Tutor/s
Dra. Clara Ràfols Llach
Departament d'Enginyeria Química i Química Analítica
Dra. Susana Amézqueta Pérez
Departament d'Enginyeria Química i Química Analítica

Grau de Química

Treball Final de Grau

Evaluation of the critical micellar concentration of the components of the simulated intestinal media by UV-Vis and fluorescence spectrophotometry

Avaluació de la concentració micel·lar crítica dels components dels medis intestinals simulats mitjançant espectrofotometria UV-Vis i de fluorescència

Ester Lopera de Llanos

January 2022

UNIVERSITAT DE BARCELONA
B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

En primer lloc, m'agradaria agrair a la Dra. Susana Amézqueta Pérez la dedicació i el seu suport continu en la realització del treball i a la Dra. Clara Ràfols Llach per haver-me guiat durant el transcurs d'aquest projecte. A tots els membres de PhysChem, en especial a la Lídia, per ajudar-me quan ho he necessitat.

Finalment, donar les gràcies a la meva mare per recolzar-me incondicionalment durant tots aquests mesos.

REPORT

CONTENTS

1. SUMMARY

The importance of the critical micellar concentration (CMC) value of biological surfactants is of great interest for the development of new drugs as, between others, it helps to predict their pharmacokinetic behavior. Intestinal fluids comprise several components, one is sodium taurocholate. The formation of sodium taurocholate micelles allows the solubilization of lipophilic drugs and enhances their absorption capacity in the body. Therefore, this study was focused on the evaluation of the critical micellar concentration (CMC) value of this surfactant.

In this work, the CMC of sodium taurocholate has been studied by UV-Vis spectroscopy and fluorescence at 25°C using neutral probes, so that they barely interfere with the formation of micelles. This study has been carried out in different media, changing the pH and ionic strength, and gradually getting closer to the desired biorelevant conditions. Next, several methods have been found to be able to calculate the value of the CMC. Here, the fluorometric method has resulted to be simpler and provides more accurate results than UV-Vis. It has been observed that CMC values are in the range of 10-4.5 mM and that ionic strength favors the micelle formation. Also, it has been noticed that the presence of components such as lecithin or oils make the surfactant aggregation and the micelle formation occur more gradually.

Keywords: Critical micellar concentration, lecithin, surfactant, sodium taurocholate, simulated intestinal fluids, UV-Vis spectroscopy and fluorescence, probe.

2. RESUM

La importància del valor de la concentració micel·lar crítica (CMC) dels surfactants biològics és de gran interès pel desenvolupament de nous fàrmacs ja que, entre d'altres, ajuda a predir el seu comportament farmacocinètic. Els fluids intestinals estan formats per diversos components, entre ells, el taurocolat de sodi. Per tant, l'estudi es va centrar en l'avaluació del valor de la concentració micel·lar crítica (CMC) d'aquest surfactant.

En aquest treball, s'ha estudiat la CMC del taurocolat de sodi mitjançant espectroscòpia UV-Vis i fluorescència a 25°C, utilitzant marcadors neutres perquè amb prou feines interfereixin amb la formació de micel·les. Aquest estudi s'ha realitzat en diferents medis, canviant el pH i la força iònica, i apropant-se gradualment a les condicions biorellevants desitjades. Seguidament, s'han trobat diversos mètodes per poder calcular el valor de la CMC. Aquí, el mètode fluorimètric és més senzill i dona millors resultats que UV-Vis. S'ha observat que els valors de la CMC es troben en el rang de 10-4.5 mM i que la força iònica afavoreix la formació de micel·les. També, s'ha observat que la presència de components com la lecitina o els olis fan que l'agregació del surfactant i la formació de micel·les es produeixi de manera gradual.

Paraules clau: Concentració micel·lar crítica, lecitina, surfactant, taurocolat de sodi, fluids intestinals simulats, espectroscòpia UV-Vis i fluorescència, marcador.

3. INTRODUCTION

3.1. MICELLES AND CRITICAL MICELLAR CONCENTRATION

Surfactants are amphiphilic molecules that have a polar head and a nonpolar tail or a nonpolar head and a polar tail. They can be cationic, anionic, or non-ionic, depending on the nature of their hydrophilic part. At low concentrations surfactants exist as monomers. When more amount of surfactant is added, the surface tension begins to decrease rapidly, until it reaches a point where the addition of surfactant molecules makes monomers cluster, and form micelles [1]. This point is called the critical micellar concentration (CMC). In general, CMC is expressed as mmol/L or mol/L.

Generally, micelles are formed when the hydrophilic heads are directed towards the polar solvent and the hydrophobic part is directed as far as possible from the water, forming a hydrophobic nucleus (as shown in Figure 1). Depending on the medium characteristics, inverted micelles can also be formed. In this case the hydrophobic part will be in contact with the non-polar solvent and the hydrophilic part goes to the center, forming a hydrophilic nucleus [2].

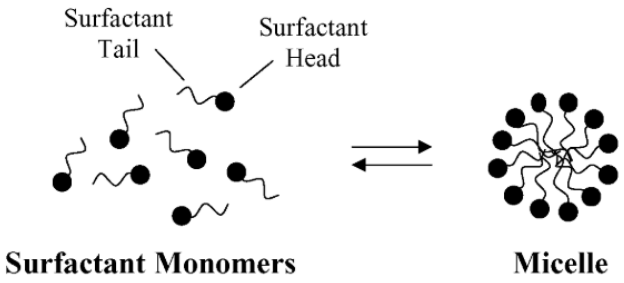

Figure 1. Representation of the equilibrium formed by the monomer-micelle surfactant molecules. The hydrophilic head is represented as a black circle and the hydrophobic tails as a curved line [3].

The physical characteristics, such as the size or geometry of the micelles, will depend on the surfactants type/s, pH, temperature and ionic streght. Surfactants can form spherical, cylindrical or planar micellar structures [3].

Surfactants are very important from the pharmacological point of view. In form of micelles, they can transport hydrophobic drugs along the organism, by improving their solubility.

Depending on the solubility of the drug and the type of micelle (classical or inverse), the drug can be transported in different ways.

In the case that the drug is hydrophilic, with opposite charge to the micelle polar head, it will be bound to the surface of the micelle (Figure 2 a). If the drug has intermediate solubility, it will be placed between the hydrophilic part and the beginning of the hydrophobic core (Figure 2 b). Finally, if the drug is hydrophobic, it will target the nucleus of the micelle which is hydrophobic (Figure 2 c) [3].

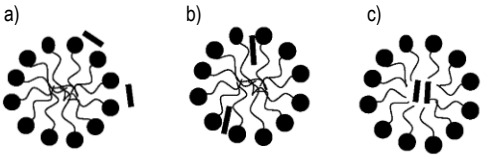

Figure 2. Representation of how the drug interacts in the micelles depending on their solubility (adapted from [3]). a) The drug is hydrophilic, with opposite charge to the micelle polar head, it will be bound to the surface of the micelle b) The drug has intermediate solubility, so it will be placed between the hydrophilic part and the beginning of the hydrophobic core c) the drug is hydrophobic, so it will target the nucleus of the micelle which is hydrophobic.

3.2. HUMAN INTESTINAL FLUIDS

Micelles are structures that improve the bioavailability of many orally administered drugs, especially of those that are poorly soluble. This kind of structures incorporate the drugs and, in this way, these last ones are transported through the gastrointestinal tract until they are absorbed. This absorption takes place mainly in the intestine, and once the drug is released from the micelle. This will happen if the absorption equilibrium is more favorable than the interaction with the micelle. Otherwise, the drug will be retained in the micelle and excreted.

Hence, the knowledge of the CMC value of biological surfactants is of great interest in new drugs development as it may help in the prediction of their pharmacokinetic behavior.

Table 1 compares the composition of human intestinal fluids with the simulated ones, in the fasted state (before a meal) and fed state (after a meal). The small intestine is divided between the duodenum, jejunum, and ileum. Its functions are the digestion and absorption of food and the

secretion of substances by bile into the large intestine. Then, the composition of the intestinal fluid is different depend on the intestinal zone.

Generally, human intestinal fluids (HIF) are mainly composed of Bile Salts, Phospholipids, Fatty acids, Monoglycerides, Cholesterol and various salts.

Bile acids are important natural surfactant. They consist of a rigid steroid ring structure with several hydroxyl groups. They are amphiphilic molecules that play a very important role in the solubilization of hydrophobic or poorly soluble substances in the body. If the CMC value is reached, they can form classic and/or mixed micelles with molecules such as phospholipids and cholesterol [4].

Phospholipids are amphiphilic molecule consist of hydrophilic head formed by a phosphate group and a glycerol group, and two hydrophobic tails formed by fatty acids. The most important phospholipids present in intestine are phosphatidylcholine (lecithin) and lysophosphatidylcholine (lysolecithin). [4]

Cholesterol it's a steroid ring formed by a hydroxyl group and a hydrocarbon chain. It can also have a significant influence on surface tension. Fat content is principally digested to monoglycerides and fatty acid that can form mixed micelles with bile salts. The solubilization of poorly soluble molecules improves with the formation of mixed micelles in the small intestine, especially in the fed state [4].

Table 1 summarizes the HIF components in the fed state and in the fasted state. A small difference in pH can be observed, as in the fed state the pH range is 5.4-6.5 and in the fasted state 5.6-7.8. There is also a big difference between bile salts and fatty acids in fed and fasted state. In fed state the value of bile salts are obtained in a range of 3.6-24 mM and fatty acids in a range of 39.4-13.2 mM. In fasted state bile salts are obtained in a range of 1.4-5.5 mM, being a much lower value. There are not observed fats in the fasting state, as most of these are found in the diet.

Table 1. Comparasion of the composition of human intestinal fluids with the simulated one.

Components and properties	HIF Fasted state		HIF Fed State		SIF Fasted and Fed State	
	duodenum	jejunum	duodenum	jejunum	FaSSIF - V2	FeSSIF - V2
pH	5.6 - 7	6.5 - 7.8	5.4 - 6.5	6.1	6.5	5.8
Bile salts (mM)	2.5 - 5.9	1.4 - 5.5	3.6 - 24	4.5 - 8	3	10
Monoglyceride (mM)	-	-	5.9	2.2	-	5
Fatty acids (mM)	0.1 mM		39.4	13.2	-	0,8
Phospolipid (mM)	0.53 ± 0.64	0.13 ± 0.04	1.2 - 6	2-3	0,2	2
Surface Tension (mN m-1)	31 - 45	28 - 46	27.8 - 35.4	30	54	48
Buffer capacity (mmol L-1 pH unit-1)	5.6 - 8.5	4	24 - 30	13.9	10	25
Osmolarity mOsmol/kg	137 - 224	200 - 300	276 - 416	NA	180 ± 10	390 ± 10
Sodium (mM)	142 ± 13		NA	NA	103.4	208
Potassium (mM)	5.4 ± 2.1		NA	NA	-	-
Calcium (mM)	0.5 ± 0.3		NA	NA	-	-
Chloride (mM)	126 ± 19		NA	NA	68,6	126
Ionic Streght (M)	0.139 ± 0.014		NA	NA	NA	-
Cholesterol (mM)	0 - 0.48		0 - 3.29		-	-

NA: Data not available

3.3. SIMULATED INTESTINAL FLUIDS

3.3.1. FeSSIF-V2 and FaSSIF-V2

Measuring the CMC of drugs using as media human intestinal fluids is ethically questionable and technically complex. Therefore, to determine this and many other parameters, simulated intestinal fluids (SIF) have been developed.

An example, Biorelevant company [5] offers different commercial gastrointestinal simulated products. They simulate gut fluids more accurately than traditional buffers and contain components that replicate conditions found in the gastrointestinal tract such as the solubilizing agents, pH, and osmolality.

Biorelevant company simulates gastrointestinal fluids in the fed and in the fasted state. As their composition changes depending on the food intake, hence it can also modify the solubility and absorption of the drugs to be studied. This company sells FaSSIF (simulated intestinal fluid in the fasting state), FeSSIF (simulated intestinal fluid in the fed state), FaSSGF (simulated gastric fluid in the fasted state), FaSSCoF (simulated colonic fluid in the fasted state) and FeSSCoF (simulated colonic fluid in the fed state). It is a product in powder format that is reconstructed in a suitable buffer with specific pH and ionic strength.

In the case of FaSSIF and FeSSIF, the products have been improved to do a better simulation of the intestinal fluids, obtaining a second version (FaSSIF-V2 and FeSSIF-V2). It should be noted that FeSSIF-V2 readjusts the amounts of bile salts and phospholipids and adds monoglycerides and fatty acids to do a better simulation of the intestinal fluids after food intake.

In the fasting state, the pH of simulated intestinal fluids (FaSSIF-V2) is 6.5. This value is within the pH range of the duodenum and jejunum (5.6-7.8 mM). The buffer capacity in FaSSIF-V2 is slightly higher (10 mM/pH) than that of both HIFs. In the case of osmolarity, no significant differences are observed. The surface tension in simulated state (54 mN m^{-1}) is slightly higher than in HIFs.

In the fed state, the pH of simulated intestinal fluids (FeSSIF-V2) is 5.8, a lower value than in simulated fasted conditions, and similar to jejunum and duodenum fluids. In the case of osmolarity, the value is within the range of the duodenum human fed conditions. To date, no osmolarity studies have been found for jejunal fluids in the fed state. As seen in the table, the values of surface tension are also higher than in human intestinal fluids fed state, and the same

behavior in FaSSIF-V2 is followed. No big differences are observed between the simulated medium and the real fluid buffer capacity.

On the other hand, in simulated intestinal fluids in the fed state, fatty acids and monoglycerides are simulated. Fatty acids are simulated by sodium oleate, although the value in the simulated case is lower than in the real case, probably due to stability problems when trying to simulate this so complex medium. Monoglycerides are simulated by glyceryl monooleate and there are no significant differences between simulated and HIF values.

As regards bile salts and phospholipids there is not much difference between the simulated state and HIF in fasted and fed conditions, sodium taurocholate simulates bile salts found in the intestine and lecithin simulates phospholipids. Although there are other parameters such as cholesterol, ionic salts or fatty acids that influence the solubilization capacity, the components mentioned above are the most important one for the solubilization of drugs.

3.3.2. Sodium Taurocholate (NaTc)

NaTc is a bile salt derivated from taurocholic acid. On its behalf, taurocholic acid (Figure 3 a) is a derivative of cholic acid (Figure 3 b), one of the major and most abundant bile acids in humans.

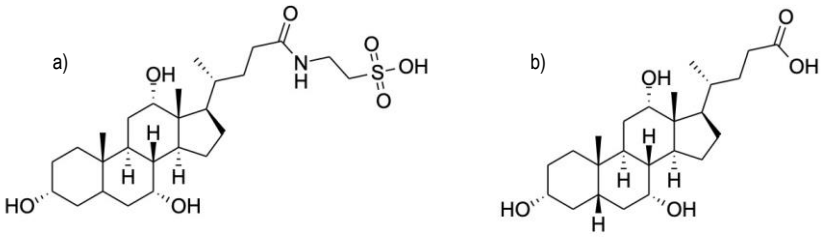

Figure 3. a) Representation of Taurocholic acid, and b) Cholic acid structures (Obtained from Chemdraw).

As explained before, NaTc is a very important component for simulating intestinal fluids, specifically bile salts. It is an amphiphilic molecule, formed by a hydrophilic and hydrophobic part (Figure 4). The first one is formed by hydroxyl groups and sulfonate group. The second one is formed by methyl groups [4].

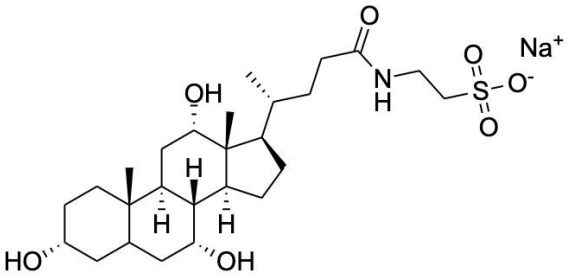

Figure 4. Representation of Sodium Taurocholate structure (Obtained from Chemdraw).

When two NaTc molecules interact, their hydroxyl groups form H-bridge bonds, creating a hydrophilic space. Methyl groups are combined by hydrophobic interactions to form a hydrophobic layer. A multilayer consisting of successive hydrophilic and hydrophobic spaces is created in which sodium ion will interact with the sulfonate group to enhance the stability. So, the final structure consists in a more or less stable micelle [4].

Bile salts are in general much different in aggregation behavior from the conventional surfactants with a long alkylchain, whose CMC becomes more evident for those with longer alkylchain. The micellization of bile salts is divided in three states, the monomer state, the small aggregates state and the stable micelles in equilibrium with the monomers. The aggregation of bile salts increases with a wide range of concentration, so the CMC range of bile salts is much broader than the conventional surfactants. For them, the aggregation occurs in a narrow range of concentration, closer to CMC [6].

3.4. EVALUATION OF CRITICAL MICELLAR CONCENTRATION

The critical micellar concentration can be measured by conductometry, tensiometry, fluorimetry, UV-Vis, viscometry, calorimetry, voltammetry, HPLC, capillary electrophoresis and NMR [7,8]. In the present work, we will focus on UV-vis and fluorescence spectrometry techniques due to the low amounts of sample required and their availability in general laboratories.

3.4.1 Fluorescence and UV-Vis based methods

Fluorescence is a phenomenon of light emission that goes from an excited electronic state to a lower electronic state with the emission of a photon [9].

The absorption of ultraviolet or visible radiation generally results from excitation of bonding electrons. All organic compounds can absorb electromagnetic radiation because they contain valence electrons that can be excited to higher energy levels [9].

The CMC of the surfactants cannot be measured directly because their fluorescence or absorbance does not undergo a change when the monomers form the micelles. For this reason, probes are used to measure the CMC. These probes are compounds that interact with micelles and that change their absorbance/fluorescence intensity in the absence and presence of micelles.

The surfactant (NaTc) used is anionic surfactant, so the surface of the micelle will be formed by a negatively charged layer and the micelle core in aqueous media (such as simulated intestinal fluids) will be hydrophobic. The electrostatic interactions between the probe and the surfactant may modify the micellization process (favor the micelle formation and decrease CMC value). Hence, in this study neutral probes will be used.

Since neutral probes are nonpolar molecules, they will be attracted to the hydrophobic tails of the surfactant. When the CMC is reached and the micelles formed, they will be introduced into the hydrophobic core. During this migration, the fluorescence or absorbance intensity of the probe will experience some changes that later will help to evaluate the CMC.

4. OBJECTIVES

The main objective of this study is to evaluate the critical micellar concentration of NaTc, under biorelevant conditions, using UV-Vis and fluorescence spectrometry techniques.

In order to achieve the main objective, different sub-objectives have been set:

- Define appropriate probes and their corresponding experimental conditions for UV-Vis and fluorescence screening.
- Set up analytical methods for the determination of CMC using SDS as model surfactant and water as medium.
- Determine the CMC of NaTc in aqueous medium and in biorelevant buffers.
- Determine the CMC of the components of a biorelevant media, FeSSIF-V2, that contains NaTc as surfactant.

5. EXPERIMENTAL SECTION

5.1. REAGENTS

Sodium Dodecyl Sulfate (SDS) ≥ 99%, Sodium Taurocholate (NaTc) ≥ 95%, Pyrene ≥ 99% were from Sigma Aldrich (St Louis, MO, USA), FeSSIF-V2 was from Biorelevant company (London, United Kingdom), Naphthalene was from Baker (Deventer, Holland), 4-nitroanisole 98% and Dimethyl Sulfoxide (DMSO) ≥ 99% were from Merck (Darmstadt, Germany). The substances were dissolved in water previously purified using the Mili-Q™ plus System with a resistivity of 18,2 MΩ cm from Millipore (Billerica, MA, USA).

Experiments were performed with buffers, using Sodium Chloride ≥ 99.5%, was from Fischer Chemical (Geel, Belgium), Maleic Acid 99,5% was from Carlo Elba (Milano, Italy), Sodium Hydroxide ≥ 98% and and Sodium Acetate Anhydrous > 99% were from Sigma Aldrich (St Louis, MO, USA), Sodium Dihydrogenphospate Monohydrate ≥ 99% and Chloric Acid (1mol/L) was from Merck (Darmstadt, Germany).

5.2. INSTRUMENTATION

Absorbance measures were done using a spectrophotometer Cary 60-UV-Vis (Agilent tecnologies, USA). Fluorescence measures were performed with a Cary Eclipse fluorescence spectrometer (Agilent techonologies, USA).

A Crison GLP 22 (Barcelona, Spain) 5014 combination electrode pH-meter was used to make all the pH measurements. The instrument was calibrated with two buffers solutions at pH 4,00 and 7,00 from Hach (Düsseldorf, Germany).

A magnetic stirrer SBS A-06 with a water bath and a thermometer to control the stirring and the temperature were used to do all the experiments.

An automated pipettes from Brand (Germany, Wertheim) in the range of 2-20 µL, 20-200 µL and 100-1000 µL were used to prepare the solutions for the experiments.

5.3. PROCEDURES

5.3.1 Preparation of buffer solutions

For the simulation of intestinal fluids, buffers at pH 5.8 and 6.5 have to be used, in accordance with fed and fasted state conditions, respectively.

Appropriate amounts of reagents were weighed and solved in water to obtain the concentrations shown in Table 2. The pH was adjusted with a 0.5 M solution of sodium hydroxide or sodium chloride to obtain the desired pH, and finally brought to a volume of 500 mL.

Table 2. Components and their concentration in each buffer.

Components	Buffer at 5.8 (mM)	Buffer at 6.5 (mM)
Sodium acetate anhydrous	145	-
Sodium hydroxide	-	34.8
Sodium chloride	63.9	68.6
Maleic acid	-	19.1
Ionic strength	238	121

5.3.2 Preparation of solutions

A stock solution of different probes was prepared in DMSO due to their limited solubility in water. Next, a working solution was prepared in the desired medium (water or buffer). The experimental conditions of the solutions are summarized in Table 3.

The amount of DMSO in the final solution is very small and does not have an influence in further experiments.

Table 3. Experimental conditions of stock and work solutions in UV-Vis and fluorescence for each probe.

Experimental	Stock solution (mM)	Working solution (mM)
UV-Vis 4-nitroanisole	10	0.05
UV-Vis Pyrene	2	$2x10^{-3}$
Fluorescence Pyrene	2	$3x10^{-5}$
Fluorescence Naphthalene	10	$5x10^{-4}$-$5x10^{-5}$

Also, a solution of surfactants was prepared. An appropriate amount of NaTc and SDS was weighed and dissolved with 1 mL of the desired medium (water or buffer) in a small beaker at a concentration of 200 or 400 mM, depending on the experiment.

Finally, a solution of FeSSIF-V2 was prepared by weighting the appropriate amount of the commercial solid and solving it in buffer at pH 5.8 to obtain a final concentration of 215 mM.

5.3.3 CMC evaluation

Three mL of the previously prepared probe solution were placed in a cuvette, and the fluorescence or absorbance was measured. The first addition of surfactant working solution (6 µL) was made and left stirring for two and a half minutes until the fluorescence and absorbance measurement was made. From this point on, small volumes of surfactant (6-10 µL) were added several times and fluorescence, or absorbance were recorded. The surfactant concentration covered the premicellar and micellar range or all the experiments (from 1 to 8-20 mM depending on the surfactant and the medium). Throughout the experiment the temperature was monitored with a thermometer so that it was 25ºC. With the help of a magnetic stirrer and ice, it was possible to lower and raise the temperature when necessary.

Absorbance measurements were done at scan 200-550 nm wavelength range and SBW spectral band width was 2 nm. Absorbance values were recorded every 1 nm with a scan rate 600 nm/min.

The experimental conditions for fluorescence using naphthalene and pyrene are summarized in Table 4.

Table 4. Experimental conditions to measure the CMC use naphthalene and pyrene as a probe.

Parameter	Conditions in Water	Conditions in Buffers	Conditions in Water
	Naphthalene		Pyrene
λ Excitation	238 nm	286 nm	270 nm
λ Emission	250 - 450 nm	300 - 450 nm	290 - 450 nm
Scan speed	Medium	Medium	Medium
Slit Excitation	5	5	5
Slit Emission	5	5	5
Temperature	25ºC	25ºC	25ºC

5.4. DATA PROCESSING

5.4.1 UV-Vis using pyrene as a probe

The method to determine the CMC in UV-Vis using pyrene as a probe, is described in the literature [10], and is based in the absorbance of two peaks, named P2 and P7. When representing P2-P7 *versus* log concentration of the surfactant, two regressions with distinctively different slopes can be observed below and above a point that corresponds to the CMC. In Figure 5 was shown the representation of the method.

These peaks were selected for determined the CMC by three reasons: P2 is the strongest peak, which gives the largest difference when another peak, the larger difference gives better resolution of the method and P7 is a weaker peak but stable and clearly measurable, the value of P2-P7 remains high at elevated concentrations of a surfactant [10].

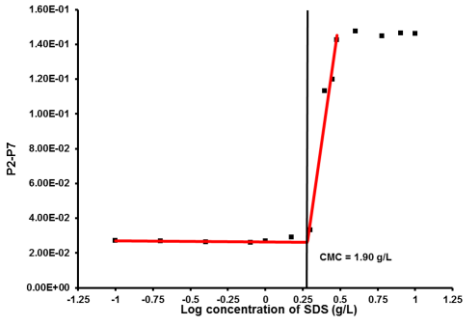

Figure 5. Graphical representation of the method to find the CMC in UV-Vis, of the SDS surfactant, in water at 22ºC, using pyrene in methanol as a probe [10].

5.4.2 Fluorescence using pyrene as a probe

The method to evaluate CMC in fluorescence using pyrene as a probe, is explained in literature [11]. This article represents the ratio of pyrene intensities of two peaks named 1 and 3 *versus* the surfactant concentration (SDS).

Below the CMC value, the 1:3 ratio corresponds to the profile of a more polar environment. Then, the ratio decreases as the surfactant concentration increases, pyrene is in a more hydrophobic environment. Finally, when the CMC value has been reached, the ratio remains constant, pyrene has been incorporated into the hydrophobic core of the micelles [11].

This method shown in Figure 6, is based on the assumption that the graph can be described by a decrease sigmoid Boltzmann type.

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/\Delta x}} + A_2 \quad (1)$$

Where A_1 and A_2 represent the initial and final asymptotes, x_0 represents the concentration of the surfactant at the center of the sigmoidal curve and Δx is directly related to the independent variable range where the abrupt change of the dependent variable occurs.

The literature recommends that if the value of $x_0/\Delta x$ is less than 10, the value of CMC can be taken as x_0 (x_{CMC1}). But if the value of $x_0/\Delta x$ is higher than 10, such as the case of SDS or NaTC, the value of CMC is $x_0+\Delta x$ (x_{CMC2}).

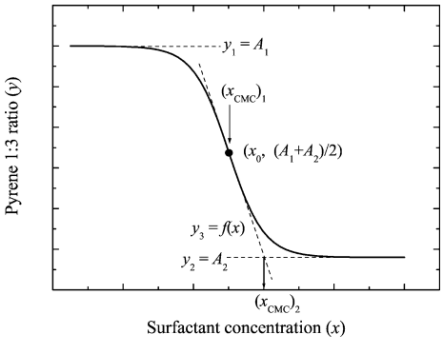

Figure 6. Graphical representation of the method to evaluate CMC in fluorescence of the SDS surfactant, in water at 25ºC, using pyrene as a probe [11].

5.4.3 Fluorescence using naphthalene as a probe

Not all probes behave in the same way when micelles are formed, which is why other methods have been defined to determine the value of the CMC.

The literature [12] proposes four different methods to evaluate the CMC by conductimetry of a cationic surfactant called CTAB, represented in Figure 7.

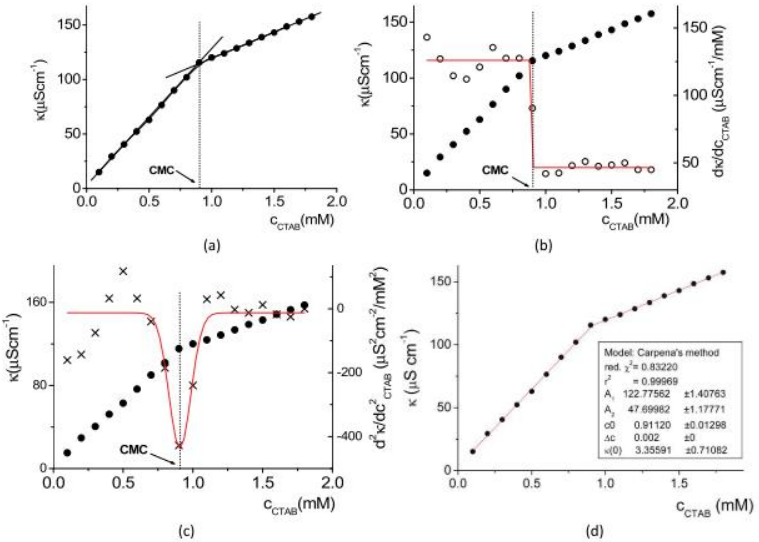

Figure 7. Graphical representation of four methods used to determinate the CMC using the conductimetry technique, of CTAB surfactant, in water at 25ºC [12]; a) Conventional method; b) The first derivative method; c) The second derivative method and d) Iteration method.

This method can be used for fluorescence determinations using probes and considering single peaks for the calculations.

The conventional method (Figure 7 a): This method represents the conductivity (or fluorescence) *versus* the concentration of surfactant. Then, the CMC would be found the intersection between the two straight lines, the premicellar zone, and the post-micellar zone.

The first derivative method (Figure 7 b): This method represents the conductivity (or fluorescence) derivative *versus* the surfactant concentration. A sigmoid that follows the Boltzmann equation is observed.

$$\frac{d\kappa}{dc} = \frac{A_1 - A_2}{1 + e^{(c - c_0)/\Delta c}} + A_2 \qquad (2)$$

Where A_1 and A_2 are the slopes of the premicellar zone and post-micellar zone, found in conventional method, c_0 the center of sigmoidal curve and corresponds to the CMC value, and Δc is the concentration range where a change of conductivity (or fluorescence) occurs.

Second derivative method (Figure 7 c): This method represents the second derivative of conductivity (or fluorescence) *versus* surfactant concentration. This function is described by the following equation:

$$\left(\frac{d^2\kappa}{dc^2}\right) = \left(\frac{d^2\kappa}{dc^2}\right)_{c=0} + \frac{A}{w\sqrt{\pi/2}}\exp\left(\frac{-2(c-CMC)^2}{w^2}\right) \quad (3)$$

Where $(d^2\kappa/dc^2)_{c=0}$ is the conductivity (or fluorescence) value when the concentration of surfactant in the solution is 0. A is the area under the curve, w is the width of the peak at half height, c corresponds the concentration of surfactant in the solution, and c_0 is the CMC value.

Iteration method (Figure 7 d): This method represents the conductivity (or fluorescence) *versus* the surfactant concentration and follows the Boltzmann equation:

$$\kappa = \kappa(0) + A_1 c + (A_2 - A_1)\Delta c ln\left(\frac{1+e^{(c-c_0)/\Delta c}}{1+e^{-c_0/\Delta c}}\right) \quad (4)$$

Where κ (0) is the value of the conductivity (or fluorescence) when no surfactant has been added to the solution. A_1 and A_2 are the slopes of premicellar and post-micellar segments, found in conventional method. Δc is the concentration range where a change of conductivity (or fluorescence) occurs, c corresponds the concentration of surfactant in the solution, and c_0 is the CMC value.

6. RESULTS AND DISCUSSION

6.1. EVALUATION CMC IN UV-VIS

First, a neutral probe that absorbs ultraviolet or visible radiation has to be selected, so 4-nitroanisole and naphthalene were proposed. An initial spectrum of each probe was recorded using water as a solvent, to get the absorbance spectrum at the same concentration. It was observed that 4-nitroanisole profile (Figure 8 b) had more intense and well-defined bands than naphthalene (Figure 8 a). Therefore, 4-nitroanisole was chosen as the probe, where the maximum is observed at 317 nm.

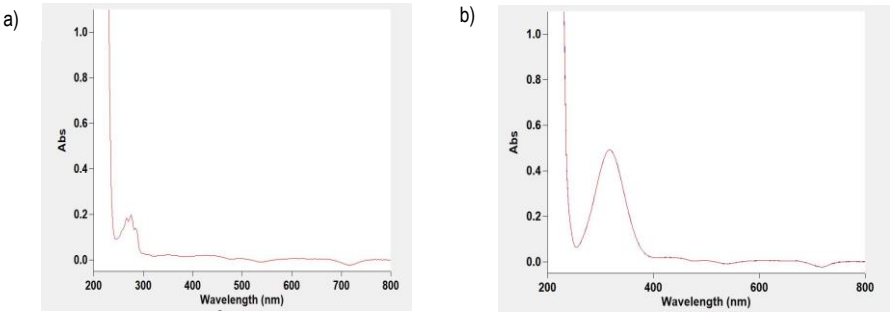

Figure 8. Representation of the absorbance spectrum of the probes proposed in water. a) Absorbance spectrum of naphthalene; b) Absorbance spectrum of 4-nitroanisole.

Before starting the experiments with NaTC, the analytical method was set up using SDS as surfactant since there is more literature available for comparison (Appendix 1).

An experiment with SDS in water at 25ºC was performed. As seen in Figure 9, no change in the absorbance of 4-nitroanisole with surfactant additions was observed. For this reason, another probe was looked for.

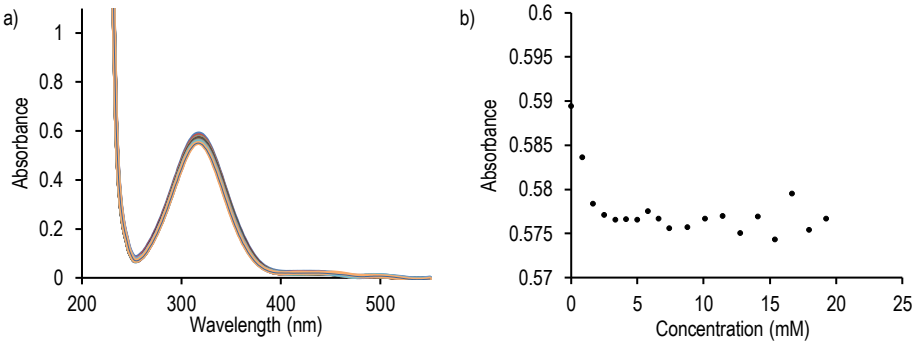

Figure 9. Representation of the experiment used to obtain the CMC of SDS surfactant, in water at 25ºC, using 4-nitroanisole as a probe; a) Representation of the absorbance in each addition of surfactant; b) Representation of the absorbance at 317 nm wavelength, in relation to the concentration of surfactant in the solution.

Hence, bibliographic research was done to find another probe. Several articles (Appendix 1) proposed pyrene as a probe in UV-Vis to evaluate the CMC of SDS. So, a measurement was made to see the absorbance behavior of pyrene in water, using the bibliography concentrations as a reference. The result is shown in Figure 10.

Five peaks at wavelengths around 240, 273, 321 and 336 nm were obtained, in concordance with the articles.

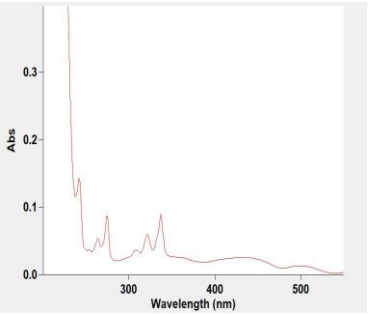

Figure 10. Representation of the absorbance spectrum of pyrene in water.

Next, successive additions of SDS were done. In the first measurements, when the surfactant was added, the absorbance remained constant. When the CMC was reached, the absorbance

increased dramatically until it remained constant again, when all the micelles had been formed (Figure 11 a).

Later, P2-P7 absorbance *versus* the logarithm of the SDS concentration was plotted (Figure 11 b).

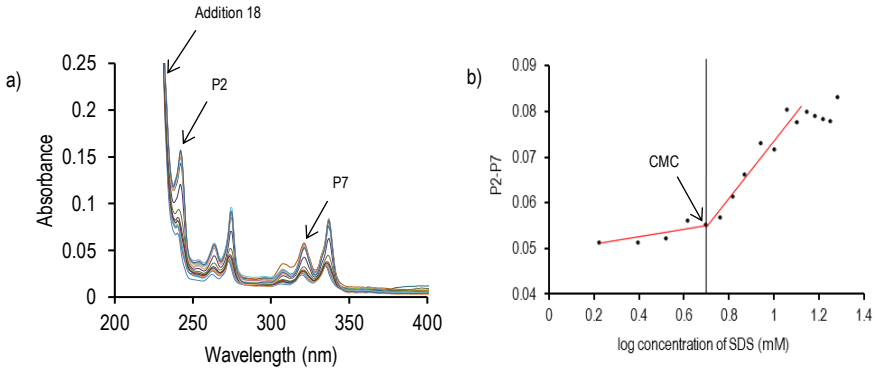

Figure 11. Representation of the experiment used to obtain the CMC of SDS surfactant, in water at 25ºC, using pyrene as a probe; a) Representation of the absorbance in each addition of surfactant; b) Representation of the absorbance of P2-P7, in relation to the concentration of surfactant in the solution.

Therefore, using this method the point where the two regressions intersected was at 4.95 mM concentration of SDS, corresponding to the value of CMC. The value obtained is 1-3 units lower than the ones reported in the literature and, as more promising results were being obtained in parallel fluorescence measurements, this method was discarded.

6.2. EVALUATION CMC IN FLUORESCENCE

First, a neutral probe that had fluorescence had to be selected, so pyrene and naphthalene were proposed.

Several articles (Appendix 1) indicate that pyrene is a good candidate as a fluorescence probe for the evaluation of CMC. Hence, a measurement was performed to fix the excitation wavelength and see the emission peaks, using water as a solvent. Three maximums are observed at 240, 270 and 335 nm (Figure 12). Although any of the three wavelengths could be useful for the aim of the experiments, 270 nm was set as excitation wavelength because the peak intensity was high and far from the Rayleigh dispersion. Two maximums emission occurred at 370 and 390 nm.

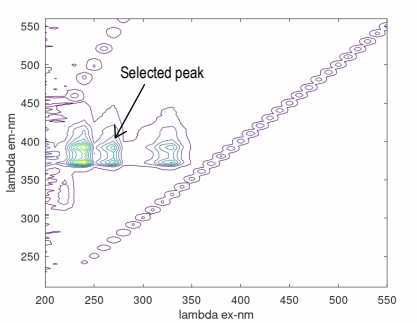

Figure 12. Representation of the excitation and emission wavelengths of pyrene in water with a contour graph.

Next, an experiment was performed using SDS as a surfactant, in water at 25ºC to test the method. It was observed that as more surfactant was added the fluorescence of the pyrene increased up to the CMC value, then the fluorescence remained constant (Figure 13 a).

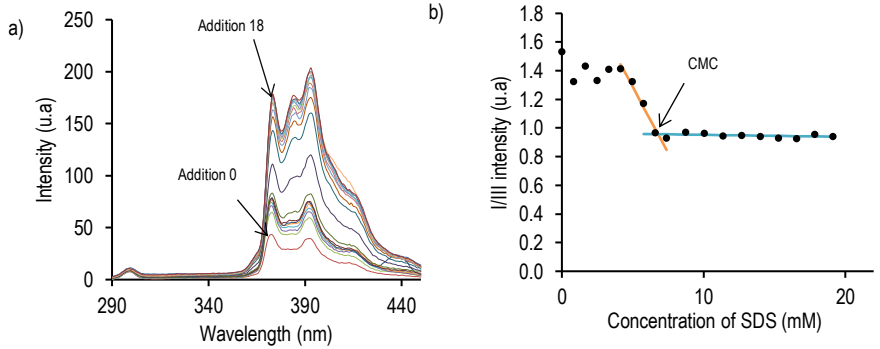

Figure 13. Representation of the experiment used to obtain the CMC of SDS surfactant, in water at 25ºC, using pyrene as a probe; a) Representation of the intensity in each addition of surfactant; b) Representation of the intensity of I/III at 370 and 390 nm wavelength, in relation to the concentration of surfactant in the solution.

When representing the I/III fluorescence intensity peaks ratio *versus* the concentration of SDS (Figure 13 b), the point at which the two straight lines intersect is at 6.8 mM. This value is similar to other values reported in the literature (Appendix 1).

Seeing the results, the same experiment was done but using NaTc as surfactant. As seen in Figure 14 b, no I/III fluorescence ratio difference can be observed as NaTc is added up to a concentration of 8 mM and next it slightly diminishes up to a concentration of 11.4 mM. Finally, it remains constant. This CMC value of 11.4 mM agrees with data in the literature (Appendix 2). However, as it can be observed, it is quite difficult to get a good sigmoid representation with so little differences in the I/III ratio. So, it was decided to look for a new probe.

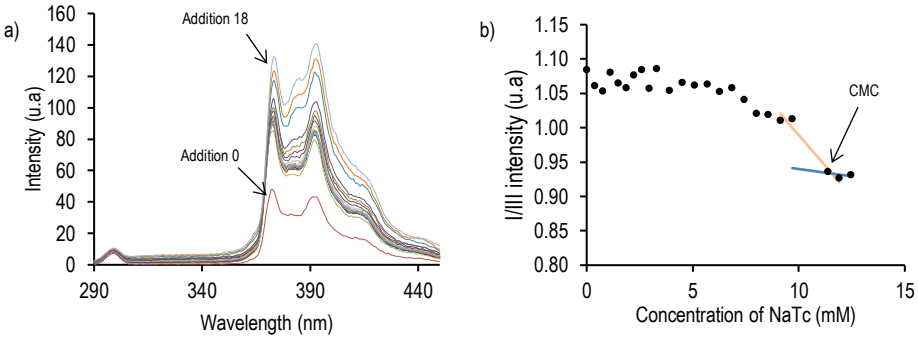

Figure 14. Representation of the experiment used to obtain the CMC of NaTc surfactant, in water at 25ºC, using pyrene as a probe; a) Representation of the intensity in each addition of surfactant; b) Representation of the intensity of I/III at 370 and 390 nm wavelength, in relation to the concentration of surfactant in the solution.

Another compound of the same family but with only two aromatic rings was selected: naphthalene. A measurement was performed to fix the excitation wavelength and see the emission peaks, using water as a solvent. The excitation wavelength is set at 238 nm. Two maximums emission are at 320 and 330 nm (Figure 15).

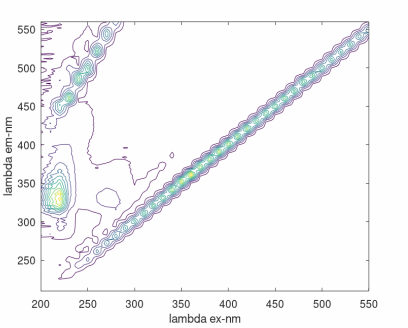

Figure 15. Representation of the excitation and emission wavelengths of naphthalene in water with a contour graph.

First, the method was set up using SDS as a surfactant, in water at 25ºC. It was observed that as more surfactant was added the fluorescence of naphthalene decreased up to the CMC value, then the fluorescence remained constant (Figure 16 a). Three replicates of this experiment were performed, and the results are shown in Table 5.

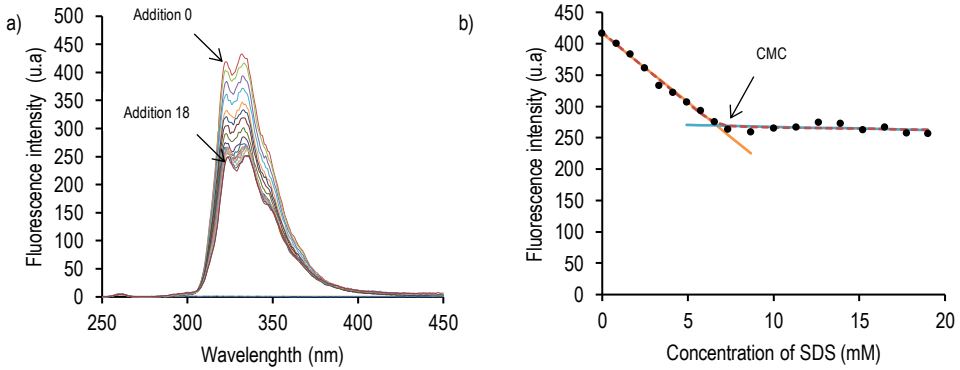

Figure 16. Representation of the experiment used to obtain the CMC of SDS surfactant, in water at 25ºC, using naphthalene as a probe; a) Representation of the intensity in each addition of surfactant; b) Representation of the intensity at 320 nm wavelength, in relation to the concentration of surfactant in the solution.

To evaluate the value of the CMC, the conventional and the iteration method, explained above, were used (Figure 16 b). The first and second derivative method were not used, as the differences in fluorescence increments are not noticeable.

Table 5. Summary of the CMC values of SDS surfactant, in water at 25ºC.

SAMPLE	CMC [mM]	CMC [mM]
	Conventional Method	Iteration Method
Replicate 1	6.7	6.6
Replicate 2	7.4	7.4
Replicate 3	6.4	6.5
$\bar{x}(s)$	6.8(0.5)	6.8(0.5)
RSD (%)	7.7	7.6

As can be seen in Table 5, the mean value of the CMC in both methods is 6.8(0.5) mM. The values in the two methods have a great similarity and no big differences are appreciated. The average value obtained is comparable with the values obtained in the literature (Appendix 1), that range from 8.2 mM to 6.4 mM.

Next, the same experiment was carried out with NaTc in water at 25ºC. It was observed that the fluorescence decreased steadily with the surfactant additions, the change in fluorescence intensity of naphthalene is a gradual process (Figure 18 a).

The value of the CMC could be determined using the conventional and the iteration method. In both methods the average of CMC value for the three replicates, was 10(0.9) mM, as shown in Table 6. This result can be compared with the literature of NMR and Fluorescence (Appendix 2). In N. Funasaki *et al.* [13] the CMC value is 8.3 mM and in S.M. Meyerhoffer and L.B. McGown [14] the CMC value is in the range of 8-12 mM. Both results are of the same order as the results obtained.

It has been observed that a lower value (5.6 mM) has been obtained by calorimetry [15], but in my laboratory it has been possible to carry out the experiment by calorimetry at the same temperature and the value obtained is in the order of 10 mM.

It has been observed in the literature that to evaluate the CMC of NaTc in water, the value is greatly affected by temperature [15] and ionic strength [6]. Hence, it is interesting to work in media that mimic the ionic strength of the intestinal fluids.

In view of the results, and in order to work using more biorelevant conditions, it was verified that the probe did not interfere with the buffers that simulate the intestinal fluid conditions (Figure 17). A measurement was performed, and it was observed that the buffer showed a very low fluorescence intensity. Also, that when solved in buffer, the maximum excitation of the probe peak was shifted to 286 nm.

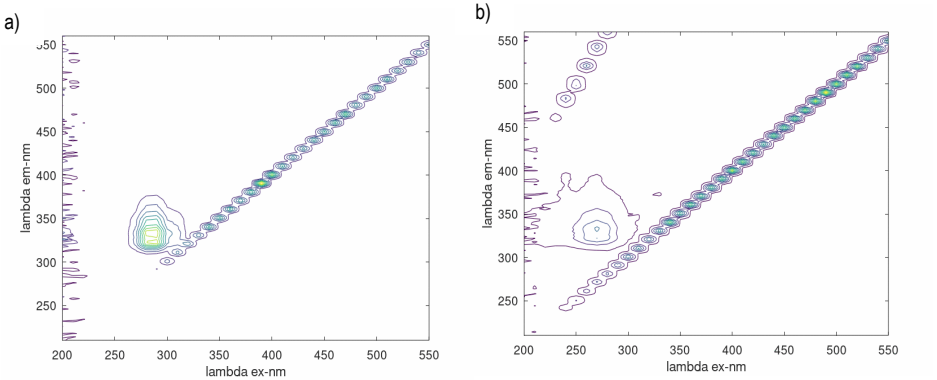

Figure 17. Representation of the excitation and emission wavelengths of naphthalene with a contour graph a) in buffer at pH 6.5 b) in buffer at pH 5.8

The experiments were performed at 25ºC, using buffers as solvents and the appropriate excitation wavelength. It was observed that the change in fluorescence intensity is more noticeable using the pH and ionic strength of the biorelevant media (Figure 18 b and c).

When using the buffer at pH 5.8 (I=238 mM), the value of CMC obtained is 4.5(0.4) mM when the conventional method is used and 4.3(0.2) mM using the iteration method (Figure 19 c and Table 6). Not too many differences are observed with the value and behavior obtained when using the buffer at pH 6.5 (I=121mM), the conventional and iteration methods have an average CMC value of 4.5(0.4) mM (Figure 19 b and Table 6). Both values are more than a half lower than the CMC of NaTc in water. Therefore, and as already stated in literature, the ion strength has a big influence in the micelle's formation. In this case, ions presence favors the surfactant aggregation. Moreover, plots show that there exists a curvature in the plot between the two slops. This curvature occurs in a narrower range of surfactant concentration for the studies of CMC in buffers, that would mean that the micellization process is less gradual, more homogenous.

Experiments in buffers at pH 6.5 and 5.8 can be compared with the values obtained in the literature [16], both around 3mM. A slightly higher value has been obtained in the present work, but the values in the literature have been carried out at 37ºC (Appendix 2).

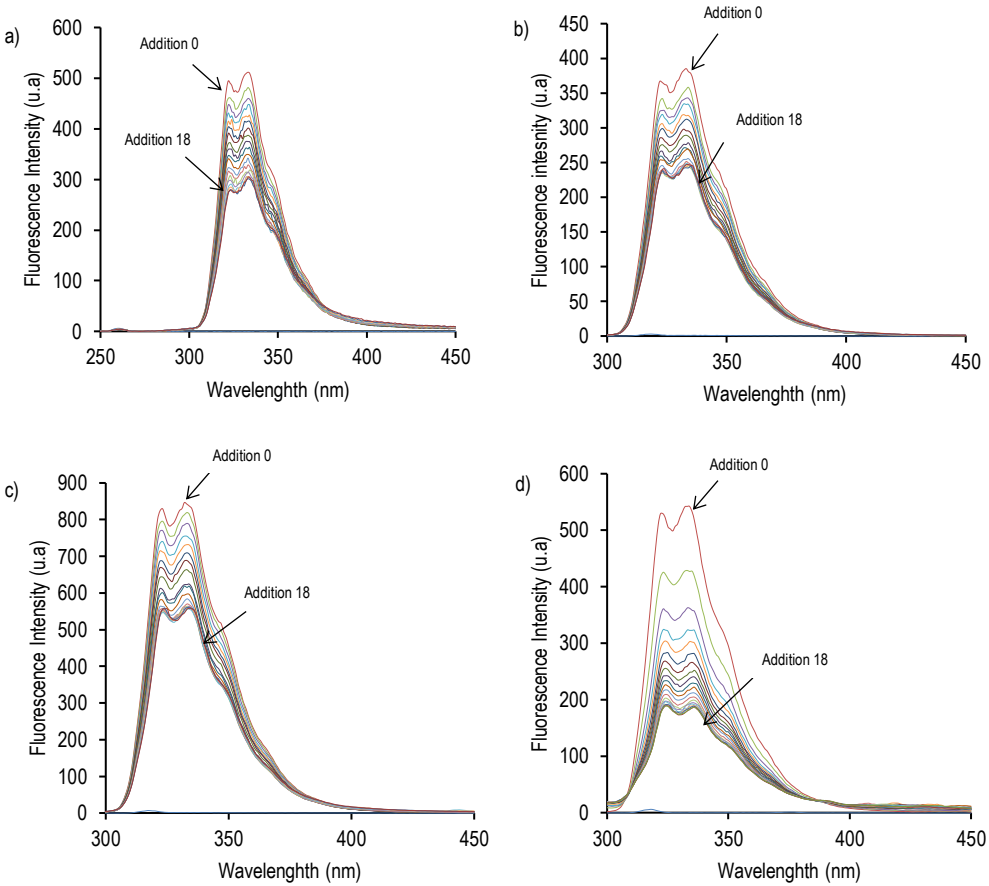

Figure 18. Representation of the experiment used to obtain the CMC of NaTc surfactant at 25ºC, using naphthalene as a probe; a) in water; b) in buffer at pH 6.5 c) in buffer at pH 5.8 d) FeSSIF-V2 in biorelevant buffer.

Finally, the CMC of NaTc mixed with other components of intestinal fluids (lecithin, fatty acids, monoglyceride) in a biorelevant buffer was evaluated. To do so, the commercial product FeSSIF-V2 was solved in the buffer at pH 5.8.

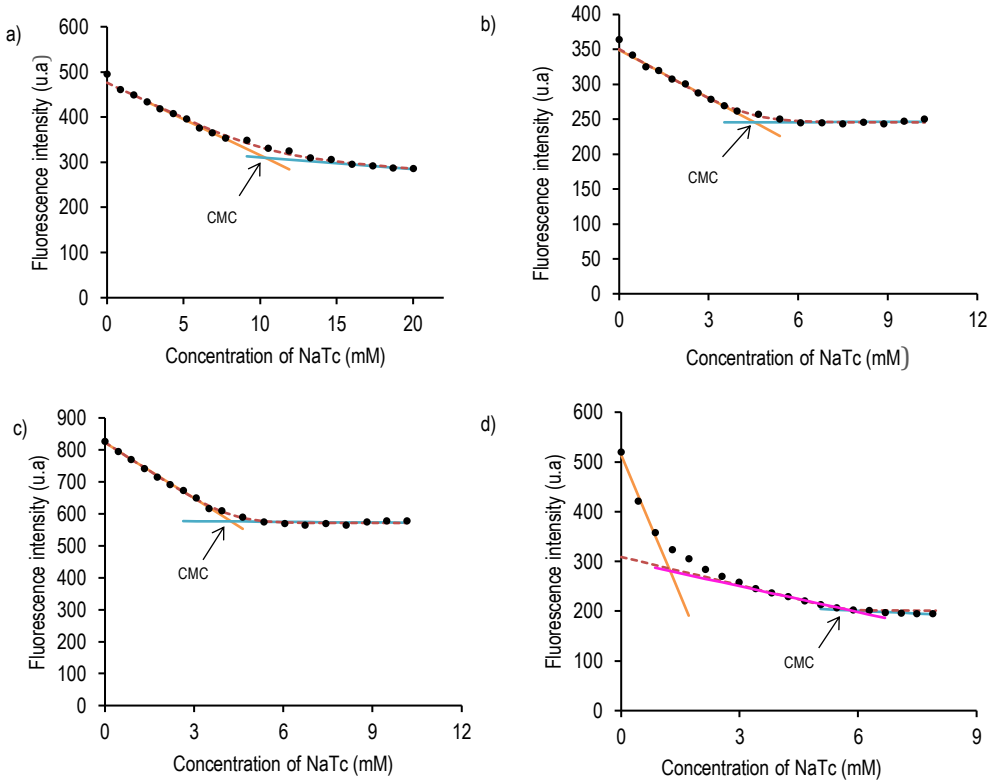

Figure 19. Representation of the experiments used to obtain the CMC of NaTc surfactant, in water and biorelevant media using naphthalene as a probe, at 25ºC; a) Representation of the intensity at 320 nm wavelength, in relation to the concentration of surfactant in the solution in water b) Representation of the intensity at 320 nm wavelength, in relation to the concentration of surfactant in the solution at pH 6.5; c) Representation of the intensity at 320 nm wavelength, in relation to the concentration of surfactant in the solution at pH 5.8; d) Representation of the intensity at 320 nm wavelength, in relation to the concentration of surfactant in FeSSIF-V2.

In Figure 18 d and Figure 19 d it can be observed that in FeSSIF-V2 the monomer aggregation process is slower, due to the incorporation of lecithin and fatty acids. There exists a first aggregation process (possibly dimers or small aggregates) at about 0.9-3 mM of NaTc and the formation of stable micelles takes place at higher concentrations.

In conventional method CMC was 5.9(0.1) mM and in iteration method it was 5.5(0.3) mM. The value reported in literature [17], was 3.4 mM at 20ºC (Appendix 2). The CMC was slightly lower, but in that experiment it was not been carried out at the same temperature and the FeSSIF used (FeSSIF-V1) contained different amounts of NaTc and lecithin than FeSSIF-V2, and did not contain fatty acids.

The study of CMC using FaSSIF-V2 is still pending.

Table 6. Summary of the values obtained in the evaluation of the CMC in the different conditions.

SAMPLE	CMC [mM]	CMC [mM]
	Conventional Method	Iteration Method
	NaTc in Water	
Replicate 1	10.3	10.2
Replicate 2	10.8	10.8
Replicate 3	9.1	9.0
$\bar{x}(s)$	10.1(0.9)	10.0(0.9)
RSD(%)	8.5	9.0
	NaTc in buffer at pH 6.5	
Replicate 1	4.5	4.3
Replicate 2	4.8	5.0
Replicate 3	4.3	4.3
$\bar{x}(s)$	4.5(0.3)	4.5(0.4)
RSD(%)	6.3	9.1
	NaTc in buffer at pH 5.8	
Replicate 1	5.0	4.6
Replicate 2	4.2	4.2
Replicate 3	4.4	4.2
$\bar{x}(s)$	4.5(0.4)	4.3(0.2)
RSD(%)	8.5	4.9
	NaTc from FeSSIF-V2 in buffer at pH 5.8	
Replicate 1	5.8	5.6
Replicate 2	5.9	5.1
Replicate 3	5.8	5.7
$\bar{x}(s)$	5.9(0.1)	5.5(0.3)
RSD(%)	1.2	5.5

10. CONCLUSIONS

In this study it was possible to set up analytical methods for the determination of CMC, using UV-vis and fluorescence spectrometry techniques. The achieved results led to the following conclusions:

- 4-nitroanisole is not an appropriate probe for UV-Vis, and, in the case of pyrene, the CMC values obtained do not agree with the available literature. So, in future studies, experimental conditions should be revised.
- Pyrene and naphthalene can be used as probes for the determination of CMC values of NaTc using fluorescence. The optimized conditions have been successfully applied using different biorelevant media.
- The use of SDS as a surfactant in water allows setting up the analytical methods for both, UV-Vis and fluorescence techniques. It has been possible evaluate the CMC of this surfactant by fluorescence satisfactorily, using pyrene and naphthalene as probes, and in agreement with other authors.
- The value of CMC using NaTc as surfactant in water at 25ºC is 10 mM, and agrees with previously reported values. The formation of micelles under these experimental conditions occurs gradually.
- The CMC of NaTc using more biorelevant conditions (buffers at specific pHs and ion strengths) is 4.5 mM, a lower concentration than when working with water. These conditions favor the formation of micelles and make the aggregation process more homogenous.
- When the CMC of NaTc is evaluated using the commercial product FeSSIF-V2, a value in the range of 5.5-5.9 mM has been obtained. This value is higher than the value in buffers. Now, the monomer aggregation process is slower due to the gradual incorporation of lecithin and fatty acids in the mixed micelles.

11. REFERENCES AND NOTES

[1] M.J. Jaycok, Critical Micelle Concentration, Exp. Phys. Chem. (1968) 343–344. https://doi.org/10.1016/b978-0-08-012541-1.50115-2.

[2] K.L. Mittal, T.A. Roth, Solution Chemistry of Surfactants, 1980. https://doi.org/10.1149/1.2129918.

[3] C. de O. Rangel-Yagui, A. Pessoa, L.C. Tavares, Micellar solubilization of drugs, J. Pharm. Pharm. Sci. 8 (2005) 147–163.

[4] X. Xie, J.M. Cardot, G. Garrait, V. Thery, M. El-Hajji, E. Beyssac, Micelle dynamic simulation and physicochemical characterization of biorelevant media to reflect gastrointestinal environment in fasted and fed states, Eur. J. Pharm. Biopharm. 88 (2014) 565–573. https://doi.org/10.1016/j.ejpb.2014.05.020.

[5] Biorelevant, Biorelevant Media, (2000). https://biorelevant.com (accessed October 1, 2021).

[6] K. Matsuoka, M. Maeda, Y. Moroi, Micelle formation of sodium glyco- and taurocholates and sodium glyco- and taurodeoxycholates and solubilization of cholesterol into their micelles, Colloids Surfaces B Biointerfaces. 32 (2003) 87–95. https://doi.org/10.1016/S0927-7765(03)00148-6.

[7] S. Mondal, S. Ghosh, Role of curcumin on the determination of the critical micellar concentration by absorbance, fluorescence and fluorescence anisotropy techniques, J. Photochem. Photobiol. B Biol. 115 (2012) 9–15. https://doi.org/10.1016/j.jphotobiol.2012.06.004.

[8] B. Tanhaei, N. Saghatoleslami, M.P. Chenar, A. Ayati, M. Hesampour, M. Mänttäri, Experimental study of CMC evaluation in single and mixed surfactant systems, using the UV-Vis spectroscopic method, J. Surfactants Deterg. 16 (2013) 357–362. https://doi.org/10.1007/s11743-012-1403-7.

[9] A. Townshen, Principles of Instrumental Analysis, 1983, Book. https://doi.org/10.1016/s0003-2670(00)84936-3.

[10] J. Fu, Z. Cai, Y. Gong, S.E. O'Reilly, X. Hao, D. Zhao, A new technique for determining critical micelle concentrations of surfactants and oil dispersants via UV absorbance of pyrene, Colloids Surfaces A Physicochem. Eng. Asp. 484 (2015) 1–8. https://doi.org/10.1016/j.colsurfa.2015.07.039.

[11] J. Aguiar, P. Carpena, J.A. Molina-Bolívar, C. Carnero Ruiz, On the determination of the critical micelle concentration by the pyrene 1:3 ratio method, J. Colloid Interface Sci. 258 (2003) 116–122. https://doi.org/10.1016/S0021-9797(02)00082-6.

[12] J.M. Goronja, A.M.J. Ležaić, B.M. Dimitrijević, A.M. Malenović, D.R. Stanisavljev, N.D. Pejić, Determination of critical micelle concentration of cetyltrimethyl- ammonium bromide: Different procedures for analysis of experimental data, Hem. Ind. 70 (2016) 485–492. https://doi.org/10.2298/HEMIND150622055G.

[13] N. Funasaki, M. Fukuba, T. Kitagawa, M. Nomura, S. Ishikawa, S. Hirota, S. Neya, Reply to the comment on “Two-dimensional NMR study on the structures of micelles of sodium taurocholate”., J. Phys. Chem. B. 109 (2005) 9851–9852. https://doi.org/10.1021/jp0455374.

[14] S.M. Meyerhoffer, L.B. McGown, Critical Micelle Concentration Behavior of Sodium Taurocholate in Water, Langmuir. 6 (1990) 187–191. https://doi.org/10.1021/la00091a030.

[15] A. Maestre, P. Guardado, M.L. Moyá, Thermodynamic study of bile salts micellization, J. Chem. Eng. Data. 59 (2014) 433–438. https://doi.org/10.1021/je400903n.

[16] C. Pigliacelli, P. Belton, P. Wilde, S. Qi, Probing the molecular interactions between pharmaceutical polymeric carriers and bile salts in simulated gastrointestinal fluids using NMR spectroscopy, J. Colloid Interface Sci. 551 (2019) 147–154. https://doi.org/10.1016/j.jcis.2019.05.002.

[17] S.M. Gómez, D.M. Cristancho, F. Martínez, Solubilization thermodynamics of ibuprofen in modified and classical FeSSIF biorelevant media, J. Mol. Liq. 179 (2013) 110–117. https://doi.org/10.1016/j.molliq.2012.12.010.

[18] A. Domínguez, A. Fernández, N. Gonzalez, E. Iglesias, L. Montenegro, Determination of critical micelle concentration of some surfactants by three techniques, J. Chem. Educ. 74 (1997) 1227–1231. https://doi.org/10.1021/ed074p1227.

[19] H. Li, D. Hu, F. Liang, X. Huang, Q. Zhu, Influence factors on the critical micelle concentration determination using pyrene as a probe and a simple method of preparing samples, R. Soc. Open Sci. 7 (2020). https://doi.org/10.1098/rsos.192092.

[20] G. Basu Ray, I. Chakraborty, S.P. Moulik, Pyrene absorption can be a convenient method for probing critical micellar concentration (cmc) and indexing micellar polarity, J. Colloid Interface Sci. 294 (2006) 248–254. https://doi.org/10.1016/j.jcis.2005.07.006.

[21] B.S. Gupta, C.R. Shen, M.J. Lee, Effect of biological buffers on the colloidal behavior of sodium dodecyl sulfate (SDS), Colloids Surfaces A Physicochem. Eng. Asp. 529 (2017) 64–72. https://doi.org/10.1016/j.colsurfa.2017.05.066.

[22] S.M. Gómez, D.M. Cristancho, F. Martínez, Solubilization thermodynamics of ibuprofen in modified and classical FeSSIF biorelevant media, J. Mol. Liq. 179 (2013) 110–117. https://doi.org/10.1016/j.molliq.2012.12.010.

[23] S.M. Gómez, D.M. Cristancho, F. Martínez, Physicochemical aspects of the solubilization of ibuprofen in biorelevant media: Modified and classical FaSSIF systems, Lat. Am. J. Pharm. 31 (2012) 1261–1269.

12. ACRONYMS

CMC: Critical Micellar Concentration

DMSO: Dimethyl Sulfoxide

FaSSIF: Fasted State Simulated Intestinal Fluid

FaSSCoF: Fasted State Simulated Colonic Fluid

FeSSCoF: Fed State Simulated Colonic Fluid

FeSSIF: Fed State Simulated Intestinal Fluid

HIF: Human Intestinal Fluids

HPLC: High Performance Liquid Chromatography

NaTc: Sodium Taurocholate

NMR: Nuclear Magnetic Resonance

SDS: Sodium Dodecyl Sulfate

SIF: Simulated Intestinal Fluids

UV: Ultraviolet

APPENDICES

APPENDIX 1: BIBLIOGRAPHIC REFERENCES OF THE CMC OF SDS SURFACTANT

Technique	Solvent	Temperature	CMC (mM)	References
Fluorescence			7.4 mM	
Conductance	Water	25ºC	8 mM	[18]
UV-Vis			7.8 mM	
UV-Vis	Water	25°C	3.46 mM	[8]
			6.60 mM	
Fluorescence	Water	25°C	6.44 mM	[19]
			6.70 mM	
UV-vis	Water	25ºC	7.09 mM	[20]
Fluorescence			7.05 mM	
Fluorescence	Water	25ºC	8.22 mM	[11]
Fluorescence	Water	25ºC	8.26 ± 0.04 mM	[21]

APPENDIX 2: BIBLIOGRAPHIC REFERENCES OF THE CMC OF NATC SURFACTANT

Technique	Solvent	Temperature	CMC	References
		NaTc surfactant		
Calorimetry	Water	25ºC	5.6 ± 0.2 mol kg-1	[15]
Fluorescence	Water	25°C	8-12 mM	[14]
NMR	Water	25ºC	6.5 mM	[13]
NMR	Buffer at pH 5	37ºC	Around 3 mM	[16]
NMR	Buffer at pH 6.5	37ºC	Around 3 mM	
		FeSSIF-V1		
HPLC	Buffer at pH 5	20ºC	3.4 mM	[22]
HPLC	Buffer at pH 5	40ºC	2.1 mM	
		FaSSIF-V1		
HPLC	Buffer at pH 6.5	25ºC	4.7 mM	[23]
HPLC	Buffer at pH 6.5	35ºC	4.2 mM	