Valorization of tannery wastes: lipoamino acid surfactant mixtures from the protein fraction of process wastewater

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Abstract

The first stages of the transformation process of hides into leather (beamhouse process) generate an important waste in the tanning industry, since a considerable fraction of solubilised proteins ends up in waste water with the corresponding increase in contamination parameters, especially when the process is carried out without hair recovery (hair-pulping process). The objective of this work was the valorisation of this waste (the separated protein fraction) which conveniently hydrolyzed to amino acid level constituted the starting material for the production of biodegradable surfactants. The lipoamino acid surfactants were obtained by acylation of the amino acids from the protein hydrolisate. These surfactants were characterized and their physico-chemical and biological properties evaluated. They exhibit very low cmc values (about 40 mg/L). These surfactants are readily biodegradable and present an aquatic toxicity significantly lower than many common commercial surfactants derived whether from renewable or petrochemical feedstock. The mixtures of surfactants obtained are able to form oil/water emulsions that remain stable for at least one year. The results obtained in this work confirmed that it is possible the production of biodegradable and efficient lipoamino acid surfactant mixtures from the protein fraction present in beamhouse process wastewaters. This study constitutes a promising approach for the reduction of the pollution load from industrial tannery wastes and its valorisation as raw material for the production of surfactants with excellent environmental properties and good technical properties.

Keywords: tannery waste; valorization; lipoamino acid surfactants; physico-chemical properties; biodegradation; ecotoxicity;

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1. Introduction

Any industrial activity generates wastes to a greater o lesser extent and the growing demands of environmental respect in the production processes force companies to reuse the most of their wastes. Besides the respect for the environment, the reuse of wastes is of great importance and interest also from an economical point of view since the lower the amount of wastes generated, the lower the management cost in specialized plants. Moreover, the reuse of wastes can represent a sustainable solution to the lack of raw material to be used for the production of energy, fuel and chemicals than can be integrated again in the industry. The production of high added value materials as bio-products, nanomaterials and bio-polymers starting from wastes underlines, even more, the interest for their reutilization [1-5].

The leather industry generates a considerable amount of wastes. However, the studies dealing with its potential valorization are scarce [6,7]. According to Sykes and Corning [8], each tonne of raw hide yields 200 kg of finished leather, 50 m^3 of contaminated wastewater and the rest are solid wastes. Therefore, only 20% is transformed into useful material. According to Aloy [9], the main pollution load is produced in the beamhouse operation (stages before tanning process): 83% of BOD_5 (five-day biochemical oxygen demand), 73% of COD (chemical oxygen demand) and 76% of toxicity. In another study, Portavella [10], states that for every 100 kg of dry raw sheepskin from Catalonia, 15 kg of solubilised proteins that are more or less structured (keratins, albumins, globulins, glycoproteins, etc) and that contain nearly 18% nitrogen will end up in waste water following beamhouse operations. Portavella [11] found that 70% of COD from the beamhouse operations is due to the skins themselves and that only 30% comes from added chemical products. Although the values of the parameters may vary as a function of the type of raw material treated, there is no doubt that the beamhouse operations produced most contamination, much of which is due to the solubilised components (proteins) of hides or skins. Therefore, separation of the dissolved protein fraction represent not only a way to significantly reduce the contamination of beamhouse wastewaters [12], with the economic savings this entails, but also provides a residue, the separated protein fraction, which conveniently hydrolyzed to amino acid level, could be used as the raw material for the production of surfactants. Protein hydrolysates from different sources have been used with the same aim [13, 14]. Other possible applications of the separated protein fraction

from the beamhouse tannery process could be the production of liquid leaf fertilizer, solid fertilizer for soils or retanning agents for tannery. However, these possible applications will be the subject of future works.

The use of this liquid waste of the leather industry as starting material for the production of surfactants is of great interest for several reasons: i) to reduce water pollution load, ii) to reduce the cost of waste disposal and iii) to provide an appropriate source of renewable raw materials not derived from petrochemical feedstocks.

Amino acids are a very interesting raw material for the chemical preparation of environmentally friendly surfactants [15]. Twenty different α-amino acids are commonly present in proteins. Amino acid based surfactants are biodegradable and biocompatible compounds that can be prepared using natural compounds as fatty acids and amino acids as starting material [16,17]. Also, several commercial firms offer the possibility of finding amino acid surfactants on the market (series Amilite® from Ajinomoto; series Aminofoam™ from Croda; series Perlastan® from Schill&Seilacher, series Lamepon® from Basf; N^{α}-Lauroyl ethyl ester (LAE) from Lamirsa S.A [18], etc).

One interesting strategy to reduce the tannery industrial pollution load as well as to obtain environmentally friendly compounds is to synthesize surfactants using the amino acids obtained by acid hydrolysis of the protein fraction recovered from the wastewater of soaking, unhairing-liming and conditioning operations of a hair-pulping (with hair destruction) beamhouse process. In the present work lipoamino acid surfactant mixtures using as raw material the protein fraction were obtained and their physico-chemical and biological properties were investigated. This study constitutes a promising approach to the reduction of the pollution load of industrial wastes and its possible reutilisation as starting material for surfactant production.

2. Experimental

2.1 Materials

Dodecanoyl chloride, decanoyl chloride, dodecanoic acid, decanoic acid, pyreno, and squalane were purchase from Fluka. L-lysine, L-serine, L-proline, L-glutamic acid, glycine, L-leucine, L-arginine and sodium dodecyl sulfate were purchase from Sigma. Acetonitrile was purchase from Fisher Chemical. Acetone, n-hexane and ethanol absolute were purchase from Panreac. Trifluoroacetic acid, n- decane and hydrochloric acid were purchase from Merck. Sodium hydroxide was purchase from Carlo Erba.

2.2 Methods

2.2.1 Preparation of the amino acid mixture from the protein fraction.

The protein fraction used in this work as starting material was obtained by acid precipitation (adding 2 M sulphuric acid solution up to the isoelectric point) [19] of the effluents of the unhairing-liming process and subsequent washings in a hair-pulping beamhouse process of hides. This protein fraction was subjected to a degreasing process with dichloromethane during 5 hours and subsequently hydrolyzed with 6 N HCl during 24 hours. The amino acid mixture was quantitatively determined in accordance with the AccQ-Tag Waters method [20] with previous derivatization with 6-AQC. A Waters 600 model with a 2487 UV detector was used for HPLC analyses.

2.2.2 Synthesis of surfactants from the amino acid mixture

Surfactants were obtained by the introduction of a fatty acid residue, as an acid chloride, to the amino acids obtained from the protein hydrolisate in a strong alkaline aqueous medium. The mixture of amino acids obtained was dissolved in acetone/water (34/66) and NaOH was added until a pH of 10. This solution was filtered to remove the insoluble residue present in the medium. The residue was analyzed by thin-layer chromatography in order to check the absence of amino acids. Next, dodecanoyl chloride or decanoyl chloride were added dropwise maintaining the pH at 10 with NaOH. After adding the acid chloride, the reaction mixture was kept at -10 ºC for 3 hours. The progress of the reaction was checked by HPLC. Solvent was eliminated using a rotary evaporator and the sample was freeze-dried. Acylation reactions were carried out for different amino acid/acid chloride molar ratios. In some cases, salt formed in the reaction was removed with dry ethanol and subsequent filtration. In other cases, this purification process was not carried out (see Table 2).

2.2.3 Synthesis of pure N^{α}-acyl amino acid surfactants

A series of standard N^{α}–acyl amino acid surfactants was prepared for the characterization of the surfactants synthesized in this work. The standard surfactants were prepared with those amino acids that were present at the highest percentage in the starting protein fraction (glycine, leucine, proline, arginine, glutamic acid, lysine and serine). Dodecanoyl chloride was selected as the acylating agent. The reaction was carried out under the above mentioned conditions. 0.5 g of pure amino acid were taken and the reaction was carried out at the 1:1 ratio. Once obtained, the surfactants were

purified by repetitive washings with n-hexane. For identification purposes, the purified standard surfactants prepared for each amino acid were added one by one to the final product of the acylation reaction. HPLC calibration curves were prepared for every pure N^{α}–acyl amino acid surfactant and then, the concentration of the major components in the mixture was calculated.

2.2.4 High Performance Liquid Chromatography (HPLC)

To check the progress of the acylation reaction and for identification purposes, HPLC analyses were performed on a VWR- Hitachi ELITE LaChrom system which consisted of an injection valve fitted with a 20 µl loop, and pump L-2200 and a UV-Vis detector L-2400 at 215 nm wavelength. A Lichrocart 250- 4, lichrospher 100 CN (5µm) column was used at room temperature. The flow-rate through the HPLC column was 1.0 ml/min. Elution was performed in a gradient system of water/acetonitrile. Eluent A was 0.1% (v/v) trifluoroacetic (TFA) in water, and eluent B was 0.085% TFA in water/acetonitrile 1:4. The initial composition A/B of the gradient was 75/25 (v/v), changing over 24 min to a final composition of 5/95.

2.2.5 Surface tension measurements

The critical micelle concentrations (cmc) of the obtained surfactant mixtures were determined by surface tension measurements. Surface tension measurements were carried out at 25 ºC in accordance with the Wilhelmy plate method [21] using a K12 Krüss tensiometer. A stock solution of 1 mg/ml in water (pH = 6) was prepared from which different solutions were obtained for cmc determination**.** The cmc of decanoic and-dodecanoic acids were also determined for the sake of comparison.

2.2.6 Spectrofluorimetry

Fluorescence measurements were carried out with a Shidmadzu RF 540 spectrofluorometer. The fluorescence emission spectra of pyrene dissolved in surfactant aqueous solutions were recorded from 340 to 450 nm after excitation at 332 nm. Pyrene exhibits fine structure in 370–400 nm regions of the steady-state fluorescence emission spectra. The nature and the intensity are extremely dependent on the polarity of the environment. The ratio of the first to the third vibronic peaks, i.e., I_1/I_3, shows the greatest solvent dependency, and can be used to obtain the cmc of the surfactant solutions [21]. A pyrene aqueous solution of 10^{-6} M (pH = 6) was used. The different

surfactant concentrations were prepared with this pyrene aqueous solution. All measurements were carried out at 25 ºC.

2.2.7 Qualitative phase behaviour

Optical microscopy was used to study the qualitative phase behaviour of binary water/L-3 and water/C-2 systems as a function of temperature. Optical observations were performed according to the "flooding" (penetration) method of Lawrence [22]. A polarising microscope Reichert Polyvar® 2 Leica equipped with a hot stage was employed. A videocamera and a PC with Leica IM 500 software were used for the image capture. In the flooding experiment, water was allowed to diffuse into anhydrous surfactants placed between a slide and a cover slip. After a short time, gradients in composition were produced and different separated mesophases developed around the crystalline surfactant [23].

2.2.8 Foaming properties: foaming capacity (FC) and foaming stability (FS)

Foaming properties were measured using a modified Padmashree's method [24-26]. Different amounts of surfactants (10, 20 and 30 mg) were mixed with 2 ml of distilled water (pH = 6) at 25 ºC in a graduated test tube. Purified and unpurified (with salts) surfactant samples were tested. Sodium dodecyl sulfate was used as control. Given its higher foaming capacity a lower amount (3 and 4 mg) was taken for the test. Solutions were continuously shaken by hand during 1 min. After 30 s shaking, the volume was measured. The foaming capacity (FC) was expressed as the percentage of volume according to the following formula:

$$FC = ((\text{Volume after stirring} - \text{Volume before stirring}) / \text{Volume before stirring})) * 100 \quad (1)$$

The foam volume was recorded at 5, 30, 60, 120 and 180 min after shaking. Foaming stability (FS) was calculated using the following formula:

$$FS = (\text{Foam volume after a time "t"} / \text{Initial foam volume}) * 100 \quad (2)$$

2.2.9 Emulsifying capacity

To check the emulsifying capacity of the surfactant mixtures, they were dissolved in water and then oil was added, thus favouring the formation of Oil in Water

(O/W) emulsions. Emulsions were prepared by adding dropwise 1 g of oil (decane or squalane) to aqueous solutions containing 10 or 20 mg of surfactant at 25 ºC. The volume of water was 0.2, 0.4 or 1 ml. During the addition of the oil, the samples were stirred with a Heidolph Reax 2000. When the addition of the oil was finished, the samples were left to stand.

2.2.10 Biodegradability assessment

The biodegradability of the surfactants under aerobic conditions was evaluated according to the ISO-14593 CO_2 headspace test [27]. This method allows the evaluation of the ultimate aerobic biodegradation (mineralization to carbon dioxide) of an organic compound in aqueous medium by measuring the increase in total inorganic carbon over time with respect to a blank without the addition of the test substance. The surfactants were tested at a concentration of 20 mg C/L. Samples were inoculated with activated sludge (10 mg dry solids/L) collected from a municipal wastewater treatment plant (Manresa, Barcelona) and then incubated in the dark at 22 ± 1 ºC in 250 mL sealed vessels (air headspace/liquid volume ratio, 1:2). Sodium n-dodecyl sulphate was used as reference substance. Three replicates of the surfactants, blank and reference substance were measured for each sampling day. The test ran for 28 days. Each sampling day, after injecting a sodium hydroxide solution to the vessels, shaking for 1 h and allowing settling, appropriate volumes were withdrawn by syringe from the liquid phase of each vessel and kept in small beakers carefully filled to the brim and covered with a cap to prevent CO_2 exchange with the air. The concentration of inorganic carbon was determined in a carbon analyzer (Shimadzu TOC-5050). The biodegradation level was expressed as a percentage of the theoretical amount of inorganic carbon based on the initial amount of the test compound.

2.2.11 Aquatic toxicity assessment

The aquatic toxicity determination of the obtained surfactants was carried out in accordance with the Daphnia magna method where the swimming incapability is the end point [28]. The pH of the medium was 8.0 and the total water hardness was 250 mg/L (expressed as $CaCO_3$), with a Ca/Mg ratio of 4/1. Tests were performed in the dark at 20 ºC. Twenty daphnia, divided into four batches of five animals each, were used at each test concentration. The concentration range was first established in a preliminary test and 10 concentrations in a geometric series were tested for each

surfactant. The percentage immobility at 48 h was plotted against concentration on a logarithmic-probability scale and a linear relationship was obtained. The Probit method was employed as statistical procedure to determine the IC_{50} (the estimated concentration to immobilise 50% of the daphnia after 48 h exposure) and the corresponding 95% confidence interval (CI).

3. Results and discussion

3.1 Amino acid composition of the protein fraction

The amino acid composition obtained after the hydrolysis of the protein fraction separated by acid precipitation from the effluents of the unhairing-liming operation in a hair-pulping beamhouse process of hides is shown in Table 1. Amino acid percentages were determined in accordance with the AccQ-Tag method [20]. As observed, the major amino acids in the protein fraction were: glutamic acid + glutamine (14.12%), serine (9.46%), arginine (9.00%) and proline (7.08%). Notice that the fraction of neutral amino acids was the most abundant (64.21%) in the protein fraction.

3.2 Synthesis of lipoamino acid surfactant mixtures

The main purpose of this work was the exploration of novel and advanced routes for the valorisation of a waste from the leather industry. The chemical procedure used to prepare surfactants from the mixture of amino acids was easy and very efficient. It consisted of the N-acylation of the amino groups of the amino acids with two different acid chlorides, decanoyl chloride and dodecanoyl chloride. This is a traditional synthetic method in which the reaction is carried out in a mixture of water/acetone, no organic waste is generated and it is not necessary to increase the temperature.

The N-acylation reaction was carried out with different amino acid/acid chloride ratios (Table 2). A pondered molecular weight was calculated for the amino acid mixtures taking into account the amino acids and their percentages. Using the 1/0.5 amino acid/acid chloride ratio it was observed that part of the amino acids remained without reacting (Figure 1 B). When the percentage of acid chloride was increased up to a 1/0.75 ratio all the amino acids present in the mixture reacted (Figure 1 C). Because of that, the proportion of acid chloride was not further increased and the ratio 1/1 was not used. This behaviour indicates that the amino acid mixture also contained some salts from the hydrolysis process.

It is important to emphasize that for each amino acid/ acid chloride molar ratio three replicates were performed and the chromatograms obtained presented always the same profile. Chromatograms for lipoamino acid surfactants with C12 alkyl chains are similar to those for lipoamino acid mixtures containing C10 alkyl chains being the retention time the only difference. Because of the enhanced hydrophobicity of the compounds with C12 alkyl chains regarding compounds containing C10 alkyl chains, the HPLC of the mixture obtained with dodecanoyl chloride contained peaks with retention times higher than those obtained with decanoyl chloride.

The retention times of the surfactants obtained ranged from 9 to 22 minutes. Given that different amino acids were present in the starting material (Table 1), the obtained mixture contained surfactants with different retention times. The retention time of the surfactant depends on the hydrophobicity of the amino acids that form the polar head. In the case of basic amino acids with two amino groups, such as lysine or arginine, it is possible to obtain amphiphilic molecules with two alkyl chains. It is also possible that the acyl chloride reacts in some cases with the hydroxyl group present on serine and threonine. Because of that, peaks of the HPLC chromatograms at high retention times (>18 minutes) are expected to correspond to the surfactants containing two alkyl chains.

The mixtures of lipoamino acid surfactants were not further purified because the aim of the work was to obtain surfactants with easy and clean technologies. The isolation of pure surfactants from these heterogeneous mixtures would require different purification methods with the use of huge quantities of solvents. The only additional process carried out in this study after surfactant synthesis was the removal of some inorganic salts. The acylation reaction produces salts, most of them can be removed with dry ethanol and subsequent filtration. With this objective, the sample was dissolved in dried ethanol and filtered through a 0.22 μm porous membranes. As expected, the chromatographic peaks of the surfactant mixtures after removing salts were sharper than those corresponding to the lipoamino acid surfactants containing salts.

3.3 Critical micelle concentration of the surfactant mixtures

In the lipoamino acid surfactant mixtures, the alkyl chain is linked to the amino group of the amino acid through an amide bond. It means that the polar groups of the surfactant mixture contain a carboxylic group that can be neutral or negatively charged.

Therefore, these surfactants can behave as nonionic or anionic depending on the pH. The micellization process of these surfactant mixtures was determined by tensiometry and spectrofluorimetry.

Values of surface tension and fluorescence as a function of the surfactant concentration are plotted for: i) different amount of acid chloride in the acylation reaction (Figure 2), ii) different length of the hydrocarbon chain (Figure 3) and iii) presence or absence of salts in the surfactant mixture (Figure 4).

Table 2 shows the cmc values for the lipoamino acid surfactant mixtures obtained from the graphs plotted in Figures 2, 3 and 4. Given that surfactant mixtures might contain dodecanoic and decanoic acids from the surfactants synthesis, the cmc values of these compounds were also determined by surface tension. Due to its low solubility in water, the cmc values of the fatty acids were determined at pH 12. The values obtained were 18.9 g/L for dodecanoic acid and 21.1 g/L for decanoic acid.

In general, the cmc values of the different surfactant mixtures determined by surface tension are in good agreement with those obtained from fluorescence measurements.

Compared to conventional anionic or cationic surfactants with similar alkyl chain length these surfactants mixtures have very exceptionally low cmc values [29]. These low cmc values can be due to the presence of double chain surfactants. It has been reported that the cmc of cationic surfactants based on quaternary ammonium polar heads with two hydrophobic chains of twelve carbon atoms is 0.04 g/L [30]. On the other hand, the presence of non-ionic surfactants could also contribute to these cmc values. Notice that the mono acyl lysine and N-acyl arginine derivatives are amphoteric surfactants given that they have one negative charge in the carboxylic group and one positive charge in the amino or guanidine group. It means that at the pH corresponding at the isoelectric point, these surfactants behave as non-ionic. Non-ionic surfactants with similar hydrophobic groups also have low cmc values, around $0.1x10^{-4}$ M [29].

Regarding the effect of the amount of acid chloride in the N-acylation reaction, the cmc values increased when the ratio amino acid/acid chloride ratios varies from 1/0.5 to 1/0.75 (Figure 2) .These results suggest that the ratio 1/0.5 is not enough to convert all the amino acids of the starting protein hydrolisate in their corresponding lipoamino acid derivatives. For this reason the acylation reaction should be carried out at 1/0.75 ratio for which the reaction of all the amino acids was confirmed (Figure 1C). With regard to the effect of the length of the hydrocarbon chain of the fatty acid

chloride on the cmc values of the surfactant mixtures obtained, that the cmc values for the reaction with dodecanoyl chloride were lower than those obtained with decanoyl chloride (Figure 3). As expected, surfactants with longer alkyl chains gave rise to lower cmc values due to their superior hydrophobicity. This behaviour is analogous to that described for different surfactant families [29,31,32]. Finally, analysing the effect of salt removal, Table 2 shows that the cmc values decreased in surfactant mixtures without salts (Figure 4). It can be attributed to the fact that these mixtures were purified which resulted in an increased total surfactant content.

It is worth to underline that the extremely low cmc values of the surfactant mixtures indicated that these surfactants form molecular aggregates at very low concentrations. For example, the sodium salt of dodecanoic and decanoic acids form micelles at concentrations two order of magnitude higher than the studied mixtures.

Two additional parameters were also determined from the surface tension plots: surface tension at the cmc (γ_{cmc}) and the concentration necessary to reduce by 20 mN/m the surface tension of pure solvent (C_{20}). These parameters are given in Table 3. The effectiveness of these mixtures (γ_{cmc}) is similar to those reported for surfactants with comparable hydrophobic groups [29]. However, the C_{20} is significantly low. This means that these lipoamino acid mixtures are efficient in reducing the surface tension of water.

From these results, it can be stated that it is possible to obtain mixtures that present excellent surface properties using a tannery waste as starting material. These mixtures reduce the surface tension of water and form micelle aggregates at very low concentrations.

3.4 Qualitative phase behaviour. Liquid crystals

Conventional surfactants aggregate in solution to form micelles because of the hydrophobic effect. At high concentration, micelles become ordered forming lyotropic liquid crystals. Liquid crystals formation can stabilise the emulsions. By accumulating at the interface, liquid crystals form a high-viscosity region. Surfactants tend to form compact films at interfaces when concentrations are lower than those for which liquid crystals form [21].

Liquid crystal formation for the binary systems L-3/water and C-2/water was determined by visual observation of the samples through crossed polarised microscopy. Qualitative phase behaviour studies by applying the flooding method revealed that

formation of lamellar liquid crystals took place for both samples. As expected, it was necessary more concentration and higher temperature to obtain lamellar liquid crystal structures when the hydrophobicity of the sample decreased. The L-3 surfactant mixture formed lamellar structures at room temperature (22ºC) (Figure 5A). This structure was stable up to a temperature of 60ºC when the liquid crystal began to melt (Figure 5B). Figure 6 shows the formation of lamellar liquid crystals and shapes of Malta cross for the C-2 surfactant mixture. This structure was clearly observed when the sample was heated at 60º C (Figure 6A) and remained stable until 70 ºC when the liquid crystal began to melt (Figure 6B). The presence of lamellar structures in these systems indicate the high hydrophobicity of the mixtures and this agree with their low cmc values. Usually, N^{α}-lauroyl arginine surfactants form hexagonal and cubic structures. The formation of lamellar crystal liquids requires longer alkyl chains (14-16 C atoms) or the presence of two alkyl chains (gemini or diacyl glycerol arginine surfactants) [23].

3.5 Foaming properties

Surfactants can be also used as foaming agents because of their capacity to absorb at the air/water interface. In this work, a modified Padmashree's method [24-26] was applied to evaluate the foaming properties. Sodium dodecyl sulphate (SDS) was used as reference foaming substance. Since C10-acylated mixtures provided unstable foams, foaming properties were evaluated only for C12-acylated mixtures (L-2 and L-3).

In general, the cmc of a surfactant is a good parameter for estimating its efficiency as foaming agent. The lower the cmc the more efficient the surfactant as foaming agent [29], consequently, the foaming capacity enhances by increasing the length of the hydrophobic group of the surfactant. This trend has been also confirmed for surfactant mixtures obtained from acylated peptides. Foaming properties of surfactants prepared from peptides obtained by the enzymatic hydrolysis of rapeseed were found to be better for C12- than for C10-acylated derivatives [33]. Similarly, for anionic surfactants obtained from pea protein, the best foaming capacity was also reported for the C12-acylated mixture [34].

The foam capacity and the foam stability of these samples were determined as a function of the surfactant concentration. The initial maximum foam height was measured after extensive shaking of the vials. The foaming capacity values are shown in Table 4. As expected, foam capacity increased with increasing surfactant concentration

for both L-2 and L-3 surfactant mixtures as well as for the SDS. It was observed that L-3 system showed better foaming efficacy than L-2 surfactant mixture. It could be attributed to the higher surfactant content in L-3 with respect to L-2 surfactant mixture due to the salt removal treatment applied to the former ones. It should be taking into account that the presence of salt inhibits the foam formation. As compared to the reference substance, the foaming efficacy of these surfactant mixtures was lower than that of SDS. Although foaming behaviour is very complex, this difference could be partially ascribed to the presence of double chain surfactants. It has been reported that double chain amphiphiles form large aggregates in aqueous solutions resulting in lower foaming capacity [25].

Stability is another important parameter when studying foaming properties. Foams are thermodynamically unstable systems. Their stability and their fracture depend on a series of complex phenomena that begin with the hydrodynamic drainage of the liquid, the dilution of the aqueous film and the coalescence of bubbles [24, 35]. Figure 7 shows the change in foam volume (v) as a function of time (t) for aqueous solutions of L-2, L-3 and SDS. In general, very stable foams were obtained for all the samples. After 180 minutes, the foaming stability was higher than 80% for all the systems.

3.6 Emulsifying capacity

Emulsions are colloidal dispersions of two immiscible liquid phases that consist of droplets of one liquid dispersed in another liquid (continuous phase). Emulsions are used in many fields (cosmetics, foods, medicines, remediation, etc) and surfactants are widely used as emulsifier's molecules. There are two different types of emulsions: oil-in-water (continuous phase water) and water-in-oil (continuous phase oil).

Given the interest of non-toxic and biodegradable surfactants in industrial applications, we studied the efficiency of the synthesized surfactants mixtures in forming emulsions. The emulsifying capacity was evaluated using decane and squalane as oil phases. The composition of the systems investigated is given in Table 5.

The visual appearance of the emulsions after one hour and one year is shown in Figure 8.

It was observed that the surfactant mixtures formed readily emulsions after vigorous shaking and these emulsions were stable for a long time (the emulsion remained stable after one year). In this study, the surfactant was first dissolved in water

and then oil was added, thus favouring the formation of O/W emulsions [36]. Differences in the refractive index between dispersed and continuous media confirmed that O/W dispersions were formed. These surfactants are water soluble and form micelles and liquid crystals in water solutions that can favour the formation O/W dispersions. A refinement of Brancroft's rule states that the preferred type of emulsion will be that in which self-aggregation of surfactant takes place [36]. It has been reported that single chain amino acid based surfactants from arginine also stabilize O/W emulsions [37]. On the other hand, dialkyl arginine based surfactants can form both O/W and W/O emulsions. This ability is ascribed to the very low solubility of these surfactants in water and oil as well as to their ability to form vesicles [23].

Emulsions containing decane were more stable than those containing squalane (Figure 8). The solubility of surfactants in no polar phases decreases with increasing molecular weight and alkyl chain length of the oils [38]. Hence, the stability of the dispersions decreases by reducing the affinity of the surfactant to the oil phase. Moreover, it is well known that short-chain oils, such as decane, have a more pronounced tendency of penetration into the hydrophilic/lipophilic interface of the surfactant [39,40]. For surfactant mixtures with alkyl chains of 10 carbon atoms (C-1 surfactant mixtures), the emulsion with the lowest water content (E2) exhibited the highest stability. Emulsions prepared with L-2 and L-3 surfactant mixtures (alkyl chains of 12 carbon atoms) were more stable with respect to coalescence. It is outstanding that, in fact, no phase separation was detected in E6 and E8 emulsions after one year. Since these surfactants have longer hydrophobic chains, they exhibit greater ability of aggregation at low concentration. Because of this, their emulsification ability is enhanced with respect to C-1 surfactant mixtures. It was also observed that the stability improved by increasing the percentage of water. Moreover, the removal of salts in the surfactant mixture (L-3) did not change the appearance or stability (see Figure 8, E6 and E8 emulsions).

The capacity to stabilize O/W emulsions for a very long time could render these surfactant mixtures appropriate for their use as green emulsifiers in different industrial applications.

3.7 Biodegradation

The biodegradability of a compound expresses the ability for microorganisms to degrade a molecule or a mixture of molecules. It is a data of great importance because it

is related to the persistence of a compound in the environment. According to REACH (Registration, Evaluation and Authorization of Chemicals) legislation, new chemicals have to pass ultimate biodegradation test in order to be marketed. Therefore, we have investigated the biodegradability of the surfactant mixtures obtained as well as the influence of the hydrophobic tail length. Biodegradation curves are plotted in Figure 9. Biodegradation curves show that both surfactants mixtures were easy and quickly mineralized by the aerobic microorganisms in the first week of the testing period. No significant differences in terms of biodegradability were observed between C12 and C10 amino acid based surfactants. It can be observed that both amino acid based surfactant mixtures passed the threshold of the biodegradation test (60%) and therefore can be classified as readily biodegradable.

3.8 Aquatic toxicity

Acute toxicity tests on freshwater crustacea (*Daphnia magna*) were carried out to assess the aquatic toxicity of the surfactant mixtures obtained. The results of the *Daphnia magna* immobilisation test [28] for C-2 and L-2 surfactant mixtures are given in Table 6.

The estimated concentration required to immobilise 50% of the crustacea population after 48 hours of exposure ranged from 69 to 245 mg/L. Data on Table 6 indicate that the toxicity to daphnia increased when increasing the alkyl chain length attached to the amino acid polar head. Thus, C-2 surfactant mixture with hydrophobic tails of 10 carbon atoms exhibited a significantly lower toxicity than L-2 surfactant mixture with C12 hydrophobic tails.

Table 7 shows the ecotoxicity hazard classification category of the surfactant mixtures synthesized on the basis of the EC_{50} values obtained from the short-term crustacea test in accordance with the OECD (2001) [41] and the US fish and Wildlife services (1994) [42]. As shown in this table, these surfactant mixtures ranged from practically non toxic to slightly toxic as a function of the alkyl chain length. On the other hand, these amino acid based surfactant mixtures are less toxic to aquatic organisms than common anionic [43,44], cationic [45] and non-ionic surfactants [46,47] derived from petrochemical feedstocks.

4. Conclusions

Lipoamino acid surfactant mixtures were obtained by acylation of the amino acids obtained from the acid hydrolysis of the protein fraction present in the waste waters of the tannery beamhouse processes. The surfactant mixtures from this type of waste show very low cmc values indicating that these surfactants form aggregates at very low concentrations. Moreover, they are very efficient in reducing the surface tension of water. The mixtures obtained form lamellar liquid crystal structure and very stable O/W emulsions and foams. In addition, these surfactants are readily biodegradable and result to be non-toxic or only slightly toxic to the aquatic environment. Consequently, bearing in mind their physical-chemical and environmental properties the surfactant mixtures could be used as green solubilizers, green emulsifiers or foaming agents in different industrial applications. Our results confirm that it is possible the valorisation of a waste of the tanning industry that entails the reduction of the pollution load from this sector. This approach also contributes to save fossil resources such as crude oil and natural gas.

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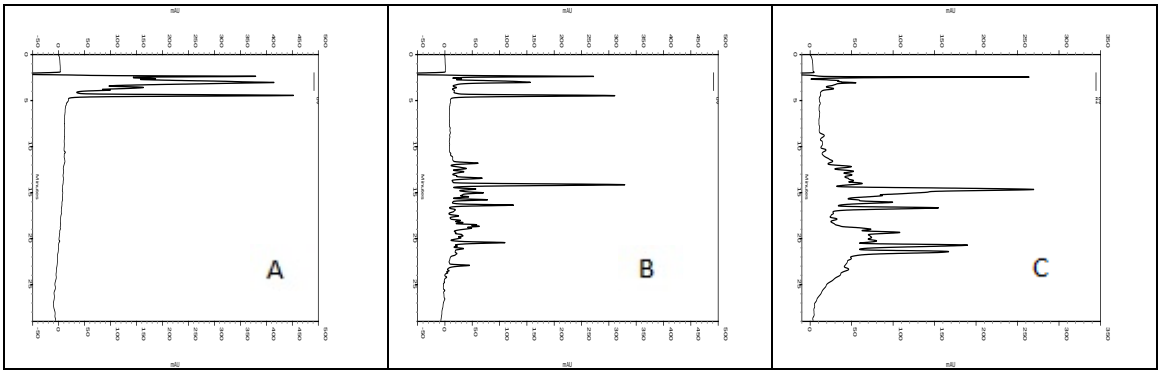

Fig. 1. HPLC chromatogram corresponding to the initial mixture of amino acids (**A**), lipoamino acid mixture L-1 (**B**) and lipoamino acid mixture L-2 (**C**).

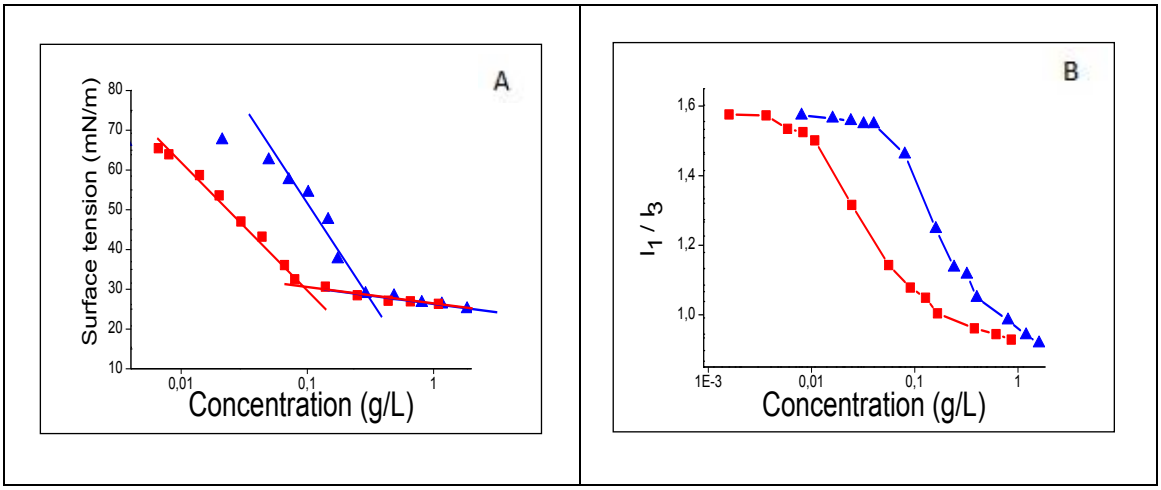

Fig. 2. Influence of the amount of acid chloride in the acylation reaction on the cmc in water (pH 6) at 25ºC of the L-1(▲) and L-2 (■) lipoamino acid surfactant mixtures (A: Surface tension; B: Fluorescence).

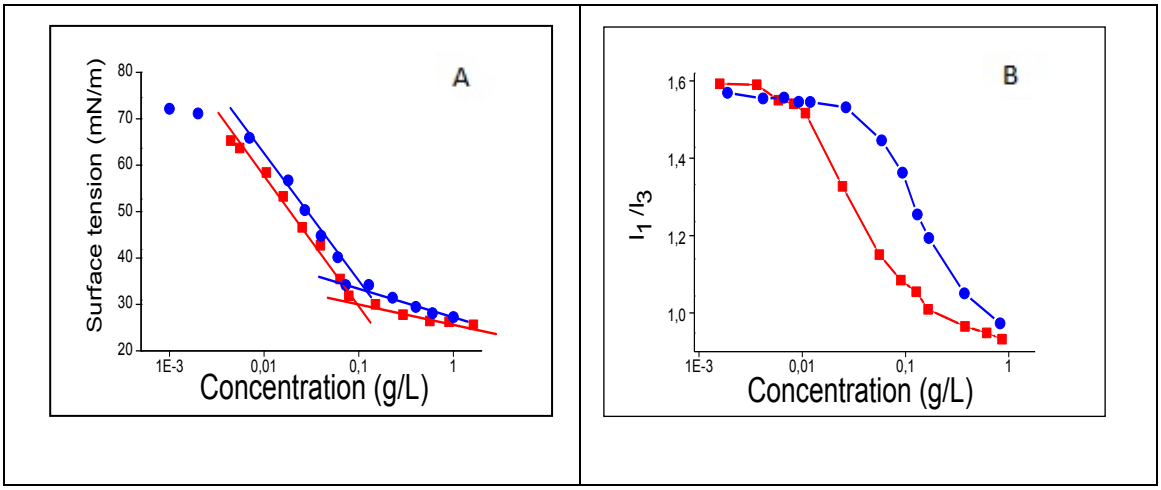

Fig. 3. Influence of the length of the hydrocarbon chain on the cmc in water (pH 6) at 25ºC of the L-2 (■) and C-2 (●) lipoamino acid surfactant mixtures (A: Surface tension; B: Fluorescence).

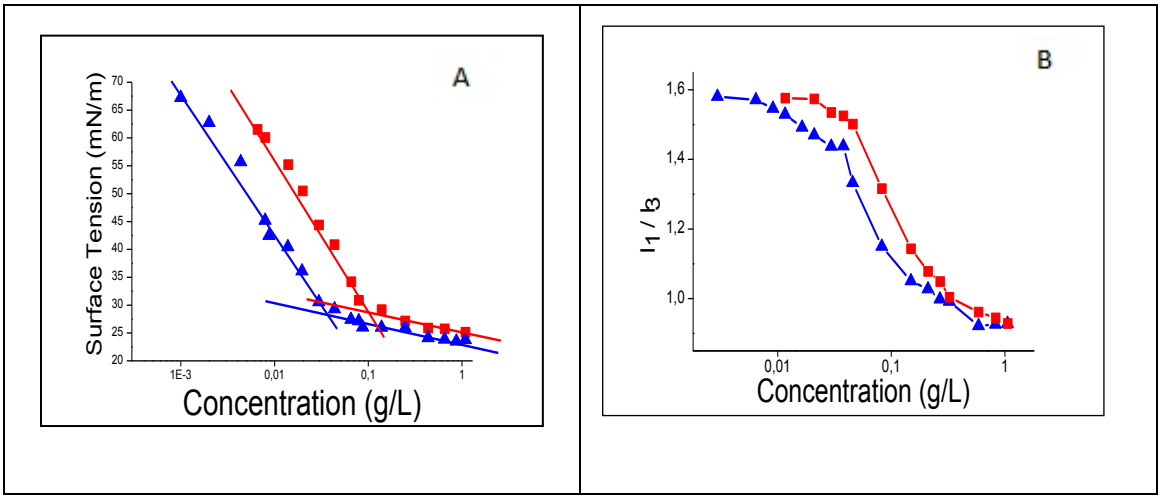

Fig. 4. Influence of the salt removal on the cmc in water (pH 6) at 25ºC of the L-2 (■) and L-3 (▲) lipoamino acid surfactant mixtures (A: Surface tension; B: Fluorescence).

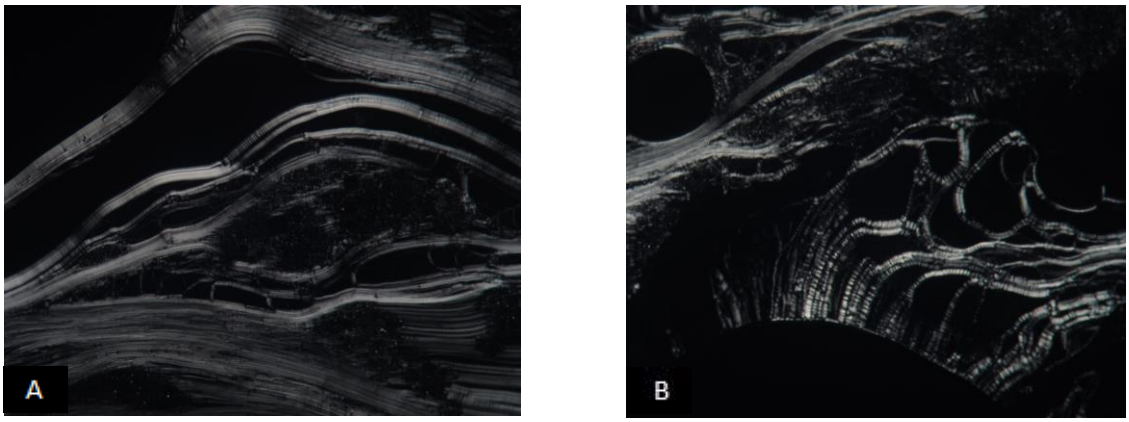

Fig. 5. Polarised microscopy of lamellar liquid crystals using the Lawrence method for the L-3/water system. (A at 22ºC and B at 60ºC)

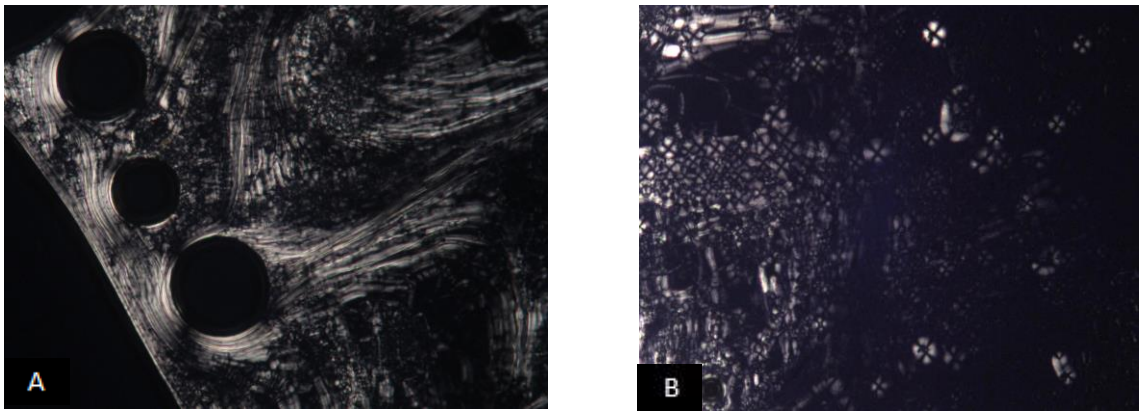

Fig. 6. Polarised microscopy of lamellar liquid crystals using the Lawrence method for the C-2/water system. (A at 60ºC and B at 70ºC)

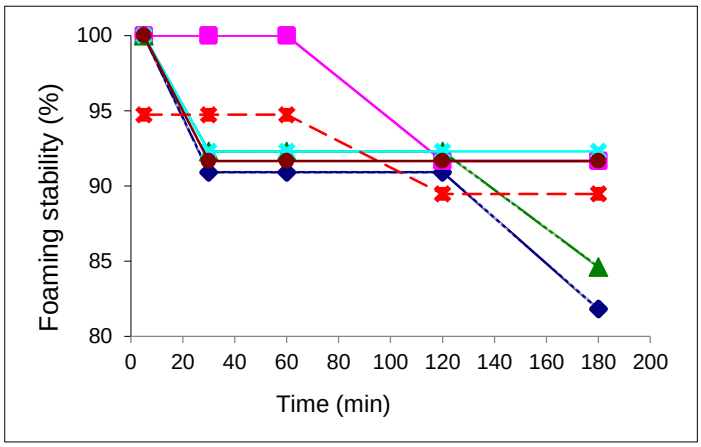

Fig. 7. Foaming stability of aqueous solutions at 25ºC: L-2 at 5 mg/mL (♦); L-2 at 10 mg/mL (■); L-2 at 15 mg/mL (▲); L-3 at 10 mg/mL (x); L-3 at 15 mg/mL (x) and SDS at 1.5 mg/mL (●)

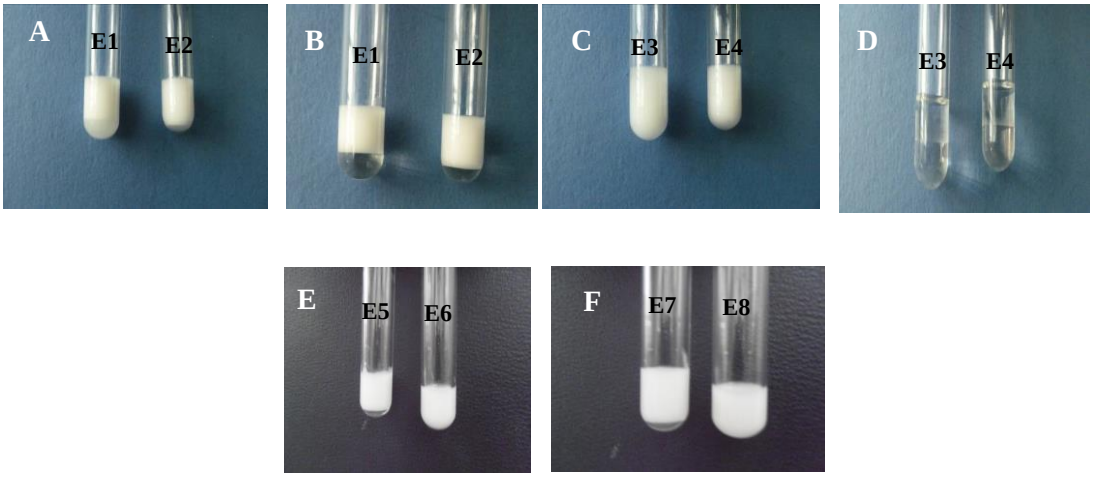

Fig. 8. Visual appearance of the emulsions investigated (see Table 5). Photographs A and C taken after 1 hour of preparation and photographs B, D, E and F taken after 1 year of preparation.

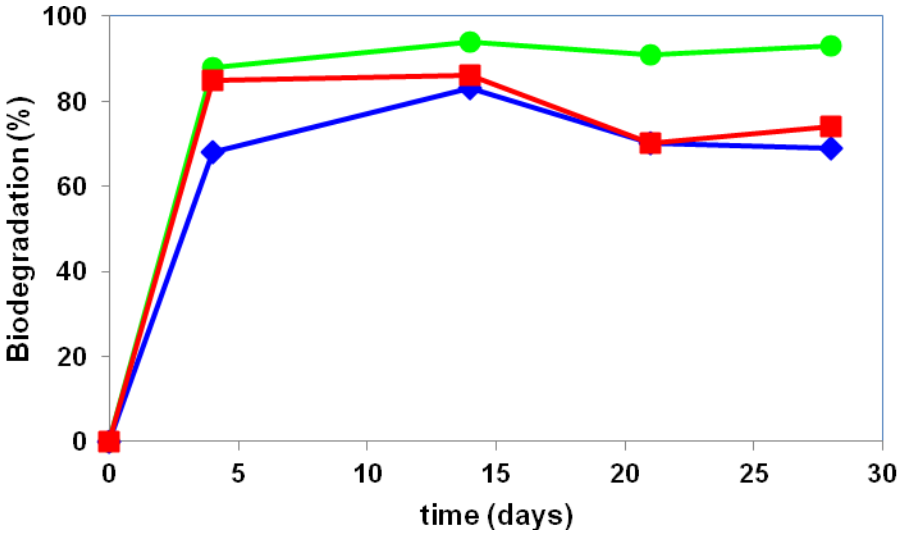

Fig. 9. Aerobic biodegradation curves of the lipoamino acid surfactant mixtures at a concentration of 20 mg C/L: C-2 (◆) and L-2 (■); reference substance: SDS (●)

Table 1. Amino acid composition of the protein fraction

Acid amino acids		Basic amino acids		Neutral amino acids	
	% ,w/w		%,w/w		%,w/w
Asp+Asn	7.07 ± 0.06	Lys	3.86 ± 0.10	Ser	9.46 ± 1.00
Glu+Gln	14.12 ±0.67	Arg	9.00 ± 0.22	Gly	5.45 ± 0.54
		His	1.73 ± 0.32	Thr	5.60 ± 0.10
				Ala	4.43 ± 0.30
				Pro	7.08 ± 0.22
				Cys	5.92 ± 0.29
				Tyr	4.84 ± 0.45
				Val	5.75 ± 0.05
				Met	0.85 ± 0.02
				Ile	3.84 ± 0.07
				Leu	7.83 ± 0.29
				Phe	3.17 ± 0.08
	Σ = 21.29%		**Σ = 14,60%**		**Σ = 64,21%**

Table 2. Abbreviations used to name the obtained surfactant mixtures, N-acylation reaction conditions and critical micellar concentration (cmc) values of the lipoamino acid surfactant mixtures obtained by surface tension and fluorescence measurements

Lipoamino acid surfactant mixture abbreviations	Alkyl chain	Amino acid/acid chloride molar ratio	Salt Removal	cmc (surface tension) (g/L)	cmc (fluorescence) (g/L)
L-1	C12	1/0.5	No	0.13	0.08
L-2	C12	1/0.75	No	0.09	0,06
L-3	C12	1/0.75	Yes	0.03	0.04
C-1	C10	1/0.5	No	0.24	0.24
C-2	C10	1/0.75	No	0.12	0.15

Table 3. Critical micellar concentration (cmc), surface tension at the cmc (γ_{cmc}) and the concentration necessary to reduce by 20 mN/m the surface tension of water (C_{20}). Values of lipoamino acid surfactant mixtures obtained by surface tension measurements in water at 25ºC.

Surfactant mixture	cmc (g/l)	γ_{cmc} (mN/m)	C_{20} (g/l)
L-1	0.135	28.4	0.038
L-2	0.094	29.2	0.015
L-3	0.034	28.1	0.004
C-1	0.24	28.3	0.022
C-2	0.12	32.8	0.021

Table 4. Foaming capacity (%) of L-2, L-3 and SDS in water at at 25ºC.

Surfactant mixture	Concentration (mg / ml)	pH	Foaming Capacity (%)
L-2	5	7	275
L-2	10	7	300
L-2	15	7	325
L-3	10	7	325
L-3	15	7	475
SDS	1,5	7	300
SDS	2	7	400

Table 5. Composition of the emulsions investigated

	Surfactant Mixture		Water content	Oil	
Emulsion	Name	Content (mg)	(ml)	Type	Content (g)
E1	C-1	20	1	Decane	1
E2	C-1	10	0.4	Decane	1
E3	C-1	10	1	Squalane	1
E4	C-2	10	1	Squalane	1
E5	L-2	10	0.2	Decane	1
E6	L-2	10	0.4	Decane	1
E7	L-3	10	0.2	Decane	1
E8	L-3	10	0.4	Decane	1

Table 6. Acute toxicity of lipoamino acid surfactant mixtures on *Daphnia magna* after a 48 h exposure time expressed as the EC_{50} value and its corresponding 95% confidence interval (95 % CI).

Surfactant mixture	EC_{50} (mg/L)	95% CI (mg/L)
C-2	245	196-326
L-2	69	43-99

Table 7. Ecotoxicity hazard classification of the surfactant mixture on the basis of the EC_{50} values of the *Daphnia magna* test

Surfactant mixture	Acute toxicity EC50	US fish and Wildlife services	OECD
C-2	>100 mg/L	Practically non toxic	---
L-2	10-100 mg/L	Slightly toxic	Acute Toxicity III (harmful to aquatic life)