

Pressurized Organic Solvent Extraction with On-line Particle Formation by Supercritical Anti Solvent Processes

Diego T. Santos , Dayane F. Barbosa , Ketllen Broccolo, M. Thereza M. S. Gomes , Renata Vardanega ,
M. Angela A. Meireles*

Lasefi/Dea/Fea (School of Food Engineering)/UNICAMP (University of Campinas) Cidade Universitária "Zeferino Vaz", R. Monteiro Lobato, 80; 13083-862, Campinas, SP, Brazil

Abstract In this work, a novel on-line process for pressurised hot organic solvent extraction of antioxidants from plants as well as precipitation of the extract with or without a carrier material in one step was developed. This process has been called OEPO, Organic solvent Extraction and On-line particle formation. With this process, different products with a very low residual organic solvent concentration (< 50 ppm) can be obtained by the use of supercritical CO₂ as anti solvent for solvent elimination. OEPO process consists of hyphenated Pressurized Liquid Extraction (PLE)-Supercritical Anti Solvent (SAS) precipitation, PLE-SAS co-precipitation and PLE-Supercritical Fluid Extraction of Emulsions (SFEE). OEPO process was successfully developed using Brazilian ginseng roots (*Pfaffia glomerata*) as a model case using ethyl acetate as extracting solvent. Results were compared, in terms of antioxidant activity or morphology, with the ones obtained by each process separately. In addition, an optimization study for antioxidants recovery was performed using ethyl acetate as extracting solvent during PLE process. Optimum PLE extracts were produced under moderate extraction temperature (373 K) and high static extraction time (15 min). Under this condition an extraction yield of 1% (dry basis, d.b.) and an antioxidant activity of 53% are obtained, which was approximately 14% higher than that observed after PLE-SAS precipitation and after SAS precipitation performed in two steps (step one - PLE extraction; step two - SAS precipitation by the use of the extract solution produced by step one stored). Similar behavior (hyphenated process producing similar products than the two step process done separately) was observed for PLE-SAS co-precipitation and PLE-SFEE indicating that the OEPO process developed in this work can be considered as a suitable and promising process to obtain, in only one step, different products (precipitated extract, co-precipitated extract or encapsulated extract in suspension), directly from plant materials.

Keywords PLE, SAS, SFEE, Supercritical Fluids, Bioactive Compounds, Hyphenated Processes

1. Introduction

Nowadays, the demand for natural bioactive compounds is increasing due to their use in the functional food industry. Natural components from plants are employed, including different functional activities, for instance, antioxidant activity, antimicrobial activity, anti-cancer, or neurodegenerative diseases prevention, among others [1]. Ginseng species is one of the most appreciated natural sources for this kind of compounds. The most known Ginseng species in the world belongs to the *Panax* genus, which have been used for thousands years by folk medicine. Asian ginseng (*Panax ginseng*), American ginseng (*Panax quinquefolius*) roots are renowned and widely used herbs in China, United States,

Canada, etc. [2].

Species of the genus *Pfaffia* (Amaranthaceae) has been commercialized as substitutes for *Panax* (ginseng, Araliaceae). Due to the similar morphology of its roots to those of ginseng, they are popularly known as "Brazilian ginseng". Around 90 species of *Pfaffia* are known in Central and South America [3]. In Brazil, 27 species have been described, being *Pfaffia glomerata* the most important species. Since besides similarity in appearance Brazilian ginseng roots (*Pfaffia glomerata*) extracts have also similar effects to ginseng, large amounts of this plant material are being exported for production of their extracts [4].

Different classical extraction techniques have been applied to obtain antioxidant extracts from *Pfaffia glomerata* roots [5-7]. Classical extraction methods are time- and solvent-consuming and may promote extract degradation during the extraction process. On the other hand, pressurized liquid extraction (PLE) technique enables the rapid extraction (less than 30 min) of analytes in a closed and inert

* Corresponding author:

meireles@fea.unicamp.br (M. Angela A. Meireles)

Published online at <http://journal.sapub.org/fph>

Copyright © 2012 Scientific & Academic Publishing. All Rights Reserved

environment under high pressures (no higher than 20 MPa) and temperatures (298–473 K). A major advantage of PLE over conventional solvent extraction methods conducted at atmospheric pressure is that pressurized solvents remain in a liquid state well above their boiling points, allowing for high-temperature extraction. These conditions improve analyte solubility and the kinetics of desorption from matrices [8].

The low stability during extraction, formulation, purification and storage of some class of bioactive compounds has influenced all these steps, which are being studied by different researchers interested in novel forms for processing these compounds with minimum degradation [9]. The most important bioactive principles of Ginseng are saponins. Gradual degradation was observed with further increase in temperature resulting in complete destruction of saponins at temperatures > 543 K. Hydrothermolysis of triterpenoid and steroid saponins occurred upon heating in water at 473–513 K resulting in the production of aglycones, prosapogenins, and sugars [10, 11]. Thus, the extracting solvent elimination step also should be done quickly and using mild operation conditions of temperature. Evaporation step usually expose the extracts to a risk of degradation of bioactive compounds catalysed by heat besides light and/or oxygen.

In the search for alternative solvent elimination processes that can keep the stability of the extracted compounds, we have focused our attention on the use of supercritical fluids. Supercritical CO₂ and organic solvents are miscible above a moderate pressure and temperature, while the compound or class of compounds are not soluble in the mixture and it precipitates [12]. Denominated as Supercritical Anti Solvent (SAS) precipitation this method has been used extensively mainly to obtain small particles with narrow particle size distribution, which can be encapsulated, if required, by its co-precipitation together a carrier material (SAS co-precipitation) or by the formulation of an emulsion [Supercritical Fluid Extraction of Emulsions (SFEE)] [13, 14].

The encapsulation of natural substances presents several advantages over the natural substance itself. First, they acquire controlled release behaviour and are able to maintain their stability for longer periods [15].

The development of hyphenated processes for combining bioactive compounds extraction with on-line particle formation is rarely reported. Ibanez group in Spain, recently, have developed a hyphenated process to obtain dried powders of extracts from natural sources in one step. This process called Water Extraction and Particle formation On-line (WEPO) similarly to ours also use PLE technique for bioactive compounds recovery, but employ water as extracting solvent. Since, in their case supercritical CO₂ are not suitable for solvent elimination due to the low solubility of water in CO₂, this fluid is used as a dispersion medium and hot N₂ is used as drying agent [16]. Due to the similarities of WEPO process we have named our process as Organic solvent Extraction and Particle formation On-line (OEPO). Differently of WEPO process, OEPO process also permits the encapsulation of the extract immediately after their

production. Indeed, OEPO process consists of hyphenated PLE-SAS precipitation, PLE-SAS co-precipitation and PLE-SFEE.

In this study the OEPO device and procedures are described in detail and successfully developed using Brazilian ginseng roots (*Pfaffia glomerata*) as a model case. Results were compared, in terms of antioxidant activity or morphology, with the ones obtained by each process separately. In addition, an optimization study for antioxidants recovery was performed using ethyl acetate as extracting solvent during PLE process and with static extraction time (5–15 min) and extraction temperature (353–413 K) as independent variables. The organic solvent ethyl acetate was chosen because it is a Generally Recognized as Safe (GRAS) solvent according to the US Food and Drug Administration (FDA) (toxicological class 3), it can be safely used in food applications [17, 18].

2. Materials and Methods

2.1. Materials

Brazilian ginseng roots (*Pfaffia glomerata*) were cultivated in the experimental field of CPQBA (Campinas, Brazil), where they were collected on March 25, 2004, being 3 years old. They were washed and dried in a forced air circulation dryer at 313 K for 5 days. The dried roots (8.9% moisture) were then comminuted in a pulse mill (Marconi, model MA 340, Piracicaba, Brazil) for few seconds. Next, the particles of higher size were milled again, this time using a knife mill (Tecnal, model TE 631, Piracicaba, Brazil) for 2 s at 18,000 rpm and finally, they were separated according to their size using sieves (Series Tyler, W.S. Tyler, Wheeling, IL). The milled roots were stored in freezer (Metalfrío, model DA 420, São Paulo, Brazil) at 263 K. For the extraction assays, particles of 7.89 µm of diameter, according to ASAE methodology [19], were used. The moisture content of the dried roots was determined by the AOAC method (Method 4.1.03) [20].

Ethyl acetate (analytical grade) purchased from Merck (Darmstadt, Germany) was used as extracting solvent. Dichloromethane (analytical grade) purchased from Merck (Darmstadt, Germany) was used to prepare the Polyethylene glycol (PEG) solutions.

Polyethylene glycol (PEG) with a mean molecular weight of 10,000 g/mol (mp: 336–338 K) (Sigma-Aldrich, Steinheim, Germany) was used as carrier material.

N-octenyl succinic anhydride (OSA)-modified starch, kindly provided by National Starch Food Innovation (Hamburg, Germany), was used as surfactant and carrier material.

Dry Carbon dioxide (CO₂), 99.9% purity (Gama Gases Especiais Ltda., Campinas, Brazil) used as the antisolvent was supplied in the liquid phase.

2.2. Pressurized Liquid Extraction (PLE)

The pressurized liquid extraction (PLE) system was designed and assembled at LASEFI/DEA/FEA (School of

Food Engineering)/UNICAMP (University of Campinas). The solvent was pumped by a HPLC pump (Thermoseparation Products, Model ConstaMetric 3200 P/F, Fremont, USA) into the extraction cell, which was placed in an electrical heating jacket at a desired temperature, until the required pressure was obtained.

Dried and milled pieces of Brazilian ginseng roots (4.5 g with a moisture content of 8.9%) were placed in a 6.57-cm³ extraction cell (Thar Designs, Pittsburg, USA) containing a sintered metal filter at the bottom and upper parts. The cell containing the sample was heated, filled with extraction solvent (ethyl acetate) and then pressurized. The sample was placed in the heating system for 6.5 min to ensure that the extraction cell would be at the desired temperature during the filling and pressurization procedure. After pressurization, the sample with pressurized solvent was kept statically at the desired pressure for the desired time (static extraction time). The pressure of the extraction was set in all experiments to 12 MPa to simulate the conditions of the OEPO process. Thereafter, the back pressure regulator (BPR) valve (Model #26-1761-24-161, Tesco, Elk River, USA) was carefully opened, keeping the pressure at an appropriate level for the desired flow (1.0 cm³/min), to rinse the extraction cell with fresh extracting solvent for 20 min (dynamic extraction time). After pressurized liquid extraction (PLE), the extracts were rapidly cooled to 268 K in ice water using glass flasks to prevent extract degradation. After extraction, depending on the aim different procedures were done. If the aim was to determine the extraction yield and the extract antioxidant activities, the solvent was evaporated using a rotary evaporator (Laborota, model 4001, Vertrieb, Germany) with vacuum control (Heidolph Instruments GmbH, Vertrieb, Germany) and a thermostatic bath held at 313 K. On the other hand, if the aim was to use it in the particle formation processes, the 20 cm³ of ethyl acetate extract solution produced in each experiment were directly stored. All extracts (dried or not) were stored (263 K) in the dark prior to the next step (analysis or particle formation).

Table 1. Summary of the extraction operating conditions

	Extraction Temperature (K)	Static Extraction Time (min)
E1	353	5
E2	353	10
E3	353	15
E4	373	5
E5	373	10
E6	373	15
E7	393	5
E8	393	10
E9	393	15
E10	413	5
E11	413	10
E12	413	15

Statistica' software (release 7, StatSoft, Tulsa, USA) was used to calculate the effects of extraction temperature and static extraction time on extraction yield and antioxidant activity (Table 1). All extractions were performed in duplicate. Statistical analyses were performed using analyses of variance (ANOVA). The mean values were considered significantly different at $p < 0.05$. The prediction of one set of optimal conditions for both response variables was done by using desirability function approach.

2.3. Supercritical Anti Solvent (SAS) Precipitation Process

The experiments for ethyl acetate *Pfaffia glomerata* roots extract precipitation were performed in a homemade Supercritical Anti Solvent (SAS) equipment designed and assembled at LASEFI/DEA/FEA (School of Food Engineering)/UNICAMP (University of Campinas) employing the ethyl acetate extract solution produced by PLE.

Liquid CO₂, antisolvent, was fed from the cylinder through a thermostatic bath (MA-184, Marconi, Piracicaba, Brazil) at 263 K to ensure the liquefaction of the gas and to prevent cavitation, then it was pumped by an air-driven liquid pump (Maximator GmbH, PP 111, Zorge, Germany) to the high pressure vessel (volume of 500 cm³; 6.8-cm internal diameter) via a nozzle. The nozzle consists of a 1/16" tube (inner diameter [i.d.]: 1.778 millimeters) for the solution, placed inside a 1/8" tube for the CO₂. Once the particle formation vessel reached steady state (temperature-313 K, pressure-10 MPa and CO₂ flow rate-0.6 kg/h), the ethyl acetate extract solution was introduced into the vessel by a high-performance liquid chromatography (HPLC) pump (Model ConstaMetric 3200 P/F, Thermoseparation Products, Fremont, USA) through the coaxial annular passage of the atomizer at a constant flow rate. The flow rate of the solution was set in all experiments to 1.0 cm³/min to simulate the conditions of the OEPO process. The vessel temperature (313 K) was maintained by a heating water bath (MA 127BO, Marconi, Piracicaba, Brazil). CO₂ flow rate (0.6 kg/h) was measured using a glass float rotameter (0.15–2.2 kg/h of CO₂ at 0.1013 MPa/293 K; 16/286A/2, ABB, Warminster, PA) coupled to a flow totalizer (Model G0,6, LAO, Osasco, Brazil). When the desired amount (20 cm³) of solution (ethyl acetate *Pfaffia glomerata* roots extract) has been injected, which enabled the collection of sufficient amount of precipitated powder for analysis, the HPLC pump was stopped and only pure CO₂ was fed. The flow of CO₂ was maintained for 20 min for the complete removal of the solvent from the precipitator, which was proven necessary by preliminary experiments.

Pfaffia glomerata roots extract precipitates were trapped by a paper filter fixed at the bottom of the precipitation vessel while the fluid mixture (CO₂+ethyl acetate) flowed to a second vessel (100-cm³ glass flask). At the end, the precipitation vessel was slowly depressurized to atmospheric pressure and particles were collected and stored in the dark in a domestic freezer (263 K; Double Action, Metalfrío, São

Paulo, Brazil) until subsequent analysis and characterization. A heating system maintained at 353 K was used to heat the micrometric valve to avoid the Joule–Thompson freezing effect that can lead to clogging of the throttling device during particle formation procedure.

2.4. Supercritical Anti Solvent (SAS) Co-Precipitation Process

The experiments for ethyl acetate *Pfaffia glomerata* roots extract co-precipitation with PEG were done in the SAS equipment previously described. The SAS co-precipitation procedure is very similar to the SAS precipitation, differing that a carrier material was added to the ethyl acetate extract solution produced by PLE. PEG, in this case, was the carrier material and dichloromethane was the solvent. Dichloromethane was the selected solvent because it is a good solvent for PEG. Thus, in this case *Pfaffia glomerata* roots extract in PEG co-precipitates were trapped by a paper filter fixed at the bottom of the vessel while the fluid mixture (CO₂+ethyl acetate+dichloromethane) exited the vessel.

The operating conditions were the same that during SAS precipitation (10 MPa and 313 K, CO₂ flow rate of 0.6 kg/h, ethyl acetate solution flow rate of 1.0 cm³/min). The mass ratio between *Pfaffia glomerata* roots extract and PEG investigated was 1:10.

2.5. Supercritical Fluid Extraction of Emulsions (SFEE) Process

Before initiating the SFEE process, an oil-in-water emulsion must be prepared. In general, these emulsions are prepared with the aid of surfactants.

Twenty cubic centimeters of the ethyl acetate solution produced by PLE was dispersed into 80 cm³ of an aqueous solution with OSA-modified starch surfactant (6 g/dm³) by the aid of a high speed-stirring mixer (IKA® magic LAB®, Staufen, Germany) with an engine power of 900 Watt processed during 4 min at 26,000 rpm. The mixer was cooled by ethylene glycol that circulates through a jacket, which allows to remove the heat generated by the equipment and to operate at temperatures lower than 298 K to avoid ethyl acetate evaporation.

The SFEE experiments were done also in the SAS equipment previously described. The SFEE procedure and operating conditions were the same that during SAS precipitation and SAS co-precipitation, only differing that instead of a solution, an emulsion with ethyl acetate extract solution produced by PLE was used as the dispersed phase. Then, in this case the suspension containing *Pfaffia glomerata* roots extract encapsulated in OSA-starch micelles remained in the precipitation vessel while the fluid mixture (CO₂+ethyl acetate) exited.

Afterwards, the suspensions obtained were further processed removing water to produce a dry powder. This was done by freeze-drying for 5 days at 60–100 μHg and at -223 K (Liobras, Liotop L101, São Carlos, Brazil).

2.6. Organic Solvent Extraction and Particle Formation On-line (OEPO) Process

The Organic solvent Extraction and Particle formation On-line (OEPO) process combines the two different processes previously described: firstly a dynamic PLE process using organic solvents and secondly the elimination of the solvent by the precipitation of the extract, using supercritical CO₂ as an anti solvent. Thus, extraction and precipitation take place in the same system with a small time delay between these two processes.

Figure 1 shows a scheme of the home-built equipment designed to carry out the organic solvent extraction with particle formation on-line (OEPO).

From the extraction cell, the extract solution is led to a T-mixer where it can be mixed with a solution containing a carrier material dissolved also in an organic solvent or with an aqueous solution of surfactant. Shortly afterwards the solution or emulsion is exited through the coaxial annular passage of the atomizer together with supercritical CO₂ into the precipitation vessel. All connections used for coupling the PLE system with the SAS equipment were made using stainless steel tubes (1/16" and 1/8").

The extraction cell was filled with dried and milled pieces of Brazilian ginseng roots. The amount inserted of plant material was calculated in order to keep the same solvent volume to feed volume ratio employed during the previous PLE experiments (20 cm³ of solvent/4.5 g of roots). The process started with a static extraction period (selected after optimization) by filling the cell with ethyl acetate at the desired temperature (selected after optimization) and pressure (12 MPa), with valve V18 closed (see Figure 1). At the same time, CO₂ was pumped through the system at the desired temperature (313 K) and pressure (10 MPa), with a constant flow rate (0.6 kg/h). The extraction continued in a continuous flow mode (dynamic extraction period) by opening valve V18 and setting the extracting solvent rate at the desired constant value. The ethyl acetate extract solution can meet first the solution containing PEG dissolved in dichloromethane or with an aqueous solution with OSA-modified starch surfactant (6 g/dm³) pumped with HPLC pump of the SAS equipment. The flow rate of both solutions was set in order to achieve a constant total flow rate of ethyl acetate extract solution plus the resulted solution or emulsion of 1.0 cm³/min. When the aimed process was PLE-SAS precipitation, the second HPLC pump was turned off, since in this process there is no need of any carrier or surfactant material addition. Afterwards the organic solvent (ethyl acetate) or solvents (ethyl acetate+dichloromethane) from the solution or emulsion are exited through the vessel precipitating the product, which can be: i) precipitated extract (product after PLE-SAS precipitation); ii) co-precipitated extract (product after PLE-SAS co-precipitation) or iii) encapsulated extract in suspension (product after PLE-SFEE).

The suspensions obtained by PLE-SFEE were further freeze-dried to produce a dry powder as previously

described.

2.7. Product Characterization

2.7.1. Antioxidant Activity (AA)

The evaluation of antioxidant activity of the extracts was based on the coupled oxidation of β -carotene and linoleic acid. The technique developed by Marco[21] consisted of measuring the bleaching of β -carotene resulting from oxidation by the degradation products of linoleic acid. In short, the substrate of reaction was prepared using 10 mg of β -carotene (97%, Sigma–Aldrich, St. Louis, USA), 10 cm³ of chloroform (99%, Ecibra, Santo Amaro, Brazil), 60 mg of linoleic acid (99%, Sigma–Aldrich, St. Louis, USA) and 200 mg of Tween 40 (99%, Sigma–Aldrich, St. Louis, USA). This solution was concentrated in rotary evaporator (Laborota, model 4001, Vertrieb, Germany), with vacuum control (Heidolph Instruments GmbH, Vertrieb, Germany) and a thermostatic bath at 323 K, being then diluted in 50 cm³ of distilled water. The oxidation reaction was conducted using the following procedure: to each 1 cm³ of substrate, 2 cm³ of distilled water and 0.05 cm³ of extract diluted in

ethanol (99.5%, Ecibra, Santo Amaro, Brazil) were added. The dilution used for AA determination was 0.02 g of extract/cm³ of solvent. The mixture was placed in thermal bath (model TE 159, Tecnal, Piracicaba, Brazil Marconi, model MA159/300, Piracicaba, Brazil) at 313 K, and the product of reaction was monitored using a spectrophotometer (Femto, model 800 XI, São Paulo, Brazil Hitachi, model U-3010, Tokyo, Japan) at 0, 1, 2 and 3 h of reaction, using absorbance at 470 nm. The antioxidant activity was determined in duplicate for each extract and calculated following the same calculation procedure done by Santos et al.[22]. Antioxidant activity for synthetic BHT (at the same concentration that the extract) was also determined for comparison.

2.7.2. Microscopy

Micrographs of the particles collected were taken by means of a scanning electron microscope (SEM) (LEO 440i, Leica, Cambridge, USA) after coating with a thin gold film with the aid of a sputter coater (Polaron, SC 7620, Ringmer, U.K.).

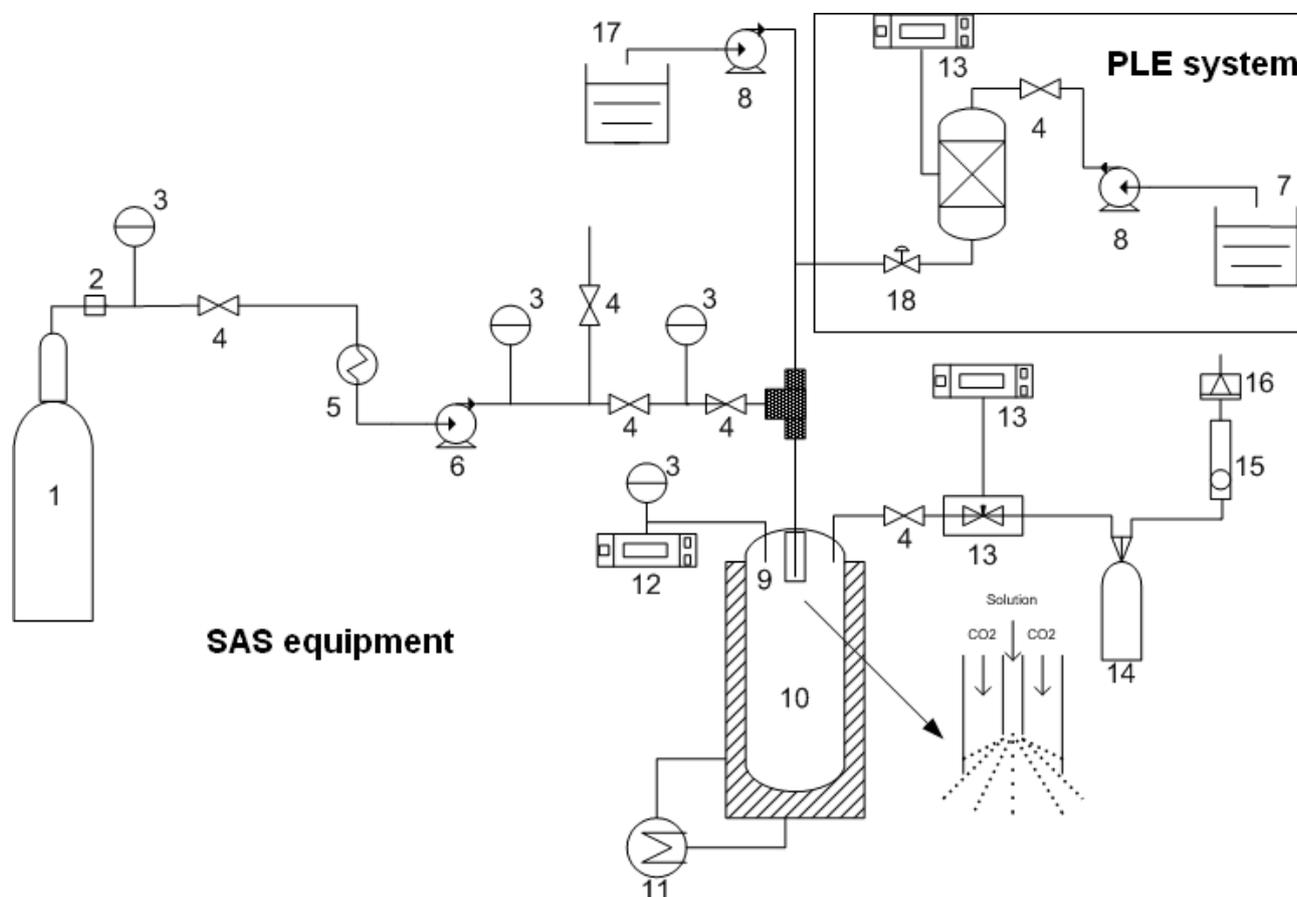


Figure 1. Scheme of the home-built equipment designed to carry out the organic solvent extraction with particle formation on-line (OEPO). 1 CO₂ Cylinder; 2 CO₂ Filter; 3 Manometers; 4 Blocking Valves; 5 Thermostatic bath; 6 CO₂ Pump; 7 Extracting solvent reservoir; 8 HPLC pumps; 9 Thermocouple; 10 Precipitation vessel; 11 Heating bath; 12 Temperature controllers; 13 Micrometric valve with a heating system; 14 Glass flask; 15 Glass float rotameter; 16 Flow totalizer; 17 Carrier or surfactant solution reservoir; 18 Back Pressure regulator

3. Results and Discussion

3.1. Effects of PLE Process Variables on the Extraction Yield and Antioxidant Activity

The effects of extraction temperature and static extraction time on the extraction yield and on antioxidant activity were evaluated. The experimental values at various experimental conditions are presented in Table 2. In the variable ranges of 353–413 K and 5–15 min, the extraction yield variable was significantly (95% confidence level, $p < 0.05$) affected by extraction temperature, static extraction time and their interaction. On the other hand, only extraction temperature was significant (95% confidence level, $p < 0.05$) with respect to the antioxidant activity.

The relationship of the extraction yield, extraction temperature and static extraction time was linear. An increase in either of temperature and static time, while the second variable remains constant, results in enhancement of the extract recovery. Moreover, the interaction between them had also a positive effect on the production of extract (Table 2). With regards of antioxidant activity, the increase of the extraction temperature beyond 373 K possibly might enhance the degradation of the bioactive compound extracted decreasing the antioxidant activity.

Table 2. Summary of the main experimental results (Mean value \pm Standard Deviation)

	Extraction yield (%) d.b.	Antioxidant Activity (%) after 3h of reaction
E1	0.49 \pm 0.03	46 \pm 3
E2	0.51 \pm 0.03	46.5 \pm 0.5
E3	0.74 \pm 0.05	48 \pm 3
E4	0.64 \pm 0.05	56 \pm 4
E5	0.73 \pm 0.05	54 \pm 4
E6	0.93 \pm 0.02	53 \pm 4
E7	0.94 \pm 0.07	42.8 \pm 0.5
E8	1.14 \pm 0.07	46 \pm 3
E9	1.45 \pm 0.01	46 \pm 1
E10	1.48 \pm 0.09	43 \pm 1
E11	1.54 \pm 0.07	38 \pm 3
E12	2.5 \pm 0.2	36 \pm 2

An increase in extraction temperature is reported to improve the efficiency of extraction because of enhanced diffusion rate and solubility of analytes in solvents; nevertheless, high extraction temperatures may simultaneously increase the degradation rate of some extracted compounds [8].

The use of high temperature pressurized solvents in saponin processing is still limited. Mazza group in Canada has been demonstrated that during PLE extraction degradation of the some saponins from cow cockle seeds occurs at 398 K [10,11]. Besides saponin degradation, the increase in the content of non-saponin compounds in the extracts with temperature can also help with the decrease in the antioxidant activity.

Although the extraction yields beyond 373K had been larger, the antioxidant activities were smaller. This confirms

that the extracts obtained under higher temperatures contain other compounds classes that can be reducing the antioxidant activity. The prediction of one set of optimal conditions for both response variables was done by using desirability function approach (Figure 2).

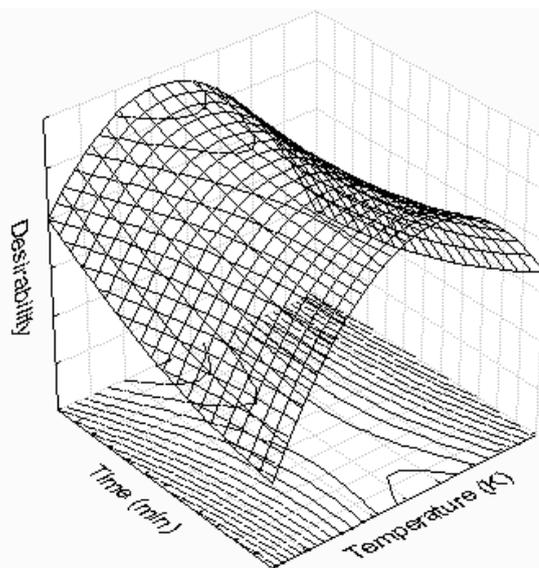


Figure 2. Three-dimensional response surface of the influence of the PLE process variables on the desirability function

In particular, high desirability, within the experimental design values, has been achieved under moderate extraction temperature (373 K) and high static extraction time (15 min). Under this condition an extraction yield of 0.934 % (dry basis, d.b.) and an antioxidant activity of 52.96 % are obtained.

Comparing with synthetic BHT (48.62 %) and with literature data (up to 25 %) employing supercritical CO₂ as extracting solvent [23], it is demonstrated the potentiality of our PLE extracts using ethyl acetate as extracting solvent.

The dynamic extraction time of 20 min was decided after preliminary studies and based on literature data [8]. Obviously, the dynamic extraction time plays an important role on the extraction yield, which will be evaluated in the future experiments.

3.2. Comparison between the PLE Extracts, SAS Precipitated Extract and the Precipitated Extract Obtained from the OEPO Process

The extract obtained under optimum PLE conditions was used as a control to compare antioxidant activity with the SAS precipitate extract and the precipitated extract obtained from the OEPO process (PLE-SAS precipitation)

The pressure during the PLE process was set in all experiments to 12 MPa, given that, this condition permits the coupling of SAS precipitation (and other anti solvent processes) after PLE.

A slightly difference (14.24% lower) was observed between the PLE and OEPO samples, meaning that the OEPO process affects the antioxidant activity of the extracts.

SAS precipitation and OEPO process presented no significant difference (< 5%) between the samples, as expected. This difference between the samples can be attributed to the solubilization of some compounds in supercritical CO₂ during anti solvent process[24]. Further improvements in our equipment in order to determine this loss will be done.

A reference point for the anti solvent processes is the mixture critical point for the binary system CO₂–organic solvent. In addition to the complete miscibility of the selected organic solvent in the supercritical anti solvent, a partial solubilization of some class of compounds can be aimed for fractionation purposes[25]. Recently, good results were obtained to fractionate the antioxidants from methanolic extract solution obtained from grape wastes without degradation and with the complete elimination of the solvent residues[26]. In our case, the fractionation phenomenon was undesired, but tuning the supercritical CO₂ density, we can probably reduce the observed difference or even improve the antioxidant activity.

3.3. Comparison between the SAS Co-Precipitated Extract and the Co-Precipitated Extract Obtained from the OEPO Process

Figure 3 shows the pictures of SAS co-precipitated extract and the co-precipitated extract obtained from OEPO process (PLE-SAS co-precipitation) obtained by scanning electron microscope (SEM).

No significant differences were observed between the samples, meaning that the OEPO process does not have positive nor adverse effect on the morphology of the co-precipitated extracts.

Recently, our research group evaluated the influence of several process variables during SAS co-precipitation of bixin-rich extract also in PEG[27]. Taking into account this study, we selected operating conditions that effectively encapsulate the extract minimizing extract and carrier material losses with supercritical CO₂ flow. Obviously, as well as occurred during SAS precipitation the extract loss

may have reduced the antioxidant activity. Once again, this antioxidant activity change can be avoided with the optimization of the anti solvent process.

3.4. Comparison between the Dried SFEE Encapsulated Extract and the Dried Encapsulated Extract Obtained from the OEPO Process

OEPO process was also effective for the production of encapsulated extract. In Figure 4, SEM images of freeze-dried encapsulated extracts suspensions in water obtained by SFEE process and PLE-SFEE process, respectively. It is observed that both produced dried particles were spherical. Otherwise, the particles produced by OEPO process presented higher degree of porosity in their surface and higher particle diameter than SFEE particles.

Briefly, in the SFEE process the emulsion and the supercritical CO₂ are injected into the precipitation vessel continuously, and CO₂ diffuses through the aqueous phase to the drop, extracting organic solvent out of the drop and precipitating the solute dissolved into the organic phase due to the anti solvent effect of the carbon dioxide[28].

In SFEE process, like in the previously reported anti solvent processes, operating conditions, in general, are selected in order to facilitate maximum extraction of the organic phase with minimum extract and carrier material losses due to dissolution in CO₂. In contrast, process variables like pressure and temperature are more closely related to the capacity to eliminate the remaining organic solvent from the products than to the final particle size[29].

The preparation of the emulsion has demonstrated to be the crucial point for the production of fine particles. The similarity between particle sizes of the initial emulsion and the final suspension suggests that the final particle size is dependent of the original droplet size of the emulsion[28-30]. Thus, the production of larger particles by OEPO process can be associated to the quality of the emulsion prepared using the T-mixer compared with that emulsion prepared using high speed-stirring mixer.

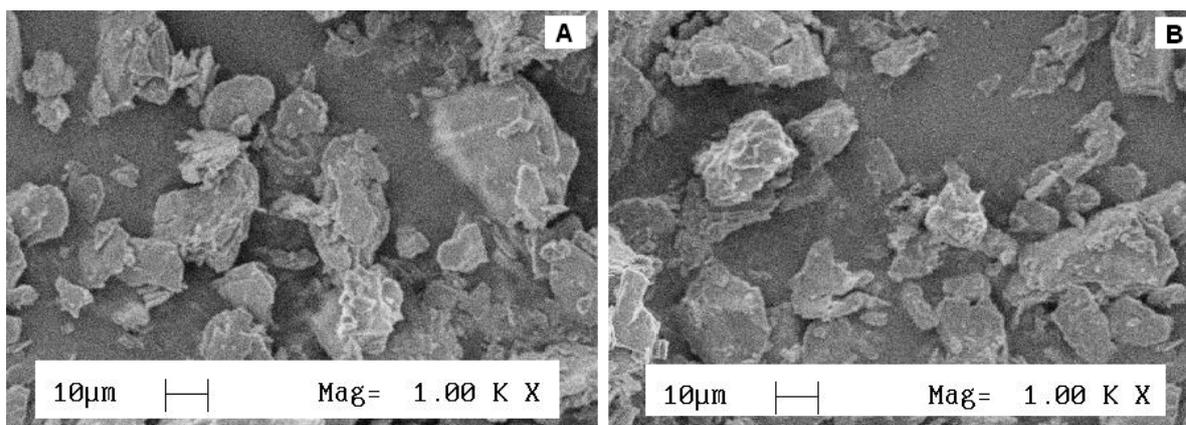


Figure 3. Scanning electron micrographs of: a) SAS co-precipitated extract; b) co-precipitated extract obtained from the OEPO process

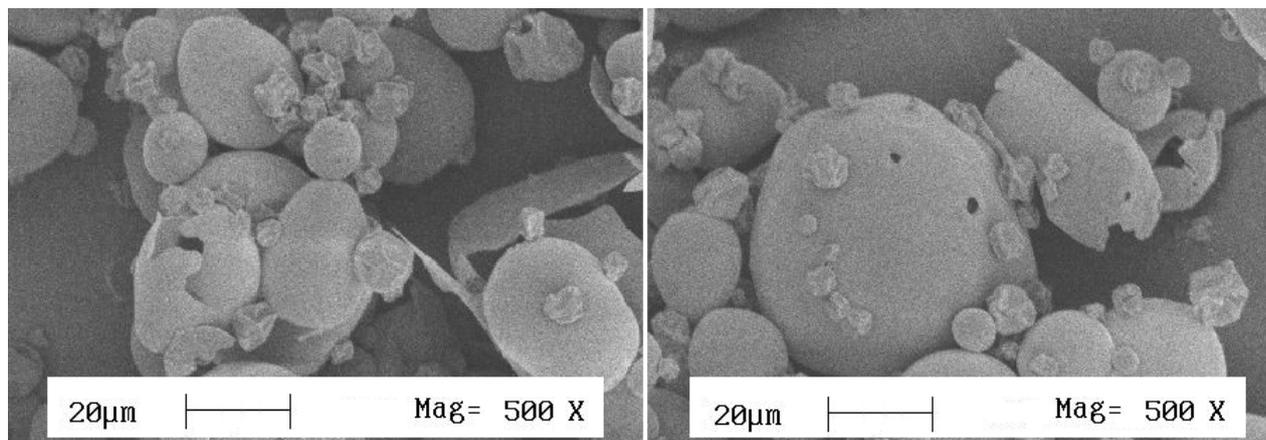


Figure 4. Scanning electron micrographs of: a) dried SFEE encapsulated extract; b) dried encapsulated extract obtained from the OEPO process

The fabrication of water-in-oil emulsions is a process with widespread applications in formulation engineering. The idea to create the emulsion in line and operate the process in a continuous mode is not new, existing several commercial in-line dispersing technologies already available [31]. Better results could be obtained with the optimization of this point during OEPO process. Further experiments will be done in this direction. In addition, the use of ultrasound irradiation in this stage and also in the PLE process in order to try to enhance the extract's antioxidant activity will be studied in the future for the improvement of OEPO process.

Evidently, the OEPO process can be used for other plant materials. The use of other organic solvents as extracting solvents though is limited since they have to be miscible in the supercritical fluid. Another limitation for the choice of the organic solvent is the international regulations regarding the safety of consumers. Others class 3 solvents (like ethyl acetate) that meet both requirements are: ethanol, acetone, Dimethyl sulfoxide, among others. With regard to the choice of the carrier material, their biocompatibility and lack of toxicity are of course important considerations as well their low solubility in the supercritical fluid. Polyethylene glycol (PEG), used in this study, has been extensively used for precipitation and co-precipitation studies with supercritical fluids due to achieve both needs [13]. A disadvantage of the use of PEG, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), among others, is that they are only dissolved in dichloromethane (class 2 solvent - which means that it can be used with products for human consumption, although subject to a legal concentration limit in the final product). Thus, biodegradable polymers that can be dissolved in lower toxic solvents such as poly-lactic acid (PLA) (dissolves in ethyl acetate) should be preferred for PLE-SAS co-precipitation [32]. For SFEE during the hyphenated PLE-SFEE process, some additional benefits can be obtained if the surfactant material can also be used as carrier material. N-octenylsuccinic anhydride (OSA)-modified starch surfactant, employed in this study, is being expansively used because they are suitable for food and nutraceutical applications and they are also capable of providing a double functionality as a surfactant for the emulsion stabilization and as a carrier

material in the final product [14]. Another versatility of our process is related to the possibility of change the pressure during the precipitation stage, which can as previously reported, promote a fractionation/purification of the extract produced. For this, we have to set the pressure of the previous stage (PLE extraction) higher or at least equal to the next stage (Anti solvent process). In this study, we have set the PLE pressure at 12 MPa (120 bar) due to the range usually used for the anti solvent processes studied be in the range of 8-12 MPa [13-15]. Obviously, together with a fractionation/purification the organic solvent elimination rate will also be changed producing products with different residual organic solvent concentrations. This analysis of the residual ethyl acetate concentration was not done in this work, but at the operating conditions employed during the anti solvent processes it is expected a concentration lower than 50 ppm, while the conventional solvent evaporation results in a residual content of around 500 ppm. [33,34].

4. Conclusions

Organic solvent Extraction and Particle formation On-line (OEPO) process was described in detail and successfully developed using Brazilian ginseng roots (*Pfaffia glomerata*) as a model case. This novel process consists of an on-line process for pressurized hot organic solvent extraction of plant materials and precipitation of the extract with or without a carrier material by organic solvent elimination in one step, based on the use of supercritical CO₂ as anti solvent.

The use of PLE conditions employing ethyl acetate as extracting solvent for obtaining antioxidants from *Pfaffia glomerata* roots set at 12 MPa and 373 K under a static extraction time of 15 min was selected for further coupling with SAS precipitation, SAS co-precipitation with PEG and SFEE using OSA-modified starch as surfactant/carrier material. Indeed, OEPO process consists of hyphenated PLE-SAS precipitation, PLE-SAS co-precipitation and PLE-SFEE. Under this PLE condition an extraction yield of 0.934% (dry basis, d.b.) and an antioxidant activity of

52.96% were obtained, which was slightly higher (14.24%) than that observed after PLE-SAS precipitation and after SAS precipitation performed in two steps (step one - PLE extraction; step two - SAS precipitation by the use of the extract solution produced by step one stored). Similar behavior (hyphenated process producing similar products than the two step process done separately) was observed for PLE-SAS co-precipitation and PLE-SFEE indicating that the OEPO process developed in this work can be considered as a suitable and promising process to obtain, in only one step, different products (precipitated extract, co-precipitated extract or encapsulated extract in suspension) with desired antioxidant activity and particle size, directly from plant materials.

ACKNOWLEDGEMENTS

Diego T. Santos would like to thank the FAPESP (2010/16485-5) for a postdoctoral fellowship. Maria Thereza M. S. Gomes and Renata Vardanega would like to thank CNPq for the doctoral and the master fellowships, respectively. Dayane F. Barbosa and Ketllen Broccoli are thankful to SAE/Unicamp. The authors acknowledge the financial support from CNPq(564721/2010-7) and FAPESP (2009/17234-9).

REFERENCES

- [1] M. Angela A. Meireles, *Extracting Bioactive Compounds for Food Products: Theory and Applications*, Contemporary Food Engineering series, CRC Press, 2008.
- [2] Guang-hua Lu, Qun Zhou, Su-qin Sun, Kelvin S. Leung, Hao Zhang, Zhong-zhen Zhao, "Differentiation of Asian ginseng, American ginseng and Notoginseng by Fourier transform infrared spectroscopy combined with two-dimensional correlation infrared spectroscopy", *Elsevier, Journal of Molecular Structure*, vol. 883-884, pp. 91-98, 2008.
- [3] Aline R. Zimmer, Fernanda Bruxel, Valquíria L. Bassani, Grace Gosmann, "HPLC method for the determination of ecdysterone in extractive solution from *Pfaffiaglomerata*", *Elsevier, Journal of Pharmaceutical and Biomedical Analysis*, vol. 40, pp. 450-453, 2006.
- [4] I. M. Júnior, "Avaliação de genótipos de *Pfaffiaglomerata* (Spreng.) Pedersen visando o seu cultivo comercial", Master's degree dissertation, Agronomic Institute of Campinas, Brazil, 2005.
- [5] Cristina S. Freitas, Cristiane H. Baggio, Samanta L. Araújo, Maria C. A. Marques, "Effects of *Pfaffiaglomerata* (Spreng) Pedersen Aqueous Extract on Healing Acetic Acid-induced Ulcers", *SciELO, Brazilian Archives of Biology and Technology*, vol. 51, no. 4 : pp. 679-683, 2008.
- [6] Cristina S. Freitas, Cristiane H. Baggio, José E. Silva-Santos, Lia Riecka, Cid A. M. Santos, Cirino C. Júnior, Lin C. Ming, Diógenes A. G. Cortez, Maria C. A. Marques, "Involvement of nitric oxide in the gastroprotective effects of an aqueous extract of *Pfaffiaglomerata* (Spreng) Pedersen, *Amaranthaceae*, in rats", *Elsevier, Life Sciences*, vol. 74, pp. 1167-1179, 2004.
- [7] Rejane Flores, Fernando T. Nicoloso Daniela Brondani, Joseila Maldaner, Verciane Cezarotto, Sandro R. Giacomelli, "Extraction of ecdysterone from roots of Brazilian ginseng", *SciELO, Ciência Rural*, vol. 39, no. 4, pp. 1223-1226, 2009.
- [8] Diego T. Santos, Priscilla C. Veggi, M. Angela A. Meireles, "Optimization and economic evaluation of pressurized liquid extraction of phenolic compounds from jaboticaba skins", *Elsevier, Journal of Food Engineering*, vol. 108, pp. 444-452, 2012.
- [9] Diego T. Santos, Juliana Q. Albarelli, Marisa M. Bepu, M. Angela A. Meireles, "Stabilization of anthocyanin extract from jaboticaba skins by encapsulation using supercritical CO₂ as solvent", *Elsevier, Food Research International*. in press.
- [10] Ozlem Guclu-Ustundag, Giuseppe Mazza, "Extraction of saponins and cyclopeptides from cow cockle seed with pressurized low polarity water", *Elsevier, LWT-Food Science and Technology*, vol. 41, pp. 1600-1606, 2008.
- [11] Ozlem Guclu-Ustundag, John Balsevich, Giuseppe Mazza, "Pressurized low polarity water extraction of saponins from cow cockle seed", *Elsevier, Journal of Food Engineering*, vol. 80, pp. 619-630, 2007.
- [12] Ernesto Reverchon, "Supercritical antisolvent precipitation of micro- and nano-particles", *Elsevier, The Journal of Supercritical Fluids*, vol.15, no.1, pp.1-21, 1999.
- [13] María J. Cocero, Ángel Martín, Facundo Mattea, Salima Varona, "Encapsulation and co-precipitation processes with supercritical fluids: fundamentals and applications", *Elsevier, The Journal of Supercritical Fluids*, vol.47, no.3, pp.546-555, 2009.
- [14] Diego T. Santos, Ángel Martín, M. Angela A. Meireles, María José Cocero, "Production of stabilized sub-micrometric particles of carotenoids using supercritical fluid extraction of emulsions", *Elsevier, The Journal of Supercritical Fluids*, vol.61, pp.167-174, 2012.
- [15] Diego T. Santos, M. Angela A. Meireles, "Carotenoid pigments encapsulation: fundamentals, techniques and recent trends", *Bentham Open, The Open Chemical Engineering Journal*, vol.4, pp.42-50, 2010.
- [16] Maria E. E. Ibanez, I., Alejandro Cifuentes., Irene Rodriguez-Meizoso, José A. Mendiola, Guillermo Reglero, Javier Señorans, Charlotta Turner, Device and process for the on-line extraction and drying of complex extracts, Spanish Patent no. P200900164, 2009.
- [17] Esther Paz, Ángel Martín, Antonio Estrella, Soraya Rodríguez-Rojo, Ana A. Matias, Catarina M. M. Duarte, María José Cocero, "Formulation of β -carotene by precipitation from pressurized ethyl acetate-on-water emulsions for application as natural colorant", *Elsevier, Food Hydrocolloids*, vol.26, no.1, pp.17-27, 2012.
- [18] Miguel A T, Cardoso, Sofia Antunes, Frederik V. Keulen, Bruno S Ferreira, Augusto Galdes, Joaquim M. S. Cabral, António M. F. Palavra, "Supercritical antisolvent micronisation of synthetic all-trans- β -carotene with tetrahydrofuran as solvent and carbon dioxide as antisolvent", *Elsevier, Journal of Chemical Technology and Biotechnology*, vol. 84, pp. 215-222, 2009.

- [19] ASAE–American Society of Agricultural Engineers, "Method of determining and expressing fineness of feed materials by sieving", in: American Society of Agricultural Engineers Standard, ASAE–American Society of Agricultural Engineers, pp. 447- 448, 1993.
- [20] AOAC-Association of Official Analytical Chemists, in: P. Cuniff (Ed.), AOAC International, Gaithersburg, Maryland, 1997.
- [21] Gino J. Marco, "A rapid method for evaluation of antioxidant", Springer, Journal of The American Oil Chemist's Society vol. 45, pp. 594, 1968.
- [22] Diego T. Santos, Priscilla C. Veggi, M. Angela A. Meireles, "Extraction of antioxidant compounds from jabuticaba (*Myrciaria cauliflora*) skins: yield, composition and economical evaluation", Elsevier, Journal of Food Engineering, vol. 101, pp. 23-31, 2010.
- [23] Patrícia F. Leal, Marina B. Kfoury, Fábio C. Alexandre, Fábio H. R. Fagundes, Juliana M. Prado, Marcos H. Toyama, M. Angela A. Meireles, "Brazilian Ginseng extraction via LPSE and SFE: Global yields, extraction kinetics, chemical composition and antioxidant activity", Elsevier, Journal of Supercritical Fluids, vol. 54, pp. 38-45, 2010.
- [24] Owen J. Catchpole, John.B. Grey, KeithA. Mitchell, Jiansheng S. Lan "Supercritical antisolvent fractionation of propolis tincture", Elsevier, Journal of Supercritical Fluids, vol. 29, pp. 97-106, 2004.
- [25] Yueh-Cheng Cho, Jia-Hui Cheng, Shih-Lan Hsu, Siang-En Hong, Tse-Min Lee, Chieh-Ming J. Chang, "Supercritical carbon dioxide anti-solvent precipitation of anti-oxidative zeaxanthin highly recovered by elution chromatography from *Nannochloropsis oculata*", Elsevier, Separation and Purification Technology, vol. 78, 274-280, 2011.
- [26] Tomas Floris, Giorgia Filippino, Stefania Scrugli, Maria B. Pinna, Francesca Argiolas, Antonio Argiolas, Mariano Murru, Ernesto Reverchon, "Antioxidant compounds recovery from grape residues by a supercritical antisolvent assisted process", Elsevier, Journal of Supercritical Fluids, vol. 54, 165-170, 2010.
- [27] Diego T. Santos, M. Angela A. Meireles, "Micronization and encapsulation of functional pigments using supercritical carbon dioxide", Wiley, Journal of Food Process Engineering, in press.
- [28] Pratibhash Chattopadhyay, Boris Y. Shekunov, Jeff S. Seitzinger, Robert W. Huff, Particles from supercritical fluid extraction of emulsion, US Patent no0026319 A1, 2004.
- [29] Facundo Mattea, Ángel Martín, Constantin Schulz, Philip Jaeger, Rudolf Eggers, Maria J. Cocero, "Behavior of an organic solvent drop during the supercritical extraction of emulsions", American Institute of Chemical Engineers, AIChE Journal, vol.56, no.5, pp.1184-1195, 2010.
- [30] Facundo Mattea, Ángel Martín, Arán Matías-Gago, María J. Cocero, "Supercritical antisolvent precipitation from an emulsion: β -Carotene nanoparticle formation", Elsevier, The Journal of Supercritical Fluids, vol.51, no.2, pp.238-247, 2009.
- [31] Benjamin Brocart, Philippe A. Tanguy, César Magnin, Jacques Bousquet, "Design of In-Line Emulsification Processes for Water-in-Oil Emulsions", Taylor & Francis, Journal of Dispersion Science and Technology, vol. 23, no. 1-3, 2002.
- [32] Fernando Miguel, Ángel Martín, Facundo Mattea, Maria J. Cocero, "Precipitation of lutein and co-precipitation of lutein and poly-lactic acid with the supercritical antisolvent process", Elsevier, Chemical Engineering and Processing: Process Intensification, vol. 47, no. 9-10, pp. 1594-1602, 2008.
- [33] Aaron S. Mayo, Balamurali K. Ambati, Uday B. Kompell, "Gene delivery nanoparticles fabricated by supercritical fluid extraction of emulsions", Elsevier, International Journal of Pharmaceutics, vol. 387, pp. 278-285, 2010.
- [34] Giovanna D. Porta, Ernesto Reverchon, "Nanostructured Microspheres Produced by Supercritical Fluid Extraction of Emulsions", Wiley, Biotechnology and Bioengineering, vol. 100, no. 5, pp. 1020-1033, 2008.