ANTIOXIDANT ACTIVITY OF BLACK PEPPER (*Piper nigrum* L.) OIL OBTAINED BY SUPERCRITICAL CO_2

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Abstract. Peppers are highly appreciated by customers in the world, moving a market in constant growth. Brazil is the second largest worldwide producer of black pepper. The constituents of black pepper oil, which contribute to the value of black pepper as a food additive, are the essential oil for the aroma and the alkaloidal compounds for the pungency. Many studies show that black pepper has antioxidant, anti-inflammatory and anti-tumor properties. Supercritical fluid extraction (SFE) provides extracts with high purity and free of solvent contamination. Therefore, the aim of this work is to investigate the quality of the black pepper extracts obtained by supercritical CO_2 at different conditions of pressures and temperatures in order to evaluate the yield behavior and the antioxidant characteristic. The moisture content of de raw material is 15.1±0.2% and the extraction yields were up to 2.9 ±0.1%, obtained at 200 bar and 313.15 K. The antioxidant aspects will be evaluated by means of the determination of the total phenolic content by the Folin- Ciocalteau method and by the antioxidant activity by the DPPH, ABTS and β-carotene methods.

Keywords: black pepper oil, antioxidant activity, process yield.

1. Introduction

Herbs and spices are considered an important part of human diet and have been used for thousands of years in traditional medicine and also to enhance color, flavor and aroma of the food [1].

Peppers are highly appreciated by customers in the world, moving a market in constant growth. Brazil is among the three largest producers in the world and has about 90% of its production exported [2].

Black pepper (*Piper nigrum*) is one of the most popular spice products in oriental countries, largely used as a flavoring agent in foods. The components of the pepper's extract that contribute to its value as a food additive are the volatile oil for its aroma and the alkaloid compounds for the pungency [3]. Black pepper is used in skin care, muscle and joint pains, and in improving blood circulation and respiratory systems. The bioactive molecule, piperine, present in pepper has major pharmacological impacts on the nervous and neuromuscular systems, exercises sedative effect and helps in digestion [4].

Antioxidants are widely used to prevent deterioration of oxidizable products such as food, cosmetics and pharmaceuticals. A tendency among modern consumers is the use of antioxidants obtained from natural sources, increased the interest in the research. The presence of active components in spices has been demonstrated over the last 30 years and their therapeutic properties have been demonstrated by the presence of many substances, including some vitamins flavonoids, terpenoids, carotenoids, phytoestrogens and minerals. The extraction processes that have been commercially employed for obtaining these substances include extraction with both polar and nonpolar solvents, steam distillation, molecular distillation and supercritical extraction with CO_2 [4, 5].

Supercritical fluid extraction (SFE) has received attention in a variety of industries, because it can provide high solubility, improved mass transfer rates, and increased selectivity with small changes in process temperature and pressure [6]. Extraction of essential oils and oleoresins by using this technology may also provide higher quality products. The supercritical CO_2 is the most common solvent used in supercritical extraction processes, due to its practical advantages: a non-toxic, non-flammable, is environmentally safe, has high availability, and achieves high purity at low cost [7].

This work aimed to evaluate the antioxidant activity and global yield for the black pepper extracts obtained by different extraction methods.

2. Materials and methods

2.1 Sample preparation

The dried black peppers were purchased in the local market. The black peppers, with moisture content of 15.1%, were ground in a knife mill (De Leo, Porto Alegre/RS - Brazil). The dried raw material was stored at 255.15 K in a domestic refrigerator until the extractions were performed.

2.2 Supercritical Fluid Extraction (SFE)

SFE from black pepper oil was performed in a dynamic extraction unit [8]. The extraction procedure consisted of placing a fixed mass of 20 g of black pepper inside the extractor cell to form the particle fixed bed, followed by the control of the process variables (temperature and pressure). The extraction was then performed and the extract collected in amber flasks after 4 hours of extraction and weighed in an analytical balance (OHAUS, Model AS200S, NJ – USA).

The SFE was performed to obtain the global yield (X_0) according to extraction conditions of 313.15 K and 323.15 K, and pressures of 150, 200 and 300 bar and constant solvent flow rate of 11±2 g min^{-1}. The SFE process used 99.9 % pure CO_2 delivered at pressure up to 60 bar (White Martins).

2.3 Low pressure extractions (LPE)

The low pressure extraction methods used in this study were Soxhlet (SOX) and ultrasound (UE), which were applied using ethanol (EtOH) as solvent, with polarity of 5.2 [9].

Soxhlet. The SOX extraction was performed according to 920.39C method of A.O.A.C. [10]. The procedure consists of 150 mL of solvent recycling over 5 g of dried sample, in a Soxhlet apparatus for 6 h extraction at the boiling temperature of the solvent used.

Ultrasound. The UE was conducted according to Freitas [11]. Briefly, 7 g of raw material and 210 mL of solvent were used for this assay, placed inside an evaporation flask connected to a condenser. The extraction time was 45 min, conducted at room temperature. The equipment used was an ultrasonic cleaner bath (Unique Ultracleaner, USC-700), which operates in a frequency of 55 kHz and potency of 220 V.

The extracts obtained by each extraction method were submitted to the solvent elimination in a rotary evaporator (Fisatom, 802, Brazil), supplied with cooling and vacuum control. The evaporation temperatures were adjusted to a level below the boiling point of the solvent in order to avoid thermal degradation of the extracts. The vacuum was adjusted at 650 mmHg. The global yield (X_0) for all method of extraction was obtained by the mean value from the duplicate experiments considering the ratio between mass of extract and mass of raw material.

2.4 Antioxidant activity

The antioxidant activity was determined for the extracts of black pepper obtained by SFE at 150, 200 and 300 bar; 313.15 K, 323.15 K, and by SOX and UE methods. The results were compared with the synthetic compound BHT (butylated hydroxytoluene). All reagents used in the antioxidant activity analysis were purchased from Sigma–Aldrich Co. (USA).

Free radical scavenging activity (DPPH). The free radical scavenging of black pepper extracts was evaluated using 1,1-diphenyl-2-picrylhydrazil (DPPH) as described by Mensor et al. and Benelli et al. [12,13]. Briefly, each extract was mixed with a 0.3 mM DPPH ethanol solution, to give final concentrations of 5, 10, 25, 50, 125, 250 and 500 µg extract mL^{-1} DPPH solutions. After 30 min at room temperature, the absorbance values were measured at 517 nm in spectrophotometer (FEMTO, 800 XI, São Paulo, SP) and converted into percentage of antioxidant activity (%AA). This activity was also presented as the effective concentration at 50% (EC_{50}), i.e., the concentration of the test solution required to give 50% decrease in the absorbance of the test compared to that of a blank solution, and expressed in µg of extract mL^{-1} DPPH. The EC_{50} values were calculated from the linear regression of the % AA curves obtained for all extract concentrations. The %AA and EC_{50} for all extracts were obtained considering the mean value of triplicate assays.

$ABTS^{\cdot+}$ radical scavenging assay. This assay was carried out according to the procedure described by Re et al. and Michielin et al. [14, 15]. The radical monocationic pre-formed ABTS•+ [2,2-azino-bis-(3-

ethylbenzotiazoline- 6-sulfonic acid)] is generated by chemical oxidation of the ABTS, and is reduced in the presence of an antioxidant compound hydrogen donor. The synthetic vitamin E, Trolox (6-hidroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma–Aldrich Co, St. Louis, EUA), was used as antioxidant reference, which was prepared in ethanol and stored as a standard solution. The ABTS was dissolved in water to a concentration of 7.0 mM, and submitted to reaction with 2.45 mM potassium persulfate for the formation of the radical ABTS. The absorbance was measured at 754 nm in spectrophotometer 6 min after the mixture of the samples to the solution of ABTS. Results were expressed as Trolox equivalent antioxidant capacity (TEAC). TEAC is defined as the mM concentration of a trolox solution which antioxidant activity is equivalent to the activity of 1.0 mM test solution. In order to find TEAC values, a separate concentration response curve for standard Trolox solutions was prepared.

Antioxidant activity with the β-carotene bleaching method. The antioxidant activity from the β-carotene/linoleic acid system was carried out according to the method described by Matthaus [16] and by Kang et al. [17]. Briefly, 40 mg of linoleic acid and 400 mg of Tween 20 were transferred into a flask, and 1mL of a β-carotene–chloroform solution (3.34 mg/mL) was added. Chloroform was removed by rotary evaporation at 313.15 K. Then 100 mL of distilled water was slowly added and vigorously agitated to form a stable emulsion. An aliquot of 5mL of this emulsion was added with 0.2 mL of ethanolic black pepper extract solution (1667 mg/mL) and the absorbance was immediately measured at 470nm against a blank consisting of the emulsion without β-carotene. The tubes were placed in a water bath at 323.15 K and the absorbance was measured every 15 min up to 120 min. The absorbance values (mean of the triplicate essays) were converted into percentage of antioxidant activity (% AA).

Total phenolic content (TPC). The TPC was determined according to the Folin–Ciocalteau method [18]. Briefly, the reaction mixture was composed by 0.1 mL of extract (concentration of 1667 mg L^{-1}), 7.9 mL of distilled water, 0.5 mL of Folin–Ciocalteau reagent (a mixture of phosphomolybdate and phosphotungstate) and 1.5 mL of 20% sodium carbonate, placed in opaque flasks. The flasks were agitated, held for 2 h, and the absorbance was measured at 765 nm. The TPC was calculated according to a standard curve, prepared previously with galic acid as standard. The results (mean value of the triplicate assays) were expressed as milligrams of galic acid equivalent (GAE) per gram of the extract (mg GAE g^{-1}).

3. Results and discussion

3.1 Global yield (X_0) of SFE and LPE

In this work, the effect of extraction condition on the extraction yield was studied. Yield of extract was defined as weight of extract divided by weight of initial sample loaded in the extractor. In order to observe the efficiency of supercritical CO_2 extraction, the yield of extract was compared with the yield of extract obtained by ethanol Soxhlet extraction.

The yield results obtained for the different extraction methods and solvents (Soxhlet, UE and SFE) are presented in Table 1, together with the CO_2 density (ρ) for SFE.

Table 1. Global yield of black pepper extracts obtained by low pressure extractions (LPE) and supercritical fluid extraction (SFE).

SFE (with CO_2)	LPE (with EtOH)	ρ (g/cm³)	X_0 (%)
150 bar/313.15 K	-	0.748	1.24 ± 0.01
150 bar/323.15 K	-	0.656	1.28 ± 0.01
200 bar/313.15 K	-	0.840	2.9 ± 0.1
200 bar/323.15 K	-	0.785	1.92 ± 0.2
300 bar/313.15 K	-	0.911	2.66 ± 0.04
300 bar/323.15 K	-	0.871	2.75 ± 0.04
-	SOX	-	10 ± 1
-	UE	-	5.3 ± 0.5

The results presented in Table 1 indicate that the best yields were obtained by the Soxhlet extraction using ethanol as solvent. The operating temperature of the recycle solvent and the interactions between solvent and plant matrix, characteristic of Soxhlet extraction, may contribute to increase the solubility of compounds of different types, raising the extraction yield [13, 15, 19].

The percentage yields of essential oil and oleoresin of black pepper are 1.0-3.5 and 5.0-15.0, respectively [4], therefore, the extraction yields obtained with SFE are within the expected range. These results are in agreement with those obtained by Ferreira [3] in the extraction of essential oil of black pepper. According Mukhopadhyay [4], pressures of around 500 bar may duplicate the extraction rate and, thereby, increase the yield and quality of the extracts.

At 323.15 K it is possible to observe the increase in yield with increasing pressure. This behavior is explained by the increase in solvent density with enhancing pressure, increasing the salvation power of CO_2 [5]. The increased pressure can lead to disruptions in the plant cells, facilitating the release of compounds that were not previously available, and thus increasing the yield of the process [19, 20]. The same behavior was observed by Perakis, Louli and Magoulas [21].

Comparing the yields obtained by supercritical CO_2 with the results obtained by conventional extraction (Soxhlet and ultrasound), it is observed that the low pressure extractions using ethanol as a solvent produced yield values superior to those achieved by SFE. These results can be explained by the extraction of more polar compounds, not soluble in CO_2, a nonpolar solvent [13, 15].

3.2 Antioxidant activity

Table 2 shows the antioxidant activity results according to analyses of TPC, ABTS and β-carotene, respectively, achieved by samples of black pepper extracts obtained from different extraction methods (SFE and LPE) and by BHT, as standard sample.

Table 2. Antioxidant activity for black pepper extracts.

Extraction	Solvent	TPC (mgGAE/g extract)[1]	AA (μgM TEAC/g extract)[2]	AA (%)[3]
SOX	EtOH	27 ± 3	104.3 ± 9.6	49.6 ± 0.1
UE	EtOH	26 ± 3	68.5 ± 5.2	42.9 ± 0.1
SFE 150 bar/313.15 K	CO_2	16 ± 2	41 ± 5	12.9 ± 0.4
SFE 150 bar/323.15 K	CO_2	19.8 ± 0.4	216 ± 11	9.3 ± 0.2
SFE 200 bar/313.15 K	CO_2	22.5 ± 5.7	-	20.5
SFE 200 bar/323.15 K	CO_2	20.3 ± 1.2	100 ± 10	12.9 ± 0.3
SFE 300 bar/313.15 K	CO_2	18 ± 4	59	26.9 ±0.3
SFE 300 bar/323.15 K	CO_2	14 ± 4	190 ± 10	25.2
BHT	-	268 ± 13	-	113 ± 7

[1] Total phenolic content.
[2] Antioxidant activity evaluated by ABTS method.
[3] Antioxidant activity evaluated by β- carotene method.

The results of antioxidant activity obtained by DPPH method are not relevant in this work because just the value of EC_{50} achieved for the Soxhlet extraction with ethanol was close to 250 μg/mL, meaning that this extract presents good antioxidant potential. The low antioxidant activity for the supercritical fluids extracts may be associated to the low amount of phenolic compounds with intermediate to high polarity present in extracts, since CO_2 as a non polar solvent does not favor the solubilization of such components. The use of a cosolvent combined with supercritical extraction is an alternative to increase the extraction yield and antioxidant activity of the products obtained [13, 19].

In the study realized by Zarai et al. [1], the best values of total phenolic contents achieved for black pepper extracts were those obtained with ethanol as solvent, as well as the one presented in this work (Soxhlet: 27 ± 3 mg GAE/g extract; UE: 26 ± 3 mgGAE/g extract). These results are below to the found for the standard sample BHT (268 ± 13mg GAE/g extract), suggesting that the extracts are not the best sources of phenolic compounds or this is not the more adequate method to identify them in this case.

The extracts obtained a 323.15 K by SFE, in all pressures tested, showed the best results for the antioxidant activity by the ABTS method, probably because the components responsible for antioxidant characteristics detected by the ABTS method were present in higher concentrations.

The antioxidant activity by β-carotene bleaching method decreases with temperature suggesting the degradation of substances responsible by the antioxidant potential. Once more, the extracts obtained by employing ethanol as solvent showed the best results, attesting that the antioxidant activity is associated with the extraction of compounds of high/intermediate polarity [22].

4. Conclusions

Black pepper is a spice known for its pungent flavor and also for its beneficial properties to health. When comparing the different extraction methods, besides process yield, it is also necessary to estimate the antioxidant potential of the product by diverse procedures and also, in the future studies, evaluate the chemical composition of the extracts. SOX and UE presented highest process yield using ethanol as solvent. SFE extracts presented lowers yields when compared to low pressure extractions, although good results of antioxidant activity by ABTS method were detected from the supercritical extracts. DPPH method not was an adequate method to determine the antioxidant activity of the black pepper extracts.

Studies will be performed to determine the potential as antioxidant for the extracts obtained in more elevated pressures. Moreover, it is extremely important to know the chemical composition of the extracts, in order to evaluate the best application of this product.

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