SUPERCRITICAL EXTRACTION OF FLAVONOIDS FROM *Piper hispidinervum*: EXPERIMENTS AND MATHEMATICAL MODELING

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Abstract. This work aims to study the flavonoid extraction process from *Piper hispidinervum* C. DC. by supercritical extraction. Flavonoid and their derivatives present various biological properties, especially antioxidant activity. The plants of the genus *Piper* are used in folk medicine for the antibacterial, antifungal and antiprotozoan properties. It is observed too the flavonoid presence in other plants of the same genus, the genus *Piper*. The goal of this work is study the effect of supercritical extraction process variables in the selectivity and yield of nonvolatile extracts of the *P. hispidinervum*. Process variables studied were temperature (313.15 K and 323.15K), pressure (90 to 250 bar), solvent (CO_2 and CO_2 + water), and raw material pre-treatment. For different process conditions were performed curves extraction, yield *versus* time. From experimental data was carried out mathematical modeling of extraction curves. The mass transfer model employed considers the one-dimensional mass balance for the solid and fluid phases. Thin layer chromatography (TLC) was performed for identification of flavonoid and their derivatives. Thereafter was performed the high performance liquid chromatography (HPLC) to quantify the compounds. From the results it can be concluded that the highest yield of flavonoids is obtained at 200 bar, 313.15K, using CO_2 + water (20% w/w in relation to CO_2). Good results were observed with relation to mathematical modeling of the extraction curves.

Keywords: *Piper hispidinervum*, flavonoids, mathematical modeling, supercritical extraction

1. Introduction

The supercritical extraction has been extremely applied in food and pharmaceutical industries in the few past years, according to Chiu [1]. Carbon dioxide is the most widely used solvent because it has the advantage of being non-flammable, non-toxic and has a low economic cost [2]. However, the greatest benefit of this technology is the easy separation of solute and solvent at the end of the process, due to its high volatility. According to Reinoso [3], the supercritical fluid has a higher diffusivity and lower density, viscosity and surface tension, compared to traditional liquid solvents.

Obtaining active ingredients from herbs and plants with enhanced reproduction of their flavors and fragrances is favored in supercritical extraction, when compared to traditional methods of extraction. The thermal degradation and decomposition of labile compounds can be avoided, due to operation at low temperatures, besides the absence of light and oxygen which prevents oxidation reactions. This last point is of particular interest when the goal is the extraction of antioxidants, ensuring the conservation of their biological properties. Compounds processed from supercritical technology need not later stages of sterilization since both Gram-positive and Gram-negative bacteria are inactivated at mild temperatures. And the high pressure gradient during expansion results in an extract free of microorganisms and their spores, giving a longer validity commercial product [3].

The solvating power of supercritical carbon dioxide can be synthesized by some principles [4]: the solubilization of compounds nonpolar or slightly polar; the ability of the solvent to dissolve compounds of low molecular weight is high, but decreases as the molecular weight increases; pigments, proteins, polysaccharides are even less soluble and minerals are insoluble. But carbon dioxide is able to extract compounds which are less volatile, have a high molecular weight or greater polarity with the increasing of the

process pressure. The use of co-solvents has been proposed as a method of increasing solubility of compounds or to increase their selectivity.

The supercritical extraction has been documented as a very effective method to prepare bioactive products from plant material [3]. However, there is little research on the extraction of flavonoids from plants of the genus *Piper*.

As presented by Bergo [5], the family Piperaceae is represented by herbaceous plants, shrubs, and rarely trees. Among the aromatic species, stands *Piper hispidinervum* C. DC., known as long pepper, found in wild conditions in Brazil. Zacaroni [6] says that *P. hispidinervum* is a species that has abundant regeneration and high production capacity. Recently, interest in this plant was awakened by the industries of cosmetics and insecticides due to the safrole obtained from the essential oil extracted from its leaves and twigs [7], used for the synthesis of heliotropine (fixative for fragrances) and piperonyl butoxide (synergistic agent) [8]. Previous studies indicate that this essential oil is rich in safrole (approximately 92%). This study aims to investigate the obtaining of heavy compounds as a means of better utilization of the raw material used. In this way is also evaluated the influence of pre-extraction of essential oil via steam distillation.

In this study was used the supercritical carbon dioxide for the extraction of flavonoids from *P. Hisperdinervum*. The effects of pressure, temperature and use of co-solvent in the extract yield were evaluated.

Recent studies show that these compounds possess antioxidant effect and exhibit various physiological activities, among them, antiproliferative effect in human cancer cells, diabetic activity and antiprotozoal activity. Wang [9] lists a number of flavonoids as responsible for a wide range of therapeutic effects. The flavonoids comprise a group of phenolic substances that are widely distributed in the plant kingdom. According to Portet [10], more than 4000 substances have been identified, many of which are responsible for color of flowers, fruits and leaves.

The flavonoids are present in all plants angiosperms and most of all gymnosperms. Sometimes, they have complex and unknown biological functions but act primarily as antioxidants protecting the plant of the auto oxidation initiated by free radicals. And despite the plant, flavonoids have a huge range of activities among mammals, as the absorption of ultraviolet radiation, inhibition of reactions with free radicals and enzymes, anti-inflammatory, among others [11]. These components are mainly used in cosmetic industry due their sedative and antioxidant actions. The action of flavonoids in the blood vessels of the skin is quite complex. Three main components can be distinguished: the protection of the vessel wall itself [12], prevention of platelet aggregation [13] and a decrease in permeability of the capillaries [14].

Investigations of Piperaceae species have shown distinct variations of flavonoids. Parmar [15] succeeded in isolating some of them, tri or tetraoxygenated, which had already been identified in several other plant families [16]. Likewise, dimetoxiflavonas have been isolated both in *Piper auritum* [17] as in *Piper sylvaticum* [18].

A method for efficient separation and quantification is needed to identify the phenolic compounds present in plant materials and their health benefits. Few methods are suitable for the simultaneous detection of flavonoids and phenolic compounds, because of the difficulty of low resolution between the two classes of compounds [19]. During the last decades, many studies have been conducted in order to develop techniques for separation and determination of phenolic constituents in various derivatives of fresh fruits and plants. The techniques previously used include UV-visible spectroscopy, thin layer chromatography (TLC) [20], gas chromatography (GC) [21] and high performance liquid chromatography (HPLC) [22].

The discovery of inhibiting lipid oxidation by phenolic compounds has contributed to the application of antioxidants in the food industry [23]. However, legislative restrictions have limited the use of such products, and encourage the development of search for natural antioxidant characteristics to be used as food additives [24]. And since ancient times, herbs have been added to different foods with the aim of expanding its organoleptic properties as well as to accentuate flavors. These plants also have high potential in the emerging nutraceutical industry, known as associated with the so-called functional foods, because its components are considered either as food or as products for medicinal purposes [25].

Antioxidants are substances capable of acting in an analogous manner, at low concentrations, inhibiting the oxidation [26]. The free radical production in humans is controlled by antioxidants, which have endogenous origin or are derived from the diet, such as tocopherols (vitamin E), ascorbic acid (Vitamin C), polyphenols, carotenoids and selenium. Antioxidants are capable of stabilizing or deactivate free radicals before they attack targets in biological cells [27].

2. Material and Methods

2.1. Plant Materials

Piper hispidinervum cultures were established in 2006, in the Center of Agriculture EMATER - Porto Alegre, state of Rio Grande do Sul (Southern Brazil; altitude of 100 m), and the aerial parts of the plant were collected in the spring season [28]. The seeds were purchased from the company Pirisa Pyrethrum Industrial Ltd. - Brazil. The plants were grown in rows 80 cm spacing within the row and 1.2 m between rows. An irrigation system was used regularly during the growing season (under glass). The amount of water used was equivalent to rainfall per year 1350 mm. The plants used in this study had four years old and pruning is done every 6 months. The processing and investigation steps are showed in Figure 1.

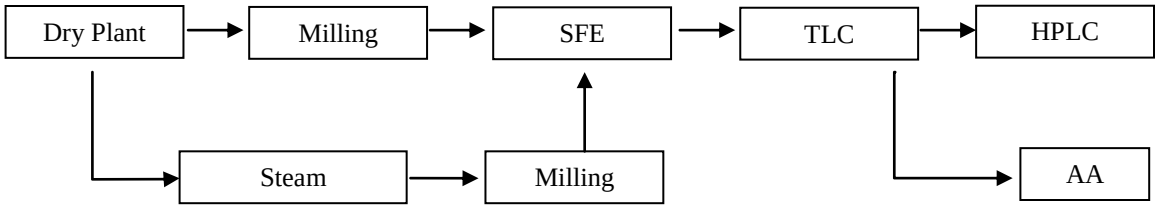

Figure 1. Scheme of the procedure employed: SFE – Supercritical Fluid Extraction, TLC - Thin-layer chromatographic; HPLC - High performance liquid chromatography; AA – Antioxidant activity.

2.2. Supercritical Fluid Extraction (SFE)

The experiments were performed in supercritical extraction pilot unit [2], shown in Figure 2. Mass of the plant used was 0.1 kg and the volume of the extraction vessel is 500 mL. The solvent used was 99.9% carbon dioxide (Air Products). The plants were dried at 313.15 K and milled in a knife mill. However, the raw material that was pre-extracted by steam distillation was just dry and not milled [29]. The experiments were performed in a temperature range of 313.15 K to 323.15 K and pressure of 90 bar to 250.0 bar. The extraction curve, defined as the yield *versus* time, was determined experimentally collecting the sample of extract every 10 min. It was also studied the influence of the water as co-solvent in extraction with a ratio of 20% mass with regard to CO_2 flow rate.

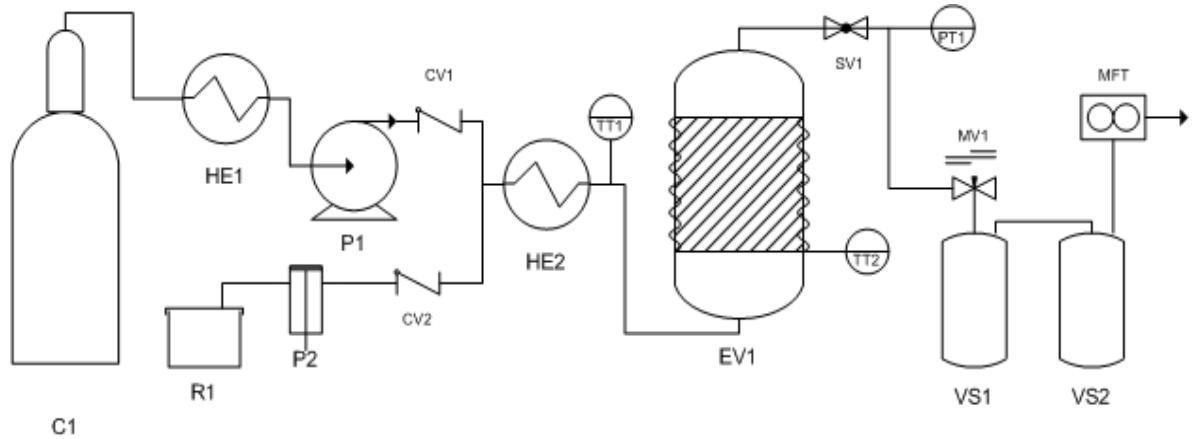

Figure 2. Supercritical extraction experimental apparatus: C1 – CO_2 Cylinder, HE - Heat Exchanger, CV – Check Valve, P1 – CO_2 High Pressure Pump, P2 – Co-Solvent Pump, R – Co-solvent Reservoir, EV – Extraction Vessel, TT – Temperature Transmitter, PT – Pressure Transmitter, VS – Separation, Vessel, MFT – Mass Flow Transmitter, MV- Micrometric Valve, SV – Shut-off Valve

2.3. TLC Analysis

The thin-layer chromatographic tests were carried out on silica gel plates with a fluorescence indicator UV 254nm (Machery-Nagel). The mixture solvent used to analyze the flavonoids was ethyl acetate, formic acid, acetic acid and water (100:11:11:26 in volume). In this system, all flavonoids cause extinction of fluorescence in UV - 254nm. Depending on their structure, flavonoids are colored dark yellow, green or blue fluorescent in length UV - 365nm [30].

2.4. HPLC instrumentation

The analysis of high performance liquid chromatography was performed on a Agilent HPLC system, model 1200 Series. The column used was a C18 (4.6 x 250 mm, 5 µm). Chromatographic conditions were as follows: the eluents used were: (A) water and (B) acetonitrile, both with 2% of glacial acetic acid. The gradient program used was as follows: elution of 20-80% B in A, from 0 to 90 minutes and returning to the initial condition for a further 5 minutes. Flavonoids and other phenolic compounds were detected at 345nm [31]. The rate was 1 mL/min and the column temperature maintained at 293.15K. All flavonoids and phenolic compounds were identified by combining the retention time and their spectral characteristics to the standards.

2.5. Antioxidant activity (AA)

The antioxidant activity analysis was performed using a spectrophotometer Model SP-220 Biospectro, which measured the absorbance of the extract at three different concentrations: 100%, 50% and 25%. Dilutions were made in a solution of absolute ethyl alcohol PA and free radical DPPH (2,2-Diphenyl-1-picryl-hydrazyl). The stabilization of the absorbance for each dilution was observed in about one hour, generating three graphs for the decreasing in absorbance with time. Each dilution corresponds to a point a new curve of absorbance *versus* dilution to be used for the calculation of IC50 (amount of extract required to reduce by 50% initial absorbance) this being a widely used parameter for comparison of the antioxidant power [32].

2.6. Mathematical Modeling

A dynamic model was used for mathematical representation of the supercritical extraction [33]. This is based on a one-dimensional mass balance for the extract, considering the hypothesis of a linear behavior for the solid vs. liquid-phase equilibrium relationship.

Supercritical Fluid:

$$\frac{\partial C}{\partial t} = -\upsilon \frac{\partial C}{\partial z} - \frac{(1-\varepsilon)}{\varepsilon}\frac{\partial q}{\partial t} \tag{1}$$

Solid phase:

$$\frac{\partial q}{\partial t} = -k_{TM}(q - KC) \tag{2}$$

The model was programmed in the Simulator EMSO [34] to solve the system of equations proposed, wherein the system is solved by a multi-stage integrator. The global coefficient of mass transfer and equilibrium constants were estimated by weighted least squares method, using a flexible polyhedra optimizer.

3. Results

The first tests on thin layer chromatography detected the presence of flavonoids in the *P. hispidinervum* extract only for this condition: 200 bar, 313.15 K, and water as co-solvent for plants with and without essential oil pre-extracted by steam distillation. All the other conditions have little evidence of flavonoids presence and the results are not conclusive.

The chemical analysis of *P. hispidinervum* extract was performed by HPLC in order to identify the compounds present, and thus determine the major fraction. The identification was based on comparison of spectra of flavonoid standards, which are listed in Table 1 with their respective retention times (RT). Analyzing the chromatogram (Figure 3) of plant extract (without pre-extraction) it could be identified the kaempferol, which has the same retention time as its standard, 19.558 minutes. This peak represents 47.38% of the total peak area. In the supercritical extract obtained from pre-extracted plant it was found a minor amount of flavonoid. The kaempferol peak represented only 2.19% of the total peak area.

Table 1. Flavonoid standards used and their HPLC retention times

Flavonoid standard	RT (min)
Caffeic acid	4.16
Ursolic acid	4.28
Coumaric acid	6.09
Quercetin	13.90
Catechin	13.87
Kaempferol	19.55

The antioxidant activity of the *P. hispidinervum* extracts obtained at 200 bar, 313.15 K, and water as co-solvent presented the following result IC_{50} = 0.1981 g/L, concentration required to reduce by 50% the amount of free radicals of the DPPH. The raw material used in the supercritical extraction did not have essential oil pre-extraction by steam distillation, because this condition presented better results with regard to kaempferol concentration.

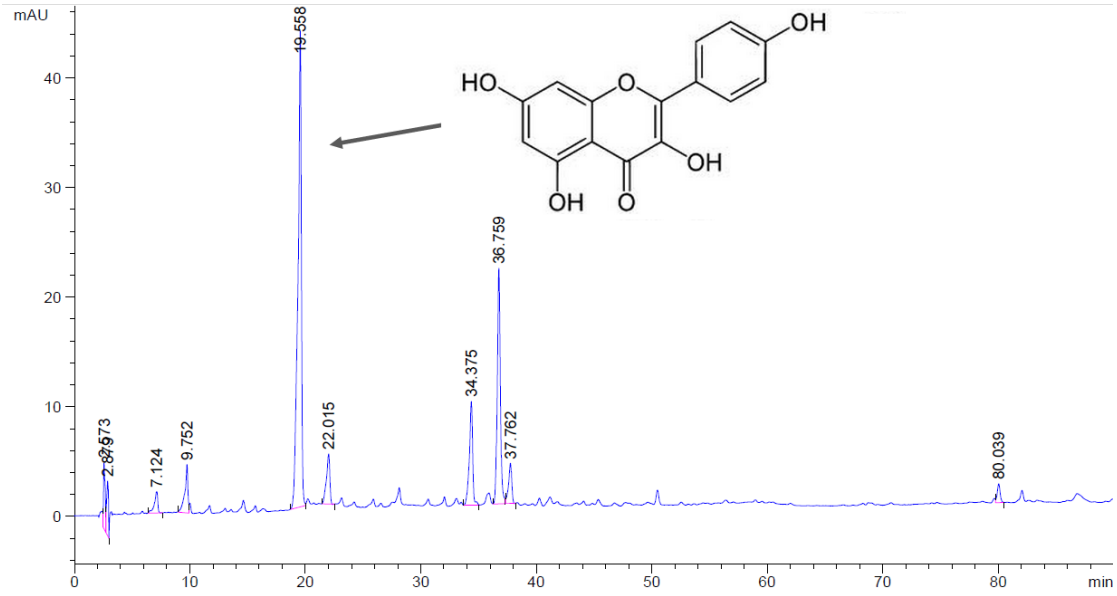

Figure 3. HPLC profile of kaempferol and supercritical extract obtained at 313.15K and 200 bar with co-solvent (without essential oil pre-extraction by steam distillation)

In Figure 4 is showed the experimental and modeling yield *versus* time curves obtained at 200 bar, 313.15 K, and water as co-solvent for raw material with and without essential oil pre-extracted by steam distillation. It is possible to observe the higher extract yield when it was used *P. hispidinervum* plant without pre-treatment (0.0273 g/g) when compared with the *P. hispidinervum* plant with pre-treatment (0.0118 g/g). Difference among the yield results was approximately 2.5 times, but the essential oil obtained in pre-extraction it is very important too, because has several applications as antifungal [6], the insecticidal activity [35; 36], and amoebicidal activity [28].

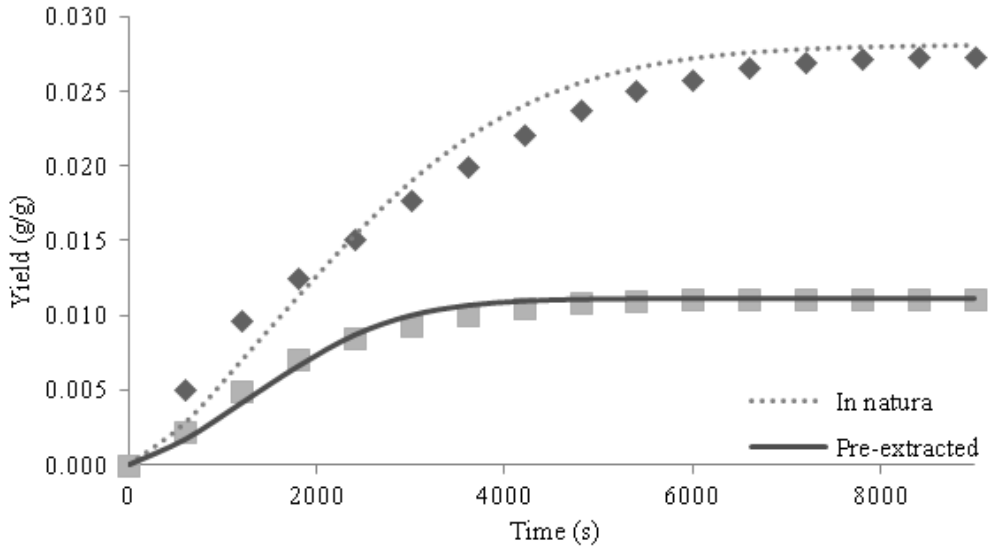

Figure 4. CO_2 supercritical extraction yield curves vs. time at 150 bar and 313.15 K

The mathematical modeling, using a dynamic model, fitted well the experimental data. The R^2 values were determined as 0.989 and 0.992 to extraction curves of the *in natura* and pre-extracted plants, respectively. The estimated parameters are shown in the Table 2.

Table 2. Fitted parameters for the mass transfer model and its coefficient of determination

Experiment	K (m^3/kg)	k_{TM} (s^{-1})
Pre-extracted 200 bar 313.15K CO_2 + water	0.00078	0.0034
in natura 200 bar 313.15K CO_2 + water	0.0018	0.0022

4. Conclusions

The use of water as co-solvent in the supercritical fluid extraction to obtain flavonoids from *P. hispidinervum* has proven effectiveness under a specific condition, 313.15K and 200 bar. The identification of a widely known flavonoid, the kaempferol, was achieved. It was also possible to verify the antioxidant activity of the extract, whereas the presence of a phenolic compound was certified. The small amount of flavonoid found in the pre-treated extract can be explained by the thermal degradation of the compounds which were submitted to high temperatures, in the order of 383.15K. The absence of flavonoids in all the other conditions can be explained by the low polarity of the solvent when it was used the carbon dioxide without water. With relation to pressure of the extraction, it is known that the solubility of high molecular weight substances in supercritical fluids increases proportionally with the process pressure. Good results were observed with respect to the simulated extraction curves. The determination of the values of the adjustable parameters of the model provides important knowledge about the supercritical extraction of *P. hispidinervum* using carbon dioxide\water mixture as solvent for future work in terms of process scale-up.

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References

[1] K-L. Chiu, Y. Cheng, J. Chen, C. Chang, P. Yang, Supercritical fluids extraction of *Ginkgo* ginkgolides and flavonoids, J. of Supercritical Fluids 24 (2002) 77-87.
[2] E. Cassel, R.M. Vargas, G. Brun, D.E. Almeida, L. Cogol, G. Ferraro, R. Filip, Supercritical fluid extraction of alkaloids from *Ilex paraguariensis*, J. Food Eng. 100 (2010) 656–661
[3] B. Reinoso, A. Moure, H. Domínguez, J.C. Parajó, Supercritical CO*2* Extraction and Purification of Compounds with Antioxidant Activity, J. Agric. Food Chem., 54 (2006) 2241-2459
[4] G. Brunner, Gas extraction, in: An introduction to Fundamentals of Supercritical Fluids and the Applications to Separation Processes, Springer. Berlin, 1994.
[5] C. Bergo, H. Mendonça, M. da Silva, Effect of time and frequency of cutting in the essencial oil production of long pepper (*Piper hispidinervum* C. DC.), Acta Amazonica, 35(2) (2005) 111-117
[6] L. Zacaroni, M. Cardoso, P. Souza, F. Pimentel, L. Guimarães, A. Salgado, Fungitoxic potential of the essential oil of the *Piper hispidinervum* (long pepper) against phytopathogenic fungi *Bipolaris sorokiniana*, *Fusarium oxysporum* and *Colletotrichum gloeosporioides*,Acta Amazonica, 39 (2009) 193-198
[7] E.M. Miranda, Caracterização e avaliação produtiva de uma população nativa de Pimenta Longa (*Piper hispidinervum*) no Seringal Cachoeira, AC. Brasil. Acta Amazonica, 32(1) (2002) 9-20
[8] C.P. Sá, M. Boyma, F. Gomes, E. Oliveira, Aspectos Agronômicos e Socioeconômicos do Cultivo da Pimenta longa para Produção de Safrol no Acre. Comunicado técnico 164. Empraba-CPAF/AC, Rio Branco, Acre 2004
[9] L. Wang, B. Yang, X. Du, C. Chun, Optimisation of supercritical fluid extraction of flavonoids from *Pueraria lobata*, Food Chemistry 108 (2008) 737-741
[10] B. Portet, N. Fabrea, R. Rozenbergb, J.L. Habib-Jiwamb, C. Moulisa, J. Quetin-Leclercqb, Analysis of minor flavonoids in *Piper hostmannianum* var. *berbicense* using liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry, J. of Chromatogr. A, 1210 (2008) 45-54
[11] J. Arct, K. Pytkowska, Flavonoids as components of biologically active cosmeceuticals, Clinics in Dermatology, 26 (2008) 347-357
[12] A. Garg, S. Garg, L. Zaneveld, A. Singla, Chemistry and Pharmacology of the citrus bioflavonoid hesperidin, Phyother Res. 15 (2005) 655-669
[13] D. Pearson, R. Holt, O. Rein, J. Paglieroni, H. Schmitz, C. keen, Flavanols and platelet reactivity, Clin. Dev. Immunol 25 (2005) 569-575
[14] J.B. Harborne, C.A. Williams, Advances in flavonoid research since 992, Phytochemistry 55 (2000) 481-504
[15] V. Parmar, K. Bisht, R. Jain, P. Taneja, A. Jha, O. Tyagi, A. Prasad, J. Wengel, C. Olsen, P. Boll, Phytochemistry of the genus Piper, Phytochemistry 46 (1997) 597–673
[16] H. Alves, G. de Oliveira, M. Zoghbi, M. Chaves, Flavonoids from *Piper carniconnectivum* C. DC.. Piperaceae, Brazilian Journal of Pharmacognosy 20 (2010) 160-164
[17] S. Ampofo, V. Roussis, D. Wiemer, New prenilated phenolics from *Piper autritum*, Phytochemistry 26 (1987) 2367-2370
[18] B. Avijit, P. Sudhir, Constituents of *Piper sylvaticum*: structure of Sylvatesmin, J. Nat. Prod. 45 (1982) 672-675
[19] B. Suárez, A. Picinelli, J. Mangas, Solid-phase extraction and high-performance liquid chromatographic determination of polyphenols in apple musts and ciders, J. of Chromatogr. A 72 (1996) 203–209
[20] F. Kaderm, B. Rovel, M. Girardin, M. Metche, Fractionation and identification of the phenolic compounds of Highbush blueberries (*Vaccinium corymbosum* L.), Food Chem. 55 (1996) 35
[21] T. Nash, Colorimetric Determination of Formaldehyde under Mild Conditions, Nature 170 (1952) 976
[22] H. Chen, Y. Zuo, Y. Deng, Separation and determination of flavonoids and other phenolic compounds on cranberry juice by high-performance liquid chromatography, J. of Chromatogr. A 913 (2001) 387-395
[23] E. Sherwin, B. Luckadoo, Studies on antioxidant treatments of crude vegetable oils, Journal of the American Oil Chemists' Society 47 (1970) 19-23
[24] A. Dapkevicius, R. Venskutonis, T. Beek, J. Linssen, Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania, J. of the Science of Food and Agriculture 77 (1998) 140–146
[25] B. Bozin, N. Mimica-Dukic, I. Samojlik, E. Jovin, Antimicrobial and Antioxidant Properties of Rosemary and Sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., Lamiaceae) Essential Oils, J. Agric. Food Chem. 55 (2007) 7879–7885
[26] H. Costa, S.M. Gallego, M.L. Tomaro, Effect of UV-B radiation on antioxidant defense system in sunflower cotyledons, Plant Science 162 (2002) 939–945
[27] B. Berenguer, L. M. Sánchez, A. Quilez, M. López-Barreiro, O. De Haro, J. Galvez, M. J. Martin, Protective and antioxidant effects of *Rhizophora mangle* L. against NSAID-induced gastric ulcers, Journal of ethnopharmacology 103 (2006) 194-200
[28] I. P. Sauter, G. E. Rossa, A. M. Lucas, S. P. Cibulski, P. M. Roehe, L. A. A. da Silva, G. L. von Poser, Chemical composition and amoebicidal activity of *Piper hispidinervum (*Piperaceae) essential oil, Industrial Crops and Products 40 (2012) 292-295

[29] V. B. Xavier, R. M. F. Vargas, E. Cassel, A. M. Lucas, M. A. Santos, C. A. Mondin, E. R. Santarem, L. V. Astarita, and T. Sartor, Mathematical modeling for extraction of essential oil from *Baccharis* spp. by steam distillation, Industrial Crops and Products 33 (2011) 599-604

[30] H. Wagner, S. Bladt, Plant Drug Analysis: A Thin Layer Chromatography Atlas, Springer, 1996

[31] S. Kumazawa, M. Yoneda, I. Shibata, J. Kanaeda, T. Hamasaka, T. Nakayama, Direct evidence for the plant origin of Brazilian Propolis by the observation of honeybee behavior and phytochemical analysis, Chem. Pharm. Bull 51 (2003) 740-742

[32] G. P. Pérez, R. M. F. Vargas e E. Cassel, Determinação de propriedades de taninos de acácia negra e tara, XIX Congresso Brasileiro de Engenharia Química, Rio de Janeiro, Brazil, 2012, p. 6710

[33] E. Reverchon, Supercritical fluid extraction and fractionation of essential oils and related products, J. of Supercritical Fluids 10 (1997) 1-37

[34] R. Busato, Modelagem, simulação e otimização de uma unidade industrial de extração de óleos essenciais por arraste a vapor. Porto Alegre: UFRGS, 2010 Faculdade de Engenharia Química, Universidade Federal do Rio Grande do Sul.

[35] M. Fazolin, J.L.V. Estrela, V. Catani, M.R. Alécio, M. S. de Lima, Insecticidal properties of essential oils of *Piper hispidinervum* C. DC.; *Piper aduncum* L. and *Tanaecium nocturnum* (Barb. Rodr.) Bur. & K. Shum against *Tenebrio molitor* L., Ciênc. Agrotec. 31 (2007) 113-120.

[36] R.K. Lima, M.G. Cardoso, J.C. Moraes, B.A. Melo, V.G. Rodrigues, P.L. Guimarães, Insecticidal activity of long-pepper essential oil (*Piper hispidinervum* C. DC.) on fall armyworm *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae). Acta Amaz. 39 (2009) 377-382.