OPTIMIZATION OF PRESSURIZED LIQUID EXTRACTION OF ECDYSTEROIDS FROM BRAZILIAN GINSENG (*Pfaffia glomerata*) ROOTS

Isabel C. N. Debien, Renata Vardanega, Diego T. Santos and M. Angela de A. Meireles*

LASEFI/DEA (School of Food Engineering)
UNICAMP (University of Campinas)
Rua Monteiro Lobato, 80; Campinas, São Paulo, Brazil

Email: meireles@fea.unicamp.br

Abstract. Species of the genus *Pfaffia* are popularly known as substitute for *Panax* due their similar morphology and bioactive properties. Among them, *Pfaffia glomerata* has received special attention due the presence of ecdysteroids. Commercially, this compound is obtained by conventional solid-liquid extraction methods, that is, using large quantities of solvents. Nowadays, more environmentally friend methods are preferred, like pressurized liquid extraction (PLE) that enables rapid extraction of bioactive compounds under high temperatures. In this work, the effects of temperature (333 – 393 K), pressure (8 – 30 MPa) and solvent [ethanol and ethanol:water (80:20 v/v)] on the global yield extraction and antioxidant activity of the extracts from Brazilian ginseng (*Pfaffia glomerata*) roots were studied. The global yield extraction increased with the increase in temperature while pressure did not affect the extraction yield. For the solvent ethanol, the global yields varied from 1.5% at 333 K to 5.0% at 393 K. For the mixture of ethanol:water, the global yields varied from 7.5% at 333 K to 25.1% at 393 K. As the temperature increased the selectivity of the solvent decreased promoting the co-extraction of other compounds besides the ecdysteroids. This behavior may explain the slightly higher antioxidant activities observed for the extracts obtained at 333 K as compared to that obtained at 363 and 393 K for the solvent ethanol; again, the pressure did not affect the response variable. A similar behavior was observed for the mixture of ethanol:water, except that at 333 K the antioxidant activities increase from 8 to 20 MPa and decreased afterwards. Also, ethanol was more selective to extract ecdysteroids, besides fractions containing up to 5 % of β-ecdysone were obtained using this solvent at 393 K.

Keywords : *Pfaffia glomerata*, pressurized liquid extraction, bioactive compounds, antioxidant activity.

1. Introduction

Plants have been the basis of traditional medicines for thousands of years, and continue to be considered valuable materials in medicines [1]. Among these plants there are the Brazilian ginseng, which has been commercialized as substitute for plants of the genus *Panax* due their similar morphology and bioactive properties. *Pfaffia glomerata* is the most important specie of the plants known as Brazilian ginseng due the presence of ecdysteroids [2]. The main constituent of *P. glomerata* is the β-ecdysone due it adaptogens effects and is used as a good marker for the differentiation of species of genus *Pfaffia* [3, 4].

Commercially, extracts from *P. glomerata* roots containing bioactive compounds are obtained by conventional extraction methods, that is, using large quantities of solvents. Nowadays, more environmentally friend methods are preferred, like pressurized liquid extraction (PLE) that has been successfully used for the extraction of several bioactive compounds from different plants [5]. 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 [6].

The use of a pressurized liquid extraction (PLE) technique is an attractive alternative because it allows for fast extraction and reduced solvent consumption. PLE enables the rapid extraction (less than 30 min) of analytes in a closed and inert environment under high pressures [no higher than 300 bar (30 MPa)] and temperatures (25–200°C). Hence, extracting solvents that are inefficient in extracting at low temperatures, may be much more efficient at the elevated temperatures used in PLE [7].

The aim of this work was study of extraction of ecdysteroids from *P. glomerata* roots by PLE. The parameters evaluated were temperature, pressure and extracting solvent. The results were evaluated based on global yield extraction (X_0), antioxidant activity and content of β-ecdysone in the extracts obtained.

2. Material and Methods

2.1. Raw material characterization and preparation

Brazilian ginseng roots (*Pfaffia glomerata*) were cultivated in the experimental field of CPQBA (Campinas, Brazil), where they were collected on November 17, 2008, being 7 years old. They were washed and dried in a forced air circulation dryer at 313 K for 5 days. The dried roots (10.8 % 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 18,000rpm 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 (Metalfrio, model DA 420, São Paulo, Brazil) at 263 K. For the extraction assays, particles of 8 μm of diameter, according to ASAE methodology [8], were used. The moisture content of the dried roots was determined by the AOAC method (Method 4.1.03) [9].

2.2. Experimental design

The effects of the following parameters were evaluated on extraction yield (X_0), antioxidant activity and content of β-ecdysone of *P. glomerata* roots extracts: temperature (333, 363 and 393 K), pressure (8, 10, 15, 20, 25 and 30 MPa) and extracting solvent [Ethanol and Ethanol:Water (80:20, v/v)]. A full factorial design (3x6x2) fully randomized was performed (Table 1). The analyse of influence of parameters was performed through the analysis of variance (ANOVA) using the software Minitab 16® (Minitab Inc., State College, PA, USA) with 95% of confiance (p-value ≤0,05).

Table 1. Experimental matrix of extraction conditions.

Extraction conditions								
Assay	Pressure (MPa)	Solvent	Assay	Pressure (MPa)	Solvent	Assay	Pressure (MPa)	Solvent
Temperature: 333 K			Temperature: 363 K			Temperature: 393 K		
1	8	EtOH	13	8	EtOH	25	8	EtOH
2	8	EtOH+H_2O	14	8	EtOH+H_2O	26	8	EtOH+H_2O
3	10	EtOH	15	10	EtOH	27	10	EtOH
4	10	EtOH+H_2O	16	10	EtOH+H_2O	28	10	EtOH+H_2O
5	15	EtOH	17	15	EtOH	29	15	EtOH
6	15	EtOH+H_2O	18	15	EtOH+H_2O	30	15	EtOH+H_2O
7	20	EtOH	19	20	EtOH	31	20	EtOH
8	20	EtOH+H_2O	20	20	EtOH+H_2O	32	20	EtOH+H_2O
9	25	EtOH	21	25	EtOH	33	25	EtOH
10	25	EtOH+H_2O	22	25	EtOH+H_2O	34	25	EtOH+H_2O
11	30	EtOH	23	30	EtOH	35	30	EtOH
12	30	EtOH+H_2O	24	30	EtOH+H_2O	36	30	EtOH+H_2O

2.3. Extraction procedure

The diagram of the PLE system is shown in Figure 1. Approximately 10 grams of *P. glomerata* roots was placed in the 415 mL extraction cell. The empty space of the cell was filled with a Teflon column. The cell containing the sample was heated by a jacket connected to a thermostatic bath until the temperature desired. The extracting solvent was pumped by a HPLC pump (Thermoseparation Products, Model ConstaMetric 3200 P/F, Florida, USA) into the extraction cell until the pressure desired was obtained. After pressurization, the *P. glomerata* roots with pressurized solvent were kept statically at the desired pressure for 10 minutes (static time). Thereafter, the block valve (Autoclave engineers, Modelo 10V2071 15000psi, Erie, Pennsylvania, USA) was opened and the pressure was keeping constant by a heated micrometric valve (Autoclave engineers, Modelo 10VRMM 11000PSI Erie, Pennsylvania, USA).

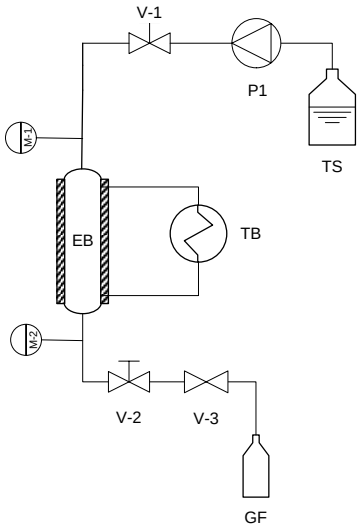

Figure 1. Schematic diagram of the pressurized liquid extraction unit. TS: Tank of solvent; P1: High pressure pump; V-1 and V-2: block valve; V-3: micrometric valve; M-1 and M-2: manometers; EB: extraction bed; TB: thermostatic bath; GF: glass flask.

The flow rate of extracting solvent was fixed in 9.5 mL/min. The extracts were collected into a glass flask immersed in ice bath at ambient pressure until to achive a S/F (solvent mass/feed mass) equal 16 (approximately 20 min) . The extracts were submitted to evaporation in vacuum-equipped rotary evaporator (Heidolph, model Laborota 4001 WB, Viertrieb, Germany) with water bath set at 50°C to eliminate the alcoholic solvent and when used a mixture ethanol:water (80:20, v/v) as extracting solvent, the remaining water was eliminate by lyophization (Liobras, model L101, São Carlos, SP, Brazil). The X_0 was calculated as the ratio between the total mass of extract and the initial mass of raw material (dry basis, d.b) fed into the extractor.

2.4. Antioxidant activity

The antioxidant activity of *P. glomerata* roots extracts was evaluated using the coupled reaction of linolenic acid and β-carotene methodology, described by Leal et al. [10]. In short, the substrate of reaction was prepared using 10mg of β-carotene (Acros, 99% purity, lot B0070864, New Jersey, NJ), 10mL of chloroform (Merck, 99.0‑99.4% purity, lot K31503045 301, Darmstadt, Germany), 60mg of linolenic acid (Sigma‑Aldrich, 99% purity, lot 054K1214, St. Louis, MO) and 200 mg of Tween 40 (Sigma‑Aldrich, 99% purity, lot 032K0104, St. Louis, MO). This solution was concentrated in rotary evaporator (Heidolph, model Laborota 4001 WB, Viertrieb, Germany) at 50 °C, being then diluted in 50 mL of distilled water. The oxidation reaction was conducted using the following procedure: to each 1 mL of substrate, 2 mL of distilled water and 0.05 mL of extract diluted in ethanol (Dinâmica, 99.5% purity, lot 52990, Diadema, SP, Brazil) were added, which were diluted in metanol (Merck, 99.8% purity, lot K28185309 035, Darmstadt, Germany). The dilution used for AA determination was 0.02 g of extract/mL of solvent. The mixture was placed in

thermal bath (Marconi, model MA127/BO, Piracicaba, SP, Brazil) at 40 ◦C, and the product of reaction was monitored using a spectrophotometer (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 by Eq. (1), proposed by Skerget et al. [11].

$$AA = 100 \times \left(1 - \frac{abs_{extract}^{t=0} - abs_{extract}^{t}}{abs_{control}^{t=0} - abs_{control}^{t}}\right) \quad (1)$$

Where abs is the absorbance at 470 nm and t is the time.

2.5. β-ecdysone quantification

β-ecdysone quantification was performed according methodology described by Leal et al [12] with modifications. The HPLC Agilent 1260 (Santa Clara, CA, United States) system used included a quarternary pump (G1311A), a photodiode array detector (G4214B), column oven (G1311A) and automatic injector (G1329B). The column used was the Zorbax Eclipse Plus (C18, 5µm, 150 x 4,6 mm, Agilent, Santa Clara, CA, United States) operating at 50°C. The mobile phases used were water with acetic acid 0.1% (A) and acetonitrile with acetic acid 0.1% (B). Gradient of B from 0 to 100% in 15 min followed by gradient of B from 100 to 0% in 10 min and maintaining A 100% during 5 min at a flow rate of 1.2 mL/min. Peaks were indentified by comparison of their retention times and UV-vis spectra with those of known standard. Quantification was based on the peak area measurements at 254nm. The linearity of the method was determined through the 20-hydroxyecdysone (≥ 93%, Sigma). The extracts were diluted in distilled water in order to obtain 10 mg/mL concentration. The solutions were filtered through a 0.45µm membrane (Milipore).

3. Results and Discussion

3.1. Global yield extraction (X_0)

The X_0 for *P. glomerata* roots extraction by PLE ranged from 1.5 to 25.0 % (d.b). Figure 2 and 3 shows the global yield isotherms (GYI) obtained in different conditions using ethanol and ethanol:water (80:20, v/v), respectively. It can be seen that when ethanol was used as extracting solvent the increase of temperature leads to an increase of X_0 (Figure 2). However, when EtOH:water (80:20, v/v) was used as extracting solvent, from 15 MPa it can be seen that the isotherms cross each other (Figure 3). This behavior is typical of systems containing water. Leal et al. [13] also observed the same behavior to sweet basil extraction by supercritical fluid extraction (SFE) using CO_2 as extracting solvent and water as cosolvent.

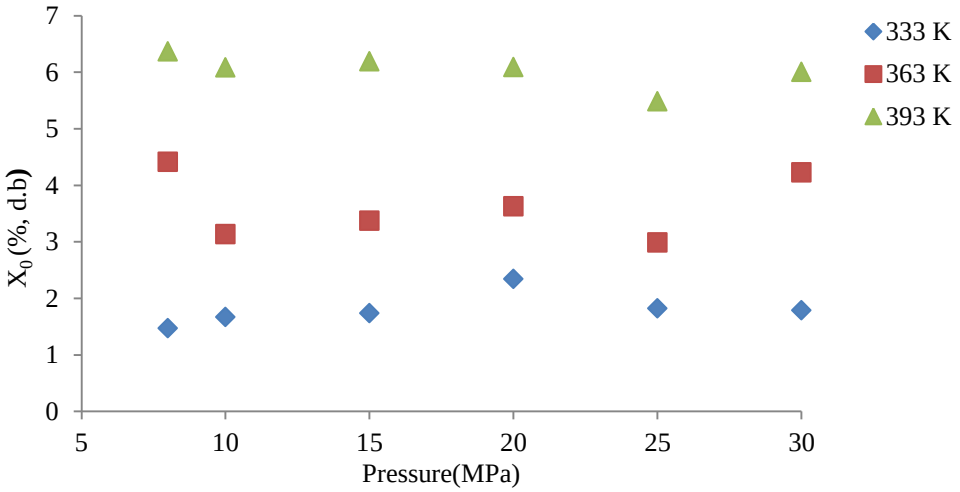

Figure 2. Global yield isotherms for *P. glomerata* roots extraction by PLE using ethanol as extracting solvent.

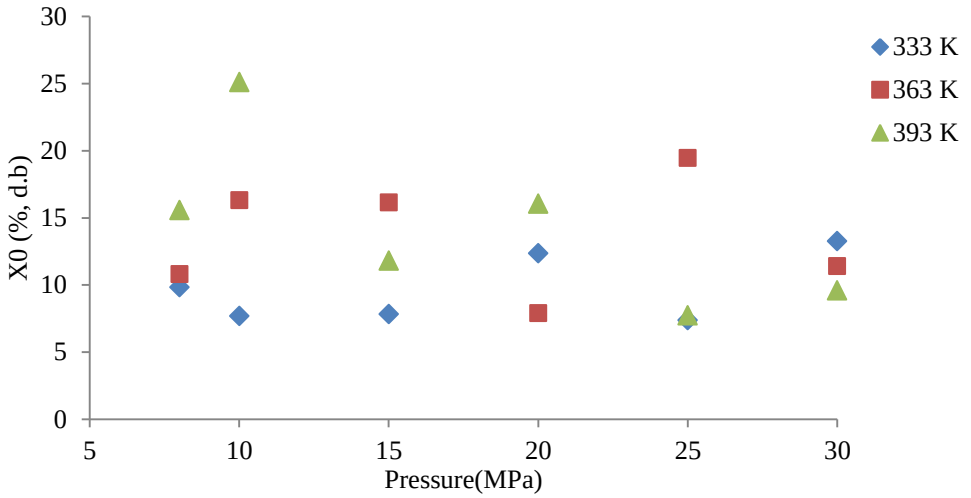

Figure 3. Global yield isotherms for *P. glomerata* roots extraction by PLE using ethanol:water (80:20, v/v) as extracting solvent.

The influence of the parameters on the X_0 was evaluated by analysis of variance (ANOVA). The parameters that influenced significantly on the X_0 were temperature (p-value = 0,041) and extracting solvent (p-value = 0,000). Figure 4 shows means values of X_0 to verify what were the levels that provide X_0 maximized. The temperature and extracting solvent that improved extraction performance were 393K and EtOH:water (80:20, v/v), respectively.

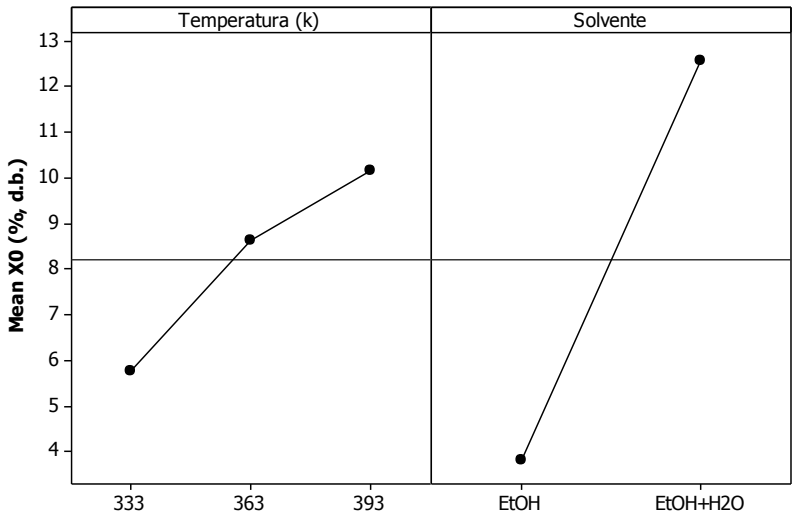

Figure 4. Principal effects of the statistically significant parameters over the X_0 of the *P. glomerata* roots extraction

It is known that higher temperatures provide an increase of the diffusivity of the solvent in the raw material and also increase the solubility of the solute from the raw material into the solvent [14]. The combined effect of these factors provides a major capacity of the extracting solvent to remove the solute from the cells, resulting in highest yield extraction.

The highest X_0 was obtained using EtOH:water (80:20, v/v) as extracting solvent probably due the polarity of this mixture (5.96) is nearly higher than that of pure ethanol (5.2). In other study of *P. glomerata* roots extraction by PLE, it was evaluated the X_0 using different solvents. The X_0 obtained ranged from 0.2 to 40.5 % (d.b.) being the highest values of X_0 obtained using water as extracting solvent [15]. These results suggest that in *P. glomerata* roots the components of polar nature are abundant.

3.2. Antioxidant activity

The antioxidant activity for *P. glomerata* roots extraction by PLE ranged from 13.3 to 48.3 %. Figure 5 shows the experimental results and it can be seen that the highest values of antioxidant activity were obtained using ethanol:water (80:20, v/v) as extracting solvent. Santos et al. [16] obtaining extracts from *P. glomerata* roots using ethanol and ethanol:water (70:30, v/v) by dynamic low pressure solvent extraction also observed that the use of hidroalcholic mixture as extracting solvent produces extracts with higher antioxidant activity (14.4 %) than those when ethanol is used (2.6 %).

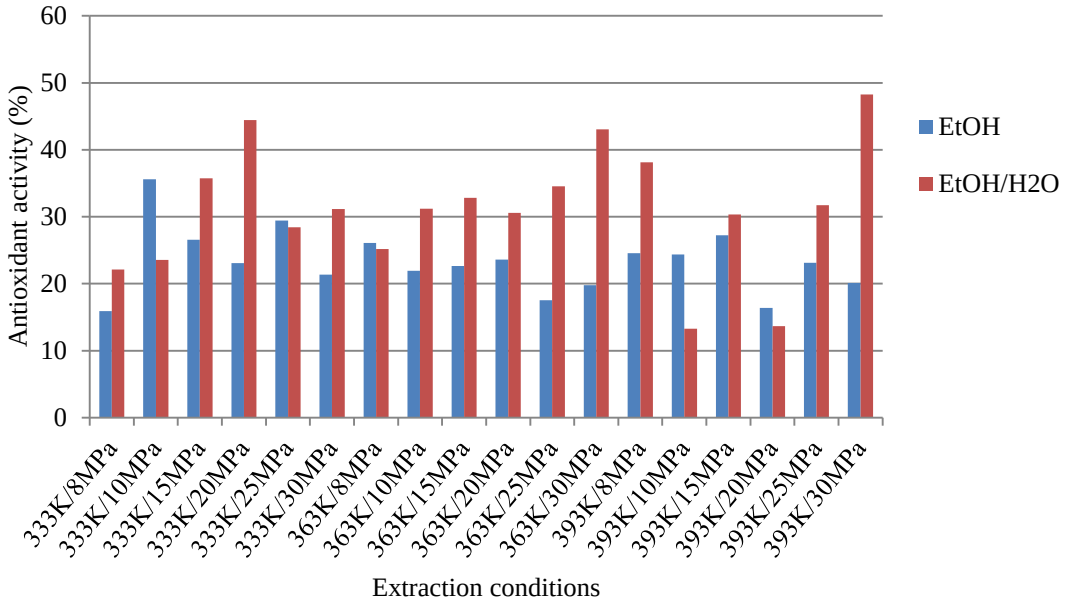

Figure 5. Antioxidant activity of *P. glomerata* roots extracts obtained by PLE in different conditions.

The influence of the parameters on the antioxidant activity was evaluated by analysis of variance (ANOVA). The parameter that influenced significantly on the antioxidant activity was only extracting solvent (p-value = 0,008). According to statiscal analysis the extracting solvent that improved extraction performance were EtOH:water (80:20, v/v). However, is possible to seen in Figure 5 that in some conditions ethanol present higher antioxidant activity values than ethanol:water (80:20, v/v).

.

3.3. β-ecdysone quantification

The β-ecdysone content of *P. glomerata* roots extraction by PLE ranged from 1.1 to 49.7 mg/g extract. Figure 6 shows the experimental results. It can be seen clearly that the highest values of β-ecdysone content were obtained using ethanol as extracting solvent. These results suggest that ethanol is more selective than ethanol:water (80:20, v/v) to extract β-ecdysone from *P. glomerata*. Comparing the antioxidant activity and β-ecdysone content of the extracts, there was no correlation between presence of β-ecdysone and antioxidant activity of the extracts. Further studies for determination of the chemical composition of the extracts are needed in order to determine the compounds responsible for its antioxidant activity.

While the *P. glomerata* roots extracts containing 4.97% of β-ecdysone were obtained in this study after around 20 minutes at optimum conditions, Leal et al [12], obtained extracts from the same plant with the same concentration of β-ecdisone (4.60%) by SFE using CO_2+ ethanol as extracting solvent after 5 hours of process.

The β-ecdysone yield in terms of raw material obtained by other authors ranged from 0.35 to 0.43% (d.b.) [2,3,17]. Leal et al [12] observed β-ecdysone yield of 0.013% (d.b.) which is 28 times higher than the one present on Patent US6224872 [18]. In this study up to 0.26 % of β-ecdysone was obtained when the best

extraction condition studied was applied: 393 K, 10 MPa and ethanol. These results shows that PLE present advantages, once higher amounts of extract containing the same β-ecdysone content were obtained in less time than those obtained by SFE [12].

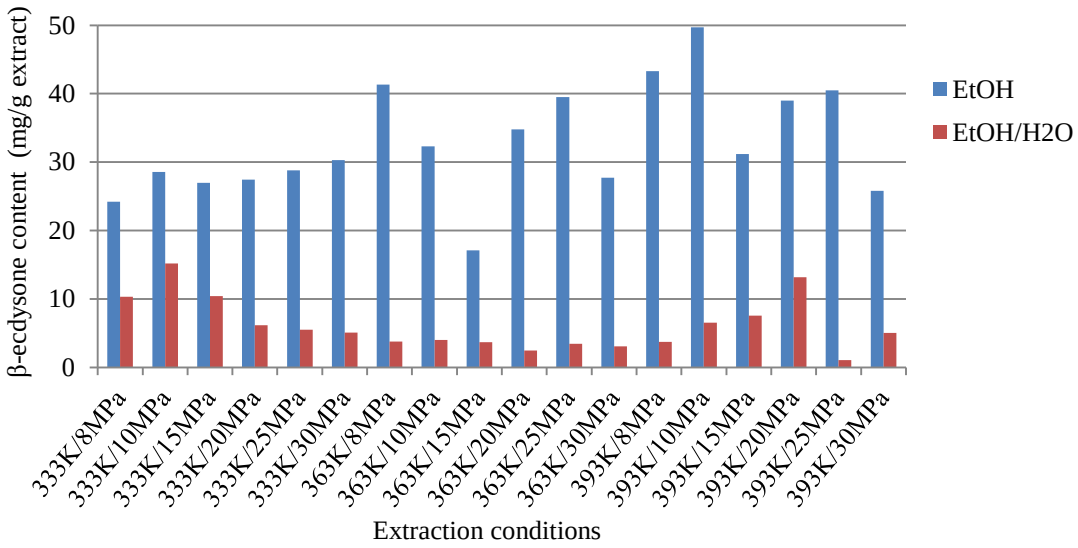

Figure 6. β-ecdysone content of *P. glomerata* roots extracts obtained by PLE in different conditions.

The influence of the parameters on the β-ecdysone content was evaluated by analysis of variance (ANOVA). The parameters that influenced significantly on the β-ecdysone content were extracting solvent (p-value = 0.000) and the interaction between temperature and extracting solvent (p-value = 0.033). Figure 7 shows means values of β-ecdysone content to interaction effects between temperature and extracting solvent to verify which levels of temperature and extracting solvent provide β-ecdysone content maximized. It can be seen that the highest β-ecdysone content is achieved when temperature was set at 393 K and ethanol was used as extracting solvent.

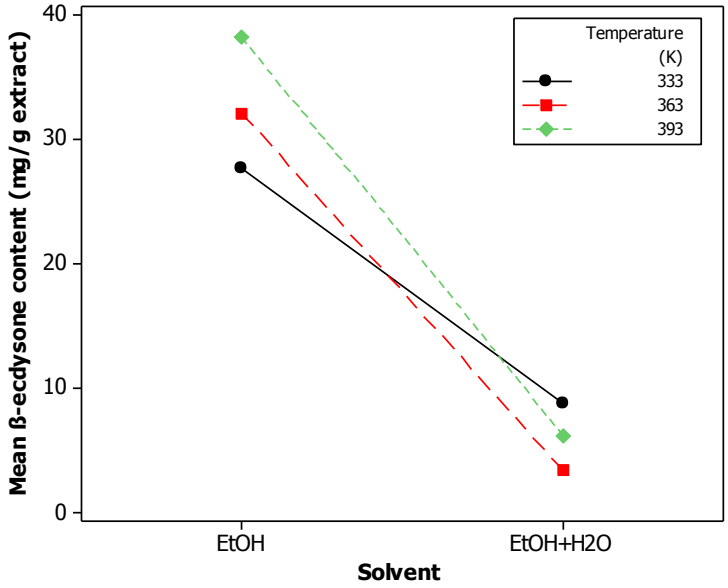

Figure 7. Interaction effect of the statistically significant parameters over the β-ecdysone content in the *P. glomerata* roots extracts.

4. Conclusion

PLE is an alternative process for obtained *P. glomerata* roots extracts rich in β-ecdysone. The extraction condition that provided the highest X_0 and antioxidant activity were 393 K, 10 MPa, ethanol: water (80:20, v/v), and 393 K, 30 MPa, ethanol:water (80:20, v/v), respectively. Among the extraction conditions studied, the best condition in terms of β-ecdysone recovery was 393 K, ethanol as extractiong solvent and 8 MPa which provided extracts containing up to 5 % of this bioactive compound.

Acknowledgements

Isabel C. N. Debien and Renata Vardanega would like to thank CNPq for the doctoral and a master's fellowships, respectively. Diego T. Santos would like to thank the FAPESP (process 10/16485-5) for a postdoctoral fellowship. The authors acknowledge the financial support from CNPq and FAPESP (09/17234-9; 12/10685-8).

References

[1] L-W. Qi, C-Z. Wang, C-S. Yiuan, Isolation and analysis of ginseng: advances and challenges, Natural Products Reports 28 (2011) 467-495.
[2] C. S. Freitas, C. H. Baggio, J. E. D. Silva-Santos, L. Rieck, C. A. M. Santos, C. Corrêa Júnior, L. C. Ming, D. A. C. Cortez, M. C. A. Maques, Involvement of nitric oxide in the gastroprotective effects of an aqueous extract of *Pfaffia glomerata* (Spreng) Pedersen, Amaranthaceae, in rats, Life Science 74 (2004) 1167-1179.
[3] A. R. Zimmer, F. Bruxel, V. L. Bassani, G. Gosmann, Jounal of Pharmaceutical and Biomedical analysis 40 (2006) 450-453.
[4] C. L. S. Vigo, E. Narita, L. C. Marques, Validação da metodologia de quantificação espectrofotométrica das saponinas de *Pfaffia glometa* (Spreng) Pedersen, Amaranthaceae, Revista Brasileira de Farmacognusia 13 (2003) 46-49.
[5] E. V. Petersson, J. Liu, P. J. R. Sjoberg, R. Danielsson, C. Turner, Pressurized hot water extraction of anthocyanins from red onion: A study on extraction and degradation rates 663 (2010) 27-32.
[6] B. E. Richter, B. A. Jones, J. L. Ezzell, N. L. Porter, Accelerated solvent extraction: A technique for sample preparation 68 (1996) 1033-1039.
[7] Z. Y. Ju, L. R. Howard, Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin 51 (2003) 5207-5213.
[8] ASAE, Standars methods of determination and expressing fineness of feed materials by sieving, ASAE, 1998, S319.3, p. 447-550.
[9] AOAC, Official methods of analysis of the association of the analytical chemists, Washington, 2000, p. 42-44.
[10] P. F. Leal, M. E. M. Braga, D. N. Sato, J. E. Carvalho, M. A. Marques, M. A.A. Meireles, Functional properties of spice extracts obtained via supercritical fluid extraction, Journal of agricultural and food chemistry 51 (2003) 2520-2525.
[11] M. Skaeget, P. Kotnik, M. Hadolin, A. R. Hras, M. Simonic, Z. Knez, Phenols proantocyanidins, flavones and flavonols in some plant materials and their antioxidant activities, Food chemistry 89 (2005) 191-198.
[12] P. F. Leal, M. B. Kfouri, F. C. Alexandre, F. H. R. Fagundes, J. M. Prado, M. H. Toyama, M. A. A. Meireles, Brazilian ginseng extraction via LPSE and SFE: global yields, extraction kinetics, chemical composition and antioxidante activity, The Journal of Supercritical Fluids 54 (2010) 38-45.
[13] P. F. Leal, N. B. Maia, Q. A. C. Carmelo, R. R. Catharino, M. N. Eberlin, M. A. A. Meireles, Sweet basil (*Ocinum basilicum*) extracts obtained by supercritical fluid extraction (SFE): Global yields, chemical composition, antioxidant activity, and estimation of cost of manufacturing, Food Bioprocess Technology 1 (2008) 326-338.
[14] T. M. Takeuchi, C. G. Pereira, M. E. M. Braga, M. R. Maróstica Jr, P. F. Leal, M. A. A. Meireles, Low pressure solvent extraction extraction (solid-liquid extraction, microwave assisted, and ultrasound assisted) from condimentary plants, Extracting Bioactive compounds from food products, CRC Press/ Taylor & Francis group , Bocca Ratum, 2009, p. 137-218.
[15] R. Vardanega, D. T. Santos, I. C. N. Debien, M. A. A. Meireles, Pressurized liquid extraction of bioactive compounds from Brazilian ginseng roots (*Pfaffia glomerata*), 16th IUFoST/ World Congress of Food Science and Technology, Foz do Iguaçu, Brazil, 2012.
[16] D. T. Santos, R. Vardanega, I. C. N. Debien, M. A. A. Meireles, Extração de compostos bioativos de raízes de ginseng brasileiro em percolador de leito fixo assistido por ultrassom: validação do processo, 9th Simposio Latino Americano de Ciência de Alimentos, Campinas, Brazil, 2011.

[17] R. Flores, F. T. Nicoloso, D. Brondani, J. Maldaner, V. Cezarotto, S. R. Giacomelli, Extração de ecdisterona em raízes de ginseng brasileiro, Ciência Rural 39 (2009) 1223-1226.
[18] T. Shibuya, T. Ario, S. Fukuda, Composition, Patent US6224872 (2001).