ABSTRACT

Key words: **squalene, *Amaranthus sp.*, supercritical extraction, short path distilation**

Research done on the topic ***Biologic active principles obtaining from vegetal sources*** aimed to obtain a squalene concentrate from *Amaranthus sp*. Seeds.

The paper is structured in two parts: Critical review of the literature in this field (3 chapters, 63 page) and Experimental work (5 chapters, 167 page). The thesis comprises also 4 annexes and a reference list totaling 341 titles.

The importance of the chosen subject, the purpose ant the objectives of the thesis are summarized in the chapter **Introduction** (3 page).

First Chapter presents a brief history of the research related to squalene discovery.

Chapter 2 (29 page, 14 tables) review the scientific literature regarding the natural sources of squalene. The traditional source, having the biggest squalene concentration (>80%) is the shark liver oil. The concerns regarding the preservation of marine life represent the first reason for identification of other sources for squalene. Another reason for searching alternative vegetal and/or microbial squalene sources is the confirmed presence of POPs, heavy metals compounds in the organism of different marine species.

Although squalene was identified in many plants, the concentrations (mg /100g) are too small to permit an effective isolation thereof. At this moment can be considered only three major vegetal sources for squalene, suitable to be commercial exploited: *Amaranthus sp.* seeds oil, olive oil deodorizer distillate and palm oil deodorizer distillate.

It is expected that the microbial squalene obtained by biosynthesis or semisynthesis to be a promising commercial alternative in the next future.

In Chapter 3 (30 page, 7 tables) there are presented the main methods for squalene analysis, as well as methods for separation, concentration and purification of this compound from the industrial relevant sources. At the end of the chapter there are summarized the most important applications of squalene in food, cosmetic and pharmaceutical industries.

Squalene emollient and moisturizing properties, as well as its biocompatibility with skin qualify squalene as an important component in cosmetic formulations (moisturizing creams, anti-ageing products). Squalene is considered as one of the best natural emollient, being well and efficiently absorbed by skin, acting in restoring the natural suppleness and flexibility of this, without any oily residues.

Naziri et al (2011) have considered that squalene has the ability to control the colonization of pathogen microbes. This property is the reason for squalene utilization in the treatment of fungal and bacterial skin infections.

One of the most important applications of squalene in pharmaceutical consisits in its utilization in the manufacture of stable emulsions, used as adjuvants in vaccine administration, for the stimulation of the immune response of the patient to the vaccine. Although there were concerns about the squalene role in the induction or rise of adverse reactions, it was demonstrated that squalene administration did not produce antibodies anti-squalene. The data published by World Health Organization (WHO, Global Advisory Committee Report on Vaccine Safety, 2008) showed that squalene was present in over 22 millions influenza vaccines distributed in all Europe since 1997 and no adverse effects were observed since then.

The experimental part of the thesis begins with the presentation of the methods used in research experiments – **Chapter 4** (20 page). In this chapter are presented the standard methods (ISO, AOAC, AOCS) used for the obtaining of the experimental data.

Chapter 5: **Obtaining and characterization of *Amaranthus sp.* seeds** (18 page, 8 tables, 18 fig.) comprised the description of the biological material, data regarding the 2012 year production for the 11 *Amaranthus sp*. varieties, seed, oil and protein production. In 2012 the average production for the eleven variants was 1861 kg/ha. The best seed production was obtained by *Amaranthus cruentus* - MT3 variety, namely 2236 kg/ha. In order to perform the chemical composition analysis, the seeds were ground. It was studied the influence of the grinding duration on the grain size distribution (determined by analytical sieves screening) for the resulted grains. In the case of a normal log distribution of particles diameters (validated by experimental data analysis), the average particle diameter varied between 660 μm (S_g=269 μm) and 396 μm (S_g= 187 μm).

For the tested varieties the seeds moisture varied between 10.9% and 13%, the protein content, determined by Kjeldahl method (transformation factor 5.85), was between 9.02 and 16.3%, the oil content from 3.69 to 5.88%. The protein protection had values between 166.44 and 333.91 kg/ha (average of 257.6 kg/ha) and the oil production was between 74.8 and 107.23 kg/ha (average value 93.69 kg/ha). Considering the results, the choice for the future experiments were the seeds of *Amaranthus cruentus* –Alegria variety, with good oil (5.78%) and protein (14.91%) concentrations and also a good seed production (1822 kg/ha in the conditions of year 2012).

In Chapter 6 (75 page, 32 table, 48 fig.) are presented the experimental results for the extraction and characterization of *Amaranthus cruentus* oil seeds.

Based on literature data and the results of preliminary tests were chosen two chromatographic analysis methods: a HPLC method and a GC one, adapted for the existent equipment and verified..

In order to determine squalene was adopted a reverse phase HPLC method, detection at 214 nm. It was used a stainless steel column 250×4.6 mm, stationary phase - Kromasil - C18 (octadecylsilane), 5μm. The mobile phase was methanol : isopropanol : acetic acid 920 : 80 : 0.5. The analysis was performed on a Merck – Lachrom chromatograph (HPLC). The retention time for squalene was 14.36 min. Linearity range was 0.063325 – 0.37995 mg/mL and the correlation coefficient 0.9934.

The simultaneous determination of squalene and fatty acids (as methyl esters) content was realized with a gas chromatograph 6890N – AGILENT, provided with a capillary column with stationary phase HP88 – (88% cyanopropyl + polysiloxane aryl), L=60 m, φ=0.25 mm, FID and autosampler 7683B. Working conditions were optimized to ensure a good separation of the main fatty acids and squalene existing in *Amaranthus* oil. Using a temperature programme in two stages: stage I 150-175^{o}C, stage II 175-220^{o}C, the total time for analysis was 45.5 min. Retention time for squalene was 39.63 min, the linearity range: 0.2344 – 4.6872 mg/mL, correlation coefficient 0.99888, quantification limit for squalene 0.015mg/mL.

Oil extraction from *Amaranthus* seeds was done with petroleum ether (SIGMA 24553), boiling point between 60–80^{o}C and the density 0.660-0.680 (20^{o}C). The extraction was performed in a laboratory Soxhlet extractor. The sample amount was 20-25 g. The moisture of the sample to be extracted was <10%.

If the extraction time is bigger or equal to 8h, the particles dimensions do not significantly influence the amount of oil extracted by Soxhlet method. The particle dimensions affect the extraction speed, being an important parameter for extraction times smaller or equal to 4h. The separation of the flour resulted from milling depending on grain size dimensions (<300 μm; 300-500 μm, > 500 μm) revealed the existence of greater concentrations of oil and protein in the fine fraction. The fraction with the average diameter <300 μm, representing about 34% of the total meal weight, had an oil concentration of 10.71% and a protein content of 20..5%, i.e. over 1.8 times more oil and 1.38 times more protein than the values obtained for the meal (unseparated by particle size).

The particles with dimensions <300 μm contain about 63% of all oil, while the coarse fraction contains only 9.3% of the total quantity of oil existing in seeds. Similar observation can be also made about the protein concentration: fine fraction contains about 47% of all protein contant while the coarse fraction, only 19% of protein. The separation by particle size does not significantly modify squalene concentration or fatty acids distribution.

A modern alternative for vegetal oils extraction is supercritical CO_2 extraction. This solvent has a minor impact on the environment, being non toxic and non flammable. Furthermore, it allows the obtaining of a ``green`` extract (without solvent traces). Supercritical CO_2 extraction of *Amaranthus* flour at 517 bar, 100^{o}C, 15 g/min CO_2 flow for 60 min, with 45 g CO_2/g seed yield to a concentration of 5.76±0.16 g oil/g seed (versus 5.86 g oil/g seed by petroleum ether extraction). The resulted oil has a squalene content of 5.03±0.083 g/100 g oil (versus 4.98 g squalene/g seed by petroleum ether extraction), acidity number 1.05±0.12 (%) and phosphor concentration of 31±13 ppm. From the data obtained it resulted that supercritical CO_2 yields to comparable values with those obtained at petroleum ether extraction for the oil content, squalene concentration and peroxide value. The acidity number and the phosphorus content are lower for the oil obtained by supercritical CO_2 extraction comparative to the values of the oil obtained by Soxhlet extraction.These characteristics demonstrate the oil obtained by supercritical extraction is higher quality than the oil obtained by the classical extraction.

As one of the most important factors influencing the oil SFE is the oil solubility in the solvent, knowing the pressure and temperature effects on the solubility and the exact determination of solubility values represents the first step for an efficient management of supercritical CO_2 extraction. The literature presents many experimental data for squalene solubility in supercritical CO_2, but there are great differences between experimental data due to the equipment and methods used for the determination of solubility. In the most of the cases, the solubility of organic substances in supercritical CO_2 is modeled with the Peng-Robinson equation, or by (semi)empirical equations based on the solvent density.

Modelling the influence of pressure and temperature on the squalene solubility in supercritical CO_2 with the model proposed by Charstil, for the entire pressure (p=100-400 bar) and temperature (T=308,15–373,15 K) ranges for which experimental data were available, yield to an *ARA* value of 56.55% (R^2=0,8347), much to higher as the model predictions could be used in simulation. After the data corresponding to the pressure of 100 bar were excluded, 91 values of solubility were retained. Charstil model parameters were optimized by linear regression so to obtain a total *ARA* value <25%.

Data for *Amaranthus* oil solubility in supercritical CO_2 were modeled by four solubility models: Charstil (1982), Adachi-Lu (1983), del Valle –Aguillera (1998), del Valle et al (2012). The best concordance between the experimental data and the theoretical predictions for the *Amaranthus* oil solubility was obtained with Adachi-Lu model.

The combination of Charstil model for squalene solubility and Adachi-Lu model for *Amaranthus* oil solubility yielded to the identification by mean of simulation of the variation range

for the most important parameters in *Amaranthus* oil and squalene extraction. Based on the results of simulation, we made extraction experiments at pressures between 200 and 400 bar, temperatures between 30-70^{o}C with CO_2 flow of 4.6 g/min, which validated the proposed models. The best conditions for the complete extraction of oil were: pressure 350 bar, time around 180 min, temperature 35^{o}C.

Amaranthus oil extraction kinetics was modeled with BIC model proposed by Sovova (1994). In order to identify the model parameters we worked at three values of CO_2 flow: 3.5; 4.5 and 6.0 g/min, pressure 300 bar and temperature 40^{o}C. There were calculated the model parameters, mass transfer coefficients in liquid phase ($k_f \cdot a_0 = 1.17\times10^{-3}$–$2.06\times10^{-3}s^{-1}$) and in solid phase ($k_s \cdot a_0$=$4{,}16\times10^{-5}$–$8{,}65\times10^{-5}s^{-1}$), a good agreement with literature data being obtained.

Chapter 7 (47 pag, 39 table, 27 fig) makes an analysis of the results of squalene separation by three process flow sheet variants at laboratory level. The first variant is based on the total extraction of *Amaranthus* flour with organic solvents, miscela concentration, oil degumming and neutralization. The oil was fractionated by short path distillation. The use of optimal parameters for each of the process steps allowed the obtaining of a squalene concentrate of 80-85 % with a separation yield of 80%. In the second variant the *Amaranthus* oil was subject to transesterification with methanol/sodium methoxide (yield of 92.24–95.1% for reaction + separation + purification). Fractionation of the organic phase by short path distillation yield to more poorly results than the first variant. The greatest squalene concentration (87.3%) was obtained in the third variant, by supercritical CO_2 extraction of the flour fraction with the average diameter < 300 μm and short path distillation at 240^{o}C, pressure (2,2–2,5) $\times10^{-3}$mbari, speed 500 rpm. As the fine fraction contains only 2/3 of all oil (and squalene) contained in the seeds, the total yield is much lower comparative with the first variant.

Chapter 8 (7 page) contains the thesis conclusions. The thesis also contains the presentation of the main own contributions and the papers published during the doctoral internship on the thesis subject.