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Highlights

- Agave bagasse compaction within the cell might hinder ultrasound intensification.
- Low mass load decreased compaction and allowed extraction intensification.
- Transducer geometry significantly affects ultrasound effect
- Multiplate transducer was efficient for SF extraction of bioactive compounds.

Original research article

Title: Effect of ultrasound intensification on the supercritical fluid extraction of phytochemicals from *Agave salmiana* bagasse

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Abstract

The aim of this work was to evaluate the effect of ultrasound on supercritical fluid extraction for the recovery of antioxidants and saponins from agave bagasse as a green extraction technique. When a mass load of 0.086 g/cm^3 was used, ultrasound effect was not observed, due to sample swelling and compaction within the cell. For 0.043 g/cm^3, the intensification effect of ultrasound was significant ($p<0.05$) and its magnitude depended on the transducer geometry. For a multiplate transducer geometry, antioxidant capacity increased from 12.18±1.01 to 20.91±1.66 µmol TE/g; and saponins from 19.05±1.67 to 61.59±1.99 µg/g, when ultrasound was applied. Although the amount of bioactives extracted is low, the use of a multiplate transducer design was able to intensify the supercritical fluid extraction of phytochemicals from agave bagasse. Consequently, this type of transducer can become an alternative for the application of ultrasound on the supercritical fluid extraction of other suitable agro-industrial by-products.

Keywords: antioxidants, supercritical fluid extraction, saponins, ultrasound-assisted extraction, ultrasound transducers

1. Introduction

Supercritical fluid extraction (SFE) using carbon dioxide has been devised as an alternative technology to reduce the use of organic solvents for the recovery of added value products from plant materials [1]. The main advantages of this technology is that the solvent physicochemical properties can be adjusted by modifying the pressure and temperature conditions within the system, therefore increasing the extraction selectivity. Additionally, with SFE it is also possible to work using solvents accepted in the food and pharmaceutical industry, and allows an easy extract/solvent separation [2]. SFE has been successfully used to recover compounds from agro-industrial wastes, such as carotenoids from a mixture of cabbage, lettuce and auyama residues [3], polyphenols from grape bagasse [4] and oil from spent coffee grains [5], using supercritical CO_2 (SC-CO_2).

The main drawbacks of supercritical fluid extraction are the slow extraction kinetics and low extraction yields compared to conventional processes. One alternative to intensify the process is the use of power ultrasound [6–9]. Ultrasound generates various effects within the system, such as cycles of fluid expansion/contraction and acoustic streaming that disrupt the vegetable tissue [10–12] and intensifies the mass transfer processes [12–15]. Ultrasonically-assisted supercritical fluid extraction (USFE) has allowed significant yield increases in the extraction of bioactive compounds on food products such as dedo de moça pepper [11] and oregano [8]. Furthermore, changes in the transducer geometry can make ultrasound application more effective during the SFE process [8].

In general, SFE is applied for the extraction of lipophilic compounds, such as fatty acids essential oils, volatile compounds or carotenoids from diverse plant sources [3,16–18]. Nevertheless, a variety of higher polarity compounds have been successfully extracted using SFE. The effect of SFE on antioxidant extraction has been studied on different plant matrices, resulting effective for the recovery of isorhamnetin glycosides from cactus pad flour [19], phenolics and antioxidants from oregano [8], anthocyanins from

blackberry [9], saponins from Brazilian ginseng [14], among others. To obtain compounds of this nature, a modifier or co-solvent, such as pure or aqueous ethanol, needs to be added to increase the supercritical fluid polarity [1].

Agave bagasse constitutes an agro-industrial waste from agave processing industries in Mexico. Agave species can be used for the production of food and beverages, such as pulque, mescal and tequila [20]. In particular, *Agave salmiana* is used to harvest the sap, a sweet, oligosaccharide-rich liquid produced by mature plants [21]. The sap can be directly consumed or used as raw material to produce an alcoholic fermented beverage known as "pulque" [20]. Sap extraction, reviewed in detail by Escalante et. al (2016), involves the agave floral bud removal (castration) to create a pit in the center of the plant, scraping the pit to promote sap flow and harvesting by suction [21]. During the scraping process, plant tissue residue (bagasse) is discarded. Reports regarding agave bagasse indicate a high fiber content, around 70-90% of the dry matter and around 4.8-5.5% ashes [22,23]. However, little is known about the particular phytochemical composition of this residue. In previous works, it was determined that agave sap is a prospective source of steroidal saponins with potential to inhibit colon cancer cell growth *in vitro* [24]. In addition, antioxidant capacity has been reported in leaf tissue of mature *Agave salmiana* (60-80 μmol Trolox equivalents (TE)/g by ORAC assay) [25]. Conventional methods to obtain extracts rich in bioactive compounds from agave leaves or other plant tissues involve the use of organic solvents, such as methanol or butanol [24,25].

Therefore, the aim of this work was to assess the effect of ultrasound on supercritical fluid extraction of added bioactive compounds from the highly fibrous material agave bagasse The effect of pressure, temperature, co-solvent proportion, and the use of ultrasound with different transducers configurations on antioxidant capacity, antioxidant compounds and saponin concentration in the extracts was evaluated.

2. Materials and methods

2.1. Plant material

Agave (*A. salmiana*) bagasse was obtained as a by-product of agave sap collection in the state of Coahuila, Mexico during the month of December 2016. It was sun dried at an average temperature of 16 °C to 11% moisture content. Dry bagasse was ground and sieved through 1 to 3 mm mesh.

2.2. Supercritical fluid extraction (SFE)

All SFE experiments were performed in a custom-designed laboratory-scale plant, built by the ASPA group of the Universitat Politècnica de València (**Fig. 1**). The plant can work at pressures up to 700 bar and temperatures of 70 °C, configured as described by Santos-Zea et al. (2018) [8]. The ultrasonic system consisted of an ultrasound transducer and an ultrasound generator (**Fig. 1**, 14; 60 W and 30 kHz, FSP300-60BTV, FSP Group Inc., Taoyuan City, Taiwan) coupled to a power meter (WT300-760401, Yokogawa Iberia S.A., Madrid, Spain). The ultrasound transducer (**Fig 1.**, 13) was composed of a metallic tail mass, a pair of piezoelectric ceramics (O.D. 36.8 mm, I.D. 12.5 mm, thickness: 5 mm), and a cylindrical aluminum (ASTM 7075) head mass (length 35.8 mm), also described in previous work [8]. For further reference, this cylindrical transducer corresponded to T_A (**Fig 2.A**).

Experiments were carried out according to a Box-Benkhen design (**Table 1**), where process factors were pressure (X_1, bar), temperature (X_2, °C) and amount of co-solvent (X_3, %). Experiments were divided in two blocks: with (USFE) and without (SFE) the use of ultrasound. Response variables were antioxidant capacity (AOXC) and saponin concentration. Each condition was evaluated once using 10 g of ground agave bagasse (mass load of 0.086 g/cm^3), placed in a 116 cm^3 extraction cartridge (**Fig 1.**, 9). The mass load was calculated by dividing the mass of material loaded into the cell by the volume of the extraction cartridge. Carbon dioxide (**Fig 1.**, 1) (99.9%, Abelló Linde S.A., Barcelona, Spain) was liquefied in a chiller reservoir at -7 °C (**Fig 1.**, 4) and pumped (**Fig 1.**, 5) to the desired pressure (150, 300, 450 bar) at a rate of 1 ± 0.1 kg/h. Ethanol (96% pharma grade, AppliChem GmbH, Darmstadt, Germany) diluted to 70% with distilled water

(**Fig 1.**, 2) was pumped at a constant flow rate (**Fig 1.**, 3) to reach the corresponding proportion of co-solvent (5, 7.5, 10 %). Liquid CO_2 and co-solvent were mixed in a T-section (**Fig 1.**, 6), heated and fed into the extractor in supercritical state (**Fig 1.**, 8) at the desired temperature (40, 50, 60ºC). In all conditions, extraction time was 60 min. The amount of 70% ethanol consumed was 60, 90 and 120 mL for experiments with 5, 7.5 and 10% co-solvent. The separation vessel (**Fig 1.**, 10) was operated at 60 ± 5 bar and at the extraction temperature considered in each experiment. The gas was returned to the chiller reservoir for liquefaction and recirculation into the system. Temperatures and pressures were monitored by thermocouples (**Fig. 1**, T) and pressure gauges (**Fig. 1**, P). The extract was collected from the bottom of the separator by a manually operated valve (**Fig. 1**, 12) and the total volume recovered was recorded for further calculations. A 1 mL aliquot was taken from each extract for antioxidant capacity evaluation and the rest was concentrated to dryness under reduced pressure at 60 °C for saponin analysis.

2.3. Effect of the mass load and transducer geometry.

To determine whether the mass load and the transducer geometry affected antioxidants and saponin extraction, additional tests were carried out at the optimal conditions for antioxidants (pressure, temperature and % co-solvent) as obtained from the response surface for the SFE block. Experimental procedure was carried out as described in Section 2.2, but to reduce mass load 5 g of ground agave bagasse were loaded into the extraction cell. Experiments were carried out both with the cylindrical transducer (T_A) described in the previous section (**Fig. 2.A**) and with a second one (T_B) which differed in the head mass design (**Fig 2.B**). T_B consisted of a multiplate circular head mass mechanized as a whole piece, with two circular steps (36.8 mm diameter, 2.2 mm thick) separated by a distance of 18.7 mm as described by Santos-Zea et al. (2018). The mass load for these 5 g experiments corresponded to 0.043 g/cm^3. According to previous characterization, T_A and T_B presented a nominal power density of 116.4 ± 7.7 and 151.6 ± 7.1 W/L (evaluated by calorimetry), respectively; as well as an acoustic pressure of 150.6 ± 20.5 and 99.3 ±

12.8 kPa (measured by hydrophone), respectively [8]. For each transducer arrangement (T_A and T_B), three independent extractions were carried out with and without the use of ultrasound.

2.4. Evaluation of antioxidant capacity

The antioxidant capacity (AOXC) of the extracts was measured by the ferric reducing/antioxidant power assay (FRAP) [26]. The absorbance was recorded at 595 nm. Ethanol mixed with all the reagents was used as blank. To carry the out the FRAP analysis a 1 mL aliquot from the total extract was used. The AOXC values were initially calculated in µM using a Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich, St. Louis, MO) standard curve. Afterwards, to express the results per gram of dry bagasse, the total volume of extract recovered after the process was used to calculate the AOXC obtained from the 5 or 10 g, according to (Eq. 1):

$$\left[AOXC\ \left(\frac{\mu mol}{g}\right)\right] = \frac{\left[AOXC\left(\frac{\mu mol}{L}\right)\right] * [total\ extract\ volume\ (L)]}{sample\ amount\ (g)} \qquad \text{(Eq. 1)}$$

AOXC was expressed as micromol of Trolox equivalents per gram of agave bagasse (TE µmol /g).

2.5. Phytochemical analysis evaluation

For compound analysis, the dry extracts were reconstituted in 4 mL of a 1:1 mixture of HPLC grade water and methanol (Tedia, Fairfield, OH, USA), and filtered through 0.25 µm PTFE membrane disk filters (Agilent Captiva Econofilter, Santa Clara, CA, USA). Antioxidant compounds and saponin quantitation were carried out by liquid chromatography coupled to diode array and evaporative light scattering detectors (HPLC-DAD-ELSD, Agilent Technologies, 1200 Series, Santa Clara, CA, USA) as in previous work [27]. Antioxidants were detected at a wavelength of 280 nm and saponins on ELSD signal [28]. The column Zorbax Eclipse XDB-C18, 150 mm × 4.6 mm I.D, 5 µm (Agilent Technologies, Santa Clara, CA, USA) was the stationary

phase used for separation. HPLC grade water and acetonitrile (Tedia, Fairfield, OH, USA) were acidified with 0.1% formic acid (CTR Scientific, Monterrey, Mexico) and used as mobile phase. A protodioscin (Sigma-Aldrich, St. Louis, MO, USA) standard curve was used for quantitation as protodioscin equivalent µg per gram of bagasse (PE µg/g). Antioxidant compounds were quantified based on relative abundance of the area under each peak at 280 nm, and their absorption spectra were recorded. Saponins were quantified as the sum of the concentration of the individual saponins identified.

Antioxidant compounds and saponin identification was accomplished by liquid chromatography coupled to a time of flight mass detector with electrospray source in positive mode (HPLC-MS-ESI-TOF, G1969A, Agilent Technologies, Santa Clara, CA, USA) as reported previously [27]. Saponin identity was assigned according to their characteristic fragmentation patterns, as recorded in previous works [24,28,29]. Antioxidant compounds were identified by comparing their UV-spectrum and accurate mass with similar compounds identified in other works regarding agave products [28,30].

2.6. Statistical analysis

Experimental data was analyzed by ANOVA and Pareto Chart to determine significance of the experimental factors, along with response surfaces to observe the effect of interactions. The response surface model was adjusted using the Least Squares Fit method. Optimization was accomplished using the desirability function, minimizing the determinant of the covariance matrix of the model estimates. Correlation analysis was carried out according to Spearman. All analyses considered a significance of $\alpha=0.05$ and were carried out using JMP 14 (2018).

3. Results and discussion

3.1. Recovery of antioxidant capacity from agave bagasse: effect of process variables.

There was no significant difference in the average antioxidant capacity obtained with (USFE) or without (SFE) ultrasound (**Table 1**), ($p>0.05$). One of the phenomena observed during the experiments was the co-solvent absorption by the fibrous agave bagasse solids. This absorption generated sample swelling during the extraction process, compacting the material within the extraction cell. Bagasse compaction interfered in the ultrasonic field propagation throughout the medium, thus hindering the ultrasound intensification mechanisms. In this regard, it has been documented that solid bed compaction can interfere in SFE due to an increase in mass transfer resistance and solvent channeling effects [1]. To visualize the sample swelling of agave bagasse, 370 mg of ground agave bagasse (1 mL volume) were placed in a graduated cylinder and after 1 h in contact with ethanol (70%, ethanol was added to cover the sample), a 100% volume increase was observed.

When considering the block where extractions were carried out without ultrasound (SFE), the coefficient of determination obtained for the response surface model was 0.78 and was statistically significant ($p<0.05$). From this model, the Pareto analysis (**Fig. 3.A**) indicated that pressure (X_1) and temperature (X_2) were significant parameters ($p<0.05$), as well as the quadratic effect of temperature (X_2*X_2, $p<0.05$). From our results, increases in both, pressure and temperature of the system allows a greater extraction of antioxidants. When pressure increases, there is an increase in fluid density, enhancing solvation power [1,12]. The effect of temperature can be more complex, since increasing temperature decreases the fluid density and therefore solvation power. However, an increased mass transfer rate from the solid to the fluid is also achieved [1]. First order interactions between pressure and temperature (X_1*X_2) and temperature and co-solvent proportion (X_2*X_3) were also statistically significant ($p<0.05$). In this case, the addition of aqueous ethanol allows an increased solubility of the compounds depending on the extraction temperature. The use of aqueous ethanol as co-solvent is recommended for an adequate solvent polarity for the extraction of antioxidants [31,32]. In this study, the individual effect of this variable was not significant. However, the interaction with temperature makes the use of a co-solvent a relevant factor for

AOXC recovery. These interactions, along with pressure and temperature parameters (**Fig. 3.A**), accounted for 83% of the variability observed for AOXC extracted from agave bagasse.

At high temperatures and pressures, a greater AOXC was observed (**Fig. 3.B**). Interaction between X_2 and X_3 indicated that a maximum AOXC value was attained at the highest temperature (60 °C) and proportion of co-solvent (10 %) (**Fig. 3.C**). The linear effect of pressure (X1) on the antioxidant extraction can be seen in **Fig. 3.B**, indicating how the increase in pressure had intensified the solvation power of SC-CO_2 [1]. On the other hand, **Figs. 3.B** and **3.C** clearly demonstrate a concave curvature in the effect of temperature (X2). In this case, increases in temperature decreases solvation power of the fluid [1]. However, temperature can also enhance mass transfer rate, which could be the mechanism controlling the extraction at temperatures over 50 °C [32]. **Fig. 3.C** also shows that the effectivity of the co-solvent is dependent on extraction temperature, assisting to increase the AOXC at higher temperatures.

Experimentally, the maximum AOXC (17.61±0.75 µmol TE/g) was obtained at 300 bar and 60 °C with 10% co-solvent (**Table 1**). On the other hand, the lowest AOXC was obtained under extraction conditions of 150 bar and 60 °C with 7.5% co-solvent (**Table 1**, 5.28±0.18 µmol TE/g). When optimization was carried out, the optimal extraction conditions within the tested ranges for antioxidant compounds without the use of ultrasound were 450 bar, 60 °C and 10% co-solvent. A predicted maximum AOXC of 18.45 µmol TE/g was obtained, with a 95% confidence interval of 15.86 to 21.04 µmol TE/g, and an optimized desirability of 0.89. For comparison purposes, in a conventional 60 min extraction, using 150 mL 70% aqueous ethanol at 60 °C, we obtained 58.3 µmol TE/g bagasse, with is 3 times the amount obtained in this work.

The response surface model adjusted for USFE of antioxidant compounds from agave bagasse, provided a statistically significant ($p<0.05$) correlation coefficient of 0.79. Temperature (X_2) and co-solvent proportion (X_3) were statistically significant in this model ($p<0.05$), as well as the quadratic effects of co-solvent proportion (X_3*X_3), pressure (X_1*X_1) and temperature (X_2*X_2) ($p<0.05$) (**Fig. 4.A**). Overall, increases in

temperature and co-solvent proportion contribute to a greater extraction of antioxidants, by increasing the mass transfer rate and solubility of the compounds in aqueous ethanol [31,32]. With respect to interactions between factors, only pressure and temperature interaction (X_1*X_2) was significant ($p<0.05$), and negatively affecting the antioxidant capacity (**Fig. 4**.**A**). Liu et al. [33] reported that at high pressures, SC-CO_2 compression generates solute-solvent repulsion, decreasing the extraction efficiency. The Pareto Chart indicated that X_2, X_3, X_3*X_3, X_1*X_2, X_1*X_1 and X_2*X_2 accounted for 91% of the observed variability on AOXC (**Fig. 4.A**).

The prediction for maximum AOXC was at the lowest pressure, highest temperature and highest co-solvent concentration (**Fig. 4.B**). In our experimental data, the highest AOXC (**Table 1**, 11.54±0.06 µmol TE/g) when applying ultrasound was obtained at 300 bar and 60 °C, with 10% co-solvent, while the lowest was at 150 bar and 40 °C, with 7.5% co-solvent (**Table 1**, 4.46±0.01 µmol TE/g). These results were in agreement with temperature and co-solvent being the strongest factors affecting the extraction. As shown in the response surface, at 150 bar (**Fig. 4.B**), a decrease in temperature decreased the AOXC about 1.5-fold with respect to the maximum, while quadratic effect of co-solvent was confirmed, observing a minimum at 7.5% ethanol. An interesting effect was observed when analyzing the response surface fixing pressure at 450 bar. While the highest temperature and co-solvent amount still provide the best recovery of AOXC, temperature presents a quadratic behavior, with the minimum near 50°C and 7.5% co-solvent (**Fig. 4.C**). While the individual effect of pressure was not among the most significant, its interaction with temperature was considered important for USFE of antioxidants from agave bagasse.

Using the statistical model, optimal conditions within the tested ranges for antioxidant recovery were estimated at 150 bar, 60 °C and 10% co-solvent proportion. The predicted maximum AOXC was of 13.35 µmol TE/g, with a 95% confidence interval of 11.86 to 14.84 µmol TE/g, and an optimized desirability of

230 0.99. Compared to the conventional extraction, in this case the amount of antioxidants extracted was 4
231 times lower.

232 With respect to the effect of ultrasound, it was expected that ultrasonic vibrations would cause cell-wall
233 rupture, enhance mass transfer in the fluid and increase the extraction efficiency. Barrales et al. (2009)
234 explained the enhancement of solute diffusivity because the local temperature changes generated by
235 ultrasound waves during *Passiflora* oil extraction [34]. However, in this work, our results suggested a
236 fundamental role of compaction from sample swelling and the high pressures on USFE, hindering the
237 ultrasonic wave vibration and limiting its intensification effect.

238 *3.2. Identification of antioxidant compounds in the extracts.*

239 When the extracts were analyzed by HPLC-UV, several compounds were detected at a wavelength of 280
240 nm (**Fig. 5**.**A**). The correlation analysis indicated that the compounds labeled on the chromatogram 1 to 3
241 were significantly correlated to the antioxidant capacity of the SFE and USFE extracts ($p<0.05$). These three
242 compounds were given tentative identification according to their UV and mass spectra (**Table 2**).
243 Compound 1 presented a UV λ_{max} at 215 and 268 nm, and an accurate mass of 142.026. With this
244 information, (1) was identified as 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-one. Other similar pyrans
245 were reported in thermally processed agave [30]. Compound 2 showed a UV spectrum with three UV λ_{max}
246 at 221, 277 and 312 nm and an accurate mass of 173.105. However, up to date, no compounds with similar
247 UV and mass spectra have been identified within the agave genus. Compound 3 had an UV spectrum with
248 λ_{max} at 282 nm, and an accurate mass of 166.063, and likely corresponds to 2-methoxy-5-(2-methylpropyl)-
249 pyrazine. This Maillard reaction derivative was previously reported in thermally processed agave products
250 [30].

251 Maillard reaction derivatives generated during the thermal process to obtain agave sap concentrate were
252 correlated to this product's antioxidant capacity [28]. 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-one (1) is

structurally similar to 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, found in agave sap concentrate [28], both belonging to the pyranone family. Additionally, pyrazine type Maillard reaction products have been recognized to exhibit antioxidant capacity due to their heterocyclic nature [35]. Therefore, it is plausible that compounds 1 and 3 were significantly contributing to the antioxidant capacity observed in SFE and USFE extracts.

3.3. *Effect of ultrasound on saponin extraction*

The average saponin concentration was not significantly different ($p>0.05$) between the SFE and USFE extracts, with average values of 10.95±1.81 μg/g for SFE and 11.92±2.15 μg/g for USFE. Moreover, it was not possible to find a response surface model that could significantly fit the experimental results neither for SFE ($p>0.05$) nor for USFE ($p>0.05$). In both cases, none of the parameters (pressure, temperature, co-solvent proportion) affected significantly the amount of extracted saponins. When extracting saponins by the conventional method, (150 mL of 70% aqueous ethanol at 60 °C during 60 min), 550 μg/g bagasse were obtained. These results indicate that yield of these compounds by SFE and USFE was very low.

There are few studies regarding extraction of saponins from plant matter using SFE. Sun et al. (2010) determined that a group of triterpenoids known as saikosaponins could be extracted by SFE at 400 bar, 45 °C, using 80% ethanol as co-solvent, and including a static extraction step. However, the authors indicated that yields were lower than for classical solvent extraction [36]. Similar results were reported for *Ganoderma atrum* triterpenoid saponins and brazilian ginseng saponins. In the first case, SFE proved to be the least efficient technique for extraction, compared to microwave-assisted and conventional solvent extraction [37]. While in the second case SC-CO_2 with ethanol modifier yielded some low polarity saponins but lower amounts than when conventional solvent extraction was attempted [14].

Two different saponin glycosides were observed in both, SFE and USFE extracts (**Fig. 5.B**). Compound 4 was identified as a kammogenin hexaglycoside, with four hexose residues and two pentoses, showing the

molecular ion *m/z* 1374.62 ($M+H_2O^+$). The fragments for the penta (*m/z* 1242.57), tri (*m/z* 931.44), di (*m/z* 769.40) and monoglycoside (*m/z* 607.34) were detected, as well as the aglycone kammogenin (*m/z* 445.30) (**Fig. 5.B**). This saponin has not yet been reported in *Agave salmiana*. Compound 5 was characterized as a mixture of two kammogenin glycosides: magueyosides A and B. The molecular ions *m/z* 1217.47 ($M+Na^+$) for the pentaglycoside magueyoside A and the tetraglycoside magueyoside B *m/z* 1085.42 ($M+Na^+$) were co-eluting. Both compounds were previously reported in fresh and concentrated agave sap, and magueyoside B presented antiproliferative potential on colon cancer cell lines [24,29].

3.4. *Effect of mass load and ultrasound transducer design on antioxidant and saponin extraction.*

Due to the sample swelling observed during SFE and USFE extraction of agave bagasse, experiments were carried out to determine if ultrasound effect could be observed when using lower mass load (0.043 g/cm^3), compared to the original 0.086 g/cm^3. Additionally, a second transducer (T_B) was used to prove if ultrasound was more effective using a different geometry. The AOXC obtained with a lower sample load (T_A load 0.043 g/cm^3, **Table 3**) was 25% higher than the average amount originally obtained in SFE and USFE (**Table 1**), which can be due to both, the higher solvent/feed ratio and the lower bed compaction. These results (**Table 3**) showed that at 450 bar, 60 °C and 10% co-solvent, the transducer type and application of ultrasound were significant factors ($p<0.05$), when using 0.043 g/cm^3 of bagasse. When T_A was used, there was no significant difference in AOXC derived from use of ultrasound (**Table 3**). However, it is interesting to denote that for T_B (0.043 g/cm^3), when ultrasound was applied, the maximum AOXC observed in all the experiments in this work (20.91±1.66 µmol TE/g) was obtained, representing a 2-fold increase in the AOXC compared to SFE without ultrasound. This amount represents 36% of the antioxidant capacity obtained using conventional extraction (58.34 µmol TE/g). These results were in accordance to the effect observed in oregano antioxidant extraction, where a greater AOXC was observed when using the multiplate transducer (T_B) in comparison to the cylindrical transducer (T_A) [8].

With respect to saponin content, a greater amount was extracted in all cases when using a 0.043 g/cm^3 mass load (**Table 3**), in comparison with all experiments carried out with 0.086 g/cm^3 of bagasse (**Table 1**). When supercritical extraction was carried at 450 bar, 60 °C and 10% co-solvent, the transducer type and application of ultrasound resulted significant factors on the amount of saponins extracted ($p<0.05$), while the interaction of both factors was not significant ($p>0.05$). The lowest amount of saponins was obtained when no ultrasound was used (**Table 3**). Reducing the mass load increased around 1.8-fold the average amount obtained when the extraction cell was loaded with 0.086 g/cm^3. With respect to the use of ultrasound, it proved to be effective when using both transducers. With T_B, saponin extraction increased 3-fold when ultrasound was used, obtaining 61.59 µg PE/g. By contrast, ultrasound was less effective with T_A, showing only a 1.4-fold increase in saponin extraction, reaching 25.93 µg PE/g. In consequence, with T_B 11% of the saponins obtained by conventional extraction (550 µg PE/g) were obtained, while with T_A only 4.7% of that amount was extracted.

It is also noteworthy to see that USFE using T_B allowed the abundant extraction of two additional glycosides (**Fig. 4.C**). Compound 6 was identified as a kammogenin tetraglycoside with a molecular ion *m/z* 1055.42 $(M+Na)^+$ and the characteristic fragments of kammogenin (**Fig. 4.C**). Compound 7 corresponded to magueyoside C, a manogenin tetraglycoside with the molecular ion *m/z* 1087.46 $(M+Na)^+$ and the typical manogenin fragments (**Fig. 4.C**). Both of these compounds were previously reported in fresh and concentrated agave sap [24,29].

The lower acoustic pressure and higher power density of T_B compared to T_A (section 2.3) indicates that the cylindrical transducer T_A provides a higher energy concentration on the plane facing the transducer's plane surface. Nevertheless, the multiplane T_B provides a higher total energy and a better distribution of acoustic field throughout the sample, resulting in a better extraction intensification. The effect of transducer design on the ultrasonic intensification efficiency was previously reported for oregano SFE [8]. Moreover, a lower

mass load allowed the intensification effect of ultrasound to be observed. Thus, when 0.086 g/cm^3 was used, no ultrasound effect was observed. However, when mass load changed to 0.043 g/cm^3, a greater amount of antioxidants and saponins were extracted, probably due to the higher solvent/feed ratio. More importantly, the effect of ultrasound intensification was significant. Therefore, the sample swelling and compaction within the cell must be taken into consideration when loading the extraction cell in USFE. Although the capacity of USFE for phytochemicals recovery in agave bagasse is much lower than that of conventional processes, transducer geometry and mass load are highly relevant factors for ultrasound intensification in SFE.

4. Conclusions

In this work, the use of an ultrasonically-assisted supercritical fluid extraction system increased the recovery yield of antioxidants and saponins from agave bagasse when a low mass load (0.043 g/cm^3) was used. Sample swelling, due to co-solvent absorption, generated compaction within the extraction cell, which limited the propagation of ultrasound and hindered its intensification effect when a mass load of 0.086 g/cm^3 was considered. Lowering the mass load by half increased 1.2 and 1.4 times the amount of antioxidants and saponins extracted under ultrasonically-assisted SFE.

The use of a multiplate ultrasound transducer further intensified the USFE process, compared to a cylindrical one, due to a better acoustic pressure distribution and higher nominal power capacity. The multiplate transducer allowed a 1.7-fold and 3-fold increase in extraction of antioxidants and saponins, respectively. The results obtained in this work point to the use of multiplate-type ultrasound transducers for USFE due to a better acoustic field distribution. Although this technology is not the most effective for recovery of phytochemicals from agave bagasse, this study shows how the use of a transducer geometry, different of the typical cylindrical commercial probe, can significantly enhance the intensification effect of ultrasound in the supercritical extraction processes.

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Figure captions

Fig. 1: Supercritical fluid extraction pilot-scale plant. (1) gaseous CO_2 tank, (2) ethanol reservoir, (3) ethanol pump, (4) liquid CO_2 tank, (5) CO_2 pump, (6) T-section for liquid CO_2 and ethanol mixing, (7) water bath with temperature control, (8) extraction unit, (9) extraction cell, (10) separation unit, (11) microvalve for supercritical fluid flow, (12) sample recovery valve, (13) ultrasound transducer, (14) ultrasound generator, (P) pressure gauge, (T) thermocouple.

Fig. 2*:* Ultrasound transducers used for SFE: (A) cylindrical head-mass transducer, (B) multiplate head-mass transducer.

Fig 3: Graphical summary of effects of pressure (X_1), temperature (X_2) and proportion of ethanol as co-solvent (X_3) on antioxidant capacity (AOXC) obtained by supercritical fluid extraction shown as: **(A)** Pareto chart, where the dark columns indicate a positive effect on the response variable, the light columns indicate a negative effect and columns above the dotted line represent the significant factors ($p<0.05$); response surface plots showing the interactions between **(B)** pressure (X_1) and temperature (X_2); **(C)** temperature (X_2) and co-solvent proportion (X_3). For each plot the third condition was fixed at the central point.

Fig. 4*:* Graphical summary of effects of pressure (X_1), temperature (X_2) and proportion of co-solvent (X3) on antioxidant capacity (AOXC) obtained by ultrasonically-assisted supercritical fluid extraction shown as: **(A)** Pareto chart, where the dark columns indicate a positive effect on the response variable, the light columns indicate a negative effect and columns above the dotted line represent the significant factors

495 ($p<0.05$); response surface plots showing the interactions between **(B)** pressure (X_1) and temperature (X_2)

496 at fixed at 5% co-solvent; **(C)** pressure (X_1) and temperature (X_2) at fixed at 10% co-solvent.

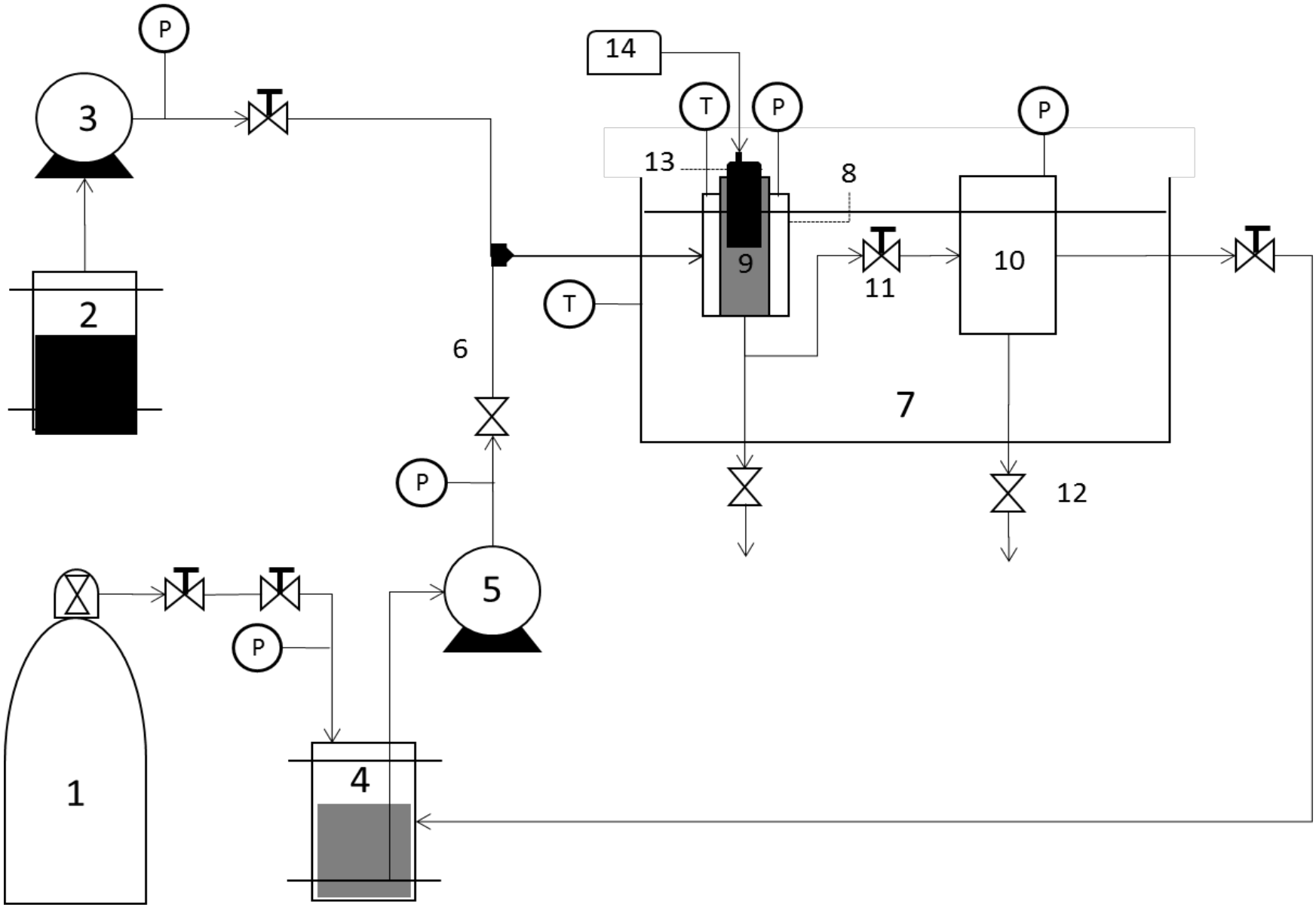
497

498 **Fig. 5:** Representative chromatograms for (A) antioxidant compounds detected at 280 nm, (B) saponins

499 obtained by SFE or USFE using 0.086 g/cm^3 of sample, (C) saponins extracted only by USFE using a

500 multiplate transducer (T_B) and 0.043 g/cm^3 of sample.

501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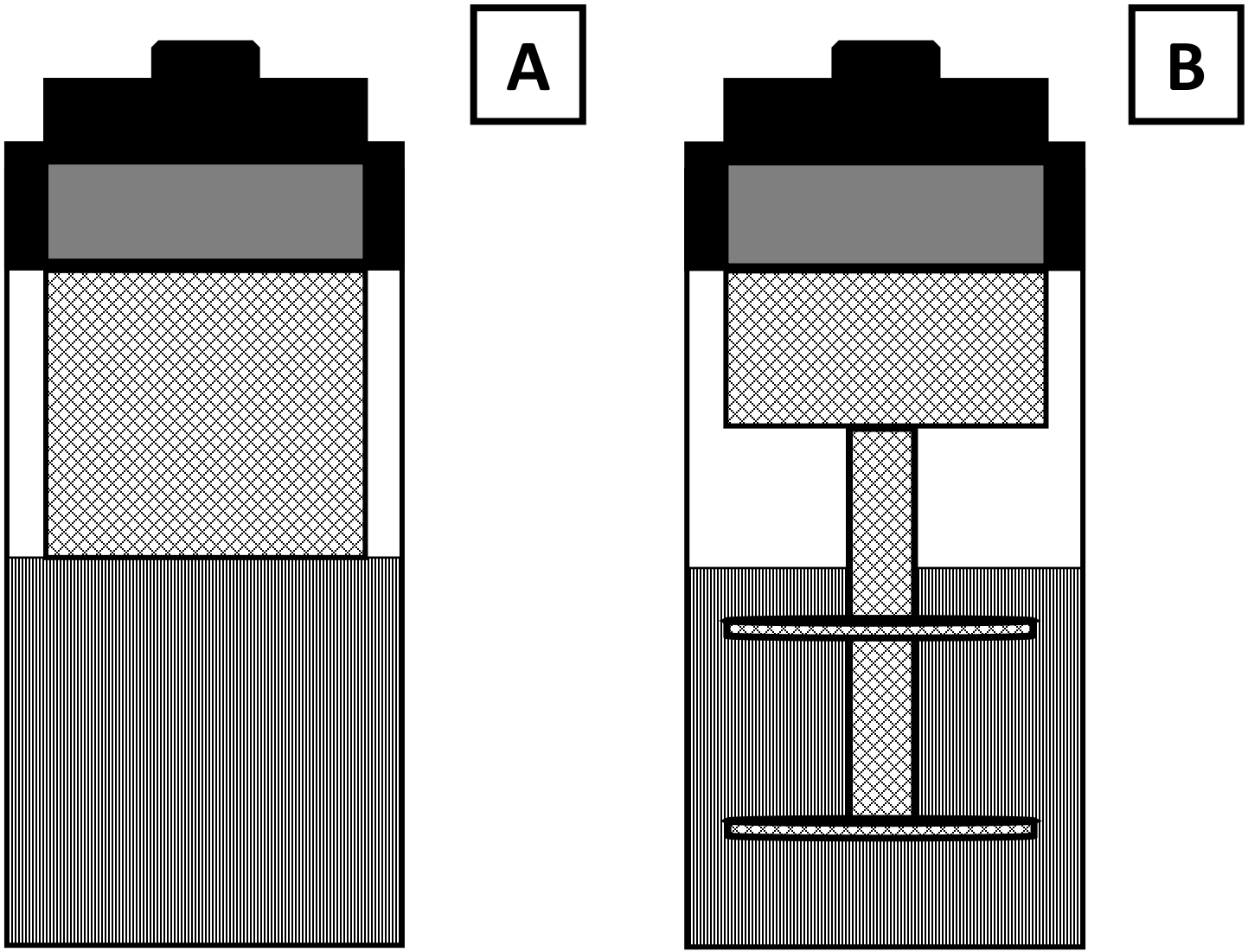
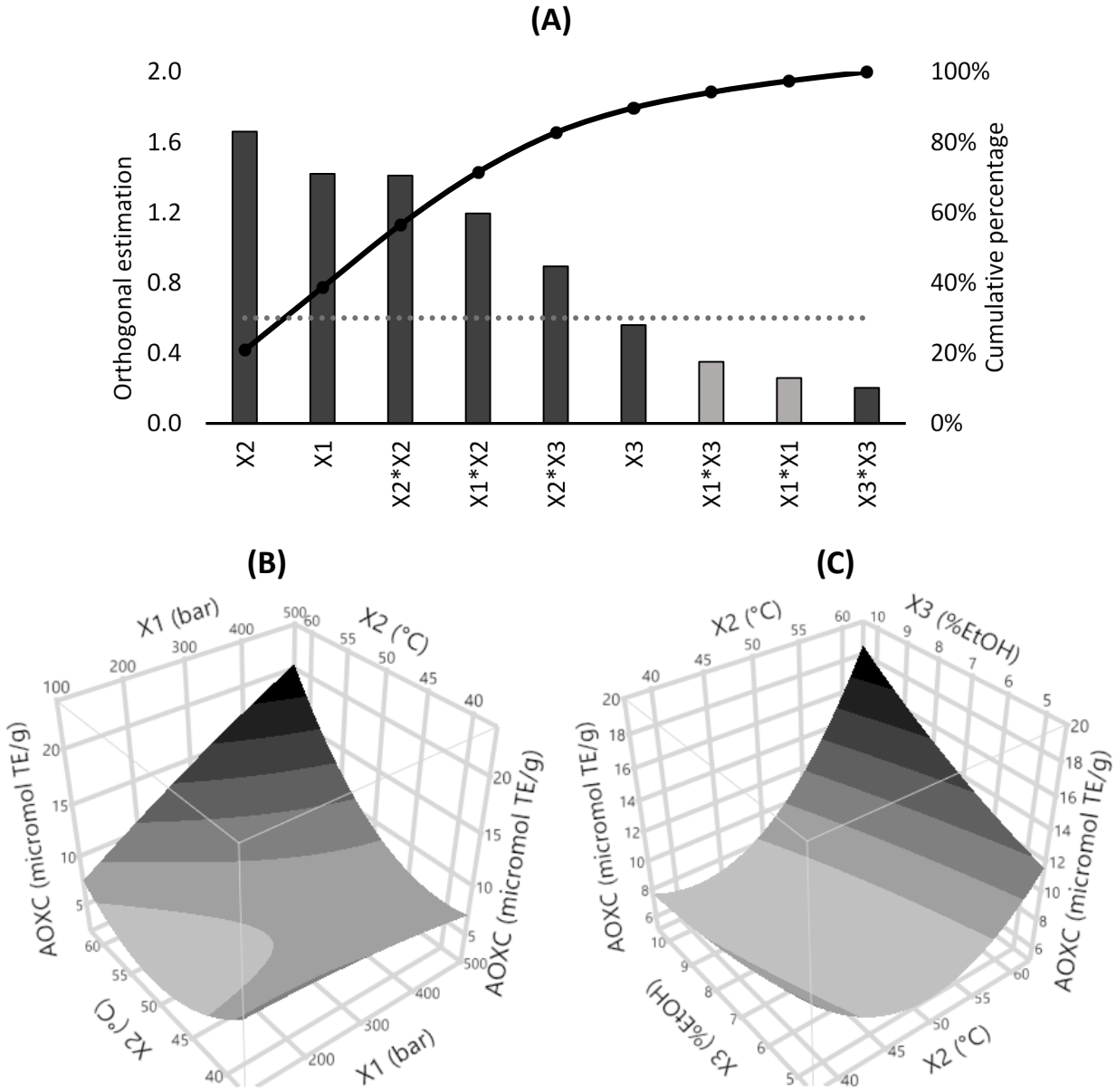
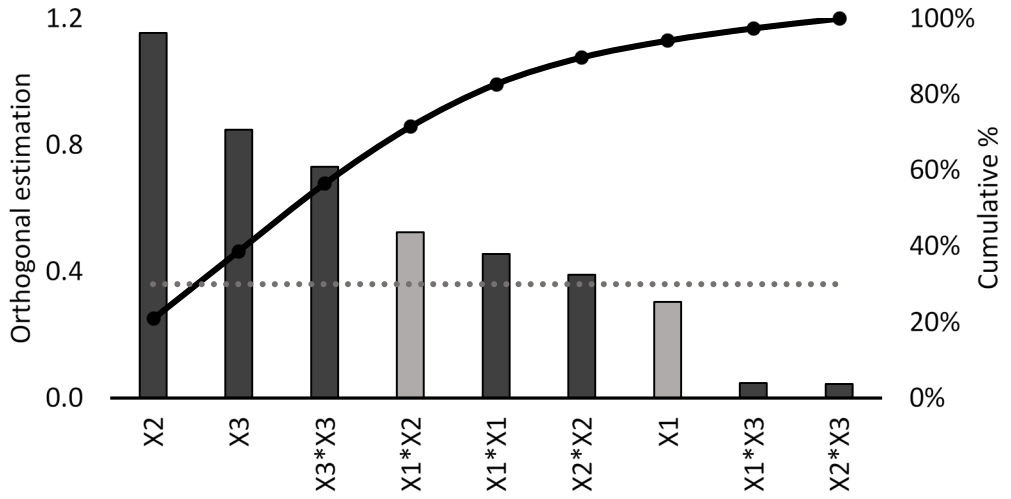
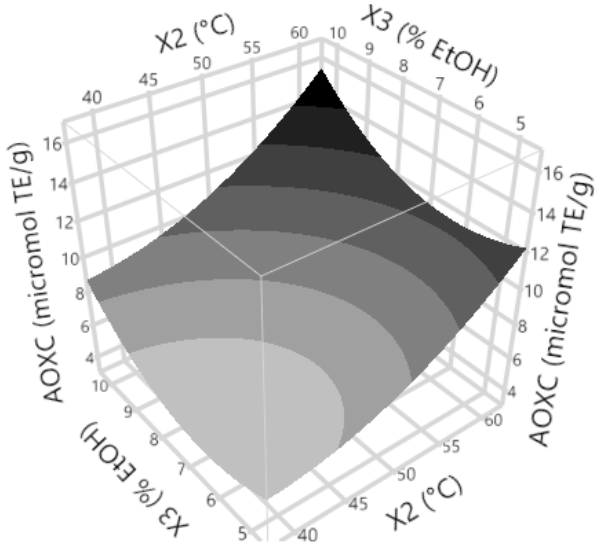

A
B


(A)
Orthogonal estimation
Cumulative percentage
2.0
1.6
1.2
0.8
0.4
0.0
100%
80%
60%
40%
20%
0%
X2
X1
X2*X2
X1*X2
X2*X3
X3
X1*X3
X1*X1
X3*X3
(B)
X1 (bar)
X2 (°C)
AOXC (micromol TE/g)
(C)
X2 (°C)
X3 (%EtOH)
AOXC (micromol TE/g)

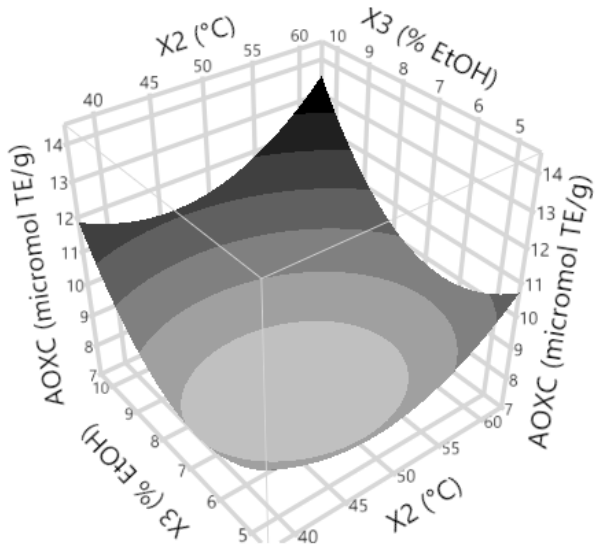
(A)

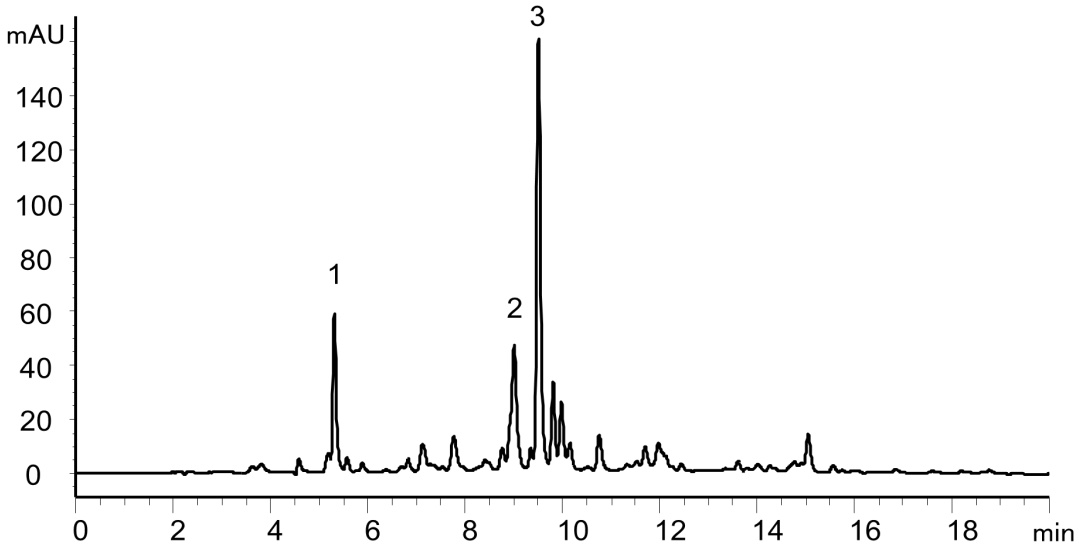

1.2
0.8
0.4
0.0
Orthogonal estimation
100%
80%
60%
40%
20%
0%
Cumulative %
X2
X3
X3*X3
X1*X2
X1*X1
X2*X2
X1
X1*X3
X2*X3

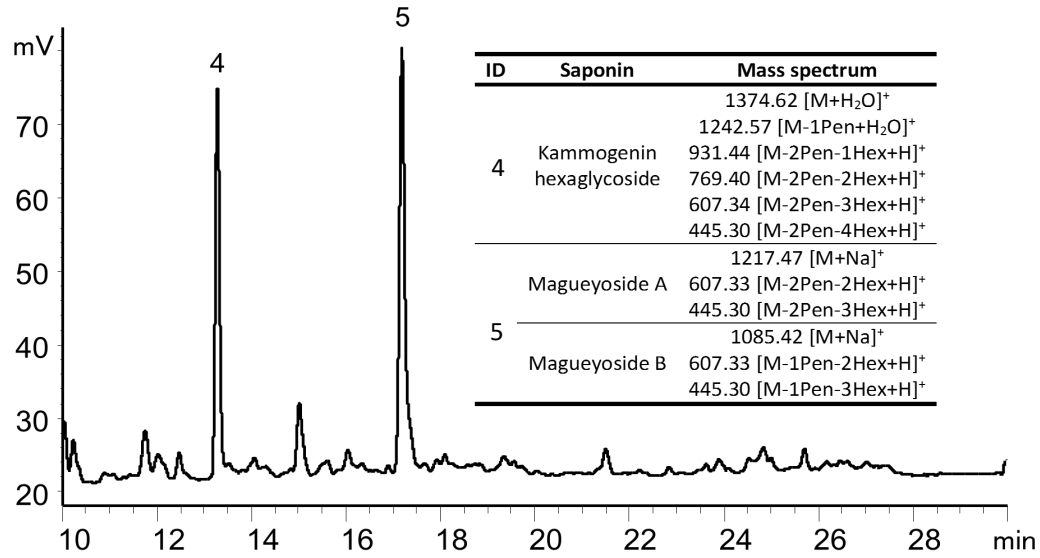
(B)


X2 (°C)
X3 (% EtOH)
AOXC (micromol TE/g)
X3 (% EtOH)
X2 (°C)
AOXC (micromol TE/g)

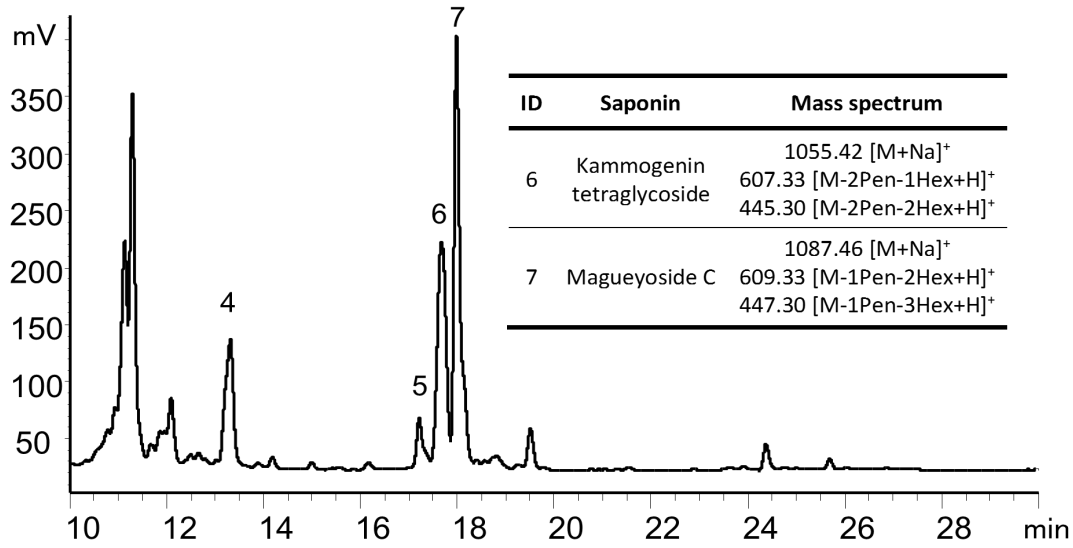
(C)


X2 (°C)
X3 (% EtOH)
AOXC (micromol TE/g)
X3 (% EtOH)
X2 (°C)
AOXC (micromol TE/g)





ID	Saponin	Mass spectrum
4	Kammogenin hexaglycoside	1374.62 $[M+H_2O]^+$
		1242.57 $[M-1Pen+H_2O]^+$
		931.44 $[M-2Pen-1Hex+H]^+$
		769.40 $[M-2Pen-2Hex+H]^+$
		607.34 $[M-2Pen-3Hex+H]^+$
		445.30 $[M-2Pen-4Hex+H]^+$
5	Magueyoside A	1217.47 $[M+Na]^+$
		607.33 $[M-2Pen-2Hex+H]^+$
		445.30 $[M-2Pen-3Hex+H]^+$
	Magueyoside B	1085.42 $[M+Na]^+$
		607.33 $[M-1Pen-2Hex+H]^+$
		445.30 $[M-1Pen-3Hex+H]^+$



ID	Saponin	Mass spectrum
6	Kammogenin tetraglycoside	1055.42 $[M+Na]^+$
		607.33 $[M-2Pen-1Hex+H]^+$
		445.30 $[M-2Pen-2Hex+H]^+$
7	Magueyoside C	1087.46 $[M+Na]^+$
		609.33 $[M-1Pen-2Hex+H]^+$
		447.30 $[M-1Pen-3Hex+H]^+$

Table 1. Box-Benkhen experimental design parameters for supercritical fluids extraction and antioxidant capacity (AOXC) obtained with (USFE) and without (SFE) the use of ultrasound.

Exp	Pressure (bar) X_1	Temperature (°C) X_2	Co-solvent (%) X_3	AOXC (µmol TE/g) SFE	AOXC (µmol TE/g) USFE
1	-1 (150)	-1 (40)	0 (7.5)	8.10±0.10	4.46±0.01
2	1 (450)	-1 (40)	0 (7.5)	9.48±0.43	9.47±0.08
3	-1 (150)	1 (60)	0 (7.5)	5.28±0.18	9.28±0.04
4	1 (450)	1 (60)	0 (7.5)	15.91±0.04	10.23±0.19
5	-1 (150)	0 (50)	-1 (5.0)	6.13±0.26	8.12±0.02
6	1 (450)	0 (50)	-1 (5.0)	9.26±0.04	6.98±0.03
7	-1 (150)	0 (50)	1 (10.0)	6.57±0.20	11.07±0.08
8	1 (450)	0 (50)	1 (10.0)	6.99±0.15	9.57±0.46
9	0 (300)	-1 (40)	-1 (5.0)	6.48±0.27	6.13±0.11
10	0 (300)	1 (60)	-1 (5.0)	10.31±0.29	9.49±0.04
11	0 (300)	-1 (40)	1 (10.0)	7.00±0.03	7.84±0.04
12	0 (300)	1 (60)	1 (10.0)	17.61±0.75	11.54±0.06
13	0 (300)	0 (50)	0 (7.5)	8.70±0.14	6.74±0.14
14	0 (300)	0 (50)	0 (7.5)	6.13±0.17	7.42±0.27
15	0 (300)	0 (50)	0 (7.5)	6.51±0.02	4.97±0.33
Average value for each block				8.71±3.54	8.22±2.08

Table 2. UV spectrum, accurate mass and ions obtained for the three most abundant antioxidant compounds observed in SFE and USFE extracts at 280 nm.

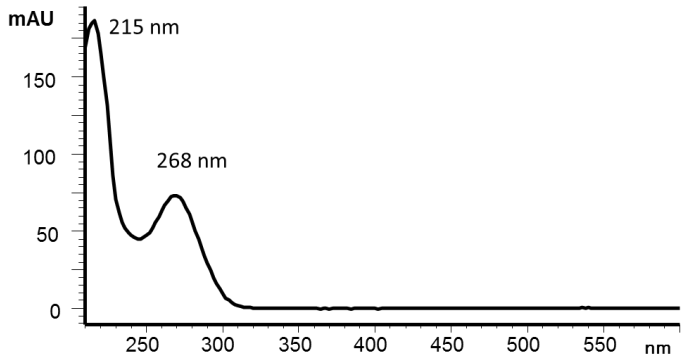
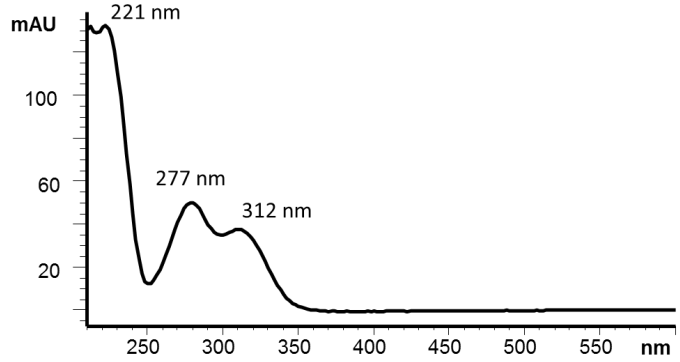
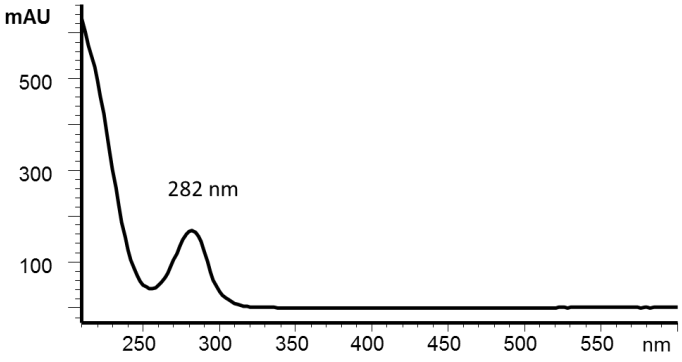
ID	UV spectrum	Accurate mass	*m/z*
1		142.03	143.04 $[M+H]^+$
2		173.11	174.11 $[M+H]^+$ 196.10 $[M+Na]^+$ 347.21 $[2M+H]^+$ 369.20 $[2M+Na]^+$
3		166.06	167.07 $[M+H]^+$ 189.05 $[M+Na]^+$ 205.03 $[M+K]^+$ 355.12 $[2M+Na]^+$

Table 3. Effect ultrasound and transducer design on antioxidant capacity and saponin extraction for 0.043 g/cm^3 mass load.

Transducer type	Ultrasound	AOXC (µmol TE/g)	Saponins (µg/g)
T_A	No	10.01±0.22^b	18.43±0.17^c
	Yes	12.42±1.29^b	25.93±1.44^b
T_B	No	12.18±1.01^b	19.05±1.67^c
	Yes	20.91±1.66^a	61.59±1.99^a

Different letters in the same column show significant differences between treatments.