

1 **Compressed fluids extraction methods, yields, antioxidant activities, total phenolics**
2 **and flavonoids content for Brazilian Mantiqueira hops**

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11
12
13 **Abstract**

14 This study compares the yields of different extractions methods for a Brazilian variety of
15 *Humulus lupulus* using scCO₂, scCO₂+ethanol, scCO₂+ethyl acetate, and compressed
16 propane. Extracts were characterized by total phenolics content (TPC), total flavonoid
17 content (TFC), and antioxidant activity (AA). The extraction yields were from 2.7 to 10.1
18 wt% when using compressed propane at 20 °C and 10 MPa and scCO₂+ethyl acetate at
19 80 °C and 25 MPa, respectively. Furthermore, the effect of adding ethyl acetate as a
20 cosolvent in a semi-batch process over the extraction time and extracts properties were
21 evaluated in comparison to the extracts obtained using scCO₂, scCO₂+ethanol, and
22 compressed propane. The results indicate that ethyl acetate increases the extraction yield
23 and produces samples with higher TPC, TFC, and AA values comparing to
24 scCO₂+ethanol and similar values compared to pure scCO₂.

26 **Keywords:** *Humulus lupulus*; antioxidant activity; supercritical CO₂; compressed
27 propane; cosolvent.
28

29 1. Introduction

30

31 *Humulus lupulus* is a dioic, perennial, herbaceous plant from the *Cannabaceae*
32 family, native to Europe, West Asia, and North America. It is classified as a vine and can
33 reach 6.1 m in height [1]. Only female plants produce the inflorescence known as hops,
34 where the lupulin glands are found. Lupulin is rich in essential oils and resins. Essential
35 oils are responsible for the aroma of the beer, and resin content provides bitterness. The
36 bitter compounds are mainly alpha and beta acids, the most abundant components in the
37 resin, and these acids become water-soluble after an isomerization reaction providing
38 bitterness to the beers [2,3].

39 Applications of hops as a medicinal plant date back to the 19th century [4].
40 Researchers have confirmed hops as an anti-inflammatory [5] and phytoestrogen source
41 [6–8]. *Humulus lupulus* flowers are used to relieve the symptoms of stress and insomnia
42 [4] and as antifungal agents [9]. In vitro studies have shown that hops have
43 anticarcinogenic and anti-fibrogenic activity in liver tissue [10,11]. The essential oil
44 obtained from hops is a mixture of more than 35 compounds, including organic acids,
45 alcohols, terpenes, esters, and phenolics [4].

46 Phenolic compounds are byproducts of plant metabolism, acting as natural
47 protection against environmental conditions. These compounds are classified as primary
48 antioxidants due to their role as reducing agents, hydrogen donors, and singlet oxygen
49 suppressants [12,13]. Hops contain phenolic compounds that are classified as phenolic
50 carboxylic acids (ferulic acid), flavonoids, and polyphenols. Among the polyphenols,
51 resveratrol and xanthohumol are the most abundant prenylflavonoids [4].

52 Methods mostly used for obtaining oil-containing extracts from vegetable raw
53 materials are the techniques involving solid-liquid extraction with organic solvents and

54 steam distillation. Methods using organic solvents demand a separation step to recover
55 the solvent, and during the solvent distillation, a significant part of the volatile
56 components can be removed from the final extract. The second mentioned method
57 demands high temperatures, which can compromise the quality of the extract due to the
58 degradation of the thermolabile compounds [14]. On the other side, supercritical fluid
59 extraction (SFE) and pressurized fluid extraction (PFE) are alternative techniques due to
60 lower temperature employed, high extraction rates. In the first case, the separation of the
61 solvent from the extract is facilitated due to the high volatility of the fluid at low-pressure
62 conditions producing a solvent-free extract [15,16]. Compressed propane has also been
63 proposed as a viable nonpolar solvent for lipids extraction from natural matters. Several
64 studies with propane as a solvent in PFE have shown good extraction yields, and high
65 antioxidant activity for the final extracts obtained [17–25].

66 The main limitation of SFE and PFE using pure supercritical carbon dioxide
67 (scCO₂) and propane concerns its low solvation capacity for polar compounds. However,
68 the addition of polar modifiers (polar cosolvents) can overcome this limitation and thus
69 contribute to increasing the overall extraction yield [26,27]. In this sense, He et al. [28]
70 showed that the use of ethanol as a cosolvent favored the extraction of flavonoids from
71 hop residues leading to an increase in flavonoid recovery from 0.5 to 7.6 mg/g at the
72 optimal concentration of cosolvent, 80 % of ethanol to sample mass ratio. However, other
73 modifiers should be tested to optimize the extraction of phenolics. Magalhães et al. [29]
74 tested five different solvents, and the methanol presented the highest recovery (92%) for
75 xanthohumol, the most abundant prenylflavonoid present in the hops, followed by ethyl
76 acetate with a recovery of 69%. Based on these previous studies, ethyl acetate as a
77 cosolvent to the carbon dioxide for hop extraction seems to be a promising technique,

78 mainly because it is a GRAS solvent with a low boiling point, and it is capable of
79 providing high extraction yields.

80 Another aspect worth mentioning is that repeated studies in the literature have
81 used hop varieties cultivated in such traditional regions as the United States, England,
82 Czech Republic, and Belgium [29–32]. This study focuses on a new variety of hops
83 developed and adapted to the climatic conditions in Brazil. This subspecies could generate
84 extracts with distinct characteristics concerning other traditional hops from temperate
85 regions. To the best of our knowledge, there are no reports in the literature combining the
86 use of this new variety of hops locally produced with developing and promoting green
87 technologies for the industry. Therefore, this work aimed to evaluate the extraction of
88 hops using supercritical CO₂ (scCO₂), supercritical CO₂ + ethanol (scCO₂+EtOH),
89 supercritical CO₂ + ethyl acetate (scCO₂+EtOAc), and compressed propane at different
90 temperatures and pressures, and compare the extracts obtained from the extraction with
91 compressed solvents to those obtained by Soxhlet extraction with different organic
92 solvents. The extraction results were evaluated in terms of extraction yield, antioxidant
93 capacity (AA), total phenolic compounds (TPC), and total flavonoid compounds (TFC).

94

95

96 **2. Material and Methods**

97

98 2.1. Samples

99 The vegetable matrix selected for this study was the flowers of *Humulus lupulus*
100 Brazilian varietal, Brazilian hops (BH), which were purchased from a small hops grower
101 cooperative in Brazil. This varietal is a mutation adapted to the Brazilian climate and
102 originated from an American hop known as Cascade, which in turn is the result of the

103 selective crossing with the Fuggle hop varietal from England. The samples were collected
104 in Tuiuti (state of São Paulo – Brazil), located at an elevation of 790 m above the sea
105 level, with a monthly average precipitation of 140 mm and temperatures varying from 11
106 to 26 °C. The coordinates of the production field are latitude S 22°49'22.6" and longitude
107 W 46°40'10.1". The hops were freeze-dried in a L101 Liobras (São Carlos, SP, Brazil)
108 equipment for 30 hours under temperature and pressure conditions of -51 °C and 13.33
109 kPa, resulting in raw material with moisture and volatile compounds of 8.6 ± 0.5 g/100 g
110 of freeze-dried hops. The samples named freeze-dried Brazilian hops (FBH) were stored
111 in polyethylene bags in a freezer at -18 °C until use.

112

113 2.2. Chemicals

114 Ethanol 95% purity, ethyl acetate 99.5% purity, methanol 99.9% purity, and n-
115 hexane 99.5% purity, all purchased from Neon (Suzano, SP, Brazil) used for extraction.
116 For the supercritical and subcritical extractions, CO₂ 99.5% purity and propane 99.5%
117 purity were purchased from White Martins (Araucaria, PR, Brazil). For the total phenolics
118 content, total flavonoids content and antioxidant activity analysis: DPPH - (2,2-diphenyl-
119 1-picrylhydrazyl), Trolox - (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)
120 standard, gallic acid standard, catechin hydrate standard, and Folin-Ciocalteu phenol
121 reagent 2 N all purchased from Sigma-Aldrich (St. Louis, MO, USA), Sodium Carbonate
122 Merck, (Darmstadt, HE, Germany) and Methanol HPLC grade Panreac (Barcelona, CT,
123 Spain).

124

125 2.3. Determination of moisture, volatile compounds, and density

126 The moisture and volatile compounds were determined by weight loss upon
127 drying, where the samples were oven-dried at 105 °C until they reached the constant

128 weight, and the moisture was calculated by dividing the mass loss by the initial mass of
129 the sample.

130 The real density of the dried raw material was determined by an automatic
131 helium pycnometer Ultrapyc 1200e Quantachrome (Boynton Beach, FL, USA) at the
132 Analytical Centre of the Institute of Chemistry at Unicamp, Campinas, Brazil.

133

134 2.4. Soxhlet extractions

135 Soxhlet extraction with different solvents was used as a benchmark extraction
136 for reference and comparison purposes. All extractions were performed in triplicate at the
137 boiling temperature of the solvent at atmospheric pressure for 8 h using 5 g of FBH and
138 180 mL of solvent. The solvents used individually for each extraction were: ethanol, ethyl
139 acetate, methanol, and n-hexane. The samples were rotary evaporated (RV 10 Digital
140 IKA, Staufen, BW, Germany) at vacuum conditions. After that, the extracts were dried
141 using an air circulation oven at 60 °C until they reached a constant weight. Equation 1
142 was used to calculate the extraction yields.

143

$$144 \quad Yield(\%) = \frac{Mass\ of\ extract}{Mass\ of\ matrix} \cdot 100\% \quad (1)$$

145

146 2.5. Extraction with compressed fluids

147 In this work, the compressed fluid extraction methods were divided into three
148 groups to evaluate the influence of different approaches when applied to hops: (i)
149 extractions using supercritical CO₂ (scCO₂), (ii) compressed propane, and (iii) using CO₂
150 plus liquid solvents, consisting of scCO₂ with ethanol and ethyl acetate liquid solvents.
151 All these extraction experiments were performed in a semi-batch approach.

152 The extraction equipment utilized in this study has been presented and described
153 in previous studies [33–35]. The laboratory-scale extractor setup consists of a high-
154 pressure jacketed-vessel, a 62.4 cm³ stainless steel extraction vessel, with 19 mm
155 diameter and 220 mm bed height, coupled to a heat circulation bath, and a syringe pump
156 (500D ISCO, Lincoln, NE, USA) coupled to a circulation bath set to 10 °C for all
157 extractions performed in this study. The solvent flow, during the dynamic extraction, was
158 controlled by modulating two valves, a needle valve and a micrometer valve couple to
159 the exit extraction vessel stream. The samples were collected in a separation flask, where
160 the compressed fluid is separated from the sample mixture at atmospheric pressure and
161 temperature due to the high vapor pressure of the compressed solvent. Sensors and
162 transducers were used to measure the temperature and pressure inside the extractor.

163 To evaluate the influence of two main variables on the overall extraction yield,
164 temperature and pressure, a 2² factorial design with triplicate determinations at the central
165 point was carried for each system. The variables studied ranged from 40 to 80 °C and 15
166 to 25 MPa for scCO₂ and scCO₂+ethanol (or ethyl acetate), 20 to 60 °C and 3 to 10 MPa
167 for compressed propane extractions. The extraction yield from each experimental
168 condition was calculated according to equation 1.

169

170 2.5.1. Supercritical fluid extraction (SFE) and pressurized fluid extraction (PFE)

171 Around 10 g of FBH was packed inside the extractor vessel, and the temperature
172 was set to extraction temperature. The pressurized fluid was pumped to the extractor until
173 the system reached the extraction pressure set point. The matrix and the solvent were kept
174 in contact for 60 min without flow, i.e., in a static extraction period to thermal and
175 mechanical equilibration.

176 The solvent flow was then adjusted until a continuous flow of $2.0 \pm 0.4 \text{ cm}^3/\text{min}$
177 of solvent, measured at the syringe pump conditions. At this point, the dynamic extraction
178 step started. In this setup, the solute-solvent mixture is depressurized through a heated
179 micrometer valve until it reaches the atmospheric temperature and pressure in the
180 separation flask. The mass of extract was collected in test tubes at predetermined times
181 and used to calculate the overall extraction yield curves, according to the procedure
182 reported by Correa et al. [36]. The total extraction time depends on each extraction
183 procedure, and they will be listed along with extraction conditions in the discussion of
184 the results.

185

186 2.5.2. Supercritical CO₂ + cosolvent extractions

187 In this study, ethanol and ethyl acetate were used as a cosolvent with scCO₂,
188 acting as polarity modifiers. Ethyl acetate was used at a cosolvent to FBH mass ratio
189 (MR) of (1:1) and (2:1). The organic solvent (ethyl acetate or ethanol) was added to the
190 raw material inside the extractor vessel before the compressed solvent was pumped. The
191 best three extraction conditions using scCO₂ + EtOAc were compared to extractions using
192 scCO₂ + EtOH, as the ethanol is the most frequent cosolvent used in SFE applied to the
193 food industry, including hop extractions [32,37].

194 For these extraction procedures, approximately 10 g of FBH was packed inside
195 the extractor bed at the extraction temperature. Then the cosolvent was added to the solid
196 inside the vessel to reach the predetermined MR. Thus, pressurized CO₂ was injected
197 using the syringe pump until the extractor reached the extraction pressure. This condition
198 was kept for 30 min to thermal and mechanical equilibration and to ensure the liquid
199 phase becomes homogeneous. As previously mentioned, this step is named static

200 extraction period. After the static extraction, the dynamic extraction took place, and the
201 extracts were collected following the same procedure described in section 2.5.1.

202 An additional step for the organic solvent separation from the extract was
203 performed in these experiments. The cosolvent (ethanol or ethyl acetate) was evaporated
204 from the extract in an air circulating oven at 40 ± 2 °C for 30 h.

205

206 2.6. Total phenolics content, total flavonoids content, and antioxidant activity

207

208 2.6.1. Preparation of samples

209 All samples were prepared and analyzed in triplicate. Extracts from hops using
210 pressurized fluids extraction methods were weighed, approximately 50 mg of each
211 sample, and then 10 mL of methanol was added before the analysis. This mixture was
212 vigorously shaken for 5 min and centrifuged for 10 min at 1010 g and 25 °C. The methanol
213 phase was used to determine the total phenolic content, total flavonoid content, and
214 antioxidant activity by spectrophotometric methods described below. All analyses were
215 conducted using a UV-VIS spectrophotometer GTA 97 Global Analyzer (Monte Alto,
216 SP, Brazil).

217

218 2.6.2. Total phenolic content by Folin-Ciocalteu reagent

219 Total phenolic content (TPC) was determined by the Folin-Ciocalteu reagent
220 method and was performed according to the procedure described by Singleton et al. [38]
221 with some modifications. Firstly, the methanol sample solution, prepared as previously
222 described, was diluted to a final concentration of 0.7 mg mL^{-1} . To determine the TPC, 0.2
223 mL of the final sample solution and methanol to complete the volume up to 0.5 mL was
224 mixed with 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10 in distilled water). The

225 mixture was kept in the darkness for 3 min. Afterward, 2 mL of sodium carbonate 7.5 %
226 was added, and the mixture was incubated in the dark for two hours. Then, the solution
227 absorbance was measured at 760 nm. The quantitative results were calculated using an
228 analytical curve of gallic acid and were expressed as mg of Gallic acid equivalents (GAE)
229 per 1 g of sample (mg GAE.g⁻¹).

230

231 2.6.3. Total flavonoid content

232 The total flavonoid content (TFC) of samples was determined based on the
233 method proposed by Zhishen et al. [39], with some modifications. The aliquots ranging
234 from 0.1 to 0.3 mL of samples were top-up with methanol reaches 0.4 mL of volume,
235 then 1.6 mL of distilled water and 0.12 mL of NaNO₂ (5% w/v) were added to amber
236 bottles and mixed. After 5 min, 0.12 mL of AlCl₃ (10% w/v) was added; and after 6 min,
237 0.8 mL of NaOH (1 molL⁻¹) and 0.96 mL of distilled water were added. The solution
238 absorbance was measured at 510 nm after 5 min. Catechin was used as the standard for a
239 calibration curve, and the results were expressed as mg of catechin equivalent (CE) per 1
240 g of sample (mg CE.g⁻¹).

241

242 2.6.4. Radical scavenging activity by DPPH[•] assay

243 The antioxidant activity (AA) was determined by the DPPH[•] assay performed
244 based on the method described by Brand-Williams et al. [40]. A 3.9 mL aliquot of a 6 ×
245 10⁻⁵ mol L⁻¹ DPPH[•] methanolic solution was mixed with 100 μL of diluted samples. The
246 DPPH[•] absorbance was monitored at 515 nm after one hour. The quantification was
247 performed using a Trolox analytical curve, and the results were expressed as mmol of
248 Trolox equivalents antioxidant capacity (TEAC) per 1 g of sample (mmol TEAC.g⁻¹).

249

250 2.7. Statistical analysis

251 Results were analyzed using analysis of variance (ANOVA) at a confidence level
252 of 95 % using the software Statistica 10 (Statsoft Inc., USA), and to plot the response
253 surfaces and evaluate the statistical model significance fits for each solvent.

254

255 **3. Results and Discussion**

256

257 3.1. Vegetable matrix and extractions

258 Vegetable matrix (FBH) used in all extraction experiments performed in this
259 study presented a moisture content (after freeze-drying) and real density of 8.6 ± 0.5 wt%
260 and 0.96 ± 0.01 g/cm³, respectively. The extraction yields in Soxhlet extraction with
261 organic solvents are presented in Table 1. The results indicate an increase in yield by
262 increasing the polarity of the solvent. The highest extraction yield was observed for
263 ethanol and methanol, around 25.8 wt% for both solvents, followed by the ethyl acetate
264 (around 11.4 wt%) and n-hexane (around 6.7 wt%). Results presented in Table 1 show
265 that this vegetable matrix presents a higher amount of extractable compounds in polar
266 solvents, using short-chain alcohols and a short-chain ester (ethyl acetate), which is a
267 food-grade compound. Therefore, based on results presented in Table 1, it can be seen
268 that the ethyl acetate (EtOAc) is a promising volatile short-chain ester to be used as
269 cosolvent and polarity modifier for hop extractions driven by supercritical CO₂
270 conditions, even presenting an overall extraction yield almost half of the values found for
271 short-chain alcohols.

272 Another critical aspect of the results presented in Table 1 is the extraction with
273 n-hexane, a nonpolar solvent. Besides, this solvent promoted the lowest extraction yield,
274 compared to the other three polar solvents, and provided a valuable recovery of extracts

275 from the hops raw material investigated. In this sense, the extractions with compressed
276 propane, a compressed nonpolar solvent, were performed, and its extracts were compared
277 to the extractions with scCO₂. These results were presented and discussed later.

278

279 Table 1 – Extraction yields of FBH extraction by different organic solvents.

Experiments	Solvent	Polarity Index^a	Yield (wt%)^b
S1	Ethanol	5.2	25.8 ± 0.9
S2	n-Hexane	0.0	6.7 ± 0.2
S3	Ethyl acetate	4.3	11.4 ± 0.6
S4	Methanol	6.6	25.8 ± 0.7

280 ^aRef. [41]. ^bExtraction yield expressed in wt% (mean ± standard deviation) based on
281 triplicate experiments.

282

283 To evaluate the influence of using different solvents and compare the results with
284 a polarity modifier-assisted extraction, extractions with pure scCO₂ (SFE), scCO₂ +
285 EtOH, and scCO₂ + EtOAc and compressed propane (PFE) were also performed. As
286 mentioned, the fixed bed for all extractions contained approximately 10 g of FBH. Table
287 2 presents the overall extraction yields, calculated at the end of the semi-batch extraction
288 process (after static extraction, plus dynamic extraction and bed depressurization steps).
289 For SFE, PFE, scCO₂ + EtOAc extractions with MR of (1:1) and (2:1), the same factorial
290 design with two factors and two levels and a triplicate at the central point was utilized.
291 The SFE showed overall extraction yields ranging from 4.7 to 7.6 wt%. Kupski et al. [17]
292 reported similar yields between 1.2 and 7.1 %, even using a different variety of hops,
293 Hallertau Mittelfrüh, in pellet form.

294 Lower overall extraction yields were observed for the extraction using scCO₂
295 and compressed propane compared with other methods. Comparing the best yield
296 condition of scCO₂ and compressed propane, the extraction yields were around 7.6 and
297 6.0 wt%, respectively, and it can be seen that the extraction yield obtained with scCO₂

298 was 27 % higher than the value obtained with propane. Extractions with compressed
299 propane reached similar Soxhlet extraction values with n-hexane, indicating that the
300 compressed propane could be used as an alternative and selective solvent for the
301 extraction of nonpolar compounds.

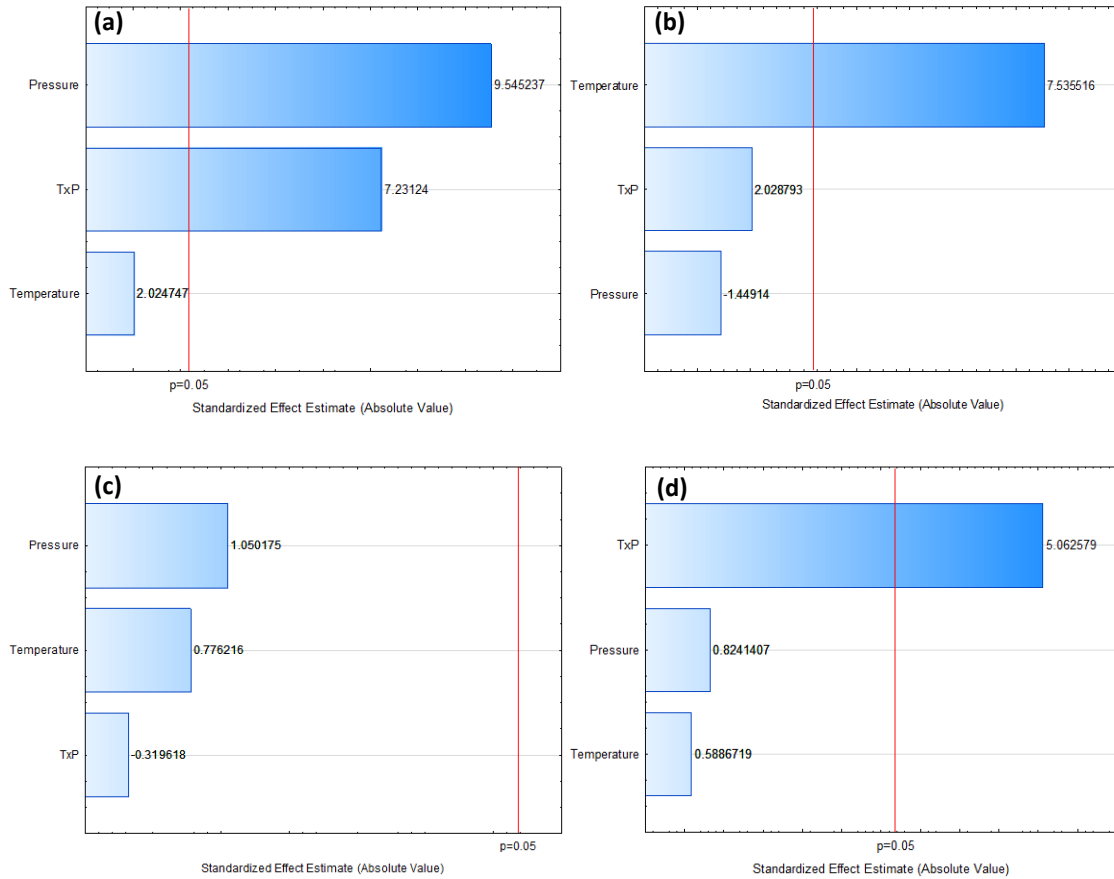
302 Table 2 also presents the results for the extractions using scCO₂ + polar solvents.
303 It can be seen that both cosolvents ethyl acetate and ethanol, presented a positive effect
304 over the overall extraction yield. EtOAc and EtOH in (2:1) MR improved the extraction
305 yield by 34 and 39 %, respectively, compared to the extraction results obtained using
306 scCO₂. The pressurized extraction techniques provided extraction efficiency of over 80
307 % for at least one set of pressure and temperature of each experimental design tested
308 (solvent type and approach used) comparing to the Soxhlet extraction (see Table 2).

309 Each extraction method was evaluated independently by analysis of variance
310 (ANOVA). Figure 1 depicts the Pareto chart for all extraction results of different methods
311 presented in Table 2. First, SFE using just scCO₂ as the solvent, where the most relevant
312 factor was the pressure, with a positive effect, i.e., the increase in the pressure of
313 extraction leads to an increase in the overall extraction yield. The temperature only
314 presented a positive effect when combined with the pressure.

315 For PFE extractions with compressed propane, a positive effect of temperature
316 and a negative effect of pressure on the yield was observed (Figure 1b), and the
317 combination of the two independent variables resulted in an increase in the extraction
318 yield. For the extraction yield of FBH using scCO₂ + EtOAc at different EtOAc to solids
319 mass ratio (MR), for both cases, temperature presented a positive effect over the
320 extraction yield for extractions at (1:1) MR, and a marginal negative effect at (2:1) MR.
321 On the other hand, pressure presented a negative effect on the extraction yield for both

322 EtOAc to solids mass ratio. Meantime, the interaction between the two factors was
323 positive for both ratios of cosolvent (MR).

324



325

326

327 Figure 1 - Pareto chart of standardized effects over the extraction yield of (a) scCO₂; (b)
328 propane; (c) scCO₂ + EtOAc at MR (1:1), and (d) scCO₂ + EtOAc at MR (2:1).

329

330 Table 2 – Overall extraction yields of FBH using SFE, PFE, scCO₂+cosolvent.

Run	Extraction Method	T (°C)	P (MPa)	MR ^c	Extraction time (min)	Extraction Yield (wt%)	Extraction efficiency (%) ^d	S/F ^e	Total compressed solvent (g) ^f
1	SFE (scCO ₂)	40	15		110	5.6	82	8.2	251
2		40	25		110	6.0	89	6.3	228
3		80	15	-	110	4.7	70	4.4	248
4		80	25		110	7.6	113	5.6	214
5, 6, 7		60	20		110	6.2 ± 0.1	92	6.1 ± 0.1	205 ± 5
8	PFE (Propane)	20	3		70	3.9	58	4.7	111
9		20	10		70	2.7	40	5.2	118
10		60	3	-	70	5.8	87	4.7	153
11		60	10		70	6.0	90	4.5	110
12, 13, 14		40	6.5		70	4.2 ± 0.2	63	4.5 ± 0.2	107 ± 6
15	(scCO ₂ + EtOAc)	40	15	2:1	60	9.6	84	8.5	167
16		40	25	2:1	60	7.8	68	8.5	177
17		80	15	2:1	60	7.7	68	12.4	162
18		80	25	2:1	60	10.2	89	6.1	107
19, 20, 21		60	20	2:1	60	8.3 ± 0.2	73	6.1 ± 0.9	126 ± 2
22		40	15	1:1	60	5.6	49	6.7	189
23		40	25	1:1	60	7.1	63	7.1	181
24		80	15	1:1	60	6.8	60	4.3	154
25		80	25	1:1	60	7.6	67	5.9	171
26, 27, 28		60	20	1:1	60	8.1 ± 0.2	71	5.9 ± 0.2	171 ± 5
29		(scCO ₂ + EtOH)	40	15	2:1	30	9.4	36	8.4
30	80		25	2:1	30	10.5	41	8.0	109
31	60		20	2:1	30	8.7	34	6.7	107

331 ^cMass Ratio = Mass of cosolvent (g) / mass of FBH (g) at the initial time of dynamic extraction ($t = 0$ min).
332 ^dExtraction efficiency = Yield of pressurized extraction / yield of Soxhlet extraction. SFE and PFE were compared to n-hexane, scCO₂ + EtOAc
333 was compared to EtOAc, and scCO₂ + EtOH compared to EtOH.
334 ^e Solvent to raw material (FBH)₀ (wt/wt), i.e., the mass of solvent injected to the extraction vessel to pressurize the system in g of solvent/mass of
335 FBH (g) at the initial time of static extraction. For SFE and scCO₂+cosolvent, the solvent is scCO₂, and for PFE, the solvent is propane.
336 ^f Total solvent consumed during the extraction. i.e., the mass of solvent injected into the vessel to pressurize the system + solvent consumed within
337 the total dynamic extraction. For SFE and scCO₂+cosolvent, the solvent is scCO₂, and for PFE the solvent is propane.
338

339 The extraction yield for scCO₂ + EtOAc at (1:1) MR showed a maximum at the
340 central point (8.1%, at 20 MPa and 60 °C, see Table 2), while for extractions at (2:1), the
341 maximum yield (10.2%) was obtained at the highest condition of pressure and
342 temperature (25 MPa and 80 °C). Comparing the results in Table 2 for extraction using
343 scCO₂ + EtOAc for the two different EtOAc to solids mass ratios, (2:1) and (1:1), the
344 overall extraction yields were similar when the same pressure and temperature condition
345 was used, except at 25 MPa and 80 °C, where the maximum yield was obtained at (2:1).
346 It is worth mentioning that 25 MPa and 80 °C was the condition that led to the highest
347 extraction yield when using pure scCO₂. Still, comparing the extraction using scCO₂ +
348 EtOAc with scCO₂ + ethanol (Table 2), the results were statistically similar, indicating no
349 difference in the extraction process using an alcohol or an ester as a cosolvent. These
350 results reinforce the idea that the scCO₂ is the leading agent for the extraction process of
351 this raw material and the liquid solvent (either ethyl acetate or ethanol) works to increase
352 the solubility of the solutes in scCO₂, increasing the extraction rates and getting higher
353 extraction performance.

354 For all cases, except by PFE, the overall extraction yield was increased by
355 increasing the temperature and pressure. For compressed propane, only the temperature
356 presented a positive effect on the extraction yield, since increasing the pressure, in a
357 subcritical state at a constant temperature, propane in the liquid state has its diffusion into
358 the matrix compromised by the increase in its density and viscosity.

359 Two main reasons for the temperature effect over the overall extraction yield
360 performance in an isobaric process are the density of the solvent and the vapor pressure
361 of the solute. As increasing the temperature increases the vapor pressure, an increase in
362 the solute solubility in the solvent occurs. On the other hand, increasing the temperature,

363 the density of the solvent decreases, reducing the solvation capacity [42]. Due to this
364 effect, extractions at high temperature and high pressure resulted in higher overall yields.

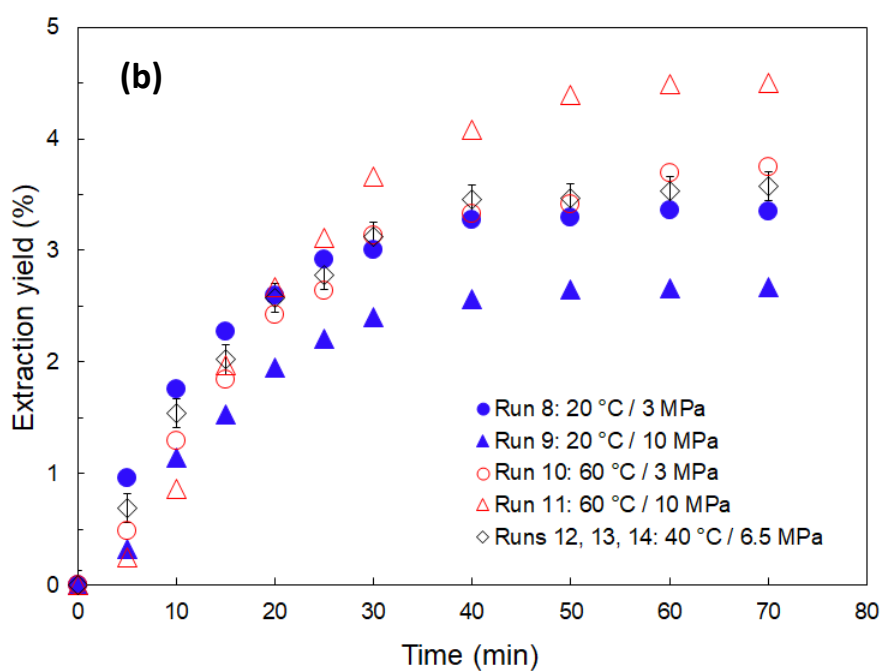
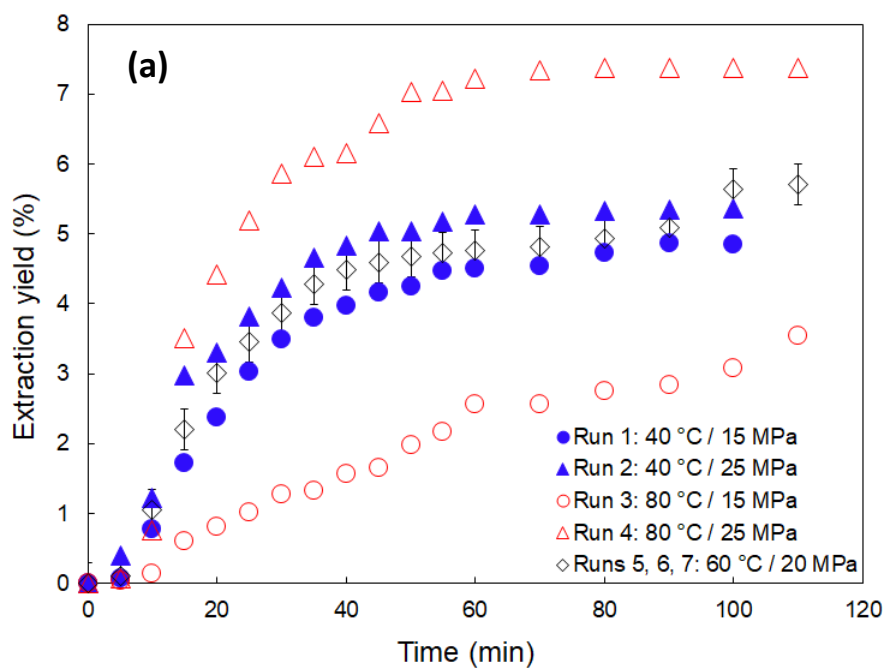
365 The results presented in this study have shown that the application of cosolvent
366 improves the extraction yield by increasing the solubility of polar compounds present in
367 the hops matrix, but it can be a factor in decreasing the selectivity of the extraction [43].

368

369 3.1.1. Overall extraction curves

370 Figure 2 depicts the overall extraction curves for extraction using scCO₂ and
371 propane. It can be observed that the highest initial extraction rate and highest extraction
372 yield for the SFE were obtained at the highest temperature and pressure levels (Run 4)
373 investigated in this work (80 °C, 25 MPa), followed by other conditions at 40 °C and then
374 at the central point. All these SFE curves presented the three extraction periods, named
375 constant extraction rate (CER), falling extraction rate (FER), and diffusion-controlled
376 period (DCP). These results observed for hop extractions (Figure 2a) are indicating that
377 the density of the supercritical fluid is the main parameter driving the extraction. The
378 temperature of the process is an important factor that is increasing the vapor pressure of
379 solutes, the diffusivity of CO₂, and increasing the solubility of compounds in dense scCO₂
380 medium, leading to high initial extraction rates.

381 For the extractions using compressed propane as the solvent, the initial
382 extraction rates were similar, indicating that the solubility of hop compounds in
383 compressed propane is, possibly, invariant within the range of temperature and pressure
384 investigated. However, the overall extraction yield is statistically significant with the
385 increase in the temperature, as already discussed.



386

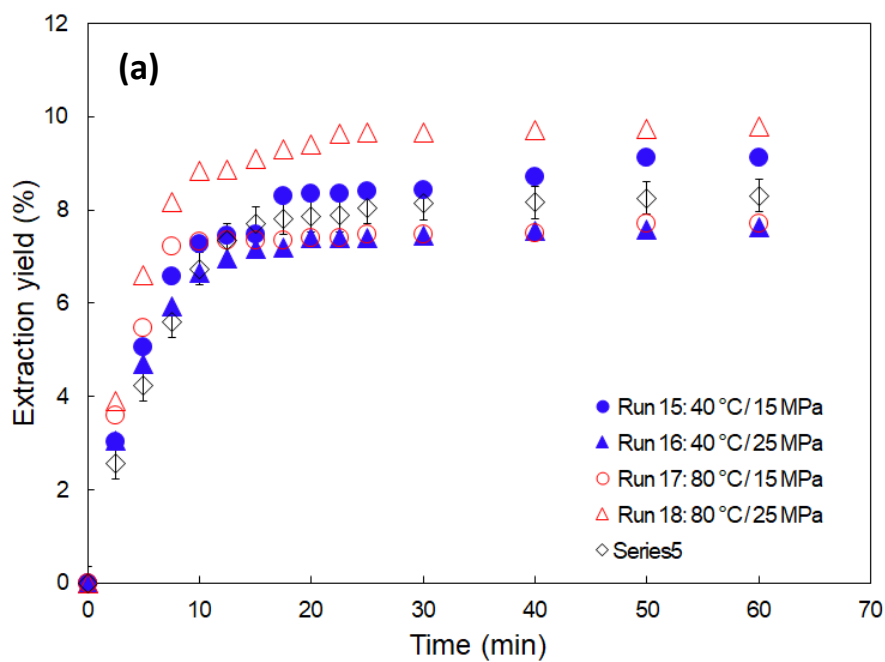
387

388 Figure 2 – Overall extraction curves of FBH using (a) scCO₂ and (b) propane as the
 389 solvent.

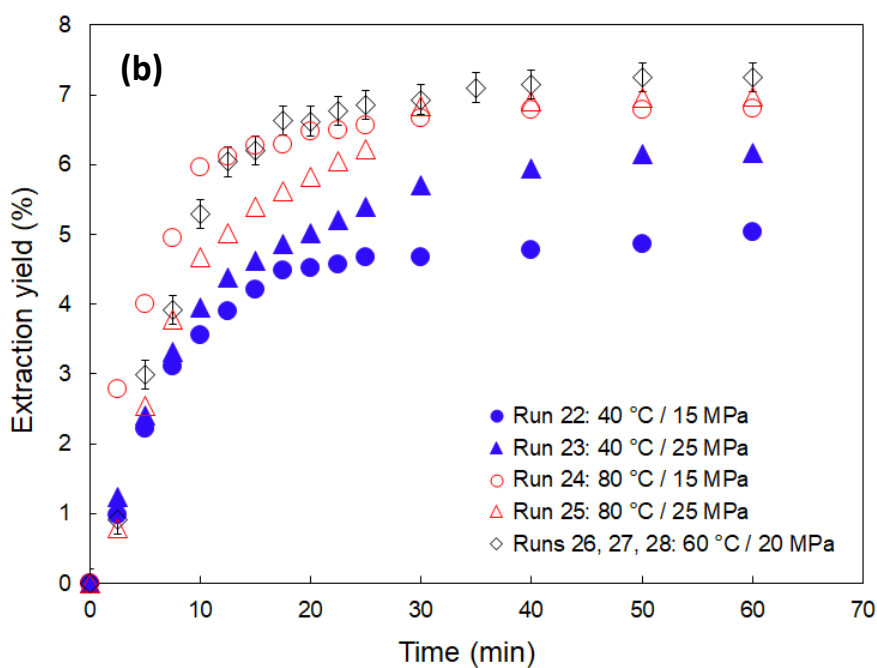
390

391 Figure 3 presents the overall extraction curves for extraction using scCO₂ +
 392 EtOAc. As presented in Figure 3(a), for the extraction with ethyl acetate (EtOAc) to FBH
 393 mass ratio of (2:1), the highest extraction rates were obtained at 80 °C and 15 MPa (Run

394 17) and 25 MPa. However, even presenting similar initial extraction rates, the condition
395 at 80 °C and 25 MPa (Run 18) presented a final higher extraction yield when compared
396 to 15 MPa. At 40 °C, an opposite effect of pressure is observed, increasing the pressure
397 from 15 MPa to 25 MPa the initial extraction rates were almost the same but decreased
398 the yield of the extraction at the end of the process. It is possible to see a strong correlation
399 between the temperature and the initial extraction rate and mainly on the overall
400 extraction yield. If the extraction is conducted at constant pressure, both the solubility and
401 diffusivity are improved by increasing the temperature, making the static extraction more
402 effective [18]. It is worth mentioning that for the results in Figure 3(a), all extraction
403 conditions presented a constant extraction rate until 15 min, approximately, and then
404 jumped to a diffusion-controlled period. Figure 3(b) presents the extraction curves for
405 conditions with EtOAc to FBH mass ratio of (1:1) (MR), and differently of extractions at
406 (2:1), this condition at lower cosolvent related to the biomass, presented the usual three
407 steps extraction, i.e., after a constant extraction rate (CER) period, the process went
408 through a falling extraction rate (FER) and then a diffusive controlled period (DCP). The
409 transition between CER and FER occurred around minute 10, and the transition to DCP
410 around minute 25. For the results presented in Figure 3(b), the highest initial extraction
411 rate was observed at 80 °C and 15 MPa (Run 24). Nevertheless, after 17.5 min, the
412 condition at the central point (60 °C and 20 MPa) provided higher extraction yields. It is
413 indicating that a lower initial amount of liquid solvent (ethyl acetate) the density of CO₂
414 has an important role in the kinetics of this extraction, and the same extraction
415 performance is obtained for extraction at 80 °C and 15 MPa (or 25 MPa) and 60 °C and
416 20 MPa.
417



418



419

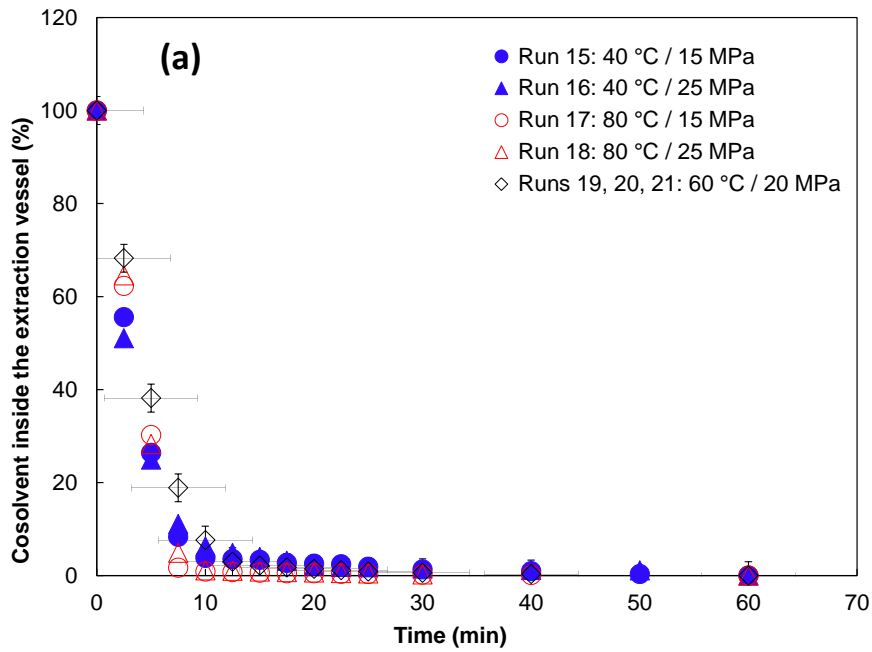
420 Figure 3 – Overall extraction curves for scCO₂ + EtOAc at different EtOAc to solids mass
 421 ratio: **(a)** (2:1) and **(b)** (1:1).

422

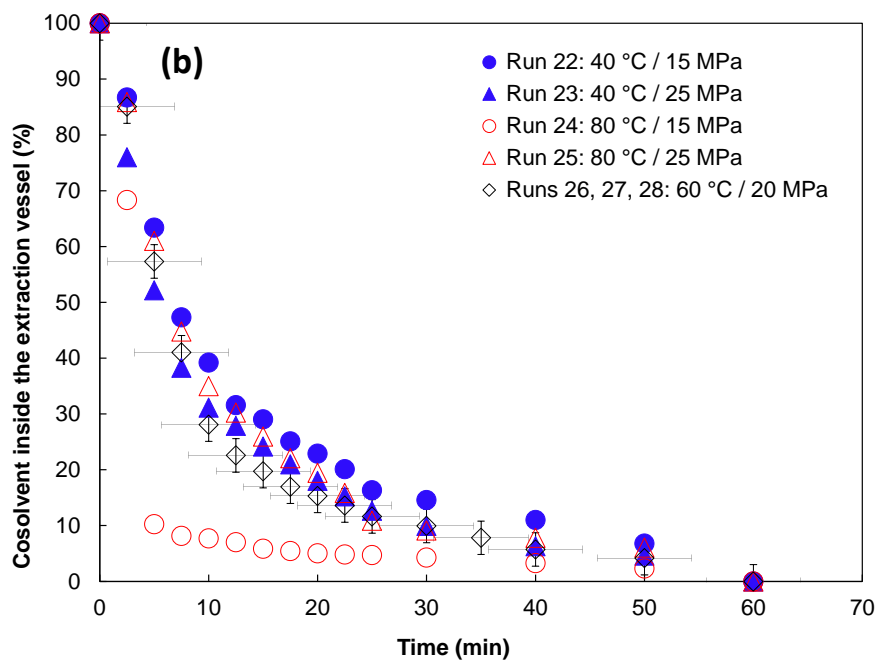
423 From the results presented in Figure 3(a), it was observed that after 15 min of
 424 extraction, the cosolvent was totally carried by the CO₂ from the extraction vessel, and
 425 then, after this point, the extraction was performed only with CO₂ being fresh pumped

426 into the extractor. Figure 4 is qualitatively showing the ethyl acetate profile inside the
427 extractor during different extraction conditions with scCO₂+EtOAc of temperature,
428 pressure, and solvent to solids mass ratio. For extractions at a solvent to solids mass ratio
429 (2:1), after an extraction time around 20 min, the liquid solvent is almost totally removed
430 from the extraction vessel by fresh scCO₂. On the other hand, for extractions at (1:1), the
431 liquid cosolvent extraction rate is slower than at (2:1), and ethyl acetate is removed from
432 the extraction vessel only around 60 min of extraction. These ethyl acetate extractions by
433 fresh scCO₂ were also visually observed during the extractions by the absence of liquid
434 solvent collected in the flasks during the extraction sampling after ~20 min and ~50 min
435 for experiments at (2:1) and (1:1) of MR, respectively. Therefore, it can be seen that in
436 this semi-batch extraction approach, when the cosolvent is removed from the extraction
437 vessel, the extraction is set back to a pure CO₂ extraction condition, which means that this
438 type of extraction could be stopped at 10 to 15 min of extraction, where the maximum
439 extraction rate and yield were observed. Thus, the results presented in this work are
440 showing that it is possible to perform the extraction of hops using scCO₂ plus ethyl acetate
441 as cosolvent, obtaining higher extraction yields in shorter times of process, comparing to
442 the extraction with pure scCO₂.

443



444



445

446 Figure 4 - Ethyl acetate (wt%) remaining inside the extractor vessel (calculated by mass
 447 balance) in semi-batch scCO₂+ethyl acetate extractions at cosolvent to solids mass ratio
 448 (MR) of (a) (2:1) and (b) (1:1).

449

450

451 3.2. Total phenolic content, total flavonoid content, and antioxidant activity

452 The results for the assays of TPC, TFC, and AA are presented in Table 3. All
453 extracts obtained from FBH presented considerable TPC values varying between 87 mg
454 GAE.g⁻¹ and 189 mg GAE.g⁻¹. These values are 20 times higher than previous studies
455 with grape using similar techniques [44,45]. These high values can be a combination of
456 different factors like the pretreatment (freeze-drying) of the raw material, storage
457 conditions, maturation stage, and growing conditions, in addition to differences between
458 extraction conditions.

459 The highest value of TPC was found in the extract obtained by SFE with CO₂.
460 All extracts of this group of extraction presented high TPC with values comprehended in
461 the range 175 – 189 mg GAE.g⁻¹. The extract with the maximum TPC was obtained at 80
462 °C and 25 MPa (Run 4). Even in higher temperature extractions, the phenolic compounds
463 are preserved.

464 The TPC values of the FBH extracts obtained by PFE with propane as a solvent
465 were independent of extraction conditions. These extracts showed similar TPC,
466 comprehended between 123 and 138 mg GAE.g⁻¹, and although these values are
467 promising, they are lower than the extracts obtained by SFE and SFE + EtOAc.

468 Ethyl acetate, combined with scCO₂, was shown to be an excellent solvent for
469 phenolic compounds. All extracts obtained using scCO₂ + EtOAc presented high TPC
470 values, comprehended between 150 and 181 mg GAE.g⁻¹. Temperature and pressure
471 conditions did not present significant differences in the outcome of the TPC. However,
472 there is a tendency to a higher concentration of phenolics in extracts obtained at lower
473 temperatures and MR = 2:1.

474 Extracts obtained by scCO₂ + EtOH presented similar values for TPC in the
475 range between 87 and 91 mg GAE.g⁻¹, presenting the lowest results for TPC comparing

476 with results obtained using ethyl acetate as cosolvent. This result was not expected
477 considering the polarity of ethanol and, consequently, the affinity with phenolic
478 compounds compared to ethyl acetate. However, the use of ethanol in scCO₂ extractions
479 may favor the extraction of other compounds from the hops matrix, such as carbohydrates,
480 reducing the concentration of phenolics in the final extract. Due to this lack of selectivity,
481 the TPC suffer dilution in these extracts (Table 2). When comparing the overall yields of
482 exhaustive extractions (Table 1), in which the yield with ethanol was 2.3 times higher
483 than that of ethyl acetate, it is indicating that each solvent extracted different classes and
484 fractions of compounds.

485 The analysis of TFC presented the tendency of an increase in values for lower
486 temperatures of extraction using scCO₂. For the PFE, the increase of temperature induce
487 a reduction of TFC as well, and the increase of pressure enhanced the extraction of
488 flavonoids. The FBH extracts with the highest TFC were obtained using the only scCO₂,
489 followed by the extracts obtained using scCO₂ + EtOAc that presents similar values
490 independently of conditions of extraction (temperature, pressure, or MR). TFC values for
491 extracts of these two methods are ranged from 60 up to 93 mg CE.g⁻¹ and 51 up to 70 mg
492 CE.g⁻¹, respectively.

493 Once more, the ethyl acetate as a cosolvent produces a richer in flavonoids
494 extract when compared to the ethanol as a cosolvent. However, in opposition to the TPC
495 results, the extracts obtained by scCO₂ + EtOH presented higher TFC (35 – 42 mg CE.g⁻¹
496 ¹) than extracts obtained by PFE (18 – 30 mg CE.g⁻¹), ratifying the higher affinity of
497 ethanol by different flavonoids.

498 All extracts have high antioxidant activity values even when compared to
499 several different matrices [46], the values ranging from 0.60 to 1.53 mmol TEAC.g⁻¹
500 corroborating all the TPC and TFC analysis. The extracts with the highest values for

501 antioxidant capacity were obtained by scCO₂ extractions, followed by scCO₂ + EtOAc.
502 This result was expected considering the values of TPC and TFC. Moreover, hops are a
503 source of phenolics compounds derived from benzoic and cinnamic acid, in addition to
504 flavonoids that can retard or prevent oxidation, acting as radical scavengers.
505

506 Table 3 - Total phenolic content, total flavonoid content, and antioxidant activity by
 507 DPPH•

Run	Extraction Method	T (°C)	P (MPa)	MR ^c	TPC (mg GAE.g ⁻¹)	TFC (mg CE.g ⁻¹)	DPPH• (mmol TEAC.g ⁻¹)
1		40	15		185 ± 9	93 ± 4	1.53 ± 0.02
2	SFE (CO ₂)	40	25		176 ± 10	90 ± 5	1.46 ± 0.04
3		80	15	-	175 ± 8	64 ± 1	1.31 ± 0.04
4		80	25		189 ± 4	91 ± 2	1.35 ± 0.02
5, 6, 7		60	20		180 ± 13	60 ± 8	1.27 ± 0.03
8		20	3		128 ± 2	25 ± 2	1.15 ± 0.03
9		20	10		128 ± 7	30 ± 0	1.10 ± 0.06
10	PFE (Propane)	60	3		131 ± 4	18 ± 1	0.95 ± 0.03
11		60	10	-	138 ± 4	21 ± 1	1.05 ± 0.04
12, 13, 14		40	6,5		123 ± 5	23 ± 1	1.02 ± 0.08
15		40	15	2:1	181 ± 2	57 ± 3	1.27 ± 0.03
16		40	25	2:1	162 ± 8	52 ± 8	1.17 ± 0.02
17		80	15	2:1	150 ± 16	58 ± 4	1.12 ± 0.03
18		80	25	2:1	156 ± 8	51 ± 3	1.14 ± 0.03
19, 20, 21	scCO ₂ + EtOAc	60	20	2:1	167 ± 20	65 ± 9	1.27 ± 0.03
22		40	15	1:1	174 ± 9	61 ± 3	1.32 ± 0.04
23		40	25	1:1	169 ± 21	70 ± 4	1.27 ± 0.01
24		80	15	1:1	164 ± 3	56 ± 1	1.18 ± 0.04
25		80	25	1:1	176 ± 5	59 ± 9	1.14 ± 0.04
26, 27, 28		60	20	1:1	176 ± 5	59 ± 1	1.23 ± 0.05
29	scCO ₂ + EtOH	40	15	2:1	87 ± 6	42 ± 2	0.62 ± 0.01
30		80	25	2:1	91 ± 9	35 ± 1	0.60 ± 0.03
31		60	20	2:1	89 ± 3	39 ± 2	0.13 ± 0.04

508 ^cMass Ratio = Mass of cosolvent (g) / mass of FBH (g) at the start of the extraction.

509

510 Conditions presented as Run 4, the best global yield for SFE, presented the best
 511 TPC and the second-best TFC. This same trend can be observed in Runs 25 and 30. Thus,
 512 it is possible to notice that the increase in yield does not compromise the quality of the
 513 extracts obtained from the FBH within the conditions evaluated in this study.

514

515

516 **4. Conclusions**

517

518 In this work, different non-conventional extractions were used to obtain extracts
519 of hops (*Humulus lupulus*) Mantiqueira variety; the addition of ethyl acetate enhanced
520 the extraction of global yield, rate, and efficiency. The best extraction was achieved using
521 scCO₂ + EtOAc at RM (2:1) and 80 °C and 25 MPa, with a global yield of 10.2 wt%. The
522 results indicated that in general terms, temperature and pressure combined have a positive
523 effect on the extraction yield.

524 The results of TPC and TFC indicate that the use of EtOAc as a cosolvent does
525 not reduce the quality of the extracts as antioxidant agents. The FBH extracts obtained
526 using scCO₂ + EtOAc presented similar TPC when compared to pure scCO₂ extraction.
527 Ethanol as cosolvent enhanced the overall extraction yield but presented a significant
528 reduction in the selectivity for phenolic compounds. All the extracts presented a high
529 antioxidant activity, expected effect due to the high concentrations of phenolic
530 compounds found in the extracts from hops.

531 This study demonstrated that ethyl acetate is a promising cosolvent in scCO₂
532 extractions due to increased yield and phenolic compound extraction in various plant
533 matrices. It is also possible to conclude that hops produced in Brazil are a rich source of
534 bioactive compounds, even those produced under different climate conditions.

535 Brazilian hop extracts obtained by the extraction techniques presented in this
536 work have great potential for use in the pharmaceutical and food industry, and a more
537 detailed study of the composition of the extracts is suggested.

538

539

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548

549 **Abbreviations**

550

551 AA Antioxidant activity

552 ANOVA Analysis of variance

553 BH Brazilian hops

554 CE Catechin equivalent

555 CER Constant extraction rate

556 DCP Diffusive controlled period

557 DPPH 2,2-diphenyl-1-picrylhydrazyl

558 EtOAc Ethyl acetate (Ethyl ethanoate)

559 EtOH Ethanol

560 FBH Freeze-dried Brazilian hops

561 FER Falling extraction rate

562 GAE Gallic acid equivalent

563 MR The ratio of cosolvent mass to Freeze-dried Brazilian hops mass at start of

564 extraction ($g_{\text{cosolvent}}/g_{\text{FBH}}$)

565 PFE Pressurized fluid extraction

566 scCO₂ Supercritical carbon dioxide

567 SFE Supercritical fluid extraction

568 TEAC Trolox equivalents antioxidant capacity

569 TFC Total flavonoids content

570 TPC Total phenolics content

571 UV-VIS Ultraviolet-Visible

572

573 **5. References**

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