Obtaining oleoresins from *Capsicum frutescens L.* by ultrasound-assisted extraction for the development of a food ingredient

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Summary

In this work, ultrasound-assisted extraction was employed to extract oleoresins in sunflower oil from Cayenne pepper powder. Time, amplitude, sunflower oil % and solid-liquid ratio were the factors investigated with respect to extraction yield of carotenoids and capsaicinoids using the follow statics designs: 2^{4-1} fractional factorial, complete factorial and a central composite design 2^2 + star with blocks. The optimum operating conditions were found to be: time, 5 min; amplitude, 40%; sunflower oil, 36 % and solid/liquid ratio, 1:6.8 for both total carotenoids content (TcrC) and total capsaicinoids content (TcpC). The predicted value for extraction yield for TCrC and TcpC were 11.6171 mg/mL and 153,969 area/volume respectively. The effect of high power ultrasound treatment on lipid fraction of samples: untreated oil (UO), sonicated oil (SO), oil-ethanol sonicated (OES) and the oleoresin extract (OlS) were studied by multivariate analysis PCA. Lipid sono-oxidation on SO, OES and OlS samples and a high content of free fatty acids in the OlS sample were detected.

1. Introduction

Eating habits have changed, consumers are looking for increasingly healthy diets and they demand higher quality food, more durable and sustainable production. Which it has resulted in a more demanding and dynamic market, a new challenge for food industries and for regulatory agencies. The norms and standards that regulate the safety and toxicity of food products for human and animal consumption are a fundamental part of the control system in the production of the industries [1]. According to the World Health Organization (WHO) [2] although government units make a great effort to implement regulatory principles and practices in touch with industries, the incidence of foodborne diseases continues to be a public health problem that has been growing in both developed and developing countries. On the other hand, the International Harmonization Council (ICH) together with other agencies such as the European Food Safety Agency (EFSA) and the Food and Drug Administration (FDA) are responsible for promoting constructive dialogues between the regulatory agencies and industries (pharmaceutical, food) on the technical requirements for the registration of new ingredients. They are also responsible for facilitating the dissemination and communication of information at a global level, in order to promote different research approaches, develop substituting techniques to conventional ones and break down trade barriers within the same integrated quality and security system of the new products [3].

Solid-liquid extraction is a very common process in the pharmaceutical, cosmetic and food industries to obtain natural ingredients such as flavorings and colorants; their main objective is to obtain molecules from a complex matrix in a sustainable way where the target-compound yield is maximized with a minimum impact on its properties, while minimizing the extraction of undesirable compounds. Therefore, in recent years a major challenge in sustainable production industries is the technological innovation of their processes, there is undoubtedly a clear need to develop safe, effective and ecologically techniques, it is important that such

techniques not only have a clean label status, but also guarantee good yields and quality in the final products [4]. Due to the constraints of conventional solid-liquid extraction techniques which imply an extensive sample preparation time, the use of petrochemical solvents, high separation costs and a large amount of wasted solvent after the process is finished, implying low yields and a harmful impact for human health and the environment [5]; emerging techniques have been developed and improved such as supercritical fluid extraction, microwave assisted extraction and ultrasound assisted extraction (UAE), they are clean alternatives to alleviate the drawbacks of conventional technologies.

The UAE is considered a safe technique for the extraction of alimentary interest compounds, its principle is based on the cavitation phenomenon. The mechanical effect of ultrasound is to accelerate the release of bioactive components of the plant due to the breakdown of plant tissues and the intensification of heat and mass transfer during the process (Annex 1) [6]. This technology have been reported better extraction yields compared to traditional techniques, in addition, have used solvents such as: ethanol, water, glycerol, edible oils, which are less polluting and safer compared to toluene, benzene, hexane and methanol, main conventional solvents used in the extraction of target compounds [7].

The pepper oleoresins are compounds of gastronomic interest, they are a rich source of carotenoids and capsaicinoids, which are valued in industry as natural colorants and flavorings [8], their functional properties in human health have been studied (Annex 2). The extraction of pepper oleoresins is usually carried out by conventional techniques, however, in recent years have been developed and improved extraction techniques that favor the obtaining of carotenoids and capsaicinoids from red pepper [9] [10], [11]. One of the most used techniques for obtaining this type of compounds has been the supercritical fluids extraction which has reported considerable extraction yields [12], [13]. However, the great cost in the implementation of this methodology is a limitation in the scaling process, with the aim of improving the performance of this technique, the coupling of assistance ultrasound has been proposed: the study carried out by Santos et al. [10] achieved to increase the global extraction of capsaicinoids from *Capsicum frutescens L.* up to 77%. Different authors propose other methods such as the selective obtaining of compounds of interest through the extraction assisted by enzymes; the work developed by Santamaría et al. [14] achieved a purity of 80% and 73% of capsaicinoids and carotenoids respectively, using ethanol as a solvent. Likewise, the comparison of both Soxhlet extraction techniques with solvents (hexane, ethyl acetate, methanol and ethanol) and the extraction by UAE using methanol and ethanol was carried out for obtaining phenolic compounds and capsaicinoids from *Capsicum baccatum L*, where the yield of the global extraction increased on 26% for UAE using methanol as solvent [9]. The work carried out by Li et al. [15] compared the UAE of carotenoids from carrots using sunflower oil and hexane as solvents, the highest concentration of β-carotene was 334.75 mg / L and 321.35 mg/L at 20 and 60 minutes respectively. Accordingly, the utilization of UAE in food technology for extraction of value metabolites, is a promissory innovate technique that involve the principles of green chemistry and successful systematic scale-up [16].

In comparison with the works described above, there are no reports of oleoresin extraction from *Capsicum frutescens L.* by using the UAE technique using edible oil as a solvent, which are allowed by the ICH [17, p. 3] to obtain food grade products. In this work, an innovative, fast and green ultrasound-assisted extraction technique for Cayenne pepper oleoresin has been developed using sunflower oil- ethanol as some alternatives solvents.

2. Materials and methods

2.1. Materials

Commercial BADIA® Cayenne pepper powder (*Capsicum frutescens L.)* and refined sunflower oil were purchased from the local market (Medellín, Colombia) and both were stored at ambient temperature. All solvents used in the extraction procedures and sample treatments were of analytical grade purchased from Merck.

2.2. Solid pre-treatment of vegetable material

In order to select the appropriate working particle size, Cayenne pepper powder was sieved using standardized Tyler sieves with 40, 50, 60 and 200 mesh sizes. 500 g of material were transferred to the top of set of sieves, assembled and fixed in a Ro-Tap H-4325 (Humbold). The sieves were kept under constant shaking for 15 min to separate the fractions and the retained material on each sieve was weighed afterwards. This procedure was carried out three times.

2.3. Obtaining oleoresins by ultrasound assisted extraction (UAE)

The experiments were conducted using a laboratory scale ultrasonic liquid processor from Industrial Sonomechanics (ISM), with (1) a 500W ultrasonic generator with output frequency of 20 kHz, (2) a piezoelectric transducer, (3) an FBH-type Barbell horn (Annex 3) with an output tip diameter of 21 mm, and (4) a stainless steel flow-through reactor chamber (flow cell) with a water-cooling jacket attached to a cold-water recirculation system from a chiller (Polyscience). The experiments were carried out in single-pass flow-through mode, utilizing an adjustable-flow pump model 07516-00 (easy-load) at 900 mL/min. 150 ml of samples were processed in a beaker, which was placed in an ice bath, which prevented the temperature of the suspension going over 22 ◦C during the sonication period. The samples obtained from ultrasound were filtered under vacuum using a Buchner Funnel, the filter discs used for filtration were of 250 µm pore size (BOECO) and the ethyl alcohol of each sample was removed using a rotavapor R-215 (BUCHI labortechnik. AG). All samples were stored in amber flasks at 22 °C.

2.4. Determination of influential factors in the extraction of oleoresins

Different parameters may affect the efficiency of oleoresins extraction using UAE such as time, amplitude percentage, solvent composition and the solid-liquid ratio. In order to develop the UAE determination method for carotenoids and capsaicinoides present in oleoresins, it was necessary to consider and optimize these variables. A half FFD was initially carried out, a half fractional factorial design (FFD) was carried out as a screening design, followed by a complete factorial (CF) design and finally a surface response methodology (RSM) based on central composite design (CCD) as a multivariate statistic technique. All designs have two response variables: total carotenoid content (TCrC) and total capsaicinoid content (TCpc) and residual normality assumptions were checked, as well as the independence of the data and the equality of variance. Design generation and statistical analysis were performed using the software package STATGRAPHICS Centurion XVI.I for Windows (Rock Vill. MD, USA).

2.4.1. Screening factors design for UAE

2^{4-1} fractional factorial design was employed with three central points, which provided protection against curvature, resulting in a total of 11 experiments from which the most significant experimental variables were screened and their interaction with the extraction of oleoresins. Table 1 shows the design factors and their corresponding selected variation levels.

Table 1. Levels and independent variables of the extraction process for fractional design 2^{4-1}.

Independent variables	Code levels	
	Low	**High**
Time (min)	5	15

Amplitude (%)	30	70
Sunflower oil (%)	60	90
Solid-liquid ratio	1:20	1:40

2.4.2. Complete factorial design

To determine the conditions that allow reaching the area of optimization for the UAE of carotenoids and capsaicinoides present in oleoresins a 2^2 complete experimental factorial design was employed with two replicas. The factors selected according to the result of the FFD were: sunflower oil content and solid-liquid ratio. The fixed conditions in the extraction were time (5 minutes) and amplitude (40%). Table 2 shows the design factors and their corresponding selected variation levels.

Table 2. Levels and independent variables of the extraction process for complete factorial.

Independent variables	**Code levels**	
	Low	**High**
Sunflower oil (%)	40	50
Solid-liquid ratio	1:10	1:15

2.4.3. Design for Response surface methodology

A Response Surface Methodology (RSM) was used to determine the optimum conditions for the UAE of carotenoids and capsaicinoides present in oleoresin. A Central Composite Design (CCD) 2^2 + star with blocks was used to conduct a second-order mathematical model which adequately represents changes in the response, depending on the input variables. A total of 20 experiments were carried out. New ranges were selected based on the results of the complete factorial design. Table 3 shows these design factors and the new ranges selected.

Table 3. Levels and independent variables of the extraction process for central composite design: 2^2 + star.

Independent variables	**Code levels**	
	Low	**High**
Sunflower oil (%)	45	35
Solid-liquid ratio	1:7.5	1:12.5

2.4.4. Determination of total carotenoids compounds (TCrC)

The total carotenoid content was determined by a spectrophotometric method at 457 nm, using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments). The samples were filtered again using syringe nylon filters 0.45 µm (Teknokroma) and then they were diluted in cyclohexane. Routine and standardization method by the laboratory according to [18]. β-Carotene (Sigma Aldrich) was used as a standard and the results were expressed as mg of β-carotene equivalent per mL of oil extraction.

2.4.5. Determination of capsaicinoids compounds (TCpC)

The extraction of the capsaicinoids was carried out as follows: 100 mg of ultrasound extract was weighed and then extracted with 1 mL of acetonitrile for 15 minutes using an ultrasound bath P60H (Elmasonic®) and centrifuged at 13000 rpm using a microcentrifuge (Kendro®). This step was repeated two more times to fully

extract the capsaicinoids before the extracts were combined. Finally, the extract was analyzed by gas chromatographic- mass spectroscopy (GC-MS) using a gas chromatograph (Agilent 7890A) equipped with a mass selective detector (Agilent 5975C) and an automatic liquid sampler (PAL RSI 120) operating in EI mode at 70 eV; the column used was a HP-5MS (30 m x 0.25 mm x 0.25 μm). The oven temperature was programmed as follows: 1 min held at 90 °C, 90- 240 °C at 11 °C min-1, 240-310 °C at 3 °C min-1, and 3 min held at 310°C. The injector temperature was 290 °C in a splitless mode, the flow rate of the carrier gas (helium) was 1 mL min−1 and the injection volume of the solution was 1 μl. Mass spectra were analyzed by both scanning mode and selected ion monitoring (SIM) mode (Table 4). Raw MS data were processed using the software the Chemstation. Routine and standardization method by the laboratory by [19] with modifications.

Table 4. Retention time, mass fragmentation and SIM mode for pattern of capsaicinoids from (*Capsicum Frutescens L.*)

Compound	Rt (min)	m/z	SIM		
Nordihydrocapsaicin	19.10	293.00	137.00	150.90	293.10
Nonivamide	19.90	279.00	137.00	151.00	293.10
Capsaicin	20.10	305.00	137.00	152.00	305.10
Dihydrocapsaicin	20.45	307.00	137.00	151.10	307.20
Homodihydrocapsaicin I	22.10	321.00	137.00	150.90	194.70
Homodihydrocapsaicin II	22.39	321.00	137.00	150.90	194.70

2.5. Technological impact on sunflower oil chemistry

The effect of ultrasound on the integrity of fatty chains, double bond, the oxidative status and the esterified/free fatty acid fraction was examined in sunflower oil samples of untreated oil (UO), sonicated oil (SO), oil-ethanol sonicated (OES) and the oleoresin extract (OlS) at the optimum conditions were analyzed for determination of triglycerides, fatty acid methyl esters (FAME´S) and free fatty acids. Others physicochemical proprieties following INVIMA norms were measured such as: relative humidity NTC 287 [20], saponification matter NTC 335 [21], unsaponification index NTC 235-2 [22] and iodine index NTC 283 [23]. The peroxide index was measured using a PeroxySafe™ STD Kit.

2.5.1. Determination of triglycerides

The triglyceride content was determined by gas chromatography with a flame ionization detector (GC-FID) using a gas chromatograph (Agilent 7890A) equipped with flame ionization, the column used was a CP-TAP CB (25 m x 0.24 mm x 0.10 μm). The oven temperature was programmed as follows: 1 min held at 180 °C, 180-350°C at 8 °C min-1, 1 min hold at 350 °C, 350-360 °C at 2 °C min-1, and 4 min held at 360°C. The injector temperature was 340 °C in a splitless mode, the flow rate of the carrier gas (air) was 1.2 mL min−1 and the injection volume of solution was 1 μl. 5 mg of each sample (UO, SO, OES, OlS) were weighed, then diluted in 700μl of n-hexane and analyzed. Routine and standardization method by the laboratory.

2.5.2. Determination of FAME´S

The FAME´S content was determined by gas chromatography with a flame ionization detector (GC-FID) using a gas chromatograph (Agilent 7890A), equipped with flame ionization, the column used was a (CP-TAP CB (25 m x 0.24 mm x 0.10 μm). The oven temperature was programmed as follows: 4 min held at 45 ° C, 45-160 ° C at 15 ° C min-1, 1 min held at 160 ° C, 160-240 ° C at 4 ° C min-1, 1 min held at 240 ° C, and 240-

310°C at 10 ° C min-1. The injector temperature was 280° C in a splitless mode, the flow rate of the carrier gas (air) was 1.2 mL min-1 and the injection volume of solution was 1 µl. 5 mg of each sample (UO, SO, OES, OlS) were weighed, then 600 µl of H2SO4-methanol at 2 % was added and held at 60 °C for 2 hours in the sample concentrator (centriVap LABCONCO®); finally, 50 µl of saturated sodium chloride solution were added. The extraction of fatty acids is carried out three times using n-hexane as follows: (1) 500 µl, (2) 400 µl, (3) 400 µl. The extracted hexanes were mixed and analyzed. Routine and standardization method by the laboratory.

2.5.3. Determination of free fatty acids

The free fatty acid content was determined by gas chromatography- mass spectroscopy (GC-MS) using a gas chromatograph (Agilent 7890A), equipped with a mass selective detector (Agilent 5975C), the column used was an HP-5MS (30 m x 0.25 mm x 0.25 µm). The oven temperature was programmed as follows: 5 min held at 120 °C, 120- 180 °C at 7 °C, 5 min held at 180°C, 180-200 at 10°C min-1, 5 min held at 200°C, 200-300 °C at 10 °C min-1, 5 min held at 300°C, 300-310 °C at 10 °C min-1, and 5 min held at 310°C. The injector temperature was 290 °C in a splitless mode, the flow rate of the carrier gas (helium) was 1 mL min−1 and the injection volume of the solution was 1 µl. 5 mg of each sample (UO, SO, OES, OlS) were weighed and then 50 µl of BSTFA + TMCS 99:01 and 50 µl of pyridine were added. The samples were held at 70 °C for 2 hours into the sample concentrator and were analyzed. Routine and standardization method by the laboratory.

2.5.4. Principal component analysis (PCA)

A multivariate analysis PCA was carried out in order to obtain a global view of the effect produced by the ultrasound and the plant material in the final product. The results obtained (physicochemical proprieties, triglycerides, FAME´S and free fatty acids) were integrated into a data matrix which was used for obtained the principal component analysis (PCA) graphs. The statistical analysis was carried out in the R studies software, using the muma package.

3. Results and analysis

3.1. Obtaining oleoresins by ultrasound assisted extraction (UAE)

The UAE is considered an innovative technique, which complies with the six principles of green extraction [24]. This includes the use of renewable vegetable resources (peppers), the use of alternative solvents (sunflower oil, ethanol), saving time, energy and costs; the biorefining concept (co-products or by-products replace waste), simplifying of operations and control, and obtaining a non-denatured and biodegradable extract without contaminants (oil extract enriched with carotenoids and capsaicinoids). All aforementioned green-extraction elements characterized the UAE process used in this work. This type of process is also reported by other lab work, which obtained oily enriched extracts under the same principles of green chemistry, seeking environmental and consumer safety [15] [25] [26]. An extract of oleoresin in sunflower oil enriched with carotenoids and capsaicinoids was obtained. Using UAE to produce Cayenne pepper extracts, quantitative determination was done of carotenoids (Annex 4) and capsaicinoids identification (nordihydrocapsaicin, nonivamide, capsaicin, dihydrocapsaicin, homodihydrocapsaicin I, homodihydrocapsaicin II) (Annex 5) by spectrophotometry and GC-MS respectively. A mixture of oil-ethanol was chosen as the extraction solvent because of its food safety and high extraction efficiency. The linear regression equation for carotenoids in the curve calibration was $y = 0.008x + 0.0046$ with regression coefficient of 0.9986.

3.2. Determination of influential factors in the extraction of oleoresins

3.2.1. Screening factors for UAE

For carrying out the UAE process, a literature review was conducted to determine the state of art variables that can affect the extraction of compounds present in plant materials such as pepper powder. The variables established for the screening design were time (A), amplitude (B), sunflower oil percentage (C) and solid-liquid ratio (D). Other intrinsic characteristics that had to be considered to achieve the expected results in the process of ultrasound-assisted extraction were the matrix parameters. The screening of the plant material was carried out in three batches of 500g approximately in order to select the appropriate working particle size (Annex 6).

It was found that 70% of the material was retained in the 200-mesh screen and 30% in the 50-mesh, 60-mesh and collector screens. Smaller particle sizes have greater surface areas, and would result in greater contact between solid and liquid phases allowing better extraction; however, no specific particle size was selected. Instead a particle diameter range between 0.297mm ≥ 0.0740mm was chosen. Because particles can be significantly reduced in size by the effects of cavitation, the subsequent grinding process was eliminated since the presence of very fine particles could hinder the filtration process [4]. Other qualitative, microbiological and nutritive properties of the raw material influencing the quality of the obtained extract were reported by the supplier (Annex 7).

A fractional factorial 2^{4-1} design was carried out in order to determine the process variables that had an effect on the response variables. A design with 11 runs was used as is presented in (Table 5). The statistical analysis of the results was carried out by means of an analysis of variance (ANOVA) with a significance of 5% and the Pareto diagram was analyzed (Annex 8). The results show that two of the variables studied favored the greatest extraction of carotenoids and capsaicinoids in the oleoresins obtained. These two variables were: sunflower oil percentage (%) and solid-liquid ratio.

Table 5. FFD design for the total amount of carotenoids and capsaicinoids of the pepper oleoresin.

	Factors				**Response variable**	
Run	**Time (min)**	**Amplitude (%)**	**% Sunflower oil (%)**	**Solid-liquid ratio**	**TCrC (mg/mL)**	**TCpC Ln(area/extraction volume)**
1	10	50	75	1:30	0.4794 ± 0.0026	8.4326
2	5	30	60	1:20	0.8913 ± 0.0046	9.6834
3	15	30	60	1:40	0.4086 ± 0.0038	8.8701
4	5	30	90	1:40	0.2297 ± 0.0017	7.9735
5	15	30	90	1:20	0.5251 ± 0.0032	8.6218
6	15	70	60	1:20	0.9828 ± 0.0029	9.7335
7	10	50	75	1:30	0.4877 ± 0.0015	8.6420
8	5	70	90	1:20	0.5543 ± 0.0010	8.8296
9	5	70	60	1:40	0.4253 ± 0.0023	8.9542
10	10	50	75	1:30	0.4710 ± 0.0040	8.6643
11	15	70	90	1:40	0.2130 ± 0.0015	7.9994
p-value carotenoids	0.8120	0.3464	**0.0002**	**0.0000**	Analysis using a significance value $\alpha = 0.05$	
p-value capsaicinoids	0.8644	0.6378	**0.0008**	**0.0022**		

*The data for TCrC are the averages of the 3 replicates. The TCpC variable was transformed with natural logarithm for statistic analysis.

It is observed that for both TCrC and TCpC the variables with the greatest significance are sunflower oil % and the solid-liquid ratio. The type, quantity and concentration of solvent and solid-liquid ratio have a prominent effect on the extraction efficiency as shown by the FFD. The effectiveness of the UAE depends on the solvent's capacity for absorbing and transmitting the energy of the ultrasound. The mechanical and cavitation effects in the extraction process cause a cellular disruption of the plant material, facilitating the penetration of the solvent into the powder, allowing to wash out extracts from the matrix until reaching the saturation point of the solution, so that by decreasing the solid-liquid ratio facilitates access of the solvent to the matrix [27]. The selected solvents in the UAE were sunflower oil and ethanol. This choice was based on the solubility of the target metabolites, but also on physical parameters that affect the phenomenon of acoustic cavitation such as viscosity, surface tension and the solvent´s vapor pressure [28]. Sunflower oil is the main extractive solvent due to the affinity it has with metabolites, mainly with carotenoids; capsaicinoids have a greater affinity for ethanol [29] [30]. By decreasing the percentage of sunflower oil in the sample, the amount of ethanol is higher, favoring the extraction of carotenoids and capsaicinoids. This resulted because of an increase of the intensity of the ultrasonic cavitation in the ethanol mixture in the presence of sunflower oil due to the increase of surface tension and the decrease of viscosity.

According to the previous results, the factors that were selected for the next step of optimization, the complete factorial design, were sunflower oil % and the solid-liquid ratio. It was found that the TCrC and TCpC values increased with a longer time and amplitude; however, as they are variables that do not have a significant effect on the UAE. They were fixed at the shortest time (5 min) to impact energy consumption and at 40 % amplitude to prevent the wearing out of the horn tip at higher amplitudes.

3.2.2. Complete factorial

Once the significant factors and the new concentration ranges were determined for the evaluation of the total content of carotenoids and capsaicinoids in the obtained oleoresin, a complete factorial design was carried out changing the ranges of sunflower oil % and the solid-liquid ratio. The corresponding ANOVA table was constructed using a significance of 5% and the table of simple effects and interactions as well as the assumptions for the analysis of variance performed were verified. The results of the complete factorial experimental design 22 are presented in (Table 6). It is observed that the content of carotenoids was between 4.4607 and 9.3707 mg/mL and the amount of total capsaicinoids represented per area was between 24142.2501 and 71886.6839 area/extraction volume. The maximum values of TCrC and TCpC were obtained by using a sunflower oil % of 40 and a solid-liquid ratio of 1:10 as shown in the interaction graphs (Annex 9).

Table 6. Complete factorial design for the total amount of carotenoids and capsaicinoids of the pepper oleoresin

Factors			Response variable	
Run	**% Sunflower oil**	**Solid-liquid ratio**	**TCrC (mg/mL)**	**TCpC (area/extraction volume)**
1	40	1:15	5.9587 ± 0.00416	48553.0525
1	50	1:10	9.2459 ± 0.01967	40283.5012
1	40	1:10	9.3707 ± 0.01572	71886.6839
1	50	1:15	4.7519 ± 0.02179	25210.7062
2	40	1:15	6.0003 ± 0.00100	39001.4915
2	50	1:10	8.5801 ± 0.01012	44596.4005
2	40	1:10	8.1640 ± 0.00709	70447.9213
2	50	1:15	4.4607 ± 0.00404	24142.2501
3	40	1:15	5.8754 ± 0.00200	38339.0656
3	50	1:10	8.8298 ± 0.00351	39498.2534
3	40	1:10	9.2459 ± 0.00794	69475.4710

3	50	1:15	5.5842 ± 0.01250	25400.8134

*The data for TCrC are the averages of the 3 replicates.

From the ANOVA (Table 7) it is observed that the two factors studied have significant effect for the total capsaicinoids and for total carotenoids only the solid-liquid ratio factor. Both the solid-liquid ratio and sunflower oil % have a negative effect in variables response as show in the Pareto diagrams (Annex 9) and in the model's equations (Table 7). This implies that the ratio of these variables should be reduced to optimize the variables.

Table 7. ANOVA analysis for complete factorial design of the content of carotenoids and capsaicinoids present in the oleoresin of pepper.

	Response variable	
Factors	**TCrC (mg/mL)**	**TCpC (area/extraction volume)**
	P-value	**P-value**
A: %Sunflower oil	0.0630	**0.0000**
B: Solid-liquid ratio	**0.0000**	**0.0000**
AB	0,0807	**0,0174**
Model equation	TCrC = 7.2902 + 0.190022*A + 0.180313*B – 0.0194183*A*B	TCpC = 341236. – 5333.86*A – 15405.6*B + 241.947*A*B
R^2	97.5549	98.1293

* Bold values indicate factors with significant effect (P-value <0.05) on the response variable

The new evaluation ranges for the optimization of TCrC and TCpC extraction were determined using lower levels of sunflower oil % and of the solid-liquid ratio. Although, the sunflower oil % had no significant effect on the response variable of TCrC, it would be possible to perform a reduction of the level without affecting the results of the response variable since the sunflower oil has not yet reached its point of saturation.

3.2.3. Response surface methodology

Once the "optimization zone" was reached according to the contours found (Annex 9) in CF and the significance of the second order model with its high correlation, Both the optimal point for each variable and the joint optimal point were proceeded. The individual optimization of each response variable and a multivariable optimization with equal weight "desirability" function were performed for the evaluated response variables. The joint response surface curve was determined for the response variables that is adapted to the experimental values of maximization of TCrC and TCpC. The results of the CCD are presented in (Table 8).

Table 8. Composite central design 2^2 + star per blocks for optimization of obtaining oleoresin from pepper.

Factors			**Response variable**	
Block	**% Sunflower oil**	**Solid-liquid ratio**	**TCrC (mg/mL)**	**TCpC (area/extraction volume)**
1	40	10	6.8408 ± 0.0021	75264.9363
1	40	10	7.0905 ± 0.0006	68792.5611
1	47.0711	10	6.0086 ± 0.0049	50205.1684
1	35	12.5000	5.8421 ± 0.0012	72187.0661
1	45	12.5000	4.5106 ± 0.0015	40786.8831
1	40	13.5355	4.4274 ± 0.0010	50725.5174
1	32.9289	10	8.7549 ± 0.0021	120641.2995

1	40	6.4645	11.0851 ± 0.0031	131370.4908
1	45	7.5000	9.4207 ± 0.0036	77725.2777
1	35	7.5000	10.3361 ± 0.0006	141317.3427
2	40	10	6.8408 ± 0.0021	82463.9675
2	40	10	6.5911 ± 0.0025	75350.5826
2	47.0711	10	6.4247 ± 0.0026	53597.7330
2	35	12.5000	5.5093 ± 0.0015	71697.2296
2	45	12.5000	4.7603 ± 0.0021	42233.5200
2	40	13.5355	4.5938 ± 0.0032	50845.8401
2	32.9289	10	8.5052 ± 0.0040	123439.2721
2	40	6.4645	11.3348 ± 0.0025	137290.2707
2	45	7.5000	8.2556 ± 0.0031	82075.0427
2	35	7.5000	11.4180 ± 0.0017	152754.1603

*The data for TCrC are the averages of the 3 replicates.

The results presented show that the total percentage of carotenoids increased by 91 % and 14 % in comparison with the maximum values obtained in the FFD and in the CF respectively. The minimum value for TCrC in CCD was approximately 4.4274 (mg/mL); this value was obtained when the level of the sunflower oil % and solid-liquid ratio were higher. The results obtained for the solid-liquid ratio show similarities with other research, for example Goula et al [31] reported that in the extraction of carotenoids from granadilla waste, the extraction yield increases until the ratio decreases to around 1:5 and Sachindra y Mahendrakar [32] indicated that the concentration of carotenoids extracted in batch-mode with sunflower oil increases with a solid-liquid ratio of 1: 0.5. Regarding the total of capsaicinoids, it was observed that the percentage increased by 88% and 42% in comparison with the maximum values obtained in the FFD and in the CF respectively. The minimum value obtained in CCD was 40786.8831 area/extraction volume.

Analyzing the data by means of the corresponding ANOVA tables (Table 9) and the Pareto diagram (Annex 10), the factors that influenced the evaluated response variables were determined. It is observed that the significant factors for TCrC are: sunflower oil %, the solid-liquid ratio and the interaction AA and BB. These last interactions have no practical implications, they are a term of adjustment to the mathematical model. For TCpC all the factors show significance, less interaction AA. In addition, (Table 9) presents the models adjusted to the response surface curves for an optimization analysis of a single variable and their respective coefficients of determination. These are the models that allow the optimization of the experiment.

Table 9. ANOVA analysis Composite central design 2^2 + star per blocks for carotenoids and capsaicinoids present in the oleoresin of pepper.

	Response variable	
Factors	**TCrC (mg/mL) p-value**	**TCpC (area/extraction volume) p-value**
A: % Sunflower oil	**0.0029**	**0.0024**
B: Solid-liquid ratio	**0.0003**	**0.0017**
AA	**0.0478**	0.1022
AB	0.0572	**0.0327**
BB	**0.0147**	**0.0435**
Blocks	0.9257	0.1853
Lack-of-fit	0.1818	0.7670
Model equation	TCrC= 54.854 - 1.18593*A - 3.26579*B + 0.0102986*A^2 + 0.0199732*A*B + 0.0761475*B^2	TCpC= 1.09313E6 - 26930.3*A - 64554.7*B + 183.382*A^2 + 734.073*A*B + 1180.5*B^2
R^2	98.3267	99.1914

* Bold values indicate factors with significant effect (P-value <0.05) on the response variable

After the independent optimization of each variable, a multivariate optimization was carried out, with which the optimal point for TCrC and for TCpC was determined regarding the factors: sunflower oil % and solid-liquid ratio (Table 10). According to the optimization carried out the maximum values of carotenoids and capsaicinoids are presented when the "desirability" is at its maximum value of 1. This analysis establishes that the required value of sunflower oil % to optimize the response variables would be 36.3051 and the value of solid-liquid ratio would be 1:6.8006 achieving a quantity of carotenoids and capsaicinoids of 11.6171 mg / mL and 153,969 area/volume respectively.

Table 10. Multivariable optimization for the amount of carotenoids and capsaicinoids present in the oleoresin of pepper.

Factor	Desirability	Conditions that maximize objective function
A: % Sunflower oil	1	36.3051
B: Solid-liquid ratio	1	1:6.8006
Response variables	**Weight in optimization**	**Optimal response**
TCrC (mg/mL)	1	11.6171
TCpC (area/extraction volume)	1	153,969

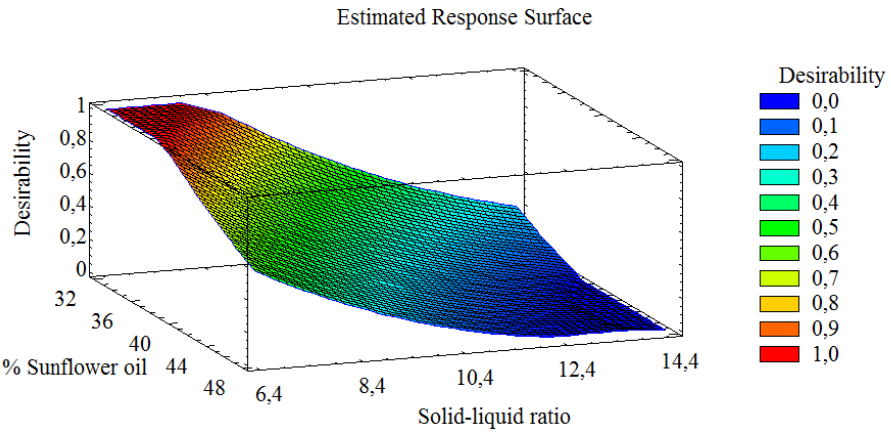

Figure 1. Response surface for multiple optimization of% sunflower oil and solid-liquid ratio in the oleoresin extraction of pepper.

Figure 1 presents the multivariate optimization and the stated values of the compounds to obtain a high "desirability". The greatest possible function of desirableness (1.0) can be obtained, there is a zone of greater "desirability", indicating the presence of an optimal point at values between 32 and 40% of sunflower oil and 1:6.4 to 1: 8.4 of solid-liquid ratio.

3.2.4. Validation of the obtained model for optimization of the UAE process

Once the optimization of the extraction process for obtaining an oleoresin rich in carotenoids and capsaicinoids was carried out, the model obtained by the surface response was validated (Table 11) and a high precision of the model was found. The percentage of error between the experimental and model values for the total carotenoids was 3.78% and for the total amount of capsaicinoids it was 2.43%.

Table 11. Concentration of carotenoids and capsaicinoids in extracted oleoresin of pepper at the optimum conditions of the process.

Response Variable	Media	% Error
TCrC (mg/mL)	12.0560± 0.0961	3.7797
TCpC (area/extraction volume)	150301,7055± 11113,5825	2.4294

*The optimization experiments were done by 3 replicates for each response variable

The experiments carried out confirm that the proposed regression model is suitable for obtaining a standardized Cayenne pepper extract in sunflower oil rich in carotenoid content and capsaicinoids from Cayenne pepper powder *(Capsicum frutescens L.)*. The main profile of capsaicinoids obtained by GC-MS and the carotenoid content in the oleoresin extract obtained at optimum conditions are showed in (Annex 11). Cayenne pepper extract obtained at the optimum conditions has both pungency and color proprieties, so it can be valued as a food ingredient.

3.3. Technological impact on sunflower oil chemistry

The impact of ultrasound was established when evaluating the lipid fractions of triglycerides, esterified/fatty acids and free fatty acids in the samples (UO, SO, OES, OlS), in addition physicochemical estimations were made such as: saponification index, unsaponification matter, iodine index, peroxide index and humidity (Table 12). The values of the physical and chemical properties found for OlS are within the range allowed by the standard, except humidity. It is necessary to improve the separation process of ethanol from samples.

Table 12. Physical and chemical properties for the samples (OES, SO, UO, OlS) and quality values required by the INVIMA standard for sunflower oil.

Samples	OES	OS	UO	OlS	INVIMA norms
Saponification index (mg KOH/g oil)	190.7400	185.1300	185.1300	187.9350	(182-194)
Unsaponification index (g/Kg)	0.6617	0.6516	0.5851	0.8612	≥ 15
Humidity (%)	0.7236	0.1536	0.0789	0.4617	0.2
Iodine index	85.3845	77.6459	96.1484	88.3481	(78-90)
Peroxide index (meqO2/Kg oil)	0.8280	0.9720	0.6920	0.8560	Up to 10

3.3.1. Determination of triglycerides

The fraction of triglycerides found for the UO sample is mainly composed of OOO, OLL, OOL, POL and PLL, which are characteristic of domestic sunflower oil composition [33]. For samples OES, SO and OlS all triglycerides are present in smaller quantities than UO, except SOO and OLL (Table 13).

Table 13. Triglycerides fraction composition percentage (%) for samples (OES, SO, UO, OlS)

Name	Code	OES	SO	UO	OlS
1,2-palmitic-3-oleic-triacylglygcerol	PPO	1.5917	1.7201	1.8518	1.7448
1,2-palmitic-3-linoleic-triacylglygcerol	PPL	2.2915	2.5328	2.7848	2.5993

1-palmitic-2-stearic-3-oleic triacylglycerol	PSO	1.2132	1.1766	1.2884	1.2338
1-palmitic-2,3-oleic triacylglycerol	POO	7.9547	8.5712	9.4833	8.6048
1-palmitic-2-stearic-3-linoleic triacylglycerol	PSL	1.5012	1.3791	1.4559	1.4497
1-palmitic-2-oleic-3-linoleic triacylglycerol	POL	10.9277	11.1945	12.2074	11.1633
1-palmitic-2,3-linoleic triacylglycerol	PLL	8.8784	8.2518	9.1209	8.2833
1-palmitic-2-linoleic-3- linolenic triacylglycerol	PLLn	1.2294	0.3720	0.4788	0.4745
1-stearic-2,3-oleic triacylglycerol	SOO	4.3728	3.3275	1.8364	3.3720
Triolein	OOO	16.7310	17.0046	18.9656	16.8188
1-stearic-2-oleic-3-linoleic triacylglycerol	SOL	2.8731	2.8251	3.2129	3.1218
1,2-oleic-3-linoleic triacylglycerol	OOL	18.6817	19.1526	13.7023	18.9611
1-oleic-2,3-linoleic triacylglycerol	OLL	14.8942	15.3718	16.5074	15.0997
Trilinolein	LLL	6.8594	7.1203	7.1042	7.0730

3.3.2. Determination of FAME´S

The esterified fatty acids found in greater proportion were (C18:1 cis n-6 and C18:1 cis n-9) for all the samples. It was found that monounsaturated fatty acids quantity (C14:1, C16:0, C16:1, C17:0, C17:1 n10, C18:0, C18:1 cis n-9, C18: n-7) is of 40% for UO and decreases to 35% for all samples. The fraction of polyunsaturated fatty acids (C18: 2 n-6, C18: 3 n-3) is 41% for UO this value increases to 45, 46 and 45% for OES, UO and OlS respectively. The sunflower oil used for the UAE process is mainly composed of unsaturated fatty acids, the saturated fraction (C14:0, C16:0, C16:1, C17:0, C18:0, C20:0, C22:0, C24:0) is approximately 15% (Table 14).

Table 14. Fatties acids methyl esters (FAME´S) fraction percentage (%) for samples (OES, SO, UO, OlS)

Name	Code	OES	SO	UO	OlS
Methyl myristate	C14:0	0.7683	0.7996	0.9834	0.8651
Methyl myristoleate	C14:1	2.0727	1.8984	2.5328	2.1662
Methyl palmitate	C16:0	1.7294	1.7151	1.9872	1.9331
Methyl palmitoleate	C16:1	1.5597	1.8243	1.6995	1.1282
Methyl margarate	C17:0	1.9452	2.3582	2.0483	1.8008
Methyl heptadecenoate	C17:1 n10	2.4442	2.2926	1.9695	2.4248
Methyl stearate	C18:0	1.6533	1.6038	1.4944	1.5147
Methyl oleate	C18:1 cis n-9	22.6668	22.4142	27.9099	21.8854
Methyl oleate	C18:1 n-7	6.2208	5.6773	5.4255	5.9003
Methyl linoleate	C18:2 n-6	37.0512	40.2592	33.9131	38.1904
Methyl linolenate	C18:3 n-3	7.7697	5.9871	7.1786	7.8389
Methyl arachidate	C20:0	1.9845	1.8925	2.1615	1.9274
Methyl behenate	C22:0	3.8030	3.4379	3.3416	3.7796
Methyl lignocerate	C24:0	3.9615	3.6409	3.5685	3.9243
Methyl nervonate	C24:1 n-9	4.3697	4.1988	3.7862	4.7210
∑ Monounsaturated		34.9642	34.1068	39.5372	33.5048
∑ Polyunsaturated		44.8209	46.2464	41.0917	46.0292
∑ Unsaturated		79.7852	80.3531	80.6289	79.5340
∑ Saturated		15.8452	15.4480	15.5849	15.7449
Saturate/Unsaturated		0.1986	0.1923	0.1933	0.1980

3.3.3. Determination of free fatty acids

The determination of free fatty acids in the samples (OES, SO, UO and OlS) shows the appearance of compounds in the samples sonicated as: glycerol, C18:1 cis n-9 free, 1-monoleoleglycerol, 1 - Estearoilglycerol and C24:0 free. The OlS sample was characterized by the appearance of other compounds such as: C12:0 free, C14:0 free, ethyl C12:0, methyl cis-9, cis-15-linoleate, methyl trans- 9, cis-15-linoleate, ethyl C18:1 cis n-9, ethyl C18:2 n-6 cis, C20:0 free and ethyl C18:2 n-6 trans, which makes it the richest sample in quantity of free fatty acids. (Table 15).

Table 15. Free fatty acids fraction percentage (%) for samples (OES, SO, UO, OlS)

Name/Code	OES	SO	UO	OlS
Glycerol	2.018	0.000	0.000	1.853
C12:0 free	0.000	0.000	0.000	0.387
C14:0 free	0.000	0.000	0.000	0.255
Ethyl C12:0	0.000	0.000	0.000	0.338
C16:0 free	2.543	5.334	16.871	31.808
Methyl cis-9, cis-15-linoleate	0.000	0.000	0.000	0.796
Methyl trans-9, cis-15-linoleate	0.000	0.000	0.000	2.856
Ethyl C18:1 cis n-9	0.000	0.000	0.000	1.857
C18:2 n-6 cis free	38.726	38.950	72.763	14.254
C18:1 cis n-9 free	41.065	42.328	0.000	11.094
C18:1 trans n-9 free	0.000	0.000	0.000	4.032
C18:0 free	4.621	4.823	7.697	11.947
Ethyl C18:2 n-6 cis	0.000	0.000	0.000	2.952
C20:0 free	0.000	0.000	0.000	1.595
Ethyl C18:2 n-6 trans	0.000	0.000	0.000	1.503
1-Monopalmitoylglycerol	0.590	0.468	1.611	1.974
C22:0 free	2.320	2.263	1.059	2.031
1-Monoleoleglycerol	7.114	4.607	0.000	7.522
1- Estearoilglycerol	0.356	0.275	0.000	0.355
C24:0 free	0.646	0.952	0.000	0.589

3.3.4. Principal component analysis (PCA)

A discriminant analysis of variables was performed in which the data matrix is normalized, the assumptions tests are applied and the data is scaled using a script (Annex 12). The correlation that exists between two independent graphs of score values (Figure 2) and loads (Figure 3) was studied, which consist of the position of the vectors (variables: physicochemical properties, triglycerides, FAME's and free fatty acids) and the position of coordinates of the samples (OES, SO, UO, OlS). These graphs explain 90% of the variance.

Sunflower oil is the main extractor solvent in the UAE process, this is characterized by having an initial lipid chemistry, which was studied and found that the numerical behavior variables of UO, undergoes changes during the sonication process. The application of high intensity ultrasound to an oil sample (SO) impacts its initial lipid chemistry (UO). This impact is reflected by the location of the samples in quadrants I and III for SO and UO respectively. Each sample is characterized by different variables, for UO the amount of monounsaturated fatty acids (Sum.Monoinsat) have a high correlation, with oleic acid being the most important monounsaturated since it is present in the most abundant triglycerides and sterified fatty acids (POO, POL, OOO, OOL, OLL and methyl oleate (C18:1 cis n-9)). Another variable to take into consideration is the iodine index which have high correlation with UO, the value for this variable have a significant change when ultrasound is applied: 98 to 78 for UO and SO respectively, this means that the unsaturated bounds in sonicated samples were affected. The

peroxide index is a variable with high correlation for OS and EOS, its behavior is inverse with the iodine index. The peroxide values found for UO, SO, OES and OlS were 0.6920, 0.9720, 0.8280 and 0.8560 (meqO2/Kg oil) respectively, this indicates that the sonicated samples undergo lipid peroxidation. Therefore, a low value in the iodine index is normal for these ones, since the quantity of double bonds decreases when suffering peroxidation due to sonication effect. Although no significant changes were observed regarding the decrease and increase in triglycerides and esterified fatty acids composition before and immediately after the treatment the ultrasound. The increase of the peroxide index in the sonicated samples is an undesirable effect due to the health implications, since the lipid peroxidation in products are highly reactive and display marked biological effects, which, depending upon their concentration, cause selective alterations in cell signaling, protein and DNA damage, and cytotoxicity [34].

The SO and OES samples are located on quadrant I, their statistical distance is short. This indicates that although the samples are different, they share a high correlation with the same cluster of variables. The saponified matter (Sindex) has a much higher correlation with SO than OES, the found values were 185.1300 mg KOH/g oil for UO and SO and 190.7400, 187.9350 mg KOH/g oil for OES and OlS respectively. For samples containing ethanol there is evidence of an increase in saponifiable matter, the high energy supplied by the ultrasound to the sample would produce an ethyl esterification of free fatty acids and it is important to note that lipid peroxidation decreases for these samples and their humidity is higher compared to OU and SO samples. The humidity behavior is independent of the sonication process. The humidity values obtained in the samples are correlated mainly with SO, OES and OlS, these values can be affected by the environment, by the recirculation in the pump, by the presence of ethanol which contributes 4% of water to the samples OES and OlS and by the presence of vegetal material which it hydrates and performs the function of absorbent. A high humidity in the samples could please the peroxidation of the lipids by the presence of OH- which is very reactive.

The SO and OES samples are located on quadrant I, their statistical distance is short; this, indicates that although the samples are different, they both share a high correlation with the same cluster of variables. The saponified matter (Sindex) has a much higher correlation with SO than with OES, the found values were 185.1300 for UO and SO and 190.7400, 187.9350 for OES and OlS respectively. For samples containing ethanol there is evidence of an increase in saponifiable matter. The high energy supplied by the ultrasound to the sample would produce an ethyl esterification of free fatty acids and it is important to note that lipid peroxidation decreases for these samples and their humidity is higher compared to OU and SO samples. The humidity behavior is independent of the sonication process. recirculation in the pump, by the presence of ethanol which contributes 4% of water to the samples, the presence of vegetal material which it hydrates itself and performs the function of absorbent. A high humidity in the samples could help to the peroxidation of the lipids by the presence of OH- which is very reactive.

The OlS sample is located on quadrant IV at 180 ° away from the rest of the samples, this is because the lipid fraction of the extracted powder contributes a chemically important change to the sample and makes it different. OlS correlates mainly with the variables that represent free fatty acids and with the unsaponification index (Insindex). The values of unsaponification index found were 0.5851, 0.6516, 0.6617 and 0.8612 g/Kg for UO, SO, OES and OlS respectively. It is evident that the non-esterified fraction increases for OlS, this is due to the significant contribution of free fatty acids from the powder. Free fatty acids can easily be oxidized by the chemistry of the system, if there are metals or enzymes present, they are available to interact with them. On the other hand, when the fatty acids are bound, they are protected by the same integrity of the structure, thus avoiding the oxidation of these and consequently the rancidity of the oil. This implicit chemistry in the oleoresin obtained implies a state of vulnerability that affects the integrity and quality of the final product [35] .

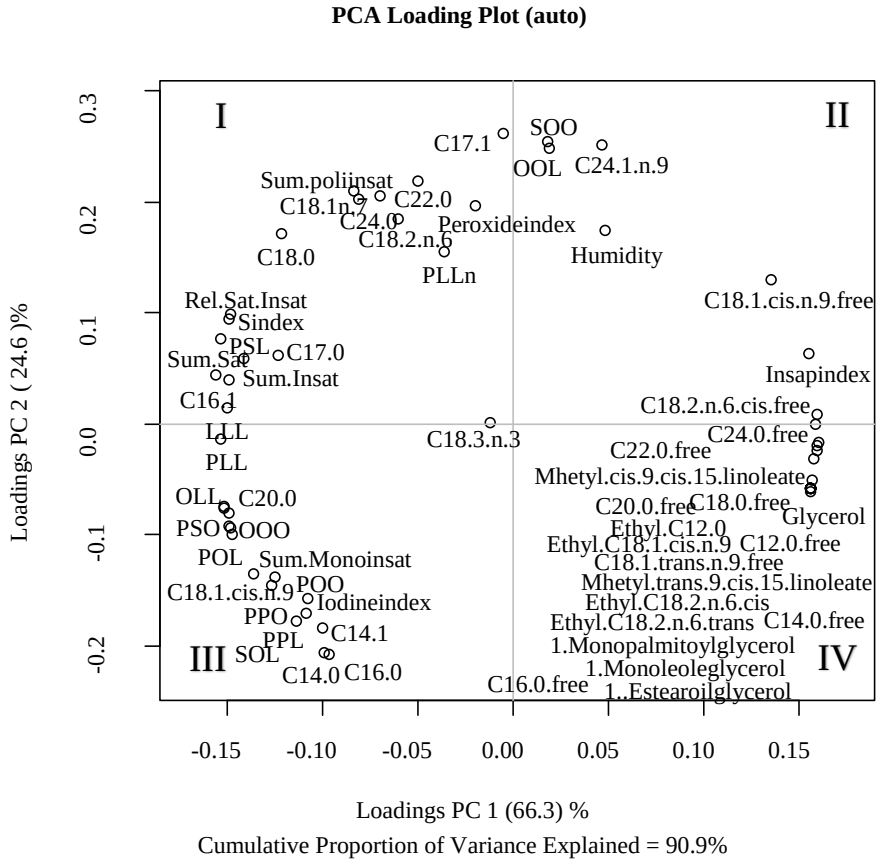

Figure 2. Position of the coordinates of the vectors (variables: physicochemical properties, triglycerides, FAME's and free fatty acids)

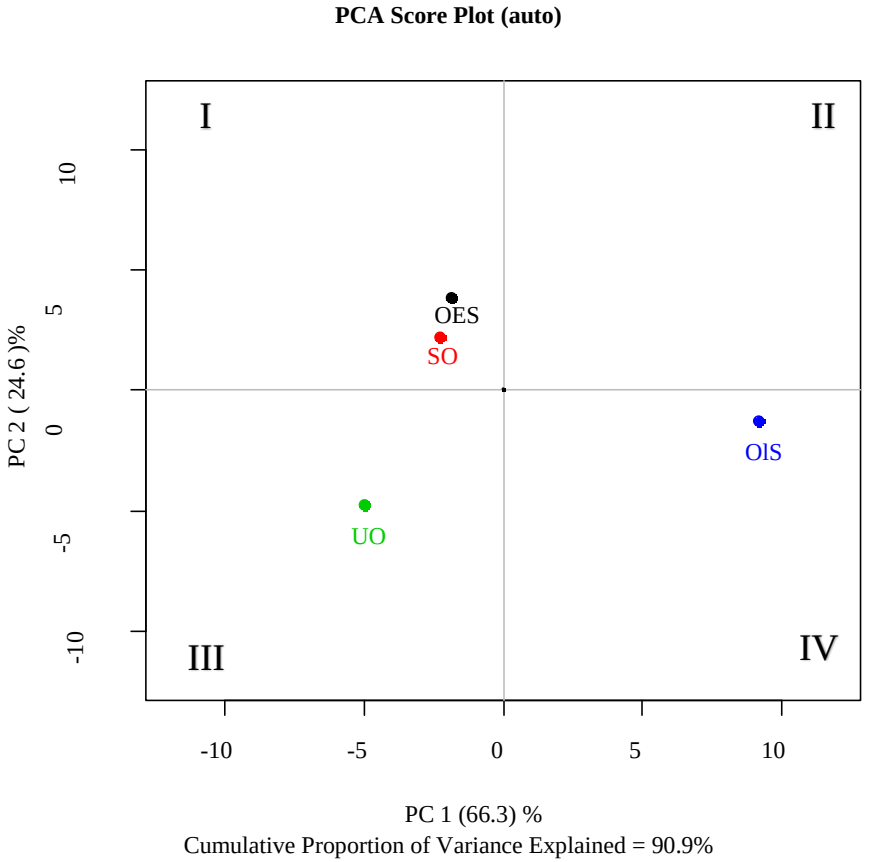

Figure 3. Position of the coordinates of the samples (OES, SO, UO, OlS)

The SO sample presented some off-flavor compounds which were not identified, however in the literature it is reported that when submitting a refined sunflower oil to high power ultrasound some typical indicators for lipid oxidation as hexanal, (E) -But-2- enal, 2-Methylfuran are found and the metallic and rancid odor detected after oil treatment by ultrasound was due to the formation of (Z) -hept-2-enal and (2E, 4E) -deca-2,4-dienal which give a fishy-sweet and a deep-fried odor respectively [36]. The OES and OlS samples did not present off-flavor. The presence of ethanol as co-solvent in oleoresin extraction, besides favoring the obtaining of carotenoids and capsaicinoids, it seems that it fulfills the function of masking the off-flavor by lipid sono-oxidation.

4. Conclusions

Ultrasound-assisted extraction process has been developed to extract oleoresins with various advantages in term of time, use of green solvents and yield. Both sunflower oil % and solid-liquid ratio are significands variables in whole static analysis, these ones were optimized. The regression model found in response surface methodology is suitable for obtaining a standardized oleoresin in sunflower oil rich in carotenoid content and capsaicinoids from Cayenne pepper powder (*Capsicum frutescens L.*).

When the samples with sunflower oil (SO, OES, OlS) were submitted to the sonication process, it was found that there was an impact on the structural and functional components up to the point of oxidation and deterioration of the lipid fraction of each sample. Although no significant changes were observed, the increase of the peroxide index in the sonicated samples and the presence of free fatty acids in OlS is an undesirable effect for safety and quality in final product. The percentage of humidity for OlS was 0.2% higher than norm specification, so it is suggested to improve the rotaevaporation process, in which the ethanol is separated. The oleoresin obtained at the optimum conditions of the process complies with the other sanitary requirements that oils and fats of vegetable or animal origin must meet to be processed, packaged, stored, transported, exported and/or sold in the country, destined for human consumption.

5. Table of Annex or Appendices

Table 16 shows a link to additional information about the project.

Table 16. Additional documents included in the project.

Name	Development (self/others)	Type of document	Google drive link (https://goo.gl/)
Annex 1	Self	Doc	http://bit.ly/2wsON4r
Annex 2	Self	Doc	http://bit.ly/2I2qjVc
Annex 3	Self	Doc	http://bit.ly/2K6GFZo
Annex 4	Self	Xml	http://bit.ly/2rw1k2n
Annex 5	Self	Xml	http://bit.ly/2KMTB89
Annex 6	Self	Xml	http://bit.ly/2rx2MR4
Annex 7	Others	Pdf	http://bit.ly/2jLQD7e
Annex 8	Self	Doc	http://bit.ly/2rwA9EB
Annex 9	Self	Doc	http://bit.ly/2Isqatu

Annex 10	Self	Doc	http://bit.ly/2KPEgmX
Annex 11	Self	Doc	http://bit.ly/2I0Omnk
Annex 12	Self	Txt	http://bit.ly/2I2UifG

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