak height and precision for each tetracycline: (a) tetracycline, (b) chlortetracycline and (c) oxytetracycline.

concentrations of 15, 10 and 10 mg L^{-1} were chosen for TC, CTC and OTC, respectively.

3.3.3. *Effect of CTAB concentration*

Preliminary experiment revealed that CTAB exhibited the enhancement in sensitivity for TCs determinations. In order to obtain the greatest sensitivity the CTAB concentration should be optimized. The influence of CTAB concentration was studied from 1.25×10^{-3} to 1.50×10^{-2} mol L^{-1}. The peak height increased with increasing concentration of CTAB. However, the increase in SIA response above 1.0×10^{-2} mol L^{-1} was not linearly related to the concentration of any tetracyclines. Therefore, the concentrations of 7.0×10^{-3}, 7.5×10^{-3} and 5.0×10^{-3} mol L^{-1} were chosen

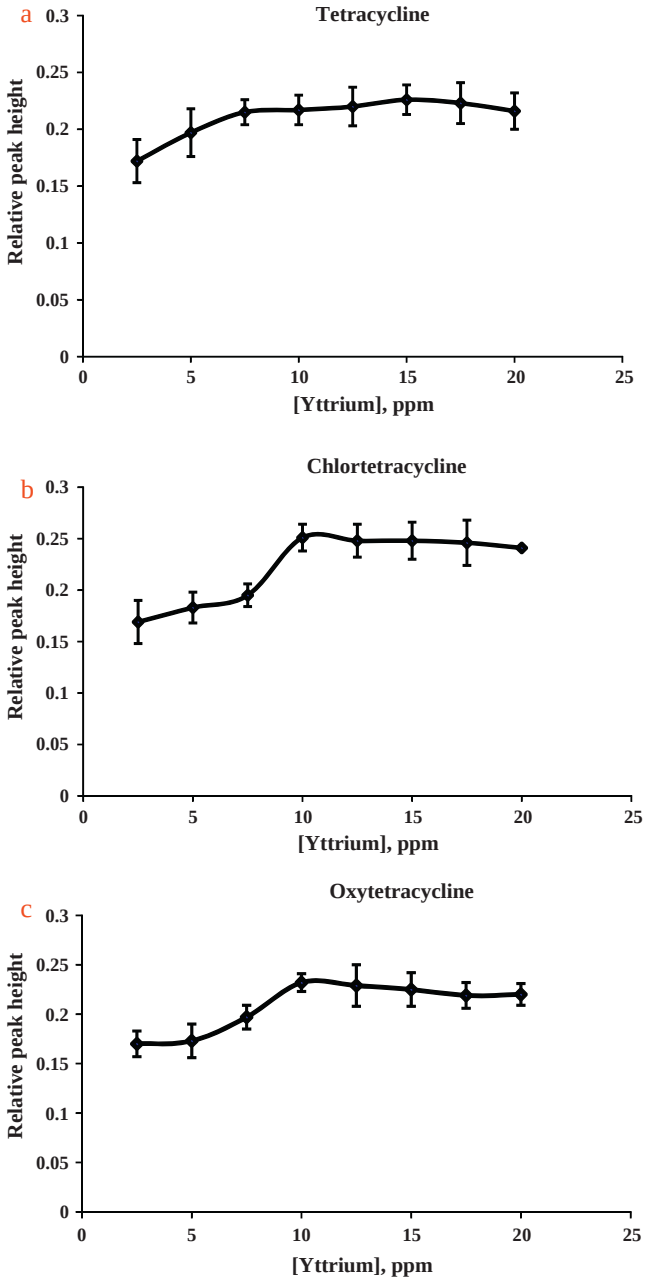

Fig. 5. The effect of yttrium (III) concentrations on peak height and precision for each tetracycline: (a) tetracycline, (b) chlortetracycline and (c) oxytetracycline.

for subsequent experiments for determining TC, CTC and OTC, respectively.

3.3.4. Aspiration volumes

Aspiration volumes of sample and reagents involve in the reaction as well as the reaction time. It is obvious that the higher aspiration volume of the sample or reagent solution leading to the longer reaction time is required. In this paper, the effect of aspiration volumes of the yttrium (III), CTAB and sample solutions were investigated over the range of 10–100 μL at every 10 μL interval. For the optimization of experimental conditions for CTC determination, initially the sample volume was studied, by keeping others reagent volumes constant at 50 μL and the sample volume aspirated into the SIA system was varied. It was shown that the peak heights for the successive increment of sample volume increase until a maximum was reached at 50 μL. Further increment of the sample volume the peak height started dipping. The suitable sample volume chosen was used for further experiment was 50 μL due to its maximum peak height and the best precision. Then, the aspiration volume of yttrium (III) solution was studied. The maximum SIA responses were obtained as aspiration volumes over the range 50–100 μL but due to the short analysis time the 50 μL was chosen. The CTAB aspiration volume was optimized by keeping the sample and yttrium (III) volumes at 50 μL. There is an increase in peak height when the aspiration volumes were altered from 30 to 80 μL followed by a decrease in peak height as soon as the aspiration volume exceed 80 μL with an improvement in precision. A volume of 30 μL was chosen since it gave the best peak height and precision. The optimum aspiration volumes for the other antibiotic drugs were investigated in the same manner. Results were shown in Table 2.

3.3.5. Flow rates

It was evident that the flow rates of the aspirated sample and reagent solutions were significant with the peak height. The reagent and sample flow rates were investigated from 15 to 50 μL s^{-1} at every 5 μL s^{-1} interval while the flow rate of delivering sample to the detector was kept constant at 50 μL s^{-1}.

For TC, as the CTAB aspiration flow rate increases the peak height increases up to 35 μL s^{-1}, where after it tends to level off. The CTAB aspiration flow rate chosen for yielding the best results was 25 μL s^{-1} (best precision) because the faster aspiration rates resulted in the bubbles in the flow line of the micellar solution stream.

The flow rates of delivering the sample solution to detector were investigated from 15 to 50 μL s^{-1} at every 5 μL s^{-1} intervals while the flow rate of aspiration of the sample and reagent were kept constant at 25 μL s^{-1}. It was observed that the peak height increased with increasing the flow rate up to 35 μL s^{-1} and then it decreased with provision of the faster flow rates. Thus, a flow rate of 35 μL s^{-1} was chosen and used for the subsequent measurements due to its highest peak height and precision. The optimum flow rates for the others drugs were examined in the same manner that were shown in Table 2.

3.3.6. Reaction coil length

The Teflon or Tygon tubing were used as flow lines and the mixing reactor that play important roles on SIA signals as well as FIA signals because peak height or peak area depends on the resident time of the sample zone in tubing with an appropriate inner diameter and length of the tubings used for making the reactor. In this experiment a coiled reactor was used. The inner diameter of the mixing tubing has to be optimized, because the dispersion of the sample zone increases with the mixing tubing diameter, and band boarding eventually results in a loss of sensitivity and a lower sam-

Table 2
Optimization of experimental parameters for determination of TC, CTC and OTC by sequential injection analysis.

Parameters	TC	CTC	OTC
Wavelength, nm	390	392	395
pH	7.0	7.5	7.5
[Yttrium], mg L^{-1}	15.0	10.0	12.5
[CTAB] × 10^{-3} mol L^{-1}	7.5	7.5	5.0
Aspiration volume, μL			
Standard/sample	40	50	50
Yttrium (III) solution	50	50	50
CTAB solution	40	30	30
Flow rate, μL s^{-1}			
Flow rate of aspiration of sample and reagents	25	25	20
Flow rate of sending sample to detector	35	25	25
Holding reaction coil length, cm	60	80	110

Table 3
Results obtained from tetracyclines determination in commercial pharmaceutical preparations in Thailand by the proposed method and reference procedure.

Drugs	Mean recovery ± SD (%)[a]		*t*-Test value[c]
	SIA method	Conventional spectrophotometric method[b]	
Hydromycin (tablet TC = 250 mg)	99.3 ± 0.58	102.1 ± 0.32	1.21
Tetra central (capsule TC = 250 mg)	98.9 ± 1.04	101.2 ± 0.88	0.95
Lenocin (capsule TC = 250 mg)	101.5 ± 1.12	101.8 ± 0.94	1.07
Aureomycin (capsule CTC = 250 mg)	100.8 ± 0.95	101.6 ± 0.56	1.24
Chlortralim (ointment 1% CTC)	101.8 ± 0.83	102.1 ± 0.78	1.03
Oxycline (capsule OTC = 250 mg)	102.43 ± 1.21	100.78 ± 1.07	1.17
Oxylim (capsule OTC = 250 mg)	98.78 ± 1.06	98.79 ± 0.91	1.31

[a] $n = 5$,.
[b] Ref. [30].
[c] t theoretical = 2.31, $n = 5$.

ple throughput [30]. The mixing tubing length for making the coiled reactor should be optimized to ensure adequate dispersion and kinetics absorbing species formation, so as to achieve the desired sensitivity. For these reasons, the inner diameter and the length of the tubings were altered over the ranges 0.5–1.3 mm i.d. and 30–120 cm, respectively for determining tetracyclines. It was noteworthy that the optimum inner diameter of the mixing tubing was 1.02 mm i.d. and that the length for the reaction coils were 60, 80, and 110 cm for TC, CTC and OTC, respectively.

3.4. Figure of merit

Under the optimized parameters listed in Table 2, the SIA system was evaluated for their response for different concentrations of standard tetracycline solutions (1.0×10^{-5} to 5.0×10^{-4} mol L^{-1}). Linear calibration curve for each drug was constructed with the following calibration equation: $Y = aX + b$, where Y = absorbance, X = concentration (mol L^{-1}), a = slope and b = intercept with a correlation coefficient (R^2). It was found that linear calibration curves for TC, CTC and OTC were established over the same concentration range of 1.0×10^{-5} to 4.0×10^{-4} mol L^{-1}. The linear regression equations for the three tetracycline were as follows: $Y_1 = 5481.5X_1 + 0.0317$, $Y_2 = 5237.2X_2 + 0.0295$, $Y_3 = 4933.7X_3 + 0.0739$ for TC, CTC and OTC, respectively with the correction coefficients of 0.9994, 0.9995 and 0.9991, respectively. The detection limit was defined as three times of the standard deviation of the blank signal (3σ) ($n = 11$)) which were 4.9×10^{-6}, 5.4×10^{-6} and 7.8×10^{-6} mol L^{-1}, respectively. The limits of quantitation (10σ) were found to be 1.6×10^{-5}, 1.8×10^{-5} and 2.6×10^{-5} mol L^{-1}, respectively. The repeatability of the method was checked for 1×10^{-5} and 5×10^{-5} mol L^{-1} standard solutions, the R.S.D. values were registered ($n = 11$ measurements in each case). It was shown that inter-day and intra-day precision (as RSD) for the three antibiotics were 1.5% and 3.3% for 5.0×10^{-3} and 1.0×10^{-5} mol L^{-1} tetracyclines, respectively. The sample throughputs for the three antibiotics were 70 h^{-1}, 67 h^{-1} and 60 h^{-1}, respectively. The sensitivity (defined as slope of calibration graph) for determining the three drugs was 5482, 5237 and 4934 mV/10^{-5} mol L^{-1} for TC, CTC and OTC, respectively.

The advantages of using yttrium (III) and cationic surfactant for the application for determining tetracycline antibiotics in pharmaceutical preparations and their residues in honey and milk samples are as follows: according to preliminary investigations, Y^{3+} as well as Eu^{3+} is promising for use as an ideal metal ion to form a light yellow, soluble complex with tetracycline antibiotics (three tetracyclines are tested, i.e., TC, CTC and OTC). The absorbance at the λ_{max} due to the colored reaction between Y^{3+} and tetracyclines can be enhanced by addition of certain cationic surfactant especially CTAB to a certain extent. A bathochromic shift of the λ_{max} is responsible for an improvement in absorbance and hence, the sensitivity. The stability and reproducibility of the Y^{3+}–TCs–CTAB ternary complex are also achieved.

This system has been successfully adopted as a basis for development of a novel, sensitive and reproducible SIA method for determining small amounts of the three antibiotics (TC, CTC and OTC) in real samples.

3.5. Analysis of real samples

In order to evaluate the method and the SIA manifold automatically controlled by the home-made software, the proposed SIA procedure has been applied for determination of TC, CTC and OTC in pharmaceutical, milk and honey samples. According to drug and food safety purposes the above three real samples were selected. Milks and honey are considered to be healthy foods the presence of TCs should be not exceeded the maximum residue limits (MRLs). For quality control these samples should be analyzed for their antibiotics contents which may cause harmful effect on consumers.

3.5.1. Determination of CTC in pharmaceutical preparations

The developed SIA method has been satisfactorily applied to the determination of TC, CTC and OTC in 7 commercial pharmaceutical preparations obtained from Chiang Mai Province, Thailand; after appropriate sample pretreatments. It was found that the % labelled amounts of TCs were over the ranges of 98.78 ± 1.06 to 102.43 ± 1.21 and 98.79 ± 0.91 to 102.10 ± 0.78 using the proposed SIA and the conventional spectrophotometric methods, respectively (Table 3).

3.5.2. Determination of TC, CTC and OTC residues in honey and milk samples

Similarly, the proposed SIA procedure has been again successfully applied for the determination of the three drug residues in 5 milk samples (MS_1–MS_5) and 6 honey samples (HS_1–SH_6) available in Thailand. The results obtained were presented in Tables 4 and 5 for honey and milk samples, respectively. It was found that all the three TCs residue in milk samples MS_1 and MS_4 were not detected. OTC (20.0 ± 0.35 ng mL^{-1}), TC (25.0 ± 0.50 ng mL^{-1}) and TC (15.0 ± 0.39 ng mL^{-1}) were found in milk samples codes MS_1, MS_2 and MS_5, respectively. However, the TCs residues found in the milk samples studied are far more less than the MRLs value (0.1 mg L^{-1}) recommended by the WHO. Regarding to TCs residues in the 6 honey samples, it was found that all the three TCs residues in the HS_4 and HS_5 honey samples were not detected. The OTC 60.5 ± 0.49 ng mL^{-1}, 75.7 ± 0.33 ng mL^{-1}, 60.0 ± 0.70 ng mL^{-1} and 105.9 ± 0.65 ng mL^{-1} were found in honey samples HS_1, HS_2, HS_3 and HS_6, respectively. The TC residues were found in honey HS_3 (7.2 ± 0.53 ng mL^{-1}) and HS_6 13.9 ng mL^{-1}. The TCs residues found in most honey samples were below the MRLs value (100 ng mL^{-1}), only the OTC residues in honey sample HS_6 was exceeded the MRLs value. Comparative determination of the three antibiotics in the

Table 4
Results obtained from tetracyclines determination in honey samples.

	TCAs	Original TCs (ng mL^{-1})		TCs standard drug added	TCs found (ng mL^{-1})		Recoveries (%)	
		SIA	STD method		SIA	STD method	SIA	STD method
HS_1	TC	ND	ND	50	49.9 ± 0.45	48.9 ± 0.45	99.8	97.8
	CTC	ND	ND	50	50.1 ± 0.53	50.0 ± 0.44	100.2	100.0
	OTC	60.5 ± 0.49	59.9 ± 0.49	100	160.7 ± 0.52	158.9 ± 0.49	102.0	99.0
HS_2	TC	ND	ND	50	49.9 ± 0.61	49.8 ± 0.58	99.8	99.6
	CTC	ND	ND	50	49.8 ± 0.70	49.8 ± 0.55	99.6	99.5
	OTC	75.7 ± 0.33	76.5 ± 0.50	100	175.6 ± 0.55	176.0 ± 0.49	99.9	99.5
HS_3	TC	7.2 ± 0.53	70.0 ± 0.55	50	57.5 ± 0.45	58.0 ± 0.72	100.6	102.0
	CTC	ND	ND	50	49.9 ± 0.39	49.8 ± 0.38	99.8	99.6
	OTC	60.0 ± 0.70	59.6 ± 0.65	100	160.2 ± 0.60	159.9 ± 0.55	102.0	100.4
HS_4	TC	ND	ND	50	50.1 ± 0.33	49.7 ± 0.75	100.1	99.4
	CTC	ND	ND	50	50.2 ± 0.47	50.1 ± 0.49	100.4	100.2
	OTC	ND	ND	100	100.1 ± 0.70	100.2 ± 0.37	100.1	100.2
HS_5	TC	ND	ND	50	50.1 ± 0.61	50.2 ± 0.44	100.2	100.4
	CTC	ND	ND	50	50.1 ± 0.51	50.0 ± 0.59	100.1	99.9
	OTC	ND	ND	100	100.1 ± 0.45	100.2 ± 0.29	100.1	100.2
HS_6	TC	13.9	14.0	50	64.0 ± 0.38	64.3 ± 0.59	100.2	100.6
	CTC	ND	ND	50	50.1 ± 0.47	49.8 ± 0.46	100.1	99.6
	OTC	105.9 ± 0.65	106.1 ± 0.70	100	206.0 ± 0.34	106.4 ± 0.81	100.1	100.3

Mean value ± R.S.D. (n = 5) ND: not detected (<LOD).

same sample solutions by the conventional spectrophotometric method [30] was also carried out. The results obtained by both methods compared favorably. The Student's i-test values indicated less than the theoretical values at a confident level of 95%.

3.6. Reaction mechanism

3.6.1. The formation of TCs–Y^{3+} binary complex

TC and its derivatives are antibiotics of the tetracycline family consisting of β-diketonate configuration as shown in Fig. 6. As reviewed in the literature, potentiometric and conductometric titrations using anhydrotetracycline and dedimethylamine tetracycline as well as the tetracycline-HCl ligand indicated that the lanthanide is complexed through the H position of the tricarbonylmethane group in the tetracycline-HCl molecule [31]. Tetracyclines have been reported as efficient and ideal ligands for a numbers of lanthanide ions for spectrofluorometric and spectrophotometric determination of TCs in pharmaceutical and biological samples for example, TC is an ideal ligand for Eu^{3+} which can be possibly sensitized the fluorescence intensity of Eu^{3+} via intermolecular energy transfer [26]. There is no information about TCs–Y^{3+} complex and its application in pharmaceutical assay.

Since Y^{3+} is one of the rare earth elements (lanthanides) its analytical chemistry should be more or less similar to those of other lanthanides such as Eu^{3+} and Tb^{3+}. Experimentally, complexation of Y^{3+} with TC in Tris-buffer pH 7.5 medium yielding the binary Y^{3+}–TC complex with the mole ratio of Y^{3+} to TC 1:1 stoichiometry. The coordination number of Y^{3+} is generally 8. The structure of the Y^{3+}–TC binary complex is shown in Fig. 3 corresponding to that proposed for the general binary Lan–TCs complexes (where Lan represents lanthanides) by Karthikeyan et al. [25]. It can be seem that the coordination of Y^{3+} is unsaturated. There are still plenty of positive charges and blank orbits in the TC–Y^{3+} complex.

3.6.2. Enhancement effect of the addition of CTAB

When the cationic surfactant (e.g., CTAB, where one end is the terminal hydrophilic groups with positive charge and the other end is hydrophobic groups with long carbon-chain) is added

Table 5
Results obtained from tetracyclines determination in mike samples.

	TCAs	Original TCs (ng mL^{-1})		TCs standard drug added	TCs found (ng mL^{-1})		Recoveries (%)	
		SIA	STD method		SIA	STD method	SIA	STD method
MS_1	TC	ND	ND	50	49.9 ± 0.29	49.8 ± 0.43	99.8	99.6
	CTC	ND	ND	50	49.9 ± 0.34	50.1 ± 0.57	99.8	100.2
	OTC	ND	ND	50	49.8 ± 0.52	49.9 ± 0.68	99.6	99.8
MS_2	TC	ND	ND	50	50.1 ± 0.45	49.9 ± 0.61	100.1	99.8
	CTC	ND	ND	50	49.8 ± 0.50	50.1 ± 0.45	99.6	100.1
	OTC	20.0 ± 0.35	21.0 ± 0.44	50	69.9 ± 0.57	70.1 ± 0.59	99.8	98.2
MS_3	TC	25.0 ± 0.50	26.0 ± 0.70	50	75.1 ± 0.46	74.9 ± 0.35	100.2	97.8
	CTC	ND	ND	50	50.1 ± 0.55	49.9 ± 0.81	100.1	99.8
	OTC	ND	ND	50	50.1 ± 0.38	50.1 ± 076	100.1	100.2
MS_4	TC	ND	ND	50	49.8 ± 0.48	49.7 ± 0.49	99.6	99.4
	CTC	ND	ND	50	49.8 ± 0.61	49.8 ± 0.52	99.6	99.6
	OTC	ND	ND	50	49.9 ± 0.54	50.1 ± 0.46	99.8	100.1
MS_5	TC	15.0 ± 0.39	17.0 ± 0.44	50	65.1 ± 0.44	65.1 ± 0.65	100.1	96.2
	CTC	ND	ND	50	49.9 ± 0.54	49.5 ± 0.45	99.8	99.0
	OTC	ND	ND	50	50.0 ± 0.45	50.1 ± 0.57	100.0	100.1

Mean value ± R.S.D. (n = 5) ND: not detected (<LOD).

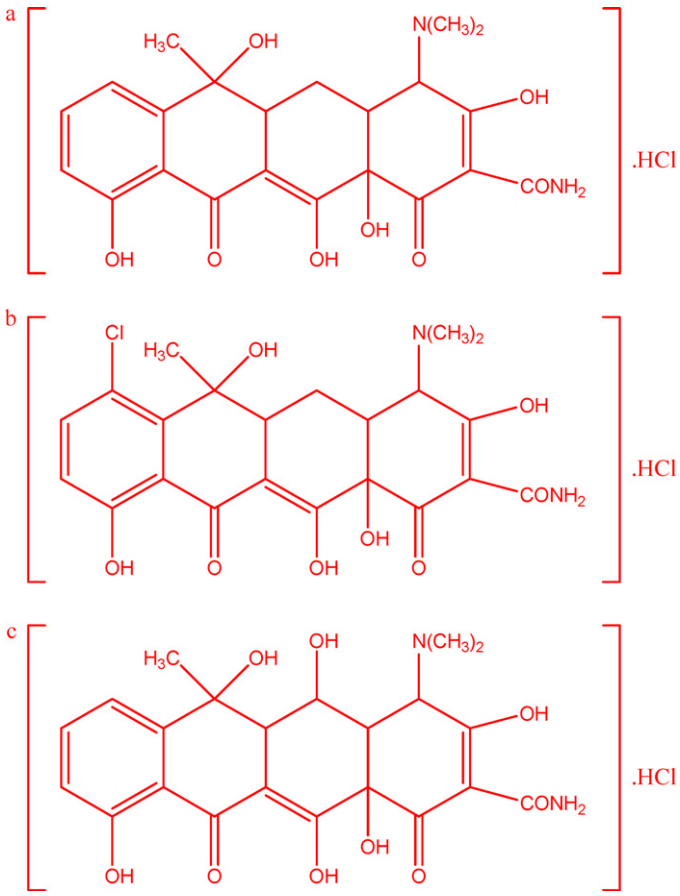

Fig. 6. Structure of TCs: (a) tetracycline, (b) chlortetracycline and (c) oxytetracycline.

in the TC–Y^{3+} system, because of the electrostatic attractions and the hydrophobic interaction, CTAB combines with the binary TC–Y^{3+} complex resulting in the ternary TC–Y^{3+}–CTAB complex. The structure of CTAB is shown in Fig. 7. It was found that in the presence of the cationic surfactant (CTAB) the complex exhibited a bathochromic shift. The stoichiometric composition of the chelates of Y^{3+} with tetracyclines (TCs) is found to be 1:1 both in the absence and presence of CTAB. The stability of the ternary complex of Y^{3+} is considerably increased in comparison to its corresponding binary complex. The increment in stability of the ternary complex may be due to the stabilization of the ternary complex in the surrounding atmosphere of cationic micelles of CTAB. The extent of bathochromic shift in λ_{max}, considerable enhancement in the sensitivity and stability of the TCs–Y^{3+}–CTAB complex in the presence of CTAB indicated the use of this miceller forming cationic surfactant in the sensitization of color reaction.

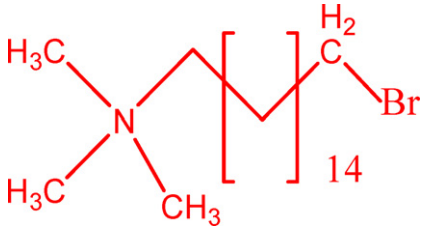

Fig. 7. Structure of cetryltrimetylammonium bromide (CTAB).

4. Conclusion

The proposed SIA procedure for determining tetracycline antibiotics (TCs) based on the complexation between Y^{3+} and TCs in the presence of cationic surfactant, CTAB using the developed SIA manifold (Fig. 1) controlled by the home-made software has proven to be feasible. The performance of the present SIA manifold has been successfully evaluated by comparative analysis of TC in the same standard solution using the proposed and the commercial SIA manifolds.

The described SIA system for determination of tetracyclines in pharmaceutical preparations and their residue in milk and honey samples has proven to be accomplished with respect to sensitivity and detection limit, simplicity, reproducibility and rapidity. In contrast with FIA, the SIA waste generations. Additional advantages of the proposed SIA method are cost effectiveness and less operator input requirements system can be controlled by means of the laboratory-made software which is relatively inexpensive. Therefore, this method is suitable for routine analysis.

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