



**HAL**  
open science

## Cleaner degreasing of sheepskins by the *Yarrowia lipolytica* LIP2 lipase as a chemical-free alternative in leather industry

Emna Moujehed, Zied Zarai, Haifa Khemir, Neila Miled, Mohamed Salah Bchir, Corinne Gablin, François Bessueille, Anne Bonhommé, Didier Leonard, Frédéric Carrière, et al.

### ► To cite this version:

Emna Moujehed, Zied Zarai, Haifa Khemir, Neila Miled, Mohamed Salah Bchir, et al.. Cleaner degreasing of sheepskins by the *Yarrowia lipolytica* LIP2 lipase as a chemical-free alternative in leather industry. *Colloids and Surfaces B: Biointerfaces*, 2022, 211, pp.112292. 10.1016/j.colsurfb.2021.112292 . hal-03494701

**HAL Id: hal-03494701**

**<https://hal.science/hal-03494701v1>**

Submitted on 20 Dec 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Received 5 September 2021; Received in revised form Cleaner degreasing of  
sheepskins by the *Yarrowia lipolytica* LIP2 lipase as a chemical-free  
alternative in leather industry**

**Emna Moujehed<sup>1,2,3,4</sup>, Zied Zarai<sup>1,5,\*</sup>, Haifa Khemir<sup>3</sup>, Neila Miled<sup>3</sup>, Mohamed Salah Bchir<sup>4</sup>, Corinne Gablin<sup>2</sup>, François Bessueille<sup>2</sup>, Anne Bonhommé<sup>2</sup>, Didier Leonard<sup>2</sup>, Frédéric Carrière<sup>6</sup>, Ahmed Aloulou<sup>1,\*</sup>**

<sup>1</sup> *University of Sfax, National Engineering School of Sfax, Laboratory of Biochemistry and Enzymatic Engineering of Lipases, 3038 Sfax, Tunisia.*

<sup>2</sup> *Univ Lyon, CNRS, Université Claude Bernard Lyon 1, Institut des Sciences Analytiques, UMR 5280, 5, rue de la Doua, F-69100 Villeurbanne, France.*

<sup>3</sup> *National Center for Leather and Footwear, 2033 Megrine, Tunisia.*

<sup>4</sup> *SO.SA.CUIR tanning company, 4070 M'Saken, Tunisia.*

<sup>5</sup> *University of Sfax, Higher Institute of Biotechnology of Sfax, 3038 Sfax, Tunisia.*

<sup>6</sup> *Aix Marseille Univ, CNRS, UMR 7281, Bioénergétique et Ingénierie des Protéines, 13009 Marseille, France.*

\* Corresponding authors: [zied.zarai@isbs.usf.tn](mailto:zied.zarai@isbs.usf.tn) (ZZ); [ahmed.aloulou@enis.usf.tn](mailto:ahmed.aloulou@enis.usf.tn) (AA)

## **Abstract**

Conventional degreasing of skins and hides in the leather industry requires high amounts of organic solvents and detergents that cause environmental issues. In this study, the LIP2 lipase from the yeast *Yarrowia lipolytica* (YLLIP2) was shown to be effective in degreasing sheepskins, thus reducing the amount of harmful chemicals. Using 10 mg of lipase/kg of raw skin, successful degreasing was achieved in only 15 min at pH 7. ToF-SIMS mass spectra of sheepskins were compared before and after degreasing (chemically and enzymatically). Results are consistent with a specific selective elimination process for the enzymatic treatment. Comparative SEM microscopy, ATR-FTIR spectroscopy and physicochemical analyses showed better properties of the enzymatically treated leather than those of the chemically treated leather. Effluent physicochemical parameters showed that the enzymatic treatment is a cleaner degreasing alternative.

**Keywords:** *Yarrowia lipolytica* LIP2 lipase; Enzymatic degreasing; Leather industry.

## 1. Introduction

The leather industry is considered as one of the most polluting activities due to the broad range and extent of hazardous chemicals that are released to the environment in wastewater. Sustainable leather manufacturing is a topic with growing importance. That's why worldwide efforts have been made towards reducing ecological risks through ecofriendly approaches in leather processing [1]. Degreasing of high-fat content animal skins is a critical step in leather industry. When processing small skins from goat and sheep, tanneries aim for a residual fat content of no more than 5 %, but they are not always able to achieve this. The problem is that the fat is embedded between the grain surface and the corium (the deep inner layer of the skin), which makes it very hard to remove. That's why dry cleaning with strong organic solvents is a widely used technique to dissolve the fat with all the inherent hazards. However, dry cleaning is expensive, and the results are not optimal due to possible damage to the skins. Detergents are a milder and safer chemical alternative but not as effective. Owing to the fast-growing enzyme industry and the diversity of products it provides, another method consists in the use of lipases, fat-degrading enzymes, that can simply be added to the drums. Lipase-mediated degreasing has emerged as a cleaner alternative in leather manufacturing [2-5]. Benefits include also increased leather uniformity and chemical uptake since lipases improve filling and dye penetration to produce a much more uniform appearance [3].

Lipases (EC 3.1.1.3) are soluble enzymes that act on natural insoluble triacylglycerols (TAG) [6]. The LIP2 lipase from the yeast *Yarrowia lipolytica* (YLLIP2, NCBI accession number CAB91111) is an attractive enzyme that can be overproduced at industrial scale up to a level of 3 g of lipase/L of culture medium [7]. YLLIP2 is the main secreted lipase by *Y. lipolytica* by exhibiting more than 97% of the extracellular lipase activity [8, 9]. It displays a 1,3-regioselectivity that allows the hydrolysis of ester bonds at external positions of the glycerol backbone of TAG [7, 10]. YLLIP2 belongs to the same gene family as *Thermomyces*

*lanuginosus* lipase (TLL), a lipase produced by Novozymes and sold under the trademarks of Lipolase® and Greasex® in the fields of detergents and leather industry, respectively, along with other biotechnological processes. Although these two lipases show a sequence identity of 30.3%, they have quite distinct biochemical [11] and structural properties [12]. YLLIP2 can catalyze the hydrolysis of both long-, medium- and short-chain TAG at high rates. At pH 8.0 and in the absence of detergents, the specific activity of YLLIP2 ( $11,751 \pm 402$  U/mg) on olive oil-gum arabic emulsion is much higher than that measured previously with TLL ( $4,975 \pm 230$  U/mg) [7, 13], and thus greater than the highest rate of hydrolysis of long-chain triglyceride previously recorded with a lipase under these conditions.

The aim of the current study was to assess use of YLLIP2 in sheepskins degreasing. Time-course and dose-dependency of YLLIP2 in degreasing were measured under conditions mimicking those prevailing in the leather industry. Lipase doses for the industrial scale degreasing were deduced from these experiments. The degreasing efficacy of YLLIP2 was tested with time-of-flight secondary ion mass spectrometry (ToF-SIMS) and compared with that of conventional detergent. Leather quality was followed by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and scanning electron microscopy (SEM). The effluent quality was monitored to determine the pollution load generated.

## **2. Materials and methods**

### **2.1. Lipase production**

Using W29 wild-type *Y. lipolytica* strain, YLLIP2 was produced in batch cultures using olive oil, the only carbon source, as inducer. Briefly, 1-L shake flasks were filled with 100 mL of YPO culture medium (1% w/v bacto yeast extract, 2% w/v bacto peptone, 1% w/v olive oil, and 0.1 M sodium phosphate, pH 5.6). Bacto yeast extract and bacto peptone solutions were sterilized by autoclaving. The sodium phosphate buffer, the D-glucose solution, and the olive oil were sterilized by filtration. Cultures were inoculated with a 24-h *Y. lipolytica* liquid preculture to get an initial optical density of 1 U at 600 nm to start the cell growth directly in the exponential growth phase, as well as to establish reproducible cell culture conditions. Cultures were then incubated at 29 °C in an orbital shaker set at 160 rpm. Media and techniques used here to grow and handle *Y. lipolytica* have been described elsewhere [14]. Biomass and lipase activity were monitored as a function of time until the lipase activity level in the culture broth reached a maximum value. After discarding cell pellets, the resulting crude supernatant was concentrated by ammonium sulfate precipitation at 65% salt saturation. After centrifugation, the lipase precipitate was stored at -20 °C until use.

### **2.2. Lipase activity assay**

YLLIP2 activity was assayed potentiometrically by automatic titration of free fatty acids released from a mechanically stirred olive oil/gum Arabic emulsion, using 0.1 M NaOH and a pH-stat device (Metrohm, Switzerland). Assays were performed at a constant pH value of 8,0 in a thermostated (37 °C) vessel containing 10 mL of olive oil/gum arabic emulsion and 20 mL of a 0.5 mM Tris, 150 mM NaCl and 5 mM CaCl<sub>2</sub> solution. The specific activity of YLLIP2 was found to be 11,751 ± 402 U/mg under these conditions [7]. One lipase international unit corresponds to one μmol of free fatty acid released per minute.

### **2.3. Degreasing assays**

Sheepskins were kindly provided by SOSA Cuir tannery (Sousse, Tunisia). After conventional delimiting and bating stages, sheepskins were randomly divided into pieces (10 x 15 cm, weight ~20 g) and distributed to different groups to assess time-course and dose-response relationships of lab-scale skin degreasing by YLLIP2 lipase. Enzymatic assays (EZ) were performed in 50 mL shake flasks, under a gentle agitation of 50 rpm, at pH 8 and 30 °C in the presence of 20 mL degreasing buffer containing 1 mM Tris. It is worth noticing that one hundred skin weight percent (100 wt %) of buffer is usually used in degreasing at the industrial scale. As a control experiment, chemical treatment (CT) was carried out with the commercial detergent Synthol (SMIT, The Netherlands) under the same conditions. For process validation, industrial degreasing of whole sheepskins was performed in tannery drums filled with 100 wt % of the degreasing buffer under optimized conditions (incubation time and lipase amount) at 50 rpm shaking frequency and 30 °C. Treated skins then underwent conventional tanning.

### **2.4. Residual fat content**

Treated sheepskins were dried at 100 °C to constant weight. Residual fats were then extracted by dichloromethane using the Soxhlet method. After solvent removal by rotary evaporation, the residual fat content percentage was calculated from the extracted fat and the initial dried sample weights.

### **2.5. Time-of-flight secondary ion mass spectrometry (ToF-SIMS)**

The leather surface analysis was carried out on a TRIFT III ToF-SIMS instrument (Physical Electronics, USA) operated with a pulsed 22 keV Au<sup>+</sup> ion gun (2 nA ion current) rastered over a 300 × 300 μm<sup>2</sup> area. The electron gun was operated in pulsed mode at low electron energy for charge compensation. The ion dose was kept below the static conditions limit. Data

were analyzed using the WinCadence software. Mass calibration was performed on hydrocarbon secondary ions.

## **2.6. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)**

ATR-FTIR analysis of the leather surface was carried out at room temperature using a NEXUS 670 spectrometer (Thermo-Nicolet, UK) equipped with mercury-cadmium-telluride (MCT) detector. Spectra were recorded in the wave number range of 4000-680  $\text{cm}^{-1}$  with a 4- $\text{cm}^{-1}$  resolution and 256 scans per sample for improved signal to noise ratio. The OMNIC software was used for spectra processing.

## **2.7. Scanning electron microscopy (SEM)**

SEM images of the leather were obtained using a VEGA<sub>3</sub> scanning electron microscope (TESCAN, Czech Republic) with a 20 kV accelerating voltage. Samples were coated with a 25-nm thin gold layer deposited by sputtering to produce an electrically conductive surface for SEM.

## **2.8. Chemical characterization**

Dried leather was pounded using a laboratory grinder. Collected powder samples were then subjected to pH, formaldehyde, and chromium VI analysis according to the International Union of Leather Technologists and Chemists Societies (IULTCS). Wastewater samples were collected at the end of the industrial degreasing process and then filtered to remove gross solids. Effluent physicochemical parameters, such as pH, conductivity, total suspended solids, five-day biochemical oxygen demand (BOD) and chemical oxygen demand (COD), were determined to assess the pollution load generated.

## **2.9. Physical characterization**

Crust leather samples were conditioned at room temperature under standard atmospheric conditions with  $65 \pm 2$  % relative humidity for 48 hours before physical tests. Samples were cut



using Hydraulic Sample Press Atom SE20 (Italy). Leather thickness was measured thrice using a Digimatic Quick Thickness Gauge (Mitutoyo™, France). Leather physical properties were determined using an Instron 4301 Tensile Strength Tester (France). Data were processed using Series IX software.

### 3. Results & Discussions

#### 3.1. Optimization of enzymatic degreasing

A layer of lipids, which are of both sebaceous and keratinocyte origin, covers the surface of the animal skin. Lipids produced by the epidermal cells are an insignificant fraction of the total extractable surface lipid on areas rich in sebaceous glands. Due to the holocrine activity of the sebaceous gland, its product of secretion (sebum) is eventually released to the surface of the skin and coats the fur as well. Lipids of epidermal origin fill the spaces between the cells. Sebaceous lipids are primarily nonpolar lipids such as TAG, wax esters and squalene, while epidermal lipids are a mixture of ceramides, free fatty acids, and cholesterol [15]. Raw sheepskin natural fat content is about 30 % of dry weight [2]. However, following bating-delimiting steps, average natural fat content was about 10 % of dry weight. Sheepskins degreasing potency of YLLIP2 was first followed by performing dose-effect and time-course relationships (**Fig. 1**). The degreasing level was evaluated by the determination of residual fat content in greasy skins. Dose-dependency study, upon 15 min incubation, showed that increased amounts of lipase led to a decrease of the residual fat content, thus reaching less than 5 % residual lipids with 6 mg of YLLIP2 per 1 kg of skin (**Fig. 1a**). Degreasing kinetics, using 6 mg of lipase/kg of skin, showed a continuous decrease in residual fat content as function of time to reach ~1 % after 1 h incubation (**Fig. 1b**). In the sake of comparison, the commercial lipase from *T. lanuginosus* (TLL, Greasex®) could be applied typically in an amount of 100,000 U per kg of skin with a reaction time of 30 min [3]. Assuming a specific activity of TLL about 10,000 U/mg, Greasex® could be added in an amount of 10 mg per kg of skin. YLLIP2 seems therefore to be as efficient as Greasex® in terms of minimum effective concentrations and reaction times to achieve sufficient degreasing.

ToF-SIMS makes possible the detailed identification of molecular structure at the top surface of materials (information depth limited to a few monolayers i.e. about 2–5 nm) [16, 17] and

was used here to gain more insight on the effect of the degreasing treatment. More precisely, this technique provides with elemental and molecular mass related signatures thanks to the bombardment of the samples using a primary ion beam and the mass analysis of the sputtered secondary ions. To study the effect of enzymatic degreasing on fat removal, both chemically and enzymatically degreased sheepskins samples were analyzed in comparison with the untreated surface in order to identify characteristic signatures before and after each respective degreasing process. Lipids with high mass as phosphatidylcholines, phosphatidylethanolamines, and sphingolipids, as well as compounds with low mass as cholesterol, fatty acids and vitamins are observed. In the negative ion mode range (**Fig. 2**), the lipid profile of untreated sheepskin surface shows triacylglycerols (TG) and fatty acids (FA) characteristic signatures that were detected at  $m/z = 255.2, 281.2, 309.2, 337.2$  and  $353.2$ . [18-20]. The results on the degreased samples are consistent with a specific selective elimination process for the enzymatic treatment. Concerning the positive ion mode (**Fig. S1**), the peak at  $m/z = 184.08$  (related to phosphatidylcholine) is well detected in sheepskins before degreasing treatment. It is well known that cell membranes are consisting of proteins, lipids and phospholipids [21]. It appears that YLLIP2 was efficient to get rid of the phosphatidylcholine signature while the conventional chemical process did not, confirming a specific selective elimination process for the enzymatic treatment. Other positive ion mode signatures were detected at  $m/z = 311.2, 313.2$  and  $339.2$  (**Fig. S2**). These peaks are attributed to MAG (16:1) as monoacylglycerols ( $C_{19}H_{35}O_3^+$ ) as well as Monoacylglycerols MAG (16:0) and MAG (18:1) corresponding to  $C_{19}H_{37}O_3^+$  and  $C_{21}H_{39}O_3^+$  respectively. The Monoacylglycerols peaks are detected in the spectra of untreated sheepskins and degreased samples, but degreasing led to some decrease of their relative intensities. The lipase YLLIP2 eliminated more specifically MAG (16:1). This suggests that, preferentially, this lipase

hydrolyzes monoacylglycerols. According to the lipid classification system determined by the lipid MAPS consortium, these MAGs are part of glycerolipids referred to as GL0101 [18].

### **3.2. Leather physicochemical properties**

Performing chemical and physical tests on leather ensures that leather products fit into the consumer market in terms quality and durability and meet legal trading requirements for the international market. Some of the treatments, used by tanneries to give leather a certain look and feel, are detrimental to the quality and durability of the leather but could go unnoticed unless the material is professionally tested. Leather manufacturers must be able to prove their products are safe for customers and the environment. Testing the chemical properties of leather products will ensure compliance, as well as the right to use the Ecolabel.

Chromium VI and formaldehyde are chemical produced during the leather tanning process. As they are known to cause severe allergic reactions on contact with skin [22], leather products must not contain chromium VI and formaldehyde in concentrations over 3 mg/kg and 50 mg/kg, respectively, of the total dry weight of the leather according to IULTCS [23]. Results showed that YLLIP2-mediated degreasing (EZ) led to much lower chromium VI- and formaldehyde-containing leather when compared to the conventional chemical treatment (CT) (**Table 1**).

Apart from chemical properties, leather's characterization and quality depend on mechanical properties such as tear strength, tear load and stitch tear resistance. The average physical values of conventional and enzymatic leather processing are given in (**Table 1**). Compared with conventionally processed leather, the tear strength resistance of enzymatically treated leather increased from  $16.5 \pm 1 \text{ N/mm}^2$  to  $21 \pm 1 \text{ N/mm}^2$ . Similarly, double edge tear and stitch tear resistance values were improved in favor of enzymatic treatment from  $62 \pm 6 \text{ N/mm}$  to  $66 \pm 5 \text{ N/mm}$  and  $60 \pm 3 \text{ N/mm}$  to  $65 \pm 2 \text{ N/mm}$ , respectively. It turned up that enzymatic

treatment enhanced strengthening leather collagen. In fact, it has been elucidated from the strength characteristics that enzymatic processed leather generates better physical values and makes stronger leather [24, 25]. Physical properties of both processed leather samples, previously described, meet the Tunisian Standards specifications 10 N/mm<sup>2</sup>, 15 N/mm and 25 N/mm respectively.

### **3.3. Leather morphology and structure**

Infrared spectroscopy study was performed to characterize leather samples surface's and to detect any chemical modification of samples after degreasing process. ATR-FTIR spectroscopic study of leather fibers, given in (**Fig.3**), showed that both FTIR data of EZ and CT samples were very similar and had the same characteristics' peaks with three important peaks corresponding to Amide I at 1637 cm<sup>-1</sup>, Amide II: 1659-1637 cm<sup>-1</sup> due to  $\nu$ C=O stretching and at 3330 cm<sup>-1</sup> due to  $\nu$ N-H stretching. Another band occurred at 2924, 2853 cm<sup>-1</sup> that corresponds to  $\nu$ CH<sub>2</sub> stretching. The 1334.59 cm<sup>-1</sup> and 1238.60- 1041 cm<sup>-1</sup> peaks corresponded to C=N of amide (III) and C-O vibrations respectively. Similar findings were reported in previous studies [26, 27]. Other studies were also performed by [28] on leather samples to analyze tannins through ATR-FTIR spectroscopy and has also determined same leather characteristics' peaks earlier identified by [29]. Since both enzymatically processed leather and conventionally processed leather spectra were identical, it was concluded that the enzymatic treatment, as revealed by FTIR, did not endure chemical changes in leather composition.

To visualize the leather's microstructure and the effect of enzymatic vs conventional degreasing, a scanning electron microscopy was ascertained. The grain surface and the cross section were given in (**Fig.4**). High magnification (2000 $\times$ ) was used to study samples binding's grain layer. Fiber's orientation and compactness with the banded structure were

clearly visualized through and the collagen polypeptide chains of enzymatically (**Fig. 4a**) and chemically (**Fig. 4a**) were obviously compacted. Leather's micrographs showed the smooth surface of samples without grease particles, which indicates the efficiency of conventional and enzymatic degreasing. The hair pores were clean without foreign particles as even reported by Saravanabhavan, S. et al., [30]. Thus, the helical conformation of the collagen was maintained for both processes (CT and EZ) as studied by Shoulders, M.D. et al., [31]. In fact, while degreasing operation, enzymes penetrated fibers and acted at molecular level. Currently, enzymes and nanozyme, such as a nanomaterial with enzyme-like characteristics and nanomaterials in general were widely applied in different sectors like medicine and analytical chemistry [32] for their interesting properties to adhere to materials for the claimed effect. According to Liu and his collaborators, the nanoparticles were conjugated to collagen and fill in fibers, which led to the improvement of leather's physical properties [33]. That explained the better properties of enzymatic treated leather from conventional processed leather in previous sections.

Concerning the cross line of enzymatically (**Fig. 4c**) and conventionally (**Fig. 4d**) treated leather, there is no significant change, fibers' orientation remained uniform in both samples. The leather structure where the layering between flesh, corium and the grain layer is well observed. CT sample presents some defibrillations that may be due the action of solvents and detergents. Therefore, these SEM results, along with FTIR results, reveal that leather structure, the surface morphology is preserved and the molecular level is not altered.

### **3.4. Environmental impact assessment of sheepskins degreasing**

The ratio BDO<sub>5</sub> to CDO was determined to assess the biodegradation capability of effluents. This study aimed to develop an enzymatic degreasing technology and reduce the chemicals pollution from leather processing, such as sodium chloride and chromium, which engendering discharge large amounts of highly polluted saline wastewater [34]. Biochemical oxygen

demand ( $BDO_5$ ), chemical oxygen demand (CDO) and suspended solid (SS) were measured to study degreasing impact and control pollution and to evaluate the environmental impact of the conventional and enzymatic processes [35]. In addition to these three parameters, the conductivity and pH were also studied [33]. Enzymatic degreasing process had certain advantages over the detergent-solvent system regarding the waste disposal issues. The results given in (**Table 2**) indicated that the CT-CDO concentration was nearly 12000, which is four times higher than EZ-CDO concentration due to the presence of hazardous contaminants from chemicals (**Table 2**). The enzymatic degreasing was carried out in one-step without detergent while conventional degreasing in local tanneries involved several processes using surfactants and solvents [36]. Thus, the CDO decreased by 75% in absence of surfactants. Water consumption of chemical-based process also reduced during the beam process where the skins were treated in aquatic environments. The proportional reduction of CDO and  $BDO_5$  in the enzymatic degreasing effluent from  $12000 \pm 600$  and  $3000 \pm 150$  to  $3520 \pm 176$  and  $2560 \pm 128$ , respectively, could be because of fat tissues present in effluent of control sheepskins. Fat destruction by detergents-solvent processes led to the raise of parameters above subsequently to pollution problems. Such findings were in line with several earlier studies. As an illustration, enzymatic process in leather manufacturing described by Sivasubramanian [37] showed that skins enzymatic proceeding is a pollution reducing process unlike conventional unhairing operation designed as the most generating waste process. The enzymatic unhairing process previously detailed was efficient to reduce  $BDO_5$  and CDO to 78% and 84% in 6 hours using an alkaline protease from *Bacillus subtilis* MTCC 6537, which provided cleaner and less toxic waste. Moreover, the suspended solids CT-SS value was higher than EZ-SS because of solid waste, which contained unloaded proteins and fatty tissues. In fact, according to Sathish et al [38], beamhouse degreasing operations generated 100 kg of solid waste per ton of hides discharged in lands that might end into water table [39]. In addition, compared to

chemical treatment, conductivity values of CT and EZ were quite similar around  $16 \pm 0.8$  and  $14 \pm 0.7$ , respectively. Neutral pH preserves the quality of the skins and prevents the denaturing of collagen fibers. The biodegradability ratio CDO/BDO<sub>5</sub> indicated that EZ effluent enzymatic degreasing is easily degradable whereas CT was not biodegradable and requires physico-chemical treatment to be finally discharged. Comparing to other international leather, new projects are focusing on the leather wastes reuse to the utmost possible. By using products with negative economic value, this green production aims to reduce pollution and industrial wastes. In agreement with other studies, the reuse of many wastes has been important in different applications, such as the retail market and poultry feed makers [36]. The degreasing solid wastes could be also used in soaps and poultry feed due to their high content in fatty tissues and proteins.

## **Conclusion**

*Y lipolytica* lipase performs sheepskins degreasing successfully, without the use of any chemical, within 10 mg lipase/kg of skin in 15 min of application. The enzymatic process is faster and powerful than the chemical process in the studied degreasing operation. It should be noted that the chemical properties of enzymatically treated leather (EZ) are better than those of the standard (CT) while no differences in leather SEM images neither in ATR-FTIR spectra were observed between the different degreasing processes applied. ToF-SIMS spectra were compared before and after degreasing (chemically and enzymatically). Results are consistent with a specific selective elimination process for the enzymatic treatment. Sheepskins leather quality has been ensured in term of physical properties. However, differences were observed in effluent analysis where CDO/BDO<sub>5</sub> ratio and solid wastes in residual baths of conventional process were higher due to enzymatic degreasing. It is challenging to note that such reductions in costs of effluent remediation were generated with



chemicals- free process due to YLLIP2 specificity to hydrolyze natural fat without damaging leather and environment.

### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

### **Acknowledgements**

Emna Moujehed's doctoral work is supported by the National Program of Research and Innovation (PNRI) & the Ministry of Higher Education and Scientific Research, Tunisia.

**Table 1.** Physicochemical characterization of chemically (CT) and enzymatically (EZ) treated sheepskins.

	<b>CT</b>	<b>EZ</b>
<b>Chromium VI (mg/kg)</b>	0.19	0.001
<b>Formaldehyde (mg/kg)</b>	25	11
<b>Tear strength (N/mm<sup>2</sup>)</b>	16.5±1.2	21±1
<b>Double-edge tear (N/mm)</b>	62±6	66±5
<b>Stitch tear resistance (N/mm)</b>	60±3	65±2

**Table 2.** Effluent quality parameters of chemically (CT) and enzymatically (EZ) treated sheepskins.

	<b>CDO (mg/L)</b>	<b>BDO<sub>5</sub> (mg/L)</b>	<b>Suspended solids (mg/L)</b>	<b>Conductivity (μs/cm)</b>	<b>pH</b>
<b>CT</b>	12000± 600	3000 ±150	3.96 ±0.2	16.06 ±0.8	8.49 ±0.42
<b>EZ</b>	3520± 176	2560 ±128	2.98 ±0.15	13.9 ±0.7	7.72 ±03.8

## References

- [1] S. Huffer, T. Taeger, Sustainable leather manufacturing: A topic with growing importance, *Journal of The American Leather Chemists Association*, 99 (2004) 424-428.
- [2] J. Christner, The use of lipases in the beamhouse processes, *Journal of The American Leather Chemists Association*, 87 (1992) 128-139.
- [3] L. Rasmussen, Q. Xu, N.K. Pedersen, Z. Zhou, An enzymatic treatment of skin and hide degreasing, in: W.I.P. Organization (Ed.) PubChem, NOVOZYMES AS, DK, 2008.
- [4] S. Saran, R. Mahajan, R. Kaushik, J. Isar, R.K. Saxena, Enzyme mediated beam house operations of leather industry: a needed step towards greener technology, *Journal of Cleaner Production*, 54 (2013) 315-322.
- [5] L. Shan, F. Luo, S. Chattha, C. Zhang, B. Peng, C. Mu, Surfactant-free beamhouse technology of leather manufacturing: Removing constraints for the breakdown of natural fats catalyzed by lipase, *Journal of Cleaner Production*, 261 (2020) 121187.
- [6] A. Aloulou, J.A. Rodriguez, S. Fernandez, D. Van Oosterhout, D. Puccinelli, F. Carriere, Exploring the specific features of interfacial enzymology based on lipase studies, *Biochimica et Biophysica Acta* 1761 (2006) 995-1013.
- [7] A. Aloulou, J.A. Rodriguez, D. Puccinelli, N. Mouz, J. Leclaire, Y. Leblond, F. Carriere, Purification and biochemical characterization of the LIP2 lipase from *Yarrowia lipolytica*, *Biochimica et Biophysica Acta* 1771 (2007) 228-237.
- [8] J. Kamoun, M. Schue, W. Messaoud, J. Baignol, V. Point, E. Mateos-Diaz, P. Mansuelle, Y. Gargouri, G. Parsiegla, J.F. Cavalier, F. Carriere, A. Aloulou, Biochemical characterization of *Yarrowia lipolytica* LIP8, a secreted lipase with a cleavable C-terminal region, *Biochimica et Biophysica Acta*, 1851 (2015) 129-140.

- [9] G. Pignede, H. Wang, F. Fudalej, C. Gaillardin, M. Seman, J.M. Nicaud, Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*, *Journal of Bacteriology*, 182 (2000) 2802-2810.
- [10] A. Aloulou, M. Schue, D. Puccinelli, S. Milano, C. Delchambre, Y. Leblond, R. Laugier, F. Carriere, *Yarrowia lipolytica* Lipase 2 Is Stable and Highly Active in Test Meals and Increases Fat Absorption in an Animal Model of Pancreatic Exocrine Insufficiency, *Gastroenterology*, 149 (2015) 1910-1919 e1915.
- [11] A. Aloulou, D. Puccinelli, A. De Caro, Y. Leblond, F. Carriere, A comparative study on two fungal lipases from *Thermomyces lanuginosus* and *Yarrowia lipolytica* shows the combined effects of detergents and pH on lipase adsorption and activity, *Biochimica et Biophysica Acta*, 1771 (2007) 1446-1456.
- [12] A. Aloulou, A. Bénarouche, D. Puccinelli, S. Spinelli, J.-F. Cavalier, C. Cambillau, F. Carrière, Biochemical and structural characterization of non-glycosylated *Yarrowia lipolytica* LIP2 lipase, *European Journal of Lipid Science and Technology*, 115 (2013) 429-441.
- [13] A. Tiss, F. Carriere, R. Verger, Effects of gum arabic on lipase interfacial binding and activity, *Journal of Analytical Biochemistry*, 294 (2001) 36-43.
- [14] A. Najjar, S. Robert, C. Guerin, M. Violet-Asther, F. Carriere, Quantitative study of lipase secretion, extracellular lipolysis, and lipid storage in the yeast *Yarrowia lipolytica* grown in the presence of olive oil: analogies with lipolysis in humans, *Applied Microbiology and Biotechnology*, 89 (2011) 1947-1962.
- [15] A. Pappas, Epidermal surface lipids, *Dermatoendocrinology*, 1 (2009) 72-76.
- [16] A. Benninghoven, F.G. Rudenauer, H.W. Werner, Secondary ion mass spectrometry: basic concepts, instrumental aspects, applications and trends, John Wiley and Sons, New York, NY, United States, 1987.

- [17] J.C. Vickerman, I.S. Gilmore, Surface analysis: the principal techniques, 2nd Edition ed., John Wiley & Sons, United Kingdom, 2011.
- [18] M.K. Passarelli, N. Winograd, Lipid imaging with time-of-flight secondary ion mass spectrometry (ToF-SIMS), *J Biochimica et Biophysica Acta -Molecular Cell Biology of Lipids*, 1811 (2011) 976-990.
- [19] M.J. Taylor, K.Y. Zhang, D.J. Graham, L.J. Gamble, Fatty acid and lipid reference spectra, *Surface Science Spectra*, 25 (2018) 025001.
- [20] M. Urbini, V. Petito, F. de Notaristefani, F. Scaldaferrri, A. Gasbarrini, L. Tortora, ToF-SIMS and principal component analysis of lipids and amino acids from inflamed and dysplastic human colonic mucosa, *Journal of Analytical Bioanalytical Chemistry*, 409 (2017) 6097-6111.
- [21] G.M. Gray, H.J. Yardley, Lipid compositions of cells isolated from pig, human, and rat epidermis, *Journal of lipid research*, 16 (1975) 434-440.
- [22] J. Buters, T. Biedermann, Chromium (VI) contact dermatitis: getting closer to understanding the underlying mechanisms of toxicity and sensitization!, *Journal of Investigative Dermatology*, 137 (2017) 274-277.
- [23] IULTCS, International Union of Leather Technologists and Chemists Societies, 1992.
- [24] S.M. Mozersky, O.D. Allen, W.N. Marmer, Vigorous proteolysis: reliming in the presence of an alkaline protease and bating (Post-Liming) with an extremophile protease, *The Journal of the American Leather Chemists Association*, 97 (2002) 150-155.
- [25] A. Dayanandan, J. Kanagaraj, L. Sounderraj, R. Govindaraju, G.S. Rajkumar, Application of an alkaline protease in leather processing: an ecofriendly approach, *Journal of Cleaner Production*, 11 (2003) 533-536.

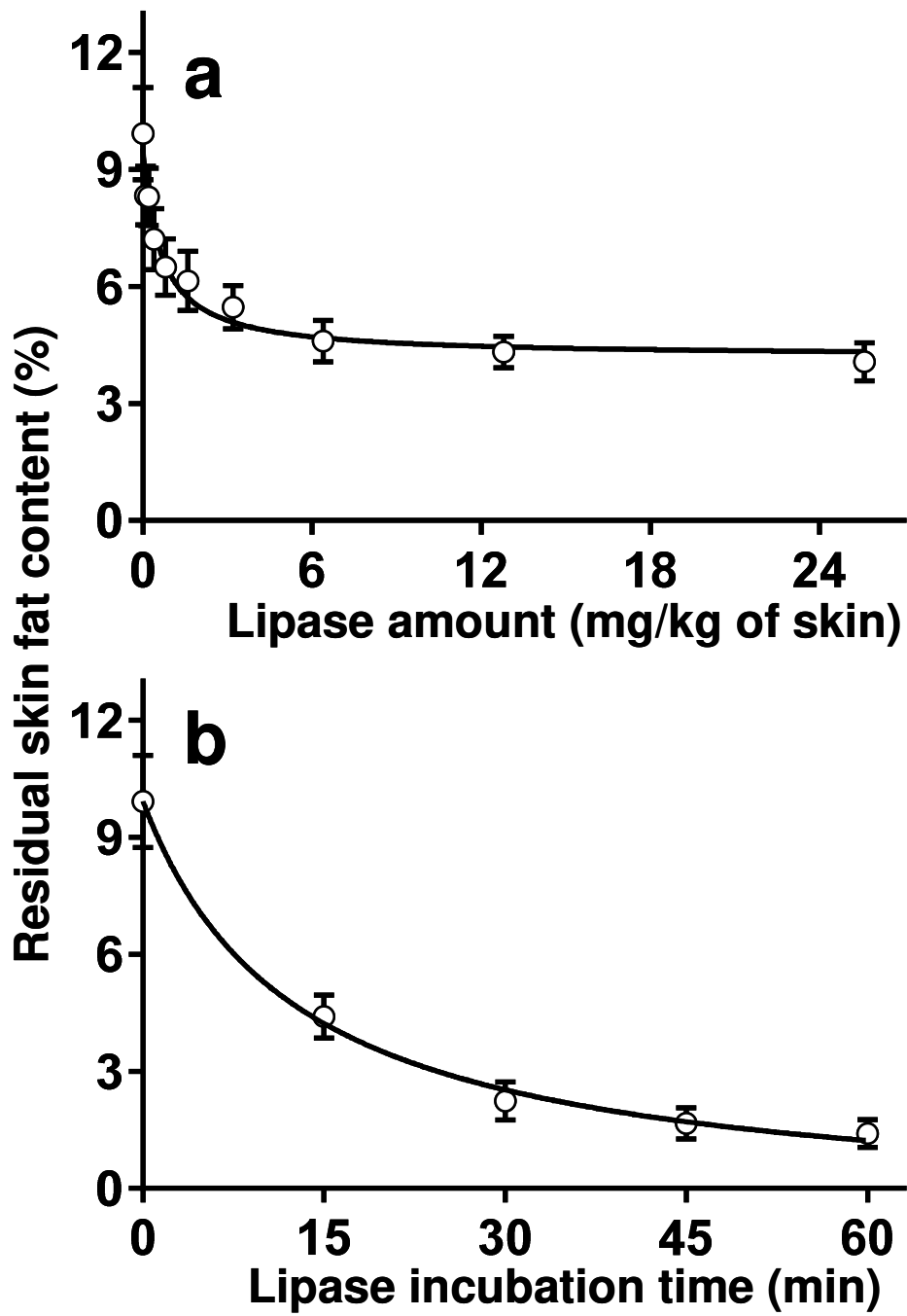
- [26] X. You, L. Gou, X. Tong, Improvement in surface hydrophilicity and resistance to deformation of natural leather through O<sub>2</sub>/H<sub>2</sub>O low-temperature plasma treatment, *Journal of Applied Surface Science*, 360 (2016) 398-402.
- [27] V. Plavan, M. Koliada, V. Valeika, An eco-benign semi-metal tanning system for cleaner leather production, *Journal of the Society of Leather Technologists Chemists*, 101 (2017) 260-265.
- [28] L. Falcão, M.E.M. Araújo, Application of ATR–FTIR spectroscopy to the analysis of tannins in historic leathers: the case study of the upholstery from the 19<sup>th</sup> century Portuguese Royal Train, *Vibrational Spectroscopy*, 74 (2014) 98-103.
- [29] M.R. Derrick, D. Stulik, J. Landry, *Infrared spectroscopy in conservation science*, Getty Publications 2000.
- [30] S. Saravanabhavan, P. Thanikaivelan, J.R. Rao, B.U. Nair, T. Ramasami, Reversing the conventional leather processing sequence for cleaner leather production, *Journal of Environmental science technology*, 40 (2006) 1069-1075.
- [31] M.D. Shoulders, R.T. Raines, Collagen structure and stability, *Journal Annual review of biochemistry*, 78 (2009) 929-958.
- [32] Q. Wang, H. Wei, Z. Zhang, E. Wang, S. Dong, Nanozyme: An emerging alternative to natural enzyme for biosensing and immunoassay, *Journal TrAC Trends in Analytical Chemistry*, 105 (2018) 218-224.
- [33] M. Liu, J. Ma, B. Lyu, D. Gao, J. Zhang, Enhancement of chromium uptake in tanning process of goat garment leather using nanocomposite, *Journal of Cleaner Production*, 133 (2016) 487-494.
- [34] O. Lefebvre, R. Moletta, Treatment of organic pollution in industrial saline wastewater: a literature review, *Journal of Water research*, 40 (2006) 3671-3682.

- [35] P.M. de Aquim, E. Hansen, M. Gutterres, Water reuse: An alternative to minimize the environmental impact on the leather industry, *Journal of environmental management*, 230 (2019) 456-463.
- [36] P. Thanikaivelan, J.R. Rao, B.U. Nair, T. Ramasami, Recent trends in leather making: processes, problems, and pathways, *Journal Critical Reviews in Environmental Science Technology*, 35 (2005) 37-79.
- [37] S. Sivasubramanian, B.M. Manohar, A. Rajaram, R. Puvanakrishnan, Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease, *Journal Chemosphere*, 70 (2008) 1015-1024.
- [38] M. Sathish, B. Madhan, K.J. Sreeram, J.R. Rao, B.U. Nair, Alternative carrier medium for sustainable leather manufacturing—a review and perspective, *Journal of Cleaner Production*, 112 (2016) 49-58.
- [39] H. Sawalha, R. Alsharabaty, S. Sarsour, M. Al-Jabari, Wastewater from leather tanning and processing in Palestine: characterization and management aspects, *Journal of Environmental Management*, 251 (2019) 109596.

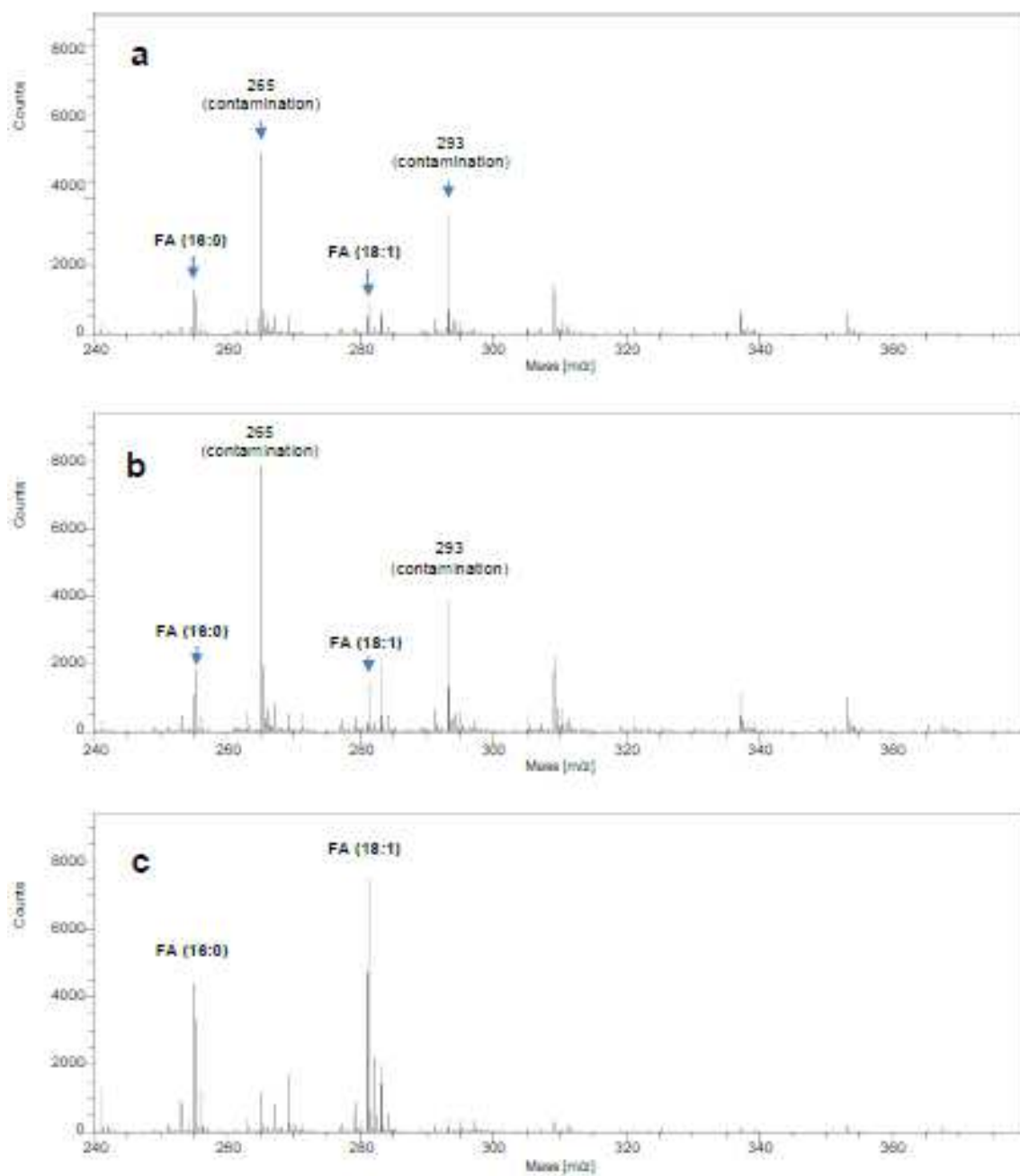


**Figures**

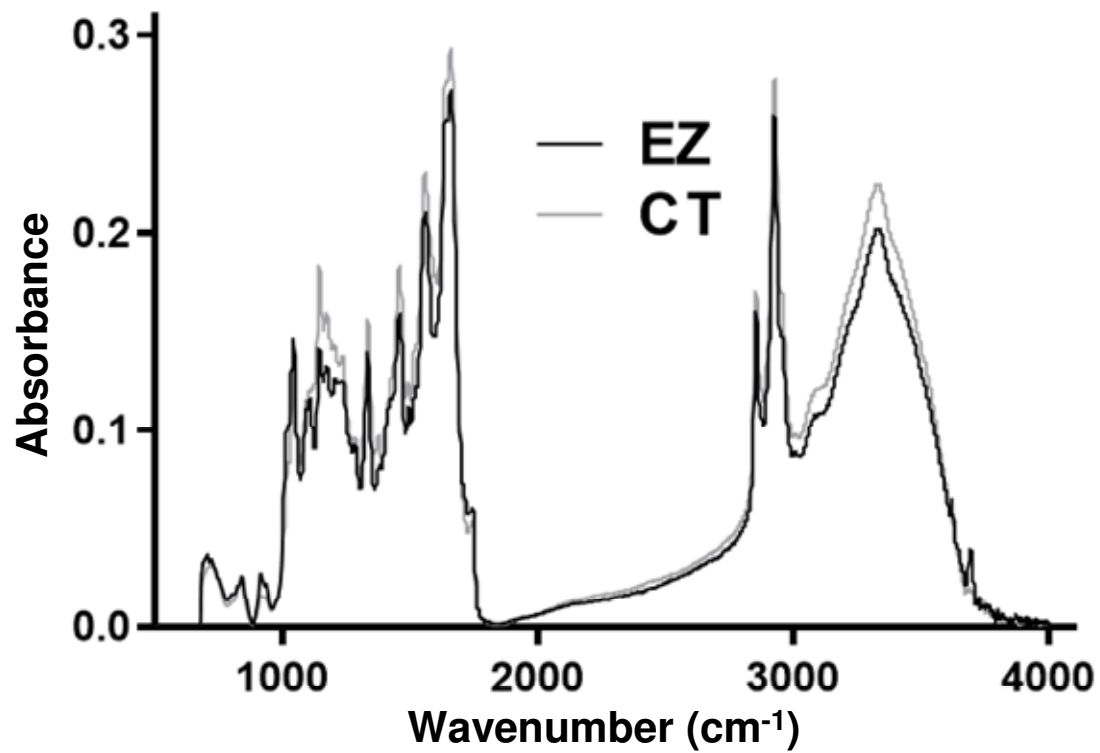
**Figure 1.** (a) Dose-response effect of YLLIP2 in sheepskins degreasing; (b) Kinetic of enzymatic degreasing.



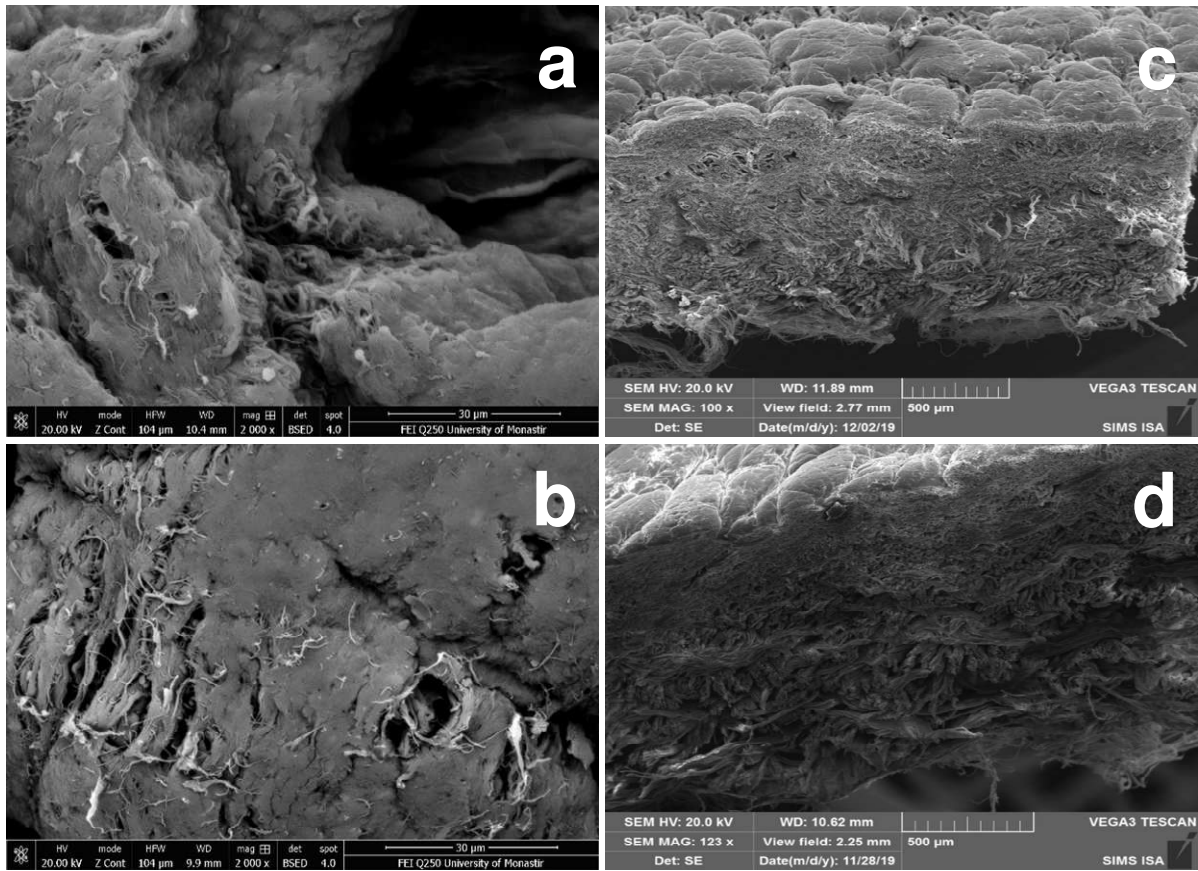
**Figure 2.** (a) Negative ion ToF-SIMS spectrum of untreated sheepskin; Chemically treated (CT) (b) and enzymatically (EZ) (c) treated sheepskins.



**Figure 3.** ATR-FTIR spectra of leather samples after conventional (CT) and enzymatic (EZ) degreasing.

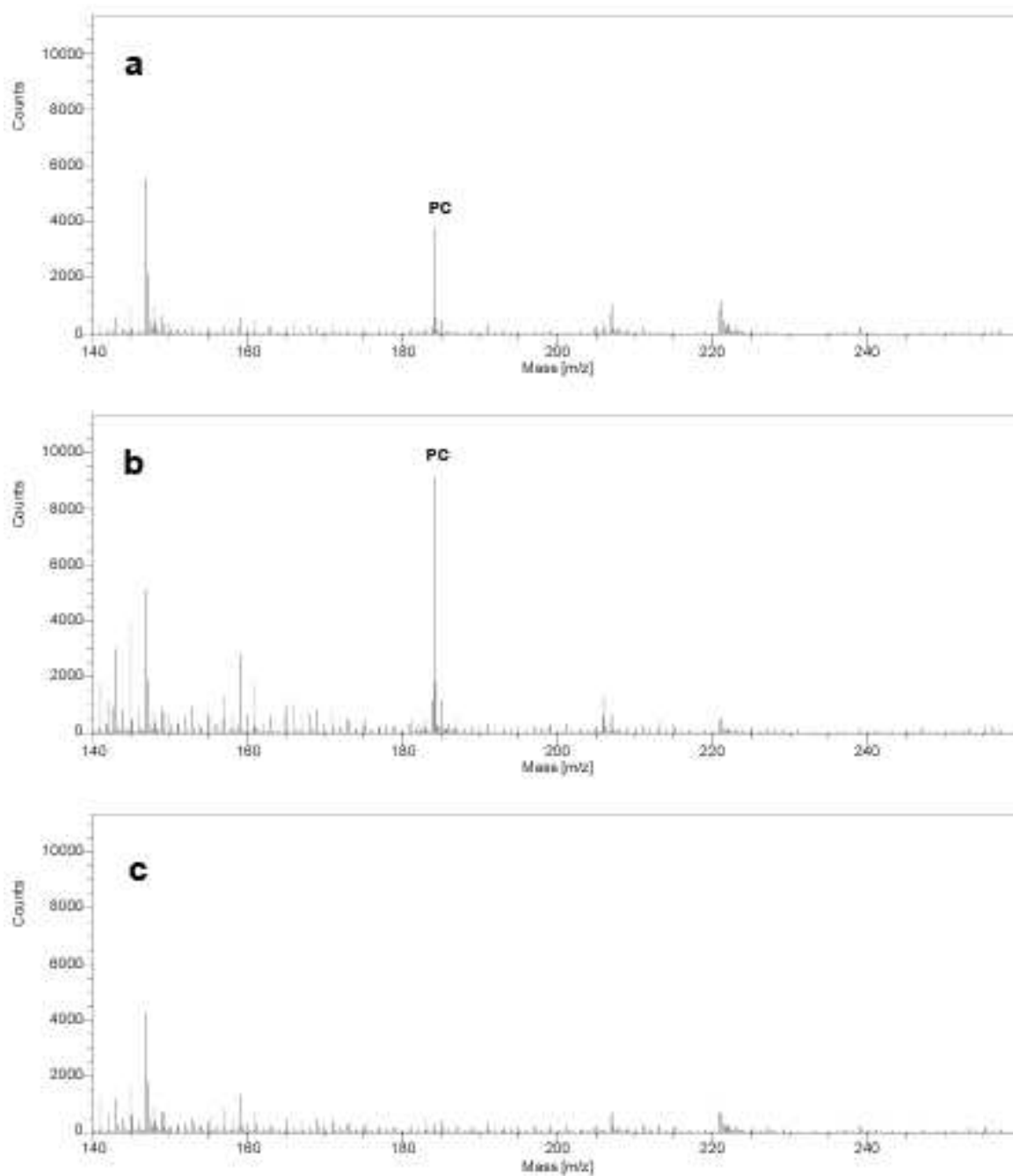


**Figure 4.** SEM images of leather samples after enzymatic (a) and conventional (b) degreasing at 2000x magnification. Corresponding cross sections are shown in (c) and (d), respectively, at 125x magnification.

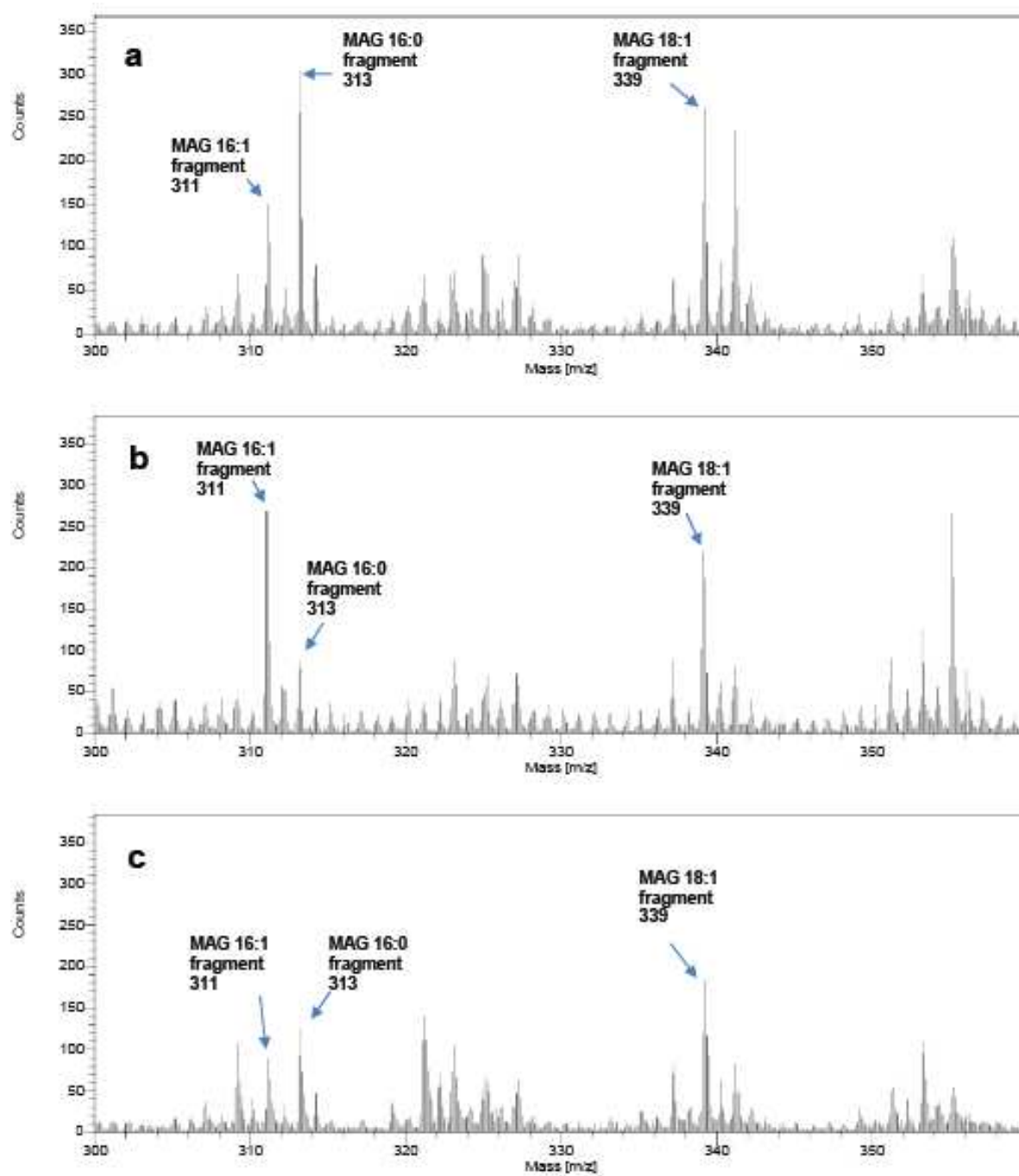


## Supplementary Data

**Figure S1:** Positive ion ToF-SIMS spectrum of untreated sheepskin (**a**) chemically treated sheepskin (**b**) and enzymatically treated sheepskin (**c**).



**Figure S2:** Positive ion ToF-SIMS spectrum in other compounds of untreated sheepskin (a) chemically treated sheepskin (b) and enzymatically treated sheepskin (c).



### **List of symbols and abbreviations**

*Yarrowia lipolytica* LIP2 lipase: YLLIP2

*Thermomyces lanuginosus* lipase: TLL

Attenuated total reflectance-Fourier transform infrared spectroscopy: ATR-FTIR

Scanning Electron Microscopy: SEM

Time-of-flight secondary ion mass spectrometry: ToF-SIMS

International Union of Leather Technologists and Chemists Societies: IULTCS

Enzymatic assays: EZ

Chemical treatment: CT

MAG: Monoacylglycerols

TAG: Triacylglycerols

Suspended solid: SS

Biochemical oxygen demand: BDO<sub>5</sub>

Chemical Oxygen Demand: CDO

