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Fatty acid profile and toxicity evaluation of stabilized *Sus scrofa domesticus Erxleben* (SSDE) fat and incorporation in animal feed and soap production.

Hermann NOUNAGNON[1], Salomé KPOVIESSI[1,*], Raymond FATONDJI[1], Bénédicta KPADONOU-KPOVIESSI[1], Bérenger LADELE [1], Pierre DOSSOU-YOVO[2], Joachim GBENOU[3],

[1] Laboratory of Organic Physical Chemistry and Synthesis (LaCOPS), Abomey-Calavi University, Faculty of Sciences and Technics (FAST), 01 BP: 4166 Cotonou, Benin
[2] Laboratory of Research in Treatment and Conservation of Halieutic products (LAREPROH), Abomey-Calavi University, Faculty of Sciences and Technics (FAST), BP: 1270 Abomey-Calavi, Benin
[3] Laboratory of Pharmacognosy and Essential Oils (LAPHE), University of Abomey-Calavi, Faculty of Health Sciences (FSS), Faculty of Sciences and Technics (FAST), 01 BP: 188 Cotonou, Benin

*Corresponding author, Laboratory of Organic Physical Chemistry and Synthesis (LaCOPS), University of Abomey-Calavi, Faculty of Sciences and Technics (FAST), 01 BP: 4166 Cotonou, Benin
E-mail: salome.kpoviessi@fast.uac.bj (S. Kpoviessi), Phone: (+ 229) 97883927

Abstract

Sus scrofa domesticus Erxleben (SSDE) fat was purified, stabilized and the GC/FID analysis of fatty acid profile determined with 48.57% of SFA, 38.59% of MUFA and 12.84% of PUFA. This fat was evaluated for toxicity against shrimp larvae and showed a value(LC_{50} 1500 µg/mL) largely higher than that of the positive control compound (camptotecine, LC_{50} = 13 µg/mL). The fat was used to make soap and it gave Marseille type soap with 19% maximum humidity, 65% minimum TFA and 0.18% maximum alkalinity. Incorporated in animal nutrition, it gave feed that had shown with rabbits, in four (4) weeks, the result obtained after eight (8) weeks of traditional nutrition.

Keywords: Stabilized pig fat, Fatty acid profile, Shrimp larvae, toxicity, Rabbits nutrition, Soap manufactory.

Introduction

Animal fats were basically extracted from adipose tissue or milk from animals. The most important sources of animal protein in Benin were fish, poultry, pig (SSDE) and beef (BTL). In general the adipose tissues of these animals, fat stock are not valued at their fair value. In our study, we will focus on SSDE fat. This animal raised throughout West Africa. The particularity in Benin is that SSDE production is fairly developed and spread throughout the country's rural and urban areas. It was present in all departments of the national territory with a high concentration in those of the South and Central (d'Orgeval et al., 1989).

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The local SSDE was the most breed by the producers and its meat, compared to that of the improved breed SSDE, was more appreciated by consumers (d'Orgeval et al., 1989). The improved breeds encountered were the Large White, the Landrace and their mixed breeds often raised in semi-modern to modern conditions. The use of SSDE meat during ceremonies such as weddings, baptisms, birthdays ..., the use of SSDE fat in traditional medicine and the frequent gathering of consumers around the many delicatessens located on both sides in the towns and villages of the country justify the high consumption of the meat of SSDE(Montcho,2014).Weighing up to 150 kg for SSDE, the carcass of the animal after slaughter can contain up to 12% lipid (Greenfield et al., 2007). The fat obtained after the melting of the adipose tissues was refined and stabilized in the laboratory (Collège des enseignants de nutrition, 2011). The fatty acid profile of refined SSDE fat revealed a high percentage of UFA (58.8%), with a high percentage of oleic acid (43.5%) (CODEX STAN 211, 1999). Palmitic and stearic acids (24.0% and 13.0%) were the majors components of SFA (Iverson et al., 1965).Under-utilized in Africa, SSDE fat was used in several industrial sectors (energy, oleochemistry, animal and human feed) in western countries (Girard, 2008).Recently, Fats of Pig and Beef from Benin were purified from adipose tissues and stabilized for future uses in our laboratory (Nounagnon et *al*., 2018). To valorize these fats, the aim of the present studies was to incorporate SSDE fat in soap manufactory and in animal feed.

Materials and Methods

Chemicals and reagents

Carbonate (Na_2CO_3) and slaked lime $Ca(OH)_2$ of High quality were obtained on the local market. 40 methylester fatty acid components and all reagents were obtained from Sigma-Aldrich (Steinhein, Germany). Absolute ethanol obtained from the labotec laboratory (Brussels, Belgium). All chemical products and reagents are of high quality and analytic grade.

Animal material

SSDE Fats purified and stabilized in our laboratory (Nounagnon et *al*., 2018) were conserved in the freezer at 4 ± 1°C for future uses.

Brine shrimp (*Artemia salina* Leach) larvae obtained from eggs incubated for 48 hours in seawater.

Two groups (A and B) of 5 rabbits aged of six (6) weeks each

Analytical method

Determination of fatty acid profile of SSDE sample by GC-FID

Fatty acid profiles of SDDE fat were obtained by gas-liquid chromatography of the fatty acid methyl ester derivatives. Fatty acids from the oil extract were methylated in a solution of KOH in methanol (0.1 mol/L) at 70°C for 60 min, then in a solution of HCl in methanol (1.2 mol/L) at 70°C for 20 min, and finally extracted with hexane. Fatty acid methyl esters (FAMEs) were separated and quantified with a gas-liquid chromatograph (GC Trace ThermoQuest, Milan, Italy) equipped with a flame ionization detector, an automatic injector and a fused silica capillary column (100m x 0.25 mm internal diameter) coated with a 0.2 mm film of biscyanopropyl-polysiloxane (Rt-2560, Restek, Bellefonte, PA, USA). The system used H_2 as the carrier gas and operated at a constant pressure of 200 kPa. Splitless injection mode was used minimizing the risk of discrimination between FAs with very different volatilities. The initial oven temperature was 80°C; it increased at 25°C/min to 175°C (held for 25 min), then increased at 10°C/min to 205°C (held for 4 min), then increased at 10°C/ min to 225°C (held for 20min) and finally decreased at 20°C/ min to 80°C. The temperature of the flame ionization detector was maintained at 255°C. Hydrogen flow to the detector was 35 mL/min and air flow was 350 mL/min. A calibration mixture of fatty acid standards was processed in parallel. The data were analyzed by using the Chromquest 3.0 software. Each peak was identified and quantified by comparison of retention times with pure FAME standards. Fatty acids were expressed as the percent of total fatty acids quantified within an individual sample. A total of forty pure FAME standards were used as reported in previous work (Nounagnon et *al*., 2018).

Determination of the toxicity of SSDE fat

The larval toxicity test of *Artemia salina* Leach, a preliminary non-clinical toxicity test proposed by (Michael et al., 1956) and later developed by (Vanhaecke et al., 1981), was used in our work.

This test was used to predict the cytotoxic activity of purified SSDE fat as there was a correlation between shrimp larval toxicity and human cell cytotoxicity; notably the pulmonary cells on the one hand and the colon cells on the other hand (San José et al., 2002).

Incubation of brine shrimp eggs and larvae obtaining.

The eggs of *Artemia salina* (10 mg) were incubated at laboratory temperature (27 ± 1 °C) in 100 ml Erlenmeyer containing seawater. The whole was

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gently shaken for 48 hours. Meanwhile, the eggs hatch and give young shrimp larvae.

Preparation of SSDE fat solutions

The stock solutions of aqueous SSDE fat were prepared in the mixture (1/1) seawater / acetone at the concentration of 25 mg / mL. In ten (10) test tubes, a range of decreasing concentrations of fats were realized by performing a dilution, in a geometric series of reason 2, of each stock solution. In the same way, two witnesses (seawater / acetone mixture and seawater alone) were realized in parallel.

Reading and counting dead larvae

After 24 hours, the test tubes are examined. The number of larvae survivors in each tube was counted and the number of dead larvae was recorded. The larvae do not receive food. To ensure that death observed in the trials was attributed solely to SSDE fat and not to hunger, the tubes are compared to control tubes containing larval solutions only. *Artemia* shrimp larvae can survive for up to 48 h without food (Michael et al., 1956) because they feed on their yolk sac (Migliore et al., 1997).

Realization of the CL_{50} of the SSDE fat

In the test tubes containing a range of decreasing concentrations of SSDE fat (65 mg / mL in the first tube and 125 10^{-3} mg / mL in the last one) we seed sixteen (16) larvae contained in 1 mL of seawater (Table 1). The whole was incubated for 24 hours at laboratory temperature 27 ± 1 °C.

Table 1 Fat concentrations tested in the toxicity test

Fat	Concentration in mg / mL									
SSDE	65	32,5	16,25	8,12	4,06	2,03	1,01	0,5	0,25	0,125

SSDE = *Sus scrofa domesticus Erxleben*

Determination of CL_{50}

The CL_{50}, concentration at which 50% of the larvae died, was determined from the graph showing the number of dead larvae as a function of the different concentrations of prepared fats (Latha et al., 2007). This value was obtained from the polynomial adjustment curve of degree 2.

Soap manufacture from stabilized SSDE fat (Caubergs, 2000)

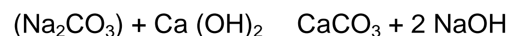
The cold process was used in soap making.

Soap preparation

The soap was made mainly from two main materials: SDDE fat and lye of alkali. We used as alkali caustic soda: NaOH. Caustic soda was the most used detergent and gives by reaction with the fatty substances a "hard" soap. In order to use local materials for the manufacture of the soap, we prepared the soda from local products using the technology developed by the Technology Consultancy Center of Kumasi University of Science and Technology (UST).

Production of caustic soda

The carbonate (Na_2CO_3) and the slaked lime $Ca(OH)_2$ were used in this preparation, according to the chemical reaction:



$$(Na_2CO_3) + Ca(OH)_2 \quad CaCO_3 + 2\,NaOH$$

In one iron bole, a layer of lime was put on, which was extinguished with water. Above this layer we put a layer of soda ash. We repeat this operation as many times wanted, according to the proportion 5:6 for example. The layers were put under water and let rest for 16 to 24 hours. The formed alkali was collected from the bottom. We can still add water and let rest as many times. However in each occasion, the strength of the collected alkali diminishes.

Preparation of lye solution:

The concentration of the lye was measured with the Baumemeter (° Be), and depends on the fatty substance and the process (cold, hot or semi-hot) of soap making, and varies from 10 to 40 ° Be. An approximate method to determine the concentration of the lye was to put an egg on it. If the egg floats on the surface, we have a solution of 24 to 25 °Be. Which means that one kilogram of lye solution contains:

- For caustic soda: 180 g of caustic soda (NaOH) + 820 g of water.

- For caustic potash: 230 g of caustic potash (KOH) + 770 g of water.

For oils other than nut oils (coconut, palm kernel), a concentration of lye of 23 to 25 °Be was required for the cold process.

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We want to transform 20 g of SSDE fat by using the cold process.

According to the saponification value of SSDE fat (191.5 mg KOH/g) 137g of NaOH were needed to transform 1 kg of SSDE fats.

137 g of NaOH for 1 kg of fat
X g of NaOH for 20 g of fat
X = (137 * 20) / 1000 = 2.74 g of NaOH

Calculation of the amount of lye solution

Since we want to use the cold process for the manufacture of soap, it requires a concentration of lye of 23 to 25 °Be.

1 kg of 23 °Be of lye solution contains 167.7 g of pure caustic soda.

167.7 g of pure caustic soda for 1000 g of lye

2.74 g of pure caustic soda for Q g of lye

Q = (2.74 * 1000) / 167.7 = 16.339 g of lye

We used the cold process for the preparation of the soap. To 20 g of SSDE fat at 40 °C, the amount of the needed lye (16.339 g) was gradually added while keeping the mixture stirring for the soap obtaining.

Characterization of the obtained soap

The obtained soap was analyzed using Beninese Standard (CEBENOR, 2002) which aims to specify the quality criteria of toilet soaps (used for cleaning the body), of the household (used for domestic and industrial cleaning) and Marseilles soaps (used for body care and for the household). These qualities are summarized in Table 2.

Determination of water and volatile matter

In general, two methods of assay were used: the azeotropic method and the parboiling method. Due to the conditions of soap preparation, it was the parboiling method which allows the removal of these materials by heating at 100 - 105 °C.

Operating mode

10 g of grated soap were weighed (P_1) in a porcelain dish and a stirrer previously tared (oven dried and cooled in a desiccator). The capsule and its contents were left in the oven. After 1 hour, stir the soap with the agitator. The capsule was returned to the oven for 1 hour longer. The capsule was removed and cooled in a desiccator and weighed. The operation was repeated until the mass difference between two successive weightings made at 1 hour interval was constant. Let P_2 be the last weighing. The content of water and volatile matter was given as a percentage of mass, by the formula:

$$\% \text{ Humidity} = \frac{P_1 - P_2}{P_1 - P} \times 100$$

P = weight capsule + stirrer
P_1 = weight capsule + stirrer + soap before parboiling
P_2 = weight capsule + stirrer + soap after parboiling

Determination of free alkali content

It was the ethanol method, which was based on the dissolution of soap in neutralized ethanol and titration of free caustic alkali with ethanolic solution of hydrochloric acid (HCl).

Operating mode

10 g of soap small pieces were weighed into a calibrated 250 mL beaker. 100 mL of 80% ethyl alcohol was added. After complete dissolution of the soap, the mixture was gently boiled for 5 minutes. Then the mixture was allowed to cool to room temperature to 70 °C.

Add a few drops of phenolphthalein and titration with 0.1 N HCl until the pink color disappears.

The content of free alkali is given by the formula:

$$\% \text{ NaOH} = \frac{V \times N \times 0{,}04}{P} \times 100$$

V = volume of the used HCl (mL)
P = test portion (g)

Determination of total fatty acid content

The purpose of this manipulation was to measure the in water insoluble fat which was collected by decomposing a soap with a strong mineral acid.

Operating mode

10 g of grated soap were taken from a 250 ml calibrated beaker. Let M be the mass. After adding 100 ml of distilled H_2O, the mixture was heated to complete dissolution. The mixture was transferred to a separating funnel and then 10 mL of 20% sulfuric acid (H_2SO_4) were added. The mixture is stirred and then allowed to stand. The acidic water is then removed, which is washed three times in succession with 50 ml of ether. The ethereal phases were reunifed. They were then washed twice with 50 ml of 10% saline solution. The ether solution is filtered in a calibrated

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flask, P. The filtrat is washed several times with ether. The ether solution is completely distilled and the residue is dissolved in 20 ml of ethyl alcohol which will be neutralized with alcoholic sodium hydroxide in the presence of phenolphthalein. The alcohol is then distilled and the soap dried in an oven at 120 °C until a constant weight P_1 (tared soap) is obtained.

The total fatty acid content is given by the formula:

$$\% AG = \frac{(P_1 - P) - 0,022\ V}{M} \times 100$$

V = added sodium hydroxide volume (mL)
P = weight of the empty vial
M = mass of the test portion (g)
P_1 = weight of vial and dry soap

NB: 0,022 = coefficient resulting from the fact that 1000 cm^3 of sodium hydroxide N neutralizes the fatty acid molecule to give 1 molecule gram of soap weighing 22 g more than the acid molecule.

Incorporation of stabilized SSDE fat into rabbits' diets

The rabbits were fed on the agropastoral farm "source of diamond" located at Sakété with an animal feed using palm kernel cake, residue of the manual extraction of the palm kernel oil, as source of lipid. For the research, feed was produced by incorporating an equivalent amount of pig fat instead of palm kernel. Two groups A and B of 5 rabbits of 6 weeks old were formed. The duration of the experiment was 8 weeks.

Usual feeding of group A rabbits (group of 5 control rabbits)

In group A, the usual feed was administered, corn mixture, palm kernel cake, rice bran, wheat bran, soybean, pulp concentrate, shell, salt.

Modified feeding and administration to group B rabbits (group of 5 experimental rabbits)

In group B, the modified feed was administered. It was the mixture of group A in which palm kernel cakes are replaced by an equivalent quantity of pig fat.

The average weight of the subjects was recorded once a week.

Statistical analysis

Student's t-test was used to test the significance of differences between results obtained for different samples, and between results for samples and controls (GraphPad Prism 4.0; GraphPad Software Inc., San Diego, USA). Statistical significance was set at $P < 0.05$ (SAS/STAT, 1990; Steel and Torrie, 1980).

Results and Discussion

Fatty acids composition of SSDE fat

Concerning the purified SSDE fat, UFA account for 51.43% of total fatty acids and this value was slightly higher than that of SFA (48.57%). The high percentage of UFA can explain in part the pasty and solid appearance of this fat at room temperature (27 ± 1°C) and at 4 ± 1°C respectively, as showed in recent work (Nounagnon et *al*., 2018).The probability of an oxidative polymerization (of SSDE fat) was high when highly heated during frying (Greenfield et al., 2007).Oleic (34.37%), stearic (21.36%) and palmitic (24.59%) acids were the major components (Table 2). Linoleic acid percentage (10.54) in this fat was closed to that obtained by Iverson (11.2 %) (Iverson et al., 1965) but the oleic acid percentage was less (43.5) and that of stearic acid was higher (13.0 %) (Iverson et al., 1965).These differences could be explained by the variation in the animal feeds composition and in the animal races (ConseilSupérieur de la Santé, 2011). The ratio SFA and UFA in SSDE fat (0.94) was lower than the recommended value (1) of dieticians (Michael et al., 1956). Among MUFA, palmitoleic acid percentage (1.11) was higher than those generally obtained in vegetal oil and fat (< 1 %) (Migliore et al., 1997).The 6 / 3 ratio of 7.23 was twice the recommended value (4) (Büchler, 2013).However, the 3 (1.56) percentage in this fat was very interesting. The trans-unsaturated fatty acids percentage (TUFA) 0.71% confirm that the studied SSDE fat was from animal origin.

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Table 2 Fatty acids composition of SSDE fat expressed in g/100g of purified fat.

Fatty acids	% TFA		RDI
Caproicacid (C6:0)	0.10	± 0.01	
Lauricacid (C12:0)	0.21	± 0.01	
Myristicacid (C14:0)	1.45	± 0.00	
Palmiticacid (C16:0)	24.59	± 0.01	
Palmitoleicacid (C16:1, c9)	1.11	± 0.00	
Margaricacid (C17:0)	0.45	± 0.00	
Stearicacid (C18:0)	21.36	± 0.05	
Elaidicacid (C18:1, t9)	**0.10**	**± 0.00**	
TransVaccenicacid (C18:1, t11)	**0.61**	**± 0.01**	
Oleicacid (C18:1, c9)	34.37	± 0.00	
Vaccenicacid (C18:1, c11)	**1.47**	**± 0.00**	
Linoleicacid (C18:2, c9c12)	10.54	± 0.02	11 - 17 g
Arachidicacid (C20:0)	0.41	± 0.00	
Gadoleicacid (C20:1, c11)	0.93	± 0.00	
Linolenicacid (C18:3, c9c12c15)	0.50	± 0.01	1.1 – 1.6 g
Eicosadienacid (C20:2, c11c14)	0.40	± 0.01	
Arachidonicacid (C20:4, c5c8c11c14)	0.34	± 0.00	
Docosapentaenoicacid (C22:5, c7c10c13c16c19)	0.34	± 0.00	
Docosahexaenoic acid : DHA (C22:6, c4c7c10c13c16c19)	0.72	± 0.08	0.5 g
6 / 3	7.23		
Total transfattyacid	0.71		
Total cis acid	50.10		
MUFA / PUFA	3.01		
SFA / UFA	0.94		

SSDE:*Sus scrofa domesticus Erxleben*; RDI: recommended daily intake; TFA: total fatty acids; MUFA: mono unsaturated fatty acid; PUFA: poly unsaturated fatty acid; SFA: saturated fatty acid; UFA: unsaturated fatty acid

The values of the total fatty acids (SFA, MUFA, and PUFA), Linoleic and Linolenic acids were compared to those of the reference (CODEX STAN 211, 1999) and other studies for SSDE (Table 4) and showed some difference between values. These differences show that the fatty acids profiles of the animals depended on their origin and consequently of their nutrition.

Toxicity of SSDE fat on the larvae of *Artemiasalina* Leach

The results of the larval test of toxicity of the fat of SSDE were summarized in the table 6. A meticulous reading of the curve of mortality according to the concentration of fat makes it possible to say that the mortality of the larvae respects a relation of amount-answer owing to the fact that the number of dead larvae increases with the concentration (Figure 1). The concentration which causes the death of half of the 16 introduced larvae (CL_{50}) corresponds to the value of 63765.25µg / mL (Table 3). This value largely higher than that of the campthotecine (13 µg/mL), proves that the SSDE fat did not have toxicity against shrimp larvae. It was shown a correlation between the toxicity of the shrimp larvae and the cytotoxicity of the human cells; in particular pulmonary cells (A-459) and cells of the colonist (HT-29) (San José et al., 2002) and the purified fat could not be toxic on these cells.

Table 3 Toxicity of SSDE fat on the larvae of *Artemia salina* Leach

Test solution	Toxicity (CL_{50}, ~g / mL)
	Artemia salina L
SSDE fat	63765,25 ± 0,02

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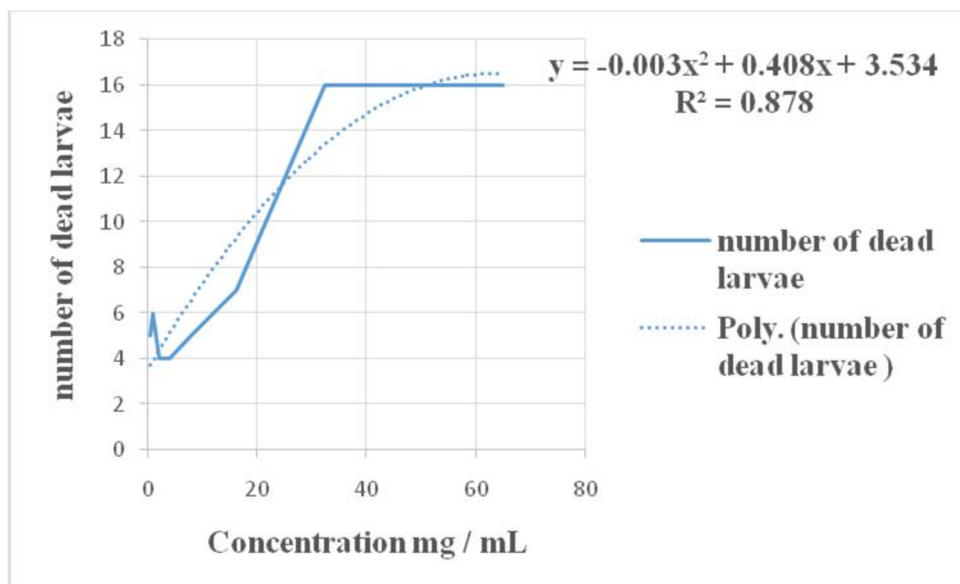

Figure 1 Variation of larval mortality according to the concentration of the SSDE fat

Manufacture of soap with the stabilized fat of SSDE

The used method had given a hard soap, white-gray with a pleasant odor, who was soft with the touch. The characteristics of the obtained soap were determined. So, it'smaximum alkalinity was 0.18 %. This value was close to that of Marseille soap (0.20), but higher than that of toilette soap (0.15) and lower than that of household soap (0.30). The percentage in the soap of total fatty acids (TFA) of 65 was within the range of 60 and 70 TFA of household soap and Marseille soap respectively. But this value was very lower than that (75% TFA) of toilette soap. The produced soap had 19 % of maximum humidity. This value was close to that of Marseille soap (20%), lower than that of household soap (25%) and higher than the value of 14% obtained for toilette soap. By comparing the determined values with those of the Standard Béninoise(CEBENOR, 2002), the soap manufactured starting from the solution of lye of 23 °Be was of Marseilles type (Table 4). This soap could be useful in several uses as to take a bath, to wash clothes and dishes ….

Table 4 Soaps quality

Parameter	standard values			Obtained values
	Sa. t	Sa. m	Sa. M	Sa. 100% SSDE fat
Maximum humidity (%)	14	25	20	19
Minimum TFA (%)	75	60	70	65
Maximum alkalinity (%)	0,15	0,30	0,20	0,18
Odor	NR	NR	NR	pleasant
Color	A	A	A	white-gray

Sa. t = toilet soap ; Sa. m = household soap ; Sa. M = Marseille type soap ; SSDE = *Sus scrofa domesticus Erxleben*

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Incorporation of stabilized SSDE fat into rabbits' diets

The stabilized fat was incorporated in the rabbit's nutrition.At the beginning of this experiment, the average weight of the subjects was about 700 grams.After two weeks, the average weight of the subjects of the group B increased. Between the second and the fourth week the increase was fast to reach the value of 1080 g (Table 5). Fromthe fifth to the eighth week, this average weight showed a slightlyincrease. Compared to that of subjects in the control group (A), the average weight of the group B was higher (Figure 2). After eight (8) weeks, the average weight of the group B reach the value of 1180 g and that of group A, in the same time, reach 1080 g. This value of 1080 g was obtained after four (4) weeks, with the rabbits of group B. The incorporation of SSDE fat inrabbits nutrition can help to save time, feed and money in their husbandry.To corroborate these observations, rabbits of each group were sacrificed. The photographs (Figure 3) showed that the rabbits of the group B accumulated in their bodies more fats than the rabbits of group of control(A).

Table 5 Subjects average rabbits weight

Subjects	Average weight (g)	
	Group A	**Group B**
Week 0	700	700
1st week	720	775
2nd week	760	798, 3
3rd week	810,5	845
4th week	870	1080
5th week	900,5	1090
6th week	940,25	1105,4
7th week	1005	1150,6
8th week	1080,7	1180,8

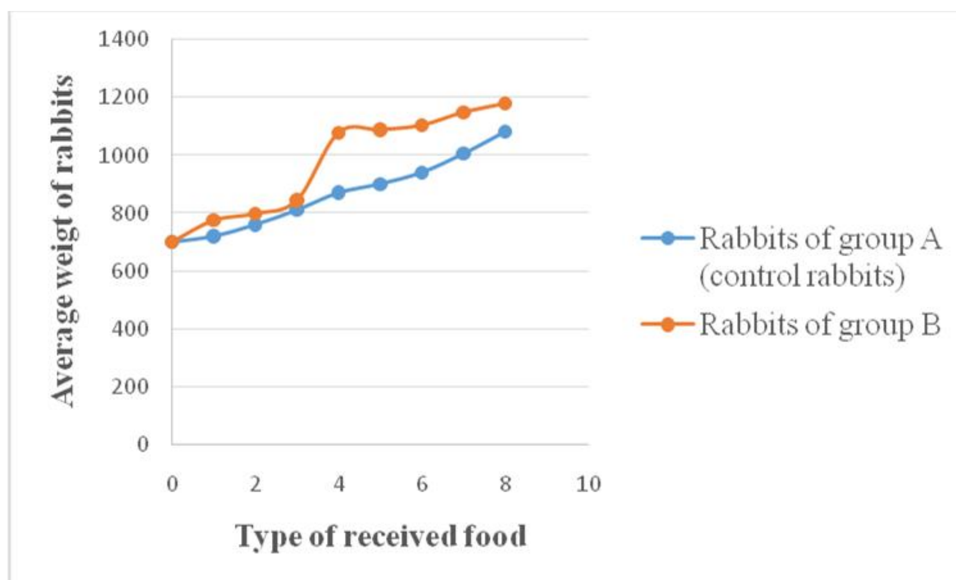

Figure 2 Curve of variation of the average weight of rabbits according to the type of received food.

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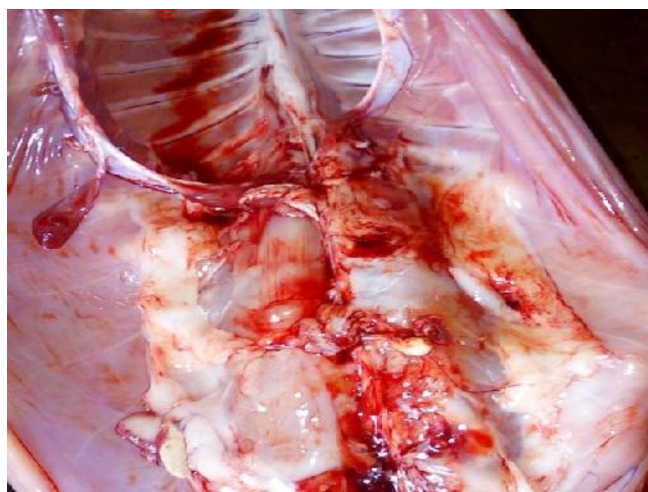

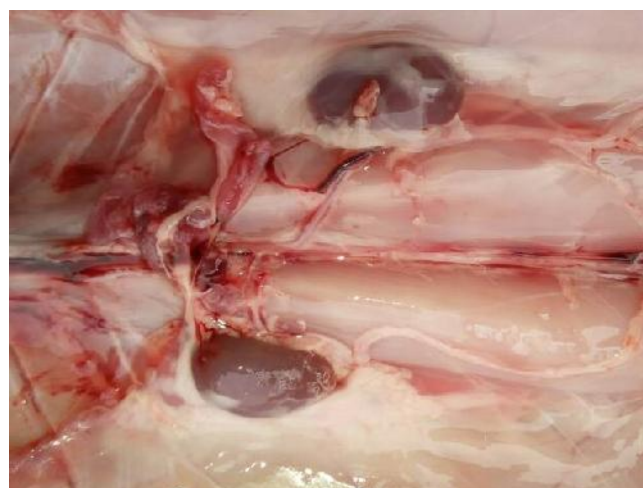

Group A **Group B**

Figure 3 Photos of the bodies of the rabbits of group A and group B.

Conclusion

SSDE purified fat showed a quantity of SFA, MUFA and PUFA in accordance with the standard values. The study of larval toxicity showed a $CL_{50}$of 63765.25µg/mL very higher than that of campthothecin (13 µg / mL), proving the non-toxicity of this fat against this larva and therefore against human cells as pulmonary and colonist cells. The fat was then use for the manufacture of soap and gave a type Marseille soap from the laundry solution of 23 °Be. Into the diet of rabbits, the stabilized fat gave in four (4) weeks the result obtained after eight (8) weeks with traditional nutrition. The stabilized fat could then be used in several industrial sectors.

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Conflict of Interest

The authors declare no conflict of interest

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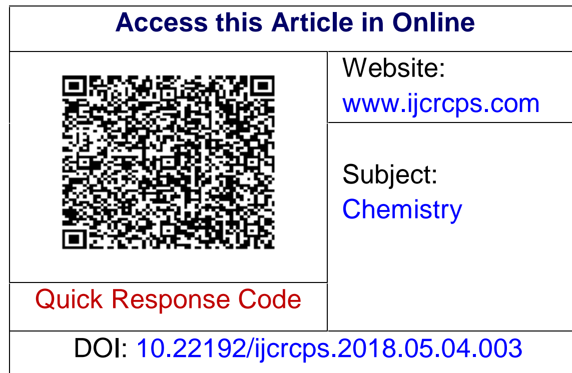

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